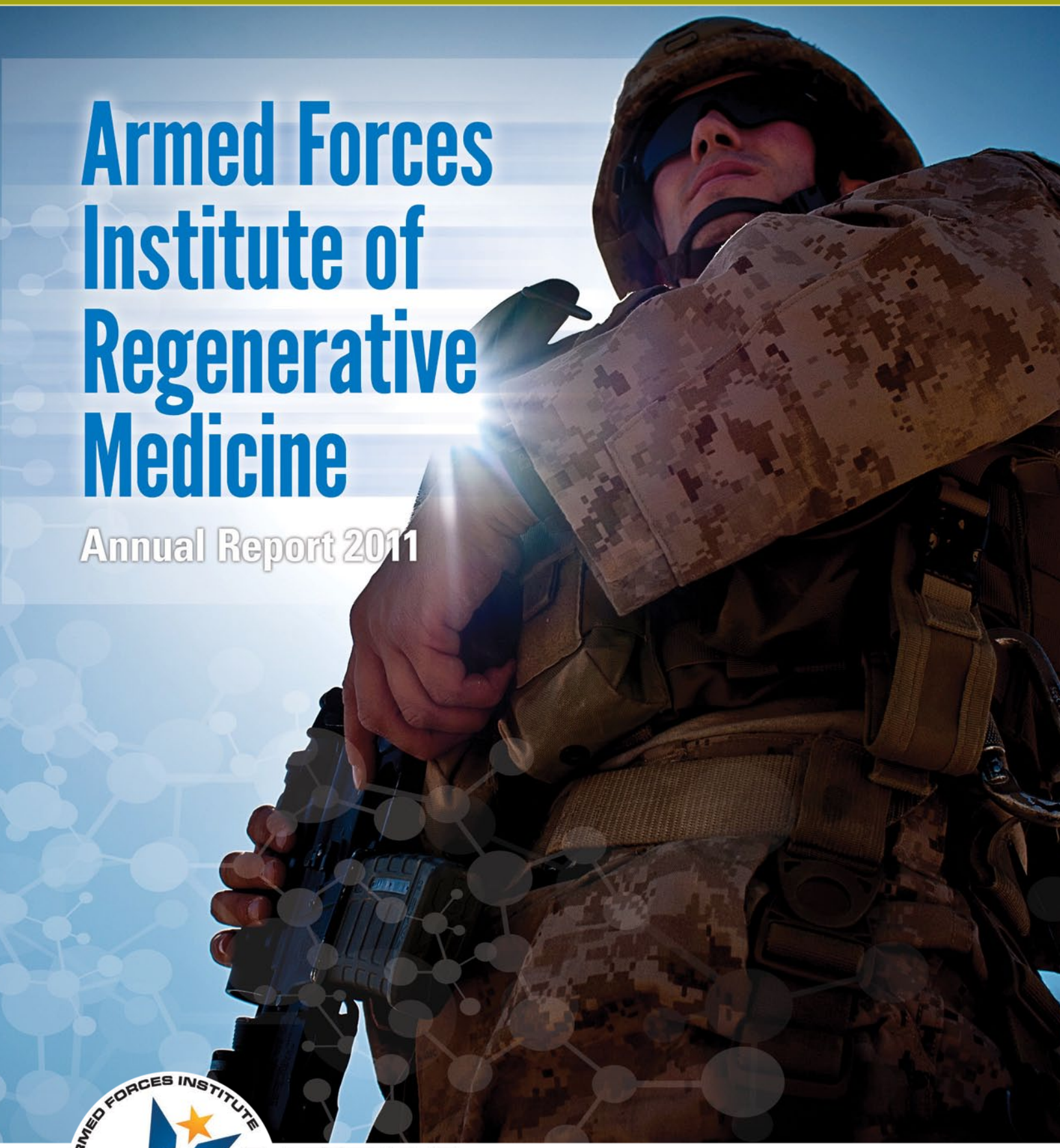
Armed Forces Institute of Regenerative Medicine

Annual Report 2011



AFIRM

our science for their healing

Armed Forces Institute of Regenerative Medicine

ANNUAL REPORT 2011

Complete Edition

This report contains detailed technical information on all currently funded Armed Forces Institute of Regenerative Medicine research projects. The report is also available in a highlights version that provides summary information.

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Table of Contents

Executive Summary vii

I: Introduction

Background 1
Research Goals 2
History 4
Funding – A Six-Way Partnership 4
Structure 5

II: Limb and Digit Salvage

Background 13
Unmet Needs 14
Areas of Emphasis 16
Bone Repair and Regeneration 17
Soft Tissue Repair and Regeneration (excluding nerve) 19
Nerve Repair and Regeneration 22
Composite Tissue Injury Repair 24
Epimorphic Regeneration (and associated methods) 25
Clinical Trials 27

Progress Reports

Bone Repair and Regeneration

Project 4.2.1, RCCC: Advanced 3-D Scaffolds for Large Segmental Long Bone Defects 30
Project 4.2.2, RCCC: Optimizing Cell Sources for the Repair of Bone Defects 36
Project 4.2.3a, RCCC: Advancing Bone Repair Using Molecular Surface Design (MSD) and/or Oxygen Delivery Systems 42
Project 4.2.3b, RCCC: Advancing Bone Repair Using Molecular Surface Design (MSD): Biodegradable Scaffolds with Tethered Osteoinductive Biomolecules 48

Soft Tissue Repair and Regeneration (excluding nerve)

Project 4.4.6, WFPC: Oxygen-Generating Biomaterials for Large Tissue Salvage 54
Project 4.5.8, WFPC: Isolation and Expansion of Native Vascular Networks for Organ-Level Tissue Engineering 60
Project 4.3.2, RCCC: Development of Tissue (Peritoneum) Lined Bioabsorbable and Fracture-Resistant Stent Graft for Vessel Trauma 64
Project 4.4.3a, RCCC: Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents 68
Project 4.4.3b, RCCC: Functional Scaffolds for Soft Tissue Repair and Joint Preservation 72

Nerve Repair and Regeneration

Project 4.4.4, WFPC: Peripheral Nerve Repair for Limb and Digit Salvage 78
Project 4.4.5, WFPC: Modular, Switchable, Synthetic Extracellular Matrices for Regenerative Medicine 88
Project 4.4.1/4.4.2, RCCC: Repair Segmental Nerve Defects 96
Project 4.4.2a, RCCC: Cells and Bioactive Molecules Delivery to Enhance the Repair of Segmental Nerve Defects 100

Composite Tissue Injury Repair
Project 4.4.3, WFPC: Spatial and Temporal Control of Vascularization and Innervation of Composite Tissue Grafts 106
Epimorphic Regeneration (and associated methods)
Project 4.4.1, WFPC: Epimorphic Regeneration Approach to Limb and Digit Reconstruction 112
Project 4.4.7, WFPC: High-Throughput Approaches Applied to Tissue Regeneration 120
Project 4.4.8, WFPC: High-Purity Magnetophoretic Sorting for Transplant Therapies 126
Clinical Trials
Project 4.4.2, WFPC: Hand Transplantation for Reconstruction of Disabling Upper Limb Battlefield Trauma – Translational and Clinical Trials 132
Project 4.4.1a, RCCC: Clinical Trial – Safety Assessment of a Novel Scaffold Biomaterial 138

III: Craniofacial Reconstruction

Background 143
Unmet Needs 144
Areas of Emphasis 145
Bone Regeneration 146
Soft Tissue Regeneration 150
Cartilage Regeneration (Focus: Ear) 152
Clinical Trials 154
Progress Reports
Bone Regeneration
Project 4.1.2, WFPC: Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects 156
Project 4.1.3, WFPC: Novel Synthetic Bone for Craniofacial Application 164
Project 4.5.1a/4.5.7, RCCC: Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Allograft Bone/Polymer Composites and Expedited Commercialization of an Injectable Bone/Allograft Composite for Open Fractures 172
Project 4.5.1b, RCCC: Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex 176
Project 4.5.6, RCCC: Vascular Tissue Engineering 182
Project 4.5.8, RCCC: Accelerating the Development of Bone Regeneration Scaffolds by 510(k) Application to FDA for Tyrosine-Derived Polycarbonate Fracture Fixation Device 186
Soft Tissue Regeneration
Project 4.1.4/4.1.5, WFPC: Soft Tissue Reconstruction/Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction 190
Project 4.1.6, WFPC: Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle 200
Project 4.1.2, RCCC: Develop Innervated, Vascularized Skeletal Muscle 210
Project 4.3.1, RCCC: Composite Tissue Allograft Transplantation Without Lifelong Immunosuppression 216
Cartilage Regeneration (Focus: Ear)
Project 4.1.1, WFPC: Engineered Cartilage-Covered Ear Implants for Auricular Reconstruction 220
Project 4.5.4a, RCCC: Engineering a Replacement Autologous Outer Ear Using a Collagen/Titanium Platform 226
Project 4.5.4b, RCCC: Regeneration of Ear Using Bioresorbable Polymers and Stem Cells 232

Table of Contents

Clinical Trials
Project 4.3.1a, RCCC: Clinical Trial – Composite Tissue Allograft Transplantation (Face) 238
Project 4.3.1b, RCCC: Clinical Trial – Anti-TCR Monoclonal Antibody (TOL101) for Prophylaxis of Acute Organ Rejection in Patients Receiving Renal Transplantation 240

IV: Scarless Wound Healing

Background 243
Unmet Needs 244
Areas of Emphasis 245
Control of Wound Environment and Mechanics 246
Therapeutic Delivery to Wounds 246
Attenuation of Wound Inflammatory Response 248
Scar Mitigation 249
Clinical Trials 250

Progress Reports
Control of Wound Environment and Mechanics
Project 4.5.1, WFPC: Mechanical Manipulation of the Wound Environment to Reduce Manifestation of Scar 252
Therapeutic Delivery to Wounds
Project 4.6.3, RCCC: Therapy to Limit Injury (TLI) and Promote Non-Scar Healing After Burns and Severe Battle Trauma 260
Project 4.7.1, RCCC: Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management 268
Project 4.5.2, WFPC: Regenerative Bandage for Battlefield Wounds 272
Project 4.5.5, WFPC: Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies 278
Attenuation of Wound Inflammatory Response
Project 4.5.3, WFPC: Multi-Functional Bioscaffolds for Promoting Scarless Wound Healing 286
Project 4.5.4, WFPC: Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration 292
Scar Mitigation
Project 4.5.6, WFPC: Peptide-Mediated Delivery of Therapeutic Compounds into Injured Tissues During Secondary Intervention 300
Project 4.5.7, WFPC: Scar Mitigation via Matrix Metalloproteinase-1 Tertiary Therapy 308
Clinical Trials
Project 4.5.9, WFPC: Neodyne's Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation – Clinical Trial 314
Project 4.7.3, RCCC: Autologous Fat Transfer for Scar Prevention and Remodeling (AFT-SPAR) Clinical Trial 322

V: Burn Repair

Background 327
Unmet Needs 328
Areas of Emphasis 330
Intravenous Treatment of Burn Injury 331
Topical Treatment of Burn Injury 331
Wound Healing and Scar Prevention 333

Skin Products/Substitutes 334
Clinical Trials 337

Progress Reports

Intravenous Treatment of Burn Injury

Project 4.6.1, RCCC: Therapy to Limit Injury Progression, Attenuate Inflammation, Prevent Infection, and Promote Non-Scar Healing After Burns and Battle Trauma 340

Topical Treatment of Burn Injury

Project 4.2.3, WFPC: Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues 346
Project 4.6.2, RCCC: Mesenchymal Stem Cells for Burn and Wound Healing 354
Project 4.6.4, RCCC: Polymeric, Antimicrobial Wound Dressing Providing Sustained Release of Iodine 358
Project 4.6.5, RCCC: Topical P12 Treatment of Burns Using Fiber Mats 362

Wound Healing and Scar Prevention

Project 4.2.2, WFPC: Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device 368
Project 4.2.4, WFPC: Artificial Extracellular Matrix Proteins for Regenerative Medicine 376
Project 4.2.5, WFPC: In Situ Bioprinting of Skin for Battlefield Burn Injuries 382

Skin Products/Substitutes

Project 4.2.1, WFPC: Tissue Engineered Skin Products – ICX-SKN 390
Project 4.2.1a, WFPC: Tissue-Engineered Skin Substitute for Burns at Organogenesis 396
Project 4.2.6, WFPC: Amniotic Fluid-Derived and Placenta-Derived Stem Cells for Burn 400
Project 4.2.8, WFPC: In Vitro Expanded Living Skin for Reparative Procedures 408
Project 4.7.2, RCCC: Engineered Skin Substitutes 416
Project 4.6.8, USAISR: Autologous Human Debrided Adipose-Derived Stem Cells for Wound Repair in Traumatic Burn Injuries 420

Clinical Trial

Project 4.2.7, WFPC: A Multicenter Comparative Study of the ReCell® Device and Autologous Split-Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries 426

VI: Compartment Syndrome

Background 431
Unmet Needs 432
Areas of Emphasis 433
Cellular Therapy of CS 434
Biological Scaffold-Based Treatment of CS 435

Progress Reports

Cellular Therapy of CS

Project 4.3.1, WFPC: Cellular Therapy for the Treatment and Consequences of Compartment Syndrome 438
Project 4.3.2, WFPC: Use of Bone Marrow-Derived Cells for Compartment Syndrome 452
Project 4.3.6, USAISR: Improving Cell-Based Approaches for Extremity Trauma 462

Biological Scaffold-Based Treatment of CS

Project 4.3.3, WFPC: Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy 466

Table of Contents

Project 4.3.4, WFPC: Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome 476
Project 4.3.5, WFPC: Material-Induced Host Cell Recruitment for Muscle Regeneration 484

VII: AFIRM Statistics

Introduction 493
Personnel 494
Honors and Achievements 496
Publications and Presentations 497
Inventions, Patent Applications, and Patents 498
Developmental Accomplishments and Milestones 499

Appendices

Appendix A: Honors and Awards to AFIRM Faculty A-1
Appendix B: Publications and Presentations B-1
Appendix C: Patent Applications and Invention Disclosures C-1
Appendix D: Acronym List D-1
Appendix E: Index of Team Leaders and Project Team Members E-1

Executive Summary

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The use of improvised explosive devices in Iraq and Afghanistan has caused a marked increase in severe blast trauma. Due to advances in body armor, quicker evacuation from the battlefield, and advanced medical care, many of the injured survive to face the challenge of overcoming severe limb, head, face, and burn injuries that can take years to treat and often result in significant lifelong impairment.

The burgeoning field of regenerative medicine provides hope for restoring the structure and function of damaged tissues and organs and for curing previously untreatable injuries and diseases. The concept of regenerative medicine—in its simplest form—is to replace or regenerate human cells, tissues, or organs to restore or establish normal function. Advanced technologies, such as tissue regeneration, novel biomaterials scaffolding, and stem cell-enabled treatments, are needed to revolutionize the clinical rehabilitation of severely injured service members.

The Department of Defense established the Armed Forces Institute of Regenerative Medicine (AFIRM) in 2008, a multi-institutional, interdisciplinary network of scientists with the mission of accelerating the development of new products and therapies to treat severe injuries suffered by U.S. service members. Centered around well-established, proven research investigators, the AFIRM has expanded the rehabilitative medicine knowledge base, developed models of injury, and tested advanced technology products.

Creating Partnerships and Collaborations

The AFIRM's success to date can be ascribed, at least in part, to the program's emphasis on establishing partnerships and collaborations. Funding for the AFIRM is a six-way partnership among the U.S. Army, Navy, and Air Force; the Veterans Health Administration; the Defense Health Program; and the National Institutes of Health. Research under the AFIRM is conducted through two independent research consortia working with the U.S. Army Institute of Surgical Research (USAISR). One research consortium is led by Rutgers, the State University of New Jersey, and the Cleveland Clinic (RCCC [Rutgers-Cleveland Clinic Consortium]) while the other is led by Wake Forest University Baptist Medical Center and the McGowan Institute for Regenerative Medicine in Pittsburgh (WFPC [Wake Forest-Pittsburgh Consortium]). Each research consortium contains approximately 15 member organizations, which are mostly academic institutions. Notably, AFIRM member organizations have established collaborations/partnerships with more than 50 academic or industrial institutions located both within and outside of the United States (including Australia, China, Finland, Germany, Italy, and the Netherlands).

Research activities are organized into five program areas: Limb and Digit Salvage, Craniofacial Reconstruction, Scarless Wound Healing, Burn Repair, and Compartment Syndrome. More than 80 projects have been funded by the AFIRM to date. A Program Synergy Group has been established to

identify collaborative opportunities and build bridges between the programs and projects.

One example of a successful collaborative effort between members of the two consortia is found in the Craniofacial Reconstruction Program. In Project 4.5.6, the RCCC team of Drs. Daniel G. Anderson, Robert S. Langer, and Nathaniel Hwang at the Massachusetts Institute of Technology (MIT) is collaborating with Dr. Michael T. Longaker's WFPC team at Stanford University to develop vascularized bone tissues. During the past year, the researchers identified an optimal biomaterial for effective bone tissue engineering, which is bioabsorbable and bioactive. They also optimized human vascular endothelial growth factor (hVEGF)-release microparticles for effective use in forming blood vessel networks in the grafts. During the next few years, the researchers will perform extended small animal studies. They will monitor the biological activity of scaffolds that have incorporated either hVEGF or hydroxyapatite. The researchers plan to perform appropriate large animal studies to confirm the biocompatibility and safety of the scaffolds with the ultimate goal of using these scaffolds in the clinical setting.

Another example of a successful collaborative effort between the consortia and military clinicians is evident in the work of WFPC researcher Dr. Peter Rubin and colleagues at the University of Pittsburgh. The Rubin team is investigating the use of autologous fat grafting, enhanced with adipose-derived stem cells (ASCs), for the treatment of soft tissue defects in the head and face (Craniofacial Reconstruction Program, Project 4.1.4/4.1.5), and for addressing soft tissue deficits in amputation stumps (Limb and Digit Salvage Program, Project 4.4.1). Both of these projects involve collaborations with clinicians at the Walter Reed National Military Medical Center (WRNMMC) to identify military patients who would benefit from the grafting procedure. Several military clinicians also are working with the Rubin team to learn the techniques of the procedure to translate the technique, albeit without cell enhancement, into broader use for wounded warriors at WRNMMC.

A noteworthy collaborative effort among scientists at four institutions is found in the Limb and Digit Salvage Program. Dr. George Muschler at the Cleveland Clinic, Dr. Michael Yaszemski at the Mayo Clinic, Dr. Aniq Darr at Rutgers University, and Dr. Linda Griffith at MIT are working together in Project 4.2.1 (RCCC) to assess and compare several new and highly promising degradable scaffolds that can facilitate the generation of bone tissue. Notably, the researchers' tyrosine-derived polycarbonate and poly(propylene fumarate) scaffolds, which were created during the first 2 years of the project, are being licensed by industry. The collaborative effort has also led to the generation of new calcium phosphate-based scaffold compositions for evaluation and advancement during the upcoming year.

Limb and Digit Salvage

The **Limb and Digit Salvage Program** ultimately seeks to develop novel solutions using regenerative medicine that will allow victims of severe extremity trauma to recover rapidly, reliably, and completely so they can return to productive lives. A total of 20 projects received funding in year 3. Projects span the following clinical challenge areas: Bone, Soft Tissue, and Nerve Repair/Regeneration; Composite Tissue Injury Repair; Transplantation; and Epimorphic Regeneration.

PhD student Chris Medberry at work in Dr. Stephen F. Badylak's laboratory (WFPC).

Year 3 Research Highlights

Research efforts over the past 3 years have led to dozens of noteworthy accomplishments; some highlights are presented as follows.

Drs. Charles J. Gatt, Jr. and Michael G. Dunn at the University of Medicine and Dentistry of New Jersey (Limb and Digit Salvage Program, Project 4.4.3b, RCCC) are developing an "off-the-shelf" tissue-engineered meniscus scaffold that can be used to replace a severely damaged knee meniscus in an injured service member. The scaffold is made of an anatomically designed, fiber-reinforced scaffold and a collagen-based extracellular matrix similar to the native meniscus. The researchers completed a rabbit study, and preliminary analyses indicated that the scaffold is biocompatible. Studies in sheep are under way. After the preclinical large animal efficacy studies are complete, the researchers will find an industrial partner to assist in the design and funding of clinical trials.

Drs. Daniel G. Anderson and Robert S. Langer at MIT (Craniofacial Reconstruction Program, Project 4.5.4b, RCCC) are learning how to regenerate the human ear using resorbable polymers and stem cells. They implanted scaffolds in mice that were co-seeded with ear cartilage cells and fat-derived mesenchymal stem cells. After 6 weeks, the co-seeded constructs contained large clusters of cartilaginous tissue with properties similar to native tissue. The researchers plan to perform large animal studies in sheep, collaborating with Drs. Cathryn Sundback and Joseph Vacanti at Massachusetts General Hospital (Craniofacial Reconstruction Program, Project 4.5.4a). They also plan to work with Concordia Biomedical to fabricate scaffolds for clinical trials.

Dr. Sandeep Kathju and colleagues at the Allegheny-Singer Research Institute (Scarless Wound Healing Program, Project 4.5.5, WFPC) are using tiny nanoparticles to deliver molecules into wounds. The researchers identified a gene (CCT-eta) that is normally decreased in healing fetal wounds but is elevated in adult wounds. They developed a nonviral, nanoparticle-mediated delivery system that selectively decreases the expression of CCT-eta in complex adult wounds.

They found that this therapy effectively inhibits scar formation without any deleterious effects on wound healing. The researchers are currently investigating whether ultrasound-mediated gene transfer can be used in conjunction with their technology to enhance their results.

Dr. James Yoo and colleagues at Wake Forest University (Burn Repair Program, Project 4.2.5, WFPC) are using inkjet technology to achieve the "printing" of skin onto an excised burn wound. They developed a portable skin printing device and achieved delivery of skin cells directly onto skin defects in a mouse model using the device. They isolated and expanded two types of skin cells from porcine skin and found that the cells remained viable when delivered through their device's printer nozzles. The printed cells participated in skin tissue formation and wound repair in the porcine excisional wound model. The researchers plan to design a bioprinter suitable for clinical application.

Dr. Kenton Gregory and colleagues at the Oregon Medical Laser Center (Compartment Syndrome Program, Project 4.3.2, WFPC) are developing a bone marrow-derived stem cell treatment regimen for compartment syndrome injuries that is aimed at shifting the balance from cellular degeneration and scar tissue formation to the generation of physiologically active cells, resulting in improved muscle and nerve function. The treatment uses autologous (one's own) bone marrow-derived stem cells. The researchers also developed a method of tracking the cells after they are implanted in an animal and demonstrated robust cell engraftment up to 3 months post-treatment. They completed a pilot study in Sinclair mini-swine and have treated 29/30 animals in a pivotal dose study. Their results to date suggest that adult bone marrow is a potential multipotent cell source for injured tissue repair.

Dr. Robert J. Christy and colleagues at USAISR (Burn Repair Program, Project 4.6.8) are using autologous ASCs and a biomaterial that mimics natural extracellular matrix as a strategy to regrow blood vessels into traumatized tissue. They found ASCs inside the matrices were viable, proliferated, and

Craniofacial Reconstruction

The **Craniofacial Reconstruction Program** aims to generate both soft and hard tissues through novel regenerative medicine approaches to reduce the impact of devastating, disfiguring facial injuries on wounded warriors. A total of 15 projects received funding in year 3. Projects span the following clinical challenge areas: Bone Regeneration, Soft Tissue Regeneration, and Cartilage Regeneration (with a focus on the ear).

Scarless Wound Healing

The **Scarless Wound Healing Program** encompasses a continuum of technologies aimed at the various stages of wound healing to find new treatment options to prevent and manage scars. A total of 11 projects received funding in year 3. Projects span the following clinical challenge areas: Control of Wound Environment and Mechanics, Therapeutic Delivery to Wounds, Attenuation of Wound Inflammatory Response, and Scar Mitigation.

RCCC researchers Drs. Stefan Salomon and Jenny Raynor process samples in the centrifuge to create therapies for burn repair.

formed microvessels. They delivered ASCs in a gel to a full-thickness excision wound in the rat and found enhanced growth of blood vessels compared to control gels lacking ASCs. The researchers plan to initiate studies in a porcine model and will use this model to develop a large total body surface area burn, which they predict will provide a stringent test for their product and for other AFIRM-related skin equivalent products.

Year 3 Program Highlights

The AFIRM program involves the efforts of nearly 450 individuals, including faculty members, postdoctoral fellows, graduate students, scientific and technical staff, and undergraduates. AFIRM faculty members are highly accomplished scientists—during the third year of the program, 52 awards/honors were conferred upon AFIRM faculty, including selection to membership or leadership positions in professional societies, honorary degrees from research/academic institutions, awards from private foundations, and recognition of exemplary AFIRM meeting presentations.

AFIRM-sponsored researchers have made substantial contributions to the scientific literature—during the third year of the program, they published 152 articles in peer-reviewed journals and produced 250 presentations and non-peer-reviewed publications. AFIRM scientists have also been making novel patentable discoveries in the field of regenerative medicine—during the third year of the program, they filed 20 invention disclosures and 14 government patent applications.

The Technology Readiness Levels (TRLs) of products generated by AFIRM-funded researchers have been steadily rising. At the start of the program, 61 products (93%) were nearly evenly distributed across TRLs 1, 2, and 3; the other 5 products were at TRL 4 or 5. By the end of the third year of the program, no products remained at TRL 1, and only 8

products were at TRL 2. The vast majority of products (47) were at TRLs 3 and 4, and the remaining 16 products were between TRL 5 and TRL 9. Notably, 27 projects increased by two or more TRLs since the first year of the program.

The AFIRM has recruited a substantial amount of young talent into the field of regenerative medicine since its inception. During the third year of the program, more than 110 students (undergraduate and graduate) received practical scientific training through AFIRM-sponsored research projects. The number of advanced degrees (masters or doctoral) awarded to students who received training through AFIRM-sponsored projects has risen steadily from 3 in program year 1 to 17 in program year 3.

From the Laboratory to the Battlefield

AFIRM-funded researchers share a strong commitment to developing commercial products that will bring therapies to wounded warriors and the civilian sector as quickly as possible. The ultimate goal of many AFIRM-sponsored projects is the conduct of clinical trials; one AFIRM-funded research team has already completed two clinical trials, seven AFIRM teams have initiated clinical trials, five AFIRM teams are set to begin clinical trials in the upcoming year, and numerous others in the program anticipate the commencement of clinical trials within the next few years. Nontechnical summaries of these clinical trials can be found at the end of the introductory section of each program-related chapter, and full progress reports of the clinical trials (when available) follow the research project progress reports in each chapter in the full-length version of this annual report. A few examples of promising translational research are highlighted as follows.

Burn Repair

The **Burn Repair Program** seeks to leverage regenerative medicine technologies to reduce morbidity and mortality in victims of severe military or civilian trauma. A total of 16 projects received funding in year 3. Projects span the following clinical challenge areas: Intravenous Treatment of Burn Injury, Topical Treatment of Burn Injury, Wound Healing and Scar Prevention, and Skin Products/Substitutes.

Compartment Syndrome

The **Compartment Syndrome Program** seeks to prevent or reverse secondary damages resulting from trauma so that repair and regeneration of wounded tissue are enhanced while healing and return of function are improved. A total of 6 projects received funding in year 3. Projects span the following clinical challenge areas: Cellular Therapy of Compartment Syndrome and Biological Scaffold-Based Treatment of Compartment Syndrome.

Dr. W.P. Andrew Lee and colleagues at the Johns Hopkins University School of Medicine and the University of Pittsburgh (Limb and Digit Salvage Program, Project 4.4.2, WFPC) have developed a protocol for hand transplantation using a patient's own bone marrow with the addition of the immunosuppressive CTLA4Ig fusion protein. This protocol has been designed to minimize the amount of maintenance immunosuppressive therapy that is needed following a transplant. The research team has performed hand transplants on five patients to date. All patients have been maintained on a single immunosuppressive drug at low levels, and they continue to have increased motor and sensory function of their transplanted hands, which correlates with their level of amputation, time after transplant, and participation in hand therapy.

Autologous fat transfer is a procedure that involves removing adipose tissue from one area of a patient's body and immediately transplanting it to a different site in the same person. Dr. Adam J. Katz and colleagues at the University of Virginia (Scarless Wound Healing Program, Project 4.7.3, RCCC) are conducting a Phase 1/2 clinical trial designed to test the safety and efficacy of using this procedure for scar prevention and remodeling. The researchers have treated eight patients so far. Total accrual planned for the trial is 52 subjects. There have been no adverse events, and all patient follow-ups and analyses have proceeded smoothly although it is still too early to draw any conclusions from the data obtained to date.

Whole biopsy of the sural nerve in the leg results in a predictable loss of function in a specific area of the foot. Reconstruction of the sural nerve in these patients can potentially restore sensation and prevent complications associated with the biopsy. Drs. Anthony Windebank and Michael Yaszemski and colleagues at the Mayo Clinic (Limb and Digit Salvage Program, Project 4.4.1a, RCCC) are seeking to repair post-biopsy sural nerve defects with a synthetic nerve conduit constructed from polycaprolactone fumarate. Their clinical trial protocol is currently under review at the Mayo Clinic's Institutional Review Board (IRB) and Human Research Protection Office. Patient recruitment is expected to begin as soon as IRB and U.S. Food and Drug Administration approval is obtained for the researchers' synthetic nerve conduits.

I: Introduction

Background

The wars in Iraq and Afghanistan have resulted in nearly 6,300 U.S. military fatalities and more than 46,800 injuries.[1] The use of improvised explosive devices in Operation Iraqi Freedom/Operation Enduring Freedom/Operation New Dawn has led to a substantial increase in severe blast trauma, which is now responsible for approximately 75% of all combat-related injuries.[2] Advances in body armor to protect the torso and vital organs, faster evacuation from the battlefield after injury, and major advances in trauma resuscitation save wounded warriors who would have died of their injuries in previous conflicts. However, those who survive often have seriously debilitating injuries. Conventional weapons and the destructive force of improvised explosive devices can ravage unprotected structures of the face, neck, head, and limbs, causing massive trauma and tissue loss.

The emerging field of regenerative medicine focuses on (1) restoring the structure and function of tissues and organs that have been damaged and (2) finding methods of curing previously untreatable injuries and diseases. Regenerative medicine holds great potential for healing military personnel with debilitating, disfiguring, and disabling injuries. Scientists working in the area of regenerative medicine use a number of different approaches to prompt the body to regenerate

[1] *October 31, 2011 http://www.defense.gov/news/casualty.pdf.*
[2] *Owens, et al. J Orthop Trauma. 2007 21(4):254-7.*

cells and tissues, often using the patient's own cells combined with degradable biomaterials. Use of a patient's own cells eliminates the possibility of tissue rejection. Technologies for engineering tissues are developing rapidly. The ultimate goal is to deliver advanced therapies, such as whole organs and engineered fingers and limbs, to injured members of the military as well as civilians.

Research Goals

The AFIRM is a multi-institutional, interdisciplinary network focused on developing advanced treatment options for severely wounded warfighters. The mission of the AFIRM is to accelerate the delivery of regenerative medicine therapies to treat the most severely injured U.S. service members. Clinical trials of several AFIRM products are under way. Inclusion of military patients in these trials is the first step in delivering advanced technologies to wounded warriors.

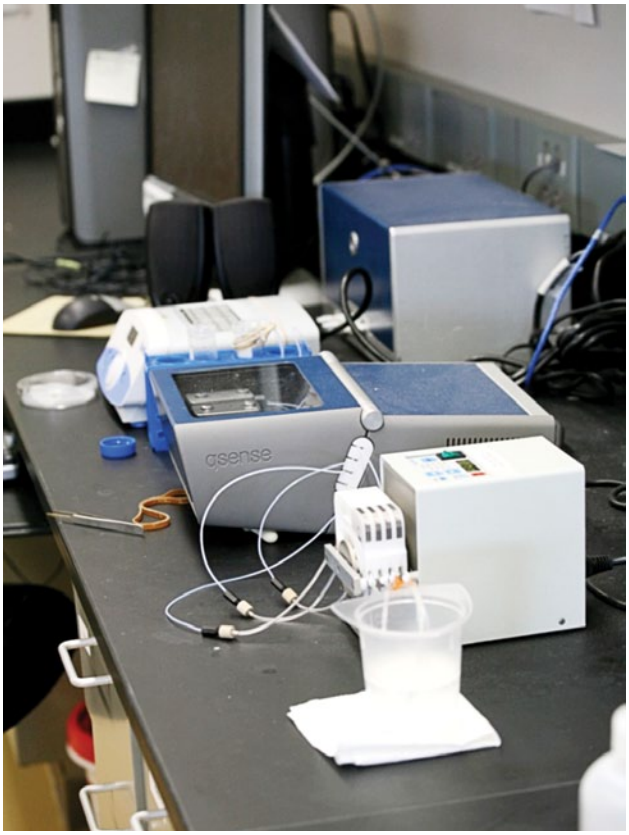
The AFIRM Has Five Major Research Programs:

Limb and Digit Salvage

Saving the limb, also referred to as "limb salvage," at a minimum requires (1) bridging large bony defects to restore skeletal integrity; (2) bridging soft tissues, such as muscle, nerves, tendons, and ligaments, to lend stability and enable movement; and (3) covering the injured area with healthy skin. The AFIRM Limb and Digit Salvage Program is dedicated to developing regenerative medicine therapies to help health care providers save and rebuild injured limbs. Enabling victims of severe extremity trauma to recover rapidly, reliably, and completely so they can return to productive lives is the ultimate goal of the program.

Craniofacial Reconstruction

Massive bone and soft tissue loss to the face and head due to blast forces is a devastating injury. The AFIRM Craniofacial Reconstruction Program is designing and developing therapies that health care providers can use to heal the wounded warfighter. These therapies will (1) regenerate functional bone and cartilage to levels of the face; (2) restore motor and sensate competencies through muscle, vascular, and nerve regeneration; (3) mitigate scar formation; (4) prevent infection; and (5) eliminate skin coverage deficits through tissue engineering. The creation and delivery of new polymers and tissues will preserve and regenerate bone and soft tissue capable of administering stem cells, growth factors, bone derivatives, and therapeutic drugs.



A QCM-D (quartz crystal microbalance with dissipation) is used by Rutgers-Cleveland Clinic Consortium researchers to measure and quantify tethered bone morphogenetic protein 2 to polymer surfaces.

Using a fluorescent microscope, Nicole Wrice of the U.S. Army Institute of Surgical Research analyzes adult stem cells used for tissue regeneration.

Scarless Wound Healing
Military trauma burns often heal with large scars that may impair normal function and cause significant disfigurement. Scars are the result of the body's complex series of wound-healing processes that begin at the onset of injury and can continue for months. This AFIRM program is investigating all phases of wound healing and scar formation to find new treatment options to prevent and mitigate scars.

Burn Repair
Severe burns are associated with substantial morbidity and mortality in spite of many advances in medical care. The AFIRM Burn Repair Program is leveraging regenerative medicine technology to (1) prevent wound infection, (2) prevent burn inflammation and injury progression, (3) speed generation of a viable wound bed and reduce reharvest time of autograft donor sites, (4) improve skin substitutes for burn wound grafting when autografts are not immediately available, and (5) prevent and manage scars.

Compartment Syndrome
Compartment syndrome is often a secondary sequela resultant from blast injuries, severe blunt or penetrating trauma, fractures, and vascular injuries. Muscles are encased in compartments of non-yielding tissue called fascia. Bleeding or tissue swelling within a muscle compartment raises the pressure in the compartment that, if unchecked, can become high enough that blood flow into the compartment is reduced or completely stopped. Prolonged interruption of blood flow can destroy the nerves and muscles within the compartment. Restoration of those damaged or destroyed tissues has no satisfactory solution with current surgical options. This AFIRM program focuses on attacking the problem of compartment syndrome through regenerative medicine therapies to stabilize tissue and reduce the onset of late effects of nerve and damage.

History

In 2005, Dr. Anthony Atala presented some of the latest advances in the field of regenerative medicine at the Advanced Technology Applications in Combat Casualty Care Conference. This talk alerted the combat casualty care research community to the near-term potential for regenerative medicine products that could make a substantial difference in the care of our wounded warriors. The following year, the Army's Director of the Combat Casualty Care Research Program, COL Bob Vandre, developed the idea of a regenerative medicine institute similar to the Department of Defense's (DoD's) Multidisciplinary University Research Initiatives but aimed at near-term, translational research. COL Vandre received U.S. Army Medical Research and Materiel Command (USAMRMC) approval in 2006 to pursue funding for the project. He subsequently briefed the DoD Technology Area Review and Analysis panel, which reviews medical research and development for the DoD. The concept received high approval from the panel.

In 2007, USAMRMC, the Office of Naval Research, the U.S. Air Force Office of the Surgeon General, the National Institutes of Health (NIH), and the Veterans Health Administration of the Department of Veterans Affairs (VA) agreed to co-fund the new institute. Taking their funds and adding in $10 million (M) from the 2007 War Supplemental bill provided $8.5M per year in funding for the AFIRM, which was deemed sufficient to proceed.

A Program Announcement was released in August 2007, and seven proposals were received in October 2007. In December 2007, two finalists were selected for oral presentations. Both received scores of "excellent" and one was selected for funding. White House staffers heard about the AFIRM and invited representatives from USAMRMC to come and discuss the new institute. After two meetings and upon hearing that there was funding for only one AFIRM finalist, the DoD was tasked to provide funding for the second AFIRM finalist. Within 1 week, an additional $8.5M per year was transferred to USAMRMC's budget lines. Both AFIRM finalists signed USAMRMC cooperative agreements in March 2008.

Funding – A Six-Way Partnership

The AFIRM is financed with basic research through exploratory development funds and is expected to make major advances in the ability to understand and control cellular responses in wound repair and organ/tissue regeneration. The program is managed and funded through USAMRMC with funding from the following organizations:

- U.S. Army
- U.S. Navy, Office of Naval Research
- U.S. Air Force, Office of the Surgeon General
- Veterans Health Administration
- Defense Health Program
- NIH

Total funding for the first 5 years of the AFIRM amounts to more than $300M:

- $100M from U.S. government funding (Army, Navy, Air Force, VA, and NIH).
- $80M from matching funds received from state governments and participating universities.
- $109M from pre-existing research projects directly related to deliverables of the AFIRM from the NIH, Defense Advanced Research Projects Agency, congressional special programs, the National Science Foundation, and philanthropy.
- $25M in additional funds provided by the Defense Health Program.

Structure

The AFIRM is composed of two independent civilian research consortia working with the U.S. Army Institute of Surgical Research (USAISR) at Fort Sam Houston, Texas. USAISR, which includes the San Antonio Military Medical Center – North (formerly Brooke Army Medical Center), serves as the AFIRM's primary government component and is home to the DoD's only burn unit. The two AFIRM research consortia are responsible for executing the management of overall therapeutic programs and individual projects within their consortia. One consortium is led by Rutgers, the State University of New Jersey, and the Cleveland Clinic, and the other is led by the Wake Forest Institute for Regenerative Medicine and the McGowan Institute for Regenerative Medicine in Pittsburgh. Each of these civilian consortia is itself a multi-institutional network, as shown in the following paragraphs.

Rutgers-Cleveland Clinic Consortium

The Rutgers-Cleveland Clinic Consortium (RCCC) is directed by Joachim Kohn, PhD, Director of the New Jersey Center for Biomaterials and Board of Governors Professor of Chemistry at Rutgers University, and co-directed by Linda Graham, MD, Staff Vascular Surgeon at the Cleveland Clinic and Professor in the Department of Biomedical Engineering at the Lerner Research Institute at Case Western Reserve University.

The RCCC consists of the following member institutions:

- Rutgers/New Jersey Center for Biomaterials
- Cleveland Clinic Foundation
- Brigham and Women's Hospital
- Carnegie Mellon University
- Case Western Reserve University
- Cooper Medical School of Rowan University
- Dartmouth Hitchcock Medical Center/ Thayer School of Engineering
- Massachusetts General Hospital/Harvard Medical School
- Massachusetts Institute of Technology
- Mayo Clinic College of Medicine

Construction continues on the new wing of the San Antonio Military Medical Center – North (formerly known as Brooke Army Medical Center), Fort Sam Houston, Texas.

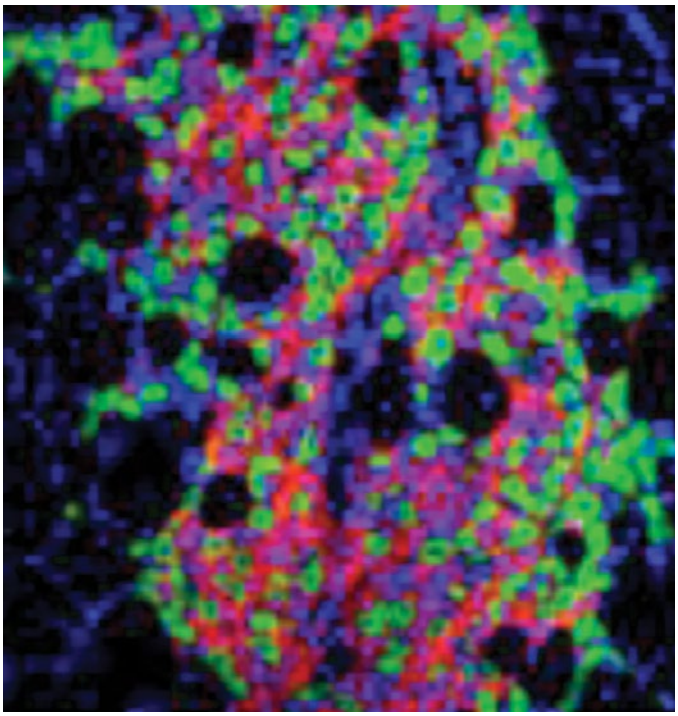
- Northwestern University
- Stony Brook University
- University of Cincinnati
- University of Medicine and Dentistry of New Jersey
- University of Michigan
- University of Pennsylvania
- University of Virginia
- Vanderbilt University

Wake Forest-Pittsburgh Consortium

The Wake Forest-Pittsburgh Consortium (WFPC) is directed by Anthony Atala, MD, Director of the Wake Forest Institute for Regenerative Medicine and Professor and Chair of the Department of Urology at Wake Forest University, and co-directed by Rocky Tuan, PhD, Director of the Center for Cellular and Molecular Engineering at the University of Pittsburgh.

The WFPC consists of the following member institutions:

- Wake Forest Institute for Regenerative Medicine/Wake Forest University
- McGowan Institute for Regenerative Medicine/University of Pittsburgh
- Allegheny-Singer Research Institute
- Carnegie Mellon University
- Georgia Institute of Technology
- Institute for Collaborative Biotechnologies (includes University of California, Santa Barbara; Massachusetts Institute of Technology; and California Institute of Technology)
- Johns Hopkins University School of Medicine
- Oregon Medical Laser Center
- Rice University
- Sanford-Burnham Medical Research Institute/University of California, Santa Barbara
- Stanford University
- Tufts University
- University of California, Berkeley
- University of Texas Health Science Center at Houston
- University of Wisconsin
- Vanderbilt University



Stem cell differentiation in an experiment by WFPC researcher Dr. Aiping Lu is studied by fluorescent colors. Cells carrying muscle markers appear in green while macrophages appear in red.

Additional Collaborators to the AFIRM
AFIRM researchers have established a wide variety of both national and international partnerships with academia and industry, which has contributed to the success of the program to date. These have included collaborations with:

- Allegheny-Singer Research Institute
- Armand Trousseau Hospital (France)
- Arteriocyte, Inc.
- Avita Medical, LLC
- Axonia Medical
- Baylor All Saints Medical Center
- Baylor College of Medicine
- Baylor University Medical Center
- Biologics Consulting Group
- Biosafe-America
- BioStat International, Inc.
- BonWrx, Inc.
- Buffalo General Hospital
- CVPath Institute, Inc.
- Cynvenio Biosystems
- Emory University
- Fidia Advanced Biopolymers (Italy)
- GID Group, Inc.
- Glycosan BioSystems, Inc.
- Healthpoint Biotherapeutics, Ltd./DFB Bioscience
- ImageIQ
- Integra Spine/Integra LifeSciences
- Johann Wolfgang University (Germany)
- Kensey Nash Corporation
- KeraNetics, LLC
- Lexmark, Inc.
- LifeCell Corporation
- LifeNet Health
- Lonza Walkersville
- Loyola University Medical Center
- Maricopa Integrated Health Systems
- Massachusetts Eye and Ear Infirmary
- MedDRA Assistance, Inc.
- Medical University of South Carolina
- Medtronic, Inc.
- Montefiore Medical Center
- Morgridge Institute for Research
- Neodyne Biosciences
- Nitinol Development Corporation
- NOVOTEC
- Organogenesis, Inc.
- Orlando Regional Medical Center
- Osteotech, Inc.
- Pennington Biomedical Research Center, Louisiana State University
- PeriTec Biosciences, Ltd.
- Philadelphia University
- Proxy Biomedical
- Queensland University of Technology (Australia)
- Radboud University of Nijmegen Medical Centre (The Netherlands)

- Rockefeller University
- Royal Perth Hospital (Australia)
- St. Barnabas Medical Center
- San Antonio Military Medical Center – North (formerly Brooke Army Medical Center)
- Shanghai 9th People's Hospital (China)
- SimQuest, LLC
- Special Operations Medical Command-Fort Bragg
- Stratatech Corporation
- Stryker Corporation
- Texas Tech University
- Tolera Therapeutics, Inc.
- Trident Biomedical, Inc.
- University of Alabama at Birmingham
- University of California, Davis
- University of California, Los Angeles
- University of Colorado, Denver
- University of Florida
- University of Indiana
- University of Kentucky
- University of Massachusetts, Lowell
- University of Michigan
- University of Minnesota
- University of North Carolina at Chapel Hill
- University of South Florida/Tampa General Hospital
- University of Tampere (Finland)
- University of Tennessee Health Science Center
- University of Texas at Arlington
- University of Texas at Austin
- University of Utah
- University of Washington
- University of Wisconsin-Madison
- Virginia Commonwealth University
- Washington Hospital Center (Washington, DC)

Programs and Projects

Within each consortium, research activities are organized into programs (Limb and Digit Salvage, Craniofacial Reconstruction, Scarless Wound Healing, Burn Repair, and Compartment Syndrome). Scientists or clinicians responsible for coordinating the research activities of an entire program are called Program Leaders. Each program consists of numerous projects, and the scientist or clinician responsible for a specific project is called a project leader.

Consortium members evaluate all levels of the operation annually to monitor progress and guide the consortium's activities. In 2009, the RCCC established a clinical trials core to emphasize standardized approaches to clinical trials at military sites and authored documents to facilitate the generation of a USAISR clinical trials manual. Active project management by each consortium has reshaped the programs, leading to the termination or reduced funding of some projects and the addition of projects that are more promising for accelerated development. Additionally, information for the public, including clinical trial opportunities, has been made available through web sites developed and maintained by the consortia.

Rutgers-Cleveland Clinic Consortium researcher John Kinol at the laboratory bench.

In addition to the three core groups (RCCC, WFPC, and USAISR), intramural researchers from the NIH and/or the Veterans Health Administration can participate in the AFIRM although none have chosen to do so as of yet. With the approval of a program leader, these intramural researchers can lead projects.

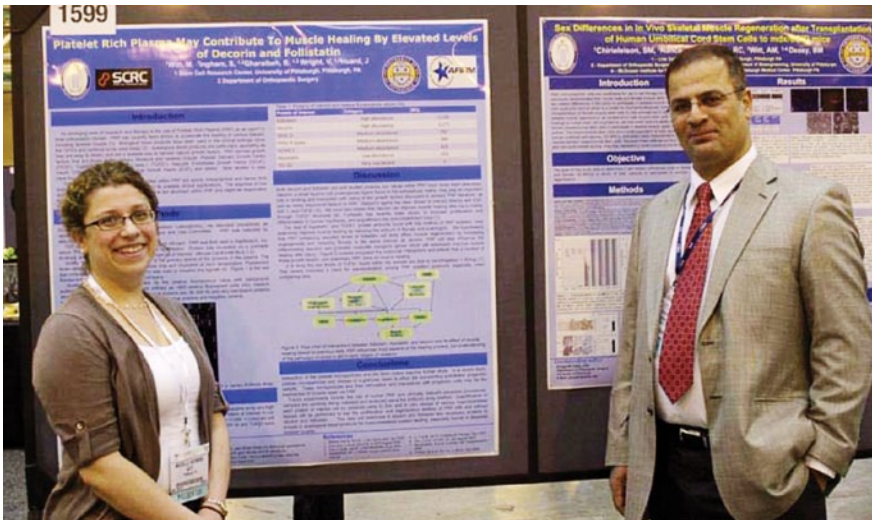
Management and Oversight

Day-to-day execution of the AFIRM's Science and Technology and Translational Science portfolio is managed by the AFIRM Project Management Office (PMO), located within the U.S. Army Medical Materiel Development Activity at Fort Detrick, Maryland. The AFIRM PMO works as part of an integrated project management team, across the AFIRM consortia, to incorporate the strategic, developmental, and tactical aspects of product management. The AFIRM PMO also functions as an accountability model to ensure execution of the AFIRM portfolio.

The AFIRM is guided by a Board of Directors (BOD) and an Integrated Project Team (IPT), which contains a Steering Group. A Program Synergy Group is responsible for research coordination and communication between the three components of the AFIRM. The roles and membership of each of these entities are described as follows.

Board of Directors

The AFIRM's BOD is chaired by the Commanding General of USAMRMC, and its members are flag-level representatives from the Army, Navy, Air Force, NIH, VA, Office of the Assistant Secretary of Defense for Health Affairs, TRICARE Management Activity, and the Uniformed Services University of the Health Sciences. The Principal Assistant for Research and Technology of USAMRMC serves as the Deputy Chair of the BOD. The main purpose of the BOD is to provide high-level guidance for the AFIRM by presiding over the IPT and the Program Synergy Group.



WFPC researchers Dr. Burhan Gharaibeh and Michelle Witt present their research findings at the Orthopaedic Research Society's annual meeting.

Integrated Project Team

The AFIRM's IPT is chaired by the Director of the USAMRMC's Clinical and Rehabilitative Medicine Research Program (CRMRP). IPT membership consists of a group of experts who represent the interests of the funding agencies, experts in military needs, external scientists knowledgeable in regenerative medicine, and specialists in contracting and product development. The overall function of the IPT is to ensure that the AFIRM meets military needs, funds superior science, and is well managed.

The specific responsibilities of the IPT are to:

- Approve the annual report and program plans that are presented to the BOD.
- Ensure that all AFIRM research projects are aligned with military requirements.
- Monitor and evaluate the activities and progress of the AFIRM programs and management and provide recommendations based on their expertise.
- Facilitate the military's evaluation and purchasing of products developed by the AFIRM.
- Assist consortia Directors and Management Teams in internal communication within the DoD and in understanding and meeting DoD regulation and reporting requirements relative to AFIRM performance.
- Facilitate the leveraging of AFIRM resources by coordinating with other funding agencies that support closely related research.

The IPT's Steering Group has day-to-day decision-making authority over the AFIRM and recommends major changes in research direction or funding to the voting members of the IPT. This group is chaired by the AFIRM Project Director and also includes the USAISR Commander, the Combat Casualty Care Senior Scientist, the Contracting Officer, and the Directors and Co-Directors of the RCCC and the WFPC. Among other activities, the Steering Group ensures that all AFIRM research projects are aligned with military

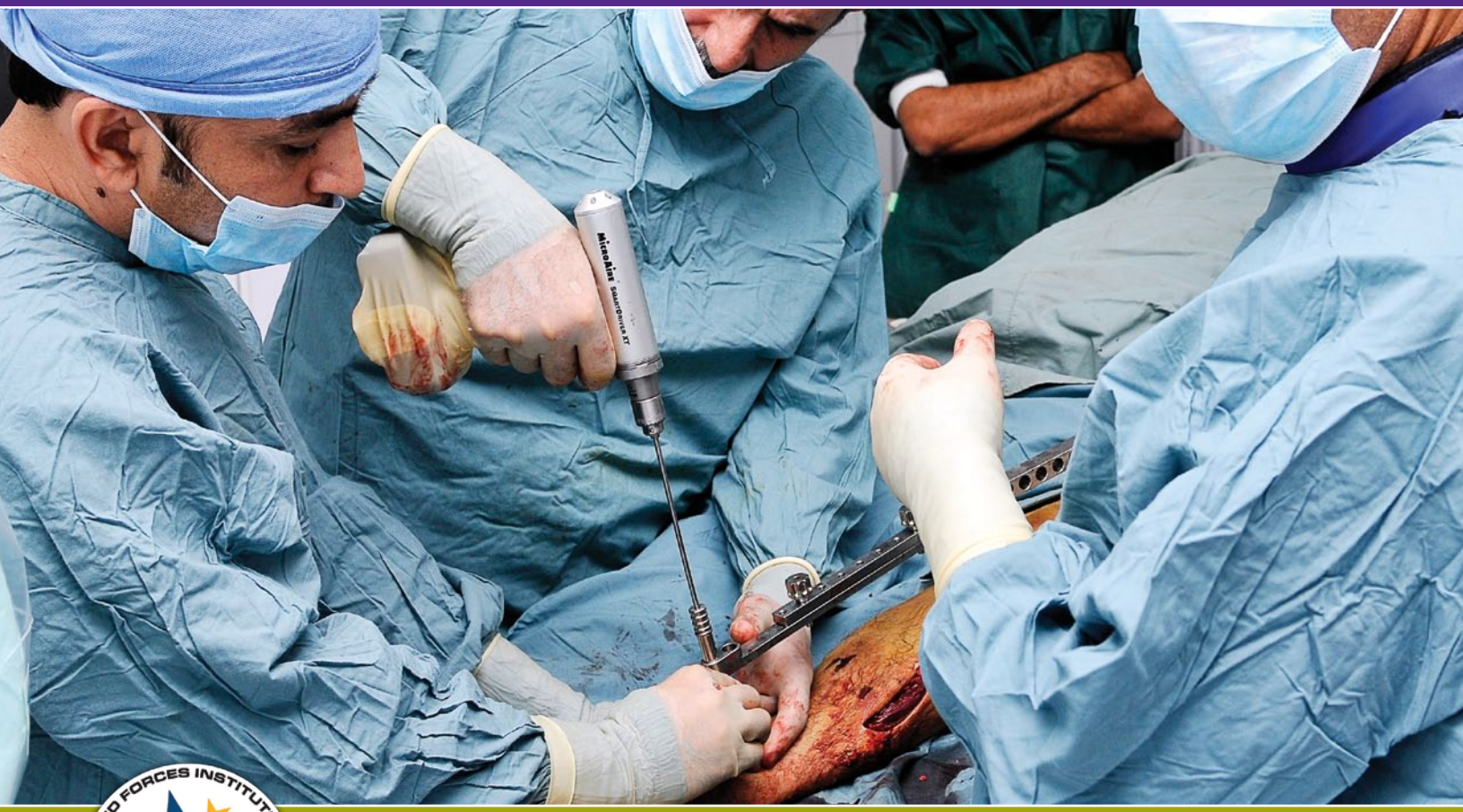
requirements, reviews AFIRM research allocation, establishes decision points and continuation criteria, assesses project and program achievements in relation to milestones and time lines, and recommends continuation or termination of programs and individual projects to the IPT.

The IPT has additional members from the Army, Navy, Air Force, and VA (one representative from each of these organizations), three representatives from the NIH (sharing one vote), and four external scientists. The IPT also has ex officio advisors from the Judge Advocate General, the DoD Human Research Protection Office, a commercialization expert, and a regulatory expert appointed by the CRMRP.

The Steering Group and the additional IPT members are voting members of the IPT. They are assisted by the ex officio members of the IPT and the Program Synergy Group to ensure that the AFIRM is progressing toward solutions for militarily relevant injuries.

Program Synergy Group

The Program Synergy Group includes representatives from each of the major programs in each of the consortia, members of the NIH or VA intramural research programs (as deemed appropriate), and USAISR. The Program Synergy Group is chaired by one of the consortia Co-Directors. It serves as a conduit for information exchange among the cores and seeks to build bridges between the programs and projects. It identifies and promotes opportunities to share or combine best practices and to accelerate existing projects or initiate new projects to bring therapies to our wounded service members. The Program Synergy Group reports its findings and recommendations twice a year to the Steering Group.



II: Limb and Digit Salvage

Background

Injuries to arms and legs following severe trauma often result in the loss of large regions of tissue in the middle portion of the limb, disrupting the healing and use of the hand or foot. Despite many advances in reconstructive surgery, current methods to reconstruct these tissues are inadequate in many settings. The AFIRM Limb and Digit Salvage Program seeks to give wounded warriors innovative solutions to the most severe, devastating limb injuries through regenerative medicine. The goals of this program are to preserve and restore damaged or missing tissue following injury, reduce the need for amputation, reduce the time and risk involved in recovery, and enable the warrior's return to an independent, fully functional life, and ideally a return to duty.

Rutgers-Cleveland Clinic Consortium (RCCC) researchers are working on a series of integrated projects focused on developing tissue-engineering solutions for bone, nerve, vessels, fascia, menisci, and skeletal muscle. They are collaborating with researchers funded by AFIRM's Craniofacial Reconstruction Program in advancing methods of immunomodulation to enable limb and face transplantation with minimal immunosuppression using composite tissue allografts when salvage is not possible. RCCC researchers are also aligned with ongoing work in AFIRM's Burn Repair Program, developing therapies for restoration of massive skin loss, including in the limb.

our science for their healing

Wake Forest-Pittsburgh Consortium (WFPC) researchers are pursuing an interdisciplinary, multipronged approach to the reconstruction/replacement of functional limb and digit tissue. Approaches they are pursuing include transplantation (composite tissue allografts), epimorphic regeneration, tissue regeneration by traditional tissue-engineering approaches, and enabling technologies. These diverse approaches represent the highly interdisciplinary background and experience of the program team leaders and the level of expertise required to address the challenging problem of limb and digit reconstruction.

Projects in the AFIRM Limb and Digit Salvage Program have established numerous industry partnerships in anticipation of commercialization, a critical step in delivering products to wounded warriors.

Unmet Needs

The unconventional weapons used in current conflicts in Iraq and Afghanistan are resulting in unconventional wounds that demand better solutions. Modern battlefield medicine is saving wounded warfighters who, in previous wars, would not have survived. These injuries often involve a massive loss of tissue, including large defects in continuity of bone (up to 20 cm), nerve, vessel, muscle, tendon, ligament, and skin. Musculoskeletal/extremity injuries are present in more than 80% of all combat injuries; fractures are present in 26%, and 82% of these fractures are open and complicated by extensive soft tissue loss.

Wounded warriors frequently sustain polytrauma (i.e., injury to several body areas and/or systems). In fact, there is a mean of 4.2 wounds reported for each wounded warrior. Wounds almost always involve injury to more than one tissue (e.g., bone, nerve, muscle, tendon, and vessel). As a result, combined approaches are envisioned. However, most often, the injury to one particular tissue becomes the limiting factor in salvage or functional restoration, most often bone and nerve, but vascular injury and muscle/tendon discontinuity are also common. Projects in the Limb and Digit Salvage Program are therefore targeted to make specific advances in these important areas of critical unmet needs: bone, nerve, artery, and soft tissue as well as composite tissue injury repair and transplantation.

Limb salvage is currently possible in a large fraction of injured warriors, but many still face amputation. Most of these are lower extremity amputations for which well-designed and well-tolerated prostheses are available. However, 20% of amputations involve the upper extremity and although prosthesis technology is advancing rapidly, fully functional, well-tolerated upper limb prostheses are still not available. The technology to salvage these limbs is progressing as evidenced by projects in this program. However, when salvage fails, the capability to provide an identically functional replacement through engineering or transplant is crucial for wounded warriors. The main obstacle to successful transplantation of composite tissue to repair segmental defects in a limb or to replace a whole limb is control of the immune response. Therefore, a critical need exists to improve immunomodulation techniques to reduce the obstacles to composite tissue transplantation.

Injuries sustained by our warfighters can be accompanied by extensive nerve loss. Autologous nerve grafts have the benefit of not being associated with immune rejection since the nerve tissue is harvested from the injured soldier's body. However, seriously injured individuals typically have very limited sources for donor material. In addition, harvesting the autografts can result in morbidity at the donor site (e.g., sensory loss,

scarring, and neuroma formation). Hence, alternatives to autografts are needed. Nerve allografts (i.e., the use of nerve tissue from an uninjured individual) represent a promising option. However, allografting requires the patient to endure approximately 18 months of immunosuppressive therapy as the nerves grow across the graft. This puts the patient at risk of developing infections or other complications. A need therefore exists to develop allografts that can be used to repair nerve defects in injured soldiers and that do not require long-term immunosuppression.

The Mayo Clinic group recently completed a systematic review of publications describing synthetic, nonbiodegradable and biodegradable conduits that have been used experimentally. Overall, the clinical performance of currently used conduits does not match that of autologous grafts. Also, commercially available nerve tubes are only being used to repair small sensory nerves in patients. The repair of motor nerves or larger nerve defects is only sporadically reported. Hence, a critical need exists for synthetic nerve scaffolds that can support regeneration across larger nerve gaps.

Areas of Emphasis

AFIRM researchers are pursuing a complementary mix of research projects focused on various aspects of limb and digit salvage. Projects can be grouped into five "clinical challenge" topic areas: Bone Repair and Regeneration, Soft Tissue Repair and Regeneration (excluding nerve), Nerve Repair and Regeneration, Composite Tissue Injury Repair, and Epimorphic Regeneration (and associated methods). Additional details on projects in each of these topic areas can be found in **Table II-1** and subsequent sections of this chapter. AFIRM researchers are also working in the area of transplantation, which has advanced to the clinical trial stage and is covered in that section.

Table II-1. Projects funded by RCCC and WFPC per clinical challenge topic area.

Clinical Challenge	Consortium/ Institution	Project No.	Project Title
Bone Repair and Regeneration	RCCC	4.2.1	Advanced 3-D Scaffolds for Large Segmental Long Bone Defects
		4.2.2	Optimizing Cell Sources for the Repair of Bone Defects
		4.2.3a	Advancing Bone Repair Using Molecular Surface Design (MSD) and/or Oxygen Delivery Systems
		4.2.3b	Advancing Bone Repair Using Molecular Surface Design (MSD): Biodegradable Scaffolds with Tethered Osteoinductive Biomolecules
Soft Tissue Repair and Regeneration (excluding nerve)	WFPC	4.4.6	Oxygen-Generating Biomaterials for Large Tissue Salvage
		4.5.8	Isolation and Expansion of Native Vascular Networks for Organ-Level Tissue Engineering
	RCCC	4.3.2	Development of Tissue (Peritoneum) Lined Bioabsorbable and Fracture-Resistant Stent Graft for Vessel Trauma
		4.4.3a	Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents
		4.4.3b	Functional Scaffolds for Soft Tissue Repair and Joint Preservation
Nerve Repair and Regeneration	WFPC	4.4.4	Peripheral Nerve Repair for Limb and Digit Salvage
		4.4.5	Modular, Switchable, Synthetic Extracellular Matrices for Regenerative Medicine
	RCCC	4.4.1/ 4.4.2	Repair Segmental Nerve Defects
		4.4.2a	Cells and Bioactive Molecules Delivery to Enhance the Repair of Segmental Nerve Defects
Composite Tissue Injury Repair	WFPC	4.4.3	Spatial and Temporal Control of Vascularization and Innervation of Composite Tissue Grafts
Epimorphic Regeneration (and associated methods)	WFPC	4.4.1	Epimorphic Regeneration Approach to Limb and Digit Reconstruction
		4.4.7	High-Throughput Approaches Applied to Tissue Regeneration
		4.4.8	High-Purity Magnetophoretic Sorting for Transplant Therapies

Bone Repair and Regeneration

Studies at RCCC

RCCC's bone program contains several integrated projects designed to address critical gaps that currently limit medical therapy options and outcomes for warriors with injured limbs whose challenges include the need to regenerate a segmental defect in an extremity. The researchers are defining optimal methods for bone regeneration, focusing on synthetic scaffold development in combination with a patient's own bone-forming stem cells to more rapidly, effectively, and reliably regenerate bone tissue in large, post-traumatic segmental defects.

In **Project 4.2.1**, the **Muschler group** at the Cleveland Clinic, the **Yaszemski group** at the Mayo Clinic, the **Darr group** at Rutgers University, and the **Griffith group** at the Massachusetts Institute of Technology (MIT) are assessing and comparing several new and highly promising degradable scaffolds that can facilitate the generation of bone tissue. During the past year, the researchers tested mineralized cancellous allograft (MCA) in a dog long bone multidefect model. They also completed a competitive analysis of the best available AFIRM scaffolds. They found that MCA performed significantly better than any polymer scaffold tested so far. MCA is therefore being advanced as the preferred scaffold on which to evaluate cell-sourcing strategies in Project 4.2.2. The researchers also successfully created new calcium phosphate (CaP)-based scaffold compositions for evaluation and advancement during the upcoming year. Notably, industry partners have licensed the researchers' tyrosine-derived polycarbonate (TyrPC) and poly(propylene fumarate) (PPF) scaffolds, which were created earlier in the award period. Trident Biomedical, Inc., is pursuing a 510(k) application on a TyrPC bone pin. BonWrx, Inc., is establishing protocols for the scale-up of the synthesis of PPF and

RCCC researcher Dr. Das Bolikal analyzes a polymer for its molecular weight using gel permeation chromatography.

the fabrication of porous three-dimensional (3-D) PPF scaffolds via Good Manufacturing Practice (GMP). The researchers anticipate that an optimized bone-generating scaffold in combination with technologies developed in Projects 4.2.2 and/or 4.2.3 will be advanced into prospective clinical trials in year 5.

Bone-forming stem/progenitor cells are severely depleted in the region of a large bone defect. The **Muschler/Zborowski group (Project 4.2.2)** at the Cleveland Clinic is investigating methods to optimize the clinical harvest, intraoperative processing, and transplantation of bone-forming stem cells to enhance bone regeneration in large defects. The researchers intend to provide surgeons treating injured warriors with effective and practical clinical methods to concentrate, select, and transplant bone-forming stem cells from a patient's own bone marrow and thereby enable and accelerate bone regeneration with minimal risk. Three basic methods for cell processing are being explored: density separation, selective retention, and magnetic separation. The researchers determined that density separation devices are no more effective than manual methods. They finalized the design of a bone marrow excavation (BME) device that can increase the yield and efficiency of marrow cell harvest. The device can now be used for testing in the dog and goat models. The researchers determined a preferred MCA composition and tested it in a dog long bone defect model. They successfully tested the hexapole magnetic separator on dog and human bone marrow samples. The research team's methods for cell sourcing and processing will be combined with top-performing scaffolds from other AFIRM projects and advanced into clinical trials, most likely in collaboration with the Major Extremity Trauma Research Consortium.

Placing a scaffold and optimal cell source in a bone defect has been shown to improve bone formation. However, under current conditions most transplanted cells do not survive. Rather, they die as a result of the profound drop in oxygen within the transplant site through a process of apoptosis (programmed cell death). This challenge becomes progressively greater as the distance from a transplanted cell to the nearest blood vessel increases from 1–3 mm (in simple fractures or small animal models) to 3–10 mm in larger defects. The **Muschler/Griffith group (Project 4.2.3a)** at the Cleveland Clinic and MIT, respectively, and the **Bushman group (Project 4.2.3b)** at Rutgers University are developing methods designed to enhance the survival and performance of transplanted cells.

In Project 4.2.3a, the researchers have been using MSD and oxygen delivery to achieve cell survival.

- MSD is a biomaterials strategy that involves tethering a growth factor or signaling molecule onto the surface of an implant to change the way that cells interact with the material. This can change cell attachment, migration, proliferation, differentiation, or survival in a controlled manner. The researchers have explored tethering of growth factors and proteins using both purely chemical strategies and a binding strategy based on a biological system. They selected epidermal growth factor (EGF) for tethering since EGF is known to help the growth of bone-forming stem cells. They have been optimizing the binding of EGF to β-tricalcium phosphate (TCP)-coated scaffolds. They have also developed an improved assay for the assessment of cell proliferation on these enhanced scaffolds. In year 4 the researchers plan safety and efficacy testing for the scaffolds tethered with EGF using a bone defect dog model. This will be a critical step in establishing a value proposition for

a commercial partner. In year 5, a product development effort could commence that may include GMP manufacturing and preliminary interactions with the U.S. Food and Drug Administration (FDA).

- Oxygen delivery within an implanted scaffold for bone repair is being developed using polymer methods that have previously been used for controlled drug delivery. Rather than using a drug, polymers are being developed that will release oxygen at very low levels over a few days following implantation. Data generated to date in this project have demonstrated that even a small amount of local oxygen delivery may have profound effects on the ability of transplanted cells to survive a rapid drop in oxygen levels. Methods enabling sustained oxygen delivery over 96 hours in a formed scaffold were demonstrated in vitro during the past year. However, this approach will not be further pursued during year 4 in part because a more advanced oxygen delivery system is being developed elsewhere.

In Project 4.2.3b, the researchers have completed work with chemically modified TyrPCs to display a tethering molecule using a highly efficient protocol. They demonstrated that the chemical modification did not alter the favorable polymer characteristics and fabrication potential. After developing and validating reproducible procedures to measure the amounts of tethered molecules, the team initiated animal studies (rabbit skull) to evaluate the efficacy of tethered bone-forming proteins in critical-size bone defects. In years 4 and 5, studies will be focused on evaluations of safety and efficacy in animal models. Experiments will be conducted to determine the necessary amount of the bone-forming biomolecules to regenerate critical-size defects in bone. Successful completion of these studies will position this technology toward GMP manufacturing and discussions with the FDA.

Soft Tissue Repair and Regeneration (excluding nerve)

Studies at WFPC

The **Harrison group (Project 4.4.6)** at Wake Forest University is developing an injectable biomaterial capable of generating oxygen—a particulate oxygen generator (POG)—that would allow the delivery of oxygen in controlled amounts to engineered tissue scaffolds or pre-existing tissue. The researchers tested the POGs for sustained release of oxygen in vitro (i.e., injected into muscles that have been removed from an animal) and feasibility of injection in vivo (i.e., injected into intact muscles in an animal). The research team demonstrated that the POGs can improve the functional response of hypoxic (i.e., oxygen starved) skeletal muscle tissue in vitro. During the next 2 years, the researchers will initiate in vivo animal studies to evaluate the efficacy of the biomaterial. POG technology will be tested in different systems—skeletal muscle, bone, nerve, and skin—to determine which areas would most benefit from this approach. Optimization of the most promising tissue systems benefiting from the technology will continue in the future along with identifying the best possible clinical application. The researchers anticipate that a clinical trial will be ready within the next few years.

Numerous tissue engineering strategies begin with the implantation of cells onto matrices followed by the attempt to create a de novo vascular system. However, researchers have fallen short in creating a viable vascular network outside of the body due to the complexities of blood vessel network formation in the living organism. The **Gurtner group (Project**

WFPC researcher Janet Reing, a staff scientist, pipets samples in the laboratory.

4.5.8) at Stanford University has developed novel strategies that utilize preformed native circulatory networks that can be supported outside the body during organ fabrication, matured using stem cell-based techniques, and then readily integrated into the systemic circulation. The researchers have demonstrated the feasibility of sustaining explanted microvascular beds (EMBs) outside of the organism for up to 7 days. They have characterized the microvascular structure of the isolated EMBs at the ultrastructural level by scanning electron microscopy (SEM). They have developed a procedure for removing cells from the tissue while preserving the matrix architecture and the macroscopic vascular structure. The researchers have seeded EMBs with murine stem cells, and preliminary results indicate successful engraftment and persistence of the cells in a functional capacity for extended time periods. In the next 2 years, the researchers plan to optimize their cell removal protocol. They will also optimize their methodology to maximize engraftment and persistence of stem cells in the EMBs. Once their procedure has been fully optimized, they will transition to human adipose-derived stem cells (ASCs).

Studies at RCCC

Current metal stents are designed for the treatment of late-stage peripheral vascular disease, leave young patients at risk for graft failure due to fracture or the recurrence of stenosis (narrowing of a blood vessel), and are not designed to last the lifetime of the individual. The **Sarac group (Project 4.3.2)** at the Cleveland Clinic is developing bioabsorbable and/or fracture-resistant, tissue-lined stent grafts for minimally invasive treatment of arterial and venous trauma in young patients. The researchers tested their fracture-resistant nitinol stent grafts in a 30-day animal trial, which was successful. They constructed five different prototypes of a fiber-based, bioabsorbable, polydioxanone (PDO) tissue-lined stent by varying the fiber diameter and weave pattern. The research team also developed a novel catheter

RCCC researchers Drs. Sanjeeva Murthy and Nava Shpaisman examine polymeric drug delivery capsules.

delivery system. During the upcoming year, the researchers will concentrate on the regenerative component of this project, the bioabsorbable stent graft. They aim to translate the fracture-resistant stent graft design for nitinol into PDO-based bioabsorbable tissue-lined stent grafts.

Dermal (skin) grafts are commonly used in clinical practice to repair abdominal wall defects and hernias. However, the implanted grafts tend to lose their integrity and strength over time, which results in complications such as bulging and hernia recurrence. Hence, engineered improvements to biologic grafts are needed to make them more suitable for abdominal wall repair and reconstruction. The **Derwin group (Project 4.4.3a)** at the Cleveland Clinic seeks to provide injured warriors with biological materials for reconstruction of abdominal defects through the generation of a fiber-reinforced biological graft. During the past year, the researchers identified a candidate material for fiber reinforcement. They demonstrated how fiber reinforcement can be used to alter the mechanical properties of biological grafts derived from extracellular matrix (ECM). In year 4, they will focus on the development of reinforced ECM constructs that maintain mechanical properties post-implantation with hopes to prevent the bulging and recurrence of a hernia. The reinforced grafts will be evaluated using burst and tensile tests. The researchers will conduct proof-of-concept studies to optimize fiber material and design of reinforced ECM grafts for abdominal wall repair applications. In year 5, the candidate graft material(s) will be evaluated in a preclinical large animal hernia model. In addition, the team's industrial collaborator will conduct manufacturing, toxicity, packaging, and shelf life studies. As the device moves into product development and a regulatory path is defined, the industrial partner will initiate human trials.

Due to limited healing capabilities, injuries to the meniscus (internal cartilage) of the knee are often treated with resection. This treatment generally leads to early symptom

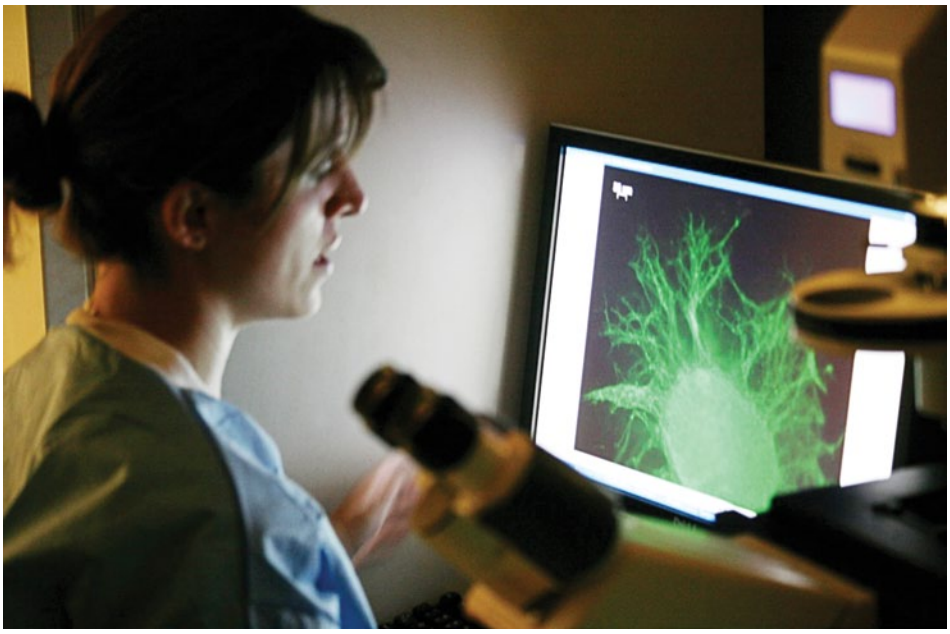
relief but frequently leads to the development of degenerative arthritis of the knee. The **Gatt/Dunn group (Project 4.4.3b)** at the University of Medicine and Dentistry of New Jersey is developing an off-the-shelf, tissue-engineered meniscus scaffold that can be used to replace a severely damaged meniscus in an injured service member. The scaffold is composed of an anatomically designed, fiber-reinforced scaffold and a collagen-based ECM similar to the native meniscus. During the past year, the research team continued to develop and evaluate its second-generation meniscus scaffold. The researchers determined that lower concentrations of glycosaminoglycans (GAGs) increased the compressive strength and decreased the permeability of the collagen matrix giving them characteristics closer to the native meniscus. The researchers also completed a rabbit study with the second-generation meniscus scaffold. Preliminary histological analysis indicates that the scaffold is biocompatible. During the next 2 years, the researchers will conduct long-term meniscus reconstruction studies in sheep using the second-generation scaffolds reinforced with resorbable fibers. They will also evaluate the efficacy of the scaffolds in a partial meniscectomy model since the medical device industry is interested in using the device to repair partial meniscectomies. After the preclinical large animal efficacy studies are complete, an industrial partner will assist in the design and funding of clinical trials.

Nerve Repair and Regeneration

Studies at WFPC

Following trauma, incomplete nerve regeneration and permanent demyelination (damage to the protective sheath surrounding nerves) may result, leading to lifelong disability. In **Project 4.4.4**, the **Marra group** at the University of Pittsburgh, the **Kaplan group** at Tufts University, and the **Smith group** at Wake Forest University is developing a proactive biodegradable nerve guide/conduit system that delivers chemical cues (e.g., growth factors) and biophysical cues (e.g., surface patterning) to regenerating peripheral nerves. The researchers identified a drug delivery strategy to deliver neurotrophic factors for up to 60 days in vivo in a rat nerve defect model. They demonstrated that their porous silk fibroin-based nerve guides, infused with bioactive glial-derived neurotrophic factor (GDNF) in a controlled manner, represent a potentially viable conduit for Schwann cell migration and proliferation in the regeneration of peripheral nerves. The researchers are now examining the use of keratin-filled nerve guides to treat a 1 cm nerve defect in the median nerve of nonhuman primates (NHPs). Their NHP results to date have been promising, which has prompted further evaluations in additional NHPs.

Functional limb and digit tissue restoration involves a hierarchically defined process that often requires precise spatial and temporal coordination among multiple biological systems and processes. The **Tirrell group (Project 4.4.5)** at the University of California, Berkeley is pursuing an approach to induce peripheral nerve growth following traumatic amputation by modulating components of the naturally occurring ECM (e.g., fibronectin and laminin). The researchers are using synthetic chemistry to construct peptide amphiphiles (PAs) with controlled physicochemical and bioactive properties, and testing their ability to promote cell adhesion, migration, and nerve regrowth. They developed several biologically active PA gels with well-defined 3-D structures and found that the gels can incorporate bioactive peptide sequences and/or growth factors. In addition, they found that the 3-D matrix system allows mammalian cell growth. They recently developed a PA-based hydrogel that contains histidine and serine, which they



RCCC researcher Dr. Basak Clements images neurons on biodegradable nerve conduits.

plan to test in a rat nerve gap injury model in collaboration with the Marra group at the University of Pittsburgh. During the next year, they also plan to enhance the bioactivities of their PA-based hydrogels by linking short peptide sequences and proteins (e.g., glial-derived neurotrophic factor) to them.

Studies at RCCC

RCCC's Nerve portfolio is composed of four parallel, synergistic, and interdependent projects (three are incorporated under Project 4.4.1/4.4.2 and the fourth is Project 4.4.2a). In aggregate, these projects seek to provide wounded warriors with injuries to large sensory and mixed motor nerves with an opportunity for recovery that does not currently exist. Novel materials are being developed, and their mechanical properties are being tested to identify those best suited to nerve repair. Biocompatibility is being tested in vitro using standardized tissue culture model systems and in vivo using rodent models.

In **Project 4.4.1/4.4.2**, the **Windebank/Yaszemski group** at Mayo Clinic, the **Kohn/Schachner group** at Rutgers University, and the **Anderson/Langer group** at MIT have developed a model for the early assessment of promising scaffolds in a human setting that involves the opportunity to repair defects of the sural nerve (a small sensory nerve on the back of the leg), which is routinely biopsied and left unrepaired during investigation of possible nerve diseases. The researchers established a standardized nerve defect and tube implantation model system in the rat that enables the competitive evaluation of polymer scaffolds across the consortia. The screening of more than 10 scaffold materials was completed resulting in the selection of a poly(caprolactone fumarate) (PCLF) tube as the candidate scaffold to move into clinical trials. The researchers developed a method for incorporating aligned electrospun fibers into the nerve scaffold in a uniform manner with no fiber aggregation. They incorporated anti-inflammatory agents in electrospun fibers to decrease the inflammatory response

to synthetic materials. They are currently completing all regulatory pathways for the initiation of a Phase 1 clinical trial that will be focused on repairing large peripheral nerve gaps in nerve biopsy patients using PCLF tubes. (See Project 4.4.1a for details on this trial.) In the upcoming year, the Mayo Clinic group will introduce Schwann cells into the PCLF nerve conduits to provide a source of growth factors to promote nerve regeneration across long nerve gaps.

Another recent accomplishment in Project 4.4.1/4.4.2 is the development by the Rutgers University group of a braided nerve conduit that is fabricated from thin TyrPc polymer fibers. These conduits have excellent mechanical properties when both dry and hydrated, and they display unique nonkinking behavior when bent at large angles. The Rutgers University team will characterize and optimize these braided nerve conduits in animal cohorts during the next 2 years. The researchers will also continue to develop bioactive peptide-conjugated hydrogel fillers. They are screening the fillers for their ability to promote nerve growth. They will evaluate the most effective filler and braided conduit combination in the rat sciatic nerve 1 cm defect model in year 4. They expect to scale up the conduit prototype for large animal testing in year 5.

Bone marrow stromal stem cells (BMSCs) comprise a heterogeneous population of cells that contribute to the regeneration of multiple body tissues. Notably, BMSCs can be placed in an individual without the need for immunosuppression, which is of value with regard to allogeneic cell therapy. The **Siemionow group (Project 4.4.2a)** at the Cleveland Clinic seeks to improve nerve regeneration by enhancing the performance of epineural nerve sheath conduits with the addition of BMSCs. Their results to date show that the inclusion of BMSCs in allogeneic nerve conduits can lead to better functional outcomes compared to saline-filled (control) conduits and can bypass the need for immunosuppression. The researchers recently obtained approval of their protocol for a study involving the repair of a long nerve defect in a large animal model (sheep). During the upcoming year, the research team is planning to complete the sheep study and, based on the results, move into clinical trials.

Composite Tissue Injury Repair

Studies at WFPC

The **Guldberg group (Project 4.4.3)** at the Georgia Institute of Technology seeks to develop and test technologies that will enable the restoration of limb function following composite tissue trauma. The researchers have established promising regenerative strategies for bone, nerve, and muscle using nanofiber mesh spatial guidance and sustained delivery of a clinically approved inductive protein, bone morphogenetic protein 2 (BMP-2). They have developed composite injury models in the rat that simulate bone/nerve, bone/vascular, and bone/muscle injuries. They have used these models to test nanofiber biomaterial delivery systems (patents pending) that provide spatial and temporal cues to guide improved bone and nerve regeneration. Notably, the researchers' rat segmental bone defect model has been adopted and used by other AFIRM investigators. The researchers next plan to initiate large animal (sheep and goat) studies of the most promising technologies. Once proof of concept has been demonstrated in a large animal model, the goal is to initiate a human clinical trial pilot study in year 5.

WFPC researchers Alice Li and Brent Uhrig (PhD students in the Guldberg laboratory) perform rat surgery.

Epimorphic Regeneration (and associated methods)

Studies at WFPC

The **Badylak group (Project 4.4.1)** at the University of Pittsburgh is investigating mechanisms for recruiting large populations of stem cells to the site of limb and digit injury and then developing strategies to induce the formation of functional limb and digit tissue to replace the damaged or missing structures. The researchers have established a model of digit amputation in an adult mouse where the second joint of a digit is amputated. In their mouse model, they have shown that treatment with peptides derived from ECM leads to the recruitment of a population of cells that express markers that are universally recognized as markers of primitive stem cells (e.g., Sox2, Rex1, and Sca1). This is an important finding because the essential first step to promoting epimorphic regeneration is the endogenous recruitment of a population of stem cells that is capable of forming all of the tissues in the missing limb or digit. During the past year, the researchers have partially characterized the source of the previously identified Sox2 cells recruited to the site of digit amputation following ECM administration. They have also shown that treatment with bioactive ECM molecules results in functional tissue formation such as bone at the site of amputation. Work in the upcoming year will focus on identifying peptides that have the ability to alter the recruitment and differentiation of stem cells toward other important tissues in the digits, such as muscle, nerve, and vasculature. They will also work on controlling the spatiotemporal pattern of treatment with the bioactive peptides at the site of amputation via a microfluidic device designed to cover the site of digit amputation. In collaboration with Dr. Peter Rubin at the University of Pittsburgh, the research team is evaluating a commercially available, FDA-approved powder form of ECM for the treatment of distal digit amputations.

WFPC researcher Vineet Agrawal, an MD/PhD student, in the laboratory.

The **Stewart/Thomson group (Project 4.4.7)** at the Morgridge Institute for Research is studying tissue regeneration using high-throughput technologies (microarrays and next-generation sequencing). During the past year, the researchers complemented the work they had completed during the first 2 years of the project by performing next-generation sequencing on RNA from the axolotl (a member of the salamander family) as a model system for limb regeneration. Certain species of salamanders and newts, including the axolotl, have the ability to fully regenerate an amputated limb as an adult, which makes these animals important models for limb and tissue regeneration. The researchers identified a number of genes that are upregulated in the axolotl blastema (a mass of cells capable of regeneration into organs or body parts). They also identified three genes that are upregulated in a spatial pattern along the axis of the axolotl limb. They are now analyzing gene expression at various locations in the adult mouse limb to determine if a similar pattern of expression exists in the nonregenerating mouse digit. It is anticipated that these studies will reveal genes that are regeneration specific.

The **Soh group (Project 4.4.8)** at the University of California, Santa Barbara is developing ways to sort and isolate cells from complex mixtures of cells. They first developed a device that allows for the purification of extremely rare cells from complex mixtures with superior cell recovery. During the past year, the researchers developed a novel microfluidic phage selection system, which is capable of efficiently isolating peptides with high affinity and specificity to target cell surfaces. Compared to conventional biopanning methods, microfluidic selection enables the more efficient discovery of peptides with higher affinity and specificity by providing a controllable and reproducible means for applying stringent selection conditions against minimal amounts of target cells without loss. Overall, it is believed that these devices will provide a critical technical solution for the isolation of the target stem cells needed for clinical therapeutics.

Clinical Trials

Several AFIRM Limb and Digit Salvage projects have technologies that are advancing to the human clinical trial stage. Additional details on these clinical trials can be found in **Table II-2** and subsequent sections of this chapter.

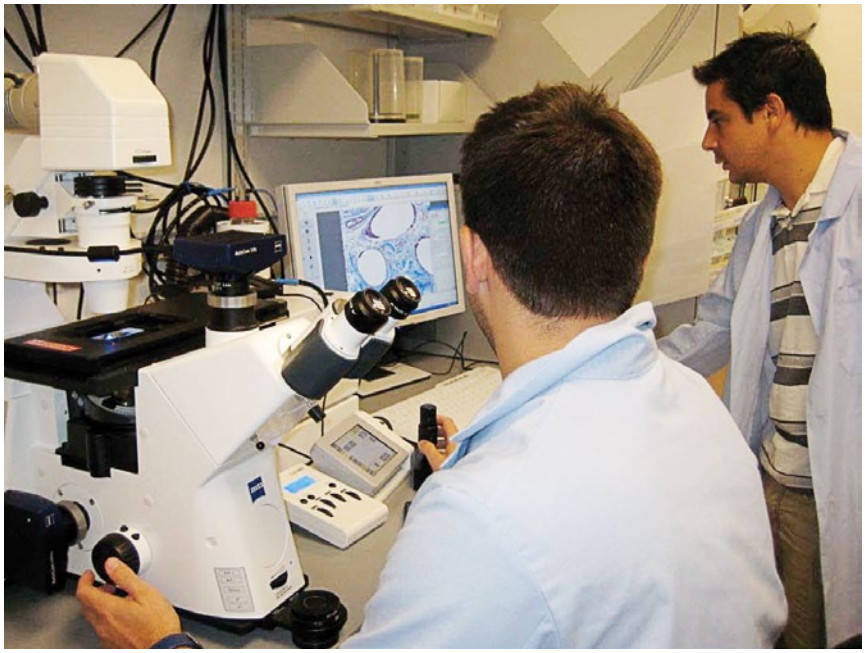
While composite tissue allografts (e.g., hand transplants) are now a clinical reality and have been performed in multiple centers worldwide, the procedure has not reached widespread clinical use because recipients require lifelong, high-dose multidrug immunosuppression to prevent graft rejection. The **Lee group (Project 4.4.2**, WFPC) at the Johns Hopkins University School of Medicine and the University of Pittsburgh is developing a protocol for hand transplantation using donor BMSCs in combination with novel fusion proteins (the "Pittsburgh Protocol") that will minimize maintenance immunosuppressive therapy. The researchers have shown that their fusion protein can prolong the survival of allografts in a preclinical hindlimb transplant model in swine. The research team has also achieved its clinical translation milestone by performing hand transplants on five patients. All patients have been maintained on a single immunosuppressive drug at low levels, and they continue to have increased motor and sensory function of their transplanted hands, which correlates with their level of amputation, time after transplant, and participation in hand therapy. In year 4, the research group aims to repeat and confirm some of its swine experiments. Clinically, the researchers are planning an optimized strategy that combines targeted modulation of the immune system, bone marrow stem cell/fusion protein induction, and topical migratory inhibitors to further reduce maintenance immunosuppression and allow weaning of systemic drug therapy. The research team hopes that this regimen will enable widespread clinical application of hand transplantation for the reconstruction of upper extremity amputations.

At the Mayo Clinic, 200–250 sural nerve biopsies are performed each year. Whole sural nerve biopsy results in predictable loss of function in the sural distribution of the foot. Reconstruction of the sural nerve in these patients can potentially restore sensation and prevent complications associated with the biopsy. The **Windebank/Yaszemski group (Project 4.4.1a, RCCC)** at the Mayo Clinic is aiming to repair post-biopsy sural nerve defects with a synthetic nerve conduit constructed from PCLF. Since the nerve is just below the skin, potential morbidity from the conduit will be readily identifiable and the device can be removed and analyzed. This

Table II-2. AFIRM-funded Limb and Digit Salvage projects with pending or active clinical trials.

Project Title	Consortium	Project No.	Trial Phase	Current Status
Hand Transplantation for Reconstruction of Disabling Upper Limb Battlefield Trauma – Translational and Clinical Trials	WFPC	4.4.2	Phase 1	Open
Clinical Trial – Safety Assessment of a Novel Scaffold Biomaterial	RCCC	4.4.1a	Phase 1	Submitted to IRB*
Epimorphic Regeneration Approach to Limb and Digit Reconstruction – Clinical Trial	WFPC	4.4.1a	Phase 1	Submitted to IRB

**Institutional Review Board*



WFPC researchers Brian Sicari (left), a PhD student, and Ricardo Londono (right), an MD/PhD student, in the laboratory.

will provide a platform for determining safety and an indication of efficacy of scaffolds for nerve repair. The researchers have been working closely with BonWrx, Inc., to complete this study. Their clinical trial protocol has been reviewed and approved by the Mayo Clinic's Department of Neurology research committee and has been submitted to the Mayo Clinic's Institutional Review Board (IRB) and Human Research Protection Office (HRPO). Patient recruitment will start as soon as IRB and FDA approval is obtained.

The **Badylak group (Project 4.4.1a, WFPC**) at the University of Pittsburgh is collaborating with researchers at the U.S. Army Institute of Surgical Research (USAISR) to initiate a 10-patient clinical trial. The proposed work involves the treatment of patients who have suffered from the loss of large amounts of functional musculotendinous tissue as a result of trauma. These patients will be implanted with one of several different forms of ECM. The clinical trial is currently in review by the IRB. The lead in the trial will be Dr. Joseph Hsu of the USAISR. A progress report is not yet available for this study.

our science for their healing

Advanced 3-D Scaffolds for Large Segmental Long Bone Defects

Project 4.2.1, RCCC

Team Leader(s): George Muschler, MD (Cleveland Clinic), Michael Yaszemski, MD, PhD (Mayo Clinic), Aniq Darr, PhD (Rutgers University), and Linda Griffith, PhD (MIT)

Project Team Members: Viviane Luangphakdy, MS, Hui Pan, MD, PhD, Kentaro Shinohara, MD, PhD, and Tess Henderson, BS (Cleveland Clinic); Ophir Ortiz, PhD, Das Bolikal, PhD, and Matthew Laughland (Rutgers University); Linda Stockdale, MAT (MIT); and Theresa Hefferan, PhD, Suzanne Segovis, Mahrokh Dadsetan, PhD, and Brett Runge, PhD (Mayo Clinic)

Collaborator(s): Sunil Saini (Integra Spine, Plainsboro, New Jersey), Deborah Schmalz (Trident Biomedical, Inc., Somerville, New Jersey), Ralph Carmichael (BonWrx, Inc., Phoenix, Arizona), and Dr. Racquel LeGeros (New York University)

Therapy: Advanced regeneration of segmental bone defects.

Deliverable(s): Advanced 3-D scaffolds for large segmental bone defects.

TRL Progress: Degradable Scaffold – 2009, TRL 3; 2010, TRL 3; 2011, TRL 3; Target TRL 4

Mineralized Cancellous Allograft – 2009, TRL 9; 2010, TRL 9; 2011, TRL 9; Target, TRL 9

Key Accomplishments: Researchers at the Cleveland Clinic completed an assessment of MCA in the canine femoral multi-defect (CFMD) model. They also completed a competitive analysis of the best available AFIRM scaffolds and compared them to the MCA in the CFMD model. Researchers at Rutgers University and the Mayo Clinic successfully created new scaffold compositions for evaluation and advancement into year 4.

Key Words: Bone, bone repair, bone regeneration, bone defect, bone graft, osteogenesis, osteoinduction, osteoconduction, calcium phosphate, allograft, polymer scaffold, degradable, bone marrow, connective tissue progenitors, canine femoral multi-defect

Introduction

Repairing large bone defects and fracture nonunion in extremity injuries represents an ongoing challenge because high-energy blast injuries from improvised explosive devices are increasingly common. Military extremity injuries traditionally comprise the majority of traumatic injuries in the U.S. armed conflicts. A significant proportion of these extremity wounds are fractures, most of which are open injuries. These fractures are frequently comminuted and complicated by extensive soft tissue loss, often with segmental bone loss of 5–20 cm. The management of acute bone loss still remains a challenge for surgeons, and methods to ensure or accelerate fracture healing are still greatly needed. Defects lacking the capacity for spontaneous healing are common, and no single existing treatment option is appropriate for all defects.

The contemporary standards of care for bone defects and complex fractures in civilian

and military practice include many options for wound management, fixation, and bone grafting. Graft options include autogenous cancellous bone, allograft bone, addition of bone marrow-derived cells (with or without cell processing), local delivery of bone morphogenetic protein or osteogenic protein 1, and occasionally bone transport procedures. These challenging settings frequently demand the use of materials in applications for which they were not specifically designed or evaluated and are therefore considered "off label," "innovative," or "out of the box." Success is not uncommon but neither is failure, and none of these options has provided more than 90% success, even in relatively common settings, particularly in large, complex defects.

Polymer-based graft substitutes have been proposed for bone replacement, such as ceramic-based grafts. They avoid the need for a harvesting procedure, subsequently avoiding chronic pain at the harvest site, and they offer structural diversity. The proper design and application of these polymer-based bone graft substitutes still remain under a multitude of investigations. Thus, the management of acute bone loss still remains a challenge for surgeons, and methods to ensure or accelerate fracture healing are still greatly needed.

RCCC's Limb Salvage and Transplantation Program will provide injured warriors with one or more new clinical methods that significantly improve the rate and reliability of regeneration of bone defects over 3 cm or bone nonunions. The goal of Project 4.2.1 is to rapidly assess and compare several new and highly promising osteoconductive scaffolds fabricated from degradable polymers and to bring forward the best new technology as a substrate that could be combined with an optimized cell source (Project 4.2.2) and an optimized modification using bioactive factors (Project 4.2.3a and b) to transform the treatment opportunities for large bone defects.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers identified, designed, and fabricated four distinct families of copolymer-based osteoconductive scaffolds for in vivo testing. They synthesized new, second-generation polyester and tyrosine-based copolymers and their composites with osteogenic inorganic particles. They fabricated complex 3-D printed scaffolds with controlled porosities from several of the copolymers and found them to be easily sterilizable. They completed all animal surgeries planned for year 1 along with micro-computed tomography (CT) scanning for statistical analysis of bone volumes and preparation of histology samples for the initial evaluation of copolymer and composite scaffolds. They achieved good in vivo performance using tyrosine-based copolymer and poly(alkyl ester) composites.

During the second year of the project, the researchers completed fabrication and characterization of three polymer-based scaffold platforms. They completed competitive assessment and downselection of scaffold materials in the CFMD model. Among the materials tested, the porogen-leached TyrPC with β-TCP performed best. The researchers also established a defined track record of historical performance standards that can be used to rapidly benchmark the performance of new or competing scaffold materials using the CFMD model.

Research Progress – Year 3

This project involves work performed across four laboratories and institutions (i.e., the Mayo Clinic, Rutgers University, MIT, and Cleveland Clinic). The Cleveland Clinic serves as the coordinating center and as the central resource for in vivo testing using the established CFMD model. MCA bone (canine) was prepared by the Musculoskeletal Transplant Foundation in the form of 3 x 3 x 3 mm^3 chips using processing methods matching established clinical practice. MCA testing was added to provide a comparison and benchmark to the current "gold standard" bone scaffold. Quantitative image analysis of micro-CT data was performed at the Cleveland Clinic. Histological assessment utilized resources at the Mayo Clinic, led by Dr. Theresa Hefferan, supplemented by the expertise of Dr. Tom Bauer at the Cleveland Clinic.

Micro-CT analysis indicated that MCA, with or without bone marrow, performed significantly better than all other test materials. Upon histological examination, defects grafted with allograft were found to have bridging bone. Active resorption of the allograft bone by osteoclasts and deposition of new bone on the allograft surface by osteoblasts was present throughout all samples. The specimens stained with hematoxylin and eosin showed no evidence of inflammation in response to MCA.

Two degradable scaffold materials, TyrPC and PPF, were developed and tested in vitro at Rutgers University and the Mayo Clinic, respectively. TyrPC scaffolds containing a small percentage of calcium minerals (as a composite or as a coating) provided overall the highest level of bone growth in the CFMD model. TyrPC scaffolds are more than 90% porous, soft, and pliable and are therefore best described as a non-weight-bearing bone void filler. TyrPC scaffolds have shown strong bone regeneration performance in both limb and craniofacial animal models.

PPF scaffolds are strong, slow degrading, and partially weight bearing. These properties differentiate the PPF technology from the TyrPC technology. The PPF scaffolds did not perform well during year 2 in either of the animal models so year 3 efforts were devoted to improving manufacturing. Since the two scaffold technologies address different clinical needs, a major development effort will be undertaken to bring the PPF technology forward pending results of studies currently under way.

In consultation with Dr. Racquel LeGeros of New York University, three new CaP minerals were produced, characterized, and then used in the fabrication of scaffolds using both TyrPC and PPF scaffolds as substrates. It is hypothesized that optimizing this parameter will result in an increase in new bone tissue formation as compared to the currently best performing TyrPC+TCP composite scaffolds.

At Rutgers University, the newly formed CaPs and TyrPC+CaP composite scaffolds were characterized using SEM, x-ray diffraction (XRD), and micro-CT. XRD was employed to confirm both CaP synthesis and the incorporation of CaPs into the composite scaffolds. SEM was used for qualitative assessment of CaP crystal morphology, pore morphology, and pore diameter measurements of composite scaffolds. SEM was also performed to obtain crystal morphology and size. The researchers performed micro-CT to obtain quantitative information on porosity and interconnectivity, and qualitative information on mineral distribution. They fabricated and characterized TyrPC+CaP composite scaffolds with all four CaPs. They obtained XRD scans for each type of fabricated composite scaffold. Obtaining XRD data of the CaPs is necessary to check for purity and

confirm the presence of characteristic peaks (fingerprints) for each of the CaPs. XRD data were also obtained after the fabrication process was determined to confirm that reproducibility was achieved.

The PPF scaffold (Mayo Clinic) surface-coating procedures were performed in consultation with Dr. LeGeros' laboratory with two different coating materials: magnesium-substituted β-TCP and carbonate hydroxyapatite (synthetic bone mineral). After the coating, the scaffolds were characterized via SEM in Dr. LeGeros' laboratory and then returned to the Mayo Clinic for further analysis. The SEM images show that surface coating alters the surface topography. The coating produces a rough surface, which resulted in improved cell attachment to the scaffolds.

Key Research Accomplishments

Cleveland Clinic:

- Completed an assessment of MCA matrix in the CFMD model.
- Completed a competitive analysis of the best available AFIRM scaffolds and compared them to MCA matrix in the CFMD model.

Rutgers University:

- Synthesized CaPs with varying solubilities and incorporated CaPs into TyrPC polymer to produce composite scaffolds.
- Identified characterization methods for the composite scaffolds to ascertain batch-to-batch reproducibility and scaffold architecture.
- Identified sterilization facilities and GMP manufacturers for polymer synthesis.

Mayo Clinic:

- Fabricated CaP-coated porous scaffolds for the rat femoral defect model.
- Established a surface-coating process in collaboration with Dr. LeGeros of New York University.
- Demonstrated enhanced in vitro performance of the surface-coated porous scaffolds.

Conclusions

A rapidly resorbing, porogen-leached TyrPC scaffold containing TCP granules and a slowly resorbing, structural PPF scaffold coated with poly(lactic-co-glycolic acid) (PLGA) and hyaluronan (HA) represent the best performing polymer scaffolds tested to date. MCA performs significantly better than any polymer scaffold tested thus far and results in both pericortical bridging and an advanced stage of bone formation and remodeling throughout the defect in the CFMD model at 4 weeks. Efforts to improve the performance of TyrPC or PPF bone scaffolds has led the team to evaluate new composite scaffolds using CaP minerals produced in consultation with Dr. LeGeros at New York University. Data summarizing the assessment of TyrPC and PPF scaffolds with CaP surface coatings in in vivo animal models are expected in the near future. These data will be used to determine if one or more scaffolds should be advanced into year 4 for in vivo assessment in an appropriate biologically and clinically relevant animal model in accordance with the guidelines that have been established within AFIRM for the use of in vivo models for bone segmental defects. MCA bone matrix has reached a level of performance where further advancement of the allograft will require testing in a more stringent biological setting of advanced models such as the chronic caprine tibial defect (CCTD) model (described in Project 4.2.2). Animal models will be used to determine if the addition of new CaP surfaces has improved the performance of

scaffold materials sufficiently to bring them into the range of comparability to allograft.

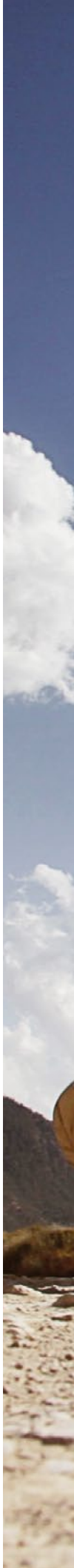
Research Plans for the Next 2 Years

Results from the scaffold testing tournaments have demonstrated that MCA bone remains the best available osteoconductive scaffold material, and as a result, it is being advanced as the preferred scaffold on which to evaluate cell-sourcing strategies in Project 4.2.2. These data also suggest that the addition of CaP-based materials can enhance the performance of polymer scaffolds. In year 4, one or more TyrPC or PPF scaffolds with CaP surface coatings are expected to demonstrate in vivo performance equal to or greater than that of MCA bone matrix. These materials will subsequently advance as substrates for further biological enhancement strategies using cell sources in Project 4.2.2 and bioactive peptides in Project 4.2.3a and b. It is expected that an optimized osteoconductive scaffold in combination with technologies developed in Projects 4.2.2 and/or 4.2.3a and b will be advanced into prospective clinical trials in year 5.

Planned Clinical Transitions

Industry partners that are currently working toward commercialization of these technologies have licensed both the TyrPC and PPF scaffolds. Trident Biomedical, Inc., is pursuing a 510(k) application on a TyrPC bone pin. This application will support subsequent bone void filler regulatory filings. A 510(k) application for a first-generation bone void filler device will be pursued. Second-generation products will include either soluble or tethered biologics. These will require a clinical trial and a Premarket Approval pathway. As the commercial partner for the technologies described previously, Trident Biomedical, Inc., is expected to pursue regulatory filings for these devices.

The Yaszemski group is currently working with its industry partner, BonWrx, Inc., to establish protocols for the scale-up of the synthesis of PPF and the fabrication of porous 3-D scaffolds from this polymer via GMP. Furthermore, testing required by the FDA (e.g., toxicology) will be initiated in a commercial testing laboratory. BonWrx, Inc., currently licenses several of the Yaszemski laboratory patents from the Mayo Clinic.



our science for their healing

Optimizing Cell Sources for the Repair of Bone Defects

Project 4.2.2, RCCC

Team Leader(s): George Muschler, MD and Maciej Zborowski, PhD (Cleveland Clinic)

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Therapy: Advanced regeneration of segmental bone defects.

Deliverable(s): Preferred clinical method for progenitor cell concentration, selection, and delivery.

TRL Progress: Selective retention (SR) with allograft – 2009, TRL 3; 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Magnetic separation (MS) – 2009, TRL 2; 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Bone marrow excavation – 2009, TRL 2; 2010, TRL 2; 2011, TRL 3; Target, TRL 5

Key Accomplishments: The researchers assessed mineralized cancellous bone and demineralized cortical bone powder (DCBP) in vitro as substrates for SR processing. They finalized the design of a BME device to increase the yield and efficiency of marrow cell harvest. The device is available for in vivo testing in the canine or caprine models. The researchers demonstrated that MS-processed cells were compatible with both SR processing and "drip soaking" using an MCA matrix. They conducted an initial in vivo assessment of MS processing using MCA in the CFMD model, which is nearing completion. They initiated a pilot in vivo assessment of SR processing using MCA in the CCTD model.

Key Words: Connective tissue progenitors, magnetic separation, cell sourcing, bone graft, hyaluronan, density separation, selective retention

Introduction

This project addresses the need to select a clinically relevant method for harvesting, processing, and transplanting the cells that form bone tissue, known as osteogenic cells (specifically, osteogenic connective tissue progenitors [CTP-Os]), which are severely depleted or missing in these bone defects and surrounding tissues.

Successful bone repair or regeneration in all settings requires CTP-Os. In settings where the local population of CTP-Os is sufficient, they may be effectively targeted using scaffolds or factors such as bone morphogenetic proteins. However, in settings where the local numbers of CTP-Os are suboptimal, as in most injuries sustained by the wounded warrior, optimizing the bone-healing response will require transplantation of CTP-Os from an alternative source.

Further advancement in CTP-O harvesting and processing is needed to better control the transplant environment and optimize

the cells' in vivo clinical performance (i.e., the cells' attachment, survival, proliferation, migration, and differentiation). Implantation of fewer cells should improve CTP-O survival by reducing the local metabolic demand, which is expected to be an important variable in large bone defects and fracture nonunion. Improved methods for CTP-O concentration and selection will enable surgeons to deliver more CTP-Os in a given site. The Muschler laboratory has demonstrated the value of SR as a way of concentrating and selecting marrow-derived CTP-Os. The clinically successful Cellect™ System (Depuy) was based on SR methods.

Project 4.2.2 was initiated to systematically evaluate three methods for CTP-O concentration and selection within a fresh marrow aspirate: (1) density gradient separation, (2) SR, and (3) MS based on HA expression.

Density separation (DS) (e.g., centrifuge) devices are available by modifying devices designed for the clinical preparation of platelet-rich plasma. DS can be used to increase the CTP-O concentration four- to eightfold; however, DS processing concentrates all nucleated cells and does not increase the prevalence of CTP-Os.

SR involves passing a cell suspension through a porous matrix. More adherent CTP-Os are preferentially retained within the matrix while nonadherent cells pass through the matrix into the effluent solution. Some formulations of clinically available scaffolds have been shown to retain 80%–90% of CTP-Os and only 20%–30% of other nucleated cells, resulting in a three- to fourfold increase in the CTP-O concentration and a two- to threefold increase in CTP-O prevalence. Further improvement in SR requires more selective surfaces or preprocessing steps.

MS systems tag cells of interest with magnetic beads and separate them in a magnetic field. CTP-Os can be enriched or depleted from a bone marrow aspirate using any marker, which provides a method for specific selection of CTP-Os. These systems are easy to use, offer high throughput, and support flexible antibody and bead combinations.

Each of these methods, alone or together, offers significant potential value to the wounded warrior by providing ways to effectively repopulate or "re-seed" the region of a bone defect with a large population of cells that is capable of regenerating the missing bone tissue.

Summary of Research Completed in Years 1 and 2

During the first year of the study, the researchers defined methods to increase the surgical yield of bone marrow harvest procedures without an increase in morbidity. Three practical methods were characterized to enable the processing of human or canine bone marrow: DS, SR, and MS.

During the second year of the study, the researchers evaluated two DS devices for concentration of bone marrow cells. They tested one of the preferred degradable scaffolds from Project 4.2.1 and found that it showed modest performance as a substrate for SR. They downselected the option of CD45 depletion as a means of enhancing the prevalence of CTP-Os in favor of HA-positive selection. They designed, fabricated, and validated a new MS system for the selection of CTP-Os. The system is simple to use, easily adaptable for intraoperative single-step procedures, capable of processing 2×10^9 cells (required for scale-up to the CFMD model), and provides improved separation over the small-scale commercial magnet used previously.

Research Progress – Year 3

Selective Retention

In year 3, the researchers focused on definitively characterizing the performance of a high-quality allograft in the CFMD model so that AFIRM scaffolds (TyrPC and PPF) or other scaffolds could be subjected to a rigorous comparison against this standard. As expected for a purely biological product, the bone regeneration performance of MCA bone was shown to be far superior to that of the synthetic polymer scaffolds tested thus far and remains as the gold standard against which AFIRM scaffolds and other outside scaffold options should be evaluated.

The project team obtained five canine marrow samples to complete in vitro SR processing, comparing all three allograft formulations using the same canine donor. The mean selection ratios for MCA, MCA+DCBP, and DCBP alone were 2.09 ± 0.86, 2.08 ± 0.81, and 1.92 ± 0.75, respectively. These data suggest that MCA chips alone provide a preferred substrate for SR processing using canine marrow. No advantage was found in adding surface area using DCBP.

Advancing future work on allografts into the caprine (goat) model, the research team designed experiments to confirm whether the findings regarding MCA as a preferred allograft scaffold could be repeated using caprine marrow. The researchers used marrow samples from four caprine subjects to compare SR processing on two allograft formulations: MCA and MCA+DCBP. The mean selection ratios for MCA and MCA+DCBP were 2.15 ± 1.75 and 1.79 ± 0.62, respectively. No benefit was seen in adding DCBP.

To further develop the BME device, the researchers evaluated three rotating needle designs. Preliminary testing in canines demonstrated that BME, when performed with a preferred prototype, significantly increased the yield of bone and marrow-derived cells and CTPs. A final prototype is now available for further in vivo testing during euthanasia of large animal subjects to refine and document this tool for increasing the cell pool that is available for intraoperative processing and transplantation.

Magnetic Selection with the Hexapole Magnetic Separator

The research team has successfully tested the hexapole magnetic separator on canine and human bone marrow samples. The hexapole magnetic separator can process 2×10^9 cells in 1 hour. The team designed a new separation chamber to better match the magnetic field geometry for high-throughput operation using a flat-bottom geometry and a central post to exclude cells from the central area of the chamber where the field strength is weakest. Cells located in the weakest area of the magnetic field required long retention times in the magnet to be retained against the tube wall; this custom channel minimizes the amount of time the cells require in the magnet while still offering a large total capacity.

The researchers loaded HA^{+++} cells onto canine MCA using either a drip soaking method or a manual SR method. Both effluent samples contained significantly less CTP-Os than the HA^{+++} fraction. This result demonstrated the important finding that CTP-Os retain their ability to attach preferentially to allograft matrix after MS processing, resulting in an increase in concentration and prevalence of CTP-Os. The researchers selected the drip soaking method for use in the first in vivo assessment of MS processed cells. They obtained HA^{+++} cells by hexapole MS and loaded them by drip soaking onto MCA. This result was compared in the CFMD model to whole bone marrow loaded by drip soaking. After loading there was, on average,

1 CTP-O per 2,770 nucleated cells retained in the MS graft compared to 1 CTP-O per 6,242 cells in the marrow aspirate graft.

CFMD Model

Work in Project 4.2.1 during the past year identified MCA as the preferred allograft substrate for in vitro assessment of the SR of processed cell populations. However, the in vivo performance of MCA in the CFMD has been found to be exceptionally good.

The research team completed an in vivo assessment of magnetically separated HA^{+++} cells transplanted on an MCA scaffold versus heparinized marrow without MS processing using the CFMD model as part of Project 4.2.2 on cell sourcing. The magnitude of bone formation in both the pericortical and intramedullary regions was slightly greater using MS processed HA^{+++} cells; however, these differences were not statistically significant. These data also illustrate that a well-processed allograft provides a consistent and high level of performance in the CFMD model. There is concern, in fact, that the magnitude of the bone formation is now limited by a "ceiling effect," making the CFMD model insensitive to further increments of improvement. It may also be true that the CFMD model is not sufficiently limited by a local deficiency of progenitor cells given the relatively rich source of CTP-Os from local periosteum, endosteum, and intramedullary marrow and perivascular cells.

At the time of this report, the analysis of the comparison of MS processed cells and heparinized marrow is not yet complete. Based on the micro-CT analysis and the initial assessment of histology in two samples of each material, the research team decided to make an additional investment in completing a quantitative assessment of histology from all samples. This analysis will characterize the relative area of new mineralized bone, residual allograft, unmineralized osteoid, fibrous tissue, fat, hematopoietic marrow, and vascular spaces. If the review of these data were to indicate that MS processed cells were associated with a more advanced stage of remodeling, enhanced reconstitution of hematopoietic marrow, or reduced fibrosis, then the recommendation would be made to advance the development of MS processing into assessment in the CCTD model.

Pilot Assessment of SR Processing in the CCTD Model

In collaboration with Dr. Joseph C. Wenke and colleagues at USAISR and Dr. Elizabeth Pluhar, DVM, at the University of Minnesota, the protocol for this pilot assessment was designed and amended to fix the single protocol that will be followed at the three treatment facilities. The team completed the series of pre-procedure surgeries for all 12 animals on April 18, 2011. The first treatment procedure was performed on April 20, 2011. The research team projects that collection of all defect samples will be completed in the near future.

Key Research Accomplishments

- Demonstrated that DS devices were no more effective than manual methods. Therefore, future assessment of DS can be done using manual methods.
- Fixed a BME device design that is ready for in vivo use to increase the cell pools that are available for processing and transplantation.
- Fixed the allograft sourcing and design specifications. Determined a preferred MCA composition and tested it both in vitro, using SR, and in vivo in the CFMD model.

- Demonstrated, via in vitro testing of magnetically separated cells, that there was no loss of selective adherence behavior on the allograft, suggesting that a combination of MS and SR processing is clinically feasible.
- Achieved scale-up of protocols for the magnetic cell separation hexapole magnetic separator system, enabling in vivo assessment.
- Completed an in vivo assessment of magnetically separated HA^{+++} cells, transplanted on an MCA scaffold, versus heparinized marrow without MS processing, using the CFMD model.
  - Bone formation using MCA with both cell sources was robust and not different than the performance of MCA alone.
  - Histological assessment is ongoing to determine the extent to which differences in remodeling or marrow tissue reconstitution may be present.

Conclusions

MCA is the preferred allograft substrate to assess, in vitro, the SR of processed cell populations. The in vivo performance of MCA in the CFMD model appears to have hit the ceiling effect after which further improvement will be difficult to achieve or detect. Therefore, the research team recommends that further advancement in evaluation and comparison of cell processing methods should be performed in the more rigorous CCTD model.

The research team's in vitro testing of magnetically separated cells demonstrated no loss of selective adherence behavior on the allografts, suggesting that a combination of MS and SR processing is clinically feasible.

The team completed its in vivo assessment of magnetically separated HA^{+++} cells transplanted on an MCA scaffold, versus heparinized marrow without MS processing, using the CFMD model. Findings to date have revealed that bone formation using MCA is very robust, regardless of the cell source, and is not different from the performance of MCA alone in the CFMD model. This presents the likely limitation of a ceiling effect, as discussed previously.

Histological assessment is ongoing to determine the extent to which differences in remodeling or marrow tissue reconstitution may be present. The decision regarding whether and how to advance MS processing into the CFMD model in year 4 will be based on this assessment.

Research Plans for the Next 2 Years

In year 4, ongoing work will further refine the preferred process parameters and loading capacity for an optimized MCA substrate. In vitro assessment of SR processing using the calcium-coated AFIRM scaffolds being developed in Project 4.2.1 is also projected.

A BME device prototype will be used at the time of euthanasia to refine and document the use of this device as a tool for increasing cell pools that are available for processing and transplantation in CCTD studies and to support the development of a clinical trial to compare bone marrow aspiration and BME methods.

The progress of MS processing is uncertain. At the time of this report, analysis of the in vivo comparison of MS processed cells and heparinized marrow is not yet complete. The research team expects that completion of the histological analysis to compare sites

engrafted with MS processed cells, versus heparinized marrow alone, will reveal an advanced state of remodeling and a more effective reconstitution of the hematopoietic bone marrow without inflammation or fibrosis. If so, the researchers hope to progress into further development and testing of MS processing using the CCTD model. If not, MS processing will be downselected from further funding in year 4 in favor of an exclusive focus on products derived from BME and SR processing.

Planned Clinical Transitions

The methods for cell sourcing and processing described in this report will be combined with top-performing scaffolds and advanced into clinical trials, most likely in collaboration with the Major Extremity Trauma Research Consortium. Depending on the preferred method, clinical trial methods involving "minimal manipulation" of autogenous cells could begin within the next year. The consortium consists of a network of clinical centers and one data coordinating center that work together with the USAISR to conduct multicenter clinical research related to AFIRM regenerative strategies in limb salvage.

Corrections/Changes Planned for Year 4

As discussed previously, histological assessment will determine if continued investment in MS processing is justified in year 4. This analysis will characterize the areas of new mineralized bone, residual allograft, unmineralized osteoid, fibrous tissue, fat, hematopoietic marrow, and vascular spaces.

Advancing Bone Repair Using Molecular Surface Design (MSD) and/or Oxygen Delivery Systems

Project 4.2.3a, RCCC

Team Leader(s): George Muschler, MD (Cleveland Clinic) and Linda Griffith, PhD (MIT)

Project Team Members: Luis Alvarez, PhD, Jaime Rivera, BS, and Linda Stockdale, MAT (MIT) and Vivek Raut, MS and Chris Heylman, BS (Cleveland Clinic)

Collaborator(s): Sunil Saini, PhD (Integra LifeSciences)

Therapy: Advancing bone regeneration in large defects using osteogenic cells, osteoconductive scaffolds, and presentation of bioactive factors using MSD strategies.

Deliverable(s): Scaffold for bone regeneration presenting tethered epidermal growth factor (tEGF) and O2-generating substrate for tissue engineering applications.

TRL Progress: 2009, Product 1; 2010, TRL 2; 2011, TRL 2; Target, TRL 5

2009, Product 2; 2010, TRL 2; 2011, TRL 2; Target, TRL 5

Key Accomplishments: The researchers engineered clinically relevant versions of the β-TCP binding peptide (β-TCPBP)-EGF fusion protein, produced at high purity, and demonstrated bioactivity. They developed two crucial assays for accurate cell counting of human mesenchymal stem cells (hMSCs) and canine/human CTP-Os on two-dimensional (2-D) surfaces and 3-D β-TCP scaffolds. They demonstrated in vitro performance of β-TCPBP-EGF tethered scaffolds in CTP-Os; no in vitro toxicity was observed. They demonstrated an increase in concentration and prevalence of CTP-Os on β-TCP scaffolds, validating methods of cell loading on scaffolds. Finally, they developed crucial assays for the in vitro analysis of TheriLok I-β-TCP crosses with and without β-TCPBP-EGF.

Key Words: Tethered EGF, connective tissue progenitors, large bone defect, scaffolds, β-tricalcium phosphate binding peptide, oxygen tension, hypoxia

Introduction

This project focuses on highly promising methods to enhance the survival and performance of CTP-Os, which can be transplanted on a scaffold. Transplanted cells face a very harsh wound environment in which oxygen levels plummet and chemical signals tend to drive cells to die via a path of necrosis or a path of programmed cell death called apoptosis. Across three laboratories, Projects 4.2.3a and 4.2.3b are developing practical methods that will improve cell survival. At MIT and the Cleveland Clinic (Project 4.2.3a), the researchers are exploring two methods that are described in this report: (1) MSD, which is used to incorporate bioactive factors onto the surface of a scaffold such that cells transplanted onto the scaffold receive signals from these factors that will inhibit early cell death and even stimulate early phases of regeneration and (2) local oxygen delivery into the area where cells are most threatened. In parallel, researchers at Rutgers University (Project

4.2.3b) are developing approaches for tethering osteoinductive biomolecules.

MSD involves linking (tethering) bioactive molecules to the surface of a biomaterial as a means of improving control over the cell and tissue response to the implant material. Tethering ensures that the signaling molecule does not easily diffuse away from the cells and enables control over the concentration and presentation of the signal so that the effect can be optimized. In this project, EGF, a pro-survival and mitotic bioactive factor, was tethered to the surface of scaffolds that contain CaP coatings (e.g., β-TCP) using high-affinity, noncovalent bonding of a novel β-TCP binding peptide (β-TCPBP).

Local oxygen delivery involves the use of biologically compatible salts that, when combined with water, will recombine to release molecular oxygen (O_2). In this project, salts with this property are incorporated into a degradable polymer in a form that can be strategically placed into the area of a large bone defect. By controlling the rate of degradation of the polymer, oxygen can be delivered over the course of a few hours to more than a week.

Product 1: Tethered EGF Delivery System

There are no current products designed to enhance the survival and proliferation of transplanted cells using the local delivery of matrix-tethered EGF. The Infuse™ bone graft with BMP-2, which is delivered by adsorption on an implanted scaffold and rapidly released in a soluble form, is thought to act on both local and transplanted cells but is not considered to be a pro-survival factor. As a result, methods for EGF delivery are projected to not only enable more effective cell transplantation but also to be synergistic with the local delivery of BMP-2 and other scaffolds and signaling technologies in the highly compromised bone defect environment.

Product 2: Oxygen Delivery System

There are no oxygen delivery products on the market. Dr. Benjamin Harrison at Wake Forest University is an AFIRM investigator who is also actively working on local oxygen delivery strategies. There are some similarities in the selection of salt reagents, but the polymer platforms being developed for controlled O_2 delivery are entirely different.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers fabricated 2-D scaffolds presenting tEGF and a fibronectin-derived peptide, P12, that enhances new bone tissue formation. They developed and implemented a protocol for spin coating 2-D scaffold surfaces with and without comb coating to which mesenchymal stem cells immortalized with the hTERT gene attached and proliferated. They were able to detect β-TCP on scaffold surfaces. They also established methods for the quantitative measurement of in vitro cell response (i.e., attachment, survival, proliferation, and migration) and methods/protocols to characterize 2-D scaffold design using radiolabeling and analytic chemistry techniques. Additionally, they developed an optimized β-TCPBP that incorporates multiple β-TCP binding sites in fusion with EGF. The researchers began in vitro MSD studies using CTP-O cells. They determined that tEGF can exert a strong proliferative response in CTP-O cells seeded on 3-D β-TCP scaffolds even while cultured under osteogenic conditions. They also determined that tEGF does not reduce the early differentiation potential of CTP-O cells as evidenced by sustained alkaline phosphate levels at day 7 versus controls. They determined that oxygen deprivation (as

opposed to glucose deprivation) is the cause of CTP-O cell death in a hypoxic cell environment. Finally, they successfully fabricated oxygen-generating microbeads and confirmed oxygen delivery from the microbeads.

During the second year of the project, the researchers showed that β-TCPBP-EGF increased proliferation of hMSCs, culture expanded on β-TCP scaffolds, without compromising the ability of hMSCs to differentiate into osteoblasts. They found that tEGF increased proliferation of MSCs while preserving differentiation while soluble EGF does not preserve differentiation of MSCs. They employed β-TCPBP technology to design new proteins for the survival and protection of CTP-Os. They demonstrated that tethered P12 increased CTP-O colony-forming efficiency and migration as well as the proliferation of hTERT cells. The research team established a system for local oxygen delivery using polymeric microparticles based on drug delivery methods. They discovered that slowing the rate of decline of oxygen is sufficient to increase CTP-O colony-forming efficiency and increase proliferation of cells and colonies.

Research Progress – Year 3

Product 1: Tethered EGF Delivery System

During the past year, the researchers at MIT designed the clinically relevant version of the β-TCPBP-EGF fusion protein (CR β-TCPBP-EGF), which will be used in clinical studies. The CR β-TCPBP-EGF protein was engineered to reduce length, remove potentially immunogenic epitopes, and simplify in-house manufacturing. Significant advances were made in the yield and purity of protein expression such that the current products are comparable to standards in the protein production field. Validation of its bioactivity (e.g., induction of proliferation, differentiation, and survival of marrow stromal cells) using hMSCs was completed, and a detailed protocol for β-TCPBP-EGF surface preparation for use in in vitro and in vivo studies was developed.

The researchers at the Cleveland Clinic conducted a series of experiments to develop a protocol for the in vitro analysis of TheriLok I-β-TCP scaffolds with and without CR β-TCPBP-EGF. This initial assessment focused on the capacity of this construct to support SR, colony formation, and proliferation of osteogenic cells.

Product 2: Oxygen Delivery System

The researchers at the Cleveland Clinic characterized CTP-O oxygen consumption in a 2-D in vitro hypoxic environment. Data suggest that oxygen consumption by the progeny of CTP-Os will decrease as oxygen tension falls in the graft site. This is predicted and may suggest that the requirements for local oxygen delivery that will be needed to prevent necrosis or apoptosis of transplanted cells may be much lower than that needed to support maximal consumption. These results were used in a mathematical model (previously described in year 2) to tailor the oxygen release kinetics of the oxygen delivery device to meet the changing needs of cell consumption following transplantation. Further, the release profile of oxygen-generating microparticles using existing oil-in-water methods were shown to be insufficient for delivering effective amounts of oxygen to CTP-Os. New methods enabling the controlled release of the oxygen over the desired time span of 96 hours have been developed and demonstrated in vitro.

Key Research Accomplishments

Product 1: Tethered EGF Delivery System

Researchers at MIT:

- Engineered, produced, purified, and quantified the purity of five clinically relevant β-TCP binding proteins: MBP-β-TCPBP-C2-EGF, MBP-β-TCPBP-C1, MBP-β-TCPBP-C1-EGF, MBP-β-TCPBP-HS, and MBP-β-TCPBP-HS-EGF.
- Determined that the protein with the C1 coil has optimal properties for clinical development.
- Developed two new assays to quantify attachment, proliferation, and spatial distribution of hMSCs in 3-D scaffolds from the time of seeding through more than 2 weeks of culture.
- Developed a protocol to determine the bioactivity of β-TCPBP-EGF by dose-response assay (pERK1/2).
- Continued to support Cleveland Clinic in vitro studies of SR and proliferation of CTP-Os on β-TCPBP-EGF-tethered β-TCP crosses.

Researchers at the Cleveland Clinic:

- Evaluated the in vitro performance of β-TCPBP-EGF, protein-tethered, 3-D TheriLok scaffolds and 2-D β-TCP coverslips using human and canine progenitor cells. These assessments demonstrated no evidence of toxicity associated with exposure to β-TCPBP-EGF protein.
- Developed crucial assays for the in vitro analysis of TheriLok I-β-TCP crosses with and without β-TCPBP-EGF.
- Developed (in collaboration with MIT) a new method to fabricate 2-D β-TCP surfaces to provide a model system to quantify CTP-O colony performance using a colony-forming unit assay.
- Developed new imaging methods for the quantification of CTP-O response on TheriLok scaffolds and 2-D β-TCP coverslips.

Product 2: Oxygen Delivery System

Researchers at the Cleveland Clinic:

- Demonstrated that oxygen consumption in human osteogenic human CTP-O cells decreased as oxygen concentration declines.
- Showed that oil-in-water emulsion techniques for SPC encapsulation were insufficient as a fabrication strategy.
- Demonstrated methods enabling sustained oxygen delivery for more than 96 hours in a formed scaffold.
- Exploring methods for increasing the oxygen-carrying capacity of SPC microparticles by further limiting burst release.

Conclusions

Product 1: Tethered EGF Delivery System

EGF is a bioactive molecule that has been shown to increase proliferation of bone-forming progenitor cells in vitro and likely in vivo when tethered to the surface of a biomaterial. EGF was chosen for tethering and MSD fabrication because it is a key effector in bone development and regeneration. EGF has been shown to be a potent mitogen for osteoblastic progenitors and is both necessary and sufficient to induce colony growth. The EGF receptor is expressed by virtually every cell type involved in bone and nerve regeneration, including angiogenic cells. EGF signaling also has potentially important

antiapoptotic (pro-survival) effects on cells under stress. EGF tethered to commercially available β-TCP-containing scaffolds (available from many different commercial sources) is expected to improve healing in a large bone defect. Methods for presenting EGF on the surface of any scaffold containing β-TCP have been established by creating a fusion protein combining a β-TCP binding protein and EGF in one molecule. A new protein construct has recently been generated with a structure that will simplify manufacturing and regulatory review. In an effort to define the effect of coil sequence on β-TCP binding affinity to obtain optimal binding, MIT produced the original MBP-β-TCPBP-C1-EGF and three versions of the clinically relevant binding proteins (along with controls lacking the EGF domain): (1) CR MBP-β-TCPBP-C1-EGF, (2) CR MBP-β-TCPBP-C2-EGF, and (3) CR MBP-β-TCPBP-HS-EGF. The high binding affinity of the fusion protein with the C1 coil made it the clear choice for the clinic. The new 3-D cell quantification assays will allow MIT to quantify proliferation of hMSCs on β-TCPBP-EGF-tethered scaffolds over almost three orders of magnitude and determine cell distributions.

Product 2: Oxygen Delivery System

A benchmark for sufficient oxygen delivery was established by quantifying CTP-O oxygen consumption as a function of oxygen concentration. Oil-in-water methods for oxygen-generating microparticles were shown to be an insufficient fabrication strategy for delivering effective amounts of oxygen to CTP-Os. Methods enabling sustained oxygen delivery for more than 96 hours in a formed scaffold have been demonstrated, which can be further advanced by additional increases in long-term oxygen-carrying capacity that further limit burst release.

Research Plans for the Next 2 Years

Product 1: Tethered EGF Delivery System

MIT will continue to support Cleveland Clinic in vitro studies of SR and proliferation of CTP-Os on β-TCPBP-EGF-tethered β-TCP crosses. Ongoing work will reverify the bioactivity of this construct and test the efficacy of β-TCPBP-EGF protein in vitro based on effects on SR of osteogenic progenitors, survival, and proliferation of CTP-Os under hypoxic conditions (0.1% O2). The overall goal of year 4 in vitro and in vivo studies is to progress the tEGF technology from TRL 2 to TRL 4. Satisfactory performance in the biologically relevant CFMD model in year 4 will position this technology for further testing in a more rigorous, clinically relevant long bone defect model. If TRL 4 is achieved and sufficient funding is provided in year 5, future work would focus on the refinement of GMP manufacturing and FDA compliance of the tEGF delivery system. This path would enable consideration for an Investigational Device Exemption (IDE) for Phase 1 clinical assessment near the end of year 5.

Product 2: Oxygen Delivery System

This project will not be pursued further under RCCC in year 4 in part because a more advanced oxygen delivery system is being developed elsewhere.

Planned Clinical Transitions

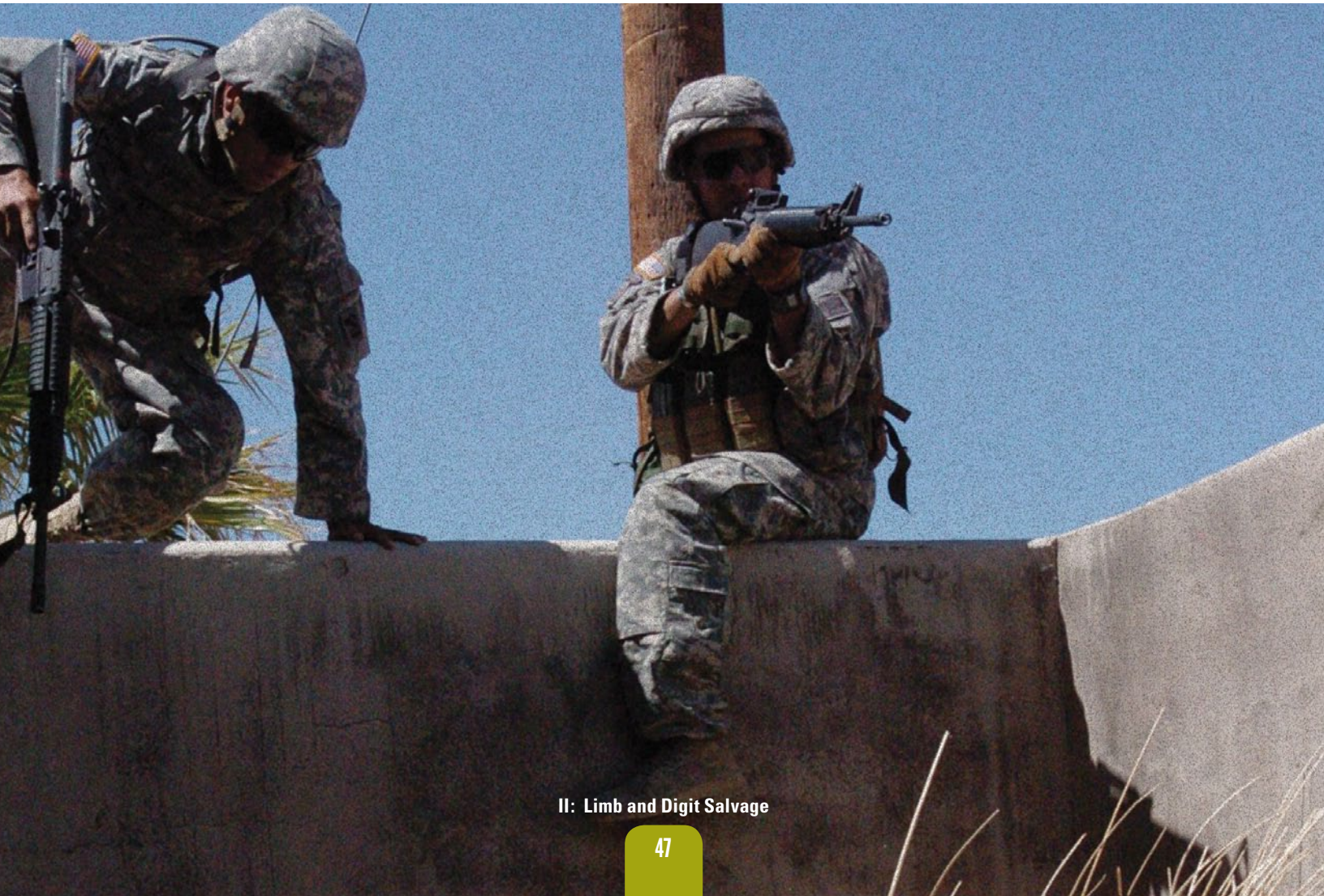
The technology transfer office of MIT is actively engaged in commercialization efforts around MSD strategies. Researchers at MIT have filed an invention disclosure and for patent protection related to the presentation of tethered biomolecules and the β-TCPBP.

Researchers at MIT and the Cleveland Clinic recognize that proof of efficacy in an in vivo setting is considered to be a critical step in establishing a commercial value proposition for a potential partner and have made plans to advance into in vivo studies. A regulatory pathway that includes a PME process must be expected in this product class. Therefore, MIT and the Cleveland Clinic have scheduled a pre-IND meeting with a representative of the FDA.

Corrections/Changes Planned for Year 4

Project 4.2.3 has closely followed the initial plan in rapidly screening potential ligands and tethering strategies related to MSD.

The development of the β-TCPBP and the opportunity to deliver tEGF using this versatile platform is a significant advancement over comb polymer methods described in the initial proposal. Systems of metrics for in vitro performance have been established to demonstrate biological feasibility and bioactivity. B-TCPBP-EGF offers a robust method of MSD of β-TCP-containing scaffolds with characteristics that afford cellular protection, enhanced proliferation, and preservation of osteogenic potential. This effect is expected to have valuable clinical implications, increasing the survival and performance of local and transplanted progenitor populations and improving the magnitude, rate, and reliability of bone regeneration in the compromised wound environment as a result.



Advancing Bone Repair Using Molecular Surface Design (MSD): Biodegradable Scaffolds with Tethered Osteoinductive Biomolecules

Project 4.2.3b, RCCC

Team Leader(s): Jared Bushman, PhD (Rutgers University)

Project Team Members: Sven Sommerfeld and Richard Farias (Rutgers University)

Collaborator(s): None

Therapy: Advancing regeneration of bone in large bone defects.

Deliverable(s): Improve control over the cell and tissue response to an implant material by tethering osteoinductive biomolecules to the scaffold surface.

TRL Progress: 2009, TRL 2; 2010, TRL 2; 2011, TRL 3; Target, TRL 4

Key Accomplishments: The researchers modified a biodegradable polymer so that large osteoinductive biomolecules can be efficiently tethered to the polymer surface. They confirmed that this polymer could be fabricated into a scaffold material and that proteins could be efficiently tethered in a complex 3-D scaffold. They also modified an osteoinductive protein for tethering, purified the modified protein, and showed equivalent activity.

Key Words: Bone, scaffold, tether, bone morphogenetic protein 2, bone morphogenetic protein 7, platelet-derived growth factor

Introduction

Significant injuries to bone occur on a large scale in military and civilian populations. It is estimated that 60% to 90% of all traumatic injuries sustained in armed conflicts involve significant injury to extremities. A large subset of these injuries involves bone fractures, some of which are accompanied by loss of surrounding soft tissue. Estimates of injuries in the civilian population vary, but hundreds of thousands of procedures are performed each year to address significant defects in the bones of extremities. Many of these injuries can be treated with conventional methods whereas some are too severe and face poor prospects for recovery. The current methods in bone repair and regeneration for large defects involve using materials that have not been designed specifically for large defects. Despite some success, the failure of these materials is commonplace. The lack of products specifically designed for these injuries is likely a reflection on the complexity of the injuries (i.e., common loss of surrounding soft tissue) as well as the necessity to include biological factors to regenerate large defects, which in turn increase the regulatory burden.

RCCC is seeking to develop therapies to treat injured warriors whose care requires the regeneration of bone in large defects

(5–20 cm). Projects 4.2.3a and 4.2.3b specifically seek to enhance the performance of implanted scaffolds used for bone repair by improving control over the cell and tissue response to an implant material using MSD in which specific bioactive ligands (i.e., recombinant osteoinductive proteins and small peptides) are tethered on a scaffold surface. These efforts are described in both this and the prior report.

Tethering of such biomolecules via MSD to scaffold surfaces has the potential to greatly reduce the quantity of the biomolecules required to achieve the desired biological response. This would in turn reduce the necessary amount of these factors, such as rhBMP-2, rhBMP-7, or rhPDGF-BB, and thereby decrease the cost and increase utilization of the device. Tethering, as opposed to adsorption or mixing, prevents biomolecules from being wasted by diffusing out of the injury site, reducing the potential for ectopic bone formation that can occur when osteoinductive proteins diffuse away from the target area. Tightly tethered biomolecules would also be less susceptible to receptor-mediated endocytosis, which would prolong the time for which they remain active in the injury site.

There has been much effort to develop a device with tethered biomolecules, yet no predicate devices exist. This is likely due to a combination of the following issues: (1) inefficiency of the tethering reaction; (2) slow speed of tethering reactions; (3) high cost of GMP biomolecules, particularly recombinant proteins; (4) reduced potency of tethered biomolecules; (5) difficulty tethering biomolecules throughout complex 3-D scaffolds; and (6) inflexibility of the tethering platform to easily adapt to a variety of biomolecules and scaffold devices. While some of these issues, such as cost of GMP proteins, are unavoidable, many of the remaining issues must be addressed to field a viable device with tethered biomolecules.

The Rutgers University group is developing a system that overcomes many of the previously described deficiencies of biomolecule tethering. This approach modifies TyrPCs to enable the tethering of bioactive molecules to complex 3-D scaffolds. The major advantages of the tethering mechanism developed at Rutgers University are (1) protein tethering is specific; (2) complete tethering is rapid and occurs in just minutes; (3) tethering is efficient, minimizing any waste to biomolecules; (4) the tethering bond is extremely strong; and (5) there is tremendous versatility as any biomolecule (or combination of biomolecules) can be tethered nearly regardless of its individual chemistry.

Summary of Research Completed in Years 1 and 2

At the conclusion of year 2, the Rutgers University group had found that TyrPCs could be successfully modified to facilitate protein tethering and that films synthesized with these polymers bound elevated levels of a fluorescent model protein as compared to unmodified TyrPC.

Research Progress – Year 3

During the past year, the Rutgers University group began an analysis of the TyrPC polymer properties to assess for any changes relative to the unmodified polymer. Analysis of the modified TyrPC found that it was similar to that of the unmodified TyrPC. The glass transition temperature and water contact angles of modified TyrPC were all very close to the values obtained for unmodified TyrPC. The modification to TyrPC also did not disrupt the favorable cell attachment characteristics

of the unmodified TyrPC with an equivalent proportion of primary hMSCs attaching to the modified TyrPC.

In Projects 4.2.1 and 4.5.1, the researchers have shown that TyrPC scaffolds are very effective at regenerating critical-size bone defects. To test whether the TyrPC modified to facilitate protein tethering could be fabricated into similar scaffolds, porogen leaching was used to fabricate 15 mm x 2 mm scaffolds that were subsequently sterilized by exposure to ethylene oxide. Measurements of polymer molecular weight found that the fabrication and sterilization processes similarly affected the modified and unmodified TyrPC polymers. The processes of fabrication and sterilization are known to decrease polymer molecular weight, and the decreases in molecular weight noted for the modified TyrPC fell within an acceptable range of less than 10%. Measurements of the dry and wet moduli also support that sterilized scaffolds fabricated from modified TyrPC are equal to unmodified TyrPC scaffolds in their potential to handle compression stress, a key characteristic for devices intended to regenerate bone. Together, these data strongly indicate that the scaffolds fabricated from modified TyrPC will perform at the same high level as those fabricated from unmodified TyrPC.

Sterilized scaffolds fabricated from modified TyrPC were next evaluated by SEM to analyze the scaffold microstructure. Control scaffolds from unmodified TyrPC have both macropores and micropores that are important for infiltration of bone-forming cells. SEM images from unmodified TyrPC and modified TyrPC showed that they had similar architectures **(Figure II-1)**. In the scaffolds made from the modified TyrPC, structure was retained for both macropores (approximately 300 µm diameter) and micropores (approximately 10 µm diameter). These structural features are thought to be integral for the thorough infiltration of bone progenitor cells throughout the scaffolds.

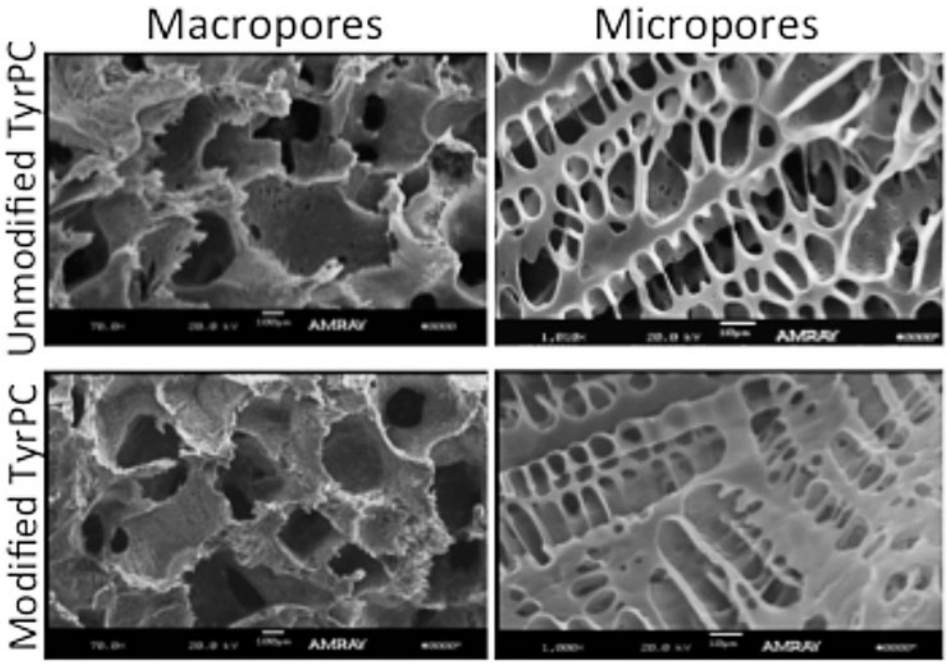


Figure II-1. SEM images of scaffolds fabricated from unmodified TyrPC and TyrPC modified to enable protein tethering. Scale bars on macropore images represent 100 µm, and micropore scale represents 10 µm.

Although beneficial for cell infiltration, the tortuous nature of porous scaffolds can be a significant hurdle for tethering proteins throughout such complex 3-D scaffolds. Using a simple syringe flow system, the researchers tested the ability to tether proteins in TyrPC scaffolds and found that uniform tethering could be achieved on the modified TyrPC scaffolds in only 30 minutes. A fluorescently labeled model protein was used in these experiments that would have the same binding propensity as rhBMP-2, rhBMP-7, or rhPDGF-BB to tether to the scaffold. Fluorescent confocal microscopy was used to determine the ability of the model protein to tether to the scaffold surface. The gain, offset, and exposure time were kept constant between modified TyrPC and unmodified TyrPC for each comparative image. A strong fluorescent signal was present from the tethered protein in all areas of the modified TyrPC scaffolds **(Figure II-2)** but not on the unmodified TyrPC scaffolds. Some macropores from unmodified TyrPC scaffolds showed little to no fluorescent protein whereas all macropores in modified TyrPC scaffolds were brightly fluorescent. These data show that tethering of the model fluorescent protein to the modified TyrPC occurs quickly and is thoroughly distributed throughout complex 3-D scaffolds. When this model protein was placed in a nontethering scaffold, it adsorbed nonspecifically to the scaffold surface but was not able to do so in a uniform manner. Additional experiments indicated that the tethering process developed at Rutgers University is also extremely efficient where nearly 100% of protein that is placed onto the modified TyrPC will specifically tether to the surface.

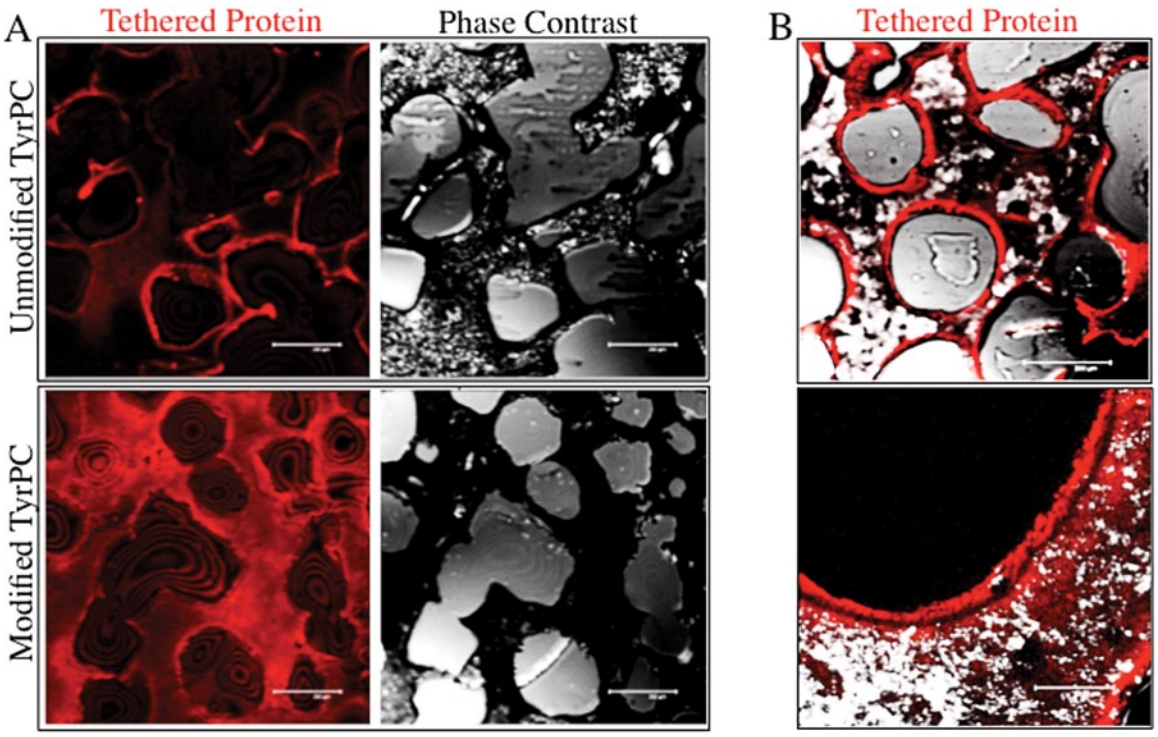


Figure II-2. High-affinity protein tethering versus nonspecific adsorption of a fluorescently labeled model protein in TyrPC scaffolds. (A) Fluorescent protein abundance and distribution after adsorption to unmodified TyrPC scaffolds and tethering to modified TyrPC scaffolds. (B) Images of a fluorescent model protein tethered to a modified TyrPC 3-D scaffold. Scale bar equals 300 µm with the exception of 75 µm for lower image on panel B.

Key Research Accomplishments

- Chemically modified TyrPCs to display a tethering moiety and found that the chemical modification did not alter the favorable polymer characteristics and fabrication potential.
- Determined that biomolecules can be tethered throughout complex 3-D scaffolds in a highly efficient manner.
- Initiated animal studies to test the efficacy of tethered osteoinductive proteins in critical-size bone defects.

Conclusions

The Rutgers University group has shown that TyrPCs can be chemically modified to enable osteoinductive biomolecules, such as rhBMP-2, rhBMP-7, or rhPDGF-BB, to be tethered to the polymer surface. The ability to tether osteoinductive biomolecules to the surface is expected to (1) increase the duration for which the biomolecules remain active to induce bone, (2) lower the dose of the costly biomolecules necessary to regenerate bone, and (3) control the localization of the osteoinductive biomolecules thereby minimizing waste and lowering the potential for ectopic bone formation. This technology is aimed at developing bone regeneration scaffolds that display osteoinductive biomolecules to allow for regeneration of large bone defects.

Research Plans for the Next 2 Years

In years 4 and 5, studies conducted by the Rutgers University group will increasingly move toward in vivo evaluations of safety and efficacy. Experiments will be conducted to determine the necessary amount of the osteoinductive biomolecules to regenerate critical-size defects in bone. These same in vivo experiments will also provide information as to the overall safety of the devices from the perspective of localized and humoral immune responses. Steps will be taken to address any concerns for the safety of the device.

It is also anticipated that the protein-tethering strategy with TyrPC could be used to act as a scaffold for tissue interfaces. In these scaffolds, multiple inductive factors could be tethered to a single scaffold in a spatially discreet manner. Infiltrating cells would be induced to become separate tissue types with an overlapping interface. The Rutgers University group will investigate this potential for creating bone-cartilage interfaces as are necessary to regenerate segments of missing joints and in damaged spinal cord.

Planned Clinical Transitions

Safety and efficacy studies conducted in year 4 will be instrumental in securing industrial partners. Several promising discussions have taken place with interested potential industrial partners, but commitment of commercialization interactions will likely be contingent upon the results of the in vivo studies to be conducted in the first months of year 4. Successful completion of these studies will prompt planning of GMP manufacturing and discussions with the FDA.

our science for their healing

Oxygen-Generating Biomaterials for Large Tissue Salvage

Project 4.4.6, WFPC:

Team Leader(s): Benjamin Harrison, PhD (Wake Forest University)

Project Team Members: Catherine Ward, BS, Sirinrath Sirvinsoot, PhD, and Zachary Haulsee, MS (Wake Forest University)

Collaborator(s): George Christ, PhD, James Yoo, MD, PhD, and Shay Soker (Wake Forest University)

Therapy: Supply temporary oxygen to hypoxic tissue.

Deliverable(s): Injectable oxygen-generating materials for tissue salvage.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 3; End Year 3, TRL 3

Key Accomplishments: The researchers have created a controllable, injectable, oxygen-generating biomaterial. They tested the oxygen-generating biomaterial for sustained release of oxygen in vitro and feasibility of injection in vivo. The research team demonstrated that the oxygen-generating biomaterial can improve the functional response of hypoxic skeletal muscle tissue in vitro.

Key Words: Oxygen, tissue engineering, tissue salvage, hypoxia, ischemia

Introduction

Replacement or restoration of tissue loss caused by traumatic injury, congenital defects, tumor removal, or severe burns is a challenge. For example, current treatment for reconstruction of volumetric muscle loss (VML) is associated with donor site morbidity and limited functional restoration. Tissue engineering and regenerative medicine offer the possibility of generating functional tissue for patients.

However, there are some significant challenges for generating large volumetric tissues. Metabolically active cells can only survive up to a few hundred micrometers away from a blood supply because of oxygen diffusion limitations. In newly formed engineered tissue, this supply is not present in the early stages and the avascular engineered tissue quickly becomes hypoxic internally. Researchers have used biological approaches to promote angiogenesis and while such approaches are able to stimulate host tissue responses associated with neovascularization, the extended time needed to establish the vascular network may be inadequate.

Preparing an injectable oxygen-generating material would allow delivery of oxygen in controlled amounts to engineered tissue scaffolds or pre-existing tissue. The ability to control the amount of oxygen delivered is important because different cell types can have different biological oxygen demands, and different oxygen tensions can trigger different biological effects in cells.

POGs are particles that have the ability to release oxygen when placed in aqueous environments such as in culture or in the body. These materials have previously been shown to aid in providing oxygen in an ischemic skin flap model in mice and in 3-D cell culture. The researchers strive to determine if POGs could provide a supplemental source of oxygen for cells and tissue experiencing a hypoxic environment similar to that seen in avascularized skeletal muscle constructs or in damaged skeletal muscle ultimately maintaining viability of the cells during the time it takes for a vascular network to be established or repaired.

The aims of the project include characterizing the novel biomaterial both in vitro and in vivo for optimal characteristics for tissue salvage and regeneration to establish its utility as an enabling technology providing oxygen in several situations.

POGs may overcome one of the major limitations in muscle salvage and tissue engineering by acting as a supplemental oxygen source in several regenerative models. Therefore, the goal is to provide oxygen at a therapeutic concentration and not necessarily to replicate standard cell culture conditions. The materials used are based on encapsulated solid peroxides that decompose upon contact with water to oxygen, water, and other biocompatible byproducts. Examples of the chemical equations governing oxygen generation are as follows:

$$4Na_2CO_3 \bullet 6H_2O_2 \rightarrow 8Na^+ + 4HCO_3^- + 3O_2 + 2H_2O + 4OH^-$$

Summary of Research Completed in Years 1 and 2

During the first year of the study, the researchers produced an injectable solution capable of in situ oxygen generation. They demonstrated that oxygen production can be sustained for up to 3 days. They also determined that the injectable oxygen-generating material is nontoxic to cells. During the second year of the project, the researchers began to use the technology developed during year 1 to establish collaborations with other AFIRM investigators and use the POG technology in relevant in vivo models.

Research Progress – Year 3

During the past year, the researchers focused on better defining the "product" that the oxygen-generating technology would be most applicable to the AFIRM. They have focused on applying this technology for skeletal muscle salvage and regeneration, specifically, the treatment of ischemic skeletal muscle. This task was accomplished by analyzing the ability of injectable POGs to aid in diminishing damage to skeletal muscle in a hindlimb ischemia model and, in addition, by analyzing the ability of 3-D POG constructs to provide a supplemental source of oxygen in a VML injury model.

The researchers first gained experience in a model system where skeletal muscle function can be evaluated. Extensor digitorum longus (EDL) muscles were removed from a rat model and assigned to treatment groups: no treatment, needle injection, saline injection, and POG injection. Muscles were mounted to a transducer between two electrodes for electrical field stimulation. Muscles were stimulated in a normoxic environment followed by stimulation in a hypoxic environment after some time. An important change in the methodology compared to typical muscle experiments of this type was the perfusion of the organ bath with nitrogen. Typically, oxygen is bubbled into the system to maximize the longevity of the muscle function. However, since oxygen generation

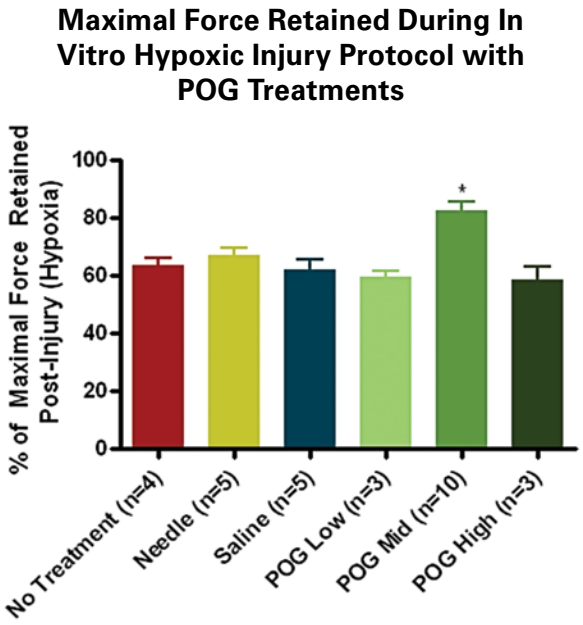


Figure II-3. Functional assessment of treatment groups. POG-injected muscles were able to maintain approximately 83% of function in a hypoxic environment. Future concentrations for POGs in skeletal muscle are based on these values.

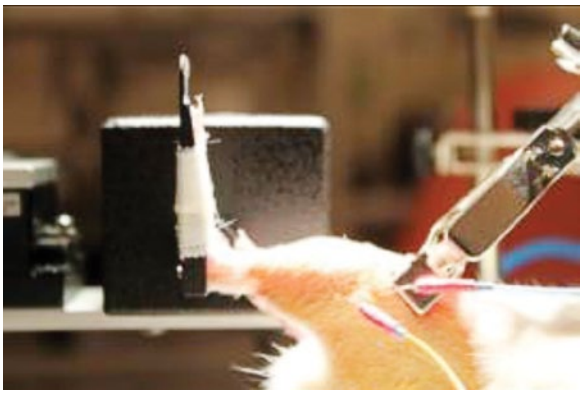


Figure II-4. In vivo servomotor with foot pedal, illustrating stimulus through electrodes positioned around nerve. Contraction of hindlimb muscles (anterior crural compartment) is measured as torque.

is the goal of the POG approach, this external source of oxygen had to be eliminated. Percentage of force retained by the muscle was then calculated. POG concentrations were calculated based on POG required for skeletal muscle in the hypoxic environment as shown in **Figure II-3**.

Hindlimb Ischemia Model with Injection of POGs

In collaboration with the Soker group (Project 4.3.1, Compartment Syndrome Program), the researchers began to use a model of induced ischemia in the hindlimb of a rat using an inflated cuff applied for 3 hours. After the application of pressure by the cuff, treatment groups included saline injections and POG injections into the injured leg. Fourteen days post-injury, both in vivo and in vitro functional assessments were performed. Functional recovery was observed in EDL muscles tested in vitro.

In vitro muscle function was analyzed using an organ bath system. Muscles were mounted onto a force transducer between two electrodes. Electrical field stimulation was performed to measure force based on increasing frequencies. To eliminate potential handling complications of removing muscles for in vitro testing, in vivo assessments leaving the muscle in place were also performed. In vivo functional assessments were taken using a customized servomotor (Aurora Scientific) with a foot pedal **(Figure II-4)**. Electrodes were placed in the limb, surrounding the common peroneal nerve, for muscle contraction. The results of the effects of POG on this compartment syndrome model 14 days after injury are shown in **Figure II-5** and **Figure II-6**.

The results did not show a significant difference between control and POG tested groups. A potential reason for the lack of difference may be due to experimental design.

It was observed by the Soker group in control tests that improvement in muscle function (i.e., less damage due to the pressure caused by compartment syndrome) occurred when a syringe needle penetrated the muscle during the application of pressure. It is theorized that the needle puncturing the muscle tissue may be performing a similar function to a fasciotomy by allowing the compartment pressure to be relieved.

In the future, improvement of the experimental design will be necessary to determine if POGs can have a significant effect in this model. In addition, a more extensive study will require a higher sample size to determine statistical effect. Finally, a supplemental study is ongoing in which an ischemic injury is created by occluding the femoral artery rather than by inflating a cuff around the entire limb. This study will allow for a more controlled injury to specific tissue and reduce swelling, which may have altered the results seen in the previous studies.

VML Injury with Tibialis Anterior Muscle Defect

Besides compartment syndrome, VML represents another significant muscle injury. In collaboration with the Christ group (Project 4.1.6, Burn Repair Program), POG-containing scaffolds were created for implantation in a tibialis anterior (TA) VML model. Scaffolds were composed of fibrin gels with specific concentrations of POG for muscle cell viability.

The researchers created 3-D POG–fibrin scaffolds to determine the ability of POGs to maintain viability in a large construct that would be used for implantation in a VML injury. Based on prior experience, a POG concentration was chosen that would be compatible with cells. One cubic centimeter scaffolds were seeded with C2C12 cells and cultured under standard cell culture conditions. At days 3, 7, and 14, scaffolds were

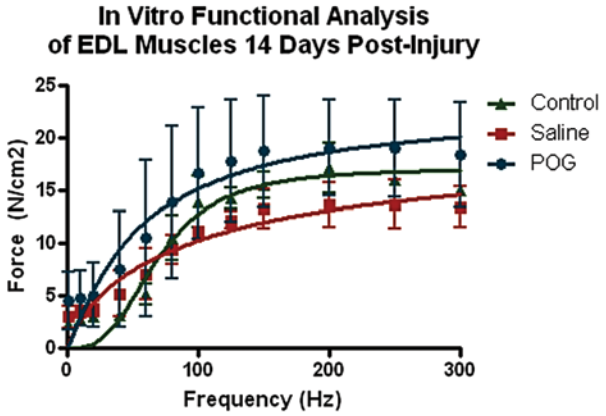


Figure II-5. Functional assessment of EDL muscles from injured hindlimb with a nonlinear fit curve. Electric field stimulation was performed with increasing frequencies. Control (or noninjured) muscles showed a characteristic force–frequency curve for EDL muscle. POG-treated muscles had slightly higher forces, and saline-treated muscles had slightly lower forces.

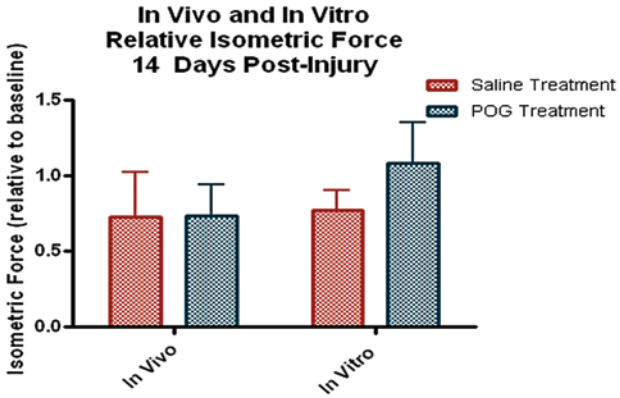


Figure II-6. Relative functional assessments for in vivo and in vitro analysis compared to baseline or control measurements. Both groups performed similarly in vivo when hindlimb was stimulated. With in vitro analysis, POG-treated muscles exhibited a greater relative isometric force when compared to control muscles. An increased sample size (currently n = 3) is needed to determine if the aforementioned trend is statistically significant.

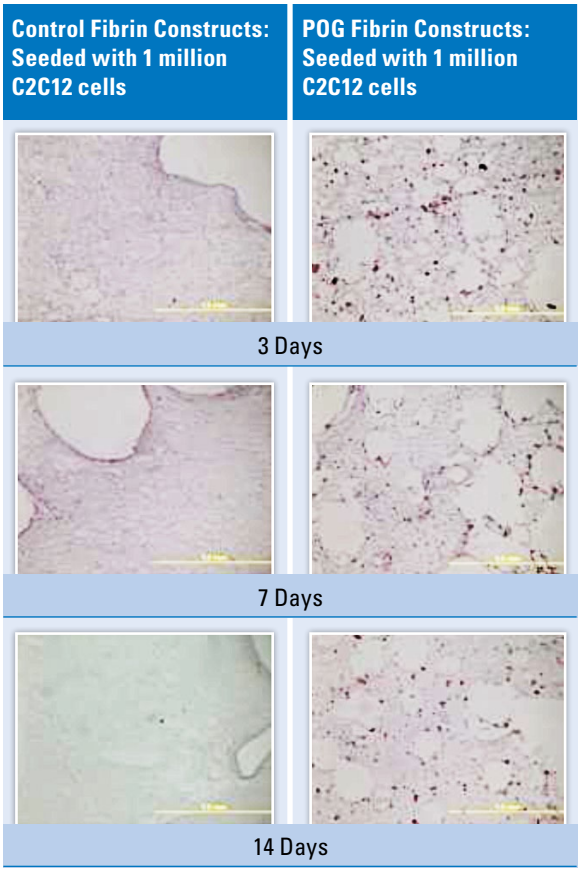


Figure II-7. Proliferating cell nuclear antigen staining of fibrin constructs illustrating live and proliferating cells. Control fibrin constructs had no proliferating cells at 3, 7, and 14 days in culture. POG–fibrin constructs exhibited proliferation throughout the 14-day study, which is within the time period it would take for this construct to be vascularized.

sectioned and stained for proliferating cell nuclear antigen to determine cell viability **(Figure II-7)**. Muscle cell viability was evident in constructs that contained POGs, suggesting that these scaffolds may support cell growth and allow for native tissue infiltration as vasculature begins to support the area.

Current work is now focused on using the POG-containing scaffolds in an in vivo model. This will involve creating a VML injury model **(Figure II-8)** by making a defect in the TA of a rat. POG scaffolds will then be implanted as a supplement to current tissue-engineering strategies once the model is established and quantified.

Key Research Accomplishments

- Demonstrated that the injectable oxygen-generating biomaterial can improve the functional response of hypoxic skeletal muscle tissue.
- Determined in vivo models for evaluating POGs for treatment of skeletal muscle damage.
- Created 3-D POG–fibrin scaffolds for implantation in a TA VML injury model, seeded the scaffolds with cells, and determined that these scaffolds support cell growth for at least 2 weeks post-seeding.

Conclusions

This project has focused on developing a chemically based oxygen delivery system. As this technology matures during year 4, the laboratory has increasingly become focused on testing the feasibility of delivering oxygen to assist in skeletal muscle salvage or regeneration. The results suggest that such materials may aid in engineering large tissue constructs that address the challenges imposed by the limits of oxygen diffusion.

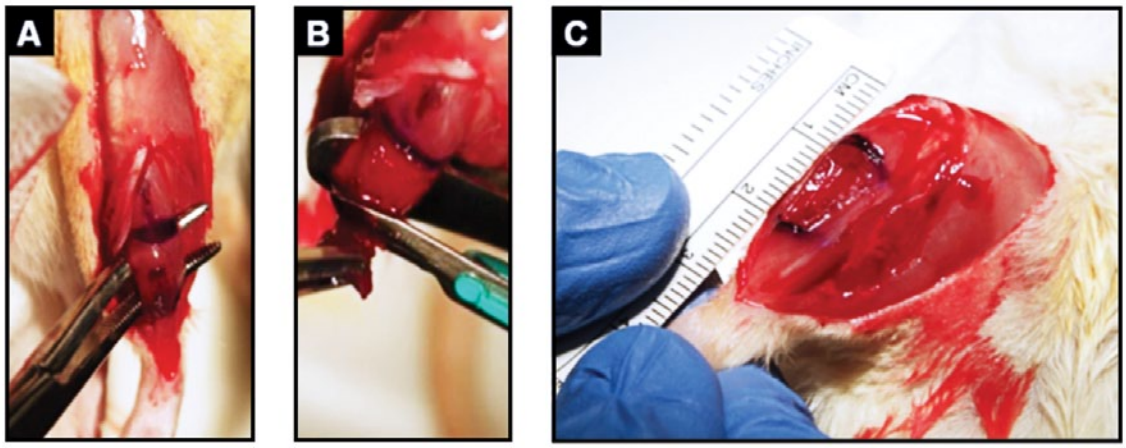


Figure II-8. Digital images of a TA VML injury in a rat. (A) Isolation of the TA. (B) Creation of muscle injury using a scalpel to remove a specified amount of tissue. (C) Injury site of TA. Scaffolds are formed to fit into the muscle defect, sutured to tissue, and the wound site is closed. Functional assessments are taken weekly to determine the recovery of injured muscle.

Research Plans for the Next 2 Years

During the next 2 years, animal studies will be initiated to evaluate the efficacy of the biomaterial. The technology will be tested in systems were hypoxia may cause detrimental effects and POGs may be beneficial. During year 4, the POG technology will be tested in different systems to determine which areas would most benefit from this approach. Because composite tissues are composed of multiple cell types, several tissues systems will be analyzed including skeletal muscle, bone, nerve, and skin. Optimization of the most promising tissue systems benefiting from the technology will continue in later years along with identifying the best possible clinical application.

Planned Clinical Transitions

While no immediate human clinical trials are currently slated under this AFIRM project, options are being explored including multiple pathways to incorporate into other AFIRM research as well as to leverage other funds to accelerate the time to clinic. It is anticipated that a clinical trial will be ready within the next few years.

Isolation and Expansion of Native Vascular Networks for Organ-Level Tissue Engineering

Project 4.5.8, WFPC

Team Leader(s): Geoffrey C. Gurtner, MD (Stanford University)

Project Team Members: Michael T. Longaker, MD, MBA (Stanford University) and Robert S. Langer, ScD (MIT)

Collaborator(s): None

Therapy: Vascularized tissue engineering.

Deliverable(s): Hydrogel-encased vascularized networks for organ-level engineering.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 2

Key Accomplishments: The researchers have initiated experiments to isolate the vascular network of rat superficial inferior epigastric artery flaps using detergents and enzymes. They have begun experiments aimed at defining the native matrix surrounding the explanted vessels and plan to test various decellularization strategies.

Key Words: Vascular engineering, hydrogel, bioreactor

Introduction

Injured or missing extremities, failing organs, and significant burn injuries continue to place a huge burden on wounded soldiers and society. Tissue engineering holds the promise of creating replacement limbs and organs outside of the human body. However, two major obstacles have hindered the development of techniques to fabricate limbs and organs: (1) the inability to adequately vascularize tissue constructs in vitro and (2) the inability to reintegrate these tissues into the systemic circulation. Many tissue engineering strategies start with cells implanted onto matrices and then attempt to induce the formation of a de novo vascular system. This approach has proven difficult because the complexities of in vivo neovascularization are difficult to recapitulate ex vivo. In contrast, the Gurtner team has developed novel strategies that utilize preformed native circulatory networks that can be supported ex vivo during organ fabrication, differentiated using progenitor cell-based techniques, and then readily integrated into the systemic circulation.

The researchers hypothesize that a native circulatory bed can be isolated and expanded ex vivo using progenitor cell- and small-molecule-based modalities to create a fundamental vascular unit from which to bioengineer limbs and organs. Their preliminary work has demonstrated the feasibility of sustaining EMBs ex vivo for up to 7 days. They have successfully transduced EMBs with nonviral vectors and demonstrated

post-reimplantation expression of therapeutic peptides. In addition, they have seeded EMBs with progenitor cells and induced sustained differentiation ex vivo following reimplantation. The technology has advanced beyond proof of principle toward a flexible, regenerative environment based on a bioreactor system. This innovative approach has allowed utilization of the pre-existing vascular system as a scaffold that can be manipulated ex vivo and subsequently reconnected to the circulatory system in vivo using standard microsurgical techniques.

Summary of Research Completed in Year 1 (funded in 2009)

During the first year of the study, the researchers initiated experiments to isolate the vascular network of rat superficial inferior epigastric artery flaps using detergents and enzymes. They also began experiments to define the native matrix surrounding the explanted vessels.

Research Progress – Year 2

The researchers are refining their technique for the isolation and maintenance of EMBs based on the rat superficial inferior epigastric vessels on an ex vivo bioreactor system to maximize preservation of the microvascular structures. They characterized the ultrastructural features of isolated EMBs using SEM and demonstrated that explanted vascular networks show a very fine and dense vascularization with microcirculatory connections between the feeding artery and vein **(Figure II-9)**, suggesting the suitability of EMBs for progenitor cell seeding. They established an in vitro decellularization protocol requiring several chemical and enzymatic digestion steps and achieved complete decellularization of tissue while preserving the matrix architecture

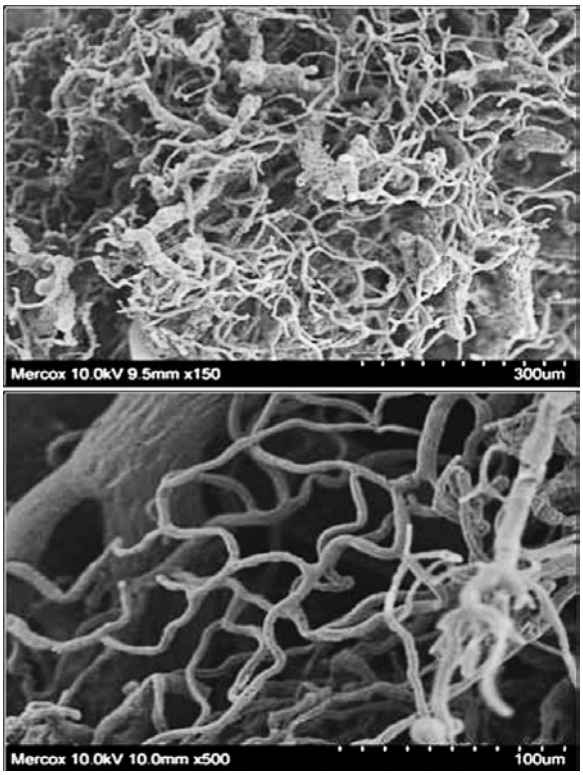


Figure II-9. SEM images of explanted native vascular networks demonstrate the finely organized microvascular structure. After explantation and preparation of feeding vessels, the flap was perfused with a casting agent (Mercox). The surrounding tissue was digested leaving a negative cast of the microvascular architecture. Scanning microscopy images demonstrate the very fine and highly organized microvascular structure at different magnifications.

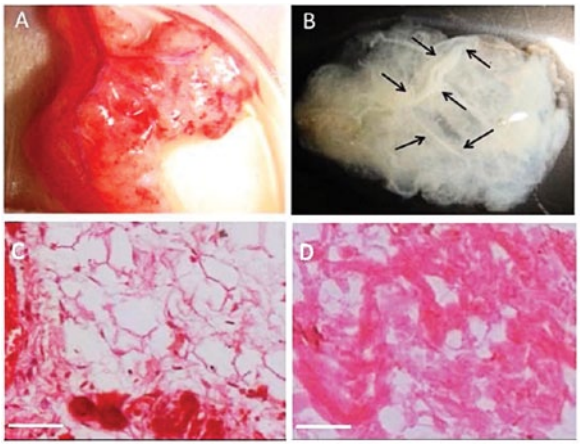


Figure II-10. In vitro decellularization of explanted vascular networks. (A) shows a photograph of the explanted vascular network before the decellularization process. After a multistep decellularization (B) the tissue becomes blanched and translucent. The intact vascular structures can still be seen macroscopically (black arrows). (C) and (D) show hematoxylin and eosin stainings before and after decellularization. While a fat globule-dense structure with cell nuclei is visible in (A), the decellularized matrix (B) appears to be more matrix dense and does not show any remaining cell nuclei.

and the macroscopic vascular structure **(Figure II-10)**. They then successfully seeded decellularized rat tissue flaps with mouse ASCs and demonstrated engraftment and persistence of these cells for extended time periods **(Figure II-11)**. The researchers were also able to improve EMB oxygenation through continuous perfusion with an oxygen carrier protein **(Figure II-12)**.

Key Research Accomplishments

- Isolated native vascular networks in rats using microsurgical techniques.
- Characterized the microvascular structure on an ultrastructural level.
- Established an in vitro decellularization protocol that preserves the matrix and vasculature while removing native parenchymal cells.
- Improved EMB oxygenation through continuous perfusion with an oxygen carrier protein.
- Seeded processed rat tissue flaps with mouse ASCs with engraftment and persistence of cells for extended time periods.

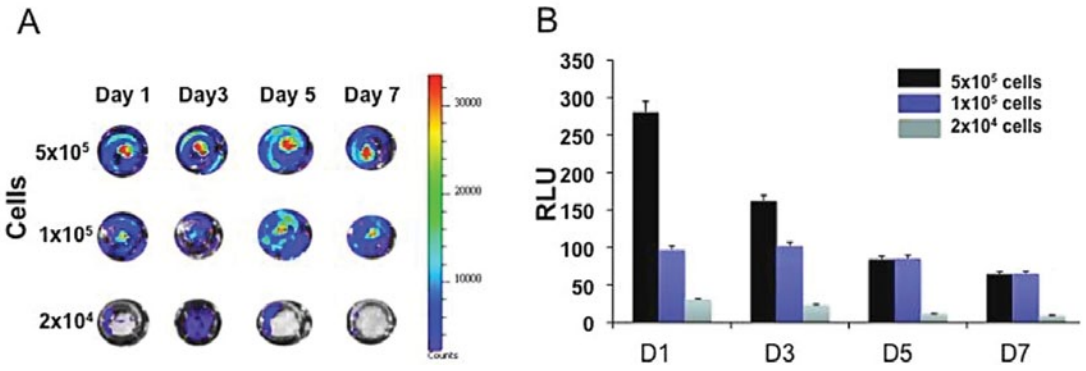


Figure II-11. Bioluminescence imaging of luciferase$^+$ mouse ASCs. Rat tissue flaps were seeded with Luc$^+$ mouse ASCs at different cell densities. Bioluminescence imaging was performed over a period of 7 days. Signal intensity increased with cell density and was the highest in the group seeded with 5 x 10^5 cells. The signal declined over the course of the experiment but was still above background levels on day 7. These data demonstrate that mouse ASCs can engraft and remain functional for extended periods following seeding onto processed EMBs.

Conclusions

The Stanford University group has successfully validated its protocol for the isolation and maintenance of EMBs based on the rat superficial inferior epigastric vessels on an ex vivo bioreactor system. The researchers have demonstrated that these explanted vascular networks exhibit a microcirculatory and macrovascular ultrastructure suitable for progenitor cell seeding. They have developed an in vitro decellularization protocol that achieves complete decellularization of tissue while preserving the matrix architecture and the macroscopic vascular structure. They have successfully seeded decellularized rat tissue flaps with mouse ASCs, and preliminary studies indicate that engrafted cells persist in a functional capacity for extended time periods.

Research Plans for the Next 2 Years

The Stanford University group plans to build on its successful external cell seeding of EMBs to develop a perfusion-based seeding protocol whereby stem cells are introduced directly through the afferent macrovasculature. The researchers will further optimize their decellularization procedure to balance the benefit of complete cell effacement with maintenance of perfusion and optimize the bioreactor system parameters to extend this ex vivo perfusion period. They will seek to integrate components of external and perfusion-based cell seeding to maximize engraftment and persistence of murine stem cells. Once these procedures have been fully optimized, they will transition to human ASCs, first demonstrating their in vitro capacity for osteogenic differentiation, and subsequently seeding these cells into nude rat EMBs for osteogenic evaluation by micro-CT.

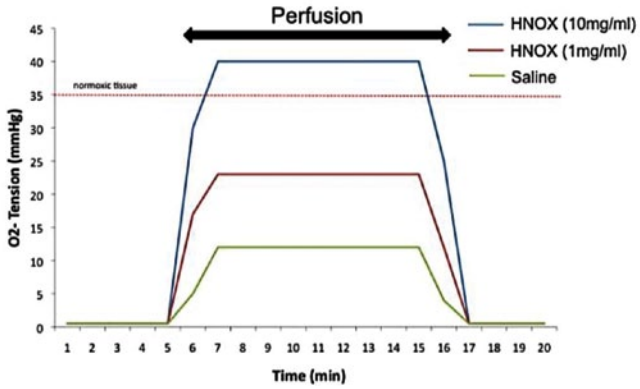


Figure II-12. Perfusion with oxygen carrier protein improves oxygenation. After harvesting the SIEA flap, the artery and vein were surgically prepared and cannulated. Oxygen tension was recorded throughout the experiment using an oxygen tension device connected to a needle probe inserted into the adipose tissue. A continuous application of either the oxygen carrier protein (HNOX 1 mg/mL, 10 mg/mL) or the control (saline) was established using a syringe connected to the arterial cannula and a flow rate of 0.75 mL/minute over a period of 20 minutes.

Development of Tissue (Peritoneum) Lined Bioabsorbable and Fracture-Resistant Stent Graft for Vessel Trauma

Project 4.3.2, RCCC

Team Leader(s): Timur Paul Sarac, MD (Cleveland Clinic)

Project Team Members: Malika Satirraju, MS and Michal Wiggins, PhD (PeriTec Biosciences)

Collaborator(s): None

Secondary Sites: Rod White, MD and Carlos Donyare, MD (University of California, Los Angeles Vascular Surgery); George Kopchak, MS (Lab Biomed, University of California, Los Angeles); Peter Gingras and Peter Mulroney (Proxy Biomedical); and Craig Bonsignore (Nitinol Development Corporation)

Therapy: Treatment of arterial and venous trauma.

Deliverable(s): Fracture-resistant and bioabsorbable tissue-lined stent.

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 4; Target, TRL 4

Key Accomplishments: The researchers have constructed several bioabsorbable PDO fiber-based, tissue-lined stent prototypes. They placed fracture-resistant, nitinol-based, tissue-lined stents into animals. All animal implants were successful after 30 days. The research team also developed a novel catheter delivery system.

Key Words: Stent, peritoneum (tissue), polydioxanone, mineral oil, blood vessel trauma, delivery system, catheter

Introduction

During the past 15 years there has been a transformation of vascular surgery toward minimally invasive therapy. Currently, the best minimally invasive therapy available to treat injured blood vessels is a stent lined with the synthetic material expanded polytetra-fluroethylene (ePTFE). However, the long-term success rates of these stent grafts are suboptimal as they are highly susceptible to infection and failure. There is no minimally invasive stent graft specifically designed to treat blood vessel injuries, taking into account the particular issues related to the trauma theater in combat and civilian populations. This is even more important as published reports from recent conflicts indicate that blood vessel injuries to our troops are five times higher than previously thought.

The purpose of this project is to develop stent grafts specifically designed to meet these needs. Year 3 goals were to develop a bioab-sorbable and fracture-resistant, tissue-lined stent graft for minimally invasive treatment of arterial and venous trauma. The stent graft has the requirement of being a durable, bio-absorbable material and/or fatigue-resistant metal structure with enough strength for the short term to cover the injury and prevent exsanguinations. Absorbable stent designs are advantageous for as the stent absorbs, the tissue heals into the arterial wall overcom-ing long-term fatigue issues. The tissue lining

utilized in this project (bovine peritoneum) is known to be resistant to acute thrombosis, intimal hyperplasia, and infection. The fracture-resistant, nitinol-based stent design may provide sufficient fatigue strength as a stand-alone delivery system. The combination of bovine peritoneum and bioabsorbable and/or fracture-resistant stent in this hybrid technology is designed to allow the body to incorporate the tissue into the vessel wall and seal the injury immediately but also to overcome long-term durability issues. The goals of this project are to extend this technology with specific development geared for long-term durability of minimally invasive stent grafts for blood vessel trauma.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the study, the researchers evaluated the tensile strength of several polymers. They chose PDO as an appropriate polymer for fabrication of a bioabsorbable tissue-lined stent to treat traumatic arterial and venous injuries, with poliglecaprone as a second choice, and polyglactin as a third choice. They identified a 3–6-month window for the degradation time line of the polymers. They discovered that mineral oil will serve as an effective storage emollient for the stent graft. They developed prototypes of two tissue-lined stent grafts and a novel anhydrous solution for the storage of bioabsorbable materials.

Research Progress – Year 3

The overall goal of this project is to have a tissue-lined stent graft to treat arterial trauma that lasts a lifetime. The aims for year 3 were to design specific stents that would accommodate tissue and treat blood vessel injuries. The specific aims were to design a fracture-resistant, nitinol-based stent **(Figure II-13)** that would accommodate tissue and be able to be used in animals, and a bioabsorbable PDO stent **(Figure II-14)** that would accommodate tissue and also be used in animals. In conjunction, a minimally invasive catheter needed to be designed to crimp the stent on the table to be user friendly to deliver the stent.

Fracture-Resistant, Tissue-Lined Stent

In conjunction with the Nitinol Development Corporation, a fracture-resistant, nitinol-based stent graft was designed specifically to accommodate tissue. In year 2, accelerated fatigue tests were performed, which documented that the design was successful. In year 3, the design was extended to smaller-length lesions (7 mm diameter and 40 mm length), which would also be sufficient for an animal iliac injury model. All animal implants were successful after 30 days.

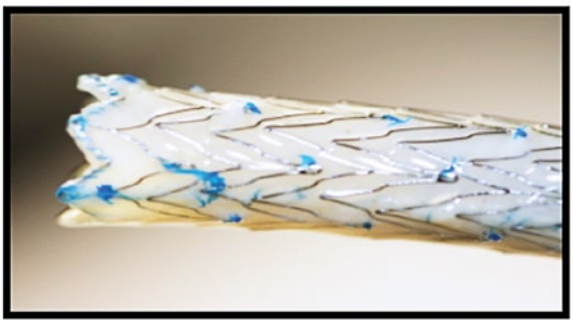


Figure II-13. Fracture-resistant nitinol stent graft.

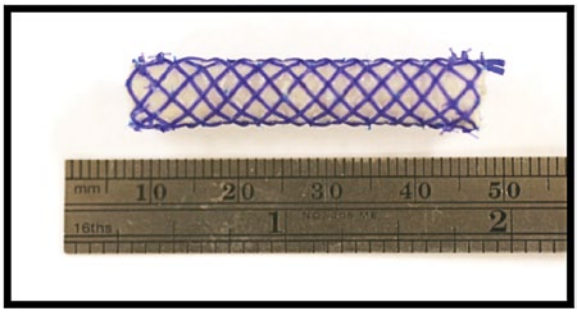


Figure II-14. PDO bioabsorbable tissue stent graft.

Bioabsorbable PDO Tissue-Lined Stent

The first prototype bioabsorbable stent grafts were designed and developed using a fiber-based technique. Five different prototypes were constructed by varying the fiber diameter and weave pattern. Changes were made in the design to accommodate tissue but also to account for radial compressive force and crimpability. It was not possible to crimp this into an adequate 9–10F profile range to be minimally invasive.

Delivery System

The first-generation catheter delivery system consists of a 96-strand mesh, which allows crimping of the stent into a cartridge, and then screws on to a braided catheter delivery system. However, this system is time consuming and cumbersome for a trauma environment and commercial application. Therefore, a second-generation catheter system was developed. Prototypes of the PDO system have been successful, and the researchers are preparing for manufacturing.

Key Research Accomplishments

- Manufactured fracture-resistant nitinol stent grafts.
- Manufactured fiber-based PDO bioabsorbable stent graft prototypes.
- Completed a successful 30-day animal trial for the fracture-resistant stent design.
- Developed a user-friendly, second-generation crimp and delivery system.

Conclusions

The proof of concept has been validated in that a fracture-resistant nitinol stent graft can specifically be designed to accommodate tissue. This concept was successfully tested in a 30-day animal trial. While many prototypes of PDO stent grafts were successfully produced, it has been more challenging to crimp the stent into a desired smaller profile. In one prototype, the fracture-resistant stent design was translated into a polymer, which can easily be crimped. The next stent will be extruded using the desired polymer, PDO, into tubes that can then be cut into the desired fracture-resistant design. A successful second-generation, user-friendly catheter delivery system was also developed.

Research Plans for the Next 2 Years

The plan for the next 2 years is to translate the fracture-resistant stent graft design for nitinol into PDO-based, bioabsorbable, tissue-lined stent grafts. The first step is to manufacture lots of PDO, which takes several months. Next, PDO tubes will be extruded of appropriate dimensions that will be cut into the fracture-resistant stent design. Following this, the cutting of the stent with an athermal laser will be tested. Otherwise, it may have to be machine-tooled. Once this is complete, the tissue will be sewed on the stents and the radial compressive force of the stents tested to be certain it could maintain laminar blood flow. The last step before in vivo implantation is to determine the degradation of the stents in mineral oil as performed in previous work. Finally, a 30-day animal trial and histology will be completed, followed by 180-day Good Laboratory Practice studies and histology. At this point a pre-IDE meeting will be scheduled, working toward a Humanitarian Device Exemption (HDE).

Planned Clinical Transitions

Since this is a platform technology that is applicable to arterial and venous occlusive disease, dialysis access failure, and liver transjugular intrahepatic portosystemic shunt procedure, the entrance into the commercial market will be hastened by using this area for commercialization with an HDE specialized for trauma. This approach has worked for predicate bovine pericardium and ePTFE-lined balloon expandable stent grafts used for coronary artery perforation after angioplasty. Currently, Peritec Biosciences has supported the project, and continued development efforts are expected. However, other potential major industry partners have been identified.

Corrections/Changes Planned for Year 4

The major change during year 4 is to specifically concentrate on the regenerative component of this project, the bioabsorbable stent graft. The commercial partner will continue to move the nitinol stent graft into a viable product. The rationale for this is that the bioabsorbable component is regenerative, is the most futuristic and desired component of the project as recommended by the Executive Committee, and has the best long-term potential for success in young soldiers.

Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents

Project 4.4.3a, RCCC

Team Leader(s): Kathleen Derwin, PhD (Cleveland Clinic)

Project Team Members: Joseph Iannotti, MD, PhD and Sambit Sahoo, MD, PhD

Collaborator(s): Stephen F. Badylak, MD, PhD, DVM

Therapy: Scaffold for repair of large abdominal wall defects and hernias.

Deliverable(s): A fiber-reinforced biologic scaffold or a combination biologic plus synthetic scaffold.

TRL Progress: 2009, TRL N/A; 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Key Accomplishments: In year 3, the Derwin group identified a candidate material for fiber reinforcement. The researchers demonstrated how fiber reinforcement can be used to alter the mechanical properties of biological grafts derived from ECM. They are currently assessing candidate stitch designs and fibers using burst and tensile tests while they await the arrival of new biaxial test equipment. Their technology is being licensed to a commercial partner.

Key Words: Extracellular matrix, abdominal wall, hernia, allograft, xenograft

Introduction

Researchers have experienced major challenges in designing methods to repair abdominal wall defects and hernias that result from traumatic injuries, surgery, or chronic diseases. Currently available synthetic and biological grafts have demonstrated limited success. Abdominal wall injuries sustained during military trauma are typically large (with massive loss of tissue) and infected, and one of every three cases fails to heal. Abdominal wall hernias also complicate 10% of all abdominal surgeries in the civilian population. While the use of synthetic meshes has reduced the risk of recurrent hernias, these repairs often require re-operation as a result of infection, bowel adhesion, extrusion, and fistulation. Significant complications can be avoided through the use of biological grafts for hernia repair. Dermal grafts are commonly used in clinical practice since they possess biomechanical properties similar to abdominal wall fascia. However, implanted dermal grafts lose mechanical strength and integrity over time, leading to complications such as bulging and hernia recurrence. Hence, engineered improvements to biologic grafts are necessary to make them more suitable for abdominal wall repair and reconstruction.

Summary of Research Completed in Years 1 and 2

During the first year of the study, the researchers developed two new, clinically

relevant bench tests for suture retention properties of scaffolds: the modified-ball-burst test and the tension-with-side-constraint test. They developed a rat subcutaneous model for evaluating the in vivo host response, degradation, and loss of suture retention following implantation of large (4 x 4 cm) scaffolds and used the model in pilot studies. They established a magnetic displacement tracking system and a multiactuated mechanical test system for human cadaver studies. Finally, they developed clinically relevant surgical methods for performing rotator cuff repair with scaffolds on cadaver shoulders, including a human cadaver model for testing the extent to which augmentation with scaffolds improves the biomechanical outcomes of rotator cuff repairs.

During the second year of the study, the researchers developed the design and testing methods for the reinforced fascia patches ECM-derived biological grafts. They finalized the rat model for dorsal subcutaneous pouch implantation. They also finalized the human cadaver model for rotator cuff tendon repair with and without scaffold augmentation. Three critical feasibility studies for rotator cuff repair are ongoing. The research team also developed and validated a fluoroscopic method for measuring tendon repair gap formation.

Research Progress – Year 3

During the past year, the Derwin group demonstrated that ECM-derived biological grafts stretch significantly and irrecoverably when loaded. The researchers showed that if the grafts were fixed under pretension (simulating surgical implantation of graft under tension), their subsequent elongation on loading was significantly reduced. The researchers also demonstrated that a reverse-cutting needle could suture thick grafts—apart from being easy to use, it reduced graft elongation and did not predispose the graft to failure upon repeated loading.

Using the group's proprietary fiber-reinforcement technique, strips of ECM-derived biological grafts were reinforced to significantly reduce their elongation at physiological loads. Fiber reinforcement also improved the stiffness of graft patches at low loads and increased the maximum load that could be borne by the patches, supporting the concept that fiber-reinforced biologic grafts could prevent bulging and recurrence and improve the long-term outcome of hernia repairs. The Derwin group is now further developing the reinforced biologic grafts to better maintain their mechanical properties post-implantation and provide longer-lasting mechanical support to the repaired abdominal wall thus preventing its bulging and reherniation.

Key Research Accomplishments

- Demonstrated how fiber reinforcement can be used to alter the mechanical properties of ECM-derived biological grafts.

Conclusions

ECM-derived biological grafts stretch significantly with loading. Graft suturing using a reverse-cutting needle, or graft fixation after application of pre-tension, significantly reduced the graft's elongation on repeated loading. Biological grafts could be fiber reinforced, using the group's proprietary technology, to improve their stiffness and reduce elongation at physiological loads and also to increase the total amount of load that the graft could bear before failing.

Research Plans for the Next 2 Years

In year 4, the Derwin group will focus on the development of reinforced ECM constructs that maintain mechanical properties post-implantation with hopes to prevent bulging and recurrence of the hernia. The researchers will conduct proof-of-concept studies to optimize fiber material and design of reinforced ECM grafts for abdominal wall repair applications. The reinforced grafts will be evaluated using both the burst/tensile and biaxial test equipment. In year 5, the candidate graft material(s) will be evaluated in a preclinical large animal hernia model. In addition, the industrial collaborator will conduct manufacturing, toxicity, packaging, and shelf life studies. The team expects a progression of TRL from 3 to 5 in years 4 to 5.

Planned Clinical Transitions

In year 4, a clinical team will be assembled to provide guidance for specific clinical indications and for the preclinical study that is anticipated in year 5. As the device moves into product development and a regulatory path is defined, the industrial partner will initiate human trials.

Corrections/Changes Planned for Year 4

The year 3 Statement of Work had also aimed to develop strategies to promote tissue adhesion and functional integration of an implanted graft with host tissues. This aim has been terminated to focus only on the primary objective: the development of a fiber-reinforced biologic graft.

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Functional Scaffolds for Soft Tissue Repair and Joint Preservation

Project 4.4.3b, RCCC

Team Leader(s): Charles J. Gatt, Jr., MD and Michael G. Dunn, PhD (University of Medicine and Dentistry of New Jersey and Rutgers University)

Project Team Members: Aaron Merriam, BS, (University of Medicine and Dentistry of New Jersey and Rutgers University)

Collaborator(s): Joachim Kohn, PhD and Sanjeeva Murthy, PhD (New Jersey Center for Biomaterials and Rutgers University)

Therapy: Regeneration of fibrocartilaginous tissue such as the meniscus of the knee.

Deliverable(s): An implantable scaffold composed of biodegradable, polymer fiber-reinforced collagen sponge for repair of knee meniscus.

TRL Progress: 2009, TRL 3; 2010, TRL 4; 2011, TRL 4; Target, TRL 5

Key Accomplishments: The researchers continued the development and evaluation of their second-generation meniscus scaffold for the treatment of moderate to severe meniscal damage. They conducted a small animal study, which demonstrated biocompatibility of the second-generation scaffold. They are preparing/beginning a functional large animal study with the second-generation scaffold.

Key Words: Meniscus, scaffold, degradable polymeric fibers, collagen, regeneration, joint preservation

Introduction

This project addresses a large burden to the military and its personnel—injuries to the musculoskeletal system, specifically, the meniscus and cartilage of the knee joint. Musculoskeletal injuries are the most common types of injuries seen in the military and occur six times more than any other injury type. A recent study on the reasons for military medical evacuation from operations found that the most common cause for evacuations was musculoskeletal and connective tissue disorders, 24%, while combat injuries only accounted for 14% of all evacuations.

Severe damage to the knee meniscus can significantly impair the normal activity of a military member. Due to limited healing capabilities, meniscal injuries are often treated with surgical resection. This treatment leads to symptom relief but in many cases results in the development of degenerative arthritis of the knee. The pain associated with damaged meniscal tissue as well as arthritis in the knee can have a debilitating effect on the ability of service men and women to perform, inhibiting their operational capability in both the short and long term.

The focus of this project is the development of a tissue-engineered meniscus scaffold for the treatment of moderate to severe meniscal damage. The overall goal is to develop an off-the-shelf clinical device that can be implanted at the site of a meniscal resection and result in knee joint preservation. The clinical role of the device is twofold: (1) to provide symptom

relief and rapid return of function for active military personnel and (2) to prevent the progression to degenerative knee arthritis that commonly requires costly total knee replacement surgery later in life. Previous successful implantations of a functional load-bearing fibrocartilaginous structure in a sheep model demonstrated implant survivability with good tissue integration and biocompatibility. Based on these findings, the Gatt/Dunn team is advancing this material platform by creating a second-generation meniscus scaffold with improved geometries, mechanical strength, and chemotactic properties.

Summary of Research Completed in Years 1 and 2

During the first year of the study, the researchers developed a novel meniscus scaffold consisting of high-strength, resorbable, tyrosine-derived polymeric fibers arranged within a collagen matrix, which promotes the synthesis of new, organized tissue when implanted as a total meniscal replacement in sheep. They completed full meniscal scaffold implantation surgeries in sheep. Half of the implants failed due to improper positioning of the posterior anchor attachment of the scaffold; the researchers developed a new surgical procedure to address this issue. They demonstrated a new synthesis of collagen at 8 weeks after meniscal scaffold implantation, which tends to organize along the longitudinal axis of polymer fibers. Immune staining demonstrated the presence of types I and III collagen, which are major constituents of fibrocartilage.

During the second year of the study, the researchers designed, fabricated, and tested a second-generation scaffold. They began a nonfunctional small animal study with the second-generation scaffold. The Kohn laboratory obtained mechanical properties and degradation profiles for five polycarbonates. Significant enhancements using automated synthesis accelerated the evaluation of wide banks of polycarbonates for the meniscus scaffolds.

Research Progress – Year 3

Altering the scaffold design will improve the long-term outcome of the meniscus replacement. This has been accomplished by changing the collagen sponge portion of the scaffold, altering the fiber pattern, adding radial tie fibers, and improving the fixation method of the scaffold.

Development of the Collagen Matrix for the Meniscus Scaffold

The researchers evaluated several techniques of altering the collagen sponge portion of the meniscus scaffold. Altering the source and concentration of the type I collagen along with the addition of GAGs had the greatest improvement on its compressive and viscoelastic properties. Sponges fabricated with bovine Achilles tendon were shown to be mechanically superior to that used in the first-generation scaffold (bovine dermis collagen). The addition of GAGs to this new source of collagen further improved the mechanics of the sponges but only when added in small concentrations (0.025%). Larger concentrations (0.05%) did not significantly alter the mechanics.

Lower concentrations (0.025%) of GAGs increased the compressive modulus **(Figure II-15)** and decreased the permeability **(Figure II-16)** of the matrix, giving them characteristics closer to the native meniscus. These sponges also had significantly higher ultimate tensile strength and toughness, when pulled in tension to failure, compared to all other sponge matrixes. Higher concentrations of GAGs had little effect.

In Vivo Nonfunctional Rabbit Model

The objective of the nonfunctional rabbit model was to observe the in vivo response to the scaffold and determine its ability to initiate cellular infiltration, proliferation, and protein deposition within the construct.

Based on the mechanical results of the new collagen sponges, two new collagen matrixes

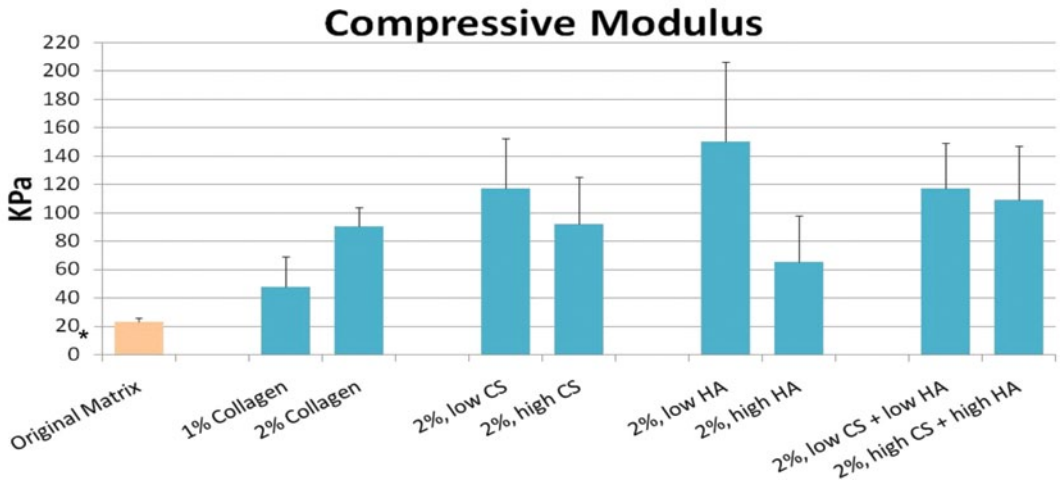

Figure II-15. Modulus during confined compressive creep of collagen sponges. Original matrix: 1% bovine dermis collagen. All others: bovine tendon collagen with GAGs.

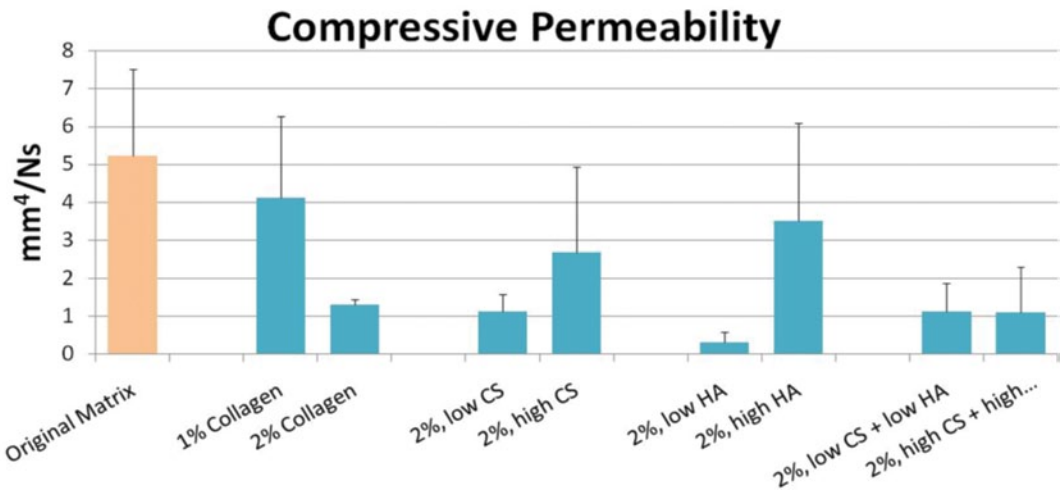

Figure II-16. Modulus during confined compressive creep of collagen sponges. Original matrix: 1% bovine dermis collagen. All others: bovine tendon collagen with GAGs.

were chosen for in vivo evaluation: (polymer fiber/2% Achilles collagen/0.025% hyaluronic acid) and (polymer fiber/2% Achilles collagen/0.025% hyaluronic acid and 0.025% chondroitin sulfate C). After implantation in the synovial space, the rabbits were sacrificed at 4 weeks (n = 5 per group, 2 groups) or 8 weeks (n = 5 per group, 2 groups) after surgery, and the implants were recovered for histological evaluation.

Preliminary investigation of the implant histology shows positive results for both new collagen matrices tested. At 4 weeks there was high cellular infiltration (mainly plasma cells), collagen matrix breakdown, and peripheral tissue ingrowth. After 8 weeks, the implants had become less cellular, new tissue deposition had occurred throughout the implant replacing the original collagen matrix, and new vascularization had formed throughout the implant. No noticeable differences were seen between the two different GAG treatments. The histology slides are currently being evaluated and graded by a pathologist at the University of Medicine and Dentistry of New Jersey, and results are pending.

Key Research Accomplishments

- Continued to develop the second-generation meniscus scaffold.
  - Changed the fixation method to interference screws for more rigidity.
  - Determined that lower concentrations of GAGs increased the compressive modulus and decreased the permeability of the collagen matrix, giving them characteristics closer to the native meniscus.
- Completed a nonfunctional rabbit model study with the second-generation meniscus scaffold.
  - Performed all nonfunctional surgeries.
  - Recovered implants at 4- and 8-week time points for histology.
  - Preliminary histological analysis indicated biocompatibility of the second-generation meniscus scaffold.

Conclusions

Overall, the researchers have shown that their novel hybrid meniscus scaffold promotes the synthesis of organized, new tissue when implanted as a meniscal replacement. Further optimization of the scaffold, as well as the surgical protocol, will improve the in vivo performance of the device and help prevent the onset of degenerative changes of the articular surfaces. The second-generation meniscus replacement is currently being implanted in a long-term sheep model. These implants will be recovered and evaluated in the upcoming year.

Research Plans for the Next 2 Years

The researchers are pursuing three objectives during the next 2 years of this project:

1. Long-term total meniscus reconstruction studies in sheep using second-generation scaffolds reinforced with resorbable poly(DTD-DD) fibers.
2. Long-term total meniscus reconstruction studies in sheep using scaffolds reinforced with resorbable fibers from the PLGA family.
3. Short-term and long-term meniscus repair surgeries in sheep using the scaffolds in a partial meniscectomy or “plug” model.

The first objective is a logical and necessary continuation of the researchers' previous implantation studies, which have shown that their novel fiber-reinforced meniscus reconstruction scaffolds are efficacious at 2 and 4 months after implantation. These studies are critical to determine long-term efficacy and satisfy regulatory requirements. The second objective is to evaluate second-generation devices with the same design but composed of polymers with a track record of FDA approval. The third objective is to evaluate both types of scaffolds in the "meniscus plug" (partial meniscectomy) model with sacrifices at 2 and 4 months post-operatively to determine the amount and type of tissue ingrowth and integration with surrounding meniscal tissue.

The research plan will address potential regulatory and commercialization hurdles for the total meniscectomy model. Scaffold efficacy in the plug model will be determined because the medical device industry is also interested in using this device to repair partial meniscectomies.

Planned Clinical Transitions

The research team is already in discussions and licensing negotiations with several major orthopedic implant manufacturers that specialize in sports medicine. The plan is to partner with one of these large established companies that will provide monetary support and regulatory guidance to bring this technology from TRL 4 through TRL 6 during approximately the next 3 years. After the preclinical large animal efficacy studies are complete, the industrial partner will assist in the design and funding of clinical trials.

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Peripheral Nerve Repair for Limb and Digit Salvage

Project 4.4.4, WFPC

Team Leader(s): Kacey Marra, PhD (University of Pittsburgh), David Kaplan, PhD (Tufts University), and Tom Smith, PhD (Wake Forest University)

Project Team Members: YenChih Lin, PhD, Mostafa Ramadan, MD, Lauren Kokai, PhD, Amir Mahan Ghaznavi, MD, Ryan Nolan, Samantha Beckowski, Danielle Minteer, BS, Wesley Sivak, MD, PhD, and Jedidiah McAtee, BS (University of Pittsburgh); Marie Tupaj and Alexander Nectow (Tufts University); and Jonathan Barnwell, MD, Zhongyu John Li, MD, PhD, Mark Van Dyke, PhD, and Lauren Pace (Wake Forest University)

Collaborator(s): Tirrell Laboratory at University of California, Berkeley; Harrison Laboratory at Wake Forest Institute for Regenerative Medicine; and University of Virginia Department of Orthopaedic Surgery (Hand Surgery)

Therapy: Combined strategy for regeneration over long (greater than 3 cm in human) peripheral nerve gaps.

Deliverable(s): Proactive biodegradable nerve guide/conduit system for peripheral nerve regeneration.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 2; End Year 2, TRL 3; End Year 3, TRL 3/4

Key Accomplishments: The researchers have established methods for incorporating pores, patterned silk films, electrospun silk fibers, growth factors, and electronics into biodegradable custom-designed silk nerve guides. They demonstrated that porous silk fibroin-based nerve conduits, infused with bioactive neurotrophic factors in a controlled manner, represent a potentially viable conduit for Schwann cell migration and proliferation in the regeneration of peripheral nerves. The researchers' results to date with keratin-filled nerve guides in NHPs have been promising, which has prompted further evaluations in additional NHPs.

Key Words: Limb regeneration, silk fibroin, nerve guides, drug delivery, keratin, functional electrical stimulation

Introduction

Approximately 1.9 million people are living with limb loss in the United States as a result of trauma, cancer, vascular problems, or congenital defects. It is well known that the presence of a copious nerve supply is a key factor in the regenerative ability among some amphibians following amputation. Peripheral nerve regeneration is a critical issue as 2.8% of trauma patients present with this type of injury. Following trauma, incomplete nerve regeneration and permanent demyelination may result, leading to lifelong disability. Several regeneration strategies are currently being employed in this project, including biophysical guidance, biochemical applications, and protein modification.

The researchers of this project are seeking to utilize the expertise and facilities of all three teams (Tufts University, University of Pittsburgh, and Wake Forest University) to (1) create a biodegradable nerve guidance system that delivers neurotrophic growth factors and biophysical guidance to regenerating peripheral nerves and (2) move this nerve guidance system to the in vivo level.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers developed a novel biodegradable conduit for nerve repair and conducted several in vitro and in vivo studies. Specifically, they examined nerve guides consisting of FDA-approved materials (collagen, silk, and polycaprolactone [PCL]) in a rat sciatic nerve defect. Neurotrophic growth factors (e.g., GDNF) were encapsulated in double-walled polymer microspheres, resulting in an extended, long-term release. These microspheres were embedded in the walls of the PCL and silk nerve guides. The researchers began to conduct sophisticated functional analyses on rats. Histological analysis of the guides included axon counting and inflammatory response evaluation. Preliminary results from a rabbit study indicated that keratin-filled collagen nerve guides could bridge a 2 cm rat tibial defect in more than half of the animals tested.

During the second year of the study, the examination of polymer/growth factor guides in a critical-size rat defect model demonstrating enhanced nerve repair resulted in the identification of a drug delivery strategy to deliver bioactive neurotrophic factors for approximately 60 days in vivo. The researchers also initiated NHP studies utilizing keratin-filled nerve guides. They completed preclinical testing of keratin biomaterials and prepared a pre-IDE package for the FDA.

Research Progress – Year 3

The researchers are testing a variety of nerve guides/fillers/drugs in this project **(Table II-3)**.

Filler Gel Assessment

Incorporation of GDNF double-walled microspheres into PCL nerve guides has demonstrated an off-the-shelf product alternative for the promotion of nerve regeneration. The University of Pittsburgh group received more keratin gels from the

Table II-3. Nerve guides/fillers/drugs that are being tested in this project. P = University of Pittsburgh; T = Tufts University; W = Wake Forest Institute for Regenerative Medicine; U = University of California, Berkeley

Nerve Guide Material	Filler	Drug (GDNF) Delivery	Animal Model
PCL (P)	Keratin gel (W)	PLGA double-walled microspheres (P)	Lewis rat, 1.5 cm sciatic nerve defect (P)
PCL (P)	N/A	PLGA double-walled microspheres (P)	NHP, 1.0 cm median nerve defect (P)
Silk (T)	N/A	Silk single-walled microspheres (T)	Lewis rat, 1.5 cm sciatic nerve defect (P)
PCL (P)	Keratin gel (W)	PLGA double-walled microspheres (P)	Lewis rat, 1.5 cm sciatic nerve defect (P)
PCL (P)	Keratin gel (W)	PLGA double-walled microspheres (P)	*Macaca fasicularis*, 1.0 cm median nerve defect (P)
O2-Generating Biomaterials (W)	N/A	N/A	Lewis rat, 1.5 cm sciatic nerve defect (P)
Peptide Amphiphile (U)	PA gel	PLGA double-walled microspheres (P)	Lewis rat, 1.5 cm sciatic nerve defect (P)

Wake Forest University team. The PCL conduit was filled with keratin gel and examined in a rat 15 mm sciatic nerve defect model. As an indicator of recovery, nerve sections were stained with S-100 and PGP9.5 antibody. The researchers found that the PCL nerve guides with GDNF-loaded, double-walled microspheres filled with keratin gel represented a potentially viable guiding material for Schwann cell and axon migration as well as proliferation in the treatment of peripheral nerve regeneration.

Neurotrophic Growth Factor and Protein Incorporation into Silk Biomaterials

Growth Factor Release Studies

During the past year, the Tufts University group continued studies that incorporated GDNF into silk biomaterials. Controlled GDNF microsphere release studies were conducted from silk in three ways: (1) GDNF microspheres alone in solution, (2) GDNF microspheres embedded in silk films, and (3) bulk-loaded GDNF in silk films. GDNF release kinetics from silk are summarized as follows:

- Group 1: Microspheres alone in solution demonstrated a high burst release (approximately 67% of GDNF was released by day 1).
- Group 2: Microspheres embedded in silk films exhibited a logarithmic release profile.
- Group 3: After day 1, linear release of GDNF was found in the bulk-loaded growth factor group with approximately 270 pg/day being released over 14 days.

Therefore, the researchers found that GDNF can be released in a controlled manner. Additionally, this system allows for the controlled release of multiple growth factors with differential release profiles.

Protein Incorporation onto Silk Films and Neuron Response Studies

The key challenge when developing a neural-material interface is identifying a neurocompatible material that can be implanted and last long term up to the lifetime of the patient. This past year, the Tufts University group worked on identifying a protein coating for its silk biomaterial that would engender nerve (and glial) attachment over the course of peripheral nerve regeneration. The researchers deposited 1 mM laminin, 1 mM gelatin plus 1 mM laminin, 0.1 M poly-d-lysine, or 1 M arginine-glycine-aspartic acid (RGD) peptides (via adsorption or chemical coupling) onto 8% silk films for the long-term assessment of nerve cell attachment. They found that silk coated with poly-d-lysine exhibited properties for increased neuron attachment over 12 days. The identification of additional peptides and proteins that increase neuron attachment has been continuing with the coupling of neural cell adhesion molecule to silk films.

Electric Field Device Design and Neural Responses

Electric fields are innate and ubiquitous in many organisms. For example, altering ion channel expression regulates phenotype during development in some animals, electric currents flow out of wounds in amputated newt and frog digits, changes in the electric properties of a tissue may be a sign of disease, and electric signals are the basis of communication and function in neuron and cardiac cells.

During the past year, the Tufts University group studied the effects on neurons exposed to electric fields at a range of field strengths. The researchers observed consistently increased expression in mid-marker nestin and late-marker B3-tubulin ($p < 0.05$,

day 6) on differentiating p19 neurons exposed to capacitively coupled alternating current fields for 45 minutes daily over 9 days. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays on differentiating neurons exposed to an inductively coupled alternating current field for 5 hours daily over 9 days revealed increased expression in B3-tubulin from day 6 to day 9 ($p < 0.05$). Increasing the field to 9 mT per day revealed significantly decreased nestin and B3-tubulin expression. Migration was consistently observed by 6 hours of exposure to 70V, DC.

The researchers also developed methods to incorporate electrodes onto flat and patterned silk films. For fabricating patterned electronic films (e-films), gold was deposited using a sputter coater onto a mask and silk film. Controllable e-film parameters included pattern dimensions, electrode dimensions, pattern/electrode orientation, and electrode material. Silk films were approximately 20 µm thick.

Electrospun Silk Fibers and Patterned Silk Films for Axon Outgrowth and Alignment

It is well known that surface topography may alter the interaction of cells, including attachment, viability, or alignment, with a biomaterial substrate. The Tufts University group has exploited surface topographies, such as patterns and fibers, for the purpose of guiding axon outgrowth. Fibers of 1–2 µm in diameter were spun onto a wheel for 1 or 5 minutes creating low- and high-density aligned fibers. Unaligned low-density fibers and unaligned high-density fibers (spin time 1 minute and 1 hour, respectively) were spun directly on a mat. Patterns were designed through laser etching "Pattern H" and "Pattern I" into the glass. Pattern H had grooves with a width of 3.5 µm and a depth of approximately 342 nm. Pattern I had grooves with a width of 3.5 µm and a depth of approximately 500 nm. Neurons were seeded at 250,000 neurons/cm^2.

Neuron axon alignment and outgrowth was quantified for all groups. The researchers found that aligned silk fibers supported the greatest amount of axon outgrowth (45 µm). Pattern I revealed the greatest alignment of 61.5%, 0–10 degrees from the direction of the groove. However, aligned electrospun silk fibers had 90% alignment within 0–20 degrees from the direction of the fiber.

Silk Nerve Guide Development

Growth Factor-Incorporated Silk Nerve Guide Designs

The researchers evaluated the efficacy of silk fibroin-based nerve guides containing GDNF in a long-gap-sized (15 mm) rat sciatic nerve defect model. Four groups of nerve conduits were prepared: (1) silk conduits with empty silk microspheres, (2) silk conduits with GDNF-loaded silk microspheres uniformly distributed in the conduit wall, (3) silk conduits with GDNF-loaded silk microspheres in a controlled fashion with the highest GDNF concentration at the distal end, and (4) isograft **(Figure II-17)**. After 6 weeks, the nerve grafts were explanted, harvested, and fixed for histological analysis. Empty microspheres (without GDNF) were evenly incorporated into silk tubes. Microsphere guides were constructed in two layers. For the inside layer, silk microspheres containing 1 µg of GDNF were mixed with a 25% concentrated silk solution and then rolled around a 1.5 mm stainless steel wire. The outside layer was gel spun using a 25% silk solution. SEM and confocal microscopy images provided nerve guide dimensions, microsphere distribution, and surface topography.

Nerve tissue stained with the S-100 and neuroendocrine marker PGP 9.5 antibodies demonstrated a significantly increased density of nerve tissue in the GDNF-treated groups compared to the empty microsphere (control) group ($p < 0.05$). GDNF-treated animals with a higher concentration of GDNF in the distal portion of the conduit possessed a significantly higher density of PGP 9.5 protein in the middle part of the conduit compared to GDNF uniform-treated animals ($p < 0.05$).

Porous Silk Fibroin Nerve Guide Designs

Silk nerve guides (approximately 1.5 mm in diameter, 10 mm or longer in length) were constructed and then imaged for assessing porosity. Groups included: gel spun nerve guides composed of a 98/2 silk/polyethylene oxide (PEO) blend, dipped nerve guides composed of a 98/2 silk/PEO blend, dipped nerve guides composed of a 90/10 silk/PEO blend, dipped nerve guides composed of a 80/20 silk/PEO blend, and dipped nerve guides composed of 75/25 silk/PEO.

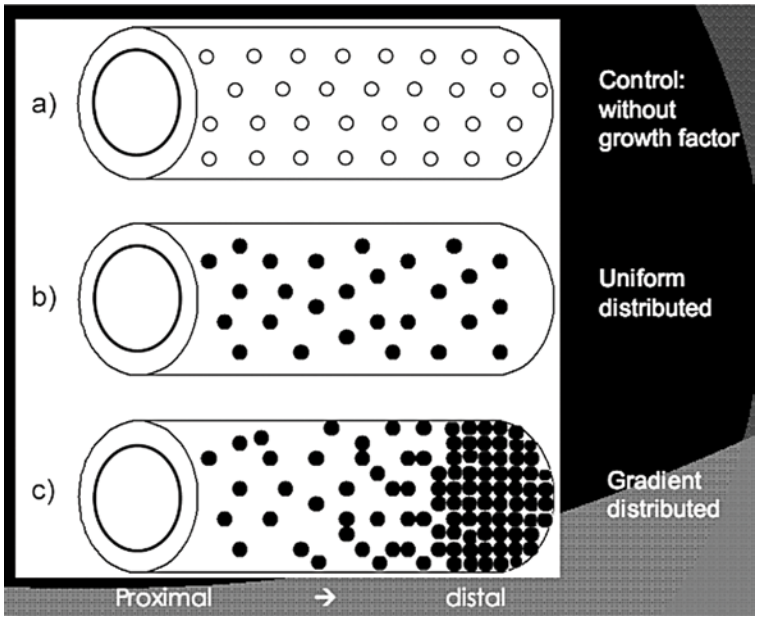
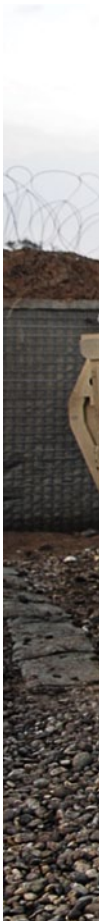


Figure II-17. The design of a spatially controlled drug release system in a silk nerve conduit.



Electrospun Porous Silk Fiber Nerve Guide Designs

For electrospun silk fiber incorporation, high- and low-density aligned electrospun silk fibers were spun onto flat silk films. Fibers were wrapped in a longitudinal direction around a stainless steel post and then treated with methanol. The outside layer was dipped into 25% silk solution containing 90/10 silk/PEO then methanol treated to induce b-sheet formation. Electrospun silk fibers 1–2 μm in diameter were incorporated into silk nerve guides.

Porous Patterned Silk Fibroin Nerve Guide Designs

Three designs of porous patterned silk fibroin nerve guides were constructed and then imaged. Design 1 consisted of a 342 nm width, 500 nm depth patterned silk film as the inside layer with a 80/20 silk/PEO blend as the outside layer. Design 2 consisted of a 342 nm width, 500 nm depth patterned silk film as the inside layer with a 90/10 silk/PEO blend as the outside layer. Design 3 consisted of a 342 nm width, 500 nm depth patterned silk film as the inside layer with a 80/20 silk/PEO blend as the outside layer.

Electronic Flat and Patterned Silk Nerve Guides (e-guides)

External platinum wire was secured to all e-films, for the purpose of an interface to external power, by using silk e-gel. Silk e-gel was formed by exposing a 25% concentrated silk solution to a 5V DC field for 10–15 minutes. The electronic film was then wrapped around a stainless steel post and methanol treated. An outer nerve guide layer consisting of 25% silk solution was created to increase the mechanical integrity of the guide. To create a porous outer layer, 7% PEO was mixed in with the 25% silk solution. The nerve guide was dipped into a 25% concentrated silk solution having a 90/10 silk/PEO ratio. Guides were left to dry overnight then soaked in water for post removal. Guides were then soaked for an additional 2 days for PEO removal.

Animal Studies

As of May 2011, there were three ongoing animal studies at Wake Forest University.

Study 1

A NHP median nerve repair study was initiated in November 2009 to evaluate long-term functional recovery in a clinically relevant model to obtain FDA approval for a clinical trial. A group of four female cynomolgus macaques underwent unilateral transection and repair of a 1 cm median nerve defect with a human hair keratin (HHK) hydrogel-filled NeuraGen® nerve conduit. In September 2010, the protocol was amended to perform bilateral median nerve repairs, and four additional animals were added to the study. This group received an HHK hydrogel-filled conduit and a saline-filled conduit. In February 2011, two more animals were added to the study. They received bilateral saline-filled conduits for a total of four uninjured controls, eight HHK, and eight saline controls. The animals underwent electrophysiology testing of the median nerve conduction velocity (NCV) prior to surgery and every 3 months as well as dexterity testing of their pinch grasp with a pegboard puzzle feeder every 6 weeks.

To date, the first four animals have been taken off the study after a 12-month period. The nerves were tested by electrophysiology and showed that the first group of HHK hydrogel conduit-grafted median nerves had an average NCV recovery, compared to the contralateral, uninjured median nerve, of 76.3% **(Figure II-18**). The nerves were then harvested, and semi-thin sections were cut, stained with 1% toluidine blue, and mounted on slides for analysis by light microscopy. Histomorphometric analysis for axon diameter and myelin thickness showed a unimodal distribution for the HHK nerves compared to a bimodal distribution for native nerve controls. Nerve area showed an average of 1.555 ± 0.8254 μm^2 (mean ± s.d.) at the midpoint of the conduit and 2.300 ± 1.017 μm^2 at the area distal to the distal graft suture.

The abductor pollicis brevis (APB) muscles (solely innervated by the median nerve) were harvested and analyzed for neuromuscular junction density and myofiber width. For neuromuscular junction density, 25 µm longitudinal cryosections were double-labeled with Alexa Fluor 568-conjugated alpha bungarotoxin and neurofilament light chain antibodies (Figure II-18). Bungarotoxin staining showed that the acetylcholine receptor density was significantly greater (p <0.01) in the control muscles compared to the HHK group. Due to the extremely small size of NHP hand

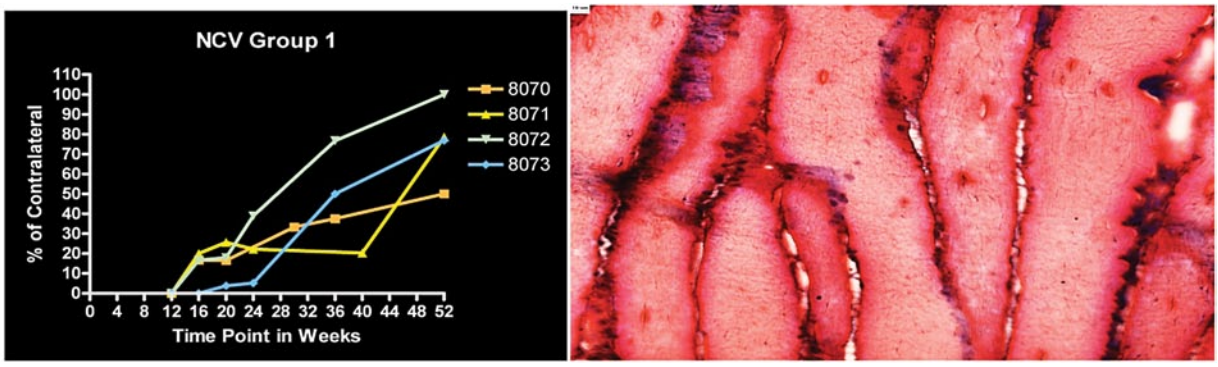


Figure II-18. NCV on four monkeys (1 cm median nerve gap). The nerves were tested by electrophysiology and showed that the first group of HHK hydrogel conduit-grafted median nerves had an average NCV recovery, compared to the contralateral, uninjured median nerve, of 76.3% (left). Histology – The APB muscles (solely innervated by the median nerve) were harvested, fresh frozen, and analyzed for neuromuscular junction density and myofiber width (right).

muscles, atrophy was evaluated indirectly by measuring the APB myofiber width using ImageJ software. Muscle analysis showed that APB muscle fiber width was significantly greater (p <0.001) in control muscles although the HHK muscles had only 19.5% atrophy after 12 months.

The second group of four animals is now at 36 weeks post-surgery. When comparing the HHK and saline groups at this time point, the HHK group has an average percentage recovery of the baseline median NCV of 45.5 ± 30 (n = 8) compared to 31.8 ± 22.7 (n = 3) for the saline group.

Study 2

Based on a biocompatibility study, the HHK hydrogel inside the conduit is resorbed within approximately 8 weeks. To assess the interaction of the HHK hydrogel with regenerative and inflammatory cells at early time points following surgery, the researchers randomized 30 male Sprague Dawley rats into saline and HHK groups. Animals underwent sciatic nerve transection and repair of a 1 cm defect with a NeuraGen conduit. Nerves were harvested at 3, 7, 14, 21, and 28 days, fresh frozen, sectioned at 10 µm, and analyzed by immunohistochemistry. Results showed that the presence of HHK in the conduit significantly reduced the number of macrophages at 3 days in the distal nerve stump with 329.3 ± 44.96 cells compared to the saline controls with 452.0 ±52.92 cells (n = 3 animals). This trend persisted in the 3-day proximal tissue although the differences were not significant.

Staining for neurofilament heavy chain (NF 200) at the 14-, 21-, and 28-day time points allows visualization of the regenerating axons from the proximal to the distal side of the conduit. Axonal growth begins 14 days post-injury for both groups but occurs more rapidly and robustly in the HHK group. Quantification of the NF 200 staining volume throughout the conduit was performed in ImageJ, and analysis showed a larger volume of regenerated axons at 21 and 28 days for the HHK group although this difference was not significant (n = 3). Cell infiltration was analyzed with DAPI staining at 3, 7, and 14 days. The HHK group had significantly more cells inside the conduit lumen at 3 days following injury with 13,429 ± 3,470 cells/1 cm graft length compared to the saline controls with 4,946 ± 2,736 cells/1 cm graft length (n = 3). To better characterize the identity of these cells and elucidate a molecular mechanism of cellular interaction with the HHK hydrogel leading to enhanced nerve regeneration, additional animals will be added to the study in the summer of 2011.

Study 3

The third series of experiments examines the natural time course of molecular and cellular events associated with nerve regeneration following injury in young versus old individuals.

MCP-1 (Monocyte Chemotactic Protein 1) Expression and Function During Wallerian Degeneration

Wallerian degeneration occurs over the first week following nerve injury and is characterized by axonal breakdown, down-regulation of myelin proteins, and Schwann cell proliferation concomitant with infiltration of hematogenous macrophages, both of which play a significant role in the clearance of myelin debris. The process concludes as Schwann cells align to guide the regenerating axons via bands of Büngner. Macrophages, possessing a diversity of functions, play important roles throughout this process. Without the efficient phagocytosis of myelin by macrophages, axonal regrowth is delayed. Furthermore, macrophages have an extensive profile of secretory molecules, including cytokines, enzymes, growth factors, arachadonic acid metabolites, and oxygen radicals. The signaling and timing of macrophage

recruitment and activation appear instrumental in the degenerative and regenerative process.

MCP-1 is expressed primarily by Schwann cells. MCP-1 peaks at 1 day post-injury during Wallerian degeneration and persists at high levels for several weeks. MCP-1 is a potent macrophage chemotactic protein that binds to the chemokine receptor (CCR2) on macrophages. The recruitment and entry of macrophages to the peripheral nervous system following injury correlates with the pattern of MCP-1 expression. The recruitment of macrophages by MCP-1 in the early phase following injury may be a key step for the initiation of Wallerian degeneration. The rate of myelin debris removal by macrophages determines the length of Wallerian degeneration. Earlier or increased expression of MCP-1 may lead to the faster completion of Wallerian degeneration, leading to an enhanced healing potential of the peripheral nerve. The researchers hypothesize that keratin hydrogels inside conduits utilized for peripheral nerve repair will lead to an earlier Schwann cell response with increased levels of MCP-1, which will shorten the length of Wallerian degeneration and lead to an enhanced regenerative potential of the nerve.

In this series of experiments, young adult rats with nerve injuries are being compared to old rats (24 months old, equivalent to approximately 70 years of age in humans). Molecular and histologic studies of both sciatic nerve as well as spinal cord are under way. Techniques for RNA extraction and real-time RT-PCR in both sciatic nerve and spinal cord were established in younger animals. Time points of 24 hours, 3 days, and 10 days post-nerve injury (crush injury) were selected to establish temporal markers for key molecular events associated with nerve regeneration. The spinal cords of the rats are being studied to provide insight into signaling responses associated with the nerve cell bodies following peripheral nerve injury. These studies are comparing the spinal cord on the injured side of rat to that of the uninjured side.

Key Research Accomplishments

- Established methods for incorporating pores, patterned silk films, electrospun silk fibers, growth factors, and electronics into biodegradable custom-designed silk nerve guides.
  - Combined methods to construct two-layer multifunctional nerve guides.
  - Characterized the properties of the multifunctional silk nerve guide designs.
- Tracked growth factor release rates from silk biomaterials and nerve cell responses to attachment proteins and peptides (i.e., laminin, gelatin, RGD, and poly-d-lysine).
- Assessed nerve cell alignment on varying silk surface topographies (i.e., patterned silk films and electrospun silk fibers) and axon outgrowth responses to electrical stimulation.
- Developed tools, including a semi-automated program for assessing axon alignment.
- Implanted PCL/GDNF guides in a 1.5 cm rat sciatic nerve defect model and assessed animals after 6 and 16 weeks.
  - Results of the 6-week study indicate that GDNF remains bioactive and successfully recruits Schwann Cells.
  - Results of the 16-week study indicate functional improvement in the rats with the GDNF microspheres.

- Examined the use of HHK hydrogel-filled nerve guides to treat a 1 cm nerve defect in the median nerve of four NHPs.
  - Additional NHP studies are under way.

Conclusions

Overall, this study demonstrates that novel, porous silk fibroin-based nerve conduits, infused with GDNF in a controlled manner, represent a potentially viable conduit for Schwann cell migration and proliferation in the regeneration of peripheral nerves. All three research groups have made significant progress during the past year:

- The Kaplan group at Tufts University utilized silk biomaterial protocols and integrative regenerative approaches to develop a biodegradable nerve guidance system. Regenerative approaches included incorporating biophysical cues (i.e., surface patterning and electrophysiology applications) and chemical cues (i.e., protein coatings and growth factor incorporation) into the silk nerve guides.
- The Smith group at Wake Forest University examined the use of HHK hydrogel-filled nerve guides to treat a 1 cm nerve defect in the median nerve of four NHPs. Those studies are completed, with promising results, and a second group of six animals is now at 36 weeks post-surgery.
- The Marra group at the University of Pittsburgh identified the spatially controlled drug delivery system and the benefit of combining biodegradable gel filler with nerve conduits in peripheral nerve regeneration. The researchers have obtained promising results in the rat critical-size sciatic nerve defect model. They have received approval from their Institutional Animal Care and Use Committee to test peripheral nerve repair in NHPs, and they have begun negotiations with both a drug company and a biomaterials company to manufacture GMP guides for the monkey study (and eventual clinical trials).

Research Plans for the Next 2 Years/Planned Clinical Transitions

The researchers will perform a 1 cm median nerve resection in NHPs (rhesus macaques) and will implant GDNF double-wall microspheres combined with PCL nerve conduits. They have scheduled surgeries on two animals in July 2011 and four additional animals in November 2011. All three groups have begun planning a pathway to clinical studies. A multicenter clinical trial has been initiated at Wake Forest Medical School with the University of Virginia as a collaborator. IRB approval has been obtained, and the trial is awaiting FDA approval of the HHK material for the nerve guide filler. FDA-approved nerve guides (NeuraGen) filled with HHK provided by KeraNetics® will be utilized. This trial will study large segmental injuries in peripheral nerves. This project is being funded through the Congressionally Directed Medical Research Programs.

Modular, Switchable, Synthetic Extracellular Matrices for Regenerative Medicine

Project 4.4.5, WFPC

Team Leader(s): Matthew Tirrell, PhD (University of California, Berkeley)

Project Team Members: Won H. Suh, MS, PhD, Katie Megley, BS, Nickesh Viswanathan, BS, and Seema Desai (University of California, Berkeley) and Brian Lin, BS, MS and Dan Krogstad, BS (University of California, Santa Barbara)

Collaborator(s): Kacey Marra, PhD (University of Pittsburgh)

Therapy: Injectable synthetic ECMs for regenerative medicine.

Deliverable(s): Task 1 (year 1) – Model PA synthesis and characterization; Task 2 (years 1–2) – Employ fibrous networks composed of worm-like micelles; Task 3 (year 2) – Neural stem cell attachment, proliferation, and differentiation; Task 4 (year 2) – Shear responsive PA development; Task 5 (year 3) – Additional PA construction; Task 6 (years 4–5) – Test injectable synthetic matrices in animal models.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 2; End Year 3, TRL 2

Key Accomplishments: The researchers have developed a peptide-based hydrogel system as injectable ECMs with nanofibrous structures. The 3-D hydrogel system can incorporate bioactive peptide sequences and/or growth factors and allows mammalian cell growth.

Key Words: Synthetic extracellular matrix, peptide amphiphile, nerve regeneration, tissue engineering, micelles, vesicles

Introduction

The Tirrell group is developing injectable, synthetic 3-D ECMs that are gel-like and designed to aid in the regenerative processes involved in peripheral nerve growth following traumatic injury by modulating components of the naturally occurring bioactive signals present in proteins such as fibronectin, laminin, and collagen. Utilizing chemistry-enabled tools, the researchers construct PAs (lipopeptides) with controlled physicochemical (i.e., defined stiffness) and bioactive (i.e., cell adhesion and proliferation) properties and produce injectable or moldable gel-like systems that can aid in the functional recovery of animals with traumatic injuries (i.e., the nerve gap injury model).

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers synthesized double-tailed PAs with controlled bioactive components and found that double-tailed PAs that incorporate RGD units can promote attachment, proliferation, and differentiation of human neural stem cells (hNSCs). They also developed an alanine-rich peptide head group containing PAs (C16-WAAAAXAAAAXAAAAXA = C16-W3X where X=Lys or Glu) that can have modulated physicochemical and biological properties based on electrostatics control, shear force modulation, and compositional changes.

Research Progress – Year 3

The Tirrell group continued to work with the Ala-Ala-Ala-Ala-X (X=Lys or Glu), incorporating PAs to make synthetic 3-D matrices in year 3. The researchers focused on: (1) developing a user-friendly and modular PA-incorporating gel system that transforms into an injectable gel within the time frame that matches most biological system processing conditions (e.g., animal surgery and cell preparation for in vivo delivery) and (2) further testing the PA formulations identified during year 2 with regard to hNSC compatibility via interfacing experiments with individual hNSCs or neurospheres **(Figures II-19–II-21)**. In addition, the group developed a multicomponent hydrogel system that can incorporate both HA and PAs **(Figures II-22–II-24)**. HA systems have been well utilized in biomedical research involving in vivo injury model studies.

During the past year, attention was also directed to developing PAs with modified head group peptide sequences that are not alanine rich but are still able to produce robust and hierarchical structures (after lipidation and self-assembly processing) that are ideal for sustaining cellular adhesion, proliferation, and regeneration. The group applied a new mode of peptide head group stabilization enabled by controlling hydrogen bonding between imidazole groups and primary alcohol side residues after cooperative assembly and with proton (H^+) concentration modulation as such controlled interactions are already present in nature (e.g., ragworm jaw). In particular, it was envisioned that PAs incorporating multiple histidine and serine amino acid groups would transform from an injectable solution to a stiffer scaffold-like macroscopic object when exposed to physiologically relevant pH ranges.

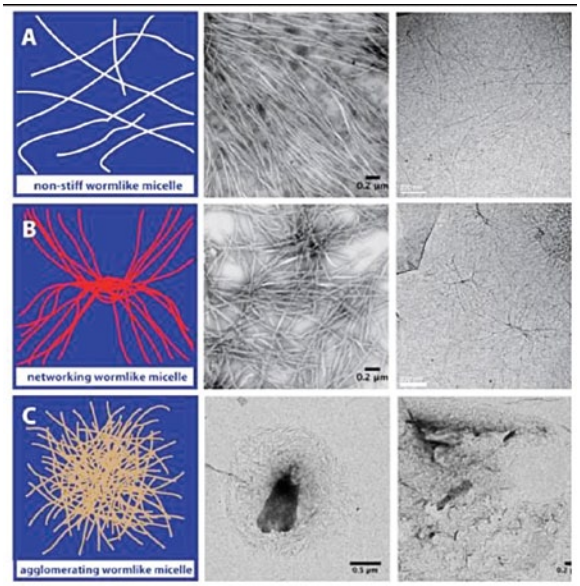


Figure II-19. Morphological analysis of the charge-mixed, alanine-rich PA system. Transmission electron microscopy (TEM) analysis: (A) Worm-like nanofibrous PA C16-W3K-RGD(S), (B) short, aggregated nanofibrous PA C16-W3E, and (C) PA mixture of 70% C16-W3E and 30% C16-W3K-RGDS.

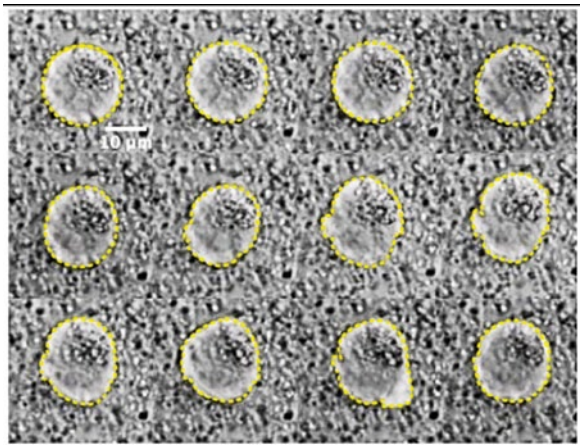


Figure II-20. Live cell imaging of an hNSC with PA particles. A 12-minute time-lapse, live-cell confocal microscopy captures images of a single hNSC interfaced to PA particles formed with 80% C16-W3E and 20% C16-W3K-RGD. Cell membrane traces are provided as dashed yellow lines, and the masked areas have been falsely processed using a graphics design computer program to highlight the movement of the interfaced single hNSC.

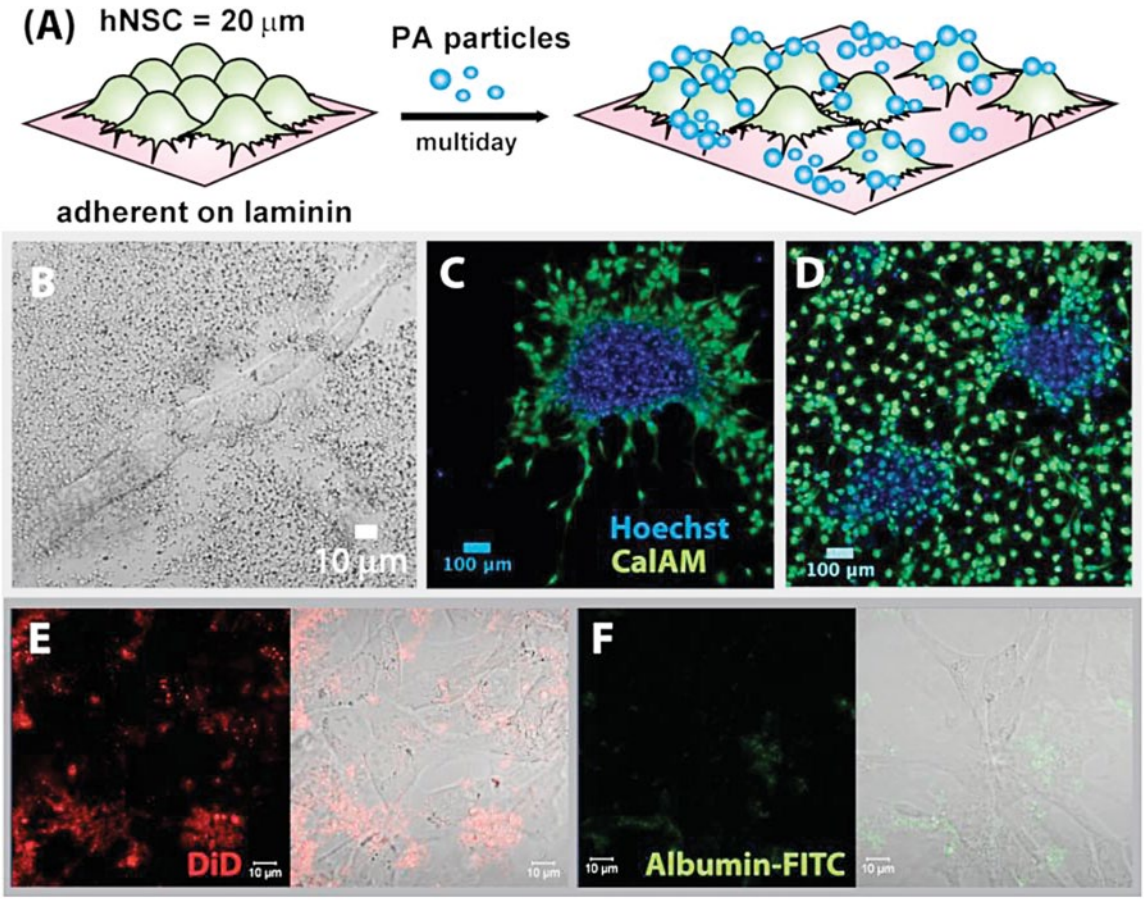


Figure II-21. Adherent stem cell compatibility of charge-mixed, alanine-rich PA system. (A) Experimental scheme of a PA particle interfacing with hNSCs cultured on a laminin-coated glass surface. (B) Time-lapse, live-cell confocal microscopy imaging snap shot of multiple hNSCs interfaced to PA particles formed with 80% C16-W3E and 20% C16-W3K-RGD (at 1 mg/mL overall PA concentration). (C) Live hNSCs cultured on a laminin surface for 1 month (CalAM stains live cells; Hoechst nuclear stain is blue). (D) Live hNSCs cultured for 1 month on a laminin surface interfaced to PA particles prepared with 70% C16-W3E and 30% C16-W3K-RGD (treatment conditions: <100 μg/mL overall PA concentration; approximate initial cell density is 100,000 cells). For C and D, living cells are stained green with calcein AM while the cell nucleus is stained with a Hoechst dye (blue). (E) DiD molecules were incorporated into PA particles prepared with 85% C16-W3E and 15% C16-W3K-RGDS. These particles were biocompatible with hNSCs cultured on laminin. (F) Albumin-FITC proteins were incorporated into PA particles prepared with 85% C16-W3E and 15% C16-W3K-RGDS. These particles were biocompatible with hNSCs cultured on laminin.

Specific Key Accomplishments for Year 3

1. The Tirrell group developed protocols that allowed 70%–85% C16-W3E and 15%–30% C16-W3K-RGD(S) PA self-assembled mixtures to form micron-sized particles and incorporate other biomolecules such as proteins and lipids. Additional procedures were developed that allowed such PA particles to (positively) interface with individual adherent hNSCs and neurospheres. PA nanostructures were analyzed using TEM (Figure II-19). The group then conducted adherent stem cell and neurosphere compatibility experiments utilizing charge-mixed alanine-rich PA systems as outlined in Figures II-20–II-22. Time-lapse, live-cell confocal microscopy imaging snapshots of multiple hNSCs interfaced to PA particles suggest that long-term stem cell experiments with or without supporting ECM proteins will be achievable in the presence of PA particles for hNSCs (Figure II-20). It was also confirmed that DiD and albumin-FITC molecules can be incorporated into PA particles formed with 70%–85% C16-W3E and 15%–30% C16-W3K-RGD(S) (Figures II-20–II-21). PA particles with and without foreign molecules were stem cell biocompatible both in the short term and long term.

2. The group also developed a new bioactive fluorescent PA gel system that is fully self-sustaining and has nanofibous structures (Figure II-23). The particular peptide sequence is KWAAAAEAAAAEAAAAEA. The fluorescent alanine-rich PA gel system transforms into a self-sustaining gel at 60°C in 10 minutes and in 1.5 days at 37°C. TEM analysis confirmed that the new fluorescent PA gel system also self-assembles into high-aspect ratio nanostructures identical to the nonfluorescent

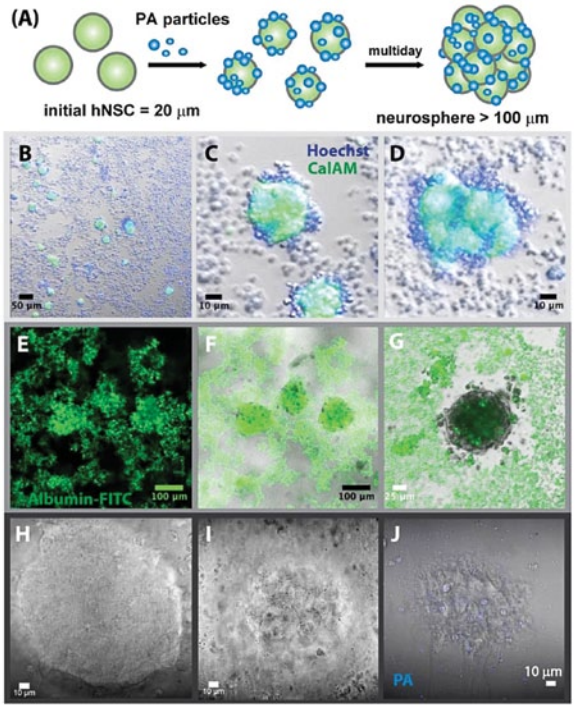


Figure II-22. Neurosphere compatibility of the charge-mixed, alanine-rich PA system. (A) Experimental scheme of PA particle interfacing to nonadherent hNSCs that transform into neurospheres. (B-D) Confocal microscopy imaging snap shots of multiple hNSCs interfaced to PA particles formed with 80% C16-W3E and 20% C16-W3K-RGD. (E-G) Confocal microscopy imaging snap shots of multiple hNSCs interfaced to albumin-FITC incorporating PA particles formed with (E, F) 100% C16-W3E and (G) 80% C16-W3E and 20% C16-W3K-RGD. Live cell imaging snap shot of (H) a neurosphere attached to a glass surface and (I) a neurosphere interfaced to PA particles. (J) PA (blue particles) incorporating neurospheres can be passaged onto laminin-coated surfaces.

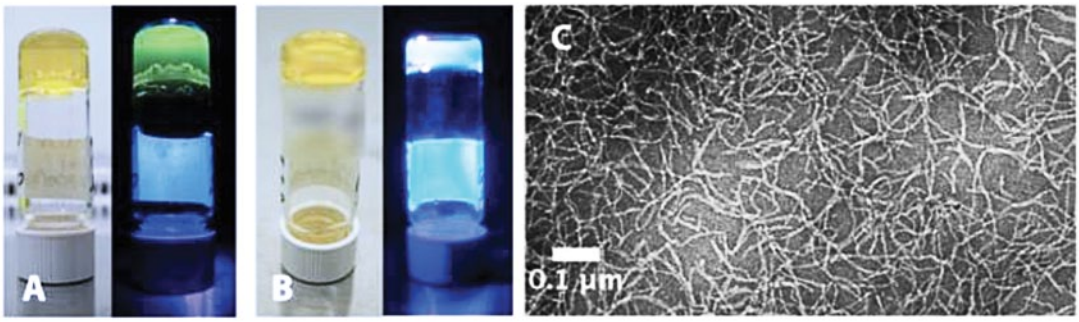


Figure II-23. Physicochemical properties of the fluorescent, alanine-rich PA gel system. (A) The new fluorescent PA gelled at 60°C in 10 minutes and (B) 37°C in 1.5 days. (C) TEM image of the new fluorescent PA gel system showing high-aspect ratio nanostructures.

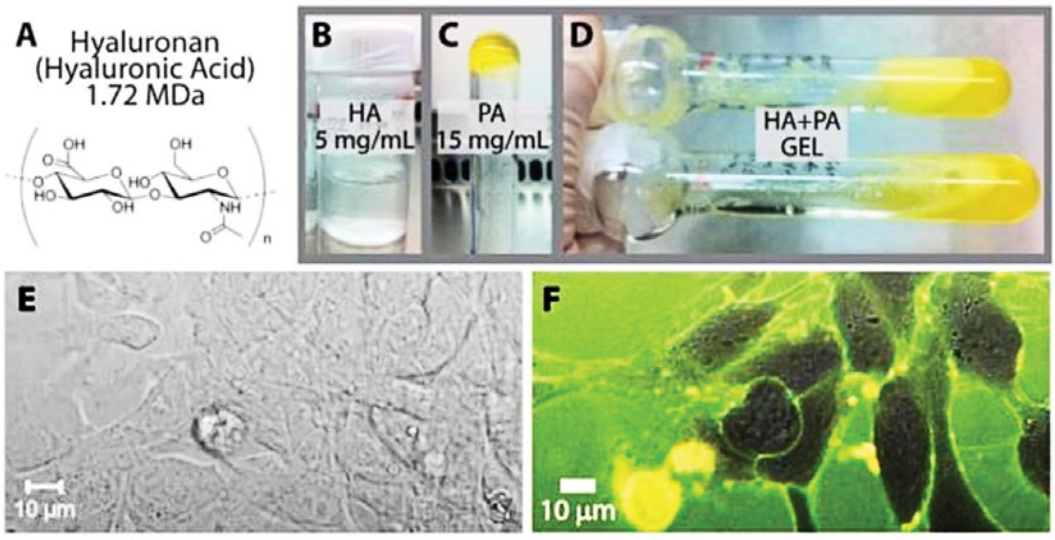


Figure II-24. HA and PA combined gel system. (A) Chemical structure and molecular weight of the HA utilized, (B) HA gel at 5 mg/mL concentration, (C) PA gel at 15 mg/mL forms a self-sustaining gel with 10 minutes of 60°C treatment, (D) HA and PA gel mixed in a tissue grinder, (E) NSCs growing inside an HA gel, (F) hNSCs growing inside a hydrogel made with 70% HA and 30% PA.

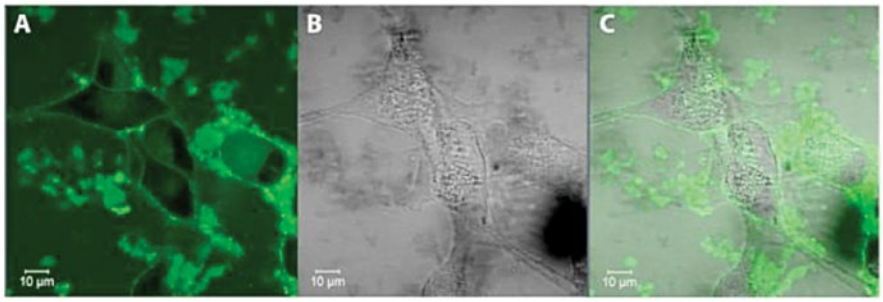


Figure II-25. hNSCs cultured and interfaced to an HA–PA hydrogel system. (A) Five hNSCs cultured inside a hydrogel composed of 67% HA and 33% PA. The overall gel concentration is approximately 6 mg/mL. The PAs are green fluorescent and have incorporated into the cell membrane and partially into the cytosol. The cell nucleus is black indicating no PA internalization. (B) Bright field image of A, (C) merged image of A and B.

versions introduced in the first 2 years of the project.

3. The self-sustaining PA gel system can be combined with an HA hydrogel system to afford a new optically transparent HA–PA gel system. Cell encapsulation and interfacing experiments can be easily performed utilizing this new gel system (Figure II-24 and **Figure II-25**). The particular molecular weight of the HA polymer is 1.72 MDa, and the chemical structure is provided in Figure II-24. The HA gel is prepared at a concentration of 5 mg/mL in nanopure water with sonication-induced solubilization and gelation. The PA gel, on the other hand, is first prepared at 15 mg/mL as a clear liquid and then transformed into a self-sustaining gel in 10 minutes at 60°C. The pre-gelled HA and PA gels are freshly mixed in a tissue grinder before interfacing experiments are performed with hNSCs. The working concentration formation for the new HA–PA gel system is HA 70–100 wt. % and PA 0–30 wt. %. The pre-gelation is important for the generation of an optically clear gel, which will enable facile microscopy-based analyses including live cell confocal imaging.
4. The Tirrell group's newly developed HA–PA hydrogel system allows the incorporation of bioactive sub-domains, such as growth factors, and fully enables 3-D and multiday cell cultures (Figures II-24–II-25). The hNSC media incorporates bioactive molecules, such as human serum albumin, transferrin at the sub-300 µg/mL concentration level, insulin, heparin at sub-10 µg/mL concentrations, and basic fibroblast growth factor and epithelial growth factor at sub-0.05 µg/mL concentrations. The HA–PA hydrogel's gel-like property does not appear to be affected to the extent where it no longer qualifies as a hydrogel with hNSC media supplementation.
5. The group developed a new biocompatible and pH responsive PA hydrogel system that incorporates histidine and serine amino acid units in the head group, which will be referred to as HS PA or His-Ser PA from this point forward. The serine-histidine peptide combination is desirable because serine possesses a side chain capable of forming stable hydrogen bonds, and the hydrogen bonding properties of histidine are tunable in the physiological pH range as its side chain pKa is 6. Below

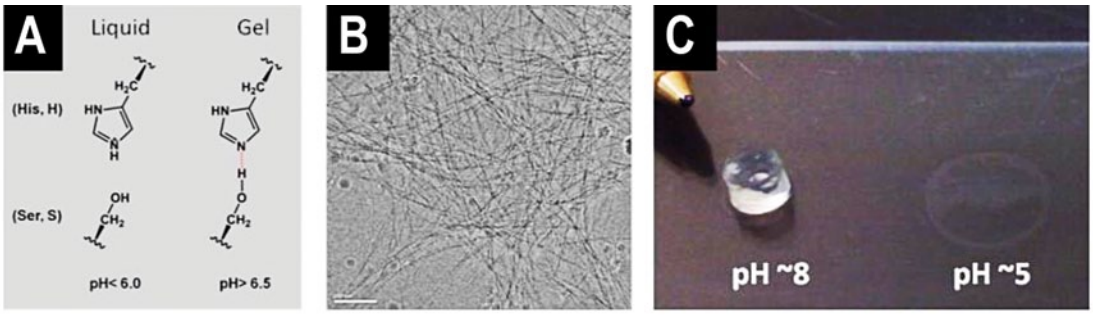


Figure II-26. Physicochemical properties of pH responsive, histidine- and serine-incorporating PA gel system. (A) Above pH 6.5, histidines are predominately basic and capable of forming strong hydrogen bonds with serines, resulting in strong hydrogels. (B) Cryo-TEM image of PA fibers. (C) 2 mg/mL of hydrogel solution cast in a glass tube and its pH was subsequently adjusted. The high pH sample resulted in a self-supporting gel, and the sample with low pH remained a liquid. Gelation is reversible with pH. The spectrum of stiffness this gel can achieve resembles synthetic systems capable of selectively supporting the growth of different cell types from neural to bone. (TEM imaging credit: D. Krogstad)

pH 6, histidines are predominately acidic and form weak hydrogen bonds with serines as proton donors. Above pH 6.5, histidines are mostly basic and form strong hydrogen bonds as proton acceptors. As a result, the PAs form weak fibers at low pH while strong fibers form above pH 6.5 **(Figure II-26)**. Weak fibers in solution resemble a low-viscosity liquid and strong fibers form self-supporting hydrogels. Gel formation is reversible. The stiffness of the bulk hydrogel is tunable by controlling the PA concentration. A stiff gel of 1 X 10^4 Pa (G') is achieved at 1% by wt (10 mg/mL) of PAs. The new HS PA system shows great promise as an injectable cell niche capable of simulating a wide range of functional tissues; the stimuli-responsive gelation properties and the stiffness ranges are dynamic.

6. The biocompatibility of hydrogels formed with HS PAs was assessed with fibroblasts as a 2-D surface culture model system. NIH 3T3 cell attachment was observed in the absence of serum at 6 hours, and the cells remained attached overnight although spreading was not observed. Long-term proliferation studies were conducted in the presence of 10% calf bovine serum with an initial seeding of 5,000 cells/cm^2. Cells became confluent after 2 hours, and dead cells were scarcely found using a live-dead cell assay. Cell activity was similar on gels modified with 30% glycine-arginine-glycine-aspartic acid-serine peptide.

Key Research Accomplishments

- Determined that electrostatically controlled, alanine-rich PA particles can positively interface to adherent or suspension cultures of hNSCs. These stem cell-compatible PA particles can also incorporate other types of lipid molecules and biomolecules such as proteins and peptides.
- Found that palmitoylated KWA4EA4EA4EA peptides can form a self-sustaining gel and can incorporate fluorescent molecules. This C16-KWA4EA4EA4EA PA, after heat-induced gelation, can mix well with HA hydrogels

to form clear gels at concentrations of up to approximately 30% PA in HA.

- Developed a new biocompatible and pH-responsive PA hydrogel system that incorporates histidine and serine amino acid units in the head group.
  - Exhibited dynamic stimuli-responsive gelation properties and the stiffness ranges.
  - Allowed fibroblast cell adhesion on a pre-gelled surface.

Conclusions

This project continues to progress toward the goal of developing an injectable gel system that will set and fix at the injury site, in situ, during the time domains critical for successful nerve regeneration.

Research Plans for the Next 2 Years

During the next 2 years, the group will focus on the development of the new His-Ser PA-based hydrogel as a matrix material for nerve damage repair. The plan is to provide enabling technologies in the form of PA-based hydrogels that incorporate peptide head groups instilled with histidine and serine. The new hydrogel system will be ideal for in vivo testing of regeneration in a rat nerve gap injury model in collaboration with the Marra group at the University of Pittsburgh. During year 4, efforts will be directed toward enhancing the bioactivities of the PA-based hydrogels by covalently incorporating short peptide sequences such as RGD and IKVAV to mimic the naturally occurring ECMs or by supplementing the hydrogels with proteins such as GDNF but without sacrificing gelation properties. Biomaterial designs will be further modified and updated based on the biological testing results arising from the in vitro Schwann cell work at the University of California, Berkeley (Tirrell group) and the in vivo rat injury model study at the University of Pittsburgh (Marra group).

Repair Segmental Nerve Defects

Project 4.4.1/4.4.2, RCCC

Team Leader(s): Anthony Windebank, MD and Michael Yaszemski, MD, PhD (Mayo Clinic); Joachim Kohn, PhD and Melitta Schachner, PhD (Rutgers University); and Daniel G. Anderson, PhD and Robert S. Langer, ScD (MIT)

Project Team Members: Robert Spinner, MD, Huan Wang, MD, PhD, Brett Runge, PhD, Jing Rui, MD, David Brogan, MD, Chandan Reddy, MD, Shuya Zhang, Andrew Knight, PhD, Glenda Evans, and Suzanne Segovis (Mayo Clinic); Basak Clements, PhD, Jack Hershey, PhD, Mindy Ezra, Shirley Masand, Jian Chen, MD, Melitta Schachner, PhD, David Shreiber, PhD, and Joachim Kohn, PhD (Rutgers University); and Paulina S. Hill, PhD and Hao Cheng, PhD (MIT)

Collaborator(s): BonWrx, Inc., for Mayo Clinic, Sanjeeva Murthy, PhD (Rutgers University), and Christopher Pastore, PhD (Philadelphia University)

Therapy: Treatment of peripheral nerve injuries.

Deliverable(s): Polymer conduits suitable to repair large motor nerve defects.

TRL Progress: 2009, TRL 3; 2010, TRL 4; 2011, TRL 4; Target, TRL 6

Key Accomplishments: The researchers developed and successfully evaluated braided nerve regeneration conduits in the rat sciatic nerve model. They developed modified biorubber conduits with superior mechanical properties. They developed an intraluminal scaffold composed of aligned fibers that are capable of releasing anti-inflammatory drugs in a controlled manner and showed that this promotes functional regeneration in a rat sciatic nerve defect model.

Key Words: Peripheral nerve, conduit, biodegradable polymer, growth factors, braiding, tyrosine-derived polycarbonates, biorubber, clinical trial

Introduction

Peripheral nerve lesions occur through a variety of injuries ranging from the battlefield, industrial, farm, and traffic accidents to violence and sports injuries. The current clinical gold standard of repairing nerve defects is the use of an autologous nerve graft, which has several drawbacks. While the current commercially available synthetic conduits have proven successful in repairing small gaps, they have shown little to no efficacy for the repair of longer defects in either civilian or military applications. The researchers in this project are developing novel biodegradable polymer nerve conduits suitable to repair large motor nerve defects to substitute autologous nerve graft.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers effectively encapsulated and released neuronal growth factors from fumarate-derived polymer scaffolds and polymer microspheres. The growth factors were released in a biologically active form and with a time course suitable to enhance peripheral nerve regeneration. The research team also showed that mesenchymal stem cells provide a potential cell-based delivery platform for growth factor delivery.

During the second year of the study, the research team found that candidate polymer PCLF and single-channel poly(xylitol-co-sebacate) (PXS) conduits promoted better functional regeneration than the

FDA-approved collagen conduit. The researchers synthesized a novel library of polyesters to further optimize the nerve guide material. They developed a method for incorporating aligned electrospun fibers into the nerve conduit lumen in a uniform manner with no fiber aggregation. They encapsulated anti-inflammatory agents in electrospun fibers to decrease the inflammatory response to synthetic materials. The researchers also established a complex soft tissue injury model and obtained approval for a clinical trial for "repair of 6 cm peripheral nerve gaps in sural nerve biopsy using PCLF tube."

Research Progress – Year 3

The Mayo Clinic group has successfully demonstrated that PCLF tubes can be autoclaved. The autoclave did not significantly change the material properties of the PCLF tubes and thus has been selected as the sterilization method moving toward FDA approval and the clinical trial. IRB pre-review of the clinical trial was completed, and IRB materials and the clinical protocol have been submitted for HRPO pre-review. Promotion of vascularity was introduced by the controlled release of the pro-angiogenesis factor, vascular endothelial growth factor (VEGF). VEGF-encapsulating microspheres have been developed that retain VEGF bioactivity and control VEGF release. The Mayo Clinic group has explored the complex ischemia fibrosis nerve injury model more characteristic of traumatic injury and clarified deprivation of soft tissue vascularity and its effect on nerve regeneration. Second-generation conduits were fabricated that allowed delivery of electric stimulation and demonstrated that electrical stimulation via a conductive polymer promotes axon growth in vitro.

The Rutgers University team developed a braided nerve conduit fabricated from thin TyrPC polymer fibers. These conduits have excellent mechanical properties when both dry and hydrated, and they display unique non-kinking behavior when bent at large angles. The conduits are porous to allow for diffusion of nutrients and growth factors, and polymers from a large TyrPC library can be selected for fabrication to fine-tune the degradation of the conduits to parallel the rate of nerve regeneration. The conduits can be fabricated in any length and diameter and hold promise for effective bridging of large nerve gaps in humans.

The MIT group successfully developed modified biorubber conduits with superior mechanical properties to the first-generation PXS and poly(glycerol-sebacate) conduits. The researchers demonstrated that encapsulation of anti-inflammatory agents into electrospun fibers decreases the local host immune response and results in considerably less fibrosis around the implant. Intraluminal scaffolds of these electrospun fibers were tested in rat sciatic nerves and were found to promote functional nerve regeneration. The MIT team is also developing lipid-based drug delivery scaffolds and has shown that these biomaterials promote axon growth in vitro.

Key Research Accomplishments

- Demonstrated that sterilization by autoclaving is compatible with material properties of PCLF conduits and will be the method used for clinical trial.
- Developed and characterized VEGF-encapsulating microspheres and showed that these microspheres are capable of controlled release of VEGF with retained bioactivity.
- Developed new polymer conduits that are conductive and showed that they support robust nerve regeneration.

- Completed a study of supplementing PCLF tube with adipose-derived stem cells (ASCs) and showed that the nerve growth inhibitory effect of ASCs was associated with the differentiation of ASCs into mesenchymal cells, which induced the development of nonfascicular tissue.
- Developed modified biorubber conduits with superior mechanical properties to first-generation polyester materials.
- Fabricated dexamethasone-encapsulated, electrospun fiber sheets and showed that they decrease inflammation at the site of injury resulting in considerably less fibrosis around the implant.
- Developed intraluminal scaffolds composed of aligned fibers that are capable of releasing anti-inflammatory drugs.
- Performed proof-of-concept in vivo testing with aligned electrospun fibers and showed that they promote functional regeneration in a rat sciatic defect model.
- Developed lipid-based drug delivery scaffolds and showed promotion of axon growth in vitro.
- Fabricated and evaluated TyrPC braided nerve conduits in a rat sciatic nerve injury model.
- Characterized the influence of incorporated bioactive peptides on functional recovery after nerve injury.

Conclusions

During the first 3 years of this project, an initial array of more than 10 polymer types have been downselected to a single polymer (PCLF) and fabrication method that is currently being translated into an accelerated clinical trial. Transferring manufacture to a GMP facility is under way, and supplemental funding was obtained to initiate a clinical trial of a 6 cm conduit in patients by the end of 2011. The regulatory pathway to final approval is anticipated to be by the 510(k) route. Development of new polymers and second-generation tubes in the Mayo Clinic, MIT, and Rutgers University laboratories is proceeding with close cooperation and coordination among the groups and continues to be supported by leveraged funding.

Research Plans for the Next 2 Years

The Mayo Clinic group will complete all of the regulatory pathways for the Phase 1 clinical trial (i.e., GMP up-scaling of PCLF tube manufacturing, filing an IDE for FDA approval of clinical implementation of the PCLF tube, and obtaining IRB/HRPO approval). Study subjects will be recruited for this clinical trial. For continuing studies outside of the clinical trial, the Mayo Clinic group will introduce Schwann cells into PCLF nerve conduits to provide a source of neurotrophic factors to promote nerve regeneration across long nerve gaps and initiate discussions with the FDA about the pathway for bringing the combination product (cells and device) to humans.

The Rutgers University team is planning to carry out sequential animal cohorts to characterize and develop the braided nerve conduits fabricated from TyrPCs during the next 2 years. In AFIRM year 4, the team will build upon the favorable mechanical properties of these conduits and optimize them for nutrient diffusion, suturability, flexibility, and kink resistance over long gaps. TyrPC composition will also be optimized for achieving desired degradation time and physical characteristics. Braided conduits will be fabricated using the chosen polymer compositions and design

criteria for evaluation in the rat sciatic nerve model.

During year 4, the Rutgers University team will also continue developing bioactive, peptide-conjugated hydrogel fillers with the aim of enhancing the speed and quality of regeneration by stimulating motor and sensory axons to their proper distal targets. These peptides and fillers are being screened in vitro for efficacy and their ability to promote neurite outgrowth, as well as in vivo in the mouse femoral nerve model, which allows the evaluation of preferential motor reinnervation. The most effective filler and braided conduit combination will be evaluated in the rat sciatic nerve 1 cm defect model during year 4. The researchers expect to scale up the conduit prototype for large animal testing in AFIRM year 5. The conduits will be fabricated to bridge long gaps (greater than 3 cm) in the sheep or monkey models of peripheral nerve injury. The goal is to show that the braided conduits combined with the bioactive fillers are superior to their clinically available counterparts in bridging long gaps, and the target is to finalize a definitive prototype and prepare for FDA approval at the end of year 5.

The MIT group plans to fabricate polyester conduits with single-layer walls; optimize the size, density, and degradation rate of electrospun, drug-releasing fibers as intraluminal scaffolds; perform in vitro controlled release testing of drugs from electrospun fibers; fabricate prototype microelectrode devices for nerve:muscle interfacing; and screen optimized materials in sciatic nerve defects and atrophying muscle.

Planned Clinical Transitions

The Mayo Clinic group works closely with BonWrx, Inc., a Mayo Clinic spin-off company that has a current Good Manufacturing Practice (cGMP) facility and substantial experience and track record in FDA filing. The Mayo Clinic group will obtain IDE approval for the clinical implementation of PCLF nerve conduits in the Phase 1 clinical trial to test the safety of PCLF tubes in the 6 cm nerve defects in post-sural nerve biopsy patients. The group will also apply for grants to support a double-blinded efficacy trial of PCLF conduits versus industry best for repair of the 6 cm sural nerve gap.

The Rutgers University group has an exclusive license with Trident Biomedical, Inc., for the library of TyrPCs, and discussions have begun to define required funding to advance the planned animal studies. The group is also working on the large-scale cGMP synthesis of TyrPCs. During year 4, the group will also be collaborating with medical device companies for the cGMP-grade fabrication of the braided conduit. The regulatory strategy is to accomplish a definitive prototype to prepare FDA 510(k) regulatory action in year 5.

Cells and Bioactive Molecules Delivery to Enhance the Repair of Segmental Nerve Defects

Project 4.4.2a, RCCC

Team Leader(s): Maria Siemionow, MD, PhD (Cleveland Clinic)

Project Team Members: Amanda Mendiola, MD, Maria Madajka, PhD, and Joanna Cwykiel, MSc (Cleveland Clinic)

Therapy: Nerve gap repair.

Deliverable(s): Method of cellular therapeutics by local administration of BMSCs into transplanted epineural tubes (epineural sheath +/- cells).

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 4; Target, TRL 4/5

Key Accomplishments: The researchers developed several biological nerve constructs and tested them in a rat nerve repair model using a combination of in vitro and in vivo methods. They identified the most effective conduit and selected it for use in the repair of a long nerve defect in a large animal (sheep) model. Testing commenced in the sheep model in the last quarter of year 3.

Key Words: Nerve repair, natural nerve conduits, epineural tubes, autogenic nerve conduits, allogeneic nerve conduits, bone marrow stromal stem cells

Introduction

Current military conflicts and antiterrorist actions have resulted in more than 30,000 wounded warfighters in the U.S. Army who require substantial surgical intervention. A major challenge in the reconstruction of these soldiers is the extensive tissue loss in the upper and lower extremities and face, and the restoration of function of the repaired or replaced tissues. In these multitrauma cases, a regional loss of bone, muscle, and skin is complicated by injury to the major nerves responsible for motor and sensory function of limbs and facial components. These segmental nerve defects can commonly exceed 20 cm, limiting access to sources of autologous nerve grafts in patients already deficient in donor tissues. Moreover, harvesting these grafts results in donor site morbidity, such as scarring, sensory loss, and neuroma formation with chronic pain at the harvest site.

Thousands of American civilians each year are affected by paralysis, a devastating injury with annual costs of $7 billion. Compared to spinal cord injury, damage to peripheral nerves is considerably more common with the excess of 50,000 peripheral nerve repair procedures performed each year. Due to these facts, there is an urgent need for an alternative to nerve autografts. Nerve allografts provide an unlimited source of nerve tissue with the potential for enhanced functional recovery. However,

allografting requires patients to be under systemic immunosuppressive therapy for approximately 18 months while regenerating axons and Schwann cells across the graft. Immunosuppression puts patients at risk for infections and other complications. On the other hand, levels of immunosuppressive drugs that are too low may result in graft rejection and hamper nerve regeneration.

To this end, a number of different natural and synthetic materials have been explored for use in aiding nerve regeneration. While synthetic scaffold conduits have shown efficacy in repairing small gaps (i.e., 3 cm or less), they have been largely ineffective for the repair of longer nerve defects. Currently, decellularized nerve allografts do not require immunosuppression although they tend to perform inferiorly to autografts in experimental models. Epineural tubes are naturally occurring conduits easily harvested from allogeneic donor nerves, and they have low immunogenic potential as they lack Schwann cells. Moreover, epineural tubes provide neuropermissive scaffolding by expressing laminin B2, a key ECM protein for nerve regeneration.

BMSCs may provide an alternative source of easily accessible and expandable neural support cells. BMSCs also express neurotropic factors that support axon growth. Moreover, BMSCs lack immunogenicity in nonimmunoprivileged sites, which is of value with regard to allogeneic cell therapy. In this project, the researchers propose to introduce novel conduits constructed from naturally occurring allogeneic epineural tubes, filled with autologous or allogeneic BMSCs, as an alternative to autograft and other means of peripheral nerve gap repair.

Summary of Research Completed in Years 1 and 2

During the first year of the study, the researchers successfully performed the transplantation of the isogenic epineural tube and the delivery of BMSCs into the transplanted tube. They began initial harvesting of transplanted tubes with positive measures of functional outcomes. Preliminary electrophysiological measurements using somatosensory-evoked potential technology showed successful regeneration over the gap; however, no significant differences between groups were noted. Preliminary immunohistochemical data supported nerve regeneration in the presence of transplanted isogenic BMSC therapy. Histomorphometric analyses indicated a positive effect of isogenic bone transplantation on nerve regeneration.

During the second year of the project, the researchers completed a study on allogeneic epineural tube repair of a 2 cm rat sciatic nerve defect. They demonstrated that allogeneic epineural tube repair without immunosuppression is a feasible method of peripheral nerve gap repair. The researchers' results were comparable to autograft repair when an epineural tube was used in conjunction with local BMSC therapy.

Research Progress – Year 3

Fibrin Gel Construct

The use of fibrin gel in peripheral nerve repair has been studied and published in the literature. Studies have demonstrated that it is a useful product for coaptation of nerves and can reduce inflammation and suture granuloma formation. It has also been shown to decrease neuropathic pain and enhance nerve regeneration. The Siemionow group explored the ability of an epineural conduit

filled with BMSCs suspended in a fibrin gel to repair a 2 cm rat sciatic nerve gap. No immunosuppression was given for any group. Experimental groups were as follows:

Group 1:

A. Isogenic epineural sheath filled with isogenic (Lewis RT1^{l}) BMSCs suspended in fibrin gel, evaluated at 12 weeks (n = 4).

B. Isogenic epineural sheath filled with isogenic BMSCs suspended in fibrin gel, evaluated at 24 weeks (n = 2).

C. Isogenic epineural sheath filled with only fibrin gel, evaluated at 24 weeks (n = 8).

Group 2:

A. Allogeneic epineural tube (ACI RT1^{a}) filled with isogenic BMSCs (Lewis RT1^{l}) suspended in fibrin gel, evaluated at 24 weeks (n = 6).

B. Allogeneic epineural tube filled with allogeneic BMSCs suspended in fibrin gel, evaluated at 12 weeks (n = 3).

C. Allogeneic epineural tube filled with allogeneic BMSCs suspended in fibrin gel, evaluated at 24 weeks (n = 3).

D. Allogeneic epineural tube filled with only fibrin gel, evaluated at 12 weeks (n = 3).

E. Allogeneic epineural tube filled with only fibrin gel, evaluated at 24 weeks (n = 3).

The Siemionow group additionally performed 11 nerve repairs with autologous nerve grafts as a positive control at 12 (n = 8) and 24 weeks (n = 3).

Assessment methods included:

- Toe spread (TS) clinical motor evaluation
  - 0 (no movement)
  - 1 (some movement)
  - 2 (abduction of toes)
  - 3 (abduction and extension of toes)
- Pinprick (PP) clinical sensory evaluation
  - 0 (no withdrawal)
  - 1 (withdrawal with PP above ankle)
  - 2 (withdrawal with PP between toes and ankle)
  - 3 (withdrawal with PP at toes)
- Somatosensory evoked potentials (SSEP) as an objective sensory evaluation
- Gastrocnemius muscle index (GMI) to gauge muscle recovery
- Histomorphometry (axon diameter, myelin thickness, G ratio, and axonal density)

In all animals, the transplanted tube appeared to be intact and was bridging the nerve gap with minimal fibrous tissue. All groups had full sensory recovery at 12 and 24 weeks with a PP score of 3. There was no statistical difference in TS between all groups at 12 weeks. The 24-week group was noted to have contractures, which precluded the evaluation of TS. With regard to the SSEP analysis, no significant differences were observed at 12 or 24 weeks. The Siemionow team did not find any statistical difference in GMI between the different groups at their respective time points. After finding no significant differences in nerve regeneration based on the histomorphometry analysis, the study was stopped.

Stromal Cell Epineural Construct

The researchers created a stromal cell epineural construct (SCEC) for the repair of a 2 cm defect in a rat sciatic nerve. They initially tested an isogenic model to assess feasibility then moved on to an allogeneic construct. No immunosuppression was given in any treatment group. Experimental groups in this study are shown as follows. Outcomes were assessed as described for the fibrin gel construct study.

Group 1:

A. Isogenic (Lewis RT1^{l}) epineural sheath filled with isogenic BMSCs, evaluated at 6 and 12 weeks (n = 14).

B. Isogenic (Lewis RT1^{l}) epineural sheath filled with saline, evaluated at 6 and 12 weeks (n = 14).

Group 2:

A. Allogeneic (ACI RT1^{a}) epineural sheath filled with isogenic BMSC, evaluated at 6 and 12 weeks (n = 16).

B. Allogeneic (ACI RT1^{a}) epineural sheath filled with allogeneic BMSC, evaluated at 6 and 12 weeks (n = 14).

C. Allogeneic (ACI RT1^{a}) epineural sheath filled with saline, evaluated at 6 and 12 weeks (n = 14).

The Siemionow group additionally performed 16 nerve repairs with autologous nerve grafts as a positive control at 6 (n = 8) and 12 weeks (n = 8). Assessment methods included TS, PP, SSEP, GMI, histomorphometry, and immunostaining.

Isogenic SCEC Results

With regard to the isogenic groups, there was no statistical difference in the PP score. The 6-week autograft repair had a significantly lower TS score than the 12-week autograft repair ($p = 0.014$). The saline/isogenic group also had a significantly lower TS score than the SCEC filled with isogenic BMSCs at 12 weeks ($p = 0.002$).

The GMI improved over time. The 12-week groups had significantly higher GMI than the 6 week groups. The saline had a lower GMI at 6 compared to 12 weeks ($p = 0.007$). Again, the isogenic SCEC had a significantly lower GMI at 6 when compared to 12 weeks as did the autograft ($p = 0.023$, $p = 0.004$, respectively). The SCEC repair was statistically comparable to the autograft repair at their respective time points.

Regarding SSEP, the P1 latencies were significantly longer in the isogenic SCEC group than the autograft group at 6 weeks ($p = 0.007$). The P1 latency significantly decreased over time with the isogenic SCEC having a lower latency at 12 compared to 6 weeks ($p = 0.043$). The saline construct had a significantly longer N2 latency at 12 weeks when compared to the autograft ($p = 0.024$). There was no significant difference in amplitudes among the different groups. The isogenic SCEC had comparable results with the autograft group at 12 weeks.

Histomorphometric analysis revealed no significant differences between the groups for G ratio or myelin thickness. The saline construct had a significantly smaller mean fiber diameter than the isogenic SCEC at 6 weeks ($p = 0.041$). The isogenic SCEC had comparable axon density at 6 and 12 weeks with the autograft repair.

Allogeneic SCEC Results

The construct created with the allogeneic epineural sheath filled with isogenic BMSCs did not demonstrate any appreciable regeneration on histomorphometry so the results are being reported with regard to the allogeneic epineural sheath filled with allogeneic BMSCs. The 6-week groups are still under observation.

Upon macroscopic observation, there were no signs of rejection. There was a significant difference between the saline, allogeneic SCEC, and autograft PP scores. The saline group had a significantly lower TS score than the autograft repair where the allogeneic SCEC was comparable. The autograft repair had significantly better GMI than the saline or allogeneic SCEC, indicating less denervation atrophy. The SSEP showed comparable P1 and N2 latencies as well as similar amplitudes among the three groups. Mean fiber diameter

and myelin thickness were also statistically similar across all groups. The autograft repair had a significantly higher axon density than the saline or allogeneic SCEC groups.

Long Nerve Defect Study

It was technically difficult to obtain and repair a 4 cm defect in a rodent. Given that a large animal model would move the project closer to clinical trials than a small animal model, it was decided that the long defect will be performed in a sheep model. The sheep protocol has been approved. Bone marrow harvesting has been performed in the first animal, and the first repair will soon be under way.

Key Research Accomplishments

- Completed a study focused on determining the ability of an epineural conduit filled with BMSCs (isogenic or allogeneic) suspended in a fibrin gel to repair a 2 cm rat sciatic nerve gap without the need for immunosuppression.
  - ♦ No significant differences were observed between the experimental and control groups on the variety of assessment methods conducted by the researchers.
- Conducted a SCEC study of a 2 cm rat sciatic nerve defect without the need for immunosuppression.
  - ♦ Initial results indicate the addition of BMSCs to the conduit leads to better outcomes than control (saline-filled) tubes.
- Obtained approval of the protocol for a study involving the repair of a long nerve defect in a large animal model (sheep).

Conclusions

The Siemionow team has proven that allogeneic epineural tube repair without immunosuppression is a feasible method of peripheral nerve gap repair. The epineural tube alone provides a neuropermissive environment for nerve regeneration by expressing laminin B2, which is a key ECM protein for axon growth. However, the addition of BMSCs results in better functional outcomes than the empty tube alone (i.e., there is a tendency for better TS scores in the BMSC groups and smaller nerve diameters in the saline groups).

In summary, an allogeneic epineural conduit combined with BMSC local therapy in a 2 cm rat sciatic nerve defect provides similar results to autograft repair. The epineural tube approach for nerve restoration is a promising new technique, which, specifically with BMSC local therapy, can provide outcomes that are comparable to autografts and superior to other techniques, including acellular nerve grafts. However, further studies are warranted to prove this efficacy in longer gap in a large animal model.

Research Plans for the Next 2 Years/Planned Clinical Transitions

The Siemionow group is planning to complete a large animal study and, based on the results, move into clinical trials utilizing two groups:

- Group 1 will include patients (n = 10) who will be considered for epineural tube nerve reconstruction after a neuroma resection when direct stump coaptation is not possible (i.e., the gap is too long) and autologous epineurium covering the

lesion can be spared during surgery after extirpation of the damaged neuroma fascicles and used as an autologous epineural conduit.

- Group 2 will consist of patients (n = 10) with traumatic peripheral nerve gaps longer than 3 cm. In this group, allogeneic epineural conduit grafting supported with autologous BMSCs will be performed.

Patients' detailed medical history will be collected with special attention paid to comorbidities that may affect nerve regeneration after repair surgery (e.g., diabetes, neuropathy, and alcoholism). Possible recipients will undergo preoperative neurological evaluation of the affected limb, including muscle strength, electromyography for muscle denervation, and sensory function testing (e.g., one-point, two-point discrimination; temperature; and light touch sensation). Magnetic resonance imaging will be utilized in patients from Group 1 to confirm the localization, dimensions, and internal structure (if possible) of the neuroma that is planned for resection. In patients with traumatic nerve gaps, magnetic resonance imaging will help to localize the proximal and distal stumps, especially in cases of proximal sciatic and brachial plexus injuries.

Regarding bone marrow harvesting and BMSC isolation and culture, the same procedure will be utilized in both groups. Between 2 and 3 weeks prior to the scheduled surgery, 40 mL of bone marrow will be harvested from the recipients' iliac crest using a standard aspiration technique under short general anesthesia. BMSC isolation and culture will be performed using standard methods. Based on the research team's previous experience in the rat model, 1×10^7 BMSCs suspended in 0.5 mL of solution will be needed per 1 cm of conduit of a median nerve at the level of the wrist.

Spatial and Temporal Control of Vascularization and Innervation of Composite Tissue Grafts

Project 4.4.3, WFPC

Team Leader(s): Robert Guldberg, PhD (Georgia Institute of Technology)

Project Team Members: Barbara Boyan, PhD, Ravi Bellamkonda, PhD, Yash Kolambkar, PhD, Nick Willett, PhD, Brent Uhrig, Isaac Clements, and Angela Lin (Georgia Institute of Technology) and Robert Taylor, MD, PhD and Natalia Landazura, PhD (Emory University)

Collaborator(s): Dietmar Hutmacher (Queensland University of Technology [QUT]), Andres Garcia (Georgia Institute of Technology), David Kaplan (Tufts University), Benjamin Harrison (Wake Forest Institute for Regenerative Medicine), Shawn Gilbert (University of Alabama at Birmingham), Thomas Clemens (Johns Hopkins University), George Muschler (Cleveland Clinic), and Josh Wenke (USAISR)

Therapy: Functional limb regeneration following severe combined bone, nerve, and vascular injuries.

Deliverable(s): Develop composite injury animal models that simulate complex military wounds. Establish and test spatiotemporal delivery strategies for regeneration of bone, nerve, and vascularity.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 3; End Year 3, TRL 3

Key Accomplishments: The researchers have established composite injury models in the rat that simulate bone/nerve, bone/vascular, and bone/muscle injuries. They have used these models to test nanofiber biomaterial delivery systems (patents pending) that provide spatial and temporal cues to guide improved bone and nerve regeneration. They have completed dose-response and clinical gold standard comparison testing. Notably, the researchers' rat segmental bone defect model has been adopted and used by other AFIRM investigators.

Key Words: Bone, nerve, vascularization, composite injury, animal model

Introduction

Traumatic injury to the extremities in combat is a significant problem for reconstruction and restoration of function. Complicated fractures and fragmented bone can cause loss of limb function even if the limb is restored esthetically. One reason for this is traumatic injury to the nerve with resulting loss of the musculature or bone tissue. Another reason is the lack of adequate vasculature needed to supply nutrients and connective tissue progenitor cells. There is a clear need for regenerative technologies that enable the restoration of limb function following composite tissue trauma. However, current preclinical testing models generally involve injury to only a single tissue type.

To address this limitation, a goal of this project is to establish animal models of composite tissue trauma that combine a massive segmental bone defect in the rat with peripheral nerve resection and/or femoral artery ligation. Importantly, quantitative evaluation methods, such as 3-D micro-CT imaging, electrophysiology, biomechanics, and gait analysis,

have been integrated into these models to provide comparison of competing regenerative strategies. The models are being used to quantitatively evaluate spatial and temporal delivery of biological cues that direct nerve, vascular, and bone growth in a synchronized manner. In addition to regenerative technologies developed at the Georgia Institute of Technology, these quantitative evaluation models are being used to test technologies developed in other AFIRM laboratories.

The specific aims of this project are to (1) develop composite injury rodent models and (2) quantitatively evaluate strategies for delivering spatial and temporal information to direct segmental bone regeneration, peripheral nerve repair, and vascular regrowth.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers established a novel spatial (nanofiber mesh) and temporal (alginate hydrogel) delivery strategy of a clinically approved osteoinductive factor (BMP-2) that can fully restore function to massive (8 mm) rat bone defects. After just 12 weeks, the fixation plates could be removed and the animals could ambulate normally on their regenerated limbs. It is important to note that the 8 mm femoral defect is 60% larger than the standard critical size (5 mm) for rat long bone defects and thus represents a highly challenging model. It was shown that a perforated nanofiber mesh design accelerates early bone repair at 4 weeks by enhancing the ingrowth of vascularity and/or osteoprogenitors from the surrounding soft tissues. This observation supported the hypothesis that composite tissue injuries would require a spatially and temporally coordinated treatment approach.

During the second year of the study, the research team established composite injury models in the rat that simulate bone/nerve and bone/vascular **(Figure II-27)** injuries. Results demonstrated that functional recovery from composite injuries is significantly more challenging than recovery from injuries to a single tissue. Using quantitative outcome measures, the researchers obtained promising results for highly translatable nanofiber biomaterial delivery systems that provide spatial and temporal cues to guide improved bone and nerve regeneration. They demonstrated that oriented nanofiber meshes can guide functional axon growth across critically sized peripheral nerve defects and that a novel nanofiber mesh/hydrogel protein delivery system fully restores the mechanical

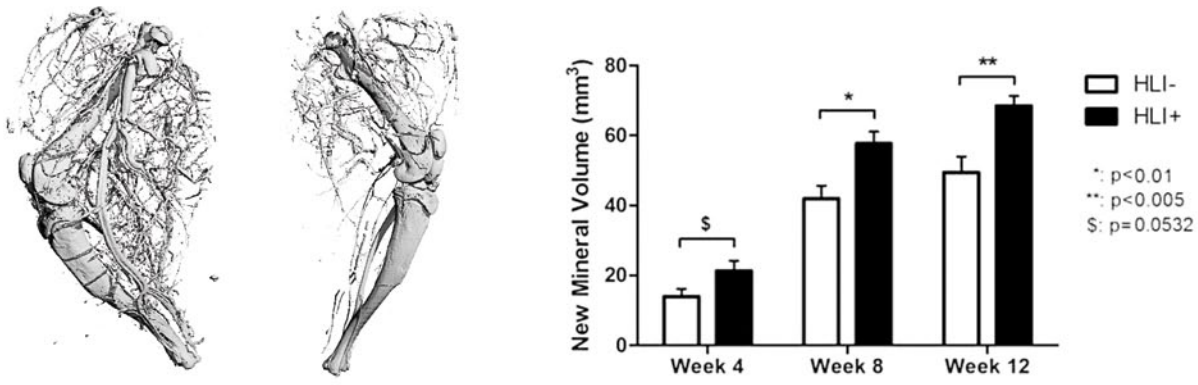


Figure II-27. Vascular image analysis of intact (left) and ischemic limbs (middle).

function of massive long bone injuries at lower doses than the current clinical standard.

Research Progress – Year 3

Over the past year, the Guldberg team made significant progress on the composite models of limb injury and hybrid construct bone repair technology. The researchers completed a study focused on quantifying the effects of ischemic injury on segmental bone repair. It was hypothesized that local ischemic injury within the thigh musculature would significantly reduce bone regeneration following the delivery of BMP-2 via hybrid nanofiber mesh/hydrogel constructs. Contrary to their hypothesis, the researchers found a stimulatory effect of hindlimb ischemia (HLI+) on bone ingrowth into adjacent 8 mm femoral bone defects. A follow-up, shorter-term study in year 4 will focus on factors expressed in the bone defect as a result of limb ischemia (e.g., hypoxia-inducible factors) to help identify potential therapeutic delivery targets that promote BMP-mediated bone regeneration in a similar manner.

The Guldberg team has also established a bone/nerve composite injury model by combining the 8 mm segmental bone defect model with a peripheral nerve injury model. A study to evaluate the effects of dual injury compared to single bone or nerve injuries treated using nanofiber delivery systems was completed during the past year. The nanofiber mesh/hydrogel BMP-2 delivery system successfully regenerated bone defects even with a 15 mm sciatic nerve injury **(Figure II-28)**. However, longitudinal gait analysis demonstrated that composite bone and nerve injury has a greater than additive effect on functional deficit compared to the effects of bone and nerve injury alone (Figure II-28). A third composite injury model involving a bone and muscle defect has also been developed and used to demonstrate that massive muscle injury significantly impairs BMP-mediated segmental bone repair (data not shown).

For peripheral nerve repair, the researchers have shown that nanofiber-based guidance channels promote robust levels of axonal regeneration even across critical-length nerve gaps. Significantly, this regeneration can occur in the absence of any exogenous ECM or trophic factors. The aligned topography of the interposed nanofiber thin-films stimulates endogenous repair processes, promoting a sequence of regenerative events that normally fail to occur over critical-length nerve gaps. The most recent studies indicate that a

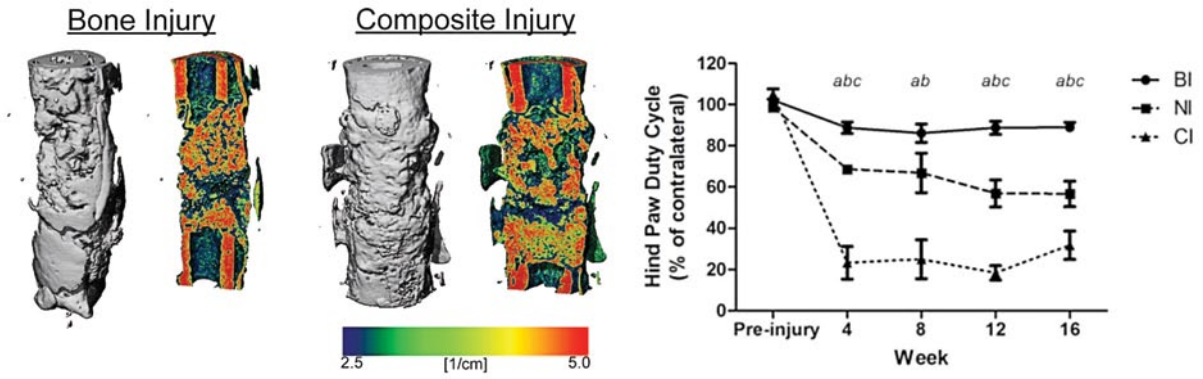


Figure II-28. Micro-CT images of bone repair at 12 weeks (left) and gait analysis results (right).

single nanofiber membrane produces superior axonal growth and organization compared to multiple membranes within guidance channels.

The 8 mm model has also been used over the past year to begin quantitatively testing a technology being developed in another AFIRM laboratory (Dr. David Kaplan, Tufts University). The 4-week only results demonstrate that silk hydrogel containing BMP can promote bone formation within 8 mm defects similar to that seen using alginate hydrogel **(Figure II-29)**. The 8- and 12-week data, including biomechanical testing results, are pending.

Key Research Accomplishments

- Established a composite bone/nerve injury model and completed a study showing increased functional deficit associated with the composite injury.
- Established a composite bone/vascular injury model and completed a study showing that transient ischemia may promote BMP-mediated repair of bone defects.
  - As part of this work, a method was developed to assess the spatial distribution of vascular ingrowth into muscle and bone and used to demonstrate a gradient reduction in vascular ingrowth from the proximal to the distal end of limb injuries.
- Established a composite bone/muscle injury model and completed a study with leveraged funds showing that massive muscle loss significantly impairs BMP-mediated bone repair.
- Initiated a pilot study of the nanofiber mesh technology in a large animal model (sheep) in collaboration with Dietmar Hutmacher at the Regenerative Medicine Institute at QUT.

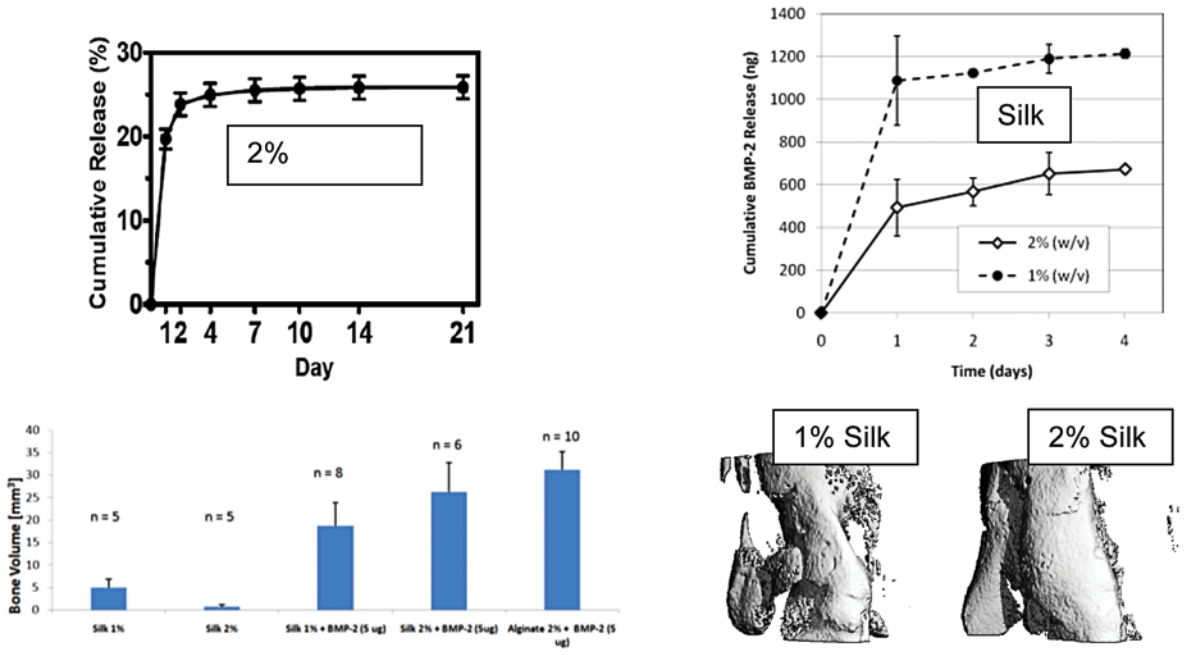


Figure II-29. 1% and 2% silk hydrogels provide slower BMP release kinetics compared to 2% alginate. At 4 weeks, the BMP groups had more bone formation than the delivery matrices alone.

- Initiated a collaborative study with Dr. Kaplan (Tufts University) to test his silk-based hydrogels for delivery of BMP in the standardized 8 mm bone defect model.

Conclusions

Composite multitissue injury models have been developed to simulate complex combat injuries and test spatial and temporal guidance strategies that take advantage of synergistic interactions among the tissues observed during development and repair. The rat model was chosen since it provides the opportunity for larger in vivo studies and is amenable to highly quantitative assessment methods (e.g., micro-CT assessment of vascularization and bone formation). Variations of the composite injury model include bone/nerve injuries, bone/vascular injuries, and bone/muscle injuries. These models are available for testing regenerative strategies developed by other AFIRM investigators. At the Georgia Institute of Technology, the researchers have established promising regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of clinically approved inductive protein (BMP-2).

Research Plans for the Next 2 Years

During the next 2 years, the researchers aim to:

- Initiate a large animal study at QUT to test the nanofiber mesh/hydrogel BMP delivery technology. Completion of this study will allow transition to TRL 4. Once the patent has been issued and the large animal study is completed, the researchers will seek out a company to license the technology.
- Perform a short-term (4 weeks) study in the bone/vascular composite injury model to assess mechanisms underlying the enhanced bone repair associated with ischemic injury.
- Complete the silk hydrogel BMP delivery study as an alternative delivery matrix that provides longer-term delivery.
- Complete a nanofiber mesh/alginate BMP delivery study using a faster degrading RGD functionalized alginate.
- Conduct follow-up studies aimed at optimizing the nanofiber mesh perforation pattern and the hydrogel degradation rate.

Planned Clinical Transitions

The next step is to initiate large animal studies of the most promising technologies. Additional funds have been identified for sheep defect studies from the Australian Research Council. Contract details for this study between the Georgia Institute of Technology and QUT are in progress. A Materials Transfer Agreement with Pfizer to provide BMP for the large animal study has also been initiated. Dr. Robert Guldberg will also coordinate with Dr. George Muschler on the development of a standardized goat bone defect model within the AFIRM.

The intellectual property will be marketed to members of the Georgia Institute of Technology industry partners program. The researchers have previously had success licensing patent rights to industry partners following successful large animal studies. Once proof of concept has been demonstrated in the large animal model, the goal is to initiate a human clinical trial pilot study in year 5.

Corrections/Changes Planned for Year 4

Large animal studies have been added to the project plan to accelerate progression toward clinical translation of the spatiotemporal delivery systems.

Epimorphic Regeneration Approach to Limb and Digit Reconstruction

Project 4.4.1, WFPC

Team Leader(s): Stephen F. Badylak, MD, PhD, DVM (University of Pittsburgh)

Project Team Members: Vineet Agrawal, Scott Johnson, Neill Turner, Alex Huber, Li Zhang, Janet Reing, and Stephen Tottey (McGowan Institute for Regenerative Medicine, University of Pittsburgh) and Ron Stewart (University of Wisconsin)

Collaborator(s): Ron Stewart and Jamie Thomson (University of Wisconsin); Susan Braunhut (University of Massachusetts, Lowell); David Kaplan (Tufts University); Eileen Moss and Muthu Wijesundara (The University of Texas at Arlington); Karen K. Hirschi (Baylor College of Medicine); and Peter Rubin (University of Pittsburgh)

Therapy: Treatment of digit loss with epimorphic regeneration strategies.

Deliverable(s): A biologic scaffold-based strategy for inducing epimorphic regeneration in limb and digit soft tissues. A biomaterial that can facilitate epimorphic regeneration in soft tissues (multiple forms, solid sheet, gel, powder, etc.).

TRL Progress: Start of Program, TRL 3; End Year 1, TRL 4; End Year 2, TRL 5; End Year 3, TRL 5

Key Accomplishments: The researchers have shown in a mouse model of mid second phalanx digit amputation that treatment with bioactive molecules derived from ECM can recruit endogenous multipotential stem cells to the site of injury. During the past year, they have partially characterized the source of the previously identified Sox2 cells recruited to the site of digit amputation following ECM administration. Additionally, they have characterized one of two isolated fractions of bioactive ECM molecules that can also recruit multipotent Sox2+, Sca1+, and Lin- cells to a site of amputation. Finally, they have shown that treatment with bioactive ECM molecules results in functional tissue formation, such as bone, at the site of amputation.

Key Words: Limb regeneration, extracellular matrix, epimorphosis, multipotential cell cluster

Introduction

The Badylak group is investigating mechanisms for stimulating nonblastemal epimorphic regeneration in tissues beyond the very few that presently exist in adult mammals. The liver, skin, bone marrow, and intestinal lining of epithelial cells are examples of tissues that exhibit nonblastemal epimorphic regeneration in adult mammals. However, virtually every other tissue does not have this capacity as a component of the default mechanism for wound healing. The present work in large part is based upon resurrecting this nonblastemal regenerative capacity in alternative tissues. The signals to facilitate this resurrection of nonblastemal regeneration reside within the ECM. Developing therapeutic strategies that can take advantage of this matrix-based approach is the fundamental objective of this project.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the research team used the C57Bl/6 mouse digit amputation model **(Figure II-30)** to show that treatment with bioactive homing signals from ECM resulted in a denser cellular accumulation at the site of amputation, which expressed multiple markers of primitive, multipotent stem cells (e.g., Sca1, CD146, and Sox2). These multipotential cells have the ability to differentiate into tissues from all three germ layers (i.e., ectoderm, mesoderm, and endoderm) and cause a significant shift in the wound-healing potential of mammals.

Research Progress – Year 3

Specific Aim 1: To identify a refined "genetic signature" for cells that participate in the formation of a blastema-like structure as opposed to the gene expression profile of cells that participate in default wound healing and scar tissue formation.

Studies during the past year have focused on further characterizing the Sox2+ population of cells at the site of amputation since Sox2 is a transcription factor associated with very primitive, pluripotent stem cells.

Treatment with bioactive molecules from ECM led to a greater number of Sox2+ cells at the site of amputation on days 10, 14, and 18, post-amputation and treatment **(Figure II-31)**. Sox2 expression was confirmed by microdissection and isolation of the cells. Fluorescence-activated cell sorting (FACS) analysis showed that the Sox2+ cells coexpressed mesenchymal stem cell markers Sca-1 and CD90 but not hematopoietic stem cell marker c-kit or dermal stem cell marker CD133 (Figure II-31). Nuclear expression of phosphorylated histone H3 confirmed that a subset of Sox2+ cells was proliferating, indicating that the Sox2+ cells at the site of amputation are functionally active.

To determine the source of the Sox2+ cells, the researchers utilized transgenic Sox2 eGFP/+ mice and bone marrow chimeric mice. In Sox2 eGFP/+ mice, all Sox2+ cells expressed green fluorescent protein (GFP). In the bone marrow chimeric mice, only bone marrow- or circulation-derived Sox2+ cells expressed GFP. Following digit amputation of Sox2 eGFP/+ and bone marrow chimeric mice and treatment with ECM

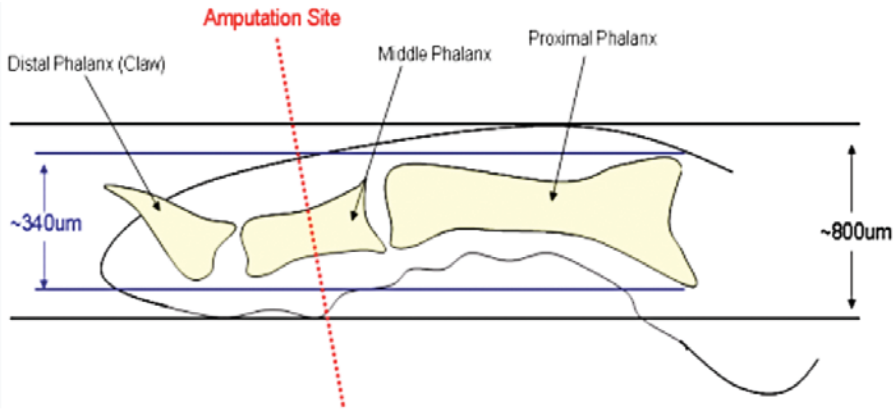


Figure II-30. Site of P2 amputation in a mouse model.

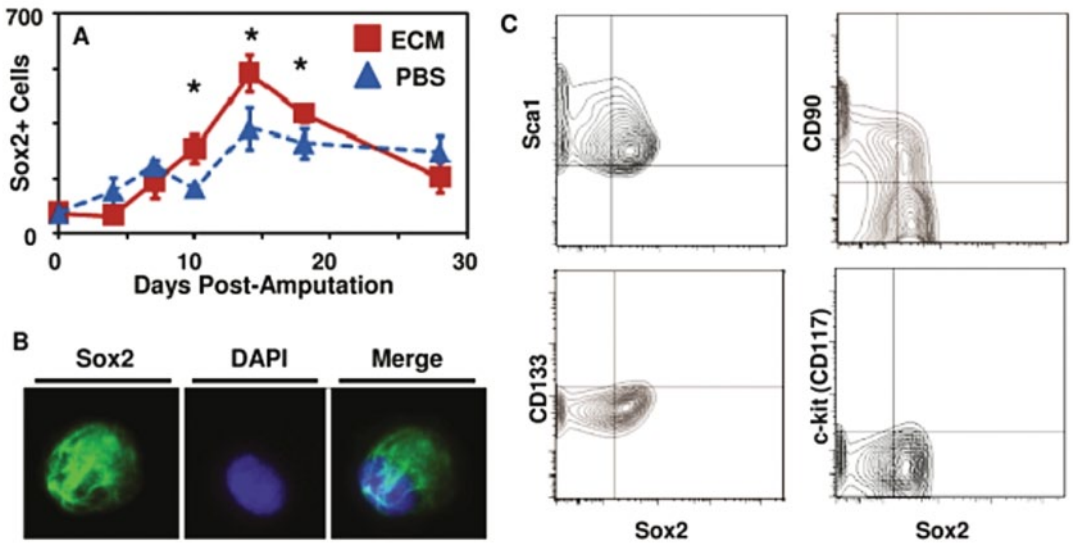


Figure II-31. ECM treatment leads to a greater number of Sox2+, Sca1+ and CD90 cells at 10, 14, and 18 days post-amputation.

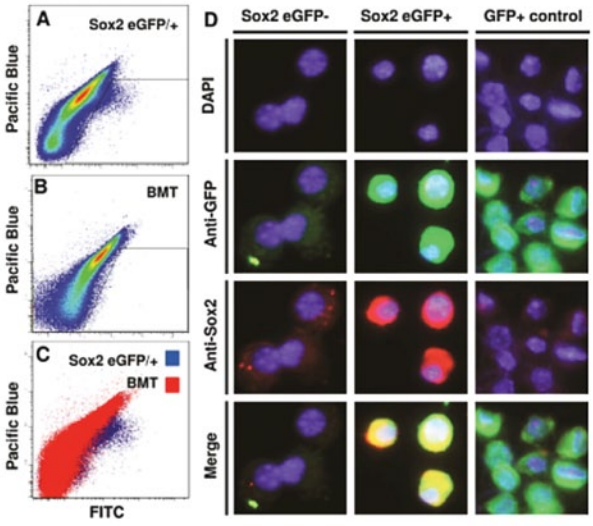


Figure II-32. Sox2+ cells are not derived from the bone marrow or circulation.

bioactive peptides, the researchers isolated the accumulated multipotential cell cluster on day 14 post-amputation by microdissection and dissociation. By FACS analysis, GFP+ (i.e., Sox2+) cells were found at the site of amputation in Sox2 eGFP/+ mice, but no GFP+ cells were found at the site of amputation in bone marrow chimeric mice. These data suggest that none of the Sox2+ cells are bone marrow derived. The GFP+ and GFP- cells were sorted, plated, fixed, and immunolabeled for GFP and Sox2 to confirm expression **(Figure II-32)**.

Specific Aim 2: To identify in vitrobioactive molecules that can instruct, facilitate, or promote the formation of a blastema-like structure following injury.

During the past year, the researchers focused on characterizing a single chemotactic peptide isolated by successive rounds of ammonium sulfate precipitation, size exclusion chromatography, ion exchange chromatography, and reverse phase chromatography. The isolated chemotactic peptide was chemotactic in a

Boyden chamber migration system for human perivascular stem cells. The peptide was identified to be a fragment of the C-terminal telopeptide of the collagen IIIα molecule, a highly conserved 12-amino acid sequence among over eight species.

The 12-amino acid peptide sequence also showed chemotactic activity for hNSCs **(Figure II-33A)**, human adipose stem cells (Figure II-33B), muscle myoblast progenitor cells (Figure II-33C), and Schwann cells (Figure II-33D). It showed relatively less activity for more differentiated endothelial cells (Figure II-33E) and epithelial cells (Figure II-33F). In vivo, the chemotactic peptide showed the ability to recruit Sox2+, Sca1+, and Lin- cells to a site of injury in an adult mouse model of digit amputation **(Figure II-34C-E)**. At 7 days post-amputation, an accumulation of cells was observed at the site of amputation following injection of the candidate peptide whereas treatment with phosphate-buffered saline (PBS) led to scar tissue deposition typical of the default response to wound healing (Figure II-34A,B).

The isolated peptide from Specific Aim 2 was further characterized for its ability to alter osteogenesis. In vitro, the isolated peptide accelerated osteogenesis of human perivascular stem cells as measured by Alizarin red staining and alkaline phosphatase activity **(Figure II-35)**. Quantitative RT-PCR showed that the peptide increases expression of both osteogenic and chondrogenic genes in vitro **(Figure II-36)**.

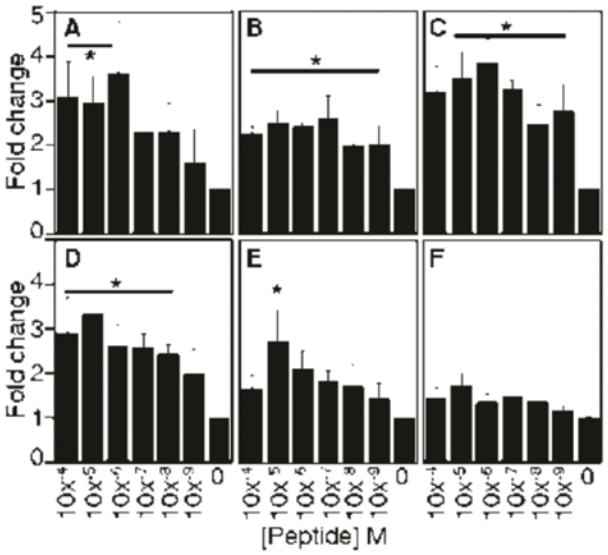


Figure II-33. Migration of (A) neural stem cells, (B) adipocyte stem cells, (C) muscle myoblast progenitors, (D) Schwann cells, (E) differentiated endothelial cells, and (F) differentiated epithelial cells toward the isolated peptide.

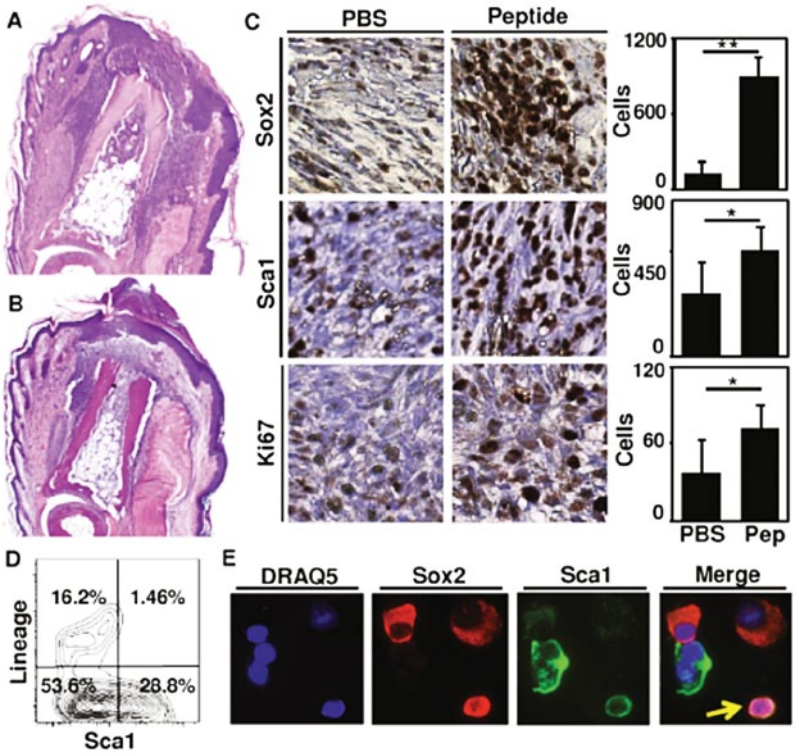


Figure II-34. Treatment with the chemotactic peptide results in the recruitment of Sox2+, Sca1+, and Lin- cells at the site of amputation whereas PBS control treatment results in scar tissue formation.

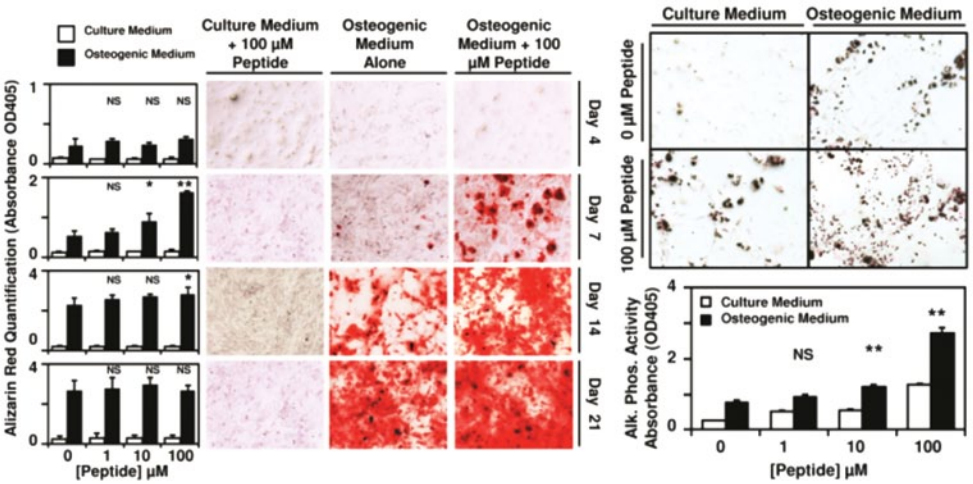


Figure II-35. The isolated bioactive peptide accelerates osteogenesis of human perivascular stem cells in vitro.

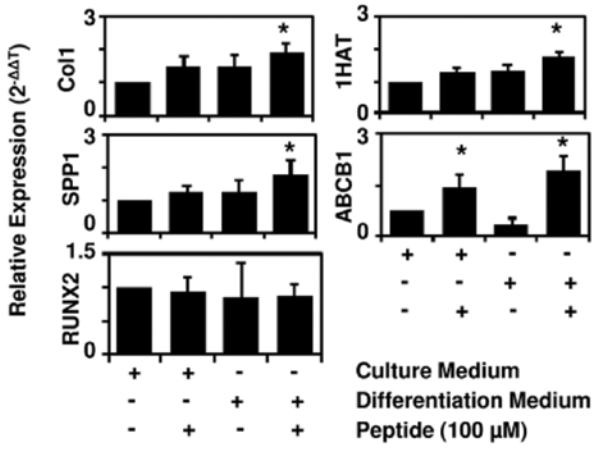


Figure II-36. The isolated peptide induces chondrogenic and osteogenic gene expression.

Finally, treatment with bioactive peptide in an adult mammalian model of digit amputation resulted in the formation of a bone nodule at the site of amputation **(Figure II-37)** consistent with the location of Sox2+, Sca1+, and Lin- cell accumulation at day 7 post-amputation. Differential calcium dye staining in vivo showed that treatment with the isolated peptide resulted in more new calcium deposition (red) as compared to old bone (green). Staining with Alcian blue stain showed that the new bone formation underwent endochondral ossification with a largely cartilaginous core that eventually matured and ossified (Figure II-37).

Specific Aim 3: To evaluate potentially therapeutic molecules for digit reconstruction in vivo.

In collaboration with the Automation and Robotics Research Institute at the University of Texas Arlington, work in the past year resulted in a prototype device designated as the BIODOME (Biomechanical Interface for Optimized Delivery of MEMS Orchestrated Mammalian Epimorphosis) that was developed for an adult mouse model of digit

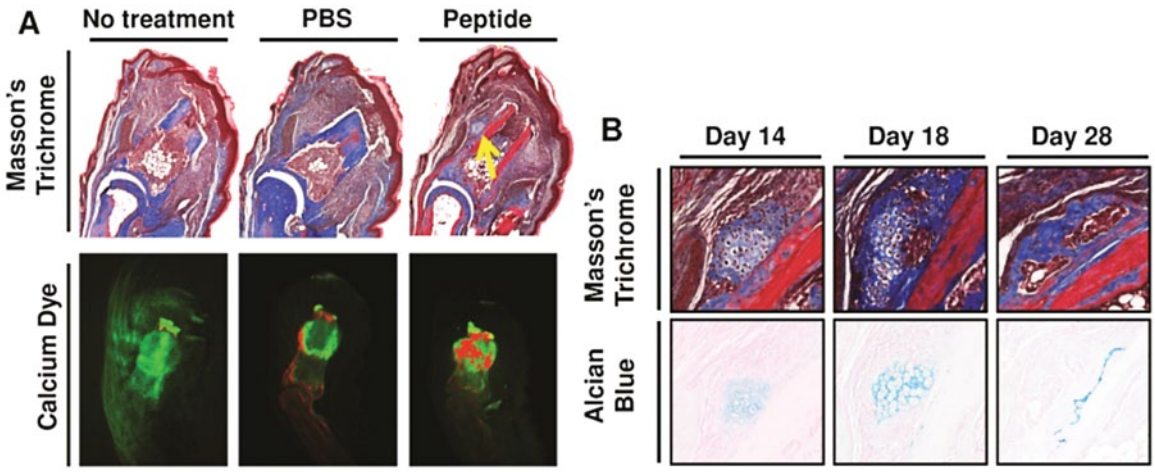


Figure II-37. (A) Treatment with the bioactive peptide in an adult mouse model of digit amputation results in new formation of calcified bone (arrow) at day 14 post-amputation. The bone nodule undergoes endochondral ossification.

amputation. The device is currently in testing in the mouse model, and optimization of the design in the mouse model will inform future designs for a BIODOME device to control digit reconstruction in a human pilot study (Specific Aim 4). The proposed work will focus on utilization of the findings of Specific Aims 1–3 and the mouse BIODOME to refine a prototype for a human BIODOME.

Key Research Accomplishments

- Established the phenotype and source of Sox2+ cells as proliferating cells that coexpress Sca-1 and CD90 that are not derived from the circulation or bone marrow but rather from tissue resident progenitor cells.
- Isolated, sequenced, and synthesized a potent ECM fraction derived from the collagen III molecule with chemotactic properties for multiple progenitor cells in vitro and in vivo.
- Identified the osteogenic activity of the isolated peptide in vitro for human perivascular stem cells.
- Demonstrated bone tissue growth and endochondral ossification at a site of amputation following injection of the isolated peptide.
- Designed a prototype BIODOME device for controlling the microenvironment at the site of amputation.

Conclusions

Progress throughout the past 3 years has demonstrated that bioactive peptides derived from ECM recruit a population of tissue resident multipotent stem cells that express markers such as Sox2, Sca-1, CD146, and CD90. Additionally, specific bioactive peptides have been identified with unique properties for stem cell migration and differentiation. These bioactive peptides are an attractive therapeutic tool by which to influence the injury microenvironment and direct local and circulating stem cells to migrate and differentiate at the site of injury into functional tissue.

Research Plans for the Next 2 Years

The researchers will continue to fractionate the ECM into constituent peptides that will be screened for their ability to induce or differentiate the multipotential cell cluster. Work in the upcoming year will specifically focus on two major aims: (1) identifying peptides that have the ability to alter the recruitment and differentiation of stem cells toward other important tissues in the digit, such as muscle, nerve, and vasculature and (2) controlling the spatiotemporal pattern of treatment with the bioactive peptides at the site of amputation via a microfluidic device designed to cover the site of digit amputation. Additionally, the researchers would like to further develop the concept of the BIODOME into a functional device with an objective of eventual use in human trials. The BIODOME now represents the rate-limiting step to clinical translation.

Planned Clinical Transitions

In collaboration with Dr. Rubin at the University of Pittsburgh, the research team is evaluating a powder form of ECM for the treatment of distal digit amputations. The product being used is a commercially available FDA-approved form of powdered ECM. The patients who are selected for the study are treated based on the findings of the work conducted during the researchers' first 3 years with the digit amputation model.

Additionally, the researchers are attempting to get a 10-patient trial started at USAISR. The proposed work involves the treatment of patients who have suffered from loss of large amounts of functional musculotendinous tissue as a result of trauma. The injury in these patients must be such that either heroic tissue transplantation procedures would be required or amputation would be necessary under the current standard of care. These patients will be implanted with one of several different forms of ECM. The clinical trial is currently in review by the IRB. The lead in that trial will be Dr. Hsu.

our science for their healing

High-Throughput Approaches Applied to Tissue Regeneration

Project 4.4.7, WFPC

Team Leader(s): Ron Stewart, PhD (Morgridge Institute for Research) and James Thomson, VMD, PhD, Diplomate ACVP (Morgridge Institute for Research, University of Wisconsin, Madison, and University of California, Santa Barbara)

Project Team Members: Srikumar Sengupta, PhD and Mitch Probasco, BS (Morgridge Institute for Research)

Collaborator(s): Stephen F. Badylak, MD, PhD, DVM (University of Pittsburgh) and H. Tom Soh, PhD (University of California, Santa Barbara)

Therapy: The long-term therapy for this work will be improved limb and tissue regenerative outcomes in mammals (including humans).

Deliverable(s): (1) Whole transcriptome measures on ECM-treated and untreated P2 amputated mouse digits. (2) Whole transcriptome measures on axolotl blastemas and portions of the axolotl limb.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 2; End Year 3, TRL 2

Key Accomplishments: During the past year, the researchers complemented the work they had completed during the first 2 years of the project by performing next-generation sequencing on RNA from the axolotl as a model system for limb regeneration. They identified genes that are upregulated in the axolotl blastema, including chromatin remodelers, high-mobility group (HMG) genes, and oncogenes. They identified three genes that are upregulated along the proximal-distal axis of the axolotl limb. They began pilot studies of the transcriptome of portions of the adult mouse limb, and preliminary data reveal patterns of gene expression along the proximal-distal axis.

Key Words: Mouse, transcriptome, regeneration, axolotl

Introduction

To complement the prior years' work on mouse digit regeneration, next-generation sequencing using the Illumina GAII has been performed on RNA from the axolotl (*Ambystoma mexicanum*) as a model system for limb regeneration. The ability to fully regenerate an adult amputated limb is found in certain species of newts and salamanders including the axolotl. This ability is unique within the vertebrate phylum and thus makes these animals important models for limb and tissue regeneration. An axolotl colony has been established in the Thomson laboratory. The team has been investigating tissue regeneration in the axolotl using high-throughput, next-generation sequencing of RNA (RNA-seq).

The research team has produced information on all of the genes that are expressed (the "transcriptome") in the axolotl blastema (the structure in the regenerating limb that orchestrates limb regeneration) during regeneration over a time course spanning 0–28 days as well as transcriptomes of various portions of the axolotl limb. The team also produced a transcriptome of the adult blastema. The longer-term purpose is to harness this knowledge in conjunction with methods from the team's prior work on the reprogramming of cells to activate or deactivate appropriate genes and gene networks to foster regeneration of mammalian tissues.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers completed a pilot microarray study of mouse digit tips, which showed that treatment with ECM factors designed to enhance regenerative capabilities led to the expression of ECM remodeling genes and genes indicative of stem cell activity. In addition, they developed methods for sample preparation and RNA amplification from small-quantity RNA samples for microarray and next-generation sequencing analysis. They established data analysis pipelines for microarray and RNA-seq analysis. They developed lentiviral-based methods for reprogramming cells based on altering transcriptional networks. They also established methods for predicting gene networks based on coregulation analysis.

During the second year of the study, the research team successfully produced a transcriptome of ECM-treated and untreated P2 amputated mouse digits over a 14-day time course. The team exceeded the deliverable in providing detailed information about the most upregulated and downregulated genes, including enriched Gene Ontologies that identified key groups of genes that are up- and downregulated on treatment. This information is useful in determining gene networks that are activated after ECM treatment.

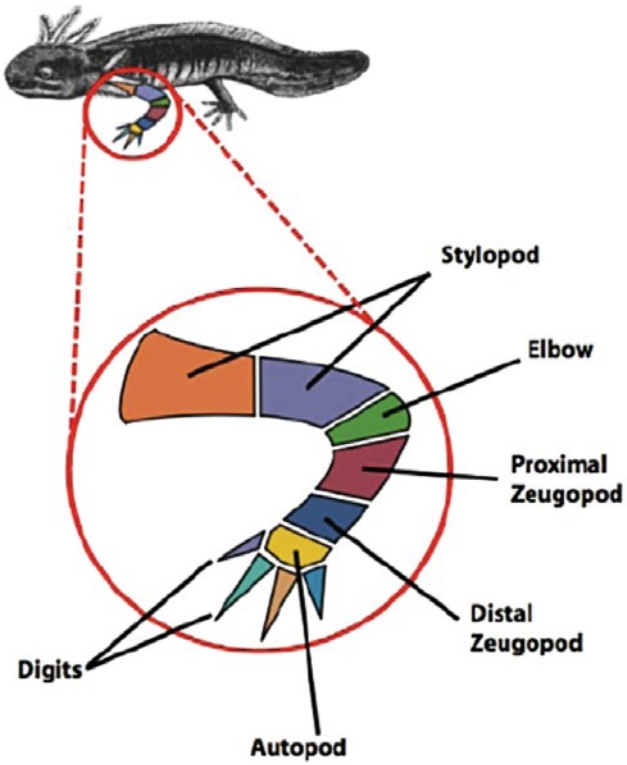


Figure II-38. Portions of the axolotl limb that were harvested for RNA-seq analysis.

Research Progress – Year 3

The team performed quality control testing on all samples for RNA-seq. All samples passed testing. Samples were processed and submitted for sequencing on the Illumina GAII sequencing platform. **Figure II-38** shows a diagram of the portions of the axolotl limb used for analysis.

Data were analyzed to identify genes enriched in the adult blastema. Identification of blastemal-enriched genes is important as it lays the groundwork for what is unique in the axolotl blastema (which mounts a regenerative response) versus the amputated mouse digit (which undergoes wound healing and scar formation but does not mount a regenerative response). The team normally studies all of the genes in the genome, but the preliminary analysis focused on transcription factors as these can set and maintain distinct cell states. Transcription factors enriched in the blastema (when compared to portions of the adult axolotl arm) are shown in **Figure II-39**.

Some salient observations include:

- HMG transcription factors (highlighted in brown-orange) are highly statistically enriched ($p < 0.01$).
- Oncogenes (purple) appear on this upregulated list. Note also that many HMGs are oncogenes.
- Polycomb repressor complex genes (green) appear on the list.
- Many genes known to be expressed in the blastema or mammalian limb bud (blue) appear on the list.

Adult Blastema-specific TFs

	symbol	description	Gene Rank (limb)		Blastema/Pod
2	HMGA2	high mobility group AT-hook 2		height in humans	11.24315285
3	PRRX1	paired related homeobox 1	52		7.88372982
4	MYCN	v-myc myelocytomatosis viral related oncogene,	139		6.685514784
5	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1			6.126971645
6	TFDP2	transcription factor Dp-2 (E2F dimerization partner 2)			5.821389846
7	HMGN2	high-mobility group nucleosomal binding domain 2		expr in limb buds	5.738047245
8	HDAC2	histone deacetylase 2			5.644422167
9	SOX4	SRY (sex determining region Y)-box 4			5.626658105
10	SUZ12	suppressor of zeste 12 homolog (Drosophila)			5.486570925
11	NCALD	neurocalcin delta			5.285675573
12	PRDM16	PR domain containing 16		limb bud: http://www.sciencedirect.	4.694029559
13	C14orf106	chromosome 14 open reading frame 106			4.584721613
14	TWIST1	twist homolog 1 (Drosophila)	42		4.521737384
15	LIMA1	LIM domain and actin binding 1			4.345202481
16	PCGF3	polycomb group ring finger 3			4.300849899
17	HMGB2	high-mobility group box 2			4.085397099
18	TCF4	transcription factor 4	174	prepatterns limb muscle: http://ww	4.082481656
19	TNRC6A	trinucleotide repeat containing 6A			3.749782035
20	TARDBP	TAR DNA binding protein			3.615127452
21	TRPS1	trichorhinophalangeal syndrome I			3.584016377
22	SUB1	SUB1 homolog (S. cerevisiae)			3.54759269
23	MLLT3	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3			3.423886672
24	DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)			3.313491393
25	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein			3.224372814
26	UBE2V1	ubiquitin-conjugating enzyme E2 variant 1			3.163877073
27	TFAP2C	transcription factor AP-2 gamma (activating enha	415		3.093388447
28	ZFPL1	zinc finger protein-like 1			3.088258587
29	EZH2	enhancer of zeste homolog 2 (Drosophila)			3.04078754
30	above have a blastema over stylo plus auto ratio of at least 3. And have low variance, adj pvalue < 0.05				

Brown--HMG (3 in top 16, pValue<< 0.01)
Purple--Oncogenes
Green--Polycomb
Blue-Blastemal or Limb Bud

Figure II-39. Blastema-enriched transcription factors.

These observations suggest that blastema formation involves substantial chromatin remodeling since oncogenes, polycomb genes, and HMG genes are all chromatin remodelers. The presence of many known blastemal and limb genes on this list increases confidence that the other genes are indeed important for the blastemal cell state. The team has performed analyses with Gene Ontology and Gene Set Enrichment Analysis that support with statistical rigor the observation of the enriched gene categories shown in Figure II-39.

When comparing the axolotl blastema to the amputated mouse digit, several genes are specifically enriched in the axolotl blastema including HMGA2. These genes are candidate genes for forced expression in the mammalian system, potentially leading to improved regenerative outcomes.

The team has also identified several genes that are expressed more highly in the distal region of the adult arm. This list includes EMX2, KLF2, and XBP1. These three genes are graded in their expression along the proximal-distal axis with highest expression in the digits **(Figure II-40)**. Notably, other researchers have implicated EMX2 to play a role in patterning in the newt.

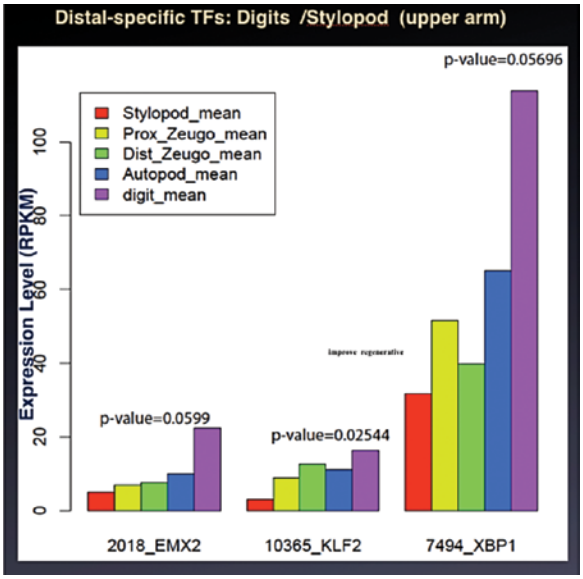


Figure II-40. Distal-enriched transcription factors.

These and other genes expressed in a region-specific manner in the adult limb are important in that they may play a role in providing instructive cues to the developing blastema as to what structures to regenerate. Understanding how this pattern forms in the axolotl is crucial to being able to reproduce, mimic, and improve regenerative behavior in the mammalian system. The team is in the process of producing transcriptomes for various portions of the adult mouse limb to determine if a similar pattern of expression along the proximal-distal axis exists in the mouse. The team has performed RNA-seq analysis in pilot studies of the mouse limb,

and preliminary data reveal patterns of gene expression along the proximal-distal axis.

Key Research Accomplishments

- Completed RNA-seq analysis of axolotl blastema time course samples, axolotl adult blastema samples, and portions of the adult axolotl limb.
- Performed detailed data analysis (included gene enrichment and Gene Ontology enrichment) of the axolotl RNA-seq data.
- Identified genes upregulated in the blastema, including chromatin remodelers, HMG genes, and oncogenes.
- Identified three genes upregulated along the proximal-distal axis of the axolotl limb (EMX2, KLF2, and XBP1).
- Began pilot studies of the transcriptome of portions of the adult mouse limb.
    - Preliminary data reveal patterns of gene expression along the proximal-distal axis.

Conclusions

The potential implications of this work are substantial. Identification of similarities and differences in the active gene networks in the mouse digit model system and the axolotl blastema will provide information on the factors needed to modulate gene expression to enhance limb/digit regeneration (and mammalian tissue regeneration in general). This work has already identified many transcription factors that are blastema enriched or enriched in various portions of the adult axolotl limb. Comparison of the axolotl data to data in the mouse digit amputation model and the mouse limb will provide clues to genes that may be regeneration specific.

This work will inform the team on how to manipulate the mouse model system to enhance digit regeneration by activating regenerative behavior within the mouse digit. Even a partial activation of regeneration would represent a substantial advance in regenerative medicine.

Research Plans for the Next 2 Years

This project will conclude on December 31, 2011.

our science for their healing

High-Purity Magnetophoretic Sorting for Transplant Therapies

Project 4.4.8, WFPC

Team Leader(s): H. Tom Soh, PhD (University of California, Santa Barbara)

Project Team Members: David Bothman, Jinpeng Wang, and Yi Xiao (University of California, Santa Barbara)

Collaborator(s): James Thomson (University of Wisconsin) and Erkki Ruoslahti, MD, PhD (Sanford-Burnham Medical Research Institute)

Therapy: Improved transplant therapy outcomes using novel microfluidic magnetophoretic cell sorting devices.

Deliverable(s): Microfluidic magnetophoretic cell sorting devices and systems for the isolation of pluripotent, rare stem cells. Magnetophoretic devices for the identification of new markers for nuclear reprogramming.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 2; End Year 3, TRL 2

Key Accomplishments: The researchers have developed a unique microfluidic phage selection (MiPS) system that is capable of efficiently isolating peptides with high affinity and specificity to target cell surfaces. They performed de novo selection of a T7 phage library expressing random, linear X7 peptides against PPC-1 cells using both conventional biopanning and the MiPS platform. They achieved efficient in vitro selection of T7 phage-displayed peptides that recognize markers expressed on live, adherent cells within a microfluidic channel.

Key Words: Magnetic cell sorting, microfluidics, peptides, aptamers, stem cell transplant

Introduction

Cell sorting is a critical technology for cell-based therapeutics wherein the rare target cells must be isolated from complex mixtures such as blood and homogenized tissue. The performance of cell sorting is benchmarked by three key metrics: purity (the fraction of target cells among collected cells), recovery (the fraction of input target cells successfully collected after sorting), and throughput (the number of cells sorted per unit time). To achieve high-performance sorting of rare cells, two key technologies are required:

- High-performance microfluidic cell sorting system. The current standard for rare cell sorting is FACS. However, due to the serial nature of its operation, FACS is inherently limited in throughput, which is insufficient for many clinical applications. Thus, the challenge in high-performance cell sorting is to decouple the three competing performance metrics and to simultaneously achieve high purity, recovery, and throughput. Furthermore, to avoid contamination by the production of stem cell products, it is imperative to integrate the cell separation with expansion. These capabilities are not currently available and need to be developed.
- Affinity Reagents. To efficiently label the target cells for sorting, there is a critical need for advanced technologies for the generation of reagents that specifically target cell surface markers because transmembrane proteins are notoriously

difficult to express in recombinant form. Conventional methods of hybridoma production or phage screening are lengthy, labor intensive, and expensive. Thus, there is a need for novel technologies that can rapidly generate affinity reagents that target cell surface markers.

The Soh group has been developing a novel MS system termed the Ultrahigh Gradient Magnetic Activated Cell Sorter (UHG-MACS). The main advantage of the UHG-MACS technology is that through the unique fluidic and magnetophoretic physics that occur only at the microscale, one can control separation forces with exquisite precision and reproducibility. This enables exceptional purity and ultrahigh recovery at high throughputs. Furthermore, since the cell-sorting process is performed in a disposable chip format, multiple assay steps can be integrated into a single, closed system. These features are highly advantageous for stem cell transplant therapies because they obviate the need for system cleaning and maintenance, and eliminate sample contamination.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers developed devices that (1) can achieve, for the first time, the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput and (2) allow purification of extremely rare cells from complex mixtures with unprecedented cell recovery. With their high-purity, multitarget MS device, the researchers demonstrated extremely high recovery of rare target cells from whole blood (92% recovery of 10 cells/mL). In addition, they successfully integrated in situ culturing capability within the separation device for contamination-free expansion of stem cell products. Finally, using their microfluidic separation devices, they obtained key results toward the rapid isolation of aptamer reagents for stem cell surface markers.

Research Progress – Year 3

Development of CT-MACS Device for Rare Cell Isolation

In collaboration with Cynvenio Biosystems, the researchers' UHG-MACS system was used to successfully capture extremely rare cells (50, 30, and 10 cells/mL) from an excess of background cells (approximately 10^9 cells/mL) with exceptional recovery (greater than 90%) **(Figure II-41)**.

The chip is optically transparent, which allows for direct optical analysis after the separation—this is a unique feature that is not available in commercial systems. In addition, the small chamber volume (300 µL) naturally offers low reagent consumption and reduced reaction time.

Development of MiPS

The researchers report the first successful directed evolution of phage libraries that target live, adherent cell surfaces using microfluidics technology **(Figure II-42)**. The ability of this system to directly target membrane-bound proteins on cell surfaces is significant since most of these proteins are notoriously difficult to produce using recombinant technology. They show that the MiPS system offers significant advantages compared to conventional biopanning methods. First, due to the fact that the selection is performed within a microchannel, significantly smaller numbers of target cells are required. This allows one to impose highly stringent mass-action selection pressure (e.g., high molar ratios between the library and target cells), yielding peptides with higher affinity. Second, control of the flow rate of fluids within the microchannel provides a continuous and reproducible means for efficiently removing weakly or nonspecifically bound phage, resulting in low background binding with minimal cell loss. The researchers demonstrate that this leads to more efficient enrichment of phage displaying high-affinity peptides to the targeted cell surface marker. Finally, they have integrated all major components of the selection process—incubation, washing, cell lysis, and lysate collection—within the MiPS device thereby reducing the risk of contamination and enabling full automation. Importantly, using the MiPS system, they are able to discover novel peptide sequences to an important cancer biomarker neuropilin 1 (NRP-1) with superior affinity and specificity to the best sequences discovered through the conventional biopanning method.

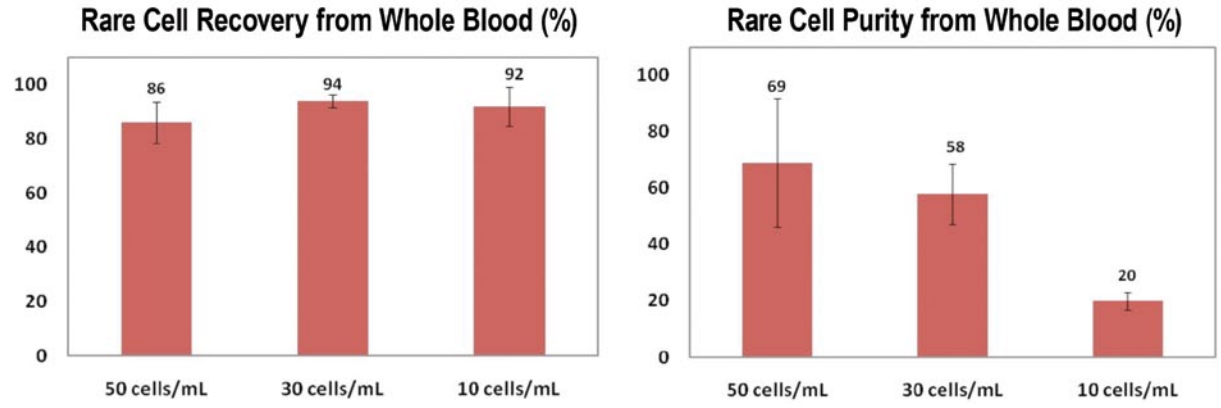


Figure II-41. The UHG-MACS system demonstrates exceptional purification performance of extremely rare cells. As a model system, a small number of MCF-7 cells spiked into whole blood (no preprocessing) and processed through the UHG-MACS system in a single pass at a throughput of approximately 10 mL/hour/microchannel.

De Novo Selection of a Random T7 Phage Library

The researchers performed de novo selection of a T7 phage library expressing random, linear 7-residue (X7) peptides (diversity approximately 5×10^8) against PPC-1 cells using both conventional biopanning and the MiPS platform. After three rounds of selection in the MiPS chip, the phage from the round 3 pool (R3) demonstrated approximately 700-fold higher binding to PPC-1 cells on chip in comparison to the initial random library **(Figure II-43)**. In contrast, conventional cell suspension biopanning yielded a 100-fold enrichment presumably due to a higher level of nonspecific background binding compared to the MiPS system (Figure II-43). The researchers believe the capability of the MiPS system in applying highly stringent mass-action selection pressure in combination with its continuous-flow washing within the microchannel offers an effective means of rapidly enriching phage that display peptides with higher affinity and specificity.

The research team investigated the compositional differences in the peptide motifs enriched by these two selection methods by randomly picking 21 individual phage clones from each R3 pool and comparing their amino acid sequences. Previous studies showed that peptides with a C-terminal (R/K)XX(R/K) sequence exhibited significantly higher affinity for NRP-1-expressing cells than peptides with a C-terminal arginine alone (XXXR). The researchers found that the MiPS system enables more efficient enrichment of phage displaying this higher-affinity motif (Figure II-43B)—90% of the phage selected using the MiPS system contained (R/K)XX(R/K) motifs while 5% displayed lower-affinity XXXR motifs. In contrast, conventional cell suspension-based panning yielded at best 52% (R/K)XX(R/K) phage and 29% XXXR phage.

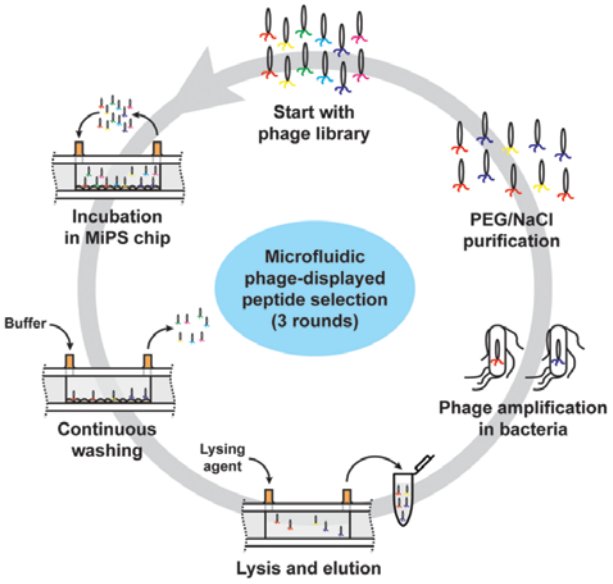
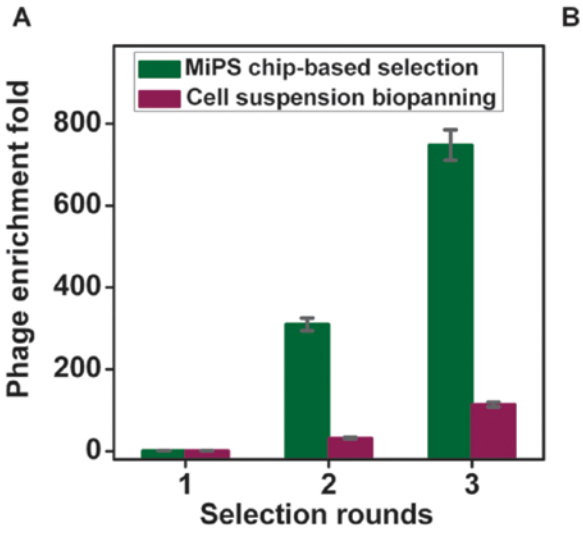


Figure II-42. Experimental scheme for microfluidic selection of phage-displayed peptides with high affinity and specificity for adherent PPC-1 cells in the MiPS system. 5×10^{10} pfu of phage library (approximately 100 copies of each unique sequence) were loaded into the device and recirculated at a flow rate of 1 mL/min for 3 hours at 4°C. The pump then delivered wash buffer at a flow rate of 1 mL/min for 90 minutes. Finally, phage-bound cells were lysed with 1 mL of 1% NP/LB solution on ice at a flow rate of 6 mL/min for 30 minutes, and the lysate was collected in a tube. The collected phage pool was amplified by infecting BLT5403 bacteria at 37°C for 2 hours followed by phage precipitation with a polyethylene glycol/NaCl solution and purification by CsCl gradient ultracentrifugation. The amplified phage pool was used as the starting library pool for the next round of selection.



B

MiPS device		Cell suspension biopanning	
A1	RWEKVPR	B1	RTPR
A2	TVKKNKR	B2	STVRRRR
A3	KVSRPVR	B3	AQVRGPR
A4	RGGRPAR	B4	MKIRPPR
A5	VSERRPR	B5	GRVRGVR
A6	LDRRPPR	B6	LKIERSPR
A7	RGGRGPR	B7	AQVRGPR
A8	VMHKPAR	B8	RRKR
A9	NKVRLPR	B9	GKPRAKR
A10	GRSKAPR	B10	AMKRVAR
A11	PSRRKLR	B11	QRKGRR
A12	GTGKRPR	B12	VRKFVIR
A13	GGKRLPR	B13	TNRVRRR
A14	RKGRPSR	B14	RGSQRAR
A15	GLTRGLR	B15	KRLNSGR
A16	RVMRGPR	B16	KMRSSLR
A17	REGRVRR	B17	IGQDSHR
A18	VMVRPPR	B18	NETRSGN
A19	LGGKKKR	B19	RKRRSVL
A20	GRKLKPR	B20	EVEGCEL
A21	ATNAEPS	B21	VGKKLP

Figure II-43. Results from de novo selection with a random linear X7 peptide library and 2×10^4 target cells in the MiPS device. (A) After three rounds of selection using the MiPS chip, the resulting phage pool (R3) demonstrated approximately 700-fold higher binding to PPC-1 adherent cells in comparison to the initial random library. In contrast, three rounds of conventional cell suspension-based biopanning yielded approximately 100-fold enrichment. (B) Sequences of peptides selected with the MiPS device (left) and conventional biopanning (right). Clones containing the high-affinity (R/K)XXR motif are shown in red, and sequences containing the weak-binding XXXR motif are displayed in blue. Clones that do not exhibit CendR motifs are shown in black. Selection in the MiPS device yielded a significantly higher fraction of phage displaying the R/KXXR motif (92%) compared to conventional biopanning (52%).

Specificity and Affinity of Individual Phage Clones

The researchers measured the equilibrium dissociation constants (Kd) of the peptide sequences for binding to the NRP-1 protein. Due to the experimental challenges in directly measuring the Kd of the native peptides, they followed a two-step process wherein they synthesized native and biotinylated versions of each peptide sequence. They first obtained the Kd of the biotinylated peptide then performed competitive binding assays to extract the Kd of the native peptide using standard methods. More specifically, to obtain the Kd of the biotinylated peptide they incubated NRP-1 protein-coated wells with different concentrations of biotinylated peptide then added streptavidin-conjugated horseradish peroxidase (HRP) to each well. The optical signal from the HRP was fitted to a kinetic model to obtain the Kd values. They confirmed that biotinylated peptides with RXXR motifs (e.g., Kd(RPARPAR) = 28.4 ± 2.9 μM) exhibited higher affinity than those containing XXXR motifs (e.g., Kd(VYKARTR) = 50.9 ± 6.4 μM) and verified that peptides that do not follow the CendR rule, such as GGRGARA and SMTARSV, show minimal affinity for NRP-1. A number of biotinylated sequences showed comparable or superior binding affinities to the biotinylated RPARPAR sequence, including NGARKPR (Kd = 28.8 ± 3.5 μM), GGKRPAR (Kd = 22.2 ± 0.3 μM), and RIGRPLR (Kd = 18.8 ± 3.2 μM).

To obtain the Kd of the native peptide sequences, the researchers utilized a competitive binding assay described in the literature. They challenged NRP-1-coated microtiter wells with mixtures containing various concentrations of native peptides and a constant concentration of biotinylated peptide

then added streptavidin-conjugated HRP to each well. Finally, they fitted the binding signal using Prism software to calculate the Kd values for each native peptide sequence. Importantly, they discovered two sequences, GGKRPAR (P4) and RIGRPLR (P7), which showed superior affinity and specificity to the well-known RPARPAR sequence (CR).

Key Research Accomplishments

- Developed the MiPS system, which is capable of efficiently isolating peptides with high affinity and specificity to target cell surfaces.
- Performed de novo selection of a T7 phage library expressing random, linear X7 peptides against PPC-1 cells using both conventional biopanning and the MiPS platform.
    - Achieved efficient in vitro selection of T7 phage-displayed peptides that recognize markers expressed on live, adherent cells within a microfluidic channel.
- Measured the Kd of isolated peptides and discovered two peptide sequences with superior binding affinity and specificity relative to the well-known NRP-1-binding RPARPAR peptide.

Conclusions

In the third year of the project, the researchers developed a novel microfluidic device that allows unprecedented efficiency in generating affinity reagents that target cell surfaces. Compared to conventional biopanning methods, microfluidic selection enables the more efficient discovery of peptides with higher affinity and specificity by providing a controllable and reproducible means for applying stringent selection conditions against minimal amounts of target cells without loss. Overall, the UHG-MACS and MiPS devices provide a critical technical solution for the isolation of the target stem cells needed for clinical therapeutics. This project is scheduled to conclude on December 31, 2011.

Hand Transplantation for Reconstruction of Disabling Upper Limb Battlefield Trauma – Translational and Clinical Trials

Project 4.4.2, WFPC

Team Leader(s): W.P. Andrew Lee, MD (Johns Hopkins University School of Medicine)

Project Team Members: Gerald Brandacher, MD, Damon S. Cooney, MD, PhD, Justin M. Sacks, MD, Stefan Schneeberger, MD, and Eric Wimmers, MD (Johns Hopkins University School of Medicine) and Vijay S. Gorantla, MD, PhD, Joseph E. Losee, MD, and Xin Xiao Zheng, MD (University of Pittsburgh)

Collaborator(s): None

Therapy: Reconstructive transplantation of upper extremity under a novel bone marrow/stem cell-based immunomodulatory protocol.

Deliverable(s): Phase 1 (Translational/ Preclinical Trials): Novel immunosuppressive protocol that combines systemic stem cell-based therapy with local immunomodulation in a swine heterotopic hindlimb model of composite tissue allotransplantation. Phase 2 (Clinical Trial): Reconstructive transplantation as treatment for hand or forearm loss under a novel cell-based immunomodulatory protocol.

TRL Progress: Start of Program, TRL 4; End Year 1, TRL 4; End Year 2, TRL 5; End Year 3, TRL 5

Key Accomplishments: In Phase 1, CTLA4/Ig fusion protein was shown to prolong survival of allografts in the swine model. In Phase 2, all five patients who underwent transplants to date have been maintained on a single immunosuppressive drug at low levels, and they continue to have increased motor and sensory function of their transplanted hands, which correlates with their level of amputation, time after transplant, and participation in hand therapy.

Key Words: Hand transplantation, immunosuppression, immunomodulation, swine

Introduction

Composite tissue allotransplantation (e.g., hand/face transplants) has become a clinical reality and is currently performed in multiple centers worldwide. To date, more than 65 hands have been transplanted globally including 8 transplants performed in 5 patients by this project team during the past 2 years. Apart from excellent and highly encouraging functional results, composite tissue allotransplantation has not reached widespread clinical use because recipients require lifelong, high-dose, multidrug immunosuppression to prevent graft rejection. These regimens carry a high risk for serious side effects. In light of these challenges, a protocol for solid organ transplantation at the University of Pittsburgh has utilized a minimization strategy consisting of recipient conditioning (induction therapy), donor bone marrow infusion, and monotherapy maintenance immunosuppression.

This project has two phases:

- Phase 1 (Translational Trials): This phase involves the development of a preclinical model of heterotopic hindlimb transplantation in SLA-mismatched Yucatan miniature swine.
- Phase 2 (Clinical Trial): This phase involves the conduction of clinical trials of human hand transplantation.

Research trials are parallel and complementary, and work in each arm will be detailed separately in this report.

Summary of Research Completed in Years 1 and 2

Phase 1: Preliminary work has shown prolonged allograft survival using a three-part immunosuppressive regimen: (1) pre-transplant induction with whole-body and thymic irradiation, (2) tacrolimus (FK506) monotherapy, and (3) donor bone marrow cell infusion. The optimal dose of bone marrow cells to be infused has never been identified in either solid organs or composite tissue allotransplantation.

In year 1, bone marrow cell infusions of 15, 30, and 60 million cells/kg were tested in the swine model. In vitro analysis including chimerism testing by RT-PCR demonstrated a statistically significant increase in microchimerism for the 60 million cells/kg group versus the 15 and 30 million cells/kg groups. Stable, long-term microchimerism was achieved at all doses at experimental end points (1×10^6), and there were no complications such as infection or graft versus host disease.

In year 2, the optimal dose of bone marrow cell infusion (60 million cells/kg) was utilized with the addition of CTLA4/Ig fusion protein. Costimulatory blockade of the CD28/B7 pathway with chimeric CTLA4/Ig fusion proteins has been shown to suppress alloimmune responses. The researchers hypothesized that the addition of the CTLA4/Ig fusion protein would diminish the dependence on calcineurin inhibitors, such as FK506, currently necessary for long-term allograft survival. CTLA4/Ig fusion protein was demonstrated to suppress alloimmune responses in an alloantigen-triggered baboon T cell proliferation assay as well as in a pig antigen-triggered baboon T cell proliferation assay. An enzyme-linked immunosorbent assay (ELISA) for measuring serum levels of fusion protein was also developed.

Phase 2: Five patients' hands have been transplanted to date. Patients continue to have increased motor and sensory function of their transplanted hands, which is detailed in the next section.

Research Progress – Year 3

Phase 1: Establish a protocol that combines systemic stem cell-based therapy with local immunomodulation enabling graft survival and minimizing systemic immunosuppressive treatment in a preclinical swine model for composite tissue allotransplantation.

The optimal dose of bone marrow cell infusion (60 million cells/kg on postoperative day 0) was applied to subsequent experiments evaluating the addition of CTLA4Ig fusion protein (20 mg/kg intravenously on postoperative days 0, 2, 4, and 6). Yucatan miniature swine (n = 9) underwent heterotopic hindlimb transplantation and received 30 days of FK506 (0.5 mg/kg) treatment. Group I (control, n = 3) received irradiation and bone marrow infusion; Group II (n = 3) received irradiation, bone marrow infusion, and CTLA4Ig fusion protein; and Group III (n = 3) received CTLA4Ig fusion protein only.

Transplanted animals in Group I rejected the skin portion of the allograft at 50, 52, and 53 days post-transplant. Remaining components of the allograft (i.e., muscle, bone, nerve, and vessel) survived indefinitely. Group II animals had unanticipated complications (unexplained weight loss) leading to sudden death (n = 1) or the need for euthanization at 28 and 35 days post-transplant. Group III animals

demonstrated significantly prolonged graft survival beyond 150 days post-transplant **(Figure II-44** and **Figure II-45)**. Skin and muscle histology in all long-term surviving animals was normal without any perivascular cellular infiltrates or C4d depositions.

The costimulatory blockade of the CD28/B7 pathway with CTLA4Ig fusion protein further optimized induction therapy, reduced maintenance immunosuppression, and prolonged graft survival. These data were unexpected and mark a potential paradigm shift in the protocol as Group III had prolonged graft survival without the addition of bone marrow infusion. To confirm this observation, it is scientifically justified to repeat this experimental group. In addition, because of the unanticipated complications in Group II, this group also needs to be repeated.

Phase 2: Establish hand transplantation as a treatment strategy for reconstruction of disabling combat injuries involving hand or forearm loss using a novel bone marrow/stem-cell based protocol (Pittsburgh Protocol) to minimize immunosuppressive therapy.

Candidates for hand transplantation continue to be screened and are accruing via an approved IRB protocol; however, no new transplants have been performed since September 2010. All of the five patients[1] transplanted to date are maintained on a single immunosuppressive drug at low levels, and they are progressively achieving greater motor and sensory function of

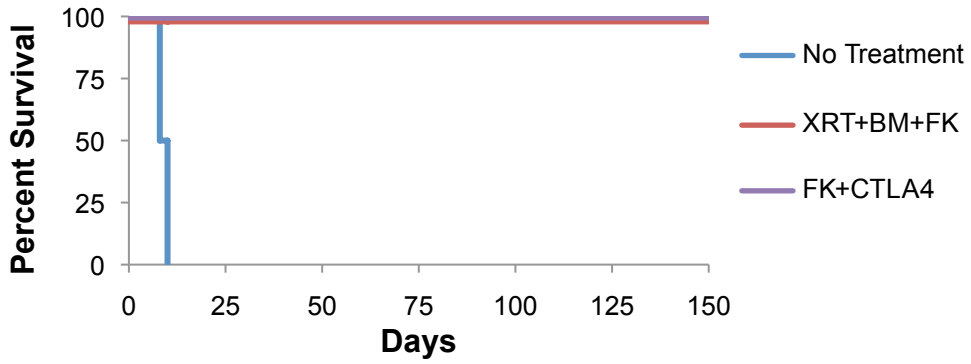


Figure II-44. Survival of the muscle component of the allograft.

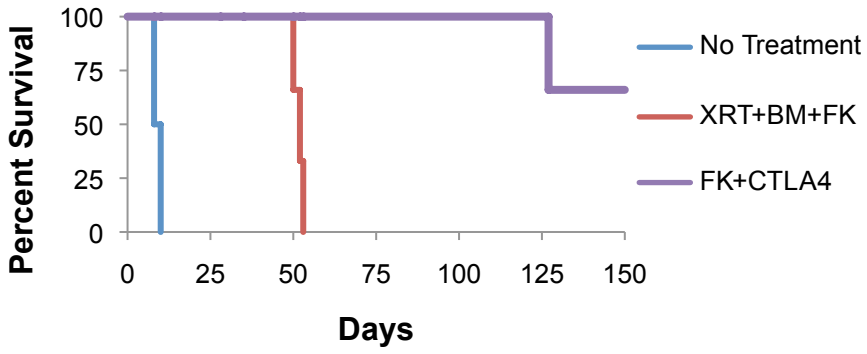


Figure II-45. Survival of the skin component of the allograft.

[1] In addition to the AFIRM, support for these transplants was provided by the Orthopedic Extremity Trauma Research Program and the University of Pittsburgh Medical Center.

their transplanted hands. Acute episodes of skin rejection were infrequent and could be reversed either with topical treatment only or with short courses of steroids. Patients demonstrated sustained improvements in motor function (e.g., range of motion, intrinsic return, and grip and pinch strength) and sensory return correlating with the time after transplantation, level of amputation, and participation in hand therapy. Side effects were few and included transient increases in serum creatinine, hyperglycemia managed with oral hypoglycemics, minor wound infection, an episode of hyperuricemia and bony nonunion in two cases. No systemic infectious (bacterial or viral) complications occurred.

Immunomonitoring revealed transient moderate levels of donor-specific antibodies, assessed by Luminex and ELISA, adequate immunocompetence (Immuknow), and no peripheral blood chimerism. Donor-specific alloantibodies were detected in four of five recipients and were associated with skin rejection in most cases. Changes in DSAs were limited to single specific haplotypes in each patient except for Patient 1. This recipient, a former Marine who lost his hand in a training accident while on active duty, initially showed DSAs against DQ7, which resolved with treatment but later recurred together with DR4 and DQ5 DSAs. Such changes coincided with an admitted period of noncompliance with immunosuppression but significantly subsided after compliance was re-established **(Figure II-46)**. This patient's noncompliance with the immunosuppression regimen, immunomonitoring follow-up, and hand therapy protocol has also resulted in regression in active hand function since the last project report (i.e., impaired motion of the wrist, metacarpophalangeal, and interphalangeal joints with stiffening and reduced mobility of the hand associated with skin dryness and soft tissue edema).

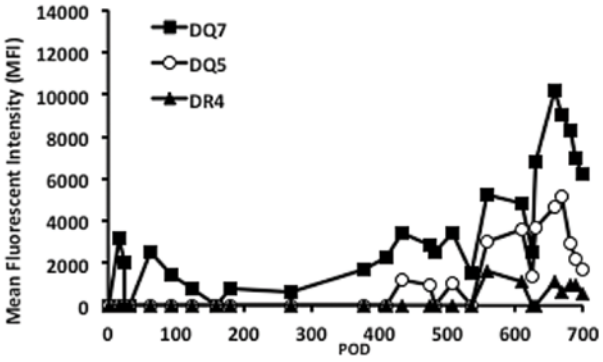


Figure II-46. Post-transplant donor-specific antibodies assessed by Luminex in Patient 1. A significant downward trend in mean fluorescent activity can be observed since compliance was re-established.

Importantly, imaging demonstrated patent vessels and soft tissue structures with no luminal narrowing/occlusion as an indirect sign of chronic rejection. Most recent protocol skin biopsies show no or minimal perivascular cellular infiltrates and are negative for C4d.

Key Research Accomplishments

- Determined that costimulatory blockade of the CD28/B7 pathway with CTLA4Ig fusion protein further optimized induction therapy, reduced maintenance immunosuppression, and prolonged graft survival in the swine model.
- Performed eight hand/forearm transplants in five patients who are all maintained on a single immunosuppressive drug at low levels and continue to have increased motor and sensory function of their transplanted hands correlating with the level of amputation, time after transplantation, and participation in hand therapy.

Conclusions

This project features the development of a preclinical heterotopic hindlimb transplant model for composite tissue allotransplantation using a novel immunomodulatory protocol. The optimal dose of bone marrow cell infusion has been determined to be 60 million cells/kg; this optimized dose has been adopted in current animal studies. Stable levels of microchimerism were achieved in swine in all groups after bone marrow cell infusion. The use of chimeric CTLA4/Ig fusion protein in the hindlimb transplant model to reduce the amount of chronic immunosuppression and augment induction shows promising early results; the animal groups are currently being repeated to confirm these data.

The results from Phase 1 studies have been applied in performing human hand transplantation using the Pittsburgh Protocol, a novel immunomodulatory strategy that aims to reduce maintenance immunosuppression necessary for successful composite tissue allotransplantation. Five patients have been transplanted with a follow-up ranging between 7 and 27 months. All recipients are maintained on a single immunosuppressive drug at low levels and continue to have increased motor and sensory function of their transplanted hands correlating with the level of amputation, time after transplant, and participation in hand therapy. The success of this experimental protocol will allow for greater clinical application of hand transplantation for the reconstruction of upper extremity amputations.

Research Plans for the Next 2 Years

Based on the striking data obtained in years 1–3, CTLA4Ig represents a potential shift in the immunosuppression protocol; as such, future groups may require significant adjustment from the originally proposed plan. In particular, if the dramatic success of CTLA4Ig with regard to tolerance induction is confirmed in the current repeated groups, then pursuit of targeted skin immunotherapy (as originally planned) will need to be reconsidered. In addition to the aforementioned repeat groups, year 4 trials will include spaced dosing of tacrolimus monotherapy followed by weaning attempted under the cover of local immunotherapy.

Planned Clinical Transitions

In Phase 2 of this project, the goal is to promote long-term hand transplant acceptance while minimizing the need for

immunosuppressive drug therapy. An optimized strategy is planned combining targeted immunomodulation, bone marrow stem cell/fusion protein induction, and topical migratory inhibitors to further reduce maintenance immunosuppression and allow weaning of systemic drug therapy. This regimen should reduce side effects related to high-dose immunosuppression and will hopefully enable widespread clinical application of hand transplantation for the reconstruction of upper extremity amputations.

Corrections/Changes Planned for Year 4

As of October 1, 2010, Dr. W.P. Andrew Lee was appointed as the Chairman of the Department of Plastic and Reconstructive Surgery at the Johns Hopkins University School of Medicine. This represents an exciting and unique opportunity for the composite tissue allotransplantation team since the university has invested significant resources to ensure patient care and basic research in this field at the highest level.

Research progress was delayed 8 months in year 3 due to transitioning the large animal trial from the University of Pittsburgh to the Johns Hopkins University. A thorough transition plan was implemented to ensure that this change in institutions does not impact the proposed research/Statement of Work. In this regard, the remaining four hand transplantation surgeries for this project will be performed at both universities: two conducted at the Johns Hopkins University and two performed at the University of Pittsburgh. All remaining translational research experiments will be conducted at the composite tissue allotransplantation Research Laboratory of the Department of Plastic and Reconstructive Surgery at the Johns Hopkins University. Codirected by Dr. Lee and Dr. Gerald Brandacher, the composite tissue allotransplantation laboratory has 1,800 square feet of state-of-the-art bench and laboratory space.

Considering the researchers' decision to repeat Groups II and III in the Phase 1 swine study (described previously in Research Progress – Year 3) as well as the 6-month delay that occurred in year 2 due to the swine vendor, Sinclair, mishandling the breeding lines (described in detail in the year 2 progress report), the decision was made to abandon the Sinclair swine and repeat the entire year 2 groups utilizing a new strain of swine, MGH miniature swine. These groups/transplants are currently under way at the Johns Hopkins University.

Clinical Trial – Safety Assessment of a Novel Scaffold Biomaterial

Project 4.4.1a, RCCC

Team Leader(s): Anthony Windebank, MD and Michael Yaszemski, MD, PhD (Mayo Clinic)

Project Team Members: Robert Spinner, MD, Huan Wang, MD, PhD, Mahrokh Dadsetan, PhD, Brett Runge, PhD, Andrew Knight, PhD, Suzanne Segovis, and Julia Lewis (Mayo Clinic)

Collaborator(s): BonWrx, Inc.

Therapy: Treatment of peripheral nerve injuries.

Deliverable(s): Neuralum, a tissue-engineered scaffold suitable to repair nerve defects up to 6 cm.

TRL Progress: 2011, TRL 4; Target, TRL 5/6

Key Accomplishments: The Mayo Clinic group has worked closely with its IRB, the RCCC clinical trial core, and its commercial partner (BonWrx, Inc.) to achieve the milestones of the planned tasks.

Key Words: Peripheral nerve, conduit, biodegradable polymer, GMP, FDA, clinical trial

Introduction

Autologous nerve grafts are the only options for major traumatic nerve injuries with tissue destruction. This type of graft has significant drawbacks: (1) loss of function in the donor sensory nerve distribution, (2) size mismatch between the autologous donor nerve and the injured nerve, (3) limited availability of donor sites, and (4) donor site morbidity, pain, and numbness.

As an alternative to nerve autografts, a number of different synthetic materials have been explored for use in repairing nerves. The Mayo Clinic group recently completed a systematic review of publications describing synthetic, nonbiodegradable and biodegradable conduits that have been used experimentally. Current FDA/CE-approved absorbable nerve conduits include NeuraGen (type I collagen, Integra NeuroSciences), NeuroMatrix and NeuroFlex (type I collagen, Collagen Matrix Inc.), NeuroTube (polyglycolic acid [PGA], Synovis), and Neurolac (poly(65/35(85/15L/D)-lactide-ε-caprolactone [PLCL], Polyganics BV). However, the clinical performance of these conduits does not match that of autologous grafts. These commercially available nerve tubes are only used for repair of small sensory nerves in patients mainly in the repair of digital nerves in the hand. Repair of motor nerves is only sporadically reported; thus, effectiveness in repairing larger nerve defects or motor nerves is inadequate. Hence, there is a critical need for new synthetic nerve scaffolds to support regeneration across larger nerve gaps.

The current approach for the introduction of nerve scaffolds typically involves both in vitro analyses to measure the material properties (i.e., degradation kinetics and mechanical properties) and in vivo testing in relevant preclinical models. The most commonly used small animal model is the rat 1 cm sciatic nerve gap. In the researcher's literature review, they identified 388 publications that described scaffold-based nerve repair in animal models. Rat was used in 285 of these studies. The sciatic nerve was used in 244; the gap was 10–20 mm in 200 of the studies. Materials used in all of the commercially available conduits were first used in this model. The studies lasted up to 20 weeks and because of the time required for tissue processing and analysis, take 1–2 years to complete. Typical large animal models involve goats, sheep, dogs, cats, and primates with nerve gaps ranging from 3–90 mm. However, large animal studies are very expensive and take 2–4 years to complete. Eighty-one different materials have been reported in in vivo preclinical studies. Only three (collagen, PGA, and PLCL) have advanced to clinical use for the repair of small nerve gaps.

The Mayo Clinic group proposes a different approach using a carefully defined clinical model. Whole sural nerve biopsies are carried out in patients with suspected peripheral neuropathy to establish definitive diagnoses that may have treatment implications. An adequate biopsy requires resection of approximately 6 cm of nerve. Dyck and Lofgren *(Mayo Clin Proc* 1966, 41(11):778–784) introduced the technique and it has gained widespread clinical use. The process results in a completely predictable 6 cm nerve gap. Sensory disturbance is expected. Long-lasting neuropathic pain develops in about 10% of patients. At the Mayo Clinic, 200–250 sural nerve biopsies are performed each year. About 25% of these patients have preserved sural nerve function based upon nerve conduction studies done prior to the biopsy. Whole sural nerve biopsy results in predictable loss of function in the sural distribution of the foot. Reconstruction of the sural nerve in these patients can potentially restore sensation and prevent complications associated with the biopsy.

The researchers therefore propose to repair post-biopsy sural nerve defects with a synthetic nerve conduit constructed from PCLF. Since the nerve is just below the skin, potential morbidity from the conduit will be readily identifiable and the device can be removed and analyzed. This will provide a platform for determining safety and an indication of efficacy of scaffolds for nerve repair.

Clinical Trial Status

GMP Scale-Up of PCLF Tube Manufacturing

The Mayo Clinic group has been working closely with BonWrx, Inc., over the past year. BonWrx, Inc., observed PCLF nerve conduit fabrication in the laboratory setting. The Mayo Clinic group created a chemicals and equipment list to be used as a checklist for equipment and chemical purchase by BonWrx, Inc., to enable manufacture of PCLF and fabrication at its GMP facility in Scottsdale, Arizona. The Mayo Clinic group also created a video for the production of PCLF scaffold and sent it to BonWrx, Inc., to be used as a guideline for setup and GMP production. The two parties also discussed the sterilization strategy, a critical step in gaining FDA approval for clinical trial and human use. The options discussed were gamma radiation, electron beam irradiation, and autoclave. It was decided to try autoclave as the sterilization for PCLF tubes due to its easy accessibility, reliability, and cost-effectiveness. The action items included testing the feasibility of autoclaving PCLF and comparing

the thermal, mechanical, and rheological properties before and after autoclave sterilization. The Mayo Clinic group has initiated this study.

Initiation and Progress of IRB Approval

Although the Mayo Clinic IRB cannot put the clinical trial protocol on the agenda for full review without IDE approval, a comprehensive "dress rehearsal" pre-review process was initiated to avoid any potential delay. Two IRB specialists, Julia Lewis and Michelle Daiss, and the physician Chair of the IRB have been working on this with the Mayo Clinic group. The specialists have been on conference calls with the RCCC clinical trials core team to get and provide real-time feedback. The clinical trial protocol and consent form were submitted to IRBe, the Mayo Clinic online IRB review site. Pre-review by the IRB has been completed. In addition, a conflict of interest (COI) committee has reviewed the clinical protocol and its monitoring strategies, and a comprehensive COI management plan has been implemented.

Initiation and Progress of HRPO Approval

The Mayo IRB and HRPO have and continue to pre-review and revise the proposal and consent form in detail and in parallel. This challenge has been mitigated as follows:

- Held a face-to-face meeting with Stan Gerson's RCCC clinical trials core group at the AFIRM All Hands 2010 Meeting and held subsequent clinical trial teleconferences. The clinical trials core also updated the OnCORE database, communicated HRPO requirements, and discussed FDA clearance strategy.
- Satisfied HRPO requirements in the preparation of IRB protocol and consent forms.
- Uploaded IRB submission materials to WorkZone for review by the RCCC clinical trials core.
- Converted pre-reviewed IRB documents from IRBe to PDF files and submitted them to the HRPO for review.
- Paula Glauber at the HRPO completed U.S. Army Medical Research and Materiel Command pre-review and recommended minimal edits and changes before HRPO final review.

Preparation of Upcoming Clinical Trial

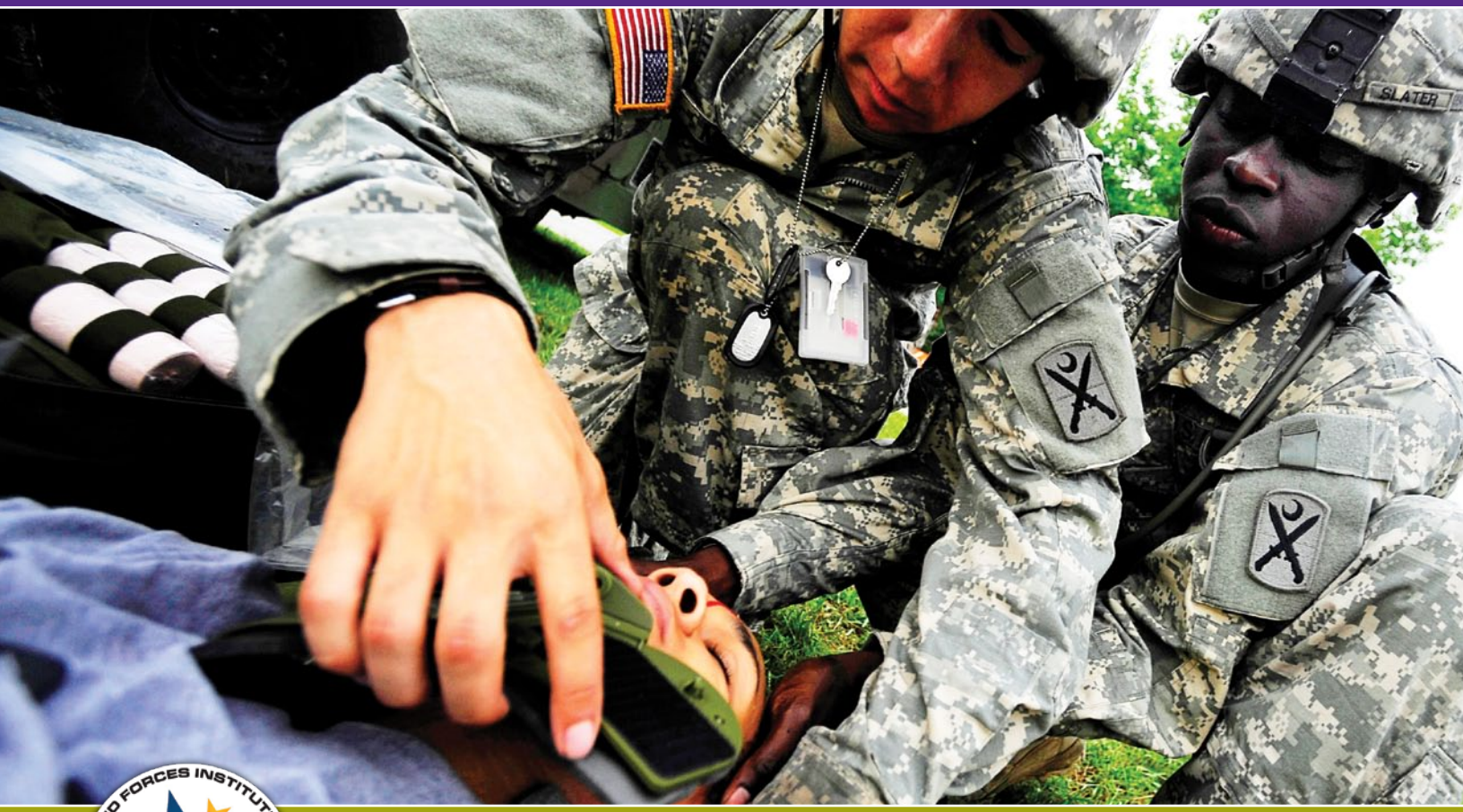
The clinical trial protocol has been reviewed and approved by the Department of Neurology research committee. Patient recruitment can start as soon as IRB and FDA approval are obtained. A clinical coordinator has been identified for the protocol.

Key Research Accomplishments

- Mitigated the risk of potential delays often associated with obtaining IRB and HRPO approvals.
- Completed IRB and HRPO pre-review of the clinical trial protocol.
- Converted the clinical protocol, consent form, and other relevant materials from IRBe to PDF files and submitted them to the HRPO for pre-review.
- Implemented a COI management plan.
- Received the approval of the Department of Neurology research committee for the clinical trial protocol, which will ensure the prompt start of patient recruitment upon regulatory approval.
- Initiated GMP scale-up and optimization of sterilization methods of the PCLF tube.

Conclusions

The researchers have worked closely with the Mayo Clinic IRB, the RCCC clinical trial core, and BonWrx, Inc., to achieve the milestones of the planned tasks.



III: Craniofacial Reconstruction

Background

More than 25% of wounded warriors treated in U.S. military facilities during the current conflicts have sustained maxillofacial injuries.[1] These injuries range from simple fractures to extensive bone defects, severe burns, and soft tissue avulsion. A warrior with a significant craniofacial injury has lost his or her interface with the world; when one's face is gone, so is one's identity. A warrior with severe facial injury may lose the ability to communicate—speech may be difficult to understand and facial expressions nonexistent—and even the ability to eat. Significant disfigurement excludes one from society—a mangled, contracted, or absent face does not invite interaction. These soft tissue and bony injuries to the face and cranium are excruciating physical injuries with devastating psychological impact. Restoring form and function to these traumatized warriors is critical to rehabilitation.

AFIRM's Craniofacial Reconstruction (CFR) Program seeks to restore wounded warriors with devastating, disfiguring facial injuries to fully functional lives, including integration with society through the application of regenerative medicine technology. The CFR Program comprises a multidisciplinary, multi-institutional collaborative research team conducting projects ranging from single-tissue regeneration to complete face transplants, from emerging products in the

[1] *Hale, Robert. Slide Presentation "The Regenerated Face for Maxillofacial Battle Injuries – Joint Theatre Trauma Registry, Oct. 19, 2001 to Dec. 12, 2007," presented May 19, 2010.*

early stages of bench research to products entering clinical trials. Drawing on the strengths of each investigator on the team, an optimal set of complementary technologies have been identified to achieve hard and soft tissue regeneration. The CFR Program will give wounded warriors a new face to present to the world.

Unmet Needs

Current treatments in craniomaxillofacial (CMF) reconstruction practices are inadequate to treat the unique and massive craniofacial deficits resulting from blast injuries. Presently, massive bone loss to the craniofacial complex incurred in combat is reconstructed with nonresorbable synthetic materials, bone implants, or metallic devices that restore anatomical form to some degree and limited function. The currently available synthetic bone materials, for example, do not remodel or integrate with host tissue and can become infected and require extensive, multiple revision surgeries. A biodynamic, biocompatible substitute for complex bone defects is a critical need in facial injury.

Current techniques and therapies neither mitigate against scar contracture nor achieve complex soft tissue coverage aesthetics of structures such as the ears and nose. Allogeneic and autogenous grafts are options with limited availability for the repair of injuries in which a prosthesis is a possible but suboptimal outcome. Current standard treatments are hindered by high donor site morbidity and poor long-term results. A readily available, or readily generated, replacement tissue for complex soft tissue structures is an unmet need in facial injury.

In the most severe facial injuries, the loss of tissue is massive. In such cases, there is no satisfactory surgical approach for facial replacement other than transplant. However, composite tissue allografts (CTAs) that constitute the face are highly antigenic and require aggressive immunosuppression regimens to prevent rejection of the transplanted tissue. This puts the host at substantial risk, both from the immunosuppressive agents and from opportunistic infections, and has led to a significantly shortened life expectancy for all transplant recipients. Development of a technique or therapy providing modulation of the immune response to allografts without indiscriminate immunosuppression is necessary to provide adequate treatment to severe facial injuries in wounded warriors.

High-order explosive devices cause severe blast injuries to unprotected craniofacial tissue, including the small muscles of facial expression and function. A severely damaged orbicularis oculi prevents eyelid closure, resulting in dry, painful, irritated eyes and potential blindness. In the catastrophically injured soldier, autologous donor sites for soft tissue transfers may be missing or compromised. Allogeneic donor muscle transplantation represents the sole near-term therapy option but requires lifelong immunosuppression with significant attendant risks. Engineered skeletal muscle restores form and function of critical tissues using a patient's own cells and fills an unmet need in facial reconstruction.

Soft tissue deficits may be treated with pedicled muscle and skin flaps and allogeneic skin substitutes. But these treatments do not restore myogenic and neurogenic competence; the warrior's face may no longer have large defects, but it does not move and does not feel. The implications for independence, communication, and integration are substantial. Restoring facial nerve and muscle competence is a critical need to significantly improve the outcome for wounded warriors with severe facial injuries.

Finally, planning the complex reconstruction and regeneration needs of wounded warriors with severe facial injuries is a critical need. AFIRM researchers are developing a visualization tool for patient-specific wounds and injuries. This will help integrate specific tissue regeneration strategies both within the CFR Program and throughout AFIRM. The approach will be staged, proceeding from the least biomechanically challenging to the most challenging zones in the CMF complex.

Areas of Emphasis

The Rutgers-Cleveland Clinic Consortium (RCCC) and the Wake Forest-Pittsburgh Consortium (WFPC) are pursuing a complementary mix of research projects focused on various aspects of CFR. Projects can be grouped into three clinical challenge topic areas: Bone Regeneration, Soft Tissue Regeneration, and Cartilage Regeneration (with a focus on the ear). Additional details of the projects in each of these topic areas can be found in **Table III-1** and subsequent sections of this chapter.

Table III-1. Projects funded by RCCC and WFPC per clinical challenge topic area.

Clinical Challenge	Consortium/ Institution	Project No.	Project Title
Bone Regeneration	WFPC	4.1.2	Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects
		4.1.3	Novel Synthetic Bone for Craniofacial Application
	RCCC	4.5.1a/4.5.7	Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Allograft Bone/Polymer Composites and Expedited Commercialization of an Injectable Bone/Allograft Composite for Open Fractures
		4.5.1b	Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex
		4.5.6	Vascular Tissue Engineering
		4.5.8	Accelerating the Development of Bone Regeneration Scaffolds by 510(k) Application to FDA for Tyrosine-Derived Polycarbonate Fracture Fixation Device
Soft Tissue Regeneration	WFPC	4.1.4/4.1.5	Soft Tissue Reconstruction/Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction
		4.1.6	Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle
	RCCC	4.1.2	Develop Innervated, Vascularized Skeletal Muscle
		4.3.1	Composite Tissue Allograft Transplantation Without Lifelong Immunosuppression
Cartilage Regeneration (Focus: Ear)	WFPC	4.1.1	Engineered Cartilage-Covered Ear Implants for Auricular Reconstruction
	RCCC	4.5.4a	Engineering a Replacement Autologous Outer Ear Using a Collagen/Titanium Platform
		4.5.4b	Regeneration of Ear Using Bioresorbable Polymers and Stem Cells

WFPC researcher Allan Henslee, a Rice University graduate student, prepares to characterize the compressive mechanical properties of a material designed for bone regeneration.

Bone Regeneration

Studies at WFPC

The **Mikos/Kasper and Wong group (Project 4.1.2)** at Rice University and the University of Texas Health Science Center at Houston, respectively, is developing porous space maintainers that will maintain the proper anatomical relationship of the tissue adjacent to a craniofacial defect. The researchers demonstrated in a rabbit jaw defect model that animals receiving porous implants, fabricated at the site of injury, experienced enhanced soft tissue healing and coverage compared to animals receiving nonporous implants. They can now control the level of antibiotic release and the amount of porosity in their constructs through the manipulation of tunable parameters of space maintainer fabrication. They have made considerable progress toward the clinical translation of their space maintainers as reflected in the treatment of three patients with the technology under physician-directed care. The research team is also developing an "in vivo bioreactor" technology that involves producing a bone/soft tissue flap with a blood supply at a site in the body away from the wound and then transplanting it into a skeletal defect once the wound has been optimized for reconstruction. The team is collaborating with investigators in China to determine the efficacy of this approach. The researchers will continue to explore this approach in a sheep model to optimize parameters for bone flap generation and to evaluate the durability of repair of jaw defects upon transfer of the bone flaps. They will also explore the delivery of growth factors from degradable material constructs to support bone regeneration and aesthetic contouring. They will soon initiate a formal clinical study of their space maintainer technology (see the Clinical Trials section for more information).

The **Sfeir/Beniash/Kumta group (Project 4.1.3)** at the University of Pittsburgh is developing nanostructured bioactive bone cements that contain the essential components to mimic bone architecture, composition, and mechanical strength while providing the bone-generating characteristics required for bone tissue regeneration. The researchers first conducted in vivo screening experiments to rapidly determine if nanostructured calcium phosphate (NanoCaP)-containing materials will be optimal in the proposed bone regeneration strategy either with or without the incorporation of the bone-inductive growth factors. Their data showed that their NanoCaP-containing bone cements were indeed very good candidates for further assessment as a therapy for bone regeneration. The research team has developed injectable forms of its bone cements. In vivo experiments in the rabbit skull model revealed that the researchers' cements alone or with the addition of bone morphogenetic protein 2 (BMP-2) had the ability to regenerate bone. Continued effort on the project will be focused on further developing the bone cements to obtain U.S. Food and Drug Administration (FDA) 510(k) clearance.

Studies at RCCC

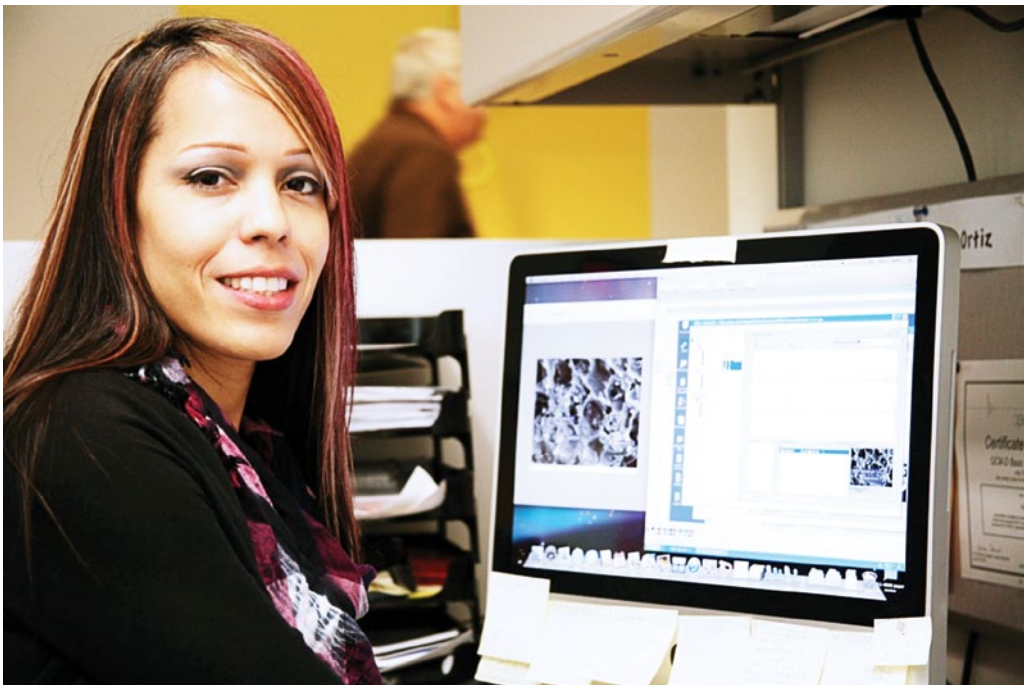
The **Guelcher group (Projects 4.5.1a and 4.5.7)** at Vanderbilt University is focused on developing and evaluating materials for treating traumatic bone defects. The researchers are pursuing two projects in collaboration with researchers at the U.S. Army Institute of Surgical Research (USAISR) and Medtronic. In the first project (4.5.1a), the researchers are developing injectable biocomposite delivery systems that release growth factors, such as recombinant human bone morphogenetic protein 2 (rhBMP-2), in damaged craniofacial bone to aid in repair. Preclinical studies in rabbit skull and jaw models of bone regeneration show that the material supports rapid cellular infiltration and new bone formation, and that the material is a useful delivery system for rhBMP2. In the second project (4.5.7), the researchers are developing an injectable allograft (i.e., tissue that is transplanted from another human donor) biocomposite bone void filler for the repair of long bone defects. They demonstrated that their injectable allograft-based bone void fillers remodeled and supported new bone formation in rabbit models. Experiments in the upcoming year for Project 4.5.1a will focus on developing the formulations; testing their feasibility in a critical-size defect (CSD), rat skull model to determine the correct dose; and validating the formulations in clinically relevant animal models. Experiments in the upcoming year for Project 4.5.7 will focus on revising the formulation, validating it in rabbit and sheep preclinical models, and conducting both current Good Manufacturing Practice (cGMP) manufacturing and International Organization for Standardization (ISO) 10993 testing to prepare for a regulatory filing with the FDA.

The **Hollinger/Darr group (Project 4.5.1b)** at Carnegie Mellon University and Rutgers University, respectively, is developing biodegradable scaffolds containing tyrosine-derived polycarbonate (TyrPC), which may provide compelling therapeutic solutions for the regeneration of craniofacial bone. The researchers added a calcium phosphate (CaP) surface coating to the TyrPC scaffolds with the goal of increasing bone formation. They found that the addition of rhBMP-2 to the TyrPC+CaP scaffolds led to substantially increased bone formation in a rabbit CSD skull model in 6 weeks. They also found that TyrPC+CaP, either with or without rhBMP-2, promoted bone formation that was similar to, if not superior to, a clinically utilized bone void filler with rhBMP-2. They determined that the TyrPC scaffolds were gradually resorbed and replaced with new bone during the 6-week period. During the upcoming year, the research team plans to identify a "frozen" composition of TyrPC+CaP, which

RCCC researcher Dr. Aniq Darr designing experiments in the office.

may contain a minimal amount of rhBMP-2, proprietary peptide, or blood component (e.g., bone marrow aspirate). They will test this composition using rabbit skull and radius models, and goat CSD calvarial models. If the researchers are successful with their rabbit and goat models in year 4, they will complete an FDA filing (e.g., 510[k]) and will design and prepare for a Phase 1 clinical study protocol in year 5.

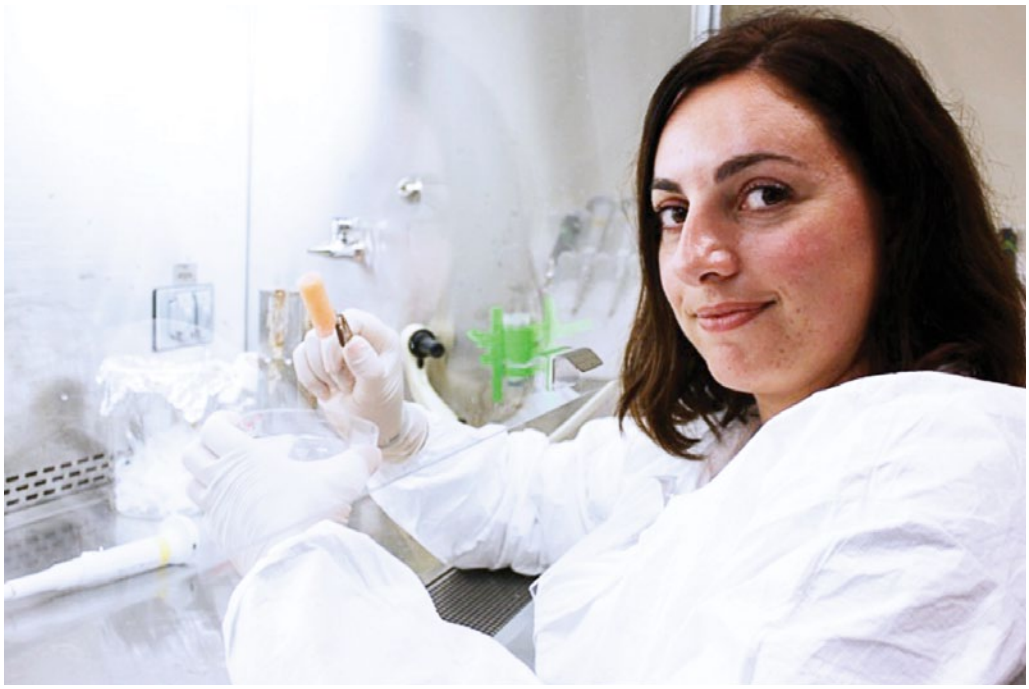
Large biologically compatible scaffolds are needed to treat sizeable bone defects, and the success of long-term engraftment of engineered tissues in humans depends on the growth from surrounding tissues of a viable blood vessel (vascular) network. In **Project 4.5.6**, the **Anderson/Langer group** at the Massachusetts Institute of Technology (MIT) is developing a novel biodegradable and bioactive scaffold system that can support stem cell growth and produce vascularized bone tissues. During the past year, the researchers optimized a scaffold system of enhanced vascular engraftment. They identified an optimal biomaterial for effective bone tissue engineering. They also optimized human vascular endothelial growth factor (hVEGF)-release microparticles for effective use in forming blood vessel networks in the grafts. During the next few years, the researchers will perform extended small animal studies. They will monitor the biological activity of scaffolds that have incorporated either hVEGF or hydroxyapatite. Toward the ultimate goal of using these scaffolds in the clinical setting, the researchers plan to perform appropriate large animal studies to confirm the biocompatibility and safety of the scaffolds. They will prepare an Investigational Device Exemption (IDE) or Investigational New Drug (IND) for clinical application upon successful completion of the large animal



RCCC researcher Dr. Ophir Ortiz analyzes the porosity and structure of biodegradable bone regeneration scaffolds.

studies. The researchers plan to work with Concordia Biomedical, a company with clinically approved biomaterials, which will provide a GMP facility for fabricating scaffolds for clinical trials.

The **Iovine/Schmalz group (Project 4.5.8)** at Rutgers University and Trident Biomedical, Inc., respectively, is focused on pursuing a 510(k) submission for a TyrPC bone fixation pin. The researchers developed and documented a robust synthesis process. They completed the development of a model that can be used to test efficacy, and they created a quality system to oversee development efforts. The researchers contracted with vendors that are working in the areas of synthesis, fabrication, packaging, and sterilization of the bone pin. The vendors have formalized the design of the pin and are in the process of validating the production processes, developing the prototype production tooling, and producing prototypes for evaluation against specifications. During the next 2 years, the research team will complete the set of tasks required for a 510(k) submission. Once FDA clearance is obtained for the tyrosine-based bone pins, the researchers could begin marketing a new line of synthetic, degradable bone fixation devices (pins) that would be a significant improvement over existing degradable bone pins. These bone pins, while not specifically targeted by the military as a high-priority product, would nevertheless have a positive impact on warfighters due to the large number of bone fractures sustained by military personnel during training and combat.



WFPC researcher Evangelia Bellas, a PhD student, prepares porous silk sponges with lipoaspirate for soft tissue reconstruction.

Soft Tissue Regeneration

Studies at WFPC

In **Projects 4.1.4 and 4.1.5**, the **Rubin/Marra group** at the University of Pittsburgh, the **Kaplan group** at Tufts University, and the **Yoo/Lee group** at Wake Forest University are seeking to develop and deliver a clinically useful, engineered soft tissue replacement that can be used as a stand-alone therapy or integrated with composite tissue regenerative medicine therapy of burns, craniofacial injuries, and extremity injuries. The researchers are generating silk-based scaffolds and injectable hydrogels that contain autologous (the patient's own) adipose-derived stem cells (ASCs), combined with carrier biomaterials, to achieve vascularized soft tissues. They have demonstrated the feasibility of using stem cell-seeded silk scaffolds for long-term soft tissue restoration. They found that silk scaffolds, either alone or seeded with ASCs, maintain their shape and size up to 6 months after implantation in the rat. They observed increased blood vessel ingrowth and new fat formation in even the unseeded scaffolds at 6 months post-implantation. During the next 2 years, the researchers plan to continue the development of silk injectables (i.e., hydrogels, sponges, and foams). They will also continue to conduct small animal studies in which the various formats will be tested. The researchers recently began a clinical trial (see the Clinical Trials section for more information).

The **Christ group (Project 4.1.6)** at the Wake Forest Institute for Regenerative Medicine (WFIRM) is developing a technological tool that preconditions and accelerates muscle tissue maturation and function. The researchers have created and implemented a rodent model of volumetric muscle loss (VML). Their model has been designed to evaluate

tissue-engineered skeletal muscle constructs generated using an innovative bioreactor system that mechanically stimulates the constructs during development. Tissue-engineered skeletal muscle constructs in the research team's initial preclinical study demonstrated clinically relevant contractile responses within 2 months of implantation, and the submaximal contractile force generated by the constructs was indistinguishable from the native muscle tissue. During the past year, the researchers' most important accomplishment was the illustration of approximately 75% functional recovery of an otherwise irrecoverable functional loss in two distinct models of VML injury within 2 months of implantation of their tissue-engineered muscle repair technology. The validation achieved through the preclinical proof-of-concept studies will facilitate the clinical translation of the bioreactor technology for skeletal muscle development to address the critical clinical need for muscle constructs capable of generating clinically relevant forces in the craniofacial complex. The researchers plan to pursue a first-in-human Phase 1/pilot study of their technology in a VML patient population and anticipate submitting an IND application to the FDA by the third quarter of 2013.

Studies at RCCC

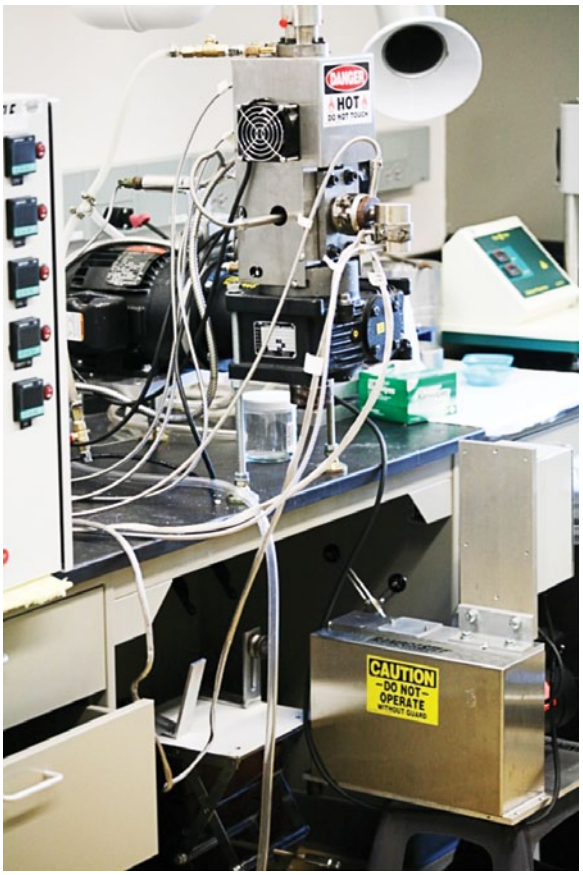
More than 10% of blast injury survivors have significant eye or eyelid injuries. Damage to the orbicularis oculi muscle prevents eyelid closure, which can result in blindness. The replacement of damaged orbicularis oculi muscles with engineered muscle will restore eyelid function, prevent blindness, and restore facial aesthetics. The **Sundback/Vacanti group (Project 4.1.2)** at Massachusetts General Hospital (MGH) is collaborating with researchers at Rutgers University and the Massachusetts Eye and Ear Infirmary to engineer skeletal muscle with physiological connections to the host's neurovascular (nerve and blood vessel) network using biodegradable polymer scaffolds. The researchers engineered three-dimensional (3-D) skeletal muscle similar to immature skeletal muscle and established protocols for the development of functional blood vessels and nerves in the tissue. They recently showed that engineered vascular networks in skeletal muscle constructs grew into the host vasculature in less than 2 days following implantation. They also demonstrated the biocompatibility of their scaffold. During the upcoming year, the research team will continue to optimize the vascular and nerve protocols of engineered muscle in rodents. In year 5, they hope to conduct a proof-of-concept study in rats to demonstrate contractile human muscle on the scale of the orbicularis oculi.

CTAs—large segments of complex, vascularized tissue—differ in their immunological responses, which raises challenges for transplant immunologists. In **Project 4.3.1**, the **Siemionow group** at the Cleveland Clinic hopes to transform standards for clinical modulation of the immune system, making transplantation of CTAs safer and more widely available to victims of disease and traumatic injury. The researchers are exploring the use of fused donor–recipient "chimeric" cells as potential immunomodulators. They recently established optimal conditions for the proliferation of the chimeric cells and improved the bone marrow preparation and isolation procedure. They are characterizing the chimeric cells in culture. They found that donor–recipient fused chimeric cells derived from the stroma (connective tissue that supports cells) possess certain stromal cell characteristics, including strong expression of collagen 1a1 and fibronectin. The researchers plan to evaluate the migratory properties, phenotype, and safety of their human donor–recipient fused chimeric cells in the nude rat model. They anticipate clinical trials involving the cells to start in 2013.

Cartilage Regeneration (Focus: Ear)

Studies at WFPC

The **Yoo group (Project 4.1.1)** at Wake Forest University seeks to accelerate the delivery of reconstructive applications to injured armed forces personnel through the development of an engineered cartilage-covered ear implant. The researchers have fabricated a flexible ear scaffold using an integrated organ printing technology, which was developed in their laboratory. Results from preclinical studies in a mouse model and initial studies in a rabbit model demonstrate the biological compatibility and structural stability of the cartilage-coated implants and point to the viability of the approach for eventual clinical translation. Indeed, the cartilage tissue-covered ear implants in mice are able to maintain device contour and placement without harming the skin. During the next 2 years, the research team plans to continue to evaluate the structural and functional integrity of its engineered cartilage ear implants in rabbits. The researchers will continue to prepare materials for FDA discussions and plan to initiate and monitor a clinical trial of engineered cartilage ear implants.



Instrument that extrudes biodegradable polymer into bone pins (RCCC).

Studies at RCCC

The **Sundback/Vacanti group (Project 4.5.4a)** at MGH seeks to expedite the development of a permanent, implantable, living external ear for the injured warfighter and to achieve cosmetic outcomes that meet patient expectations. The researchers engineered robust neocartilage in vivo using two approaches aimed at reversing and preventing cartilage cells, from undergoing "de-differentiation"—an unwanted process by which cartilage cells, after extensive expansion, return to a more primitive type of stem cell and lose their potential to form neocartilage.

They demonstrated maintenance of size and shape of neocartilage in a new generation of adult size, human ear-shaped scaffolds in a rodent model. The researchers also optimized neocartilage formation in the autologous sheep model.

To provide an adequate number of cells for tissue engineering of the ear and maximize the formation of cartilage tissue, the **Anderson/Langer group (Project 4.5.4b)** at MIT has been investigating how to combine ASCs with optimal soluble factors and growth factors from chondrocytes (cartilage cells). The researchers have successfully created in vivo systems for efficiently forming cartilage using ASCs combined with biodegradable, synthetic, polymeric materials. During the next 2 years, they will perform large animal studies in sheep, collaborating with the MGH group (Project 4.5.4a). Upon successful completion of large animal studies, the researchers will prepare an IDE or IND for clinical application. Furthermore, once the stem cell-based approaches to engineering cartilage are optimized in large animal studies, the research team plans to work with Concordia Biomedical, a company with clinically approved biomaterials, which will provide a GMP facility for fabricating scaffolds for clinical trials.

Clinical Trials

Several AFIRM CFR projects have technologies that have advanced to the human clinical trial stage. Additional details on these clinical trials can be found in **Table III-2** and subsequent sections of this chapter.

In **Project 4.3.1a**, the **Siemionow group** at the Cleveland Clinic is performing CTA face transplantation, which provides a single-stage reconstructive procedure for patients with severe cranial facial injuries and spares them from multiple surgical procedures over many years. To date, the researchers developed a workflow process for transplant evaluation and referral, developed a research database, and enrolled three patients in their CTA face transplantation clinical trial.

The **Siemionow group** in **Project 4.3.1b** is using a therapeutic antibody, TOL101, as a conditioning agent prior to transplantation to enhance allograft tolerance. The researchers are testing the safety and tolerability of TOL101 in patients who are undergoing their first kidney transplantations. A total of seven subjects have been enrolled in the Phase 1/2 clinical trial to date. The planned regulatory pathway for TOL101 includes the design of a Phase 3 trial at the conclusion of the current study.

In **Project 4.1.4/4.1.5**, the **Rubin/Marra group** at the University of Pittsburgh, the Kaplan group at Tufts University, and the Yoo/Lee group at Wake Forest University has begun a clinical trial at the University of Pittsburgh titled "Autologous Adipose-Derived Stem Cell Therapy for Soft Tissue Reconstruction after Facial Trauma." To date, 14 subjects have undergone lipoaspirate as a fat graft. The researchers will soon move on to stem cell-seeded fat grafts. A progress report is not yet available for this clinical trial.

The **Mikos/Kasper and Wong group (Project 4.1.2)** at Rice University and the University of Texas Health Science Center at Houston, respectively, has recently received approval by the Institutional Review Board (IRB) of the University of Texas Health Science Center at Houston for its protocol for a clinical study of the porous space maintainer technology. A progress report is not yet available for this clinical trial.

Table III-2. AFIRM-funded CFR projects with pending or active clinical trials.

Project Title	Consortium	Project No.	Trial Phase	Current Status
Clinical Trial – Composite Tissue Allograft Transplantation (Face)	RCCC	4.3.1a	Phase 1	Open
Clinical Trial – Anti-TCR Monoclonal Antibody (TOL101) for Prophylaxis of Acute Organ Rejection in Patients Receiving Renal Transplantation	RCCC	4.3.1b	Phase 1/2	Open
Soft Tissue Reconstruction/Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction	WFPC	4.1.4/4.1.5	Phase 1	Open
Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects	WFPC	4.1.2	Phase 1	Protocol Approved by IRB

Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects

Project 4.1.2, WFPC

Team Leader(s): Antonios G. Mikos, PhD and F. Kurtis Kasper, PhD (Rice University) and Mark E. Wong, DDS (University of Texas Health Science Center at Houston)

Project Team Members: Allan Henslee, BS, Lucas Kinard, BS, James D. Kretlow, PhD, Meng Shi, PhD, and Patrick Spicer, BS (Rice University) and Nagi Demian, DDS, MD and Simon Young, DDS, PhD (University of Texas Health Science Center at Houston)

Collaborator(s): Shanghai 9th People's Hospital, Shanghai, China and Radboud University of Nijmegen Medical Centre, Nijmegen, The Netherlands

Therapy: Staged reconstruction of large osseous defects in the craniofacial region restoring function and esthetics.

Deliverable(s): (1) Biocompatible, antibiotic-releasing implants to maintain bony wound spaces, (2) "in vivo bioreactor" that will allow for the generation of vascularized bone, and (3) injectable system for delivery of growth factors necessary for bone regeneration and wound healing.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 4; End Year 2, TRL 4; End Year 3, TRL 5

Key Accomplishments: The researchers made considerable progress over the past year toward the clinical translation of porous poly(methyl methacrylate) (PMMA)-based space maintainers as reflected in the treatment of three patients with the technology under the physician-directed combination of clinically available products.

The researchers received IRB approval to begin a formal clinical study of the technology. They demonstrated in a rabbit composite mandibular defect model that in situ-formed porous PMMA implants resulted in enhanced soft tissue healing and coverage relative to groups receiving nonporous implants. The researchers completed in vitro studies characterizing the release of antibiotics from PMMA-based space maintainers incorporating gelatin microspheres, which may allow for intraoperative antibiotic loading. They demonstrated that construct porosity and antibiotic release can be controlled through manipulation of tunable parameters of space maintainer fabrication.

Key Words: Craniofacial bone reconstruction, space maintenance, bone flap, controlled drug delivery, in vivo bioreactor

Introduction

Ballistic injuries resulting in significant soft and hard tissue loss and devitalization are commonly encountered clinical scenarios in the current U.S. military combat theaters Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF). In this project the researchers seek to develop a method to facilitate effective staged reconstruction of large osseous defects in the craniofacial region and extremities of injured military personnel thus restoring function and esthetics in these individuals.

Alloplasts such as PMMA are often used clinically as space maintainers due to their approved regulatory status and ability to

be molded intraoperatively to fill complex bony defects. Wound dehiscence over these implants is a commonly encountered problem, typically occurring at later time points and thus indicating insufficient wound strength rather than a deficiency in wound healing. It was hypothesized in the current project that a contributing factor in such cases of late wound dehiscence could be a lack of tissue ingrowth or adhesion around the currently used nonporous implants, resulting in the creation of shear planes around the implant after wound healing. Consequently, the researchers seek to apply materials currently regulated for clinical use, namely PMMA and a gelatin-based porogen, in novel combinations to produce porous space maintainers for the dual purpose of maintaining the bony defect space without dehiscence and releasing antibiotics in a controlled manner to mitigate wound infection.

The surgical transfer of autologous bone tissue to a bony defect site is the "gold standard" for augmentation of bone regeneration. The procedure is generally successful; however, the amount of autologous bone tissue available for transfer is limited and often not of the desired shape to reconstruct the complex bony contours of the craniofacial complex. Additionally, the recruitment of distal bone from the patient requires the introduction of a second defect and creates a risk of donor-site morbidity. The employment of allogeneic bone tissue provides an alternative method to autografts. Although allograft material is more plentiful than autologous bone for grafting, processing of the allogeneic tissue limits its osteoinductive properties and does not eliminate the risk of pathogen transmission. The researchers seek to apply an existing technique pioneered in an animal model in their laboratory using the body as a "bioreactor" to produce vascularized bone flaps in chambers comprising clinically regulated materials (e.g., PMMA and gelatin) at secondary sites for transplantation to a bony defect. The vascularized bone flap technology has already seen investigational clinical use to treat bony defects in the CMF complex.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers fabricated and characterized in vitro a variety of porous space maintainer formulations presenting a range of porosities and mechanical properties. They evaluated the implants for efficacy in maintaining a bony defect space while supporting soft tissue healing in a rabbit mandibular defect model in vivo. In vitro studies demonstrated that antibiotics could be incorporated into space-maintaining implants and released in a controlled fashion. The researchers also found that the release kinetics could be altered by tunable construct fabrication parameters. Initial results from an ongoing in vivo study in a sheep model suggested the feasibility of the in vivo bioreactor approach for the generation of vascularized autologous bone flaps and the transfer of the flaps to fill mandibular defects.

Research Progress – Year 3

Bone loss in the craniofacial complex following battlefield trauma remains a difficult task to address, particularly when one considers the unique structural and functional aspects of the craniofacial complex. Current solutions to the problem typically involve autologous bone grafting, free tissue transfer, and/or the placement of alloplastic materials for long-term stabilization and structural support. Very few regenerative medicine techniques are currently utilized in treating these injuries; a primary factor in this may be the presence of complicating factors (e.g., lack of soft tissue

coverage and infection) that may prevent the successful use of more traditional tissue engineering approaches to treatment as these often involve the placement of temporary, porous scaffolds that could serve as a nidus to infection. Furthermore, many tissue engineering approaches to bone regeneration require significant delays in treatment such that autologous cells can be harvested, expanded, seeded onto scaffolds, and then implanted to repair or regenerate the defect. While such a delay may allow time for the treatment of infections or soft tissue healing or grafting, it may also allow time for scarring and subsequent contracture of tissues that could be otherwise useful in the definitive reconstruction of the injured craniofacial complex.

The strategy currently being investigated to facilitate a regenerative medicine approach to treating traumatic bone loss involves two technologies—a porous PMMA-based space maintainer and an in vivo bioreactor to be used for vascularized bone flap fabrication. The overall scenario being envisioned is one in which, following injury, stabilization, and transfer to an appropriate care facility, an injured soldier would have a temporary PMMA space maintainer surgically implanted to approximate lost craniofacial bone thus supporting surrounding structures and preventing contraction into the void space once occupied by bone. At the same time, a chamber filled with a material facilitating the ingrowth or generation of new bone will be implanted elsewhere in the body to begin generating what will later be a vascularized bone flap for transfer. After some time, likely dependent on the injuries suffered and material used in the bone chamber, the space maintainer would be removed, and the vascularized bone flap would be transferred to replace the space maintainer and ultimately the bone that was lost.

A number of studies were completed or continued in the past year investigating and optimizing the technologies envisioned for application in this approach. First, an in vivo study was performed to evaluate the effects of porous PMMA space maintainers molded in situ on the healing of surrounding soft tissue in a rabbit mandibular defect model. A previous study characterized the tissue response to porous PMMA space maintainers formed prior to the surgical procedure and found improved wound healing with porous space maintainers when compared to nonporous constructs. However, the intraoperative formation of implants in situ is critical for clinical translation of the space maintenance approach to address craniofacial bone defects, such as those arising from battlefield injuries, which are rarely of regular or predetermined size or shape. Additionally, investigation of the potential impact of parameters associated with the in situ fabrication of the implants, such as the exothermic polymerization reaction, residual reactants, and the surface properties of the implants themselves, upon soft tissue healing was necessary to enable clinical translation of the approach.

In this study, the researchers specifically analyzed the effect of porous PMMA implants formed in situ on the mucosal healing and long-term histologic response in a rabbit composite mandibular defect model against similarly formed solid implants **(Figure III-1)**. The porous implants showed enhanced soft tissue healing and coverage of the implant relative to the nonporous implant groups. Additionally, the increased surface area and presence of a gelatin-based porogen did not appear to result in a prolonged, unresolved inflammatory response.

Second, in vitro studies characterized drug release from porous PMMA-based space maintainers incorporating antibiotic delivery vehicles. Previous studies demonstrated that

porous PMMA-based constructs successfully maintained the bony space within a nonhealing rabbit mandibular defect model and provided a template for improved wound healing when compared to solid PMMA implants. To modify the system to enable local antibiotic delivery, drug-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres were also incorporated into the PMMA-based constructs, creating porous constructs capable of extended antibiotic delivery. To simplify the antibiotic-releasing, porous PMMA-based constructs, gelatin microparticle-incorporating PMMA constructs (i.e., a PMMA/gelatin/antibiotic construct) were developed in which the gelatin microparticles serve as both the drug carrier and porogen. Specifically, PMMA/gelatin/antibiotic constructs with varying gelatin incorporation and antibiotic (i.e., colistin, an antibiotic effective against *Acinetobacter baumannii)* content were investigated to elucidate the relationship between material composition and construct properties, such as porosity and drug release kinetics. The researchers found that porosity of constructs could be tailored by tuning both the amount of gelatin incorporated and the amount of drug solution added for gelatin swelling. The constructs released colistin continuously over 10 or 14 days with an average release rate per day above 10 μg/mL, which is well above the minimum inhibitory concentration of colistin against *A. baumannii* (0.5 μg/mL). They envision that these formulations and others under investigation will allow for controlled, local delivery of antibiotics and/or growth factors to optimize the tissue microenvironment for the later transfer of the vascularized bone flap.

Third, ongoing in vivo studies are evaluating the efficacy of antibiotic release from intraoperatively fabricated porous PMMA-based constructs in a rabbit mandibular defect model seeded with *A. baumannii.*

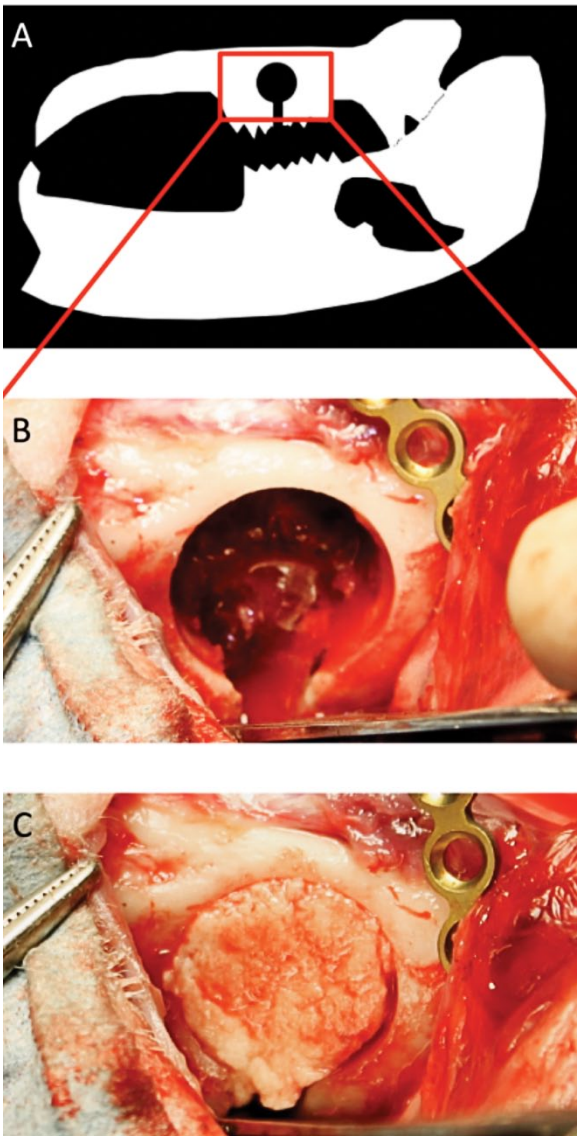


Figure III-1. Schematic (A) and photograph (B) of a 10 mm diameter defect in a rabbit mandible. Photograph (C) of the rabbit mandibular defect filled with a porous PMMA-based implant formed intraoperatively.

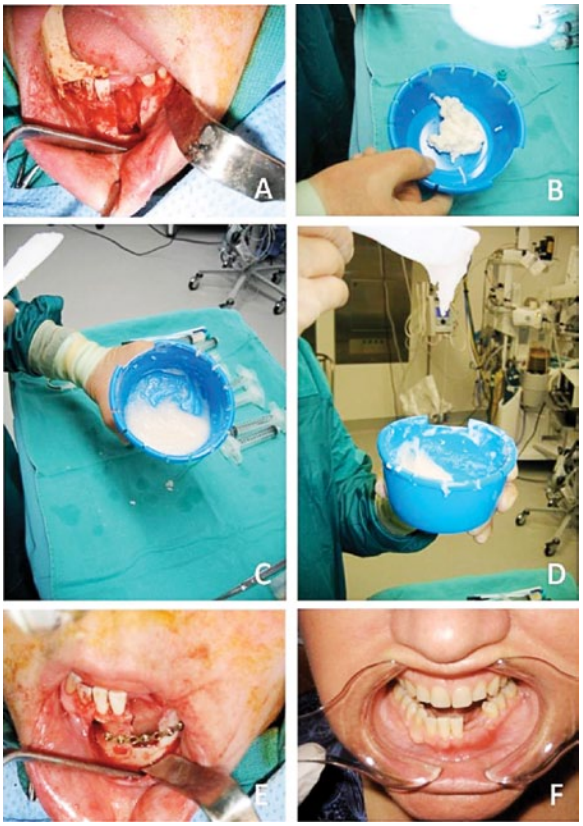


Figure III-2. Intraoperative photographs from a clinical application of the porous space maintainer technology under physician-directed combination of two clinically available products, including a mandibular defect upon removal of a benign tumor (A), a gelatin-based clinical product that serves as a porogen in the fabrication of the constructs (B), and addition of the components of a clinically available bone cement product (C) resulting in initiation of a polymerization reaction and increasing viscosity of the mixture (D). The bony defect filled with the space maintainer and stabilized with a bone plate is illustrated in (E) while (F) depicts the well-healed oral mucosa 2 weeks after the surgery.

Fourth, work was continued to investigate the de novo formation of vascularized bone flaps. In collaboration with colleagues at the Shanghai 9th People's Hospital in Shanghai, China, leveraging non-AFIRM funds, the ongoing study is designed to determine how the filler material affects bone growth during flap generation and following flap transfer. Bone is being generated within chambers placed adjacent to the cambial side of the periosteum covering a sheep's rib, and the generated bone is then transferred to a bone defect created in the sheep's mandible. The effect on bone growth of having a flap transferred with a pedicle is being compared to transferring the generated bone as a nonvascularized graft. Samples have been harvested from the sheep involved in the pilot study, and analysis of the tissue is currently under way.

Fifth, a protocol was approved by the IRB of the University of Texas Health Science Center at Houston to initiate a prospective clinical study of the porous PMMA-based space maintainer technology. The study will commence once Human Research Protection Office (HRPO) approval of the protocol has been issued. However, the technology was applied recently in the treatment of three patients through physician-directed combination of clinically available products **(Figure III-2)**.

Key Research Accomplishments

- Completed a study investigating whether in situ PMMA-based construct polymerization has an effect on the tissue response to space maintainers in a nonhealing mandibular defect in a rabbit model (Figure III-1).
    - ♦ Demonstrated that the in situ-formed porous implants resulted in enhanced

soft tissue healing and coverage relative to groups receiving nonporous implants.

- Completed in vitro studies characterizing the release of antibiotics from PMMA-based space maintainers incorporating gelatin microspheres, which may allow for intraoperative antibiotic loading.
  - Demonstrated that construct porosity and antibiotic release can be controlled through manipulation of tunable parameters of space maintainer fabrication.
- Completed in vitro studies characterizing the physical properties of PMMA-based space maintainers incorporating a clinically available gelatin-based product as a porogen, which facilitated clinical translation of the approach in the treatment of three patients under physician-directed care (Figure III-2).
  - Received IRB approval to initiate a formal clinical study, which will commence immediately upon receipt of HRPO approval.

Conclusions

Considerable progress has been made over the course of the past year toward the clinical translation of porous PMMA-based space maintainers as reflected in the treatment of three patients with the technology under physician-directed combination of clinically available products. Additional progress has been made toward the development of antibiotic-releasing implants for bony space maintenance. Specifically, the intraoperative in situ formation of porous PMMA-based constructs was shown to positively influence the tissue response to the implants in a nonhealing rabbit mandibular defect model relative to similarly fabricated nonporous controls. Further, in vitro studies demonstrated that the antibiotic colistin can be released from PMMA-based space maintainers incorporating gelatin microspheres in a controlled fashion and that the release kinetics can be modulated through manipulation of tunable parameters of the construct fabrication. Leveraging gelatin microspheres as antibiotic delivery vehicles may enable intraoperative loading of antibiotic(s) in the constructs. Initial results from an ongoing in vivo pilot study employing a sheep model demonstrate that PMMA chambers filled with osteoinductive materials can be effectively employed in an in vivo bioreactor strategy to generate vascularized, autologous bone flaps and that these flaps can be transferred to fill mandibular defects.

Research Plans for the Next 2 Years

Future research plans involve continued characterization of the efficacy of antibiotic release from porous PMMA-based space maintainer constructs in eradicating target species in infected nonhealing rabbit mandibular defects. The in vivo bioreactor approach for vascularized bone flap generation will continue to be explored in a sheep model to optimize the parameters for bone flap generation and to evaluate the durability of repair of mandibular defects upon transfer of the bone flaps. Additional studies will continue to evaluate controlled growth factor delivery from degradable material constructs to support bone regeneration and esthetic contouring. Finally, the IRB-approved clinical study of the space maintainer technology will commence immediately upon receipt of HRPO approval.

Planned Clinical Transitions

The researchers have retained a regulatory consultant to guide the team in the appropriate pathways for clinical translation and regulatory clearance and in the development of commercialization strategies for the technologies. Through the ongoing interactions with the regulatory consultant, it has been determined that FDA regulation may not be required for the porous space maintainer technology as it can be applied through a physician-directed combination of existing clinical products. Accordingly, a twofold approach for clinical translation of the porous space maintainer technology has been developed under the guidance of the regulatory consultant. The first approach involves the development of protocols to expedite the clinical translation of the technology through physician-directed combination of existing clinical products, which may obviate FDA regulation. Leverage of existing clinical products through physician-directed application of the porous space maintainer technology will circumvent the need to manufacture the constituent materials as a new product. The second approach, which is being developed in parallel with the protocols associated with the first approach, involves the identification of appropriate pathways for clinical translation, regulatory approval, and commercialization of new products based on the technologies under development through this project. For example, Synthasome, Inc., has agreed to work with the researchers to develop the concept of the porous space maintainer with a slow-release antibiotic for the treatment of mandibular injuries into a product.

Toward the goal of clinical translation, a protocol has been approved by the IRB of the University of Texas Health Science Center at Houston to initiate a clinical study of the porous space maintainer technology. Additionally, physician-directed combination of the clinical PMMA bone cement and gelatin-based products, as outlined in the submitted IRB protocol to produce a porous PMMA-based space maintainer, was applied recently in three clinical cases upon resection of benign mandibular tumors.


our science for their healing

Novel Synthetic Bone for Craniofacial Application

Project 4.1.3, WFPC

Team Leader(s): Charles Sfeir, DDS, PhD, Elia Beniash, PhD, and Prashant Kumta, PhD (University of Pittsburgh)

Project Team Members: Abhijit Roy, PhD, Shinsuke Onishi, DDS, PhD, and Sabrina Noorani, MS (University of Pittsburgh)

Collaborator(s): None

Therapy: Bone tissue-engineered therapy.

Deliverable(s): New porous bone void filler for rapid bone regeneration.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 4; End Year 3, TRL 4

Key Accomplishments: The researchers have developed and characterized novel nanostructured apatitic bioactive bone cements and scaffolds based on the use of natural extracellular matrix (ECM)-derived polymers that incorporate NanoCaPs. In vivo experiments in the rabbit calvarial model revealed that the cements alone or with the addition of BMP-2 had the ability to regenerate bone.

Key Words: Bone regeneration, craniofacial defects, synthetic bone, NanoCaPs, bioresorbable

Introduction

This project focuses on developing novel bone regeneration strategies for CFR by exploiting the combined attributes of nanoscale inorganic bioactive cements and naturally derived polymer hybrid materials that possess excellent bioreactivity, biocompatibility, safety, and regenerative capability. This combination of materials would result in structural and functional bone free of infection for injured military personnel. The proposed technologies will also be used for regenerating large osseous defects in the extremities where bone fracture is a major clinical problem contributing to nearly 50% of injuries in armed forces personnel.

The researchers are developing a synthetic bone-like environment that involves bioactive nanostructured amorphous and/or nanocrystalline CaPs, nanostructured CaP-based bioactive bone cements, and ECM-derived materials such as urinary bladder membrane (UBM). These nanostructured systems contain essential components to mimic bone architecture, composition, and mechanical strength while providing the osteoinductive and osteoconductive characteristics required for bone tissue regeneration. The combined nanoscale hybrid system will incorporate BMP-2 or BMP-7, which are known for their bone regeneration ability. This strategy will create an organic/inorganic scaffold system that would simulate the unique composition and architecture of bone.

There are three specific aims for this project:

Specific Aim 1: Synthesize and characterize nanostructured apatitic bioactive bone cements and scaffolds based on natural ECM-derived polymers that incorporate NanoCaPs (amorphous and/or nanocrystalline). These hybrid scaffold systems will incorporate BMP-2 or BMP-7 and ECM from the porcine UBM.

Specific Aim 2: Preclinical assessment of the regenerative capacity of the bone cements and ECM-derived polymers in a well-established rabbit, critical-size, calvarial defect model.

Specific Aim 3: Initiation of clinical testing of synthetic bone for craniofacial defects.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers developed and characterized novel nanostructured apatitic bioactive bone cements and scaffolds based on the use of natural ECM-derived polymers that incorporate NanoCaPs. They initiated the in vitro assessment of the cements and scaffolds and found that the synthesized forms of the cement materials are biocompatible. Preliminary experiments in a rabbit ulna defect model with bone cements containing BMP-2 showed some bone regeneration (bridging) at 8 weeks post-surgery. Overall, data indicated that the resorbable cements containing NanoCaPs with and without BMP-2 are good candidates for further assessment of the system as a bone regeneration therapy.

Research Progress – Year 3

Bone Cement Synthesis

The first cement developed by the researchers was based on CaP powders and other acidic and basic calcium salts (CaP-cements). To introduce NanoCaP-based nanocarriers into the bone cements, a colloidal solution containing NanoCaPs was used as a liquid for the cement-forming reaction. The researchers found that the in vivo resorption rate of CaP-cements could be improved by introducing controlled numbers of micropores (to allow circulation of body fluid) and macropores (to provide a scaffold for blood cell colonization). The micropores could also facilitate cell attachment, migration, and proliferation, enabling good uptake of the carriers. This was achieved by using a certain amount of water-soluble recrystallized polyol (porogen). The introduction of 40% (by weight) of the porogen into the CaP-cements did not alter the initial and final setting times, cohesion time, and injectability characteristics of the cements. However, the injectability of this cement was poor (less than 60%); therefore, the researchers used a cohesion promoter to improve its injectability. Addition of the cohesion promoter led to the injectability increasing to greater than 95% and the cohesion time under phosphate-buffered saline (PBS) to be less than 4 minutes.

The x-ray diffraction patterns of porogen-containing injectable cements (PC-cements) showed that the PC-cements mostly converted into calcium-deficient hydroxyapatite (CDHA) within 15 days. The scanning electron microscope images of the PC-cements clearly showed the formation of micro- and macropores after dissolution of the porogen and the formation of nanocrystalline hydroxyapatite. The porosity percentages, pore size distribution, and pore characteristics of the PC-cements **(Figure III-3)** were further studied using mercury porosimetry, which clearly

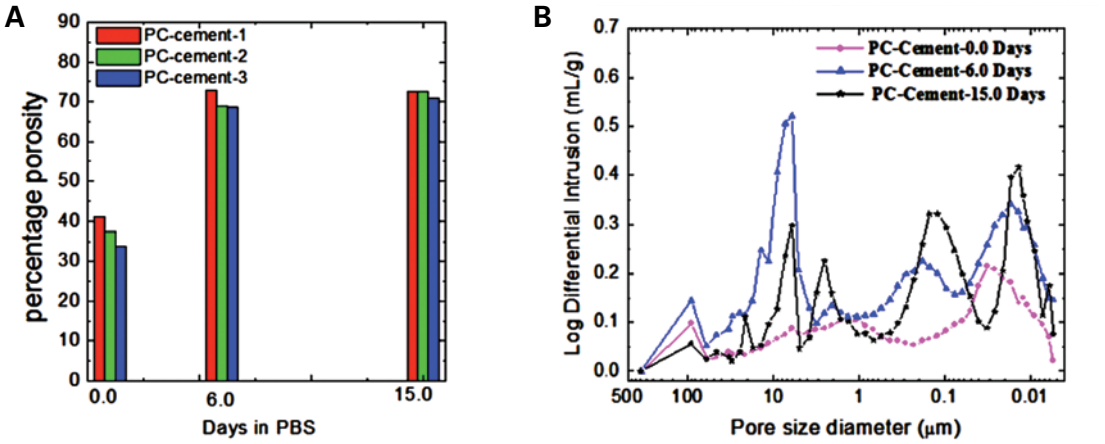


Figure III-3. (A) Percentage porosities of PC-cements after different aging times in PBS and (B) Micro- and macropore size distributions as determined from the mercury porosiometer of PC-cements after different aging times in PBS.

demonstrated that the PC-cements contained large numbers of macropores between 1 and 100 μm. High porosities of the PC-cements together with the formation of nanocrystalline CDHA may dramatically improve the in vivo dissolution rate of these cements.

To improve the bioactivity and porosity of the PC-cements, the researchers added an organic matrix, UBM, in the PC-cements. The presence of adhesion molecules, structural proteins, and growth factors inside the organic matrix may enhance the bone regeneration process while contributing to the porosity. The addition of the organic matrix into the PC-cements (henceforth described as organic UMB-cements) led to an increase in setting times of the cements. The phase analyses of the UBM-cements using x-ray diffraction showed that the cements immersed in PBS completely converted into CDHA within 3 days, which was considerably shorter than the CaP- and PC-cements. However, the morphology of the CDHA formed in these organic UBM-cements was found to be very similar to the CaP- and PC-cements. These results show that the addition of UBM into the cement reaction mixture has a profound influence on the kinetics of the cement-setting reaction.

In Vitro Assessment of the Bone Cement

Proliferation Assay

For all of the cements, the researchers assessed MC3T3 cell proliferation with the nontoxic Alamar Blue dye as shown in **Figure III-4**. This test gives a precise indication of cell function because of the reduction of Alamar Blue by intracellular enzyme activity. Two and 5 days after the cell seeding, and just before cell counting, 20 μL of the diluted fluorescent dye was added in each well. The proliferation results show that the cells grow well in all of these cements; however, the PC-cements showed the best cell growth.

Cell Attachment Assay

The research team assessed cell attachment and viability using a trypan blue assay. MC3T3 cells were plated onto wells of a 48-well dish filled with either PC-cement, organic cement, or a control substrate. The researchers found that cell viability after 6 hours of culture on the three different substrates was approximately the same.

Cell proliferation was also determined using a methyl tetrazolium assay. MC3T3 cells were plated onto wells of a 48-well dish filled with either PC-cement, organic cement, or a control substrate. The number of cells on the organic cement remained approximately the same over the 7-day study indicating limited proliferation. The number of cells was greatest on the control substrate. The proliferation rates of the control and PC-cement seemed to be approximately equal since the slopes between the time points were similar.

Release Kinetics

The wells of a 96-well dish were coated with either the researchers' cement, a commercial cement (Stryker HydroSet), or no cement (tissue culture plastic). The three substrates were coated with either a high or low concentration of fluorescently labeled bovine serum albumin (BSA). Solutions containing 0.8 or 0.33 mg/mL BSA in PBS were prepared. The researchers found that the commercial cement and tissue culture plastic adsorbed lower amounts of BSA in comparison to their cement. The commercial cement and tissue culture plastic showed an initial release at 1 and 4 hours whereas their cement exhibited a sustained release over a span of 46 days.

In Vivo Experiments Using the Rabbit Calvarial Model

A pilot in vivo experiment (no AFIRM funds were used) using the rabbit ulna model was previously carried out to screen several versions of the synthesized cements. The researchers found that the cement alone (without BMP-2) could regenerate bone. They therefore decided to focus on the cement alone as a technology to move forward to the clinical arena.

The research team tested its injectable cements in the rabbit calvarial model in four treatment groups as shown in **Figure III-5** and **Figure III-6** as well as an empty control

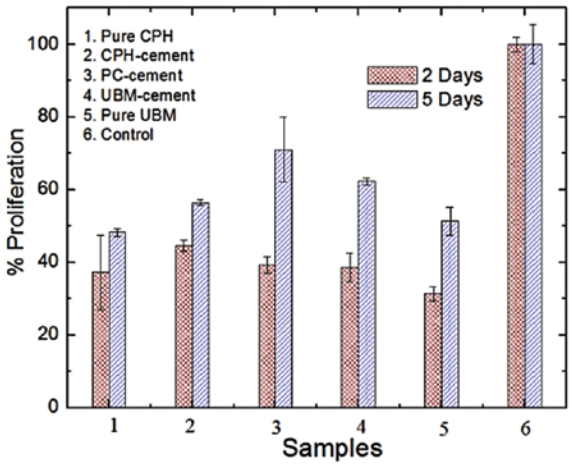


Figure III-4. MC3T3 proliferation on different bone cements.

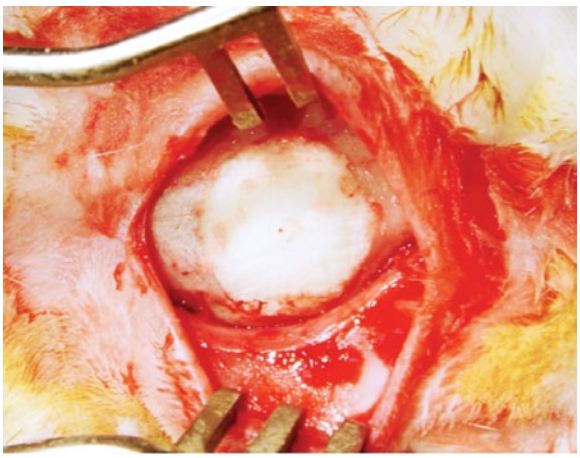


Figure III-5. Completion of the cement implantation in the defect after the CSD was created.

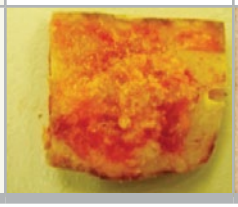
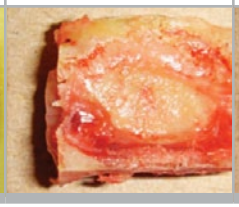
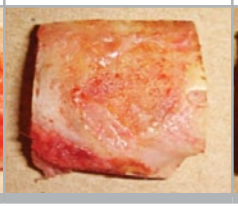
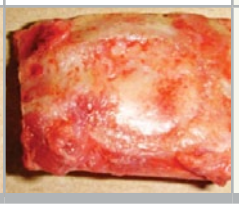
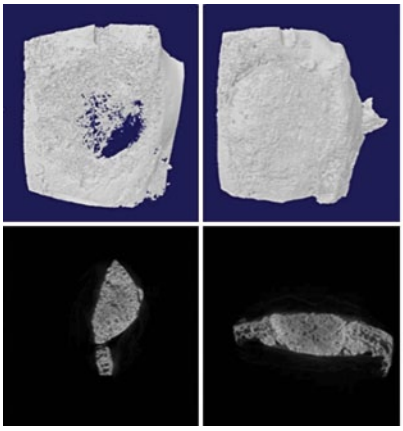
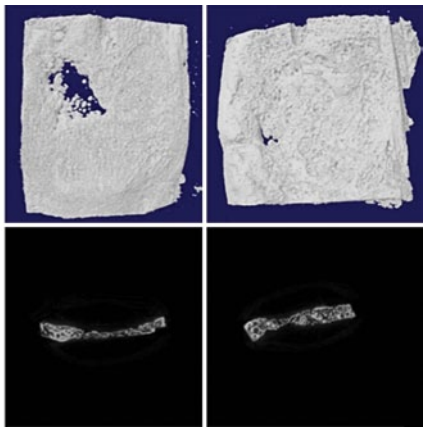
Control	A 60% Cement + 40% Mannitol	B 60% Cement + 40% Mannitol + 10% UBM	C 60% Cement + 40% Mannitol + 35 µg BMP-2	D 60% Cement + 40% Mannitol + 10% UBM + 35 µg BMP-2	Commercially Available Cement
Empty Defect					Predicate

Figure III-6. Rabbit calvariae with four different treatments retrieved at 8 weeks after implantation of cement.



Cement + 40% Mannitol



Cement + 40% Mannitol + 35 µg BMP-2

Figure III-7. 3-D models and sagittal sections generated from the micro-CT data.

(no implant) and a commercially available implant (predicate). A circular CSD (15 mm in diameter) was created in the calvaria of a male New Zealand White rabbit. The dura mater was left intact or undamaged as much as possible. After creating the defect, the cement was implanted into the defect. The periosteum and skin were subsequently closed separately by suturing (Figure III-5). Eight weeks after the implantation the rabbits were sacrificed and dissected. Figure III-6 shows samples (calvarial bone) representing each treatment group after dissection. The prepared samples were then fixed in 10% formalin and scanned with micro-computed tomography (CT) for quantification of the newly formed bone in the defects.

Figure III-7 shows 3-D models and sagittal sections of the rabbit calvarial samples created from micro-CT data. These data suggest that the cement alone may regenerate bone in the defect. **Figures III-8–III-11** show histologically that implants containing either cement alone or cement plus BMP-2 resulted in bone regeneration while the commercially available cement and the treatment group without an implant did not support bone regeneration.

Ongoing experiments involve biomechanical tests, which are important for assessing the strength of the newly formed bone/tissue

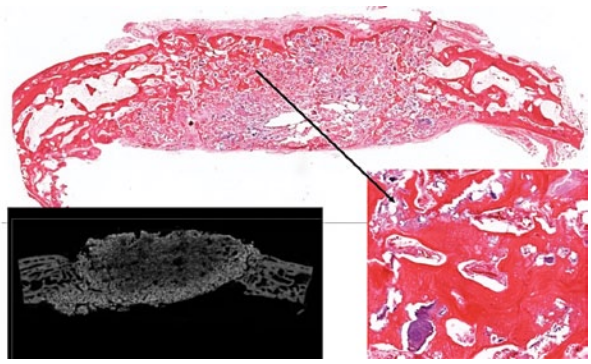


Figure III-8. Histological assessment of cement-alone implants. Bone regeneration was achieved, indicating that the cement has the ability to regenerate a CSD without the addition of any growth factors.

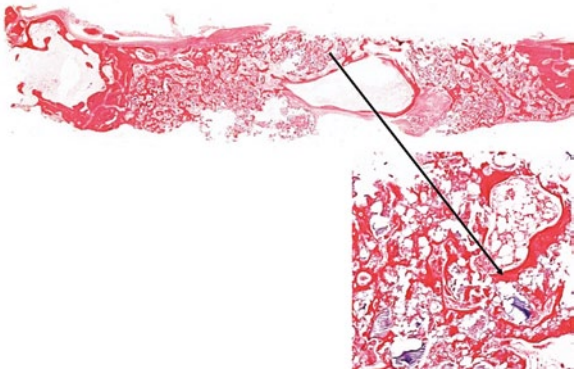


Figure III-9. Histological assessment of cement + 35 µg of BMP-2 implants. Bone regeneration was achieved, indicating that the cement can successfully support the delivery of BMP-2.

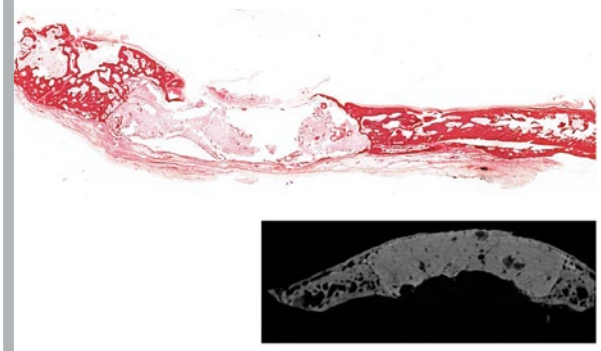


Figure III-10. Histological assessment of commercially available cement (predicate). As seen on histological sections, the material did not resorb and did not support bone regeneration.

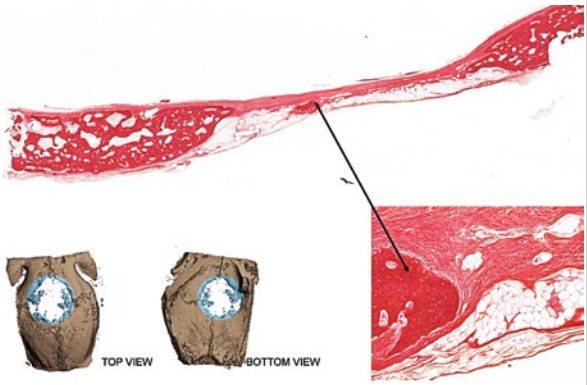


Figure III-11. Histological assessment of the control group (no implant). No bone regeneration can be seen as expected in a CSD.

in the defect compared to the strength of natural bone. Therefore, the researchers also performed surgery on another six rabbits and implanted their cement (cement + 40% mannitol) alone.

Key Research Accomplishments

- Developed and characterized novel, injectable, high-surface-area, nanostructured, apatitic bioactive bone cements and scaffolds containing NanoCaP carriers of growth factors based on the use of natural ECM-derived polymers.
- In vitro assessment shows that the synthesized cement materials are biocompatible.
- In vivo data show that the bioresorbable cements containing NanoCaPs with and without BMP-2 are excellent scaffolds for bone regeneration therapy.

- Performed in vivo experiments in the rabbit calvarial model using four different treatment groups; assessed calvarial samples histologically and by micro-CT.
    - Initial data indicate that the cement alone or with BMP-2 has the ability to regenerate bone as was previously seen in the ulna bone defect model.

Conclusions

In conclusion, the researchers have successfully developed and characterized novel, nanostructured, apatitic, bioactive bone cements and scaffolds based on the use of natural ECM-derived polymers containing NanoCaPs. The inorganic cements with or without BMP-2 appear to perform very well in vitro as well as in vivo. The researchers will therefore focus their efforts on expanding preclinical in vivo experiments to further develop these cements and gather more data to proceed further for FDA 510(k) submission and approval. In vivo experiments using the rabbit calvarial model were executed, and further analysis of bone regeneration will be conducted.

Research Plans for the Next Year

Currently, this project is set to conclude December 31, 2011. The researchers plan to continue surgeries using the rabbit calvarial model using a slightly different formulation of PC-cement. They would like to use a smaller amount of cohesion promoter to avoid any immunogenic response. The researchers would also like to use much smaller amounts of growth factor (BMP-2, 10 µg/scaffold) to avoid any complicacy related to BMP-2. However, this amount should not compromise the rate of new bone generation.

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Allograft Bone/Polymer Composites and Expedited Commercialization of an Injectable Bone/Allograft Composite for Open Fractures

Project 4.5.1a/4.5.7, RCCC

Team Leader(s): Scott A. Guelcher, PhD, Pamela Brown-Baer, DDS, COL Robert Hale, DDS, and Joseph C. Wenke, PhD (Vanderbilt University)

Project Team Members: Kasia Zienkiewiczm, MS, Margarita Prieto, BS, and Jerald Dumas, PhD (Vanderbilt University)

Collaborator(s): USAISR and Medtronic, Inc.

Therapy: Injectable bone void filler, injectable drug-delivery system.

Deliverable(s): Injectable allograft biocomposite (i.e., bone void filler made from donor tissue combined with man-made resin materials), allograft biocomposite putty, injectable rhBMP-2 delivery system, injectable rhGDF-5 delivery system.

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 4; Target, TRL 4

Key Accomplishments: The researchers developed injectable and putty synthetic biocomposites with mechanical properties comparable to or exceeding those of trabecular bone for use in delivery systems for recombinant human growth factors. In addition, they regenerated bone in rabbit models using injectable and putty allograft-based bone void fillers with added rhBMP-2. They also identified the optimum porosity of their injectable allograft biocomposites.

Key Words: Bone void filler, delivery system, injectable, growth factor, biocomposite

Introduction

Unmet Need

Military Personnel

Recent studies have examined the battle injury patterns and resource impacts of injuries for OIF and OEF. These studies have highlighted the significance of the injuries to the head and neck. Owens, et al. reported that the frequency of head and neck injuries has increased in the current conflicts in Iraq and Afghanistan, compared to previous U.S. wars, and that head and neck injuries now account for 29.4% of all battle injuries sustained in OIF and OEF *(J Trauma* 2008;64[2]:295-9). Looking at the same cohort, Masini, et al. found that head and neck injuries required 20% of all treatment resources, had the highest mean disability rating (52%), and will command 27% of all total projected benefit costs *(J Orthop Trauma* 2009;23[4]:261-6). Further research has been conducted to characterize the CMF battle injuries of OIF and OEF. Lew, et al., in characterizing the facial fractures in the current conflicts, found that the highest percentage, 36%, were to the mandible *(J Oral Maxillofac Surg* 2010;68[1]:3-7). They also reported that 76% of the CMF battle injuries were classified as open fractures. The high incidence of open fractures and the associated high complication rate result in a large patient population that requires improved treatments.

The current methods of CMF bone repair incorporate the use of autografts or allografts (transplanted tissue from either a patient's own body or from another human donor, respectively). In addition, newer surgical techniques, such as distraction osteogenesis (which lengthens or expands bone to help reconstruct injuries or defects), have been successful in restoring smaller portions of the mandible. However, due to the size and geometry of battle injuries, this technique has limited application. Autografts have been the standard of care but have the downside of causing morbidity of the donor site, which is generally the iliac crest, or in larger injuries, a composite tissue autograft from the fibula. In addition, the autologous implants may resorb (dissolve back into the body) and at times fail to integrate especially without sufficient site vascularization. Considering the frequency and characteristics of these injuries, with the resultant mean disability rating, it is hypothesized that the current surgical techniques are not sufficient to treat these injuries.

Civilian Population

A concurrent need exists to repair CMF defects that are, in the civilian case, caused by trauma, disease, and cancer surgery reconstruction. Injectable bone void fillers are used to repair bone defects in the civilian population. However, currently available bone void fillers (e.g., CaP cements) have brittle mechanical properties and remodel slowly.

The RCCC Solution

The Vanderbilt University group is focused on evaluating improved materials for treating traumatic bone defects. The researchers are pursuing the following two projects in collaboration with USAISR and Medtronic:

- Project 4.5.1a: Develop injectable rhBMP-2 and rhGDF-5 biocomposite delivery systems for repair of lateral mandibular body defects. (The injectable biocomposite delivery systems for rhBMP-2 will be useful in the civilian population for repair of craniofacial bone, such as sinus lift and ridge augmentation procedures.)
- Project 4.5.7: Develop an injectable allograft biocomposite bone void filler for repair of metaphyseal bone defects.

Benefits over the Standard of Care and Other Competitive Technologies

There is currently no commercially available, injectable, weight-bearing bone graft for the delivery of recombinant growth factors, such as rhBMP-2. The drug delivery system that the research group is developing should meet this compelling clinical need. The advantages of this product are: (1) administration using minimally invasive surgical techniques, (2) mechanical strength approximating that of host mandibular bone (approximately 10 MPa) that provides space maintenance and prevents soft tissue prolapse, and (3) sustained release of rhBMP-2, which is anticipated to lower the required dose by a factor of 5–10. Considering that no product with all of these attributes is currently available, the research team expects that its drug delivery system will be a technology that positively impacts the clinical management of open fractures in the mandible.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the research team fabricated injectable, porous bone particle/polymer composites with tunable porosities, mechanical properties, and working times. The researchers showed that these materials could remodel in a rabbit distal femur model. They achieved sustained controlled release of the osteogenic agent from the bone particle/polymer composites for up to 21 days. They fabricated low-porosity, injectable bone/polyurethane (PUR) composite cements with high bone content that produced wet compressive strengths of up to 60 MPa more than five times stronger than the first-generation, high-porosity material. The researchers demonstrated that their bone particle/PUR composites passed the ISO 10993 systemic toxicity test. In a nonsurvival test, they injected the bone particle/PUR bone void filler into a rabbit calvarial defect and found the working and tack-free times to be comparable to those observed in vitro.

During the second year of the project, the researchers identified the final formulation for the Plexur LV® injectable bone void filler. They also identified lead candidate weight-bearing composites for testing in the mandible. They showed that the Plexur LV allograft/PUR bone void filler incorporating rhBMP-2 regenerated bone in the rabbit calvarial model.

Research Progress – Year 3

In Project 4.5.1a, the research team developed injectable, synthetic biocomposites that deliver recombinant human growth factors (specifically rhBMP-2). These materials have mechanical properties similar to those of trabecular bone and will be evaluated in a rat CSD model in year 4.

In Project 4.5.7, the researchers developed an injectable allograft biocomposite bone void filler and putty with mechanical properties comparable to trabecular bone. When injected into critical-size calvarial defects in rabbits, the biocomposites supported cellular infiltration and new bone formation. Adding rhBMP-2 to the injectable allograft bone void filler led to an enhancement of new bone formation in 15 mm rabbit calvarial defects at 20% of the dose recommended for the absorbable collagen sponge. The research team also identified the optimum porosity of its injectable allograft biocomposites.

Key Research Accomplishments

- Developed injectable and putty synthetic biocomposites with mechanical properties comparable to or exceeding those of trabecular bone for use in delivery systems for recombinant human growth factors.
- Demonstrated that injectable and putty allograft-based bone void fillers remodeled and supported new bone formation in rabbit models.
- Demonstrated that rhBMP-2, when added to the injectable allograft bone void filler, enhanced new bone formation in 15 mm rabbit calvarial defects at 20% of the dose recommended for the absorbable collagen sponge.
- Identified the optimum porosity of the injectable allograft biocomposites.

Conclusions

The research team developed injectable and putty synthetic biocomposites with mechanical properties equal to or greater than those of trabecular bone. They demonstrated that injectable and putty allograft-based bone void fillers remodeled and supported new bone formation in rabbit models. Due to the limitations of the allograft biocomposites for delivery of recombinant human growth factors, the synthetic biocomposites will be used for growth factor delivery in year 4. Experiments in year 4 will focus on accelerating the rate of allograft resorption and slowing the rate of polymer degradation to improve the design so that implants will last longer in their future clinical application in human reconstructive surgery in both the military and civilian arenas.

Research Plans for the Next 2 Years

Project 4.5.1a

The research team's objective is to advance the technology from TRL 2 to TRL 4 by year 5. The research activities will focus on developing the formulations; testing their feasibility in a CSD, rat calvarial model to determine the correct dose; and validating the formulations in clinically relevant animal models.

Project 4.5.7

Using AFIRM and supplemental funding, the research team's objective is to advance the allograft injectable and putty biocomposites from TRL 4 to TRL 9 by year 5. The research activities will focus on revising the formulation, validating it in rabbit and sheep preclinical models, and conducting both cGMP manufacturing and ISO 10993 testing to prepare for a regulatory filing.

Planned Clinical Transitions

Vanderbilt University, USAISR, and Medtronic, Inc., are partnering to commercialize the injectable allograft bone void filler products by the end of year 5. The regulatory pathway will be a 510(k) device application. The injectable delivery system products are longer term and will require a Premarket Approval Application.

Corrections/Changes Planned for Year 4

The research team revised the Statement of Work (SOW) for year 4 in response to Medtronic, Inc.'s acquisition of Osteotech.

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex

Project 4.5.1b, RCCC

Team Leader(s): Jeffrey Hollinger, DDS, PhD (Carnegie Mellon University) and Aniq Darr, PhD (Rutgers University)

Project Team Members: Jinku Kim, PhD, Sean McBride, BS, and Pedro Alvarez, BS (Carnegie Mellon University); Hanshella Magno, Ophir Ortiz, PhD, Matthew Laughland, and Das Bolikal, PhD (Rutgers University); and Brett Runge, PhD and Mahrokh Dadsetan, PhD (Mayo Clinic)

Collaborator(s): Amit Vasanji, PhD, Rick Rozik, and Brett Hoover, MBA, MS (ImageIQ) and Pam Brown-Baer, PhD (USAISR)

Therapy: Bone regenerative therapies for the cranio-mandibulo-maxillofacial complex.

Deliverable(s): TyrPC scaffolds for bone regeneration, fabricated by salt leaching, and supplemented with either rhBMP-2, other growth peptides, or combinations of the two.

TRL Progress: 2009, TRL 2; 2010, TRL 3; 2011, TRL 4; Target, TRL 5

Key Accomplishments: The researchers added a CaP surface coating to TyrPC scaffolds with the goal of increasing bone formation. They identified appropriate quality assurance methods. They validated the in vivo biocompatibility and efficacy of the TyrPC+CaP-coated scaffolds to support bone regeneration. The researchers found that the addition of rhBMP-2 to the TyrPC+CaP scaffolds led to substantially increased bone formation in a rabbit calvarial CSD model in 6 weeks. They also found that TyrPC+CaP, either with or without rhBMP-2, promoted bone formation that was similar to, if not superior to, a clinically utilized bone void filler with rhBMP-2.

Key Words: Bone regeneration, cranio-mandibulo-maxillofacial, tyrosine-derived polycarbonates, BMP, B2A peptide

Introduction

In a review of approximately 28,000 patients injured in Afghanistan and Iraq over a period of roughly 2 years from late 2001 to early 2004, head and neck injuries ranked second to extremity injuries in incidence. Although the head and neck comprise only 12% of the body surface area, 64% of combat deaths are due to these injuries. Historically, head and neck injuries have encompassed between 16% and 21% of the total injuries in major U.S. conflicts (i.e., World War II, Korea, and Vietnam), and a recent survey of warfighters wounded in OIF/OEF from 2001 to 2007 showed that 26% of the trauma to military service members occurred in the CMF region. CMF injury is a serious problem not only in numbers but also in intensity because wounded warriors who have suffered traumatic brain injury or blindness are more severely handicapped and have more difficulty returning to active duty and productive life than those with extremity injuries. The clinical need for craniofacial bone regeneration is urgent, and more than 30,000 surgical procedures are performed each year to repair craniofacial bone defects in the United States.

Restoring the CMF anatomy's form and function with a regenerative implant will be a significant improvement over contemporary options. Current synthetic materials do not remodel, fail to integrate with host tissue, may become infected, and can require extensive, multiple revision surgeries. Contemporary polymeric or metallic devices

also lack the ability to deliver biological factors (e.g., antibiotics or growth factors) in a controlled fashion. Current treatment options do not regenerate tissues and produce less than satisfactory aesthetic and functional outcomes. Biomaterials, such as TyrPCs and poly(propylene fumarate), have the potential to become the foundation for bioresorbable CMF implants. The expected outcome will be tissue regeneration and restoration of form and function. The biomaterial implant design would incorporate precise calibration of the important parameters of scaffolding materials (i.e., degradation rate and physical properties).

The researchers hypothesize that scaffolds built from TyrPC containing minerals Ca, Mg, or Zn will not cause adverse host responses and will regenerate bone in rabbit CSD calvarial and radius models within weeks when combined with rhBMP-2. To test this hypothesis, the researchers fabricated and characterized porous 3-D TyrPC scaffolds. They found that bone regenerated in standardized CSD rabbit calvarial and radius models using micro-CT and histology/histomorphometry.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers prefabricated tyrosine-based copolymer scaffolds to fit into a rabbit calvarial CSD. Their preliminary results showed that tyrosine-based 3-D scaffolds induced significant osteogenic differentiation and mineralization of preosteoblasts compared to two-dimensional (2-D) tissue culture surfaces. In vitro cyto-compatibility and cell attachment data with the tyrosine-based scaffolds showed no cytotoxicity and robust cell attachment. The researchers initiated a study on the effect of ethylene oxide sterilization on molecular weight loss and began nuclear magnetic resonance analysis of ethylene oxide sterilized scaffolds. Gel permeation chromatography analyses of scaffolds that have been subjected to ethylene oxide sterilization and degassed showed approximately 35% loss in molecular weight. Finally, both injectable and implantable compositions based on two types of polyester copolymers were identified for craniofacial applications.

During the second year of the project, the researchers demonstrated the in vitro and in vivo biocompatibility of TyrPC and poly(ε-caprolactone fumarate) scaffolds and efficacy of the scaffolds containing rhBMP-2 for bone regeneration using the rabbit CSD calvarial model.

Research Progress – Year 3

The specific aims for this project in year 3 were to: (1) regenerate parietal bone in a rabbit CSD model, complete preclinical correlations, and test in 145 rabbits and (2) regenerate radius in a rabbit CSD model and test in 60 rabbits.

The Rutgers University team developed and optimized a solution precipitation method for surface coating TyrPC with osteoconductive minerals. The researchers created scaffold fabrication conditions that produced the desired mechanical and degradation properties. They identified quality assurance methods that can be used to evaluate batch-to-batch variations and reproducibility of scaffold fabrication and then fabricated and conducted quality control characterization of more than 400 sterile scaffolds that were provided to collaborators within project 4.5.1. Specifically, the researchers developed and fabricated second-generation synthetic bone grafts based on TyrPC coated with CaP (TyrPC+CaP) or a bone mineral mix of Ca, Mg, and Zn (10% w/w) for bone regeneration

in the rabbit calvarial CSD model (with 15 mm diameter) and radius CSD model (with 15 mm length ostectomy).

The Carnegie Mellon University group determined the in vivo biocompatibility and efficacy of these TyrPC-based scaffolds using standardized rabbit models. The researchers had implanted 85 scaffolds into rabbit calvarial defects and 20 scaffolds into rabbit radius defects as of May 2011. Notably, the TyrPC+CaP scaffolds significantly increased new bone formation in the rabbit CSD model as determined by micro-CT and histology/histomorphometry **(Figure III-12)**. At 6 weeks, the following outcomes were determined:

- The scaffolds containing rhBMP-2 had more trabecular bone volume than scaffolds without rhBMP-2;
- TyrPC+CaP with 50 µg rhBMP-2 had significantly more trabecular bone volume than the other groups; and

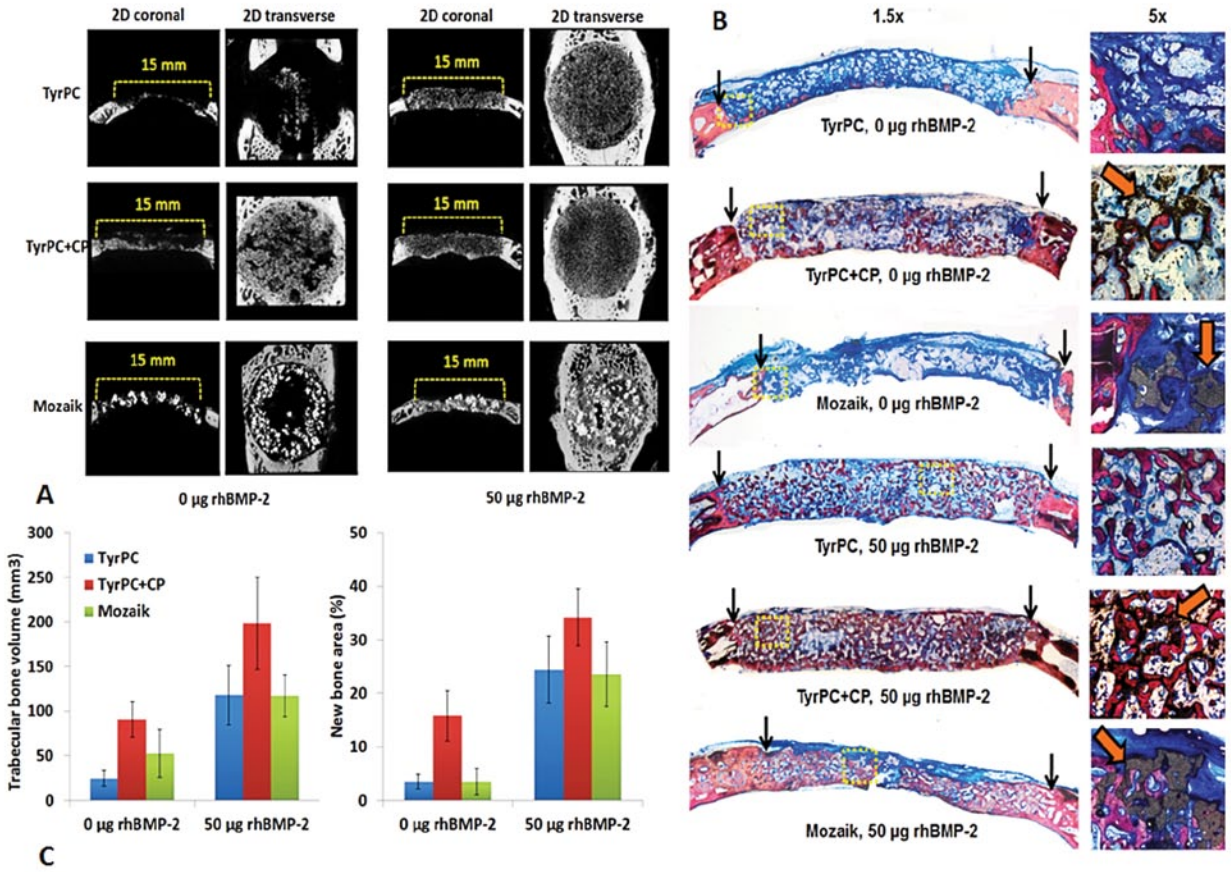


Figure III-12. (A) Representative micro-CT and (B) histology images of rabbit calvarial bone regeneration. Two-dimensional sections (coronal and transverse) of micro-CT calvarial specimens of each treatment showed that without rhBMP-2, Mozaik™ and TyrPC scaffolds barely induced new bone formation, whereas rhBMP-2-treated scaffolds (50 µg/scaffold) appeared to induce substantial bone regeneration at 6 weeks. Histology images confirmed micro-CT findings. Black arrows outline the defect site, and a substantial amount of remaining Mozaik and TyrPC+CaP scaffolds are visible (orange arrows). (C) Quantitative bone regeneration data as determined by the image analyses. These data are reported as a mean ± standard deviation for n = 4–6.

- TyrPC+CaP alone (without rhBMP-2) had more trabecular bone formation than TyrPC and promoted new bone formation that was similar to, if not superior to, a clinically utilized bone void filler treated with rhBMP-2.

The researchers have obtained Institutional Animal Care and Use Committee and Animal Care and Use Review Office approvals for their protocols for the rabbit radius model. Finally, the Hollinger group has begun working on a rabbit CSD radius model (30 rabbits planned and 20 rabbits operated upon).

Key Research Accomplishments

- Validated the in vivo biocompatibility and efficacy of TyrPC scaffolds coated with CaP (TyrPC+CaP, second generation), with and without rhBMP-2, for bone regeneration using the rabbit calvarial CSD model.
- Completed the rabbit surgeries and necropsies for the studies to determine the amount of bone regeneration of TyrPC scaffolds (including TyrPC+CaP) with and without rhBMP-2 in a rabbit CSD calvarial model. At 6 weeks, the following outcomes were determined:
  - Scaffolds containing rhBMP-2 had more trabecular bone volume than scaffolds without rhBMP-2;
  - TyrPC+CaP with 50 µg rhBMP-2 had significantly more trabecular bone volume than the other groups; and
  - TyrPC+CaP alone (without rhBMP-2) had more trabecular bone formation than TyrPC and promoted new bone formation that was similar to, if not superior to, a clinically utilized bone void filler treated with rhBMP-2.

Conclusions

The researchers have optimized a method for coating the surface of TyrPC scaffolds through the solution precipitation of minerals both for CaP and a synthetic bone mineral mix. They found the surgical handling properties of TyrPC-based materials to be much better than the controls (Integra Mozaik or Chromos™), which were friable and particulate. At 6 weeks all implants in the rabbit CSD model appeared to be biocompatible. In addition, the scaffolds degraded in register with bone regeneration during the 6-week study period.

The exciting class of biomaterials, TyrPCs, may be used either alone—with precisely calibrated physical, chemical, and biological properties—or may be combined with rhBMP-2. They may provide a compelling synthetic biomimetic material for regenerating bone in challenging clinical conditions.

Research Plans for the Next 2 Years

The project's focus is to evaluate biomaterials that may support implants to restore form and function in the CMF region. All remaining surgeries will be finished by July 31, 2011, and the specimens will be subsequently harvested and analyzed by micro-CT and histology/ histomorphometry. The researchers also plan in year 4 to identify a "frozen" design composition of TyrPC scaffolds using the rabbit calvarial/radius models (and possibly a goat calvarial model). To further enhance bone regeneration, scaffolds will be augmented with a minimal amount of rhBMP-2, a proprietary peptide, or a blood component. A total of 120 rabbits and 30 goats will be used to identify a frozen design composition. If the researchers are successful with

the rabbit (and goat) models in year 4, they will complete an FDA filing (e.g., 510[k]) and will design and prepare for a Phase 1 clinical study protocol in year 5.

Planned Clinical Transitions

The researchers may deliver a bone regeneration therapy for a small Phase 1 clinical trial as early as year 4 and will prepare a regulatory strategy with both the industrial partner, Trident, and the Rutgers University-retained regulatory consultant. The regulatory strategy may include a 510(k), Humanitarian Device Exemption, and combination of IND/IDE.

Corrections/Changes Planned for Year 4

During the past year, the researchers have shown the potential of both TyrPC and TyrPC+CaP for bone regeneration therapy. They now plan to identify a frozen composition of TyrPC+CaP, which may contain a minimal amount of rhBMP-2, a proprietary peptide, or a blood component (e.g., bone marrow aspirate). They will test this composition using rabbit calvarial and radius models and goat CSD calvarial models.

our science for their healing

Vascular Tissue Engineering

Project 4.5.6, RCCC

Team Leader(s): Daniel G. Anderson, PhD and Robert S. Langer, ScD (MIT)

Project Team Members: Nathaniel Hwang, PhD (MIT)

Collaborator(s): Michael T. Longaker, MD, MBA (Stanford University)

Therapy: Development of bioactive scaffolds for vascular tissue engineering.

Deliverable(s): (1) Fabricate bioactive microparticles to induce vasculogenesis (growth of blood vessels), (2) engineer vascularized tissue constructs using novel surface modification, and (3) analyze the integration of vascularized bone constructs in vivo.

TRL Progress: 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Key Accomplishments: During the past year, the researchers optimized a scaffold system of enhanced vascular engraftment. They identified an optimal biomaterial for effective bone tissue engineering. They also optimized hVEGF-release microparticles for effective use in vasculogenesis.

Key Words: VEGF scaffolds, adipose-derived mesenchymal stem cells, hydroxyapatite, vasculogenesis, tissue engineering

Introduction

Serious wounds suffered by military service members during combat or by civilians who suffer trauma or disease may require large bone grafts to repair a patient's body functionally and cosmetically. Often there is not enough transplantable tissue in an individual's body to cover the defect area. Transplants from other human beings may require constant uptake of immune-suppressing drugs. Blood vessel recruitment into engineered tissue is the pivotal step toward the long-term engraftment and survival of engineered tissue in the clinical setting. Treatment of large, segmental defects would require large scaffolds, and the success of long-term engraftment of engineered tissues in humans will be highly dependent on anastomosis with surrounding host tissues (i.e., growth of a successful vascular network).

To address these needs, the Anderson/Langer group at MIT is developing a novel, biodegradable, and bioactive scaffold system that can differentiate progenitor cells to produce vascularized bone tissues. The aims of this project are to develop two products: (1) bioactive scaffolds for vascular tissues and (2) vascularized bone tissue constructs with stem cells. Currently, the researchers have utilized microparticle-based scaffold systems that can either control the release of or immobilize growth factors (e.g., hVEGF) to promote growth of a vascular network within the biodegradable scaffold system.

Few suitable cell types can be expanded sufficiently to generate functional bone tissues, and this problem hampers the clinical application of tissue-engineered bone constructs.

Previously, Dr. Longaker's group at Stanford University showed that ASCs could be isolated and grown in culture. The group demonstrated that these cells could differentiate into osteoblasts in vitro and could subsequently form bone tissues in preclinical animal experiments. Recent evidence indicates that supplementation of endothelial progenitor cells with mesenchymal stem cells (MSCs) can augment the osteogenic differentiation of the MSCs. Therefore, the MIT group proposes to utilize a coculture system and functionalized bioactive scaffolds to engineer vascularized bone grafts. The project team anticipates that a vascular network created in vitro, via functionalized scaffolds, will promote the integration and calcification of tissues in vivo.

Research Progress – Year 1

Currently, the MIT group has utilized microparticle-based growth factors in vitro, with controlled release and/or chemical immobilization, to promote growth of a vascular network within a biodegradable scaffold system. The researchers will further tailor this technology to improve bone and vascular network formation. To improve blood vessel networks, they have modified the surface of porous scaffolds with bioactive molecules such as hVEGF, which promotes blood vessel growth. The researchers hypothesized that hVEGF immobilization could enhance the angiogenic potential of "seeded" cells and allow host blood vessel recruitment into the scaffolds.

Toward the surface immobilization of bioactive molecules, the researchers have developed a novel method to immobilize various bioactive molecules on biodegradable scaffolds. They can achieve a polydopamine substrate coating and functionalization via a single procedure **(Figure III-13)**. It is known that dopamine can be self-polymerized under alkaline solution and that the polymerized-dopamine (polydopamine) acts as a surface-coating agent due to its surface-adherent property. In the research team's approach, polydopamine was used as a multitasking agent for surface coating. This platform technology allowed the researchers to introduce special functionality onto scaffold surfaces.

For the vascularized tissue engineering approach, scaffolds were immobilized with a bioactive component, such as hVEGF or hydroxyapatite. The researchers monitored the efficacy of the compounds in supporting and maintaining the isolated vascular bed and supporting MSC differentiation to produce large vascularized bone in vitro. The surface immobilization with hVEGF allowed increased osteogenic differentiation of stem cells and formation of blood vessels within the scaffolds.

Key Research Accomplishments

Regarding the development of platform technology for bioactive scaffolds for vascular tissue engineering, the researchers:

- Effectively utilized dopamine chemistry to modify biodegradable scaffolds with hVEGF.
- Developed a platform technology for surface modification on porous scaffolds.

Regarding the development of vascularized bone graft, the researchers:

- Demonstrated an osteogenic response of stem cells on hVEGF immobilized scaffolds.
- Observed an osteogenic response of stem cells on hydroxyapatite scaffolds.

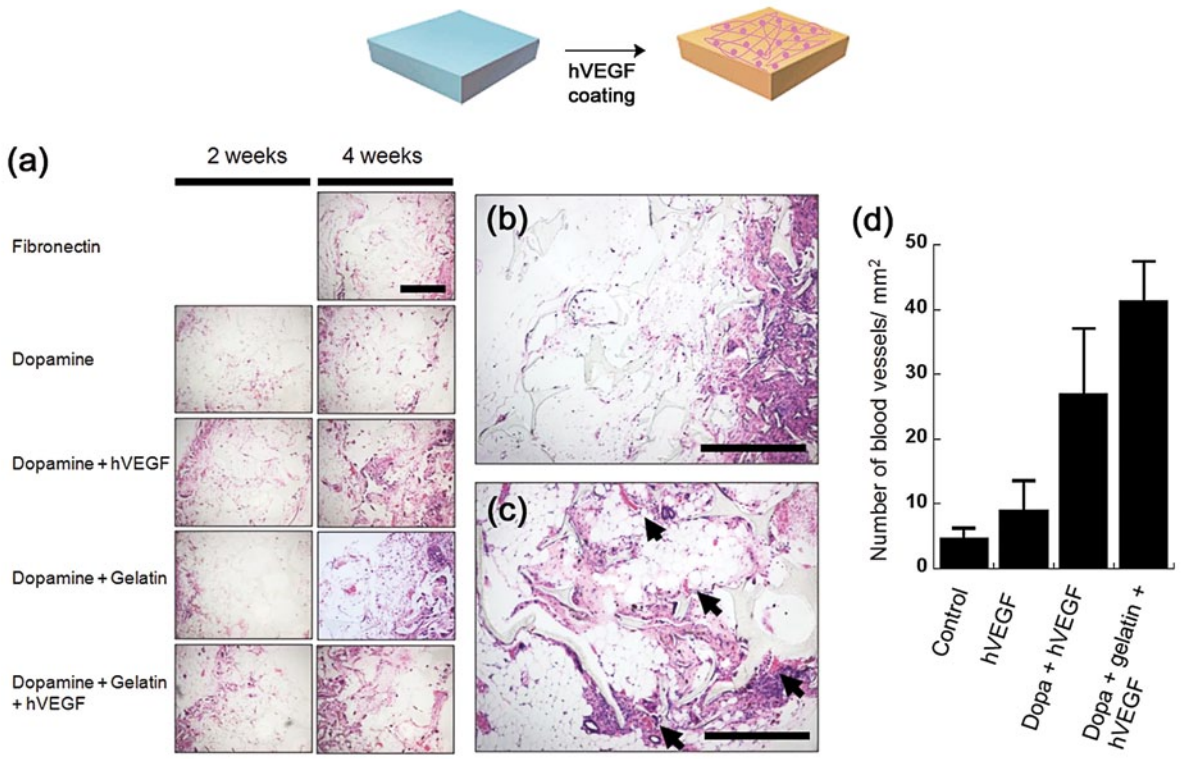


Figure III-13. Histological analysis of blood vessel recruitment and host cell migration into the hVEGF immobilized scaffold with polydopamine coating at 4 weeks. (a) hVEGF and/or gelatin were immobilized with polydopamine coating, resulting in significantly higher cellularity and blood vessel formation within the scaffold compared to the fibronectin coating and dopamine coating alone. (b) Dopamine-coated scaffolds resulted in minimal number of blood vessels in proximity to the scaffolds. (c) hVEGF immobilization with dopamine showed blood vessels in proximity to the bioactive scaffolds (arrows). (d) Quantification of blood vessel numbers within the scaffolds. Bar = 200 mm.

Conclusions

Successful treatment of large, segmental defects will require large scaffolds, and the success of long-term engraftment of engineered tissues in human patients will be highly dependent on anastomosis with surrounding host tissues (i.e., whether a successful vascular network develops around the graft). The MIT group's strategy is to create a bioactive proangiogenic microenvironment, which will promote a vascular network within the engineered tissues. Toward this end, the researchers have successfully demonstrated the bioactivity of scaffolds after simple immobilization with hVEGF. They will further develop this technology for a vascularized bone graft utilizing large animal models.

Research Plans for the Next 2 Years

In the next few years, the MIT group will perform extended small animal studies in collaboration with the Stanford University group. Long-term stability of the blood vessel network is a prerequisite for the success of engineered tissues; therefore, the researchers are planning to monitor the biological activity

of scaffolds that have incorporated hVEGF. Toward the goal of engineering vascularized bone grafts, the researchers are also incorporating hydroxyapatite scaffolds in conjunction with hVEGF for bone tissue engineering with stem cells derived from fat. They will also monitor whether any calcification or tissue resorption occurs in the animal models. They will further fine-tune the bioactive hVEGF scaffolds to try to achieve a low inflammatory response in immunocompetent animals.

Planned Clinical Transitions

The researchers are currently utilizing FDA-approved materials for scaffolding materials. However, dopamine coating and the use of hVEGF have not yet been approved for clinical trials. Toward the ultimate goal of using hVEGF scaffolds for vascularized tissue engineering in the clinical setting, the MIT group plans to perform appropriate large animal studies to confirm the biocompatibility and safety of this device. In addition, the MIT and Stanford University groups will prepare an IDE or IND for clinical application upon successful completion of large animal studies. Once the optimal composition of biomaterials has been identified in large animal studies, the researchers plan to work with Concordia Biomedical, a company with clinically approved biomaterials, which will provide a GMP facility for fabricating scaffolds for clinical trials.

Corrections/Changes Planned for Year 4

In the MIT group's prior annual report, the research team had proposed to complete animal studies in 2010. However, the team now hypothesizes that long-term, large animal studies are needed to demonstrate the efficacy of stem cell-based cartilaginous tissue formation on fully biodegradable scaffolds. Therefore, the MIT group has changed the time frame for initial clinical trials accordingly.

Accelerating the Development of Bone Regeneration Scaffolds by 510(k) Application to FDA for Tyrosine-Derived Polycarbonate Fracture Fixation Device

Project 4.5.8, RCCC

Team Leader(s): Carmine Iovine, MS (Rutgers University) and Deborah Schmalz (Trident Biomedical, Inc.)

Project Team Members: Das Bolikal, PhD, Aniq Darr, PhD, Matthew Laughland, Veena Bolikal, and Shaili Saini, MD (Rutgers University); Bob Marcus and Dietmar Hirt (Trident Biomedical, Inc.); and Howard Schrayer (Independent Contractor, Regulatory Advisor)

Collaborator(s): Joachim Kohn, PhD (Rutgers University)

Therapy: Small bone fracture repair.

Deliverable(s): 510(k) submission for a TyrPC bone fixation pin.

TRL Progress: 2010, TRL 4; 2011, TRL 4; Target, TRL 6

Key Accomplishments: The researchers developed and documented a robust synthesis process. They completed vendor selection for fabrication, sterilization, safety and toxicity, and efficacy testing. They identified an efficacy model, and they created a quality system to oversee development efforts. The researchers also finalized pin design as well as kit and packaging design.

Key Words: 510(k), bone pin, tyrosine-derived polycarbonate, fracture repair

Introduction

Unmet Need

There is a clinical need to improve orthopedic devices that are used to repair and regenerate bone specifically by making bone grafts osteoconductive (i.e., promoting the attachment and growth of bone at the bone-implant interface). Degradable biomaterials are used regularly in orthopedics for internal fixation of fractures, management of osteotomies (i.e., surgeries in which bone has been cut to foster healing), and in bone void fillers. All currently used small-bone fixation devices and degradable orthopedic implants are made of materials that are not osteoconductive and have yielded limited clinical results. The development of an osteoconductive polymer, one that is based on TyrPCs, which are both osteoconductive and tissue integrative, would significantly improve the clinical performance of degradable orthopedic devices for both the military and civilian populations.

Military Personnel

Extremity injuries account for the preponderance of combat-related injuries. In October 2011, Department of Defense statistics reported 46,800 injuries to service members in the current U.S. conflicts in Iraq and Afghanistan. Eighty-two percent of these

battlefield-injured soldiers suffered at least one extremity injury. Penetrating soft tissue wounds and open fractures accounted for many of these wounds.

Civilian Population
The orthopedic sector has paid little attention to the fact that more than 4 million patients report bunion problems annually. It is estimated that about 1 million of these patients are treated surgically each year. Civilians need bone repair and reconstruction also for defects that result from trauma or surgery to remove cancerous tumors or repair birth defects in both adults and children.

The RCCC Solution

Currently, two orthopedic products that may help the wounded warfighter are bone regeneration scaffolds for maxillofacial reconstruction and bone regeneration scaffolds for treating defects in long bones. Two products that serve these applications, based on a tyrosine-derived polymer, are moving toward commercialization within the AFIRM. Developing a successful and efficient FDA approval strategy is critical in advancing any of the AFIRM therapies toward clinical use in a timely manner. It is widely recognized that the process of gaining FDA market clearance for a fundamentally new (and therefore potentially high risk) device can be significantly longer and more costly than getting to the same end goal in a series of incremental steps. The risk of bringing polymer-based bone regeneration scaffolds to the FDA is that the FDA approval process may require years to come to completion. During this time, further development of the polymer technology as well as the commercial success of the company underwriting the product development remain on hold.

To address this risk, the approval process was separated into two steps. The first step aims to familiarize the FDA with the use of a tyrosine-derived polymer in an orthopedic implant. The subsequent FDA filing can then focus on the efficacy of the bone scaffold since the issues relating to the safety of the polymer used in the device will already have been largely addressed in the first step. The best way to conduct the first step is to select a relatively simple device for which there exists a successful "predicate" device. A synthetic, resorbable bone pin used in fracture fixation has been identified as this predicate device, and this project is focused on completion of the tasks required to submit the 510(k) application required to gain regulatory approval. While this resorbable bone pin is an intermediate step toward the overall goal of regulatory approval for the bone regeneration scaffolds, it offers an improved treatment option for extremity injury in both military and civilian populations.

A large benefit of this project is associated with the accelerated development of new small bone fixation devices and other degradable musculoskeletal implants that will directly impact the large number of military personnel who suffer small bone fractures and other musculoskeletal injuries (such as rotator cuff injuries). Such injuries are surprisingly common as part of training activities and accidents. Both injured service members and civilians will benefit from improved therapies for small bone fixation, soft tissue anchors used in the treatment of rotator cuff injuries, and degradable, small bone intramedullary rods used in the alignment of fractures in the hand and foot.

Research Progress – Year 1

The researchers have developed a series of biodegradable, tyrosine-derived polymers of which the polycarbonates are the most advanced. TyrPCs possess several advantages: (1) they have highly tunable degradation rates (ranging from days to years),

(2) they are biocompatible, and (3) they provide engineering properties appropriate for a broad range of medical applications. TyrPCs are ideal biomaterials for orthopedic implants, such as bone fixation devices and bone regeneration scaffolds. The portfolio of patents covering the use of TyrPCs has been licensed from Rutgers University to Trident Biomedical, Inc. (Trident), a newly founded technology company. Trident is dedicated to exploring the use of TyrPCs as orthopedic biomaterials.

The Kohn laboratory and Trident are collaborating on this project. This development project will result in a Premarket Notification (510[k]) Application filed with the FDA for clinically useful, degradable bone pins made of the same tyrosine-derived polymer that is currently being used in two different bone regeneration scaffolds that are part of the AFIRM preclinical research and development effort. These AFIRM projects are moving toward potential clinical trials in 2011. The successful filing of a 510(k) application requires the project team to establish a manufacturing facility for the tyrosine-derived polymer and to collect substantial documentation relating to the polymer chemical device and its characteristics—specifically its mechanical properties, biocompatibility, resorption mechanism and time frame, effects of sterilization, polymer safety, and device efficacy.

The development methodology for this project aims to provide sufficient evidence of the bone pin device's substantial equivalence to other pin devices in intended clinical use. Trident's overall work plan includes: (1) managing the process of optimizing polymer production, (2) managing the development to formalize the design of the pin and validating the production processes, (3) managing the development of the prototype production tooling, (4) managing the production of samples for evaluation according to specifications described during the development phase, and (5) contracting with vendors to carry out biocompatibility testing, in vivo functional testing, pathology, histomorphology, sterilization validation, sterility testing, monomer and polymer synthesis, packaging design and testing, and process validation.

To date, Trident has contracted with vendors that have begun work in the areas of synthesis, fabrication, packaging, and sterilization of the bone pin. These vendors have formalized the design of the pin and are in the process of validating the production processes, developing the prototype production tooling, and producing prototypes for evaluation against specifications. Additionally, Trident has completed kit design, packaging, and processes for sterilization.

Key Research Accomplishments

- Developed and documented a robust synthesis process.
- Selected vendors for prototype fabrication, terminal sterilization, shelf life, safety and toxicity, and efficacy studies.
- Completed development of an efficacy model.
- Created a quality system to oversee development efforts.
- Finalized pin design.
- Finalized kit and packaging design.

Conclusions

As the commercial partner, Trident is pursuing the 510(k) regulatory filing described previously for a TyrPC bone pin that is made from the same polymer used in the TyrPC scaffold devices. Subsequently, following

the progression of AFIRM Projects 4.2.1 and 4.5.1, this project team will pursue 510(k) application(s) for first-generation bone void filler device(s). The team's submission to the FDA will contain reports of the results of material characterization studies, biocompatibility and efficacy studies in animals, and descriptions of manufacturing and processing procedures, including packaging and sterilization. Additional information to be submitted includes the results of shelf life/stability testing, proposed labeling and directions for use, and references to the history of FDA approval of "substantially equivalent" devices. The execution of the precise work plan required to complete this submission is under way, on schedule, and expected to be complete within 18 months.

Research Plans for the Next 2 Years

For the next 2 years, the team will complete the set of tasks to demonstrate substantial equivalence as required for a 510(k) submission. The project team will transition the polymer technology, specifically the synthesis and fabrication of the specific TyrPCs for orthopedic applications, to a commercial cGMP manufacturing site thus making available cGMP polymers for all ongoing orthopedic research and development efforts using TyrPCs within AFIRM. These tasks will continue to be managed under a quality system developed by vendors, consultants, and the Trident team.

Planned Clinical Transitions

When the 510(k) submission that is the focus of this project has been approved, all regulatory requirements relating to the tyrosine-derived polymer will have been addressed. Most importantly, this initial FDA clearance of a tyrosine-derived polymer bone pin will reduce the time required to bring the AFIRM bone scaffolds to patients by about 4 to 10 months.

Upon successful 510(k) approval, Trident plans to commence marketing the tyrosine bone pins and would use part of the revenue derived from the sale of the bone pin product to further underwrite the development costs of the bone regeneration scaffolds. In this way, the relatively straightforward and lower risk 510(k) Premarket Notification process could have significant advantages. Once FDA clearance is obtained for the bone pins, the following benefits could be derived:

- Trident could begin marketing a new line of synthetic, degradable bone fixation devices (pins) that would be a significant improvement over existing degradable bone pins. These bone pins, while not specifically targeted by the military as a high-priority product, would nevertheless have a positive impact on warfighters due to the large number of bone fractures sustained by military personnel during training and combat.
- Revenues generated from the sale of the Trident bone pins would help underwrite the development of the AFIRM bone regeneration scaffolds thereby reducing the investment of taxpayer money needed to bring a significant AFIRM therapy to the injured warfighter.
- The successful early clinical use of tyrosine-based bone pins would make orthopedic surgeons aware of the new technology and would facilitate the clinical adoption of the new bone regeneration scaffolds once they become available.

Soft Tissue Reconstruction/Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction

Project 4.1.4/4.1.5, WFPC

Team Leader(s): Peter Rubin, PhD and Kacey Marra, PhD (University of Pittsburgh), David Kaplan, PhD (Tufts University), and James Yoo, MD, PhD and Sang Jin Lee, PhD (Wake Forest University)

Project Team Members: Evangelia Bellas, BS and Bruce Paniliatis, PhD (Tufts University), Rachel Hoyer and Donna Ward, PhD (University of Pittsburgh), and Chang Mo Hwang, PhD, Weijie Xu, PhD, Sang-Hyug Park, PhD, and Young Min Ju, PhD (Wake Forest University)

Collaborator(s): Jeff Gimble (Louisiana State University, Pennington Research Center) and Stephen F. Badylak, MD, PhD, DVM (University of Pittsburgh)

Therapy: Long-term soft tissue restoration of traumatic defects with cell based-degradable scaffolds resulting in sustained shape and volume over time.

Deliverable(s): (1) Engineering of vascularized connective tissue and fat pad; (2) development of implantable and injectable vascularized soft tissue composed of connective tissue and fat; (3) demonstration of the applicability of using implantable and injectable soft tissue composites for limb, burn, and craniofacial applications in a large animal model; and (4) initiation of clinical testing of soft tissue replacement for small defects.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 3; End Year 2, TRL 4; End Year 3, TRL 4

Key Accomplishments: In the past year, the research team conducted numerous preclinical studies using silk and human adipose-derived stem cells (hASCs). In a long-term in vivo silk scaffold study, silk scaffolds, alone or seeded with hASCs or lipoaspirate, maintained their shape and size up to 6 months post-implantation. More blood vessel ingrowth was seen in all groups compared to that seen at 3 months. Though volume retention was just 38% in the hASC-seeded group and 29% in the lipoaspirate-seeded group at 1 year after implantation (compared to 72% volume retention in the unseeded group), greater amounts of new fat could be seen encasing both seeded groups.

Key Words: Adipose tissue, adipose-derived stem cells, lipoaspirate, regeneration, silk scaffold

Introduction

The current standard of care for fat deposit regeneration has relied on three approaches: (1) surgical flaps that move adipose tissue from one site to another while maintaining an intact blood supply; however, these techniques are associated with medical risks, high costs, scarring, and functional loss; (2) artificial fillers, such as Teflon® paste, silicone implants, and bovine collagen, that lack any metabolic activity; and (3) free fat transplants that involve the implantation of autologous adipose tissue fragments without an intact blood supply. Often, the free fat transplants lose volume over time, and this is attributed to traumatic rupture, avascular necrosis, apoptosis of the adipocytes, inflammation secondary to cell death, fibrosis and contraction of the graft, and/or delipidation of the adipocytes with subsequent volume loss.

The restoration of traumatic soft tissue defects must start with a strategy that will restore tissue size and shape to near normal dimensions. Furthermore, this goal must be addressed with a strategy that will provide sustained retention of such improvements for at least 1 year while the body gradually remodels and regenerates the site into tissue with semi-normal or normal structure and function.

Nondegradable scaffold systems have been used to provide rapid restoration of morphological features; however, these often fail to integrate and regenerate native tissue thus remaining a barrier to tissue function. The use of degradable scaffold systems based on collagen or PLGA has provided some benefits, but many fail within about 3 months due to premature degradation and subsequent loss of transport, leading to necrosis and collapse of the soft tissue. Thus, silk biomaterials have been employed for soft tissue reconstruction because of their tunable degradation rates, their ability to be processed into various formats (e.g., sponges and gels), their ability to function well in water for facile delivery of bioactive components, and their robust mechanical properties.

The specific aims of this study are as follows.

Aim 1: Biomaterial-Based Scaffolds for Adipose Tissue Regeneration – This first deliverable will be an injectable scaffold designed to provide sustained morphology and structure for at least 1 year while supporting cellular and vascular ingrowth to restore functional tissue.

1.1 Development of an injectable sustainable silk protein biomaterial scaffold that can be combined with lipoaspirate to serve as a template for soft tissue regeneration.

Aim 2: Cell-Based Scaffolds for Adipose Tissue Regeneration – The second-generation scaffolds will include adipose stem/progenitor cells (hASC, with or without transfection with VEGF/fibroblast growth factor genes) in combination with the scaffolds from Aim 1 to accelerate adipose tissue regeneration.

2.1 Development of quantitative analytical methods for cell characterization.

2.2 Development of an implantable sustainable silk protein biomaterial scaffold that can be cultured with hASCs, adipocytes, or lipoaspirate to serve as a template for soft tissue regeneration.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers developed four types of silk fibroin scaffolds and demonstrated that each scaffold could support the growth of soft

tissue. They developed a collagen gel delivery system that releases VEGF with the goal of enhancing vasculogenesis in vivo. They implanted cell–hydrogel constructs in athymic mice and began to examine the dimensional changes in the mice at various time points post-implantation. They also developed polymeric microspheres containing proteins known to promote angiogenesis; these microspheres are expected to enhance tissue formation by the cell–hydrogel constructs.

During the second year of the project, novel quality assurance/quality control markers for flow cytometry analysis were explored. Coculture systems containing silk scaffolds and adipocytes were scaled up from 2-week to 6-month static and dynamic cultures. Functional outcomes (i.e., glycerol release and leptin release) and adipogenic transcript levels (i.e., GLUT4 and PPAR gamma) were seen in these cultures, as in natural adipose tissue, for at least 6 months in vitro. In addition, hallmarks of adipose tissue development were seen in the cultures using histological techniques. The cell/scaffold constructs in dynamic culture increased in diameter by 1 mm during this culture period. Importantly, immunohistochemistry for CD31 (an endothelial cell surface marker) indicated that vascular cells were present in these cultures and had organized into vessel-like structures over the 6-month culture period. Long-term in vivo studies using silk sponges (sponges alone and sponges seeded with hASCs or lipoaspirate) commenced during the past year. The research group also successfully combined silk-based scaffolds and cellular elements (both mature cells and progenitors) to form stable injectable and implantable soft tissue constructs in a small animal model.

Research Progress – Year 3

Target Product Profiles

The researchers have focused on three products: silk scaffolds, lipoaspirate scaffolds, and polymeric scaffolds. They are examining all of these products in preclinical studies with and without hASCs.

Major Tasks and Milestones

Novel quality assurance/quality control markers for use in flow cytometry were investigated for improving the analysis of freshly isolated hASCs, cultured hASCs, and hASCs differentiated into adipocytes. Data from the new markers tested—Pref-1, Glut-4, and AdipoRed—were not conclusive. However, CD34, a marker for the hematopoietic lineage, was present (74% positive) in freshly isolated cells and was also present at lower levels in cultured hASCs after several passages. Large numbers of CD34-positive cells were also seen when the hASCs were differentiated into adipocytes over several passages.

In Vivo Silk Scaffold Study

A long-term in vivo study using silk scaffolds, either alone or seeded with in vitro cultured hASCs or fresh lipoaspirate, is ongoing **(Figure III-14)**. This study is being performed in a nude rat subcutaneous pocket. Porous silk scaffolds contain pores that are 500–600 μm in size. Autoclaved scaffolds were implanted bare or coated with laminin to increase initial cellular adhesion. The hASC-seeded scaffolds were cultured ex vivo with ASCs for 1 month in adipogenic media. To seed scaffolds with lipoaspirate, the scaffolds were immersed in freshly harvested lipoaspirate for 1 hour prior to implantation. The residual lipids and blood were removed by centrifugation from the lipoaspirate before the scaffolds were immersed in it. The lipoaspirate was readily absorbed by the silk scaffold

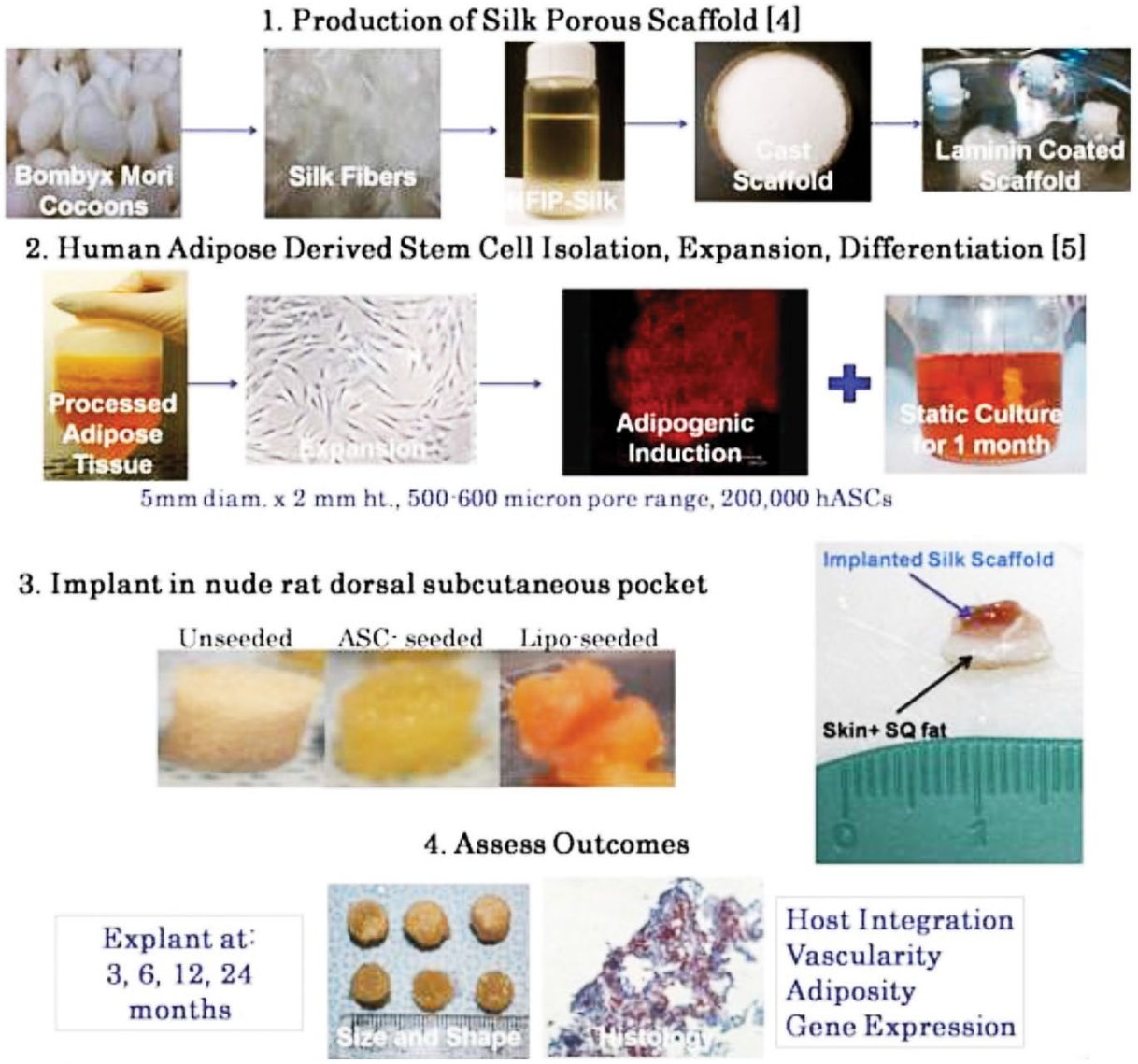


Figure III-14. Experimental outline for the 1-year in vivo study in the rat subcutaneous model.

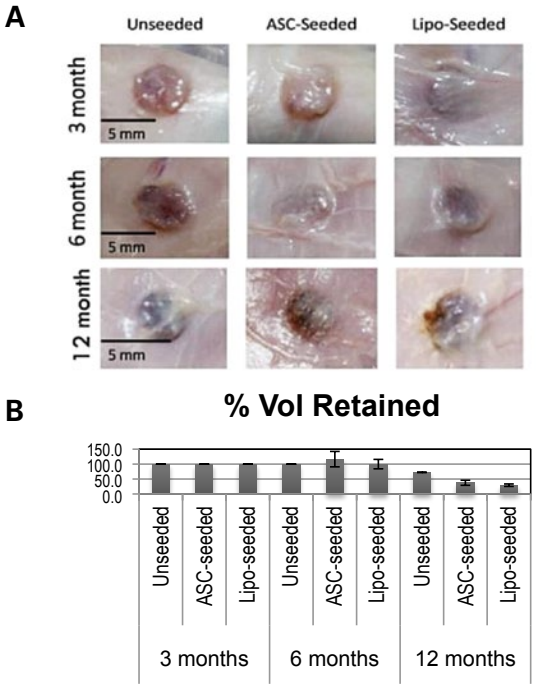


Figure III-15. (A) Macroscopic images of explanted subcutaneous silk scaffolds at 3, 6, and 9 months. Note: implants are magnified to the same size to show explant details. (B) Percentage volume retention of explanted subcutaneous silk scaffolds at 3, 6, and 12 months.

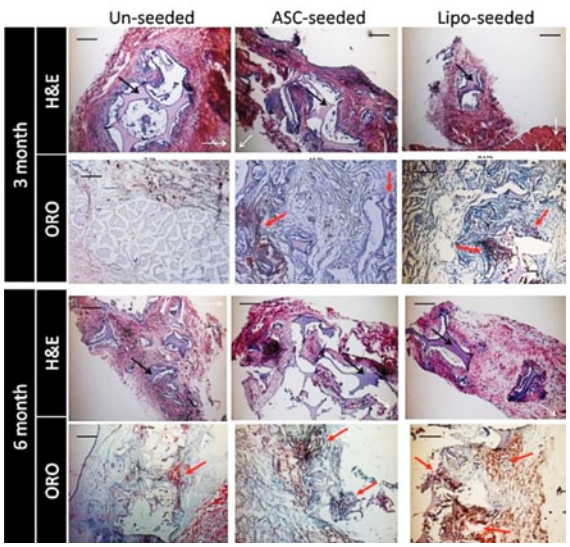


Figure III-16. Representative histological hematoxylin and eosin (H&E) and Oil Red O images at 3 and 6 months of explanted silk scaffolds. Scale bar – 200 μm. Black arrows – silk scaffold. Red arrows – Oil Red O-positive areas. White arrows – direction of skin for orientation.

and immediately filled every pore (step 3 in Figure III-14).

At 3 and 6 months after implantation in vivo, no degradation or loss of volume was seen in any group **(Figure III-15A)**. By 1 year, some degradation had begun while the aspect ratio was maintained (Figure III-15A, III-15B).

At 3 months, both study groups in which seeded scaffolds were used maintained cellularity while in the unseeded group many of the scaffold pores had not yet been infiltrated by host cells **(Figure III-16)**. Additionally, no Oil Red O staining (which stains lipids in mature adipocytes) was present in or at the periphery of the unseeded scaffold group, but red staining was seen in both seeded groups. At 6 months, these trends were no longer evident as high cellularity and Oil Red O staining could both be seen in the unseeded group as well as the two seeded groups. At the time of this report, the histological analysis for the 1 year time point was not yet complete.

At 6 months after implantation, more blood vessel ingrowth was seen in all groups as compared to that seen at 3 months. Quantification of the number of vessels per mm^2 is ongoing. These vessels appear mature and functional as red blood cells can be detected within them. This delayed vessel ingrowth at 6 months after implantation is of interest given that most grafted materials lose most of their volume by 6 months. Some macrophages and giant cells were present likely reflecting the scaffold remodeling process.

At 1 year after implantation, blood vessels in the underlying muscle (along the rib cage) were observed to be directly feeding into the lipoaspirate-seeded scaffolds **(Figure III-17)**. This was not seen in either of the other groups or at any other time point studied thus far. However, in all groups and at all time

points, blood vessels were observed leading into the scaffolds from the subcutaneous area (Figure III-15A).

Currently, the researchers are also developing a muscle construct from hASCs to enable the building of more complex tissues and to assess adipose integration with other soft tissues. Preliminary studies are being conducted to optimize media conditions for myogenic differentiation of the hASCs.

In Vivo Silk Gel Studies

An injectable silk scaffold for soft tissue reconstruction has been fabricated and tested in vivo in an athymic mouse model to study volume maintenance and development of vascularity over time. These studies have provided the framework for second-generation scaffolds to be developed by allowing optimization of the protein concentration of the silk gel and the identification of an appropriate stem cell density. Specifically, a 4% silk gel was combined with lipoaspirate at various ratios (0:1, 1:0, 1:1, 3:1, and 1:3). Some of the gel-lipoaspirate constructs were also seeded with various numbers of green fluorescent protein-labeled ASCs (0, 1, 2, or 4 million cells/mL). These mixtures were injected bilaterally into the dorsal region of athymic nude mice at 1 mL per injection. Mice were sacrificed at 3 or 6 weeks after implantation. At each time point, the graft explants were assessed in terms of mass, volume, and histology. This work revealed that over time a decrease in graft volume occurred with increasing volumes of silk and increasing numbers of seeded ASCs.

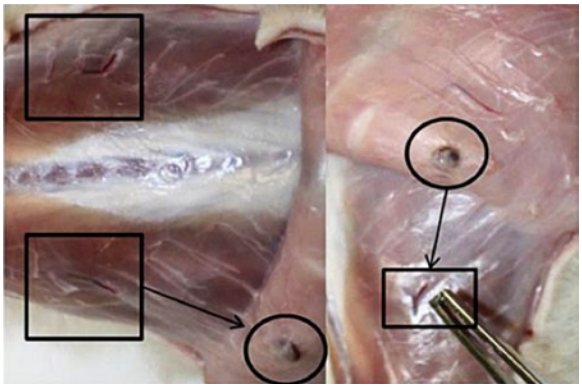


Figure III-17. Vessel (boxed) from underlying muscle feeds lipo-seeded silk scaffold (circled).

In Vitro Silk Gel Studies

Based on the in vivo studies using 4% silk, various percentages of silk were assessed in vitro to determine a more optimal formulation for graft retention in vivo. Specifically, 4%, 8%, and 12% silk solutions were made and combined with ASCs at 1 million cells/mL

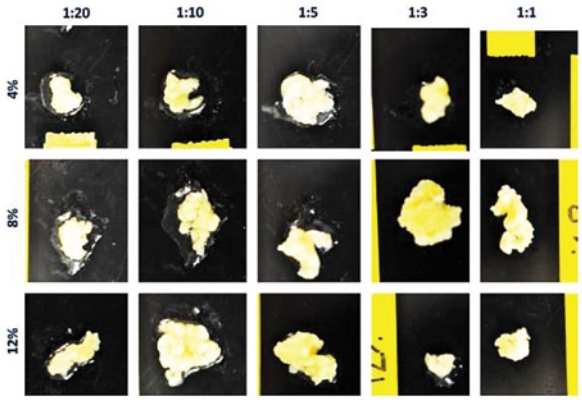


Figure III-18. Varying protein concentrations of silk solution at varying ratios of silk to lipoaspirate.

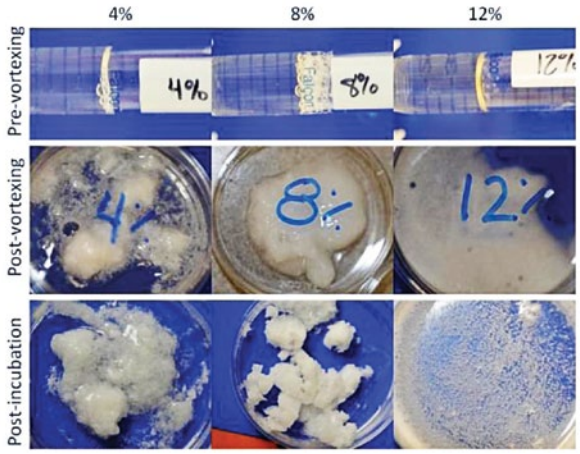


Figure III-19. Varying protein concentrations of silk solution following gelation.

or combined with lipoaspirate at various ratios (1:20, 1:10, 1:5, 1:3, or 1:1) **(Figure III-18)**. Gels with ASCs were assessed for live and dead cells and cellular distribution while those mixed with lipoaspirate were assessed for handleability, homogeneity, and maintenance of shape. Results indicated that 8% gels mixed at 1:1, 1:3, and 1:5 ratios of gel:lipoaspirate provided the most desirable characteristics **(Figure III-19)**.

Previously, the researchers developed a porous hydrogel system using gelatin microspheres, which resulted in a hydrogel with many large pores. Fibrin hydrogels containing gelatin microspheres were significantly more porous than the fibrin hydrogels without the microspheres, and when these porous hydrogels were implanted in vivo, they resulted in greater amounts of collagen deposition. These findings indicate that gel porosity likely improves new tissue formation and facilitates integration with the host tissue. However, the use of gelatin microspheres to make the pore structures resulted in limited interconnectivity between pores. Thus, the research team developed a novel porous hydrogel system using gelatin microfibers. A micro-channeled structure in the hydrogel was generated after dissolving these gelatin microfibers. They showed that dermal fibroblasts proliferated and spread in these microchannels, and this configuration resulted in higher cell survival over time. To evaluate cell–hydrogel constructions in vivo, they used a mouse subcutaneous model. Briefly, cell–hydrogel constructs containing fibroblasts were injected into the subcutaneous space of athymic mice. The constructs were retrieved at 1, 2, and 4 weeks after implantation for analyses. The retrieved cell–hydrogel constructs were characterized by evaluating dimensional changes, histology, cell survival, vascularization, and the levels of ECM production within the construct.

Gelatin microfibers mixed with the hydrogel solutions could be dissolved, resulting in a significantly more porous structure than the fibrin hydrogel only group. The gelatin microfiber-containing hydrogels resulted in better cell proliferation and ECM production in vivo than the nonporous hydrogels **(Figure III-20)**. These findings indicate that interconnected pores within a hydrogel improve new tissue formation and facilitate integration with the host tissue.

Fabrication of Microvessel-Like Structures in Hydrogels Prior to Implantation

Injectable hydrogels containing encapsulated cells have an advantage in terms of tissue formation in vivo after injection. However, cell proliferation and tissue formation in injectable hydrogel systems are still limited when the regeneration of large, thick tissue is involved. Recently, the research team has developed a microchanneled structure in a hydrogel. Cell-encapsulated microfibers can be fabricated using gelatin without chemical cross-linking, and these structures are physically dissolvable under physiologic temperatures. Endothelial cells were encapsulated in gelatin microfibers in this manner, and these microfibers were mixed into a fibrin hydrogel to form microvessel-like structures in vitro. The gelatin microfibers dissolved after 1 day of culture, and this resulted in the formation of endothelial cell-covered microchannels in vitro. These endothelial cell-lined channels in the hydrogel showed microvessel-like lumen formation at 1 week in culture. Further culture in vitro showed that these microchannels expanded with time due to dissociation of the fibrin matrix by an endothelial cell-driven mechanism **(Figure III-21)**. When pericytes were added to the gelatin microfibers, the researchers found that the resulting microchannels were more stable, and this did not change microvessel size significantly.

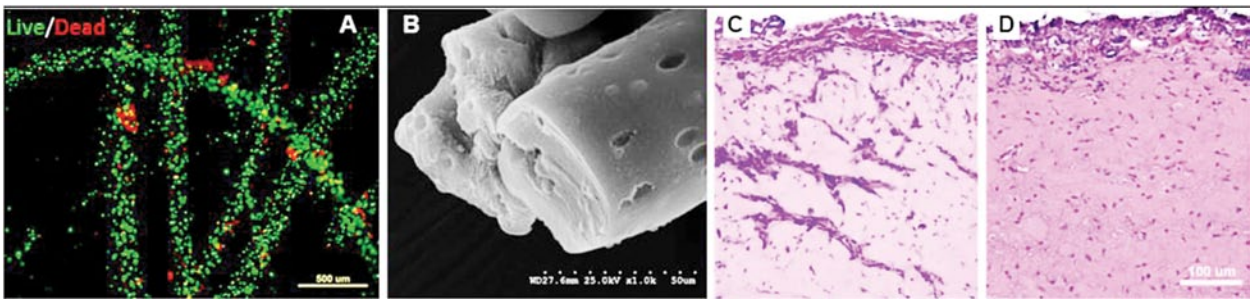


Figure III-20. (A) Fluorescence image and (B) scanning electron microscope image of cell-encapsulating gelatin microfibers. In vivo result showing cell proliferation and ECM production in the hydrogels: (C) microfiber-contained and (D) control hydrogels.

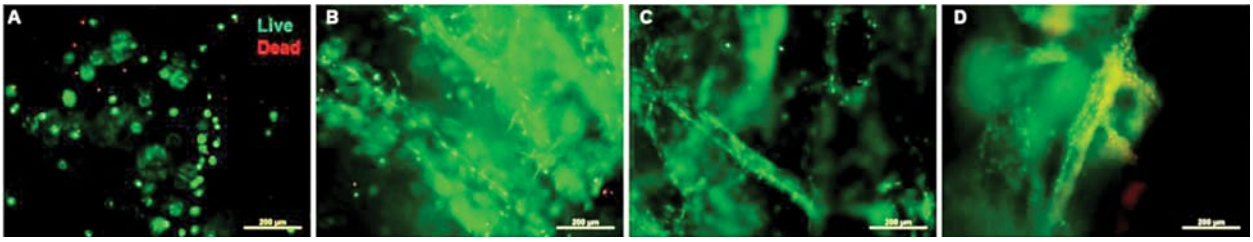


Figure III-21. Microvessel-like structure formed in vitro after (A) 1 day, (B) 3 days, (C) 1 week, and (D) 5 weeks in culture of endothelial cell-encapsulating microfibers in fibrin hydrogels stained with calcein AM (green, live cell) and ethidium homodimer (red, dead nuclei).

Porous fibrin hydrogels produced using dissolvable gelatin microfibers allow higher cell proliferation and increased cell spreading within the microchannels of the hydrogel. This resulted in improved tissue formation in vivo and will be important for soft tissue reconstruction following craniofacial injury.

Key Research Accomplishments

- In the long-term in vivo silk scaffold study, found that silk scaffolds (alone, with hASCs, and with lipoaspirate) maintained their shape and size up to 6 months after implantation (100% volume retention).
  - At 6 months, observed increased blood vessel ingrowth and new fat formation in even the unseeded scaffolds as shown by positive Oil Red O staining.
  - At 1 year, discovered that volume retention was 72% in the unseeded group, 38% in the ASC-seeded group, and 29% in the lipoaspirate-seeded group. However, greater amounts of new fat could be seen encasing both seeded groups.
  - Observed that host integration increased with cell seeding.
  - Observed no negative biological responses over the long term.
- Demonstrated long-term survival and integration of a silk sponge in a rat model.
- Determined that silk gels with and without ASCs have potential in soft tissue repair.
- Found that development of a more porous structure enhanced cell proliferation and ECM production in the hydrogels.
- Determined that fabrication of microvessel-like structures in the hydrogel using vascular cells encapsulated in gelatin microfibers can be performed prior to implantation and may increase vascularization of the construct in vivo.

Conclusions

This project has demonstrated the feasibility of using stem cell-seeded silk scaffolds for long-term soft tissue restoration. Silk scaffolds, either alone, with hASCs, or with lipoaspirate, maintained their shape and size up to 6 months after implantation. Though volume retention was just 38% in the hASC-seeded group and 29% in the lipoaspirate-seeded group at 1 year post-implantation (compared to 72% in the unseeded group), greater amounts of new fat could be seen encasing both seeded groups. A clinical study has been initiated in which 14 subjects have undergone lipoaspirate as a fat graft, and a stem cell-seeded fat graft clinical trial is awaiting regulatory approval and is under review by the FDA.

Research Plans for the Next 2 Years

The research group will continue to optimize its coculture systems and silk scaffold constructs both in vitro and in vivo. Studies to develop dynamic culture systems to improve cellular ingrowth and vascularity of the construct are ongoing. Immediate future plans include improving the vascularity of the constructs by transfecting the stem cells used to create microvessels with angiogenic factors such as VEGF and/or fibroblast growth factor.

In vivo studies using cocultured constructs and lipoaspirate-infused silk scaffolds are still in progress and will be carried out for up to 2 years in a mouse model. Success will be measured in terms of sustained morphology

of the construct and the level of integration of the engineered tissue with the host tissue.

Studies designed to enhance translation of this technology to the clinic include development of cell culture systems that do not include the use of xeno factors, such as fetal bovine serum, and development of a method in which ASCs could be differentiated into both adipocytes and endothelial cells so that a single (and autologous) source of cells would be sufficient for all parts of the process.

The researchers plan to continue development of silk injectables (i.e., hydrogel, sponges, and foams) over the next 2 years.

Small animal studies are under way in which various formats are being tested in physiologically relevant models (i.e., subcutaneous, submuscular, and intramuscular sites). Options for development of soft tissue defect models will be assessed. Silk sponge implants will be tested in a large animal model study.

Planned Clinical Transitions

Initial clinical trials are under way at the University of Pittsburgh. Discussions have been initiated on additional large animal trials and possible clinical trials with Allergan, Inc.

Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle

Project 4.1.6, WFPC

Team Leader(s): George Christ, PhD (WFIRM)

Project Team Members: James Yoo, MD, PhD, Sang Jin Lee, PhD, Benjamin T. Corona, PhD, and Masood A. Machingal (WFIRM)

Collaborator(s): Tom Walters, PhD (USAISR) and David Kaplan, PhD (Tufts University)

Therapy: Autologous bioengineered skeletal muscle implant for functional reconstruction/repair of complex craniofacial injuries.

Deliverable(s): An implantable tissue-engineered muscle repair (TEMR) construct capable of restoring clinically relevant force/tension following a VML injury.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 3; End Year 3, TRL 4

Key Accomplishments: The researchers completed and published a seminal study on a murine model of surgically created trauma (i.e., VML) in which 50% of the latissimus dorsi (LD) muscle was removed, and within 2 months of implantation of a TEMR, the retrieved LD muscle containing the tissue-engineered construct was capable of generating nearly 75% of the maximal contractile response observed in native LD muscle from the same animal. In addition, the researchers developed a proof-of-concept rodent tibialis anterior (TA) VML injury model. Once again, implantation of the TEMR construct led to robust functional recovery of dorsiflexion of the foot following stimulation of the peroneal nerve in vivo, which documents functional reinnervation of the leg implanted with the TEMR construct. This observation stands in stark contrast to findings with retrieved LD muscle or stimulated TA muscle that contained only the scaffold or was unrepaired.

Key Words: Tissue engineering, skeletal muscle, bioreactors, muscle precursor cells, biomaterials

Introduction

Current management of injuries resulting in VML in terms of tissue coverage and augmentation involves the use of existing host tissue to construct muscular flaps or grafts. In many instances, this approach is not feasible, delaying the rehabilitation process as well as restoration of tissue function. In fact, the inability to engineer clinically relevant functional muscle tissues remains a major hurdle to the successful skeletal muscle reconstructive procedures required to repair the complex facial injuries suffered by warfighters.

The long-term goal of this project is to create a skeletal muscle tissue implant capable of generating clinically relevant force/tension. Engineering skeletal muscle tissues de novo with the patient's own cells would accelerate wound healing with cosmetic augmentation of the tissue defect and, thus, enhance restoration of tissue function. This project will continue the development of a technology to further probe the feasibility and applicability of creating contractile skeletal muscle tissues using a bioreactor system in conjunction with novel biomaterials/scaffolds and optimized bioreactor protocols. The overall goal is to utilize this technology in injured soldiers to assist with rehabilitation and restoration of skeletal muscle function. The initial clinical application will be the repair and restoration of craniofacial battlefield wounds.

The specific aims of this project are to (1) demonstrate proof of concept for engineering functional (i.e., contractile) skeletal

muscle tissue for craniofacial defects, (2) conduct a feasibility study involving the implantation of engineered skeletal muscle in a rat skeletal muscle replacement model, (3) conduct an applicability study involving the implantation of engineered skeletal muscle in a large animal (dog) model of craniofacial defects, and (4) determine the feasibility of using biopsies from human patients for the engineering of functional skeletal muscle.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers focused on the first specific aim. As such, they generated a set of standard operating procedures (SOPs) for (1) isolation of rat muscle precursor cells, (2) bladder acellular matrix (BAM) scaffold generation, (3) scaffold seeding and culture conditions, and (4) bioreactor preconditioning conditions. All of these methodologies required standardization to create a reliable and reproducible skeletal muscle tissue-engineered construct. Using these standardized procedures, they successfully generated an organized muscle tissue in vitro (Task 1.1). Additionally, they optimized the scaffold and bioreactor preconditioning in comparison to their previously reported methods for engineering contractile skeletal muscle (Task 1.2). Last, they began to develop a mouse LD model of VML. They established a surgically created LD muscle defect in which tissue-engineered skeletal muscle constructs may be implanted to replace up to 50% of excised LD muscle. The establishment of this LD defect model enabled the experiments proposed for the current year (this report) to characterize engineered muscle tissue following in vivo implantation (Task 1.3).

During the second year of this project, the researchers focused on characterizing their first-generation TEMR constructs to restore tissue function in a rodent LD model of VML (Task 1.3). The results of these studies indicated that 2 months after implantation, TEMR constructs promoted functional recovery to 70%–75% of the force generated by uninjured muscle while untreated, VML-injured LD muscle produced only approximately 50% of uninjured force values. The functional recovery observed 2 months post-injury required a cellular component as the implantation of a BAM scaffold alone did not support functional recovery. Histological examination of VML-injured LD muscles treated with TEMR constructs revealed the presence of myotubes and mature muscle fibers within the implanted scaffolding material as well as vascular and neural structures required to support functional tissue formation. The research team has recently published an in-depth description of these findings.

Research Progress – Year 3

During the past year, the researchers primarily focused on performing preclinical studies in a rat model of VML injury. They also explored the use of commercially available scaffold materials as possible alternatives to the currently used BAM scaffold since an FDA-approved, commercially available scaffold material would accelerate the regulatory path. As such, suitable FDA-approved, sheet-like acellular scaffolds, which are similar to the currently used BAM scaffold, are being tested for functional bioequivalence. Moreover, in preparation for a second-generation technology, the researchers are exploring the utility of a novel 3-D silk scaffold made by the Kaplan team at Tufts University. The rationale is that these scaffolds provide the advantage of tunable mechanical properties (degradation kinetics and porosity) and spatial dimensions that may better approximate the nature, form, and volume of VML

injury wounds. Last, the researchers have collaborated with the WFIRM GMP facility to lay down the framework for the translational manufacturing process of this technology in human cells. In this coordinated work, feasibility studies of human muscle progenitor cell (MPC) expansion have been conducted.

Tissue Engineering Model Overview

To address the functional debilitation imparted by VML injury in craniofacial muscles, the researchers have developed a skeletal muscle tissue engineering model. In this model, MPCs derived from whole skeletal muscles are prompted to proliferate in culture. These proliferating cells are seeded on an acellular scaffold material and then subjected to standard serum starvation to induce differentiation and allow the cells to fuse together forming myotubes. Once myotubes form, the constructs are placed in a bioreactor that exerts uniaxial strain on the construct. This final bioreactor preconditioning stage prompts cellular alignment along the axis of strain. Following this culture and preconditioning process, aligned constructs composed primarily of multinucleated myotubes are formed.

Completion and Publication of a First-Generation TEMR Construct in the Murine LD VML Injury Model

The research team completed its physiological and histological analyses of the LD VML injury model at the 1- and 2-month post-implantation time points and published this work in the journal *Tissue Engineering*. This seminal study documented approximately 72% recovery of an otherwise irrecoverable injury within 2 months of implantation of the TEMR technology and demonstrated remodeling of the TEMR construct as well as the presence of desmin-positive myofibers, blood vessels, and neurovascular bundles within the TEMR.

Rat TA Model Development

In collaboration with Thomas J. Walters, PhD, and his team at USAISR, an irrecoverable VML injury model in the rat TA muscle was created and optimized. Following the development of this model, the first-generation TEMR constructs were implanted at the site of VML injury in the left TA muscle of female Lewis rats, which have a competent immune system. Since the cells that comprise the TEMR construct are derived from male Lewis rats, the TEMR construct is a syngeneic treatment closely approximating the ideal autologous treatment of VML injury in humans.

When creating the rat TA VML injury model, three goals were set: (1) The VML injury must impart irrecoverable functional deficits, (2) functional assessment of the injured muscle must be reliably made via neural stimulation in vivo to unequivocally document the restoration of functional neuromuscular junctions, and (3) the functional assessment must be made in vivo and repeated in the same rat over a prolonged period of time (e.g., every 2 weeks for 2 months), permitting longitudinal evaluation of the recovery process without having to terminate the experiment.

With these goals in mind, the research team found that the anterior crural muscle unit is an exemplary model system. The anterior crural muscles reside on the anterior aspect of the lower leg in both humans and rodents (hindlimb) and function in dorsiflexion of the foot. The anterior crural muscle unit is composed of the TA muscle, the extensor digitorum longus (EDL) muscle, and the extensor hallicus longus (EHL) muscle. All three muscles are innervated by the common peroneal nerve, which is readily accessible for electrical stimulation via percutaneous needle electrodes. The primary muscle, by weight and function, of the anterior crural muscles is the TA muscle (i.e., approximately 80% wet weight) although the EDL (approximately

18% wet weight) and EHL (approximately 2% wet weight) muscles also contribute to dorsiflexion of the foot.

Surgical creation of VML injury in the TA muscle was performed as outlined in **Figure III-22**. An estimated 20% of the mass of the TA muscle is excised from the middle third of the superficial aspect of the muscle, which creates a wound bed with approximately 1 x 0.5 x 0.5 cm dimensions. Less than 2 weeks following surgical VML injury creation, as well as synergist muscle excision, rats ambulate normally, exhibiting the ability to bear full weight on the injured leg while standing to reach food and to dorsiflex the foot during walking. Functional assessment of the anterior crural muscles was performed by electrically stimulating the common peroneal nerve and measuring torque using a servomotor system (Figure III-22E). Using this method of functional assessment, reliable (i.e., less than 5% variability) torque measurements can be made in the same animal over a prolonged period of time (Figure III-22F). One technical difficulty is discrimination of torque elicited by the muscle of interest (TA muscle) and the other synergists (EDL and EHL muscles). This limitation was removed by ablating, or surgically excising, both the EDL an EHL muscles at the time of VML injury creation in the TA muscle. Thus, in vivo functional assessment, solely of the TA muscle, was performed in the same animal following ablation of the synergists.

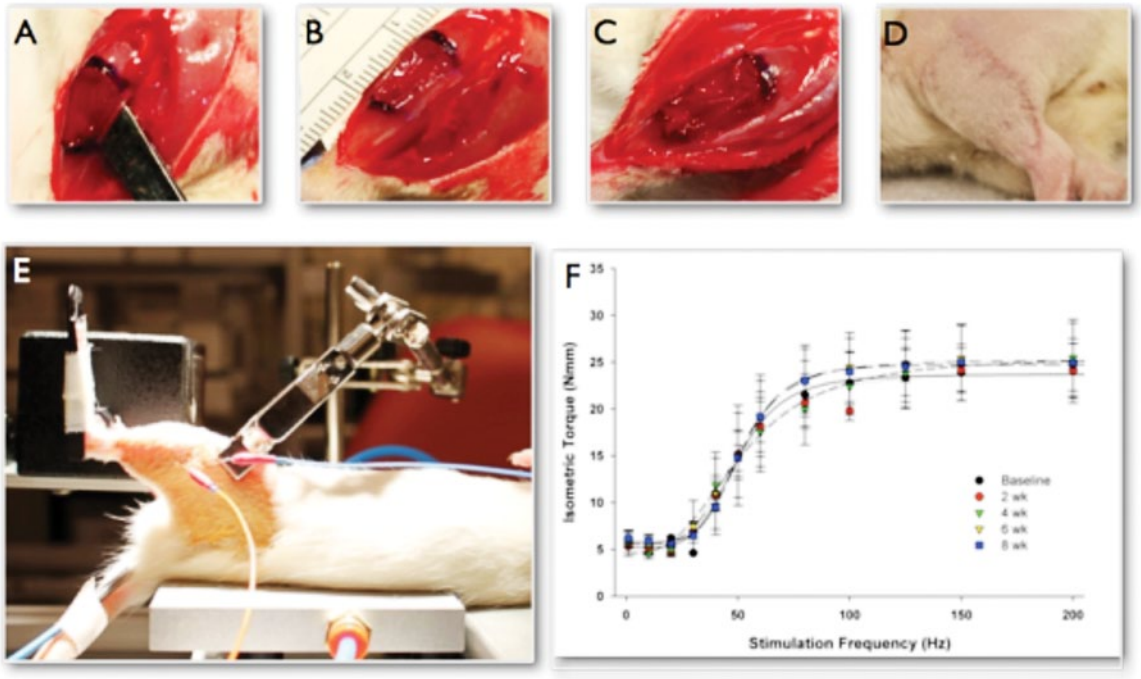


Figure III-22. Rat TA muscle VML injury model development and in vivo functional assessment. The VML injury model involves making a 1 x 0.5 x 0.5 cm (L x W x D) defect in the middle third of the TA muscle (A and B). TEMR constructs are then sutured into the void created by the defect (C). Two weeks after surgery the incision is healed (D), and the animal exhibits the ability to fully bear weight on the injured limb. Functional assessment of the anterior crural muscles (primarily TA muscles) is performed in vivo by attaching the foot to a servomotor and then depolarizing the common peroneal nerve via electrical stimulation (E). In vivo torque of uninjured anterior crural muscles (TA muscle) was measured as a function of stimulation frequency (F). For a single animal, less than 5% variability is observed among peak force over five assessments spanning 2 months.

RAT TA Muscle Morphology After TEMR Treatment

Upon retrieval of TA muscles after at least 2 months of implantation, there were noticeable effects of VML injury on gross muscle morphology. The untreated VML-injured tissue exhibited an atrophic appearance. In contrast, TEMR implanted tissues exhibited a more normal appearance with regard to shape and mass of the muscle. Moreover, the TEMR construct was visibly present to varying degrees in the majority of treated VML-injured muscles **(Figure III-23A)**. In both untreated and TEMR-treated VML-injured muscles, a longitudinal fissure was occasionally observed in the middle third of the muscle where the surgical defect was originally created. The wet weights of TA muscles from experimental legs were normalized to that of contralateral control legs (Figure III-23B). The uninjured and synergist ablation-only groups approximated a ratio of 1.0 indicating that neither the repeated functional testing nor the synergist ablation significantly altered TA muscle wet weight. In contrast, VML injury significantly reduced TA muscle wet weight to approximately 17% of uninjured group values, suggestive of little tissue generation (i.e., approximately 20% of muscle mass was removed via surgery) in the absence of treatment and, furthermore, supportive of the visual assessment of atrophy in this group. TEMR-treated, VML-injured TA muscles exhibited a wet weight ratio that was within approximately 10% of, and not statistically different from, uninjured values, indicating that TEMR implantation supported recovery of skeletal muscle mass during the months post-injury (Figure III-23B).

Rat TA Muscle Functional Assessment

Isometric torque was measured in response to neural stimulation in vivo as described in Figure III-22. Before injury, all groups produced similar peak isometric torque. Eight weeks post-injury, TEMR-treated TA muscles displayed a nearly 40% improvement in isometric torque when compared to the untreated injured muscles **(Figure III-24A)**. Moreover, the total degree of functional recovery at 2 months post-implantation in the TEMR-treated groups was approximately

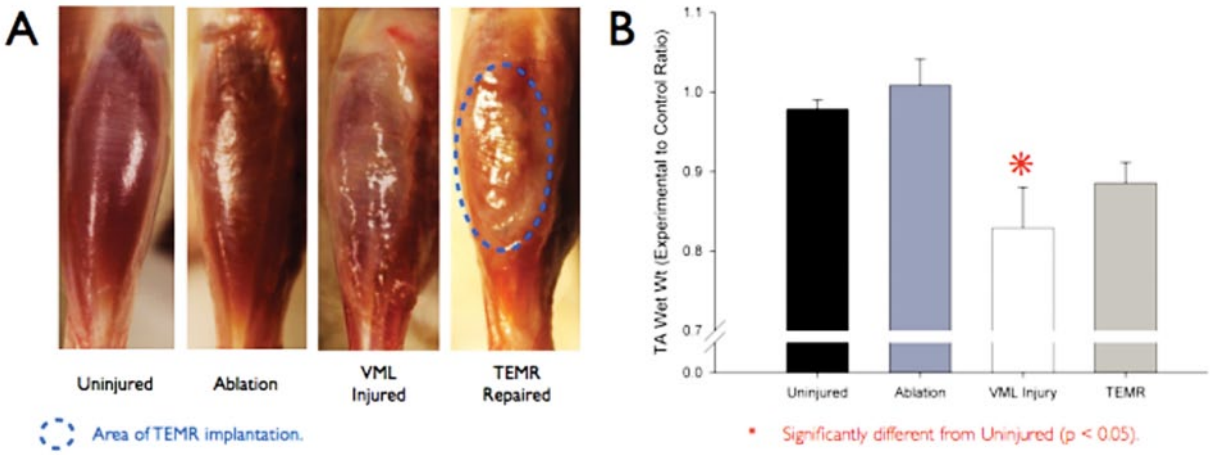
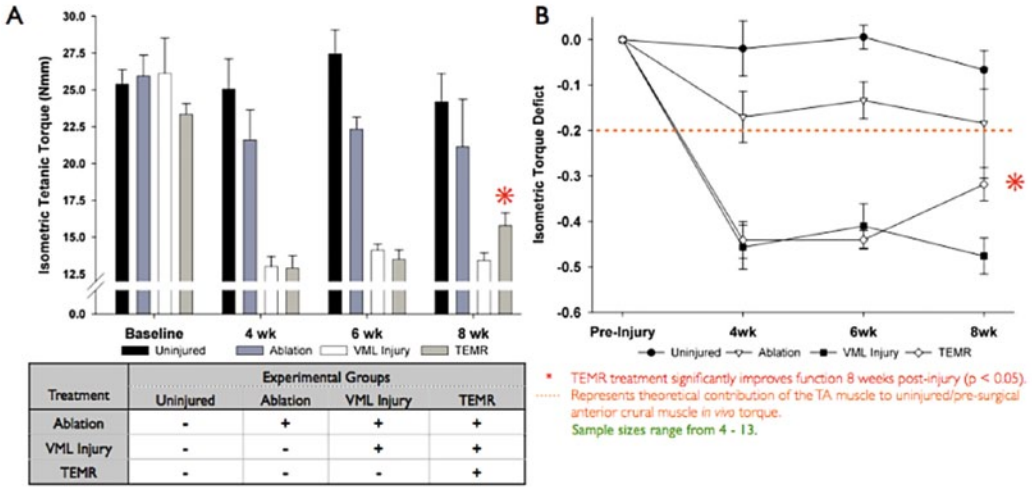


Figure III-23. TA muscle gross morphology and wet weight. TA muscles were retrieved at least 2 months post-injury. (A) Gross morphological assessment revealed an atrophied appearance with the VML-injured muscles. Also, for the TA muscles with TEMR implantation, BAM scaffolding material was still present to varying degrees. (B) Wet weights of TA muscles from treatment groups were normalized to their contralateral control muscles. Only the VML injury wet weight ratio was significantly reduced compared to uninjured values ($p < 0.05$).

70% of uninjured anterior crural muscle peak torque (includes all muscles), which is nearly identical to the functional recovery recently published for the impact of TEMR implantation in the murine LD VML injury. Also of note is that this degree of recovery actually underestimates the actual recovery since removal of the synergistic muscles provides a much better index of TA functional recovery (in this instance an average to approximately 80% functional recovery; Figure III-24B).

Rat TA Muscle Histological Assessment

To date, tissue morphology has been assessed via Masson's trichrome staining **(Figure III-25)**. In line with the observed gross tissue atrophy and the functional



Treatment	Experimental Groups			
	Uninjured	Ablation	VML Injury	TEMR
Ablation	-	+	+	+
VML Injury	-	-	+	+
TEMR	-	-	-	+

Figure III-24. TA muscle peak isometric tetanic torque is improved at 8 weeks post-injury with TEMR implantation. Isometric tetanic torque was elicited via neural stimulation of the common peroneal nerve (0.05–0.1 ms pulse width; 400 ms strain; 100–200 Hz). (A) Baseline values represent torques from all anterior crural muscles (TA, EDL, and EHL muscles) while the experimental groups' (ablation, VML injury, and TEMR) values at 4–8 weeks represent torque produced solely by the TA muscle. The experimental manipulations included for each group are denoted in the table. At 8 weeks, absolute torque values for the TEMR treatment groups were significantly greater than untreated VML injury groups (*, $p < 0.05$). (B) Absolute isometric torque was normalized to each animal's pre-injury baseline measure. Eight weeks post-injury, the functional recovery for TEMR-treated TA muscles was significantly greater than for untreated VML-injured TA muscles (*, $p < 0.05$).

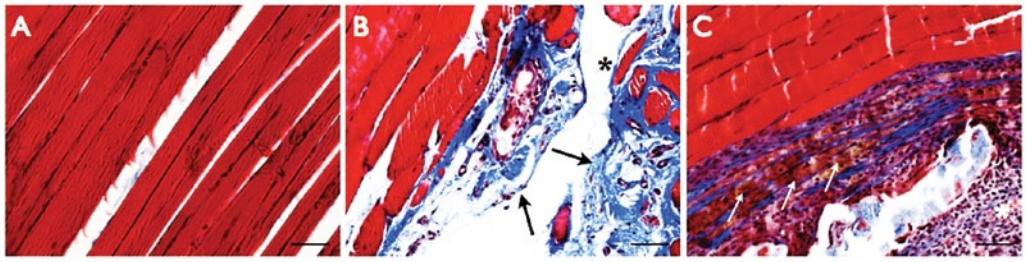


Figure III-25. TA muscle histology following VML injury and TEMR implantation. Longitudinal sections were cut from formalin-fixed, paraffin-embedded TA muscles. Sections were stained with Masson's trichrome and counterstained with hematoxylin, which indicates the presence of skeletal muscle tissue (red), collagen (blue), and nuclei (black). Uninjured muscle (A) presents little collagen deposition, peripheral nuclei, and striations. VML-injured muscle (B) presents with collagen deposition (arrows), suggestive of fibrosis at the wound site, with immature skeletal muscle tissue formation (*). TEMR-treated, VML-injured muscle (E) presents skeletal muscle tissue formation (arrows) at the site of injury and within the implanted scaffold; however, there is significant infiltration of mononuclear cells (e.g., macrophages) in the external region of the remaining BAM scaffold (*).

impairment, VML-injured tissue presented with collagen deposition and incomplete tissue regeneration at the site of injury (Figure III-25B). In general, TEMR treatment of the VML injury resulted in cellular presence at the site of injury that was not observed in untreated muscle (Figure III-25C). In close proximity to the native tissue there appears to be regeneration of elongated multinucleated muscle cells. Qualitatively, the width of these cells suggests that these cells remain immature. Additionally, mononucleated cells appear in presumptive residual BAM scaffolding, indicative of an immune response.

Exploration of Commercially Available, FDA-Approved Scaffolds for the Second-Generation Technology

As the logical next step toward a planned pre-pre-IND conversation with the FDA concerning the clinical development of this technology, the researchers have sought Animal Care and Use Committee approval for a short series of studies to evaluate the utility of commercially available, FDA-approved decellularized scaffolds (including porcine BAM, porcine small intestine submucosa Cook Wound Matrix, and bovine pericardium B. Braun Lyoplant®). They expect these bioequivalence studies to be under way shortly. They will compare the BAM scaffolds to the commercially available sheet-like scaffolds. In short, they will implant TEMR constructs created from BAM as well as the alternative scaffolds (noted previously) in the rat TA VML injury model. They will evaluate functional recovery in vivo at the same time points illustrated in Figure III-24 and, furthermore, retrieve the tissue after 2 months and conduct detailed histological analyses.

Evaluation of a Novel 3-D Silk Scaffold

Additionally, the research team has begun testing a novel silk-based, 3-D, aligned scaffold for the treatment of VML injury **(Figure III-26)**. These silk scaffolds offer an advantage to sheet-like scaffolds as they may better approximate the form (i.e., shape) and volume of VML wounds in craniofacial muscles, and the material properties may be modified (i.e., the degradation characteristics as well as porosity are tunable). The first of these novel scaffolds has been implanted in the rat TA VML injury model.

Evaluation of Phenotype and Scalability of Human MPCs

To evaluate the applicability of this technology to wounded warriors, the researchers have begun, in collaboration with the Regenerative

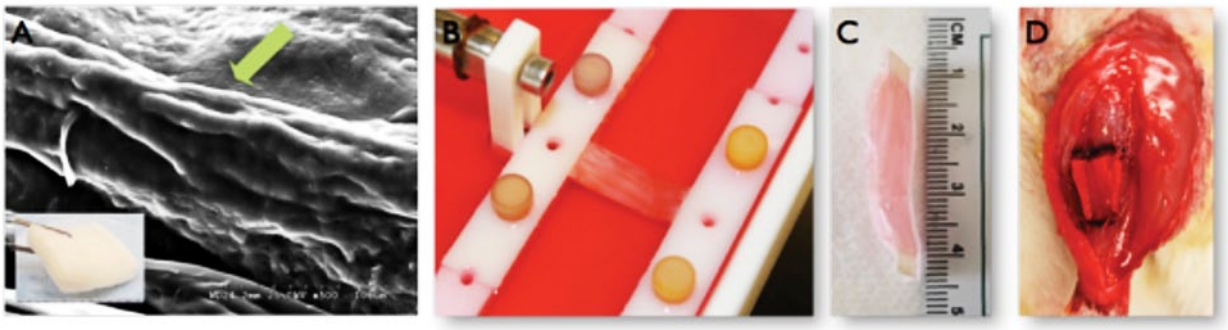


Figure III-26. Aligned silk sponge scaffold TEMR construct. Silk scaffolds are designed and tested in collaboration with Dr. David Kaplan at Tufts University. An aligned silk sponge scaffold has been tested for cellular adherence (A) and bioreactor compatibility (B, C), indicating the utility of this material for use as a TEMR platform scaffold for the treatment of VML injury. Current studies are under way testing the silk scaffold-based TEMR construct in a rat TA muscle VML injury (D).

Medicine Clinical Center team at WFIRM, to characterize the expansion, proliferation, and phenotype of passaged human MPCs. The overall goal is to develop an SOP for the human MPCs that yields a TEMR construct with equivalent properties in vitro to those previously described by the research team.

Key Research Accomplishments

- Completed and published the proof-of-concept findings (physiological and histological) documenting the approximately 72% functional restoration of the murine LD VML injury model observed 2 months post-implantation of the TEMR construct.
- Created and optimized a reliable VML injury model in the rat TA muscle in collaboration with Dr. Walters and his team at USAISR.
    - Preliminary results indicate that implantation of the TEMR construct in the TA VML injury model is associated with a similar degree of functional recovery as observed in the murine LD VML injury model. This observation provides further evidence for functional reinnervation of the TEMR construct following implantation in a VML injury in vivo.

Conclusions

The research team has made significant progress and remains on target with respect to completion of all milestones/deliverables. All assays and SOPs are in place for rapid completion of the proof-of-concept rat studies in the TA VML injury model and quick translation toward Good Laboratory Practice (GLP)/GMP studies upon obtaining guidance from the FDA. The most important accomplishment this past year is the illustration of approximately 75% functional recovery of an otherwise irrecoverable functional loss in two distinct models of VML injury within 2 months of implantation of the TEMR technology.

Research Plans for the Next 2 Years

The researchers are prepared to make a final decision on the scaffold for their second-generation technology pending completion of the bioequivalence studies by the second quarter of year 4. At that point they will have had a pre-pre-IND conversation with the FDA. In anticipation of their second-generation technology (and beyond), they have also begun exciting work on a novel, tunable, 3-D silk scaffold system and are well on their way to developing an SOP for the human MPC-derived TEMR technology.

Planned Clinical Transitions

The researchers plan two key development events for the upcoming year: completion of the bioequivalence studies and subsequent FDA conversations. More specifically, based on current progress and expected time lines, they anticipate to hold a pre-pre-IND teleconference with the FDA. Once their final scaffold decision is made, they will finish a definitive toxicology study prior to preparing an IND submission. They anticipate that they will need additional funds in the first 6 months post-AFIRM I to complete this study. With this information in hand, they will be in an excellent position to submit the IND by the end of the third quarter of 2013.

In addition, the researchers anticipate moving forward with a second-generation technology that includes SOPs designed to increase cellular density/phenotype during bioreactor conditioning and prior to implantation. Moreover, they are confident that the FDA

will accept the rat TA VML injury model as the definitive preclinical study required prior to human trials. They believe that they have established the scientific rationale for this approach, and if they obtain FDA consent, they could eliminate the canine studies planned for year 5 and accelerate the remaining toxicology, manufacturing, and second-generation studies.

Finally, the researchers plan to pursue a "first-in-man" Phase 1/pilot study of their technology in the VML patient population described in the recent article by Grogan and Hsu *(J Amer Acad Ortho Surg* 2011;19 [Suppl. 1]:S35-37). Patients with temporal wasting following the raising of coronal flaps (atrophy of the temporalis muscle following devascularization of the muscle where it may be possible to add a muscle flap to augment the result) or perhaps the repair of cleft lips where there is a quantitative decrease in muscle in the lip may also be candidates for the research team's current TEMR technology. Another attractive application of the TEMR technology is in the treatment of Bell's palsy. The researchers will continue to discuss and refine the clinical development plan internally at WFIRM as well as in consultation/collaboration with their colleagues at USAISR and within the CFR Program consortium.

Corrections/Changes Planned for Year 4

The researchers plan three main course corrections for the upcoming year. Specifically, they will:

- Focus on the development of silk scaffolds as the synthetic alternative to a decellularized scaffold and not pursue the polycaprolactone/collagen scaffolds at this time. The 3-D silk scaffolds are aligned and tunable, and furthermore, the silk scaffolds are in hand and appear to have all of the requisite mechanical and structural requirements for the use of TEMR in VML injuries.
- Discuss with the FDA the possibility of using the rat TA VML injury model as the definitive preclinical model prior to initiation of clinical trials. As noted previously, the rat TA VML injury model confers numerous advantages with respect to the longitudinal functional measures that can be made and, moreover, coupled to detailed histological investigations.
- Begin to evaluate the use of a second-generation technology based on the simulated "exercise" paradigm and multiple bioreactor-derived, cell-seeding protocols. The researchers have used leveraged funding mechanisms to develop this technology and propose to begin to evaluate it in the later years of AFIRM I and the beginning years of AFIRM II.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Develop Innervated, Vascularized Skeletal Muscle

Project 4.1.2, RCCC

Team Leader(s): Cathryn Sundback, PhD and Joseph Vacanti, MD (MGH)

Project Team Members: Craig Neville, PhD, Olive Mwizerwa, BS, Jake McFadden, BS, Mei Li, MS, Eric Finkelstein, PhD, Caitlyn Dickinson, BS, and Kenneth Rask, MBA (MGH); Douglas Henstrom, MD and Tessa Hadlock, MD (Massachusetts Eye and Ear Infirmary); and Sanjeeva Murthy, PhD and Joachim Kohn, PhD (Rutgers University)

Collaborator(s): David Sachs, MD (MGH Transplantation Biology Research Center)

Therapy: Replacement of severely injured facial skeletal muscles.

Deliverable(s): Innervated, vascularized skeletal muscle.

TRL Progress: 2009, N/A; 2010, TRL 3; 2011, TRL 3; Target, TRL 4

Key Accomplishments: The researchers determined that the vascular network in implanted, engineered, vascularized skeletal muscle grew into the host vasculature in less than 2 days. They engineered 3-D skeletal muscle with human myoblasts and identified soluble factors and ECM proteins that enhanced myotube hypertrophy. They demonstrated that 2-D cocultures of myotubes and fibroblasts were innervated in vitro. Finally, an improved in vivo biocompatibility of prototype scaffolds was demonstrated.

Key Words: Tissue-engineered skeletal muscle, vascularization, innervation

Introduction

Traumatic injuries to the head, neck, and particularly the face have been major contributors to the mortality and morbidity of military personnel in the current conflicts in Iraq and Afghanistan. Up to 13% of all blast injury survivors have significant eye and eyelid injuries involving perforations from high-velocity projectiles. A severely damaged eyelid muscle (orbicularis oculi) prevents eyelid closure, potentially resulting in blindness. Autologous tissue transfer is the standard of care, but the outcome is suboptimal. Engineered replacement orbicularis oculi muscles will restore functionality of the eyelid, prevent blindness, and restore aesthetics of the face. This therapy would improve the wounded warrior's quality of life and maximize eyelid function to allow for return to duty. In civilian populations, eyelid reconstruction with replacement muscle would significantly benefit patients with extensive eyelid skin cancers, major facial trauma, and long-standing facial paralysis with both neural and muscular deficits.

The MGH-Rutgers University team is developing a muscle construct with native muscle morphology. Myotubes, differentiated from a coculture of myoblasts and fibroblasts, self-assemble to form a 3-D muscle on the scale of a fascicle (500–1,000 μm in diameter). The resulting construct has the complex, multinucleated muscle architecture of native neonatal skeletal muscle. The project team designed the muscle constructs to be rapidly perfused and scaled to a muscle the size of the orbicularis oculi. Using a vasculogenesis approach,

a prevascular network within the engineered muscles in vitro was established. Upon implantation, this vascular network readily grew into the host vasculature. To scale the muscle size, the researchers developed a highly porous sleeve scaffold from TyrPC, a bioresorbable, biocompatible material. Leveraged funding from the Congressionally Directed Medical Research Programs supported the scaffold development work.

The guiding hypothesis of this project is that the scaled-up, 3-D, engineered, innervated, prevascularized muscle can develop sufficient muscle contractility to replace orbicularis oris function.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers tested biodegradable polymer scaffolds that improved the ability to handle engineered myooids for skeletal muscle regeneration. They produced vascular-like networks and immature muscle in vitro using biopolymer gels. They employed MSCs to support the formation of the prevascular network. They also implanted engineered immature muscles into adipose tissue of mice and found explanted muscle constructs continue to express muscle contractile proteins.

During the second year of the project, the researchers engineered vascularized skeletal muscle in vitro by co-seeding endothelial cells and MSCs along with muscle cells in the 3-D muscle fabrication process. They developed an innervation model in immunocompromised rats and demonstrated effective innervation in control studies in which neuromuscular junctions were re-established in denervated native muscle. Finally, they developed prototype scaffolding to support scale-up of the engineered muscles from the size scale of human muscle fascicles to an orbicularis oculi.

Research Progress – Year 3

The specific aims for year 3 were to (1) develop protocols to vascularize and innervate engineered muscle in immunocompromised rodents, (2) translate the self-assembly muscle protocol from rodent cell sources to human cell sources, and (3) continue scaffold development to scale-up the engineered muscle to the size of the orbicularis oculi muscle.

The researchers produced immature skeletal muscles in vitro using a coculture of primary myoblasts and fibroblasts, which were seeded, proliferated, and differentiated on fibrin gel. Differentiation induced alignment and fusion of myoblasts to form long, multinucleated myotubes. Contraction of the fibrin gel induced the cell sheet to roll, ultimately forming an immature muscle 10 days after differentiation. Fibrin is an appropriate scaffold material as it supports cellular proliferation and differentiation, and is readily degraded during muscle construct self-assembly.

Human Cell Sources

The researchers engineered 3-D muscles using the self-assembly process, substituting mouse myoblasts with human myoblasts. The genes and proteins expressed to support muscle contraction in human myotubes were similar to those expressed in engineered mouse myotubes. The engineered human myotubes were multinucleated, aligned, and striated; however, the human myotubes were smaller in diameter than their mouse counterparts, and the engineered muscle tissue was less dense than mouse muscle. The researchers are testing multiple approaches to increase the diameter and density of human

myotubes; these include optimizing fibroblast number and type, using electrical stimulation, adding small molecules to the culture medium, and optimizing the initial culture gel.

Vascularization

To induce rapid perfusion of the muscle construct, the project team established a prevascular network within the engineered immature muscle prior to its implantation. A coculture of human endothelial cells and human MSCs formed a construct with an endothelial-lined network that was supported and maintained by the MSCs. The researchers then implanted the prevascularized, self-assembled muscles into nude mice and explanted them 2 to 7 days later. At all time points, explanted muscles were viable and expressed normal muscle contractile proteins. Human blood vessels that contained red blood cells were observed throughout the muscle. Connection of the host and engineered vasculatures occurred less than 2 days after implantation.

Innervation

Nude rats were originally selected for innervation studies. This immunodeficient rodent model can easily accommodate single and scaled-up engineered muscles at locations where motor nerves are easily accessible. Researchers expected the immune response of nude rats to engineered muscles to be similar to that observed in nude mice as both immunodeficient rodent models have similar genetic mutations.

In side-by-side tests, engineered muscles were implanted into nude mice and nude rats and explanted at 1 or 3 weeks. Muscles implanted into nude mice remained viable and maintained their muscle phenotype. CD68+ macrophages were not recruited to the implantation site. Muscles implanted into nude rats were not viable 1 week after implantation. CD68+ macrophages homed to the implantation site by week 1 and fully engulfed the implanted muscle by week 3. Macrophages, along with natural killer cells, significantly contribute to xenograft rejection. The MGH group is collaborating with Dr. David Sachs from the MGH Transplantation Biology Research Center to implement approaches to dampen the immune response.

Until the nude rat model is fully developed, the project team is conducting 2-D innervation studies in vitro with rat spinal cord explants. Neurites grew out of the spinal cord explants and formed neuromuscular junctions with engineered myotubes. The researchers will incorporate spinal cord explants into the muscle self-assembly process to examine in vitro innervation of 3-D engineered muscle.

Muscle Scale-Up

The project team plans to engineer a muscle on the scale of the human orbicularis oculi muscle by bundling self-assembled 3-D muscles. The researchers will enclose the engineered muscles within a highly porous braided sleeve, which was designed and fabricated by the Kohn laboratory from a biocompatible, biodegradable synthetic material. During year 3, the Rutgers University team refined the sleeve design, the degradation time of the scaffold material, and the fabrication protocols. The MGH team conducted biocompatibility testing, and the inflammatory response to the selected material and cleaning protocol was minimal to moderate.

Key Research Accomplishments

- Demonstrated that engineered vascular networks in skeletal muscle constructs grew into the host vasculature in less than 2 days following implantation.

- Engineered 3-D skeletal muscle with human myoblasts; identified soluble factors and ECM proteins to enhance myotube hypertrophy.
- Demonstrated that 2-D cocultures of myotubes and fibroblasts were innervated in vitro.
- Demonstrated an improved in vivo biocompatibility of prototype scaffolds.

Conclusions

Toward the goal of engineering replacement orbicularis oculi muscles, the researchers have engineered 3-D vascularized skeletal muscle with morphology and maturation metrics similar to those of immature skeletal muscle. Upon implantation, the engineered vascular networks in the muscle constructs connected with the host vasculature in less than 2 days. The innervation work was delayed because the nude rats exhibited a strong immune response to implanted, engineered muscle. While they implement a strategy to suppress this response, the researchers are conducting innervation studies in vitro with spinal cord explants using first 2-D and then 3-D differentiated muscle. The Rutgers University team has further refined prototype scaffolding to support scale-up of the MGH engineered muscle to the size of the human orbicularis oculi muscle; the biocompatibility of the scaffold was confirmed by the MGH team in year 3. Ultimately, clinical implementation of this muscle will provide return of function to the injured warfighter and establish a platform for engineering other facial muscles.

Research Plans for the Next 2 Years

The research plans for years 4 and 5 have been scaled back because of the decrease in AFIRM funding in year 4.

Year 4

The researchers will continue to optimize the vascularization and innervation protocols of engineered muscle in immunocompromised rodents. The MGH team is collaborating with Dr. Sachs' group at the Transplantation Biology Research Center to suppress the rejection response of nude rats. Human cell sources have replaced rodent cell sources in the 3-D engineered muscle protocol; the project team will continue to optimize processing protocols. The researchers will assess muscle contractility as a function of in vitro processing parameters, vascularization, and innervation.

Year 5

Assuming that leveraged funding is obtained for years 4 and 5, a proof-of-concept study in immunocompromised rats will be conducted to demonstrate contractile human muscle on the scale of the orbicularis oculi. In parallel, preparations will be made to engineer an autologous rabbit sphincter muscle. The researchers will engineer a 3-D rabbit vascularized skeletal muscle in vitro. The Rutgers University team will develop scaffolding to support formation of a sphincter muscle.

Planned Clinical Transitions

An agreement will be signed in year 5 with an industrial partner to fabricate the scaffold used to bundle the engineered muscles for scale-up. Trident Biomedical, Inc., will be the likely partner if a TyrPC is demonstrated to successfully support muscle scale-up.

Engineered muscle is a combination product. The researchers will submit a Request of Designation to the FDA Office of Combination Products to determine the regulatory path. Subsequently, the research team will hold a pre-IND or IDE meeting with the appropriate FDA agency to review existing data and receive guidance on documentation preparation. At the current funding levels, conversations with the FDA are planned for AFIRM II.

Corrections/Changes Planned for Year 4

In year 3, the most significant challenge was the inflammatory response of nude rats to implanted engineered muscle. Nude rats were originally selected for innervation studies as this rodent model can easily accommodate single and scaled-up engineered muscles. However, nude rats have a significant population of natural killer cells and macrophages, which together initiate an attack on implanted engineered muscle. The researchers are collaborating with Dr. Sachs in the MGH Transplantation Biology Research Center to implement a strategy to suppress the natural killer cell/macrophage response in the rat model. While researchers test approaches, they are also conducting innervation experiments with engineered muscle, both in vitro with spinal cord explants and in vivo in nude mice.

As originally planned in year 4, the engineered muscle was to be scaled up to a muscle the size of the orbicularis oculi using a highly porous sleeve manufactured by the Rutgers University team. Because of this project's funding cut, this aim has been delayed until leveraged funding can be obtained.

Last, the self-assembly protocol must be optimized for human cell sources. Engineered muscle fabricated from human cells has fewer myotubes and a less dense structure than engineered muscle fabricated from rodent cells. The researchers will optimize the myotube number and hypertrophy by using in vitro electrical stimulation, soluble factors, and ECM proteins. A clinically relevant human fibroblast cell source will replace the current mouse-based fibroblast cell source.

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Composite Tissue Allograft Transplantation Without Lifelong Immunosuppression

Project 4.3.1, RCCC

Team Leader(s): Maria Siemionow, MD, PhD (Cleveland Clinic)

Project Team Members: Joanna Cwykiel, MSc, Agata Matejuk, PhD, Maria Madajka, PhD, Jacek Szopinski, MD, Bahar Bassiri Gharb, MD, and Antonio Rampazzo, MD (Cleveland Clinic)

Collaborator(s): Jim Herrman, PhD (CEO, Tolera Therapeutics, Kalamazoo, Michigan)

Therapy: Chimeric, cell-supportive therapy for allotransplantation (transplanting tissue from another human donor who is not genetically identical) without lifelong immunosuppression.

Deliverable(s): Demonstration of a therapeutic effect of ex vivo created donor–recipient chimeric cells on prolonged vascularized skin allograft survival.

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 3; Target, TRL 4

Key Accomplishments: The researchers performed a phenotype characterization of donor–recipient chimeric cells. They discovered a new subpopulation of donor–recipient chimeric cells. They also tested the therapeutic potential of the adhesive subpopulation of donor–recipient chimeric cells and evaluated the migratory potential of chimeric cells.

Key Words: Chimeric cells, vascularized skin allograft survival, cell fusion, tolerance induction, prevention of transplant rejection, cellular therapy

Introduction

The conditions of modern warfare require novel approaches for treating injured warriors. One-third of all warfighters are exposed to blast explosions during their service. Although there have been significant advances in the safety standards of the equipment and military body armor, blast trauma is responsible for roughly 80% of all injuries. Battlefield body injuries involve a massive loss of tissue, including large defects in the continuity of bone, nerve, muscle, tendon, ligament, soft tissue, and skin. The combination of protective body armor, effective immediate care, and rapid evacuation to the theater hospital and then outside of the war zone have improved survival of soldiers injured from high-order explosives commonly used by insurgency forces in Iraq and Afghanistan. Due to more efficient medical care, the ratio of wounded to dead U.S. soldiers in Iraq and Afghanistan increased (1:7.3) compared to the wars in Vietnam (1:2.6) or Korea (1:2.8).

Currently, the most common wounds are severe limb and craniofacial injuries as well as large area burns that in the past would have been fatal. Statistics show that 70% of all combat injuries are musculoskeletal and 55% involve extremities. Massive tissue loss occurs either at the time of the initial trauma or during subsequent surgical wound debridement.

Reconstructive surgery can help patients who have suffered tissue loss; however, if the damage is severe, there is little possibility of performing autograft (i.e., transplanting the patient's own tissue from another body region). While the field of medical engineering is evolving, it still cannot provide tools for the efficient treatment of large-scale traumas. Currently, the only treatment option for patients with severe tissue loss is tissue transplantation from an unrelated donor. The surgical technique is an established procedure; however, the patient is continuously in danger of rejecting transplanted tissue. The transplant patient must take lifelong medication to prevent this immune reaction, but such "immunosuppressant" drugs have negative side effects, including making the patient vulnerable to serious illnesses and even cancers. Some patients decline the transplants because such side effects are too severe. Researchers are now focusing efforts on engineering a transplant method that does not make a patient have a severe immune response or require him or her to take lifelong immunosuppressant drugs.

Vascularized composite allografts, typically used in transplants, are a challenge for scientists. One of the vascularized composite allograft elements, skin, is considered to be the most immunogenic tissue in the human body (i.e., the most likely to provoke an immune response). Also, lymphoid tissue, such as lymph nodes and bone marrow, may generate a high immunological response. During the first face transplant, performed in France, donor bone marrow transplantation was first used in clinical practice as a supportive therapy to improve the outcome after CTA transplantation. Currently, clinical, technical, and biological criteria for the use of bone marrow transplantation as a part of cell therapy protocols are still being investigated. Frequent complications include graft failure and a long time period for the body to regenerate blood (hematopoietic) cells especially when the number of such cells is limited. An alternative transplant method, which may not require immunosuppressant drugs, is supportive therapy with donor–recipient chimeric cells. The term chimera refers to one body containing cells from two genetically distinct individuals—in this case, donor and recipient cells are fused together and transplanted into the recipient body in the hope of reducing or eliminating the recipient's immune response and subsequent need for immunosuppressant medication and its negative side effects.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the research team confirmed that ex vivo creation of donor–recipient chimeric cells by cellular fusion was a feasible method in a rat model. In a series of experiments, the researchers confirmed the chimeric cells' polyploidy (i.e., having more than two copies of each chromosome) by karyotyping and polymerase chain reaction (PCR) analysis (reported previously). The chimeric cell's proliferative potential was proven by colony forming unit assays. In an in vivo model, the research team confirmed the therapeutic potential of ex vivo fused chimeric cells as a supportive therapy combined with a selective, short-term immunosuppressive protocol of anti-αβ T cell receptor (TCR) monoclonal antibody and cyclosporine A in the rat model of CTA transplants. The researchers observed a significant improvement in the survival of fully major histocompatibility complex-mismatched vascularized skin allografts, compared to the survival of vascularized skin allografts only, under a short-term, immunosuppressive protocol.

Research Progess – Year 3

Determination of Cellular Markers Present on the Surface of a "Hematopoietic" Lineage of Donor–Recipient Fused Chimeric Cells

The researchers improved the preparation and isolation procedure for bone marrow cells. Results of these in vitro experiments confirmed that the culture time they had previously used for chimeric cells was optimal for the cells' growth and for the further therapeutic application of chimeric cells. Using flow cytometry analysis, they characterized the phenotypes of the donor–recipient fused chimeric cells. They evaluated the semi-adherent and nonadherent populations of bone marrow-derived cells that had undergone fusion. They found that in chimeric cells up to 90% of cell subpopulations presented adherent properties whereas only about 15% of cells had no adhesive properties.

Further flow cytometry characterization of donor–recipient fused chimeric cells revealed that both subpopulations of semi-adherent and nonadherent chimeric cells were, as expected, hematopoietic cells of myeloid origin, indicated by positive staining with CD90, CD45, CD18, and CD54. The lack of CD11a and CD31 expression and low level of CD3 expression show that semi-adherent and nonadherent chimeric cells do not have lymphocytes or stromal cell characteristics. By design, chimeric cells are kept in an undifferentiated state, which will allow them to differentiate in the facilitated environment of the recipient lymphoid organs such as thymus or lymph nodes.

The researchers confirmed the previous results using PCR analysis of RNA transcript expression of both subpopulations of chimeric cells (nonadherent and semi-adherent). They evaluated the expression of the following marker genes: CD31, collagen 1a1, fibronectin, CD45, CD18, CD90, and beta-actin as reference gene. The project team chose these particular genes for characterizing chimeric cells to determine the hematopoietic or stromal origin of the cells. Transcripts of these genes were present at comparable levels in each subpopulation, independent of the adhesive properties. As expected, chimeric cells cultured in hematopoietic lineage-specific medium did not possess the typical characteristics of stromal cells, such as strong expression of CD31 or collagen 1a1.

Based on phenotype characterization results, the researchers hypothesized that directing chimeric cells to the stromal cell lineage through cell culturing would (1) increase the number of chimeric cells and (2) improve the mechanism of action by modulation of the immune response.

They performed phenotype characterization of stromal donor–recipient chimeric cells by PCR analysis of RNA transcript expression. PCR evaluation of stromal donor–recipient fused chimeric cells showed that these chimeric cells possessed some of the characteristics of stromal cells, such as strong expression of collagen 1a1 and fibronectin. (In comparison, there was weak expression of these markers on the surface of hematopoietic donor–recipient fused chimeric cells). However, stromal donor–recipient fused chimeric cells showed weak expression of CD31.

Key Research Accomplishments

- Completed phenotype characterization of hematopoietic lineage ex vivo donor–recipient fused chimeric cells.
- Developed new therapeutic cells—stromal donor–recipient fused chimeric cells—and began an in vivo evaluation of these cells.

Conclusions

In summary, the researchers established optimal conditions for fusion cell proliferation and improved the bone marrow preparation and isolation procedure. They evaluated semi-adherent and nonadherent populations of bone marrow-derived cells that had undergone fusion. They found that in the semi-adherent cell subpopulation up to 90% of cells were chimeric and in the nonadherent cell subpopulation up to 15% of cells were chimeric. The researchers demonstrated that both subpopulations of semi-adherent and nonadherent chimeric cells were hematopoietic cells of myeloid origin. However, no CD11a lymphocyte marker was detected. Finally, they showed that stromal donor–recipient fused chimeric cells possess some stromal cell characteristics, such as strong expression of collagen 1a1 and fibronectin.

Research Plans for the Next 2 Years

The possibility of a clinical trial based on work performed by the Siemionow group will depend on results obtained in year 4 when characterization and migratory properties of human donor–recipient fused chimeric cells in the nude rat model will be performed. By that time, the project team will have established the technique of ex vivo cell fusion and will have determined the proper in vitro culture conditions. The researchers will evaluate the viability and phenotype of human fused chimeric cells. They will test the ability of human fused cells to maintain therapeutic potential after cryopreservation. High survival rate of human chimeric cells after cryopreservation will allow storage of these cells in the future in a human chimeric cell storage bank.

Planned Clinical Transitions

Clinical trials will follow the laboratory's successful accomplishment of in vitro and in vivo studies on human chimeric cells. The researchers will evaluate the migratory properties, phenotype, and safety of human donor–recipient fused chimeric cells in the nude rat model. Clinical trials are expected to begin in 2013.

Engineered Cartilage-Covered Ear Implants for Auricular Reconstruction

Project 4.1.1, WFPC

Team Leader(s): James Yoo, MD, PhD (Wake Forest University)

Project Team Members: Sang Jin Lee, PhD, John Jackson, PhD, Chang Mo Hwang, PhD, Bukyu Lee, DDS, PhD, Young Min Ju, PhD, Weijie Xu, PhD, and Tanner Hill, BS (Wake Forest University)

Collaborator(s): Greg Sword (Porex Surgical, Stryker)

Therapy: Reconstruction of the external ear.

Deliverable(s): Engineered cartilage tissue covering the commercially available alloplastic implant.

TRL Progress: Start of Program, TRL 3; End Year 1, TRL 3; End Year 2, TRL 4; End Year 3, TRL 4

Key Accomplishments: The researchers have fabricated a flexible ear scaffold using an integrated organ printing technology, which was developed in their laboratory. They also optimized cell isolation and culture procedures by characterizing auricular chondrocytes from fresh ear cartilage tissue biopsies from New Zealand White rabbits in terms of cell growth, maintenance of phenotypic and functional characteristics, and the quality of neocartilage formation. The results of this project to date indicate that cartilage tissue-covered ear implants are able to maintain device contour and placement without causing skin necrosis in mice.

Key Words: Auricular cartilage, alloplastic ear implant, reconstruction, tissue engineering

Introduction

Traumatic injuries constitute a major cause of morbidity and mortality for the armed forces. The incidence of craniofacial injuries has been rapidly increasing due to frequent ballistic and explosive injuries on the battlefield. Protruding tissues such as ear and nose are frequently affected in these injuries. Although the loss of ear tissues does not pose a life-threatening danger, it is functionally and cosmetically debilitating and hinders an injured soldier's return to society.

The standard treatment method for auricular reconstruction uses autologous costal cartilage as a graft material. However, autologous costal cartilage is limited in supply, provides inadequate dimensions, and is progressively absorbed after implantation. Currently, alternative approaches utilize alloplastic ear implant devices composed of silicone or polyethylene. These implants are approved by the FDA and are nontoxic, cause minimal foreign body reactions, and possess adequate mechanical properties for use in non-load-bearing tissues of the craniofacial region. Although alloplastic ear implants are able to effectively eliminate the morbidity associated with the costal cartilage graft, the use of these implants is often related to complications that include inflammation, infection, erosion, and dislodgement. As a result, implant extrusion occurs frequently due to the limited vascularization and constant abrasion against the surrounding tissues. A common practice

to overcome these complications includes the use of a temporoparietal tissue flap from the side of the head to cover the implant, which provides a vascularized tissue cushion against the abrasive implant.

In this project, the researchers have developed an engineered cartilage designed to entirely cover the abrasive ear implant, which prevents implant exposure and extrusion while maintaining the appropriate mechanical properties **(Figure III-27)**. Creation of this cartilage tissue using a soldier's own cells would bring benefits and minimize the morbidity associated with implant dislodgement. The researchers plan to further refine and optimize the processing system for the cartilage-covered implant to ensure a smooth translation for soldiers who require auricular reconstruction.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers prepared fibrin hydrogels with various concentrations of fibrinogen and thrombin, mixed cultured chondrocytes with the hydrogels, and implanted the constructs subcutaneously into athymic mice. They harvested the mice at various times post-implantation. While nontreated ear implants resulted in severe skin necrosis at 2 weeks post-implantation, cartilage-covered ear implants were able to maintain device contour and placement without causing skin necrosis.

During the second year of the project, the researchers demonstrated the in vivo structural stability of their engineered cartilage-covered ear implants for clinical application.

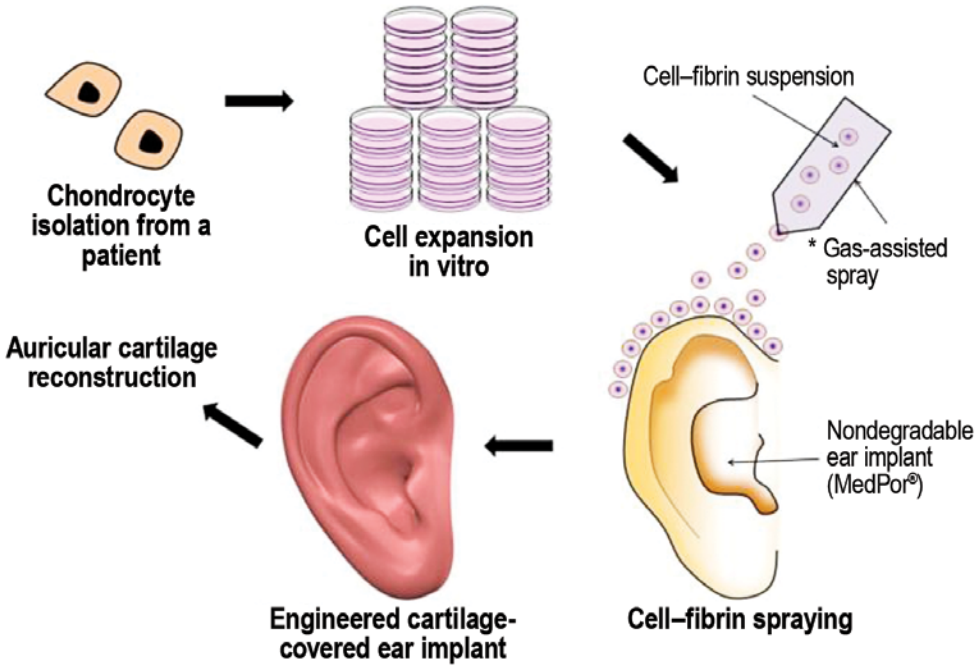


Figure III-27. Strategy for engineering cartilage tissue-covered ear implants for auricular reconstruction. Engineered cartilage tissue created using a patient's own cells is able to coat a heterogeneous, ear-shaped implant when it is applied using a gas-assisted spraying system containing a cell–fibrin suspension.

The engineered cartilage homogenously covered alloplastic ear implants by a cell-spraying method. Their research began to show that cartilage tissue-covered ear implants could maintain device contour and placement without causing skin necrosis when implanted in vivo.

Research Progress – Year 3

In Vivo Evaluation of Engineered Cartilage-Covered Ear Implants in Mice

Rabbit auricular chondrocytes were isolated from ear cartilage by collagenase digestion and were expanded in vitro. A fibrin hydrogel was used as a cell delivery vehicle, and the alloplastic ear implants (MedPor®) were coated with the chondrocyte–fibrin suspension in vitro. To optimize the stability of the chondrocyte–fibrin construct, various fabrication conditions were tested. In addition, the surfaces of some of the alloplastic ear implants were also modified to enhance the hydrophilicity of the surface. The optimized cell–fibrin suspension was sprayed onto the surface of the modified ear implants. The ear-shaped constructs were then implanted into the dorsal subcutaneous space of athymic mice (n = 20, 30–50 g, male). The animals were periodically observed and then sacrificed at 12 weeks after implantation for histologic evaluation. Unmodified, uncoated MedPor ear implants served as a control. Grossly, the engineered cartilage-covered ear implants placed in athymic mice showed no evidence of skin necrosis, implant exposure, or extrusion. However, the nontreated ear implants (control) resulted in severe skin necrosis after implantation **(Figure III-28)**.

To date, the researchers' data indicate that their cartilage-covered ear implants are able to maintain device contour and placement without resulting in skin necrosis.

Histomorphological evaluations consistently showed neocartilage formation on the ear implants. H&E staining revealed the presence of evenly dispersed triangular and ovoid-shaped chondrocytes that inhabited normal-appearing lacunae, and these were surrounded by perichondrium. However, the animals in the control group developed only thin fibrotic tissue on the ear implants **(Figure III-29A)**. Figure III-29B shows the cartilage tissue formation on the entire ear implant. From these images, it is obvious that the engineered cartilage tissue provided better structural integration between the host skin and the ear implants (Figure III-29C-D).

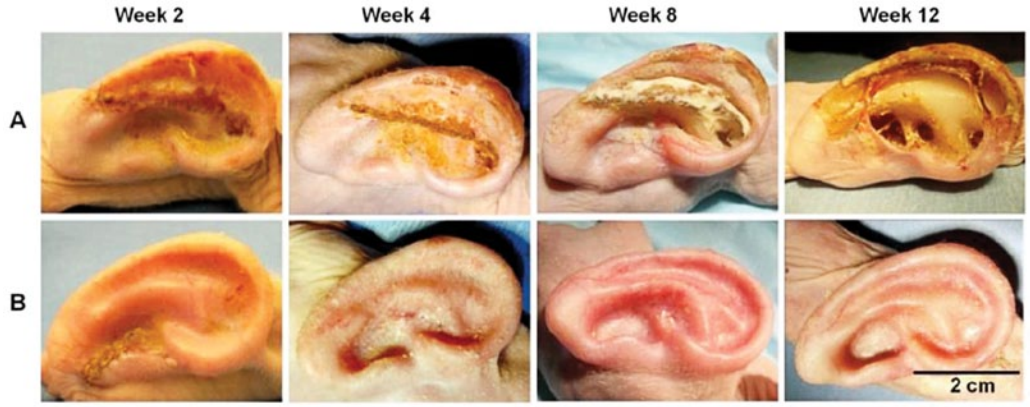


Figure III-28. Gross examination of the ear implant only (as a control) and engineered cartilage covered implant at 2, 4, 8, and 12 weeks after implantation.

In Vivo Evaluation of Engineered Cartilage-Covered Ear Implants in Rabbits

To demonstrate the clinical applicability of this approach, the researchers previously added a task that involves the subcutaneous implantation of an engineered cartilage-covered ear implant (human size 6 cm in length) that had been prepared using autologous cells. Toward this goal, they selected a rabbit auricular implantation model. Autologous chondrocytes were harvested under sterile conditions from adult male New Zealand White rabbits. The cells were expanded and combined with the fibrin-based hydrogel to cover the ear implants. The engineered cartilage-covered ear implants were then implanted subcutaneously in rabbits **(Figure III-30)**. The engineered cartilage-covered ear implants are currently being characterized for cartilage tissue formation via histological evaluation.

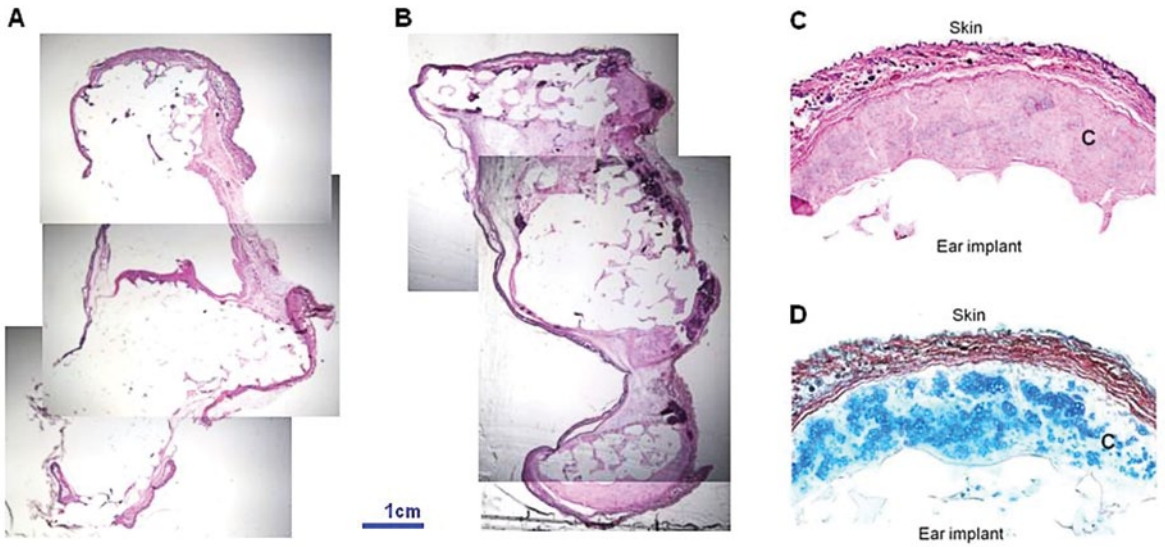


Figure III-29. Histological evaluations of engineered cartilage-covered ear implants at 12 weeks after implantation: (A) control ear implant and (B) engineered cartilage-covered ear implant. Histological staining indicated that the implants were covered by auricular neocartilage tissue consisting of chondrocytes inhabiting typical appearing lacunae and perichondrium as shown by (C) H&E staining and (D) Alcian blue staining.

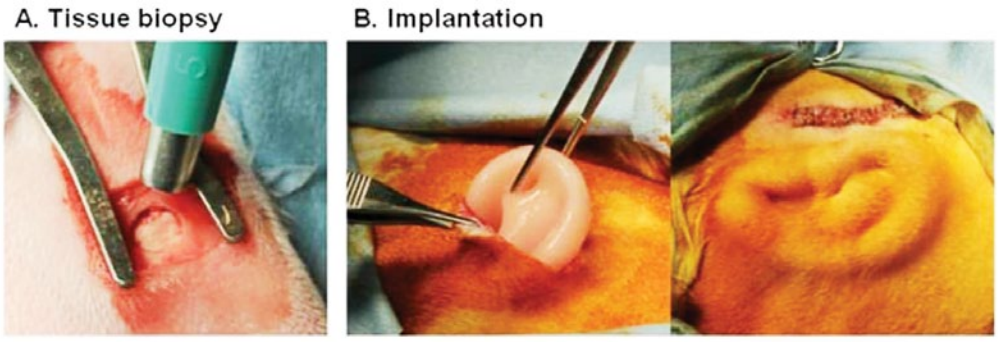


Figure III-30. Surgical procedure for implantation of autologous cell-seeded ear implant in a rabbit.

Bioprinted Flexible Ear Scaffold for Reconstruction

The integrated, 3-D printing system consists of four delivery systems that can process multiple types of materials, including synthetic polymers and a cell/gel mixture. The materials are precisely dispensed by controlling the air pressure within each delivery system. A heating unit has been employed to obtain dispensable synthetic polymers. The performance of the system in terms of 3-D patterning was investigated by fabricating a porous structure having the shape of a human ear **(Figure III-31)**.

Optimization of Chondrocyte Isolation and Culture

To optimize the cell isolation and culture procedures, the researchers obtained auricular chondrocytes from fresh ear cartilage tissue biopsies from New Zealand White rabbits. After expansion, cells were characterized in terms of cell growth, maintenance of phenotypic and functional characteristics, and the quality of neocartilage formation. The phenotype of these cells was confirmed by immunocytochemistry for types I, II, and IV collagen **(Figure III-32)**.

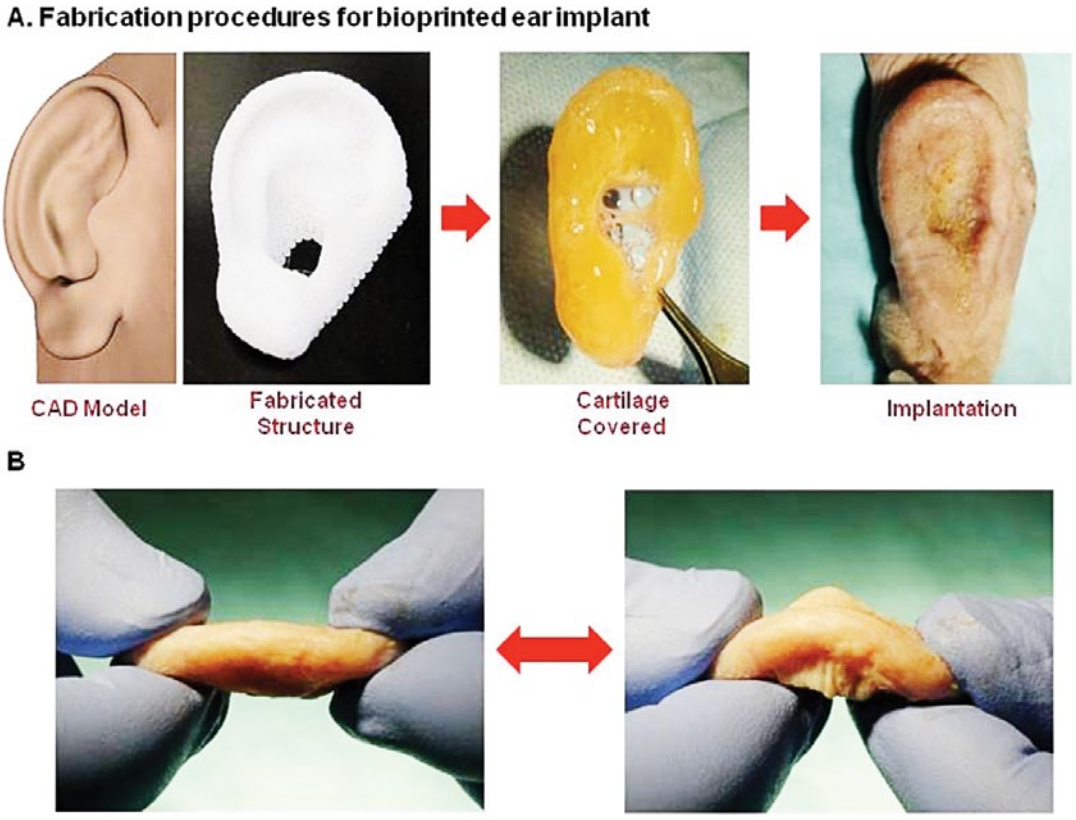


Figure III-31. A bioprinted ear implant was fabricated and implanted in nude mice. The 3-D printing technology allows the ear scaffold to be customized for each patient's unique size and shape. In addition, this system could provide novel scaffolds with similar mechanical characteristics (i.e., elasticity) to those of natural cartilage tissue.

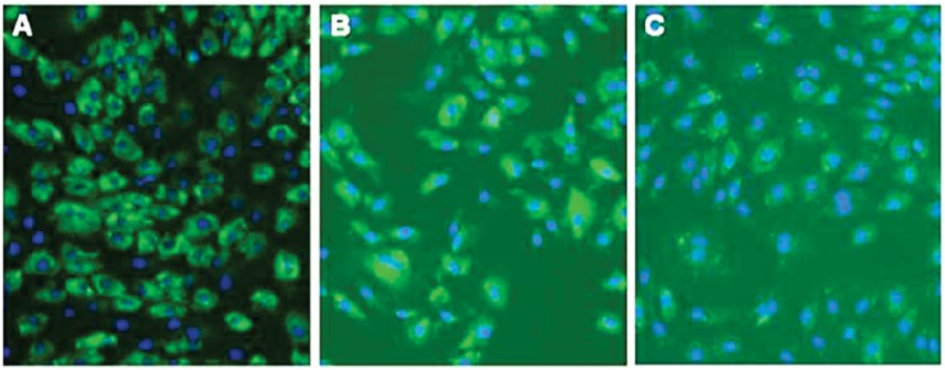


Figure III-32. Immunofluorescent images of isolated ear chondrocytes for (A) collagen I, (B) collagen II, and (C) collagen IV.

Key Research Accomplishments

- Optimized a cell–fibrin spraying device.
  - ♦ Fabricated a porous structure having the shape of a human ear and implanted it in nude mice.
- Demonstrated the structural and functional integrity of engineered cartilage ear implants in mice.
  - ♦ Determined that engineered cartilage tissue provided better structural integration between the host skin and the ear implants.
- Optimized cell isolation and culture procedures by characterizing auricular chondrocytes from fresh ear cartilage tissue biopsies from New Zealand White rabbits in terms of cell growth, maintenance of phenotypic and functional characteristics, and the quality of neocartilage formation.

Conclusions

The research team has fabricated a flexible ear scaffold using an integrated organ printing technology, which was developed in their laboratory. The results of this project to date indicate that cartilage tissue-covered ear implants are able to maintain device contour and placement without causing skin necrosis in mice.

Research Plans for the Next 2 Years

During the next 2 years, the researchers plan to continue to evaluate the structural and functional integrity of engineered cartilage ear implants in rabbits. They will continue to analyze cell isolation efficiency, cell growth, maintenance of phenotypic and functional characteristics, and ECM production of isolated chondrocytes. They will continue to prepare the materials for FDA discussions. They will begin and monitor a clinical trial of engineered cartilage ear implants.

Planned Clinical Transitions

The researchers plan to continue to develop a system that requires a minimal tissue biopsy for cell isolation and expansion from different cell sources, including ear, nose, and rib cartilage tissues. They will continue to develop SOPs for autologous cell sourcing, an associated expansion system, and a surgical procedure. They will also refine the cell delivery system to facilitate clinical translation of the engineered cartilage-covered ear implants.

Engineering a Replacement Autologous Outer Ear Using a Collagen/Titanium Platform

Project 4.5.4a, RCCC

Team Leader(s): Cathryn Sundback, PhD and Joseph Vacanti, MD (MGH)

Project Team Members: Mack Cheney, MD, Tessa Hadlock, MD, and Douglas Henstrom, MD (Massachusetts Eye and Ear Infirmary, Facial Plastic Surgery); Irina Pomerantseva, MD, PhD, Craig Neville, PhD, Kenneth Rask, MBA, Erik Bassett, Alan Tseng, and Danai Chagwedera (MGH Tissue Engineering); and Mark Randolph and Matt Johnson (MGH Plastic Surgery Research Laboratory)

Collaborator(s): Nicholas Roscioli (Kensey Nash Corporation)

Therapy: Tissue-engineered cartilage constructs for ear replacement.

Deliverable(s): A permanent, implantable, engineered, living external ear replacement for the wounded warfighter.

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Key Accomplishments: The researchers engineered robust neocartilage in vivo using two approaches aimed at reversing and preventing cartilage cells from undergoing "de-differentiation"—an unwanted process by which cartilage cells, after extensive expansion, return to a more primitive type of stem cell and lose their potential to form neocartilage. They demonstrated maintenance of size and shape of neocartilage in a new generation of adult-sized, human ear-shaped scaffolds in a nude rat model. They optimized neocartilage formation in the autologous sheep model using in vitro culture.

Key Words: Tissue engineered ear, cartilage, porous collagen, chondrocytes

Introduction

Limited reliable options exist for reconstructing the external ear following blast injuries to the head and neck. Current surgical approaches for reconstructing the external ear include attaching one of two designs: (1) a cartilage framework that surgeons hand-carve in the operating room from autologous tissue (i.e., tissue taken from elsewhere in a patient's body, such as rib bone) or (2) ear-shaped implants made of porous polyethylene (MedPor). Both reconstructive options are prone to complications, require multiple surgeries, and have unpredictable and often poor cosmetic outcomes. The MGH team has shown initial success in engineering ear-shaped cartilage using biodegradable scaffolds and chondrocytes in immunocompromised animals.

Tissue-engineered autologous ear replacements would combine the best of both current clinical approaches—the precisely defined architecture of MedPor implants and the autologous properties of carved cartilage (i.e., since tissue is taken from a patient's own body, it does not provoke an immune response). For this new MGH design, the required number of surgeries to complete ear reconstruction could be reduced from four to two. A critical, remaining design goal is to maintain the complex 3-D structure of this largely unsupported cartilage when it is subjected to the mechanical forces of the surrounding tissues during cartilage maturation

and wound healing after implantation. These forces are much greater in a human or a large animal than in a rodent model. The goal of this project is to expedite the development of a permanent, implantable, living external ear for the injured service member and to achieve cosmetic outcomes that meet patient expectations.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers developed an ovine model for subcutaneous implantation of ear-shaped constructs. They engineered cartilage from both ovine and human cartilage cells and MSCs in immunocompromised mice. They also designed ear-shaped scaffolds using computer-aided design principles and tested the scaffolds with numerous materials.

During the second year of the project, the researchers demonstrated robust autologous cartilage formation on porous fibrillar collagen scaffolds in an immunocompetent sheep model without a significant inflammatory or foreign body response. In a proof-of-concept study in nude mice, they demonstrated the effectiveness of internal support in maintaining the size and shape of human ear-shaped neocartilage constructs.

Research Progress – Year 3

Cell Source Development

Considerable success has been achieved in engineering cartilage in laboratory animals using primary (freshly isolated) chondrocytes (cartilage cells) from unrestricted amounts of donor tissue. However, in human patients, the cartilage biopsy size is limited by its availability and/or quality, aesthetic considerations, or donor site morbidity. The chondrocyte yield from human auricular cartilage, the best source for engineering ear cartilage, averages 9.7 ± 0.8 million cells per gram of tissue. Therefore, an estimated 0.3 million chondrocytes could be harvested from a 30 mm^3 (30 mg) biopsy. Using current methodologies, 100–150 million chondrocytes, a 300–500-fold increase, would be required to seed an adult-sized, human ear-shaped scaffold. The desired cell number can be achieved by extensive chondrocyte proliferation in vitro. However, with repeated passaging, cartilage cells de-differentiate, or return to a more primitive type of stem cell, and lose their potential to form neocartilage.

Recovering the Ability of Expanded Sheep Chondrocytes to Form Cartilage by Mixing Them with Freshly Isolated Chondrocytes

Sheep auricular chondrocytes were expanded in vitro, and an increase more than 8,000 times in cell numbers was observed by passage 3 (P3). Freshly isolated (passage 0, P0) chondrocytes were preserved in liquid nitrogen during this time and had 80% viability when thawed. Porous collagen scaffolds were seeded with P0, P3, or a 20:80 mixture of P0+P3 cells and implanted in nude mice. After 6 weeks in vivo, explanted P0 and P0+P3 constructs grossly resembled cartilage. Histologically, neocartilage formation was observed. In P3 constructs, only a few small areas stained positively, indicating that most chondrocytes remained de-differentiated (also confirmed by abundant staining for collagen type I) and failed to produce cartilage-specific ECM. These results confirm and further expand the results published earlier by other researchers who demonstrated the ability of the P2 bovine articular chondrocytes to form cartilage-specific ECM when cocultured with 20% P0 chondrocytes in vitro.

Preventing Sheep and Human Chondrocyte De-Differentiation During Expansion by Supplementing Culture Medium with Basic Fibroblast Growth Factor

Sheep auricular and human nasal chondrocytes were expanded in vitro with the addition of 5 and 10 ng/mL basic fibroblast growth factor (bFGF) to culture medium. By P3, the number of cells increased more than 148,000 times (sheep) and 23,000 times (human) regardless of bFGF concentration. Porous collagen scaffolds seeded with expanded chondrocytes were implanted in nude mice. After 6 weeks in vivo, all explanted constructs grossly resembled cartilage with white, glistening surfaces. Histologically, staining confirmed that neocartilage had formed. These data demonstrate that P3 chondrocytes, cultured in the presence of bFGF, are able to form neocartilage that is histologically indistinguishable from neocartilage formed by freshly isolated P0 (or moderately expanded P1) chondrocytes. The results acquired by the MGH team corroborate and complement earlier publications by other groups that studied the effects of bFGF on chondrocyte re-differentiation in vitro. In a clinical setting in Japan, bFGF was used to expand human primary chondrocytes; the group engineered cartilage for the reconstruction of microtia.

Fabrication of Human Ear-Shaped Scaffold

The MGH team demonstrated human ear-shaped cartilage formation in nude mice. By incorporating an internal titanium wire support into the collagen scaffold, the team proved that the outer shape of the engineered ear could be maintained; however, the central features of the ear were not sufficiently well defined. The team developed a next-generation scaffold that has enhanced these central features and approximates the size of an average adult human ear cartilage (5 cm in length and 1.2 to 2.4 mm in thickness). These scaffolds were tested in nude rats. Upon sacrifice at 5 weeks, the size and shape of the constructs were maintained. The MGH team assessed flexibility by repeatedly bending the construct, which recovered its original shape. Positive staining for cartilage matrix demonstrated that neocartilage had formed in tissue from three biopsies taken throughout the construct.

Cartilage Formation in a Sheep Model

In year 2, the MGH team observed inconsistent autologous neocartilage formation accompanied by a surprisingly severe inflammatory response and foreign body reaction in sheep. The research team hypothesized that the collagen scaffold material originating from a bovine source may have been the cause. To overcome this unwanted reaction (done in year 3), the researchers matured cartilage ECM on the collagen scaffolds in vitro prior to implantation, therefore embedding scaffold collagen fibers into cartilage ECM and minimizing the fibers' exposure to the host. Maturation of cartilage ECM was achieved by increasing in vitro culture time and by supplementing the culture medium with two growth factors, insulin-like growth factor (IGF) and bFGF. After 6 weeks in vivo, cartilage made without adding growth factors resembled that engineered by the MGH team in prior studies; thicker cartilage was seen in the IGF/bFGF group. Mild cellular inflammatory reactions and foreign body responses were found in all groups at the periphery of the implants. Weak staining for elastin was observed in constructs that had been cultured with the addition of both IGF/bFGF. Increasing in vitro culture time from 6 to 12 weeks had no beneficial effect on neocartilage formation during the 6-week in vivo period.

Key Research Accomplishments

- Completed pilot studies for optimization of cell sources. Results indicated that both approaches—mixing culture-expanded P3 chondrocytes with freshly isolated P0 chondrocytes and expansion in bFGF-supplemented medium—resulted in robust neocartilage formation in nude mice.
- Demonstrated that the size and shape of neocartilage were maintained in a proof-of-concept study using a new-generation, adult-sized human ear-shaped scaffold in a nude rat model.
- Demonstrated formation of engineered autologous cartilage in a sheep model using extended in vitro culture and addition of growth factors to the culture medium. It will be critical for the project team to optimize autologous cartilage formation in the autologous model to further advance the project and demonstrate ear shape retention.

Conclusions

Clinically relevant methodologies can be used to obtain massive populations of chondrocytes necessary for engineering adult-sized human ear-shaped cartilage in vivo without the loss of the ability to form quality cartilage. The new scaffold for adult-sized human ear-shaped cartilage was successfully manufactured and tested in a nude rat model, demonstrating retention of size and shape after 5 weeks in vivo. In vitro culture of seeded constructs prior to implantation improved neocartilage formation in the autologous sheep model. The development of a more mature cartilage matrix prior to implantation possibly may minimize the inflammatory response to scaffold material.

Research Plans for the Next 2 Years

In year 4, the MGH team will continue developing the cell sources for an engineered ear. Primary chondrocytes will be expanded in culture. Up to 100–150 million cells are required for one human-sized ear scaffold. Several strategies to prevent de-differentiation and/or to induce re-differentiation of culture-expanded chondrocytes will be evaluated in vitro. Cartilage formation from re-differentiated chondrocytes will be confirmed in nude mice and in sheep. Neocartilage stability and retention of the size and shape of ear-shaped scaffolds will be assessed in sheep but, due to limited funding currently, only in short-term studies. If additional funding becomes available, long-term studies will be initiated in sheep.

The next generation of full-sized, ear-shaped constructs will be tested in the proof-of-concept studies in sheep, which the project team will complete in year 4 to assess size and shape retention in an immunocompetent sheep model. In preparation for clinical trials, by the beginning of year 5, the researchers will perform a GLP preclinical trial in sheep to demonstrate the safety and efficacy of the engineered ear. Upon obtaining data and analyzing results, the research team will submit a Request for Designation to the FDA to determine the regulatory path for the engineered ear. The team will develop a protocol for a pilot clinical trial and submit it to the local IRB and the U.S. Army Medical Research and Materiel Command's HRPO.

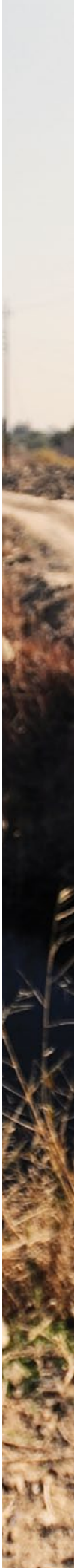
Planned Clinical Transitions

The engineered ear is a combination product; therefore, the MGH team will submit a Request of Designation to the Office of

Combination Products of the FDA to determine the regulatory path. Consequently, the researchers will hold a pre-IND or IDE meeting with the appropriate agency of the FDA and will prepare and file the required documentation. MGH team representatives will audit Kensey Nash Corporation, the manufacturer of the scaffolds, and the Harvard Medical School GMP facility, where cells and seeded scaffolds will be cultured. The research team will develop a clinical study protocol, which will include preclinical data from small and large animal studies, and submit it to the local IRB. After approval from the local IRB is obtained, the researchers will submit the clinical protocol to the U.S. Army Medical Research and Materiel Command for review.

Corrections/Changes Planned for Year 4

The MGH team needs to further develop the source of cells for the engineered ear. Originally it was proposed that MGH collaborators at MIT would supplement primary chondrocytes with MSCs and that this would yield a sufficient cell population for seeding scaffolds the size of the adult human ear. Their results are encouraging; however, it remains unclear whether it will be feasible to use stem cells for auricular cartilage repair in a clinical trial within 2 years. The MGH team is refocusing its attention on expanding primary chondrocytes in vitro and addressing the de-differentiation/re-differentiation issues. The research team will assess the neocartilage stability and retention of the size and shape of ear-shaped scaffolds in sheep initially in short-term studies and, pending further funding, in long-term sheep studies. A preclinical GLP study in sheep cannot be initiated in year 4 with the current level of funding and will be conducted in year 5.




our science for their healing

Regeneration of Ear Using Bioresorbable Polymers and Stem Cells

Project 4.5.4b, RCCC

Team Leader(s): Daniel G. Anderson, PhD and Robert S. Langer, ScD (MIT)

Project Team Members: Nathaniel Hwang, PhD (MIT)

Collaborator(s): Joseph Vacanti, MD, Cathryn Sundback, PhD, Irina Pomerantseva, MD, PhD, Kenneth Rask, MBA, and Gwen Owens (MGH) and Mark Randolph and David Bichara (MGH Plastic Surgery Research)

Therapy: Development of a tissue-engineered ear for clinical applications.

Deliverable(s): Reconstruction of the outer ear using engineered autologous cartilage.

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Key Accomplishments: The researchers have successfully created in vivo systems for efficiently forming cartilage using ASCs combined with biodegradable synthetic polymeric materials.

Key Words: Ear, auricular cartilage, scaffolds, chondrocytes, mesenchymal stem cells, tissue engineering

Introduction

Total external ear reconstruction for traumatic amputation or congenital microtia remains one of the greatest challenges to plastic and reconstructive surgeons. To date, no perfect biomaterial has been fabricated for the replacement of the elastic cartilage normally found in the ear. In addition, the optimal cell populations and the clinically relevant methodology to expand the cells need to be evaluated. The objectives of the MIT group during the past year were to (1) develop a clinically practical, tissue-engineered cartilage that would overcome the limitations of cell-source problems and (2) apply the technology in a preclinical model. To meet these objectives, the researchers initially developed an optimal scheme for expansion and commitment of ASCs in vitro. They also developed a coculture methodology to create phenotypically stable cartilage in vivo. Finally, the team evaluated the properties of engineered cartilage with ASCs and examined whether it would be applicable for use in cartilage regeneration.

In summary, the MIT group is pursuing the following specific aims to achieve the overall deliverable of auricular tissue engineering:

- To utilize morphogenetic factors from chondrocytes.
- To coculture auricular chondrocytes with ASCs.
- To fabricate and evaluate biodegradable scaffolds.

Summary of Research Completed in Years 1 and 2

In year 1, this project was part of Project 4.5.4a. The research team developed an ovine model for subcutaneous implantation of ear-shaped constructs. They engineered cartilage from both ovine and human cartilage cells and ASCs in immunocompromised mice. They also designed ear-shaped scaffolds using computer-aided design principles and tested the scaffolds with numerous materials. During year 2 of the project, the researchers optimized both in vitro and in vivo systems for efficient cartilage formation using ASCs, chondrocytes, and resorbable materials. They identified an optimal biomaterial composition (poly l-lactic acid, poly(l-lactide-cocaprolactone-co-glycolide). They also demonstrated cartilage formation in an animal model.

Research Progress – Year 3

ASCs expanded with growth medium (GM) showed no cartilaginous tissues even with 10 days of in vitro stimulation with transforming growth factor-β1. For ASCs that were expanded in conditioned medium (CM), cells formed small clusters of cartilage throughout the constructs. The pericellular regions of individual cells that had been stained positive for safranin O were distributed throughout the construct **(Figure III-33)**.

Co-seeded constructs with ASCs and auricular chondrocytes resulted in larger clusters of cartilaginous tissue in the constructs. The ASCs adopted a chondrocyte-like phenotype and resulted in a cartilage-like tissue. Furthermore, co-seeded constructs with chondrocytes and CM-expanded ASCs resulted in even more homogeneous safranin O staining throughout the construct with a more even distribution of cartilage ECM. It was possible to observe a clear chondrocyte phenotype with cell lacunae formation particularly in intense safranin O-positive regions in co-seeded constructs. Chondrocyte-only constructs showed intense matrix staining although it was more localized to the central sections of the constructs. A detectable level of elastin was observed near the tissue–scaffold interface in all engineered constructs except for the ones with GM-expanded ASCs (Figure III-33C).

The research team also examined the properties of the tissue-engineered cartilage with or without primed ASCs **(Figure III-34)**. The primed ASCs (i.e., ASCs expanded with CM) and the constructs co-seeded with chondrocytes resulted in significantly high glycosaminoglycan (GAG) and collagen content. Both GAG contents, measured by the dimethylmethylene blue assay, and collagen contents, measured by the hydroxyproline assay, were higher in cartilage tissues formed by the ASCs that had been expanded in the chondrocyte-conditioned medium. In addition, the biochemical contents of the co-seeded constructs were similar to those of scaffolds seeded with chondrocytes alone, indicating that primed ASCs yielded a biochemical contribution similar to that of chondrocytes. This result demonstrated that ASCs primed in a chondrocyte-conditioned medium could undergo in vivo chondrogenic commitment. In addition, when GAG content was normalized to the amount of DNA (i.e., GAG produced by each cell), constructs co-seeded with primed ASCs and chondrocytes resulted in the highest GAG accumulation.

These studies demonstrate that chondrocyte-secreted morphogenetic factors can be used for directing and generating efficient in vivo cartilaginous tissue from ASCs. However, previous reports have indicated that the microenvironment produced during chondrogenesis regulates the subsequent osteogenic

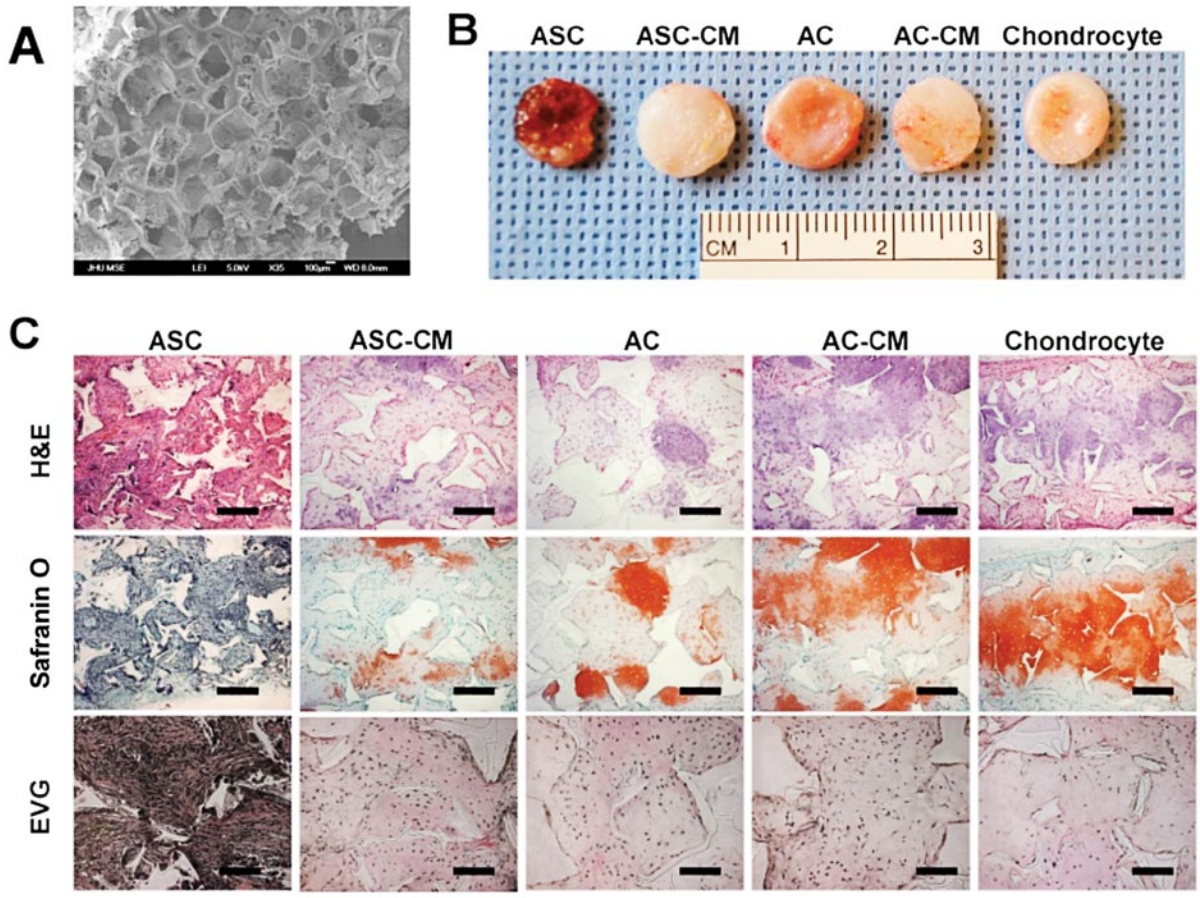


Figure III-33. In vivo engineered cartilage with primed ASCs. (A) Scanning electron microscopy of the scaffold. (B) The gross appearance of engineered tissues after 6 weeks in vivo. (C) Histological finding stained with H&E, safranin O/fast green (safranin O), and Elastica Verhoeff-van Gieson (EVG) after 6 weeks in vivo. ASC: GM-expanded ASCs, ASC-CM: CM-expanded ASCs, AC: Co-seeded constructs with GM-expanded ASCs and chondrocytes, AC-CM: Co-seeded constructs with CM-expanded ASCs and chondrocytes. Bar = 100 mm.

differentiation of MSCs, suggesting that soluble factors from chondrocytes may play a significant role in multiple steps of endochondral ossification. Likewise, in vitro differentiation of MSCs into cartilaginous tissues has been shown to promote tissue hypertrophy and eventual calcification, leading to failure after transplantation. Therefore, the use of ASCs may result in inevitable problems, such as the induction of hypertrophic differentiation of transplanted cells, which can lead to endochondral ossification, which in turn results in stiff and brittle tissues and ultimate failure. However, no hypertrophy of engineered cartilage was observed in this study, indicating that coculture with fully differentiated chondrocytes may provide inhibitory effects on the hypertrophy and endochondral ossification process.

A number of researchers have performed coculture experiments with articular chondrocytes and MSCs and reported that chondrocytes may secrete factors, such as parathyroid hormones/parathyroid hormone-related protein, to prevent hypertrophy of mesenchymal cells. Similarly, the MIT group observed that CM-expanded ASCs resulted

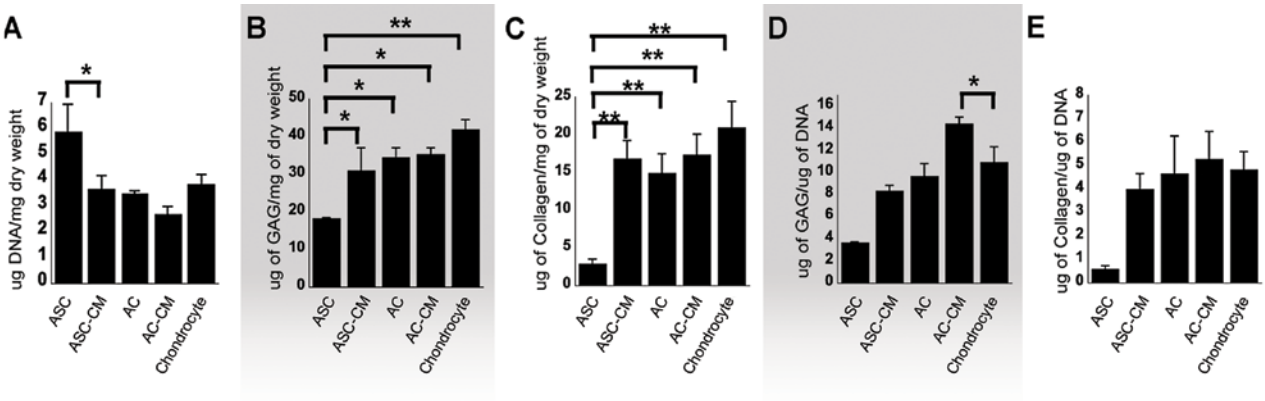


Figure III-34. Analysis of the tissue-engineered cartilage implants harvested after 6 weeks in vivo. (A) DNA amounts, (B) GAG, and (C) collagen were normalized against the dry weight of engineered tissues. In addition, (D) GAG and (E) collagen contents were normalized against total DNA. Values represent the mean ± standard error of four different samples. ASC: GM-expanded ASCs, ASC-CM: CM-expanded ASCs, AC: Co-seeded constructs with GM-expanded ASCs and chondrocytes, AC-CM: Co-seeded constructs with CM-expanded ASCs and chondrocytes. *p <0.05, **p <0.01.

in hypertrophic matrices while chondrocyte co-seeded constructs resulted in no detectable level of type X collagen. The researchers demonstrated that priming the ASCs with CM during expansion and co-seeding the primed ASCs with chondrocytes prior to the in vivo transplantation resulted in optimal cartilage tissues without the evidence of hypertrophic progression of the engineered cartilage.

Key Research Accomplishments

- Optimized a stem cell population for auricular tissue engineering.
- Successfully engineered auricular tissues with stem cells in vivo with biochemical properties similar to native tissues.

Conclusions

To provide an adequate number of cells for tissue engineering of the ear and maximize the formation of cartilage tissue, the MIT group has been investigating how to combine ASCs with (1) optimal soluble factors and (2) morphogenetic factors from chondrocytes. During the past year, the researchers identified a subset of FDA-approved polymeric scaffolds with enhanced cartilaginous tissue formation. They also demonstrated that chondrocyte-secreted morphogenetic factors could be used for directing and generating efficient in vivo cartilaginous tissue from ASCs. No hypertrophy of engineered cartilage was observed in this study, indicating that coculture with fully differentiated chondrocytes may provide inhibitory effects on the hypertrophy and endochondral ossification process. The project team observed that CM-expanded ASCs resulted in hypertrophic matrices while chondrocyte co-seeded constructs resulted in no detectable level of type X collagen. The researchers demonstrated the significant clinical potential of utilizing CM expansion and coculture for auricular tissue engineering, an important result, as the study limited the use of growth factors for the chondrogenic commitment of stem cells.

Research Plans for the Next 2 Years

During the next 2 years, the researchers will perform large animal studies in sheep, collaborating with the MGH group (Project 4.5.4a), which has been working on the autologous use of stem cells for auricular tissue engineering. They are supplementing the chondrocytes with autologous ASCs, and this protocol may hopefully alleviate cell-source issues. For the stem cell-based engineered tissue to be successful, the researchers must monitor the stability and maintenance of the tissue phenotype. Therefore, they will also monitor whether calcification or any tissue resorption is seen in the sheep model.

Planned Clinical Transitions

The researchers will prepare an IDE or IND for clinical application upon successful completion of large animal studies. Furthermore, once the stem cell-based approaches to engineering cartilage are optimized in large animal studies, they plan to work with Concordia Biomedical, a company with clinically approved biomaterials, which will provide a GMP facility for fabricating scaffolds for clinical trials.


SQUIER
GILLEN

Clinical Trial – Composite Tissue Allograft Transplantation (Face)

Project 4.3.1a, RCCC

Team Leader(s): Maria Siemionow, MD, PhD (Cleveland Clinic)

Project Team Members: Cheryl Smith (Reconstructive Research Coordinator, Cleveland Clinic)

Collaborator(s): COL Robert Hale, DDS (USAISR)

Therapy: CTA transplantation (face).

Deliverable(s): Application of a human facial allograft transplant to three subjects who have sustained a life-changing disfigurement with massive facial deformities to provide adequate coverage, aesthetic appearance, and functional outcome.

TRL Progress: 2009, TRL 6; 2010, TRL 6; 2011, TRL 7; Target, TRL 7

Key Accomplishments: The researchers have enrolled three subjects in the study to date. They also developed a workflow process for transplant evaluation and referral, and developed a research database.

Key Words: Composite tissue allograft, immunosuppression

Introduction

The Cleveland Clinic performed the first U.S. face transplant in December 2008. The patient is doing well with no critical untreatable events over the 2 years following her transplant. She is living independently at home in southern Ohio.

The clinical trial Composite Tissue Allograft Transplantation (Face) will provide a means to repair and restore critical facial functions and components that are generally not obtainable with conventional reconstructive surgery. There is an unmet medical need for providing restoration of complex, war-related craniofacial defects in a single-stage procedure. Composite face/bone allograft may provide the restoration of complex, extensive injuries from trauma, burn, and deformity.

Research Progress

The researchers have enrolled three subjects in the clinical trial to date. The process for enlisting a subject for face transplantation is lengthy and complex, and careful consideration is taken to select suitable candidates. They also hired a Reconstructive Transplant Coordinator. The Reconstructive Transplant Coordinator recently transitioned into the Research Coordinator role, and the Transplant Coordinator position has been re-posted.

The researchers developed a workflow process for transplant evaluation and referral. They also developed a research database.

Key Research Accomplishments

- Enrolled three patients in the clinical trial.
- Developed a workflow process for transplant evaluation and referral.
- Developed a research database.

Conclusions

Face transplantation provides a single-stage reconstructive procedure for patients with severe cranial facial injuries, sparing the patient from multiple surgical procedures over many years with superior functional outcomes. U.S. and worldwide success with this procedure has been demonstrated with 15 face transplants currently reported.

Clinical outcomes have been reported in publications and most recently reported at the Tenth Meeting of the International Hand and Composite Tissue Allotransplantation Society. Physicians from France, Spain, and the United States reported on the face transplant recipients living worldwide. As expected with solid organ transplant, side effects of the immunosuppression have been reported as well as rejection. Rejection has been treated, and the side effects are being successfully managed.

Research Plans for the Next 2 Years

Research plans during the next 2 years include the project team's continued evaluation of candidates for face transplant. The researchers also plan to enlist and subsequently perform transplants on two additional patients.

Clinical Trial – Anti-TCR Monoclonal Antibody (TOL101) for Prophylaxis of Acute Organ Rejection in Patients Receiving Renal Transplantation

Project 4.3.1b, RCCC

Team Leader(s): Maria Siemionow, MD, PhD (Cleveland Clinic)

Project Team Members: Stuart Flechner, MD, David Goldfarb, MD, Titte Srinivas, MD, Richard Fatica, MD, Jon Myles, MD, and Cheryl Smith (Cleveland Clinic)

Collaborator(s): University of Colorado, Denver, Baylor University Medical Center, Medical University of South Carolina, St. Barnabas Medical Center, University of Michigan, University of Utah, University of Kentucky, Baylor All Saints, Montefiore Medical Center, and Buffalo General Hospital

Therapy: TOL101, an antibody that may help prevent early-stage organ rejection and reduce unwanted side effects, such as infections, in patients receiving kidney transplants.

Deliverable(s): Kidney biopsy analysis of approximately 172 samples for the clinical trial: A two-part, Phase 1/2, safety, pharmacokinetic, and pharmacodynamic study of TOL101.

TRL Progress: 2009, TRL 4; 2010, TRL 5; 2011, TRL 6; Target, TRL 7

Key Accomplishments: The researchers have finalized the pathology services agreement and SOW for central pathology services (kidney biopsy samples) for the clinical trial protocol. Agreements are being signed. A total of seven subjects have been enrolled in the Phase 1/2 clinical trial to date.

Key Words: TOL101, anti-TCR monoclonal antibody, renal transplantation

Introduction

To reconstruct facial defects, surgeons often transplant tissue from an unrelated human donor. As with other organ transplants such as kidney transplants, the patient who receives the transplant will often have a severe immune response as his or her body tries to fight or reject the foreign tissue. To prevent this response, transplant recipients must typically take drugs for the rest of their lives. This unfortunately makes them more prone to infections or even cancers over time. To solve this problem, researchers are seeking new ways to prevent acute organ rejection that also reduce the incidence of side effects.

TOL101 is a promising new monoclonal antibody that may target immune cells that are critical in the acute organ rejection response. TOL101 will be evaluated in this study for preventing acute organ rejection when used as part of an immunosuppressive regimen that includes steroids, mycophenolate mofetil, and tacrolimus, an immunosuppressive drug used to lower the risk of rejection, in first-time kidney transplant recipients. This report describes the clinical trial of TOL101, formally called Protocol TTI-121, A Two Part, Phase I/II, Safety, PK and PD Study of

TOL101, an Anti-TCR Monoclonal Antibody for Prophylaxis of Acute Organ Rejection in Patients Receiving Renal Transplantation.

The main hypothesis of the clinical trial is that by using a selectively blocking TCR antibody, TOL101 will result in a lower incidence of opportunistic infections; faster recovery of the immune system in kidney allograft recipients, and better functional outcomes as confirmed by kidney function assays and kidney biopsies. The Cleveland Clinic Pathology and Laboratory Medicine Core Laboratory is contributing to this clinical trial through its analysis of the kidney biopsy samples. The project team expects to continue through all phases of the kidney clinical trial, and if results are positive, TOL101 would then be applied as an induction therapy for various kinds of transplants, including composite face/bone allografts. TOL101 could help surgeons to reconstruct complex craniofacial defects for both military service members and civilians by providing a safer antibody that would potentially reduce side effects, such as opportunistic infections, cytokine release syndrome (a type of inflammatory response), and the development of myeloproliferative diseases (i.e., diseases that cause abnormal growth of blood cells in the bone marrow).

Research Progress

To date, seven subjects have been enrolled in the Phase 1/2 clinical trial. The researchers are testing the hypothesis that a more specific approach, using TOL101 to prevent acute organ rejection, may provide similar or better efficacy than the currently used induction antibodies (such as anti-thymocyte globulin or thymoglobulin) while carrying fewer risks in terms of opportunistic infections, malignancies, and adverse effects. The core laboratory is in the process of being set up to receive the kidney biopsy samples for this clinical trial.

The researchers held a meeting to finalize the pathology services agreement and SOW for central pathology services (kidney biopsy samples) for protocol TT1-121. Agreements are in the process of being signed. The researchers also conducted meetings to review the protocol and finalize procedures for core laboratory operations. In addition, discussions were held between Tolera Therapeutics, Inc., and Jane Reese, Clinical Trials Core Database Administrator, regarding the AFIRM clinical trials database.

Key Research Accomplishments

- Enrolled seven subjects in the Phase 1/2 clinical trial to date.
- Finalized the pathology services agreement and SOW for central pathology services for the clinical trial protocol.
- Conducted meetings with Pathology and Laboratory Medicine and Tolera Therapeutics, Inc., to review the protocol and finalize procedures for core laboratory operations.

Conclusions

The TOL101 Phase 1/2 clinical trial will assess the safety and tolerability of TOL101 in patients undergoing their first renal transplantation. Cleveland Clinic Pathology and Laboratory Medicine will serve as the central core laboratory to analyze all of the biopsy samples for the clinical trial. The project team expects to continue through all phases of the kidney clinical trial, and if results are positive as expected, TOL101 will be applied as an induction therapy for various kinds of transplants, including composite face/bone

allografts. TOL101 could help surgeons to reconstruct complex craniofacial defects in both military and civilian patients by providing a safer antibody that would reduce the incidence of side effects, including opportunistic infections, cytokine release syndrome, and the development of myeloproliferative diseases.

Research Plans for the Next 2 Years

During the next 2 years, the project team will conduct central pathology services for Protocol TTI-121. The central pathologist will receive two H&E slides, one Masson's trichrome slide, one periodic acid Schiff slide, and two unstained paraffin slides (day 180/end of study and for-cause biopsy samples only). The central pathologist will stain unstained paraffin slides for C4d, read the biopsy samples according to the Banff 2007 criteria, and report findings to Tolera Therapeutics, Inc. The biopsy reporting form will be used to enter data into the Oncore database of RCCC, located at Case Western Reserve University, according to SOPs. The research plans include completion of all core laboratory services within the next 2 years.

Planned Clinical Transitions

TOL101 is currently in clinical trials beginning with adults undergoing renal transplantation. The planned regulatory pathway includes the design of a Phase 3 trial for this indication at the conclusion of the current study.

IV: Scarless Wound Healing

Background

Scar formation following injury is a major biomedical burden for the U.S. health care system. Both soldiers and civilians suffer from the consequences of dysregulated wound repair, which can lead to severe functional disability and disfigurement. The costs associated with treatment of fibrosis[1] in the United States are estimated to be more than $4 billion per year. Current treatment regimens involving surgery, silicone sheeting, anti-inflammatory medications, and laser/radiation have been disappointing. This is largely due to a lack of understanding of the fibrotic process. The pathophysiology of scar formation suggests the need to regulate numerous aspects of the wound environment, including cells, extracellular matrix (ECM), mechanics, and biochemical signaling.

Wound healing proceeds through overlapping and well-defined phases of repair. This process continues for months and often results in irreversible scar formation with resultant contractures and disfigurement. For any therapeutic approach to be truly successful, it must be comprehensive and encompass the myriad inputs regulating wound healing. Studies of tissue regeneration have implicated the inflammatory environment, matrix components, mechanical context, and cellular players in producing a "scarless" wound profile. The approach taken by AFIRM researchers encompasses a broad continuum of technologies aimed at modulating the tissue response

[1] *The formation of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process.*

RCCC researcher Dr. Marius Costache packages polymer for regenerative medicine devices.

to injury. Collectively, the AFIRM projects represent a collaborative effort to address every aspect and stage of wound repair in a single research program with the overarching aim of developing a more effective wound management paradigm.

Unmet Needs

Effective strategies to promote wound regeneration and prevent scar formation are needed, especially given the increasing survival of injured soldiers returning from the battlefield. The burden of scarring that follows the 230 million surgical procedures performed worldwide each year is enormous. Although the exact incidence of pathologic scarring is unknown, soldiers and civilians continue to suffer from functional disabilities caused by wound contracture and severe disfigurement from hypertrophic scarring. In some instances, the scars become so thick that they limit movement of joints and greatly restrict a patient's ability to move.

Multiple factors are known to influence wound repair (e.g., inflammation and oxygen tension), but therapeutic modalities aimed at these targets have been largely unsuccessful. Although antifibrotic biomolecules have demonstrated effectiveness in vitro, a major hurdle for clinical translation has been the ability to maintain drug release and bioactivity in a complex wound environment. There is also a lack of effective animal models to study scar formation. Therefore, the development of more appropriate and clinically relevant animal models of hypertrophic scarring is an unmet need.

Sometimes battlefield injury progression and/or impaired healing occurs secondary to ischemia or repetitive ischemia/reperfusion (I/R) injury. Currently, no therapy exists to mitigate ischemia- or I/R-induced impaired wound healing. Hence, a critical unmet need is the limitation of impaired healing secondary to ischemia or I/R injury.

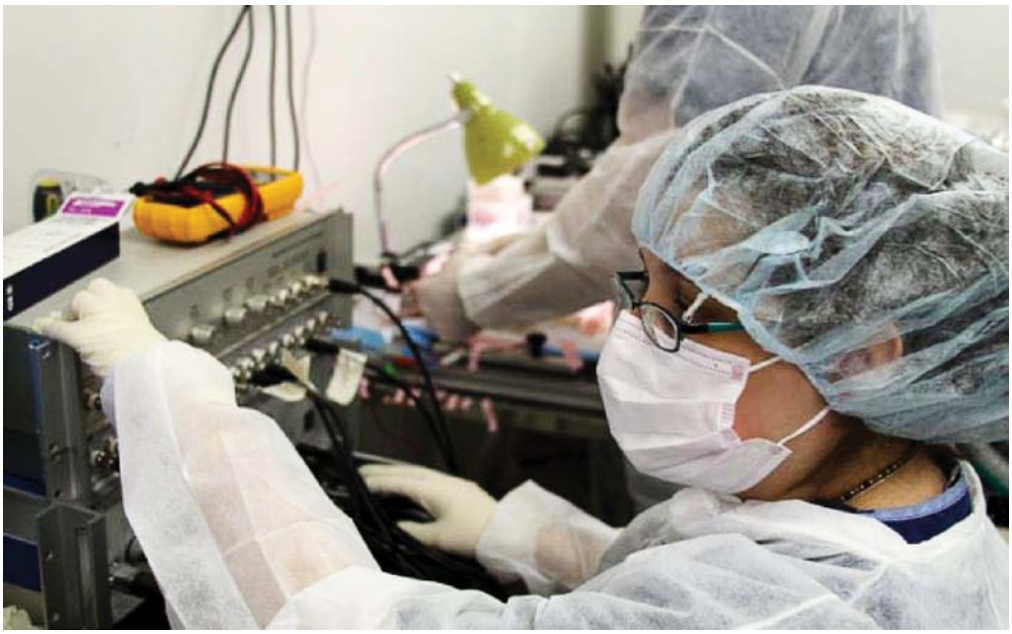
Areas of Emphasis

The AFIRM Scarless Wound Healing Program consists of a synergistic combination of nine leading research groups focusing on every aspect of scarless wound healing. Industrial partners have contributed to the initiation of two clinical trials. This program uses complementary approaches (i.e., device, pharma, and biotechnology) to balance short- and long-term objectives. Projects can be grouped into four "clinical challenge" topic areas: Control of Wound Environment and Mechanics, Therapeutic Delivery to Wounds, Attenuation of Wound Inflammatory Response, and Scar Mitigation. Additional details on projects in each of these topic areas can be found in **Table IV-1** and subsequent sections of this chapter.

Table IV-1. AFIRM-funded projects per clinical challenge topic area.

Clinical Challenge	Consortium/ Institution	Project No.	Project Title
Control of Wound Environment and Mechanics	WFPC*	4.5.1	Mechanical Manipulation of the Wound Environment to Reduce Manifestation of Scar
Therapeutic Delivery to Wounds	RCCC**	4.6.3	Therapy to Limit Injury (TLI) and Promote Non-Scar Healing After Burns and Severe Battle Trauma
		4.7.1	Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management
	WFPC	4.5.2	Regenerative Bandage for Battlefield Wounds
		4.5.5	Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies
Attenuation of Wound Inflammatory Response	WFPC	4.5.3	Multi-Functional Bioscaffolds for Promoting Scarless Wound Healing
		4.5.4	Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration
Scar Mitigation	WFPC	4.5.6	Peptide-Mediated Delivery of Therapeutic Compounds into Injured Tissues During Secondary Intervention
		4.5.7	Scar Mitigation via Matrix Metalloproteinase-1 Tertiary Therapy

**Wake Forest-Pittsburgh Consortium*
***Rutgers-Cleveland Clinic Consortium*



A WFPC researcher measures muscle force in injured muscle after treatment of experimental animals with antifibrotic medicines.

Control of Wound Environment and Mechanics

Studies at WFPC

Using a mouse model of hypertrophic scarring based on increasing the skin stress of healing wounds, the **Gurtner group (Project 4.5.1)** at Stanford University found that the skin's biomechanical properties correlated with the amount of scarring following wounding. They have developed a pressure-sensitive, "stress-shielding" device that can modify mechanical forces to control scar formation in the red Duroc pig model. In an exploration of the molecular mechanisms underlying this process, the researchers have focused on the molecular target focal adhesion kinase (FAK). They have shown in preliminary studies that FAK is a key mediator of load-induced fibrosis and scar formation.

Over the next 2 years, the Gurtner team will continue to investigate the molecular mechanisms underlying the development of scar formation using a novel mouse model. The researchers will attempt to define clinically relevant pathways that lead to post-injury scarring.

Therapeutic Delivery to Wounds

A novel approach is needed to alter the trajectory of wound healing in the initial days following injury to promote the regeneration of tissue. Such an approach could involve the delivery of cells, molecules, proteins, or genes to the wound surface.

Studies at RCCC

The **Mustoe group (Project 4.6.3)** at Northwestern University is investigating the wound-healing capability of curcumin in a rabbit ear model of I/R injury, which involves deficient blood flow to a tissue due to the injury followed by the return of blood to the damaged area. The researchers found that two micromolar doses of intravenous curcumin accelerated healing and reduced scarring in the rabbit I/R model. They also

found that they could apply curcumin via tiny nanospheres to the wound site in the rabbit I/R model with no apparent toxicity. However, curcumin nanospheres applied to I/R wounds in the rabbit model had no apparent effect on epithelialization. During the upcoming year, the researchers will begin testing the efficacy of intravenous curcumin in a porcine flap model under normal and compromised perfusion conditions. They will also coordinate plans for a clinical trial with Dr. Richard Clark's group at Stony Brook University.

The **Katz group (Project 4.7.1)** at the University of Virginia is developing regenerative therapies using adipose-derived stem cells (ASCs). The researchers are developing a "Regenerative Wound Paste" (RWP) platform for skin repair and replacement that involves the combination of ASCs (and other cells) and a cell-free dermal scaffold. They have initiated their wound paste strategy and formulation studies. They formulated RWP with cell-stabilizing solutions that maintained cell viability and growth over a 2-week period in culture. They also determined that cell-enhanced RWP reduced wound contraction in a mouse wound model more effectively than wound paste lacking cells. In addition, they found no evidence of detrimental effects related to delivery of a high dose of ASCs in mice in a long-term tumorigenesis and migration study. Over the next 2 years, the researchers plan to advance the RWP technology into Phase 1 clinical trials. In the upcoming year, they will carry out a strategic analysis of potential regulatory paths and will hold a pre-Investigational New Drug (IND) meeting with the U.S. Food and Drug Administration (FDA) to clarify the design and completion of specific preclinical studies necessary for clinical trials.

Studies at WFPC

The **Gurtner/Longaker group (Project 4.5.2)** at Stanford University is capitalizing on the ability of wounded fetal tissue to regenerate with minimal scarring by developing a regenerative bandage that contains a fetal-like matrix and wound progenitor (stem) cells. The goal is to maintain an acute wound in a state that favors regeneration instead of scarring, fibrosis, and infection. The researchers have developed a novel, modifiable hydrogel scaffold that can deliver matrix components, cells, and/or wound-healing drugs. They have determined that this composite matrix is highly biocompatible with numerous cell types important for wound repair, including endothelial cells, fibroblasts, and mesenchymal stem cells (MSCs). They seeded the hydrogels with MSCs and observed increased expression of genes and cytokines that are characteristic of stem cells and are known to be involved in wound healing. Over the next 2 years, the research team will continue to optimize its stem cell-seeded hydrogel construct to maximize its regenerative profile in the living organism. The researchers will begin to transition from murine MSCs (mMSCs) to human ASCs, which represent an ideal tissue source for candidate patients. The team will similarly begin to transition its scaffold construct toward therapeutic utility in battlefield situations with the goal of engineering portable, dry hydrogels that can rapidly expand to porous scaffolds when placed in an injured soldier.

The **Kathju group (Project 4.5.5)** at the Allegheny-Singer Research Institute continues to progress in the use of tiny nanoparticles as a nonviral means of delivering molecules into wounds. The researchers identified a gene (chaperonin-containing T-complex polypeptide [CCT-eta]) that is normally decreased in healing fetal wounds but elevated in adult wounds. They developed a nonviral nanoparticle-mediated delivery system using small inhibitory RNAs (siRNAs) that can selectively decrease the expression of CCT-eta in complex adult wounds. They found that this therapy could effectively inhibit scar formation without any deleterious effects on wound

healing. The research team also found that a novel probiotic therapy using the bacterium *Lactobacillus plantarum* for infected burn wounds could effectively abolish pseudomonal (and possibly other) infections and significantly decrease the scarring that can ensue following the infection of burn injuries. Over the next 2 years, the researchers will continue to explore even more efficient means of delivering their siRNAs to wounds. They will also determine whether ultrasound-mediated gene transfer can be used in conjunction with their technology to improve results. The research team will also test the ability of probiotics to reduce local and systemic inflammation elicited by an infected burn wound and determine how to counteract other burn wound pathogens with probiotics.

Attenuation of Wound Inflammatory Response

Following injury, an intense inflammatory response ensues and is necessary for normal wound healing. However, aberrations in this process result in chronic wounds and have been strongly implicated in fibrotic scar formation. Redirecting this process toward a regenerative outcome requires controlling the inflammatory response and is the focus of two AFIRM projects.

Studies at WFPC

The **Washburn group (Project 4.5.3)** at Carnegie Mellon University is developing hyaluronic acid (HA) biogels that contain monoclonal antibodies or peptides (short versions of proteins) with specific affinities for cytokines and other mediators of inflammation to absorb proinflammatory cytokines and decrease inflammation. The researchers have identified that a biogel with HA conjugated to an antibody against tumor necrosis factor-alpha (TNF-α) can effectively inhibit burn progression and control inflammation in a rat burn model. They plan to determine the minimal number of doses necessary to achieve reduced burn progression in their rat model as well as gain an improved understanding of the mechanism of their conjugates in reducing burn progression. They also plan to validate their conjugates in a pig burn model, which is the gold standard in the regulatory approval process for burns. Due to fundamental similarities in early inflammatory responses across species, they expect a similar response in pigs and (eventually) humans. The research team's planned clinical transition will involve Washburn Therapeutics. This company has an exclusive license for patents filed by Carnegie Mellon University and has formed a partnership with a manufacturer of anti-TNF-α that can be used in humans.

The **Hebda group (Project 4.5.4)** at the University of Pittsburgh's McGowan Institute for Regenerative Medicine is working to clarify fibroblast and inflammatory mediator interactions with the goal of developing novel anti-inflammatory therapies to improve the quality of healing. The research team demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and prostaglandin E2 (PGE2) attenuated the wound inflammatory response, which led to the promotion of healing. However, the researchers found that at longer time points (3 or more weeks), wound tensile strength tended to be similar among vehicle (control) and treatment groups; that is, the vehicle "caught up" to the treatments for this outcome measure. They feel that this phenomenon may be due to the advanced healing capacity of the rat, which warrants the use of other animal models. The researchers also showed that early, one-time topical treatment with isogenic ASCs, fetal skin fibroblasts (embryonic day 15), and even adult skin fibroblasts, leads to reduced scarring and increased healing. In the next 2 years, the

USAISR researcher SPC Jaffster Daus removes boxes containing rabbit adipose stem cell samples from a liquid nitrogen storage container. The stem cells are being used to study wound healing in collaboration with RCCC researchers Dr. Thomas Mustoe and Dr. Seok Jong Hong.

researchers will combine anti-inflammatory treatments with donor fibroblast delivery to see whether donor fibroblasts (of favorable phenotypes) have a greater impact on healing outcomes. The focus of these studies will be the basis for developing a wound treatment regimen for a future Phase 1 clinical trial. These clinical studies will likely commence after year 5 of this project.

Scar Mitigation

AFIRM researchers are studying transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase 1 (MMP-1) in scar mitigation. TGF-β1 has been shown to be a major factor in wound repair and skin fibrosis while MMP-1 has been implicated in the repair of muscle scars and has been shown to improve muscle regeneration when directly injected into fibrotic muscle.

Studies at WFPC

The **Ruoslahti group (Project 4.5.6)** at the Sanford-Burnham Medical Research Institute has made substantial progress in identifying peptides that home to wounds and can deliver a therapeutic payload to the wounds and other injured tissues. The researchers found that treatment of mice with skin wounds with the wound-homing CARSKNKDC (CAR) peptide promotes wound healing. They generated a smaller form of the CAR peptide, tCAR, which is biologically more potent than CAR. They also fused the CAR peptide with the wound-homing decorin protein and found that the CAR-decorin fusion protein selectively reduced levels of the scar-inducing forms of TGF-β. Over the next 2 years, the researchers plan to focus on the cell- and tissue-penetrating properties of the CAR peptide and its use in enhancing wound and scar penetration of co-administered compounds. They

will also focus on characterizing tCAR and improving the properties of the CAR-decorin fusion protein to facilitate regulatory approvals and commercial production. Additionally, the researchers will study the molecular basis of the wound healing-promoting activity of the CAR peptide to facilitate the transition of this treatment into the clinic.

The **Russell/Koepsel group (Project 4.5.7)** at the University of Pittsburgh is examining MMP-1 collagen matrix interactions in vitro and in preclinical animal studies. The researchers developed a method for manufacturing active human MMP-1 that results in a single homogeneous product without degradation products, something not previously attainable. Because of disappointing results with a chemically modified enzyme (loss of activity), the researchers decided to proceed with the native enzyme for future studies. Their results to date suggest that a single dose of MMP-1 will not be sufficient to remove a scar and that multiple doses will be required. The researchers recently developed and validated a mathematical model for the residence time and potential activity of a bolus injection of MMP-1 at a scar site that is based on the kinetic behavior of the enzyme. This model has allowed the researchers to bypass a planned dose-ranging preclinical trial protocol in rats and proceed directly to a multiple-dosing phase of the preclinical plan.

Clinical Trials

Several AFIRM Scarless Wound Healing projects have technologies that have advanced to the human clinical trial stage. Additional details on these clinical trials can be found in **Table IV-2** and subsequent sections of this chapter.

The **Beasley group (Project 4.5.9)** at Neodyne Biosciences, Inc. (Neodyne) has collaborated with the Gurtner group (Project

Table IV-2. AFIRM-funded Scarless Wound Healing projects with pending, active, or completed clinical trials.

Project Title	Consortium	Project No.	Trial Phase	Current Status
Neodyne's Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation – Clinical Trial	WFPC	4.5.9	Phase 1 First-in-Man	Completed
			Phase 2 (Second-Generation Device)	Completed
			Phase 3 (Third-Generation Device)	Protocol Approved by IRB*
Autologous Fat Transfer for Scar Prevention and Remodeling (AFT-SPAR) Clinical Trial	RCCC	4.7.3	Phase 1/2	Open

*Institutional Review Board

4.5.1) at Stanford University to complete a Phase 1, first-in-man study demonstrating safe and dramatic reduction in hypertrophic scar formation using its polymeric stress-shielding devices. The study outcomes demonstrated a significant and dramatic reduction in scar formation in treated wounds compared to untreated within-patient controls. The researchers also completed a pilot clinical study with a second-generation device on 61 patients after abdominoplasty, scar revision, breast lift, and/or breast reduction procedures. Results of the second-generation pilot study suggested that there is a narrow range of strain levels that provide optimal scar reduction, which supports the need for precise control of skin strain. The researchers subsequently finalized the design of an improved, third-generation, stress-shielding device. They received approval from the Human Research Protection Office (HRPO) and IRB to conduct a clinical trial to test the new design and are preparing to begin the clinical trial. During the next 2 years, they will further refine the stress-shielding device to custom-design treatments for various size wounds and tension states. This will allow for body-specific, regional stress-shielding to address a wide variety of surgical wounds.

Autologous fat transfer (AFT) is a procedure that involves removing adipose tissue from one area of a patient's body and immediately transplanting it to a different site in the same person. The **Katz group (Project 4.7.3)** at the University of Virginia is conducting a Phase 1/2 clinical trial designed to test the safety and efficacy of using AFT for scar prevention and remodeling (AFT-SPAR). Patient enrollment began in July 2010. To date, the researchers have treated eight patients within the study protocol and enrolled three additional patients for treatment. They have completed numerous revisions to the study protocol as needed, and their Manual of Operations is nearly finalized. They are currently focused on increasing enrollment through local advertisements and regional networking, and by establishing the U.S. Army Institute of Surgical Research (USAISR)/Brooke Army Medical Center (BAMC) as a second treatment site.

Mechanical Manipulation of the Wound Environment to Reduce Manifestation of Scar

Project 4.5.1, WFPC

Team Leader(s): Geoffrey C. Gurtner, MD (Stanford University)

Project Team Members: Michael T. Longaker, MD, MBA and Reinhold Dauskardt, PhD (Stanford University)

Collaborator(s): Neodyne Biosciences, Inc. and the Biomaterials and Advanced Drug Delivery Center at Stanford University

Therapy: Control of wound environment to minimize scarring.

Deliverable(s): Novel molecular targets in scar mechanotransduction and drug-eluting mechanomodulatory scaffolds capable of mitigating fibrosis.

TRL Progress: Start of Program, TRL 4; End Year 1, TRL 4; End Year 2, TRL 6; End Year 3, TRL 7

Key Accomplishments: The researchers validated their excisional wound model, showing that their novel stress-shielding polymer device could be effective for wounds more complex than linear incisions. They examined cellular, vascular, and myofibroblast densities in the following four states: unwounded, stress-shielded, natural physiologic stress, and elevated stress incisions. They confirmed histologically that stress-shielded wounds and unwounded skin exhibited a similar pattern of fibrosis while elevated and physiologic stress incisions had a wound phenotype consistent with scar formation. The research team identified a key role for the molecular target FAK in scar mechanotransduction. The researchers have begun cell-based and rodent studies to test their hypothesis that FAK drives the mechanical activation of fibrosis.

Key Words: Hypertrophic scarring, wound device, mechanotransduction, fibrosis

Introduction

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. Multiple factors are known to influence wound repair (e.g., inflammation, oxygen tension, and ischemia), but therapeutic modalities aimed at these targets have been largely unsuccessful. Mechanical force has long been recognized to influence cellular behavior in vitro, and clinical observations based on Langer's lines and hypertrophic scarring corroborate this phenomenon in vivo. Recently, the Gurtner laboratory published the first murine model of hypertrophic scarring based on increasing the skin stress of healing wounds. They found that intrinsic skin mechanics correlated with scarring phenotype following wounding as low mechanical stress fetal wounds exhibit minimal fibrosis, and stiffer human skin displays robust scarring. These findings prompted the researchers to examine the role of mechanical stress in scar formation and to develop a novel device to actively control wound environment mechanics to mitigate fibrosis. The researchers ultimately aim to create battlefield-ready, region-specific devices for different wounded areas of the body capable of precision stress shielding of mechanical forces to minimize scar formation.

There are currently no commercially available wound care products that specifically address the mechanical stress state of healing wounds to reduce scarring. Elastic bandages and pressure dressings provide a widely

variable range of compressive forces and are generally used for hemostatic purposes, not directly for scar attenuation. Negative pressure wound sponge devices (WoundVac) are used on large, open exudative wounds but require elaborate components and an electrical energy source. In contrast to existing wound care options, this technology enables precision stress shielding of area-specific wound forces through a portable, ready-to-use, simple pressure adhesive dressing that can be readily employed on the battlefield immediately following injury. This technology has not been achieved elsewhere, and the researchers continue to make significant progress in this application.

Current mechanotransduction literature implicates a central role for cell matrix interactions in scar mechanotransduction. Specifically, FAK has been identified as a potential target in the mechanical activation of inflammation and fibrosis. This molecular target may be a driving force in the formation of human hypertrophic scarring.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers determined that the Duroc pig is an ideal choice for biomechanical skin studies. They developed the first generation of safe, durable, and modifiable simple pressure adhesive dressings that could modify mechanical forces and alter scarring and fibrosis after injury. They determined that the dressings could be used on incisional swine wounds to effectively and reliably regulate wound stress and fibrosis following injury. During the second year of this project, the researchers completed a first-in-human study, which demonstrated that mechanical forces can be effectively off-loaded with a polymer device to prevent hypertrophic scar formation. They found a significant reduction in scar formation in wounds treated with the stress-shielding device compared to untreated wounds. Overall, these findings demonstrate that mechanotransduction pathways are critical in scar formation.

Research Progress – Year 3

The researchers validated their excisional wound model, demonstrating that their novel stress-shielding polymer device can be effective for wounds more complex than linear incisions **(Figure IV-1)**. They examined cellular, vascular, and myofibroblast densities in the following four states: unwounded, stress-shielded, natural physiologic stress, and elevated stress incisions. They confirmed histologically that stress-shielded wounds and unwounded skin exhibited a similar pattern of fibrosis while elevated and physiologic stress incisions had a wound phenotype consistent with scar formation **(Figure IV-2)**.

The research team performed bioinformatics analysis of several animal models of hypertrophic scarring and identified a central role for FAK. The researchers generated a fibroblast-specific FAK knockout mouse strain to test the mechanical activation of scarring using their hypertrophic scar model. Validation studies confirm temporal and spatial control of FAK expression both in vitro and in vivo **(Figure IV-3)**. Preliminary hypertrophic scar model studies indicate that FAK critically regulates the mechanical activation of scar formation **(Figure IV-4)**.

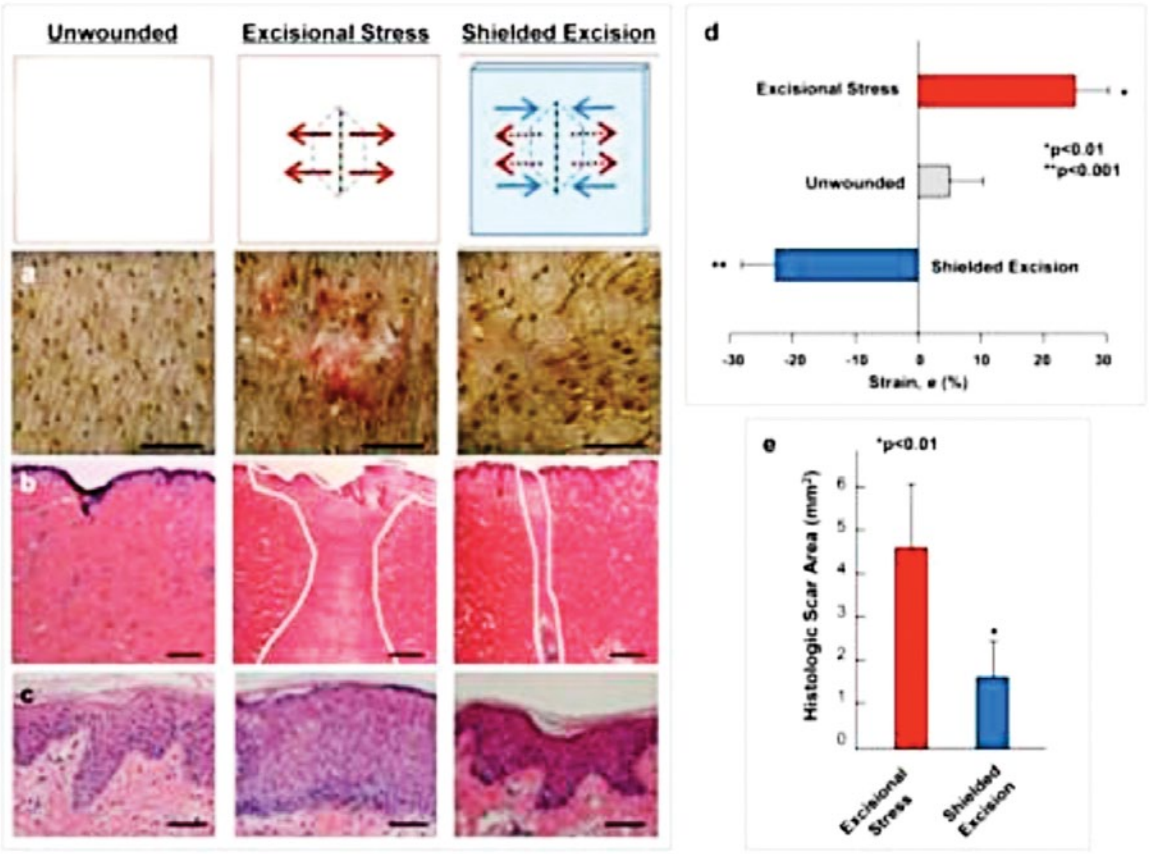


Figure IV-1. Validation of the excisional wound model. Excisional wounds closed under elevated mechanical stress demonstrated significant gross and histologic scar formation. However, when the same excisions were stress shielded, there was virtually no scar formation. Further, the epithelial architecture of stress-shielded wounds recapitulated the form of unwounded epidermis (row C), suggesting that wound regeneration is occurring.

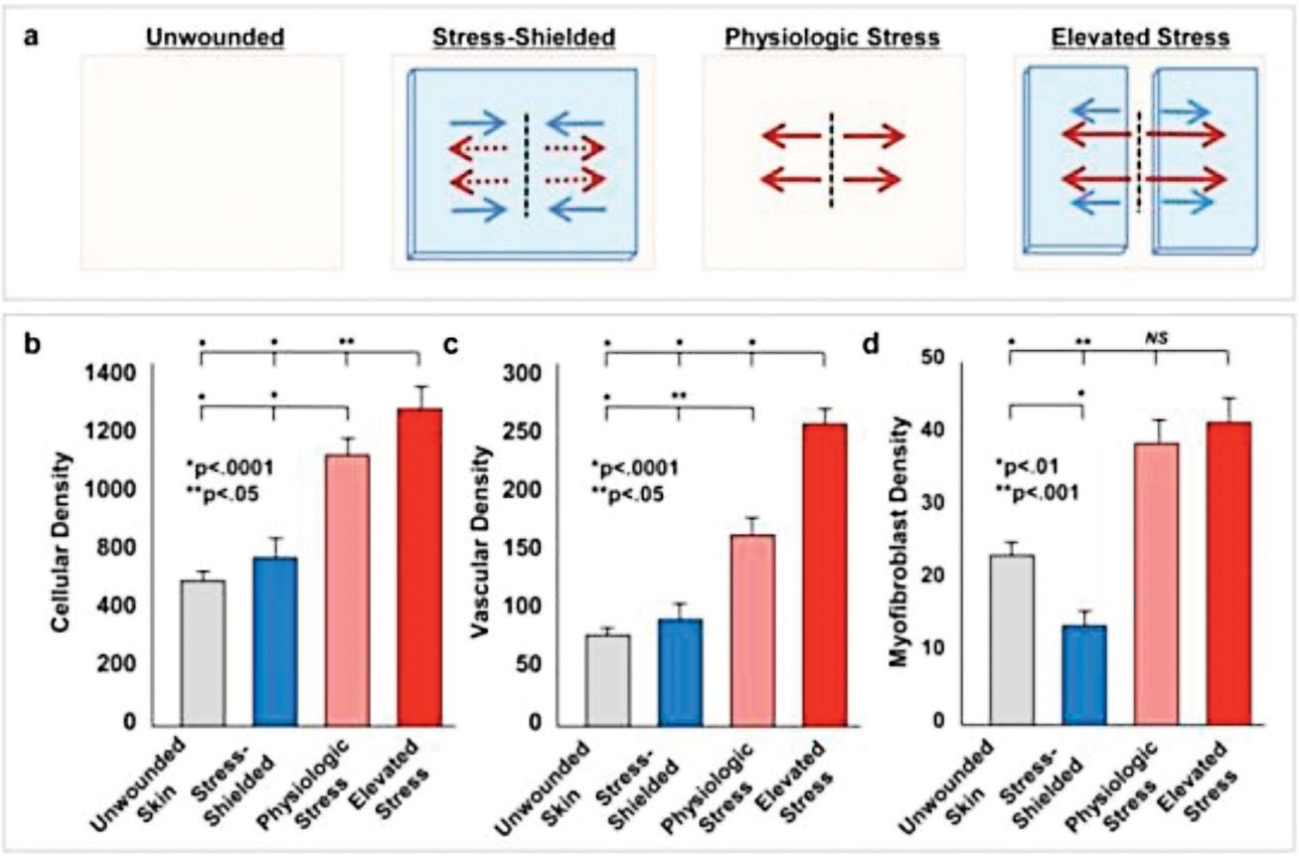


Figure IV-2. Histologic markers of fibrosis are reduced by stress shielding. The cellular, vascular, and myofibroblast densities were calculated for each wound/stress state: unwounded, stress-shielded, natural physiologic stress, and elevated stress incisions. Histologic quantification confirmed that stress-shielded wounds had a fibrotic phenotype similar to that of unwounded skin while elevated and physiologic stress incisions had a typical wound phenotype consistent with scar formation. These data support the hypothesis that mechanical wound forces can be manipulated to affect fibrosis and repair, and sets the stage to test this device in humans.

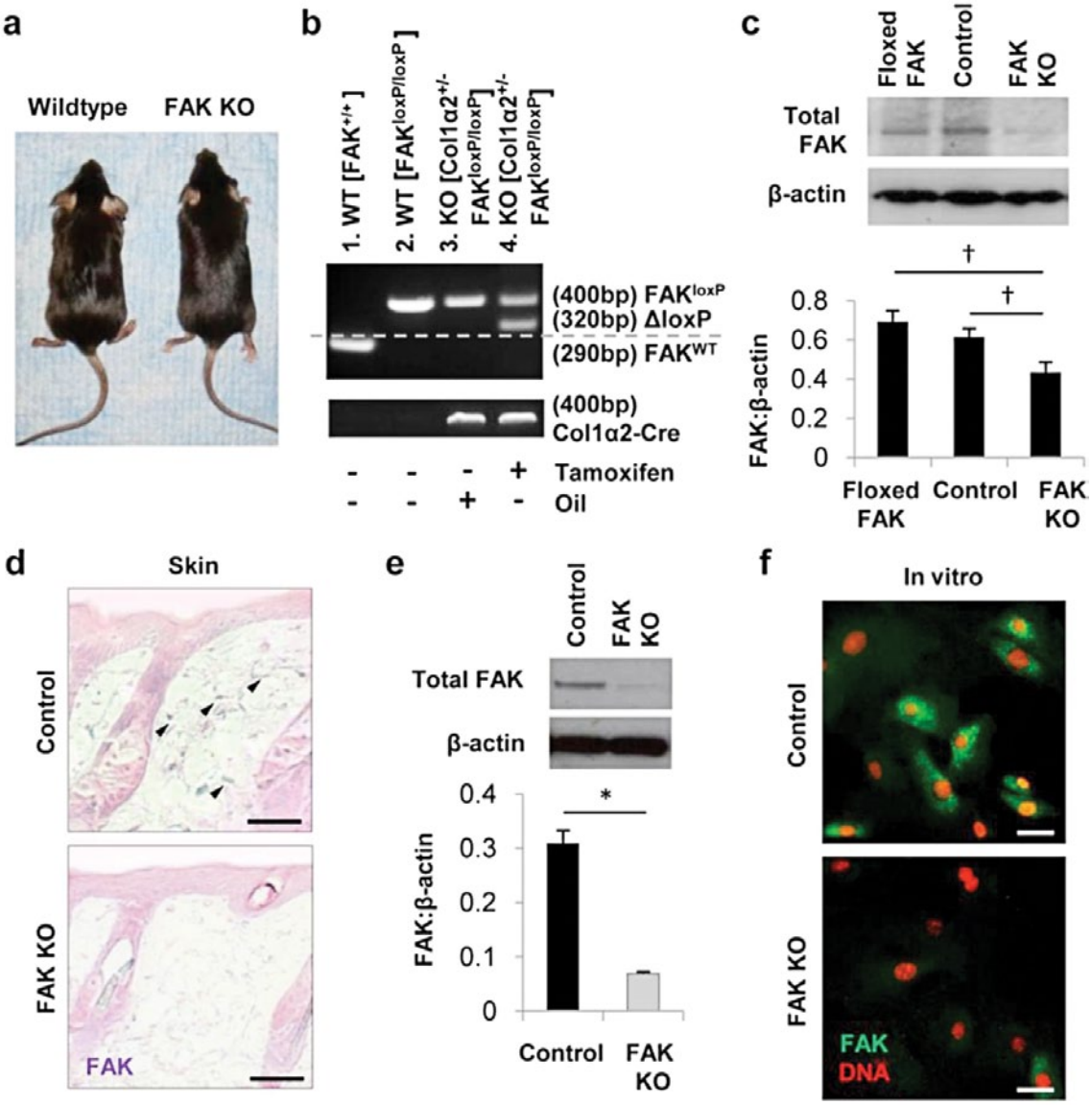


Figure IV-3. Validation of FAK knockout mice. (a) Photograph of age-matched wild-type and FAK knockout mice. (b) Polymerase chain reaction (PCR) confirmation of tamoxifen-dependent excisive recombination of FAK. 'FAK^{loxP}' is floxed FAK band, 'ΔloxP' is recombined band after FAK excision, and 'FAKWT' is wild-type band. (c) Quantification of FAK densitometry from unwounded skin, n=6. (d) Immunolocalization of FAK in unwounded skin. Arrowheads point to dermal FAK signal. Scale bar 50 μm. (e) Quantification of FAK densitometry from harvested primary dermal fibroblasts, n=6. (f) FAK immunofluorescence images of adult dermal fibroblasts in vitro. Scale bar 50 μm. Values represent means ± s.e.m. *p <0.001; †p <0.01.

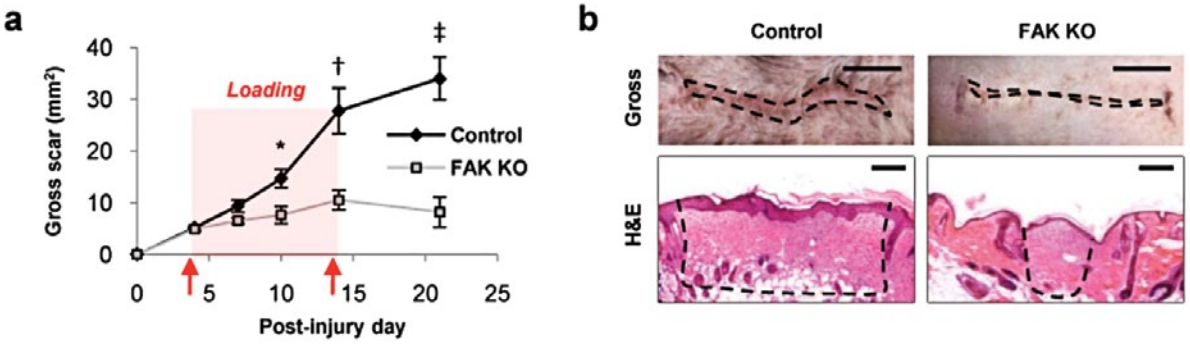


Figure IV-4. (a) Load-induced hypertrophic scar model applied to control and FAK knockout mice. (b) Gross photographs and histologic images of post-injury day 10 scars. Scar boundaries demarcated with dashed black lines. Note the significant decrease in scar formation in fibroblast-specific FAK K=knockout mice. Values represent means ± s.e.m.; scale bar 0.5 cm gross photographs; 200 μm in b. *p <0.05, †p <0.01, ‡p <0.001.

Key Research Accomplishments

- Validated the excisional wound model, which showed that the novel stress-shielding polymer device could be effective for wounds more complex than linear incisions.
- Examined cellular, vascular, and myofibroblast densities in the following four states: unwounded, stress-shielded, natural physiologic stress, and elevated stress incisions.
  - ♦ Confirmed histologically that stress-shielded wounds and unwounded skin exhibited a similar pattern of fibrosis while elevated and physiologic stress incisions had a wound phenotype consistent with scar formation.
- Identified a central role for FAK in hypertrophic scar formation using bioinformatics analysis.
- Generated and validated fibroblast-specific FAK knockout mice.
- Showed that application of the hypertrophic scar model to FAK knockout mice has a significant decrease in wound fibrosis.

Conclusions

The Stanford University group has successfully extended preclinical studies to Phase 1 clinical trials during the past 2 years. Proof-of-concept research in the red Duroc pig has yielded significant insight into the ability of polymeric devices to externally manipulate the mechanical wound environment. The researchers have developed models to study the role of mechanotransduction in fibrosis in a large animal known to scar in a similar fashion to human hypertrophic scarring, and these models will highly benefit the scientific research community. The polymeric device has proven extremely safe and effective in human patients, and the research team has achieved a dramatic and significant reduction in scar formation in a Phase 1 trial. To study the molecular mechanisms underlying this process, the Stanford University group has identified the molecular target FAK. The researchers have generated fibroblast-specific FAK knockout mice, and preliminary studies indicate that FAK is a key mediator of load-induced fibrosis.

Research Plans for the Next 2 Years

During the next 2 years, the researchers have several goals related to the basic science of skin mechanobiology and the characterization of pig and human biomechanical forces using noninvasive technologies. They plan to investigate the molecular mechanisms underlying skin mechanofibrosis using a novel mouse model and to define clinically relevant pathways that lead to post-injury fibrosis. They will utilize finite element methods and digital image correlation (DIC) technologies to predict tensional states of various wounds in different regions of the body. This will guide the development of their stress-shielding polymer device and to expand its clinical indications.

Planned Clinical Transitions

Neodyne is in the process of starting Phase 2 trials that will recruit a larger patient population. In conjunction with the Materials Science and Engineering department at Stanford University, the researchers will further refine the polymeric device to custom-design treatments for various size wounds and tension states. This will allow for body-specific regional stress shielding to address a wide variety of surgical wounds.


our science for their healing

Therapy to Limit Injury (TLI) and Promote Non-Scar Healing After Burns and Severe Battle Trauma

Project 4.6.3, RCCC

Team Leader(s): Thomas Mustoe, MD (Northwestern University)

Project Team Members: Sheng-Xian Jia, Seok Jong Hong, Mathew Geringer, and Marina Vracar-Grabar (Northwestern University)

Collaborator(s): Richard Clark, MD (Stony Brook University) and Larisa Sheihet (Rutgers/New Jersey Center for Biomaterials)

Therapy: Curcumin, enhance healing and attenuate scarring.

Deliverable(s): Intravenous treatment with curcumin.

TRL Progress: 2009, TRL 3; 2010, TRL 4; 2011, TRL 4/5

Key Accomplishments: The researchers obtained evidence of intravenous curcumin treatment efficacy in the I/R pre-injury rabbit ear model. They found that the effectiveness of nanosphere-gel technology and topical delivery of curcumin in the rabbit ear wound healing model is inconclusive.

Key Words: Burn, wound healing, hypertrophic scarring, ischemic injury, ischemia/ reperfusion, rabbit ear wound model, curcumin

Introduction

Battlefield and burn patients require specialized care and large amounts of resources. Thermal injuries are a significant source of morbidity and mortality constituting 5%–20% of all injuries and 4% of deaths. Blast injuries affect multiple body organs and systems and have devastating acute and chronic consequences. If wound epithelialization cannot be completed as quickly as needed, there is often significant scarring and wound contracture, ultimately leading to physical deformity, discomfort, and poor quality of life.

One of the unmet needs is the prevention of injury progression upon reperfusion when blood supply returns to the tissue after the period of ischemia (restriction in blood supply resulting in tissue damage). Reestablishment of blood flow generates oxidative stress that leads to cellular damage and subsequent inflammatory response. To date there is no successful therapy that could be used at the time of injury or soon thereafter to diminish the severity of I/R injury and sustain healing. Curcumin, an agent with proven anti-inflammatory, antiapoptotic, and antiproliferative properties, could potentially fulfill that role.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers demonstrated the wound-healing potential of systemic pure curcumin and the significant reduction of scarring in the nonischemic rabbit ear model. They developed an I/R injury rabbit ear model to study the therapeutic effect on wound healing in compromised vascular conditions. They found that xenogeneic cells in the rabbit ear model induced inflammation and scarring. Finally, systemic delivery of fibronectin peptide P12 in the rabbit ear model had little or no effect on wound healing, suggesting the predominant mechanism is limiting injury progression rather than accelerating wound healing.

Research Progress – Year 3

Aim 1. Effect of Intravenous Delivery of Curcumin in the I/R Rabbit Ear Model

To confirm initial observations, researchers completed the histologic evaluation and data analysis of additional experiments with a 2 µM curcumin dose (n=16). Two animals had the procedure to create wounds and received repeated I/R cycles. Following the procedure, tissue was collected and processed for histological evaluation. Curcumin given intravenously at 1 and 2 µM blood concentrations significantly promoted wound epithelialization and sustained healing when compared to wounds receiving I/R cycles but no drug treatment **(Figure IV-5)**.

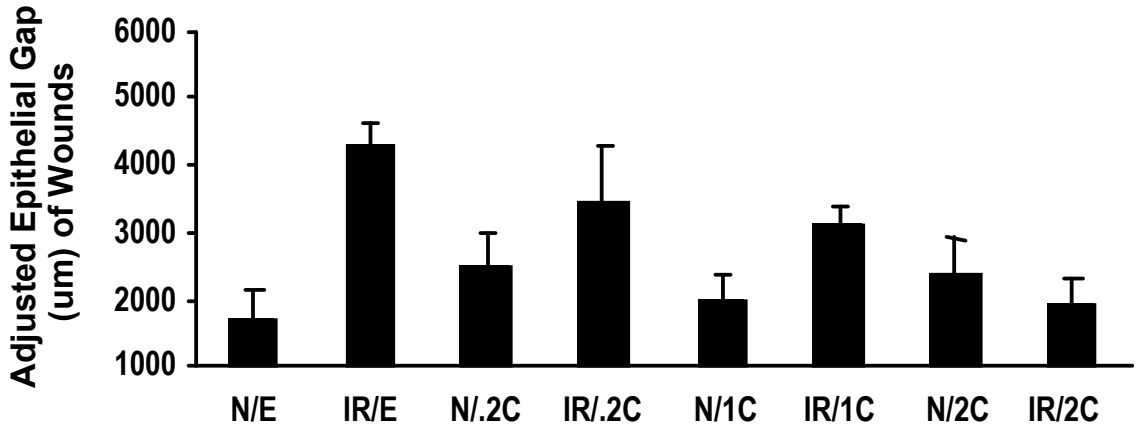


Figure IV-5. Wound epithelialization in the I/R injury rabbit ear model upon curcumin treatment. The 1 and 2 µM concentrations of curcumin had a statistically significant beneficial effect on wound healing in the I/R model. t-test, $p \leq 0.05$; p-value IR/E versus IR/0.2C = 0.364; IR/E versus IR/1C = 0.016; IR/E versus IR/2C = 0.0003. Control non-I/R (N/E) refers to the group that had not received I/R cycling nor curcumin treatment (wounding and Et-OH/phosphate buffered saline [PBS] vehicle injection only); IR/E refers to the group that received I/R but no curcumin; N/.2C refers to non-I/R control that received 0.2 µM curcumin; IR/.2C refers to I/R cycling and 0.2 µM curcumin; N/1C refers to non-I/R control that received 1 µM curcumin; IR/1C refers to I/R cycling and 1 µM curcumin; N/2C refers to non-I/R control that received 2 µM curcumin; IR/2C refers to I/R cycling and 2 µM curcumin.

Epidermal area as a measure of epidermal thickness and keratinocyte proliferation did not show consistent results across several curcumin doses tested in the I/R model. Granulation tissue formation was markedly increased with a 2 µM curcumin dose (**Figure IV-6**, not statistically significant), suggesting that curcumin stimulates new dermal tissue formation in I/R injury.

For optimal clinical applicability, it is useful to know if curcumin is also effective when given at a specific time after injury. In a preliminary set of experiments, curcumin was administered after surgery to induce I/R injury, and later after I/R injury had occurred, to test the possible protective effect of curcumin. The control ear was intact and had not undergone ischemic surgery or I/R cycles. When compared to control non-I/R ears, those receiving I/R cycles upon curcumin treatment re-epithelialized better as measured by the size of the epithelial gap. Curcumin given intravenously on days 4 and 6 postoperatively significantly improved wound epithelialization and sustained healing in the I/R injury. Histological evaluation of granulation tissue formation indicates that there was no difference between I/R wounds and control wounds upon curcumin treatment.

Aim 2. Effect of Topical Delivery of Curcumin Nanospheres in the Rabbit Ear Model

A tyrosine-derived nanosphere drug delivery approach for wound healing was studied in collaboration with researchers at Rutgers University, who provided the curcumin nanosphere formulation. The goal was to validate targeted delivery of curcumin and the efficiency of the nanospheres-gel. Ten rabbits were used in this particular set of experiments in an attempt to compare the effect of topical application of the nanospheres delivery vehicle in a gel, aqueous solution and the same loaded with curcumin. Wounding was performed in a nonischemic rabbit model. Previous experiments indicated a possible inhibitory effect of the gel itself; therefore, a nanospheres-curcumin formulation without the gel (aqueous) was included in the study.

The same animal was unilaterally treated with curcumin nanospheres-gel (0.9 µM, 1.5 µM, 11.2 µM, and 23.5 µM) or nanospheres-gel alone. Additional animals received control treatments with nanospheres-gel alone and/or aqueous solution. To confirm the positive effect of curcumin on wound healing and validate the nanospheres delivery approach, curcumin nanospheres (1.5 µM, aqueous) and

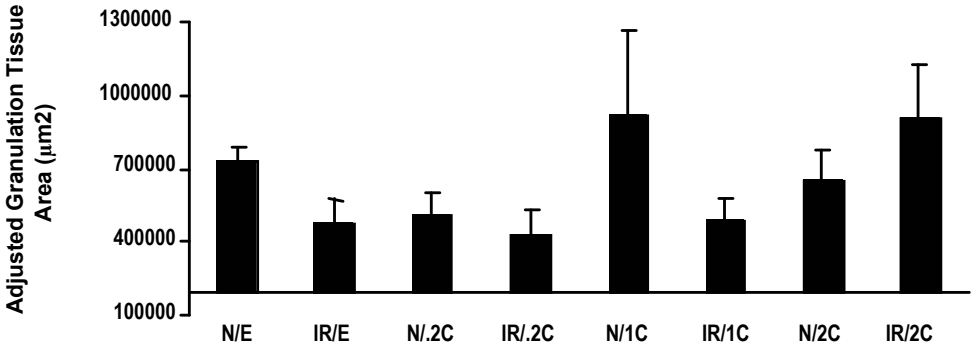


Figure IV-6. Granulation tissue formation in the I/R injury rabbit ear model upon curcumin treatment. The 2 µM concentration of curcumin improved new dermal tissue formation. However, these data were not statistically significant; t-test, *p*-value: IR/E versus IR/0.2C = 0.71; IR/E versus IR/1C = 0.91; IR/E versus IR/2C = 0.10. See Figure IV-5 for a description of abbreviations.

nanospheres-gel were tested. Gross appearance of the wounds showed no signs of acute toxicity even with the highest curcumin concentrations tested.

Histological evaluation revealed the following:

1. Treatment with control nanospheres-gel in comparison to aqueous nanospheres suggested a statistically significant inhibition of wound re-epithelialization by the gel based on measurement of the size of the epithelial gap **(Figure IV-7)**. The effect of both the nanospheres alone or with gel is comparable to control (blank) wounds with nanospheres alone supporting wound re-epithelialization. However, these data are not statistically significant. The result again suggested a possible inhibition by the gel and the need to further explore the direct effect of the vehicle components.
2. Curcumin nanospheres-gel (0.9 µM) appeared to reduce epithelial gap formation in comparison to control nanospheres-gel in rabbit ear wounds (Figure IV-7). The nanospheres-gel control was less effective than the aqueous nanosphere control although results were not statistically significant. It is possible that the beneficial effect of curcumin cannot overcome the inhibitory effect of the delivery vehicle (gel) since the epithelial gap is still larger than the gap observed in nanospheres-treated wounds alone. Doses above 0.9 µM of curcumin nanospheres-gel failed to promote healing.
3. The curcumin nanospheres aqueous solution (1.5 µM) in comparison to the gel equivalent suggested a possible beneficial effect on wound re-epithelialization; however, again this was not statistically significant (Figure IV-7). The effect was similar to 0.9 µM curcumin nanospheres-gel treatment. No obvious effect on granulation tissue formation was observed.

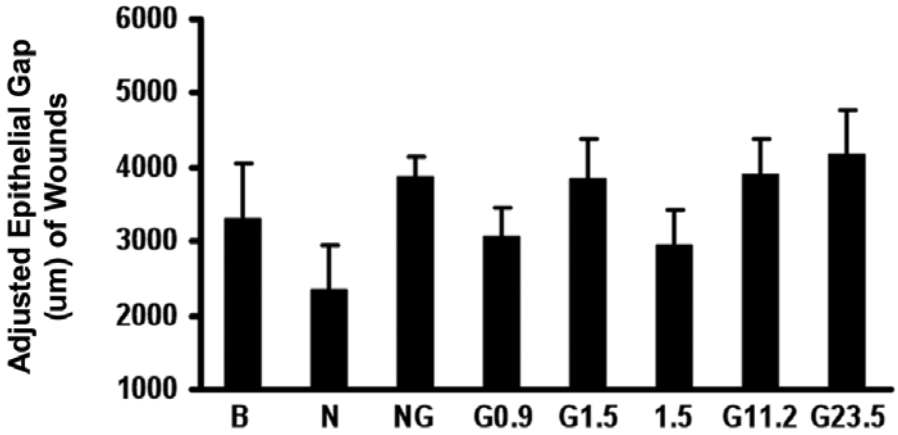


Figure IV-7. Effect of curcumin nanospheres formulation (gel and aqueous) on wound re-epithelialization. B – control untreated (blank) wounds (n=5); N – aqueous nanosphere alone (n=6); NG – nanospheres-gel (n=25); G0.9 – curcumin nanospheres-gel 0.9 µM (n=8); G1.5 – curcumin nanospheres-gel 1.5 µM (n=10); 1.5 – curcumin nanosphere aqueous solution 1.5 µM (n=7); G11.2 – curcumin nanospheres-gel 11.2 µM (n=6); G23 – curcumin nanospheres-gel 23.5 µM (n=7); t-test, $p \leq 0.05$; N versus NG $p=0.04$; G0.9 versus NG $p=0.1$; G0.9 versus G23.5 $p=0.2$; NG versus B $p=0.5$.

Effect of Nanospheres-Gel on Wound Healing

To rule out a possible inhibitory effect of the vehicle component of the nanospheres-gel formulation, additional experiments were performed in four rabbits. Wounds were created as described previously, and nanospheres diluted in PBS alone (Na) or a blank (no treatment) were applied onto two rabbits. The other two rabbits are unilaterally treated with nanospheres-gel with or without the propylene glycol (PG) component. Treatment of the wounds with nanospheres-gel containing PG was performed in the same animal to minimize individual variation. There was no statistically significant effect on wound epithelialization or granulation tissue formation between the gel containing PG or the one without PG **(Figure IV-8** and **Figure IV-9)**. Nanospheres-PBS-treated wounds, in comparison to blank wounds, did not show statistically significant increases in wound epithelialization or granulation tissue formation (Figures IV-8 and IV-9); however, PBS-diluted nanospheres had a better, although not statistically significant, effect on wound healing compared to the gel formulation.

The effect of the nanospheres-gel alone (without PG) on wound healing was also tested in subsequent experiments. To avoid individual variation, each rabbit was unilaterally treated with nanospheres-gel or curcumin-loaded nanospheres (0.9 μM). Therefore, the treatment was directly compared to the blank wounds of the same animal. HPMC/PBS-based nanospheres-gel can be used as a vehicle for the topical delivery of curcumin. PBS-based nanospheres-gel has the desirable effect of the gel to increase retention of the drug at the site of application and also has no apparent inhibitory effect on wound healing.

The results were in agreement with previous data that the presence of the HPMC component did not have an inhibitory effect on wound healing. In addition, granulation tissue formation was not statistically different between the curcumin-loaded nanospheres-gel treatment and blank wounds. These results suggest the need to evaluate different doses of curcumin nanospheres to see protective or stimulatory effects on wound healing and the effects on re-epithelialization and granulation tissue formation.

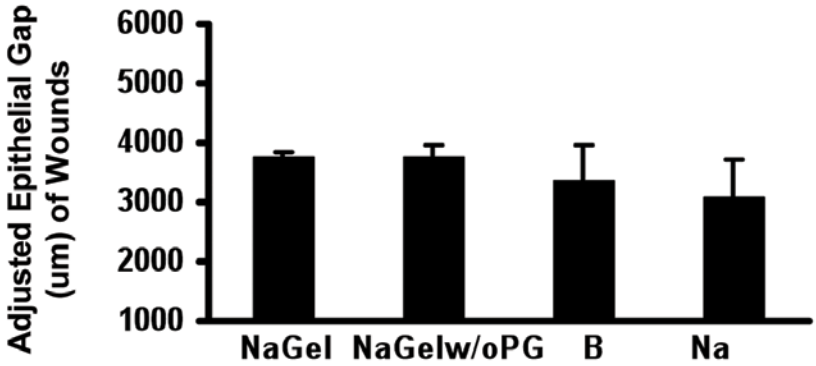


Figure IV-8. Wound epithelialization. NaGel – nanospheres-gel (PG and hydroxypropyl methylcellulose [HPMC] gel); NaGelw/oPG – nanospheres-gel without PG; B – blank wounds (wounds without any treatment); Na – nanospheres dispersed in PBS. N = 8. There is no statistically significant difference in wound epithelialization between NaGel or NaGelw/o PG, $p = 0.21$ or B versus Na, $p = 0.3$. Sample Na: diluted with PBS in the ratio 1:2 (v/v) – nanospheres alone. B: dispersed in 1% w/v HPMC gel under sterile conditions (1:2 [v/v]). NaGel:HPMC gel was prepared in PG:PBS (80:20 v/v) solution. NaGelw/oPG:HPMC gel was prepared in PBS alone.

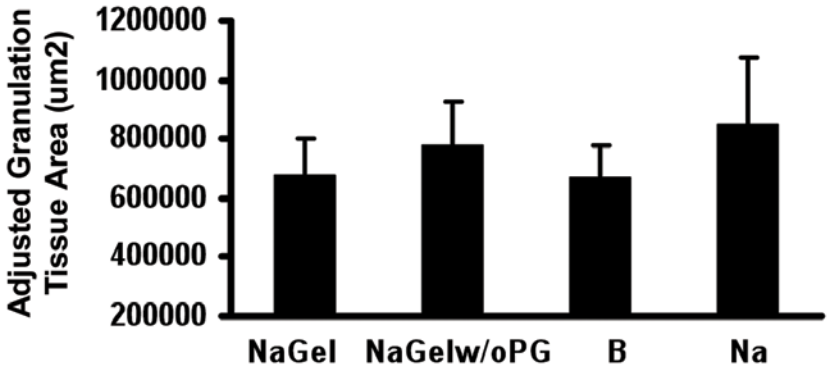


Figure IV-9. Granulation tissue formation. NaGel – nanospheres-gel (PG and HPMC); NaGelw/oPG – nanospheres-gel without PG; B – blank wounds (wounds without any treatment); Na – nanospheres dispersed in PBS. N=8. There is no statistically significant difference in granulation tissue formation between NaGel or NaGelw/oPG, p=0.19 or B versus Na, p=0.53. Sample Na: diluted with PBS in the ratio 1:2 (v/v) – nanospheres alone. B: dispersed in 1% w/v HPMC gel under sterile conditions (1:2 [v/v]). NaGel:HPMC gel was prepared in PG:PBS (80:20 v/v) solution. NaGelw/oPG:HPMC gel was prepared in PBS alone.

Effect of Curcumin Nanospheres on Ischemic Wound Healing

The efficacy of the topical application of curcumin nanospheres was tested in an ischemic rabbit ear model. The range of concentrations tested was as follows: nano-HPMC–nanospheres-gel (NC) control, NC 0.49 µM, NC 1.9 µM, and NC 4.8 µM. Curcumin nanospheres were applied immediately after surgery and the tissue was harvested at day 7. Histological evaluation revealed no apparent statistical difference in wound epithelialization across all concentrations tested **(Figure IV-10)**. Granulation tissue formation was stimulated with 1.9 µM curcumin nanospheres treatment; however, the difference was not statistically significant when compared to the paired control in the same animal **(Figure IV-11)**.

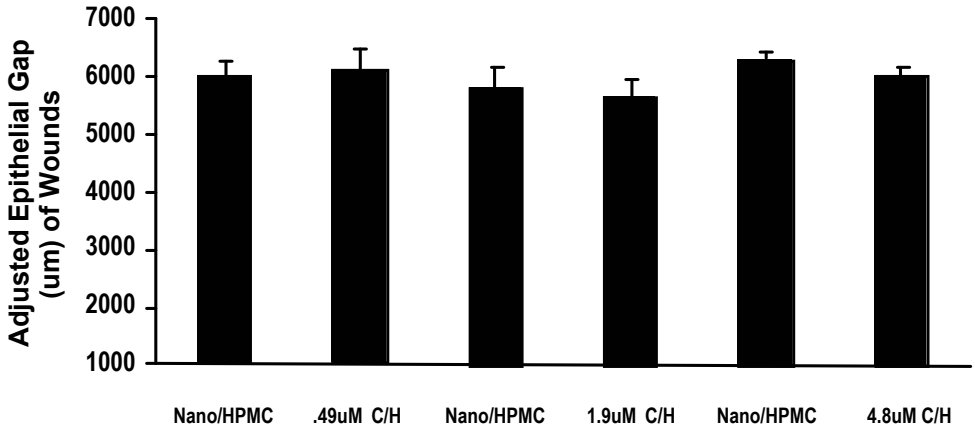


Figure IV-10. Effect of topically applied curcumin/HPMC on ischemic wound epithelialization. Adjusted epithelial gap was not significantly different between control and each of the tested curcumin nanospheres concentrations of 0.49, 1.9, and 4.8 µM. N=6, t-test, nano/HPMC versus 4.8 µMC/H p=0.34; nanoHPMC versus 1.9 µMC/H p=0.66.

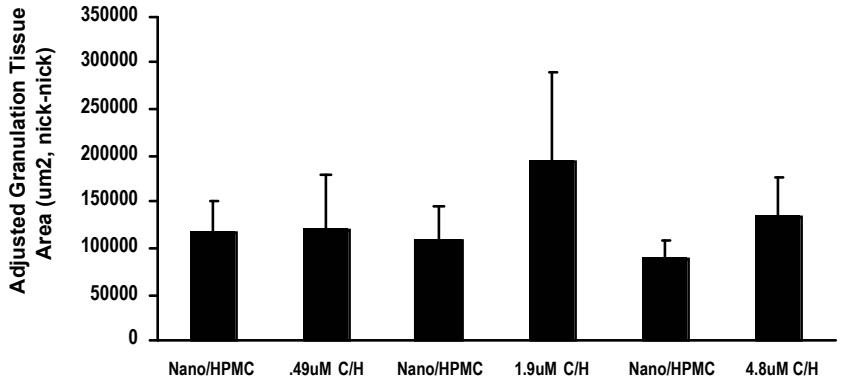


Figure IV-11. Effect of topically applied curcumin/HPMC on granulation tissue formation in ischemic wounds. No significant difference could be detected between control and each of the tested curcumin nanospheres concentrations of 0.49, 1.9, and 4.8 μM. N=6, t-test, nano/HPMC versus 4.8 μMC/H $p = 0.38$; nanoHPMC versus 1.9 μMC/H $p = 0.20$.

Key Research Accomplishments

- The initial results of intravenous curcumin treatment (2 μM tissue concentration) indicated a beneficial effect on epithelialization and wound healing in the I/R model. However, there was no difference in granulation tissue formation, suggesting the need to further explore the effect of curcumin in preventing/diminishing I/R injury progression.
- Evaluation of the nanospheres-gel (without PG) formulation confirmed that HPMC/PBS-based gel did not have an inhibitory effect on wound healing. Wound re-epithelialization and granulation tissue formation was similar in wounds treated with 0.9 μM curcumin nanospheres and control wounds.
- Topically applied curcumin/HPMC nanospheres on ischemic wound healing had no apparent effect on epithelialization or granulation tissue formation.

Conclusions

Intravenous curcumin treatment had a significant beneficial effect on wound healing in the I/R model when applied at the time of wounding (pre-injury). It is reasonable to explore multiple doses of curcumin at later time points after I/R injury to provide evidence of efficacy to prevent injury progression or support wound healing. The topical nanosphere drug delivery approach in the rabbit ear model provided evidence of nontoxicity of the delivery vehicle components tyrosine nanospheres, PG, and HPMC. A nanospheres-gel formulation containing PG had no inhibitory effect on wound healing in the rabbit ear model. A nanospheres-gel aqueous solution (nanospheres diluted in PBS/HPMC) could be used as a delivery vehicle.

Research Plans for the Next 2 Years

Curcumin efficacy (i.e., evaluation of dosing, timing, and toxicity) in a large animal model will commence in year 4. If proven successful, this will advance the technology to TRL 5.

Planned Clinical Transitions

Plans for a clinical trial will be coordinated through Dr. Clark's group at Stony Brook University.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Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management

Project 4.7.1, RCCC

Team Leader(s): Adam J. Katz, MD (University of Virginia)

Project Team Members: Adam J. Katz, MD, Ning Yang, PhD, and Hulan Shang, MS (University of Virginia)

Collaborator(s): Glycosan BioSystems, Inc., The GID Group, Inc., LifeCell, Inc. (Material Transfer Agreement), and Integra Lifesciences (Material Transfer Agreement)

Therapy: Autologous adipose cell-based therapies for wound healing and scar prevention and management.

Deliverable(s): RWP with autologous ASCs for full-thickness dermal defects.

TRL Progress: 2009, TRL 2; 2010, TRL 3; 2011, TRL 4

Key Accomplishments: The researchers initiated wound paste strategy and formulation studies. They downselected wetting solution/cell stabilization "recipes" to the two most favorable formulations after screening multiple permutations. They formulated RWP with cell stabilization solutions that maintained cell viability and proliferation over a 2-week period in culture. The research team determined that cell-enhanced wound paste reduced wound contraction in a mouse model with full-thickness wounds more effectively than acellular wound paste. Finally, they found no gross or systemic evidence of detrimental effects related to a high dose of ASCs in immunocompromised mice in a long-term tumorigenesis and migration study.

Key Words: Adipose stromal cells, autologous cell therapies, stromal vascular fraction, wound healing, wound contraction

Introduction

Essentially all battlefield wounds involve some component of skin and/or soft tissue injury, and in many cases the tissue loss/damage can be quite extensive. In general, the amount (percentage of thickness) of dermal component contained with a graft or construct primarily determines its propensity for secondary contraction. Secondary contraction can lead to scar contracture, causing deformity and functional deficit. As such, the thicker the graft (e.g., full thickness), the less contraction and the better the functional and aesthetic outcome. However, full-thickness grafts are limited by availability, donor site morbidity, and a relatively increased risk of failed engraftment.

The working hypothesis of the Katz group is that constructs composed of autologous adipose-derived leuko-vascular-stromal cells and human acellular dermal matrix can achieve expedient closure of open wounds while limiting wound contraction and favorably impacting the final aesthetic and functional outcome. The researchers' objective is to develop a dermal equivalent/replacement that is akin to a full-thickness graft but uses readily available adipose tissue as a cell source. By minimizing the need for dermal harvest from the patient, one can minimize donor morbidity and/or lessen the time needed

for cell expansion and product manufacture thereby enhancing therapeutic availability. By providing a full-thickness dermal equivalent, one can minimize wound contraction and still provide the best possible texture and color match for wound healing and tissue repair.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers made substantial progress in developing a point-of-care therapy whereby ASCs are isolated, seeded onto/combined with a dermal scaffold, and applied to an open wound all within the context of a single operative session. They determined that multicell spheroids could undergo spontaneous, self-directed migration into a bilaminar-like orientation when placed onto dermal scaffold. They also demonstrated the ability to seed and subsequently proliferate suspensions of ASCs and/or spheroids onto intact and/or microperforated acellular dermal scaffolds and the ability of such constructs to assist in wound healing in vivo while also decreasing wound contraction. Finally, they developed a novel product formulation, wound paste, composed minimally of ASCs and particulated acellular dermal scaffolds, and demonstrated the feasibility and proof of concept of its delivery to open wounds and its ability to incorporate into and close wounds while minimizing wound contraction.

Research Progress – Year 3

The research team has developed a novel platform for autologous point-of-care wound therapy using ASCs. This RWP platform involves a combination of cells, dermal scaffold, ECM hydrogel, and wetting solution all within an operating room setting. A unique, minimalist cell stabilization/wetting solution was systematically developed with the goal of optimizing cell viability within the paste during the initial days after application. Additional studies have focused on defining the benefit and role of the other wound paste components on handling properties as well as dispersion, viability, proliferation, and activity of cells within the formulation. These studies validated the benefit of using an ECM hydrogel within the paste formulation by demonstrating definite cell survival and support activity. The researchers' results supported the inclusion of HA in the formulation. They are also developing mechanistic and potency assays for specific wound paste formulations that have the greatest translational potential. These formulations are being assessed for growth factor secretion (via enzyme-linked immunosorbent assay [ELISA]) as well as the ability of the formulations to influence migration and ingrowth of targeted host wound cells into the pastes.

The research team has explored the in vivo feasibility of wound paste for the prevention of wound contraction in a mouse model with full-thickness wounds. Wound paste was formulated with or without cells, and wounds were randomly assigned to three groups: open wound, RWP without cells, and RWP with cells. Wounds were measured every week to evaluate contraction. Results indicate that wound paste significantly reduced wound contraction compared to control (wounds allowed to heal on their own), and cell-enhanced paste demonstrated a more effective trend toward this end point than acellular paste as well as generation of a notably exuberant granulation bed.

Following discussions with commercial partners, the research team also investigated the tumorigenic and migratory potential of the implanted cells in anticipation of safety studies required by the FDA. Nude mice were injected with 6 million human

ASCs at a dosage of at least 240,000 cells/gram, and vehicle-injected mice served as sham controls. Animals were monitored for 12 months for signs of systemic illness and/or gross tumor growth. Weight was monitored, and the study end point organs (i.e., brain, kidney, heart, lung, spleen, liver, and tissue around delivery site) were harvested and evaluated for gross appearance, weight, and gross tumor formation. Select organs were evaluated for the presence of migratory cells using PCR detection of human-specific gene transcript ERV-3. Of the cell-injected animals, 11/12 animals survived the entire year of the study with one infection-related death occurring 8 months into the study. All cell-treated animals maintained their weight over the 12-month period, with no significant difference from control animals, despite having received a large burden of human cells. In addition, there was no gross evidence of any tumor formation either at the sight of injection or in any of the organs harvested. PCR analysis of lung and spleen tissue harvested after sacrifice did not show any evidence of the migration/presence of human cells. These results provide strong evidence that human ASCs did not generate tumors or migrate systemically after implantation in vivo even when delivered at very high doses and when formulated as spheres composed of cells and self-generated ECM.

Key Research Accomplishments

- Initiated wound paste strategy and formulation studies.
- Downselected wetting solution/cell stabilization "recipes" to the two most favorable formulations after the screening of multiple permutations.
- Formulated RWP with cell stabilization solutions that maintained cell viability and proliferation over a 2-week period in culture.
- Determined that cell-enhanced wound paste reduced wound contraction in a mouse model with full-thickness wounds more effectively than acellular wound paste.
- Found no gross or systemic evidence of detrimental effects related to a high dose of ASCs in immunocompromised mice in a long-term tumorigenesis and migration study.

Conclusions

The research team has progressed toward a thorough understanding of the studies and methods needed to achieve transition of its RWP platform to TRL 5 and beyond. The researchers have become familiar with the details of CMC (Chemistry, Manufacturing and Controls), preclinical, and clinical information that will be expected as part of an Investigational Device Exemption/Biologics License Application combination product review and have now focused all studies toward addressing these regulatory review "action items." They have considerably defined the specific components of a final formulation and have begun to transition preclinical studies to the use of uncultured, minimally manipulated ASCs (i.e., stromal vascular fraction cells).

Research Plans for the Next 2 Years

In the coming years, the research team aims to transition RWP into clinical trials (TRLs 5–6). In the near term, the researchers plan to complete a pre-IND meeting with the Center for Biologics Evaluation and Research

office of the FDA to clarify the design and completion of specific preclinical studies necessary to justify transition of RWP into clinical trials. The team will then focus all efforts on the completion of such studies/activities (several currently under way) to support and submit an IND packet as soon as possible (TRL 5). The researchers have discussed the RWP development plans with several potential commercial partners, all of whom are supportive of the project and have expressed interest in the ultimate commercialization of the platform.

Planned Clinical Transitions

The researchers have progressed in planning for transition to clinical trials. A request for designation has been obtained from the FDA's Office of Combination Products, and plans are under way for a pre-IND meeting. Similarly, the research team has established and initiated several strategic relationships with potential commercialization partners, some of whom are providing leveraged funding through in-kind services or material.

Regenerative Bandage for Battlefield Wounds

Project 4.5.2, WFPC

Team Leader(s): Geoffrey C. Gurtner, MD and Michael T. Longaker, MD, MBA (Stanford University)

Project Team Members: Anthony Oro, MD, PhD (Stanford University)

Collaborator(s): None

Therapy: Improved wound healing and reduced scarring.

Deliverable(s): Regenerative bandage that promotes fetal-like wound healing instead of scarring.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 3; End Year 3, TRL 4

Key Accomplishments: The researchers have developed a novel biomaterial scaffold with modifiable open porosity and matrix components. This composite matrix is highly biocompatible with numerous cell types important for wound repair, including endothelial cells, fibroblasts, and MSCs. The research team characterized the morphology of MSCs seeded within a hydrogel in vitro. After incubating MSCs within the hydrogel, they observed increased expression of stemness genes and cytokines known to be involved in wound healing. The researchers also used a transgel migration assay to determine that the hydrogel can function as an effective stem cell delivery system for wound applications.

Key Words: Dermal matrix, wound healing, fetal skin

Introduction

Wounded soldiers returning from Iraq have sustained significant trauma to the head, neck, face, and limbs. Timing is critical to optimize salvage of traumatic wounds; once wounds are surgically debrided, coverage is important to reduce a prolonged inflammatory state, infection with subsequent contraction, and disability. A novel approach is needed to minimize this inflammatory and fibrotic cascade in the initial days following injury while promoting tissue regeneration. The research team's technical approach begins immediately post-injury with a regenerative bandage consisting of a fetal biomimetic matrix and human progenitor cells to maintain an acute wound in a pro-regenerative state of "suspended animation" and prevent the onset of scarring, fibrosis, and infection. Utilizing their knowledge of fetal skin development, scarless repair, and burn therapy, the researchers hope to preserve wounds in a "fresh state" by recreating a fetal-like wound-healing milieu to promote regeneration and optimize the results of definitive therapy provided in the United States.

There are several commercial products used for skin engineering based on human or pig skin. These decellularized matrices are effectively used in a variety of surgical and wound settings, and clinical results are improved in many cases compared to no treatment at all. However, natural skin sources are limited by availability, cost, and risk of disease transmission. Further, clinical results using skin substitutes remain suboptimal due to poor cosmetic and functional outcomes. Synthetic skin substitutes offer the promise of a widely available, disease-free, cheaply produced

replacement skin that can potentially improve current clinical outcomes.

The Stanford University group has developed hygroscopic dressings mimicking unwounded dermal micropatterning. This engineered construct significantly improved cutaneous wound healing in a mouse model and demonstrated potent immunomodulatory properties that enhanced wound vascularization. The researchers are now focused on using this regenerative template to maintain progenitor cells in suspended animation for delivery into wounds and to develop a battlefield-ready, rapidly expanding hydrogel that can be used as a regenerative bandage and vehicle for autologous stem cell delivery.

Summary of Research Completed in Years 1 and 2

During the first year of this study, the researchers developed porous, modifiable, pullulan-collagen hydrogel scaffolds. They modified hydrogels to deliver small molecules (deferoxamine) into murine wounds. They observed that molecules maintain their efficacy and are effectively released by the dressing biomaterial. They determined through preliminary in vivo studies that pullulan-containing hydrogels are biocompatible, maintain their architecture, do not incite a robust inflammatory response, and allow cellular incorporation. During the second year of the project, researchers modified their synthetic bioscaffold to mimic the structure of fetal and unwounded murine skin. The porous biomaterial was fabricated using salt-induced phase inversion techniques and displayed predictable swelling, degradation, and rheologic behavior. In vitro studies demonstrated high biocompatibility with endothelial cells, fibroblasts, and MSCs. In vivo studies showed a predictable degradation profile and no impairments in wound healing when used in a subcutaneous position. When used in a humanized excisional wound model in mice, there was significant improvement in early wound healing, possibly related to the induction of granulation tissue formation.

Research Progress – Year 3

The Stanford University group has begun to add MSCs to its carbohydrate-based collagen hydrogel dressing and has investigated this construct in vitro. The researchers first evaluated the optimal method of seeding MSCs into the hydrogel comparing dynamic seeding, static seeding, and direct injection of cells. Based on superior cell density and uniformity throughout the scaffolds and optimal cell viability after seeding, they determined that static seeding of cells onto hydrogel scaffolds was the optimal seeding method; thus, this method will be used for all subsequent experiments. They have shown that cells seeded within the hydrogel have a predominantly rounded morphology with a smaller percentage of cells attaching and spreading out to conform to the three-dimensional hydrogel topography **(Figure IV-12)**.

The researchers have also assessed changes in gene expression in MSCs grown within the hydrogel compared to standard cell culture conditions and have shown that there is a significant increase in gene expression of stemness genes in MSCs cultured within the hydrogel based on real-time PCR analysis **(Figure IV-13A)**. They analyzed gene expression of various cytokines by MSCs in the hydrogel scaffolds and have shown a significant upregulation of vascular endothelial growth factor A (VEGF-A) and monocyte chemoattractant protein 1 (MCP-1) by MSCs seeded within the hydrogel **(Figure IV-14)**. They have also demonstrated that hydrogel-seeded MSCs exhibit a markedly diminished rate of proliferation consistent with their theory of hydrogel-induced quiescence (Figure IV-13).

Finally, the researchers performed a transwell migration assay in which MSCs were seeded onto a hydrogel scaffold, and platelet-derived growth factor (PDGF) was used to stimulate migration out of the hydrogel **(Figure IV-15)**. They found that the MSCs were capable of migrating out of the hydrogel and across the porous membrane, which suggests that the hydrogel can function as an effective stem cell delivery system for wound applications.

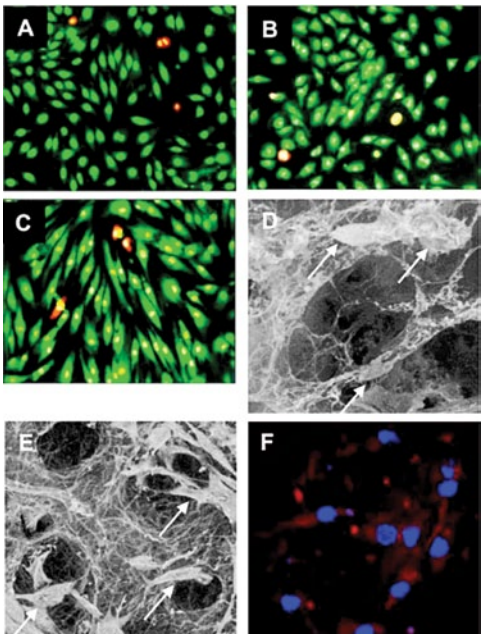

Figure IV-12. In vitro hydrogel biocompatibility and cellular incorporation: Hydrogels were biocompatible with fibroblasts (A), bone marrow-derived MSCs (B), and endothelial cells (C) for up to 7 days as determined by a live/dead assay. Scanning electron micrographs from within 5% collagen-pullulan hydrogels seeded with fibroblasts (D, arrows) and MSCs (E, arrows) show cellular incorporation within the hydrogel matrix. Confocal imaging shows human dermal fibroblasts incorporated three-dimensionally within a 5% collagen-pullulan hydrogel 24 hours after seeding (F). These data demonstrate the biocompatibility of the hydrogel scaffold with several skin cell types critical in wound repair and regeneration.

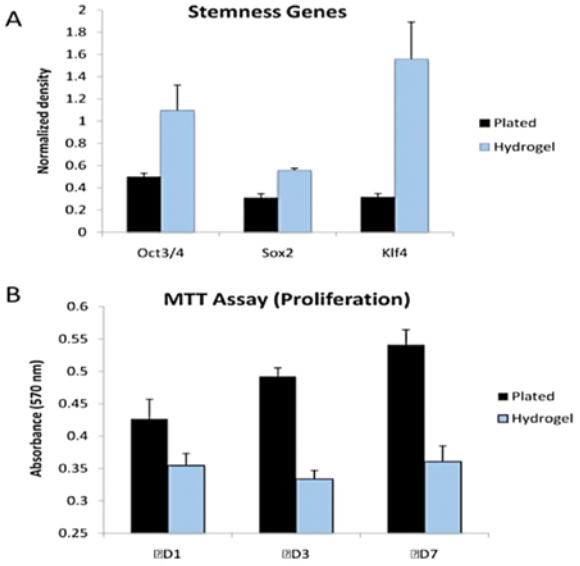

Figure IV-13. (A) Analysis of stemness properties regulated by the hydrogel scaffold system. Seeding of MSCs onto hydrogels for 24 hours significantly augmented the expression of the stemness genes Oct3/4, Sox2, and Klf4, suggesting that the hydrogel environment is highly conducive to maintaining the pluripotent state of delivered stem cells. In contrast, plating of MSCs onto standard tissue culture had an inferior stemness profile compared to hydrogel seeding. (B) Methyl tetrazolium (MTT) proliferation assay confirms that hydrogel-seeded MSCs have a lower rate of proliferation (consistent with the quiescent state induced by the hydrogel) compared to plated MSCs for up to 7 days in culture. $p < 0.05$.

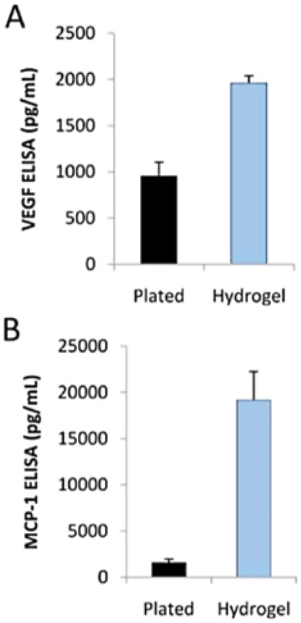

Figure IV-14. (A) ELISA cytokine analysis of VEGF levels in vitro. Hydrogel-seeded MSCs exhibited greater secretion of the angiogenic cytokine VEGF compared to plated MSCs. (B) MCP-1 levels were significantly elevated in hydrogel-seeded MSCs compared to plated MSCs. These findings are consistent with the transcriptional analyses, suggesting that the hydrogel scaffolds augment the provascular effects of MSCs and may be highly effective in promoting wound repair. These differences are significant ($p < 0.05$).

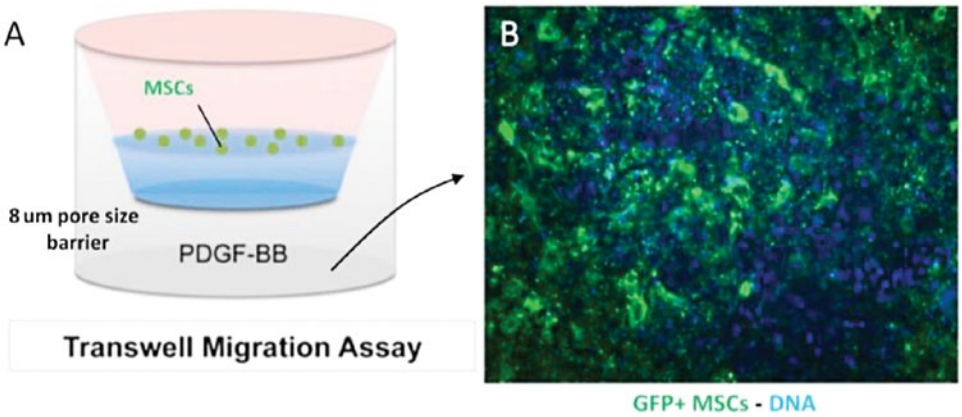

Figure IV-15. Transwell migration assay. (A) MSCs were seeded onto the hydrogel scaffold for 24 hours and then placed over an 8 µm pore size filter. PDGF was used as a migratory stimulus at the bottom of the well to induce MSC migration out of the hydrogel similar to an inflammatory wound environment. (B) Immunofluorescence imaging of the bottom of the well demonstrated the presence of green fluorescent protein (GFP)-positive cells, indicating that MSCs are capable of migrating out of the hydrogel and across the porous membrane.

Key Research Accomplishments

- Determined that static seeding methods of MSCs onto hydrogel scaffolds provided optimal cell density, viability, and uniformity.
- Characterized the morphology of MSCs seeded within hydrogels in vitro.
- Demonstrated increased expression of stemness genes by MSCs incubated within their hydrogel compared to standard cell culture.
- Demonstrated increased expression of VEGF-A and MCP-1, two important cytokines involved in wound healing, by MSCs in hydrogels.
- Confirmed that seeded MSCs remain in a quiescent state while within the hydrogel scaffold through MTT proliferation assays.
- Used a transgel migration assay to determine that the hydrogel can function as an effective stem cell delivery system for wound applications.

Conclusions

The Stanford University group has successfully fabricated a biocompatible dermal scaffold based on the architecture of fetal murine skin. This material is highly modifiable with predictable swelling, degradation, and rheologic properties. Preliminary in vitro studies have demonstrated significant biocompatibility with several wound repair cell types, including endothelial cells, fibroblasts, and MSCs. This wound dressing does not impair wound healing in a subcutaneous model and predictably degrades over a period of 3 weeks. In a humanized excisional wound model, the pullulan-collagen composite hydrogel significantly improved early wound healing, potentially by stimulating granulation tissue formation.

Research Plans for the Next 2 Years

During the next 2 years, the Stanford University group will continue to optimize its stem cell-seeded hydrogel construct to maximize its in vivo regenerative profile. The researchers will characterize the mechanisms underlying the marked improvements in neovascularization for recipient murine wound beds, and they will use this information to refine their seeding protocol and scaffold structural parameters. They will begin to transition from mMSCs to human ASCs, which represent an ideal autologous tissue source for candidate patients, but this transition necessitates more challenging murine experiments using nude mice due to immunologic incompatibility. The group will similarly begin to transition its scaffold construct toward therapeutic utility in battlefield situations with strategies aimed at engineering portable, dry hydrogels capable of rapid expansion to porous scaffolds in vivo.

Planned Clinical Transitions

The Stanford University group plans to continue in vivo small animal studies to further characterize the observed improvement in early wound healing. The researchers plan to file a 510(k) for a cell-free matrix if continued positive results are obtained. This could set the path for clinical trials using this dressing on open wounds to enhance granulation tissue formation.

Corrections/Changes Planned for Year 4

The Stanford University group is expanding its use of progenitor cells to include adipose- and bone marrow-derived stem cells to seed into these biomatrices. The use of these cells has greater clinical applicability compared to embryonic stem cells (as initially proposed), and their use bypasses ethical concerns regarding the use of embryonic tissues. Autologous cells can potentially be harvested from injured patients and used to seed biomatrices in vitro for subsequent use as a regenerative wound bandage.

Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies

Project 4.5.5, WFPC

Team Leader(s): Sandeep Kathju, MD, PhD (Allegheny-Singer Research Institute)

Project Team Members: Latha Satish, MSc, MPhil, PhD (Allegheny-Singer Research Institute)

Collaborator(s): None

Therapy: Formulation containing siRNAs that can be applied to wounds to mitigate scar formation. Probiotic therapy for burn wounds to inhibit pathogenic infection and reduce scar.

Deliverable(s): A formulation of molecular agents that can be applied to healing wounds such that they repair with diminished or absent scar formation using nanoparticulate technology. To employ probiotic therapy to reduce infection and scar in a burn wound scenario.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 3; End Year 3, TRL 3

Key Accomplishments: The researchers are using siRNA in novel nanoparticulate formulations to mitigate scar formation in healing wounds. They found that siRNA versus CCT-eta applied to incisional wounds significantly decreased collagen deposition in the wound bed and actually increased the tensile strength of the healing wound. In addition, the researchers found that a novel probiotic therapy for infected burn wounds using *Lactobacillus* decreased the length and severity of pathogenic infection and also decreased resulting fibrosis as evidenced by decreased collagen deposition in the burn wound.

Key Words: Scarless wound healing, nanoparticles, siRNA, probiotics, burns

Introduction

The purpose of this project is to arrive at technologies that will enable the reduction of scar formation after injury. Scar, while useful in sealing an injured area, is also the source of significant morbidity, including restriction of movement (e.g., in tendons and muscle), narrowing of viscera, entrapment of nerves, and the psychosocial damage associated with severe facial disfigurement that can result from scarring. Burn injuries are particularly prone to extensive and crippling hypertrophic scarring.

This laboratory has investigated mammalian fetal wound healing as a model of scarless healing after integumentary injury. Mammalian fetuses (until the beginning of the third trimester) heal their injuries regeneratively without attendant scar deposition. The research team has previously used differential display, PCR suppression subtraction hybridization, and microarray analysis to identify multiple candidate genes that are differentially expressed in healing fetal wounds. The ultimate goal is to use these gene products to modulate the adult wound environment so as to abolish or mitigate scar formation in healing adult wounds.

To this end, the research team has chosen the eta subunit of the CCT-eta for their initial test candidate gene. Since CCT-eta was found to be specifically reduced in healing fetal wounds, the researchers have sought to evaluate technologies that would allow for efficient transfection of their candidate molecular agents into healing skin wounds. In particular,

they have evaluated nanoparticle-complexed formulations as a nonviral means of molecular delivery in animal wound models.

Summary of Research Completed in Years 1 and 2

The research team first established in situ hybridization and immunohistochemical protocols for determining levels of CCT-eta genes and proteins, respectively, in adult wounds. They determined that CCT-eta is increased in healing adult integumentary wounds, which is in contrast to levels of CCT-eta in healing fetal wounds. They identified multiple cell types that upregulate CCT-eta in response to wounding in an adult organism. They also determined that multiple nanoparticulate carriers of molecular constructs, including atelocollagen and agarose, can be used to modulate gene expression in a wound milieu. The researchers designed and validated an siRNA-expressing plasmid vector that can be used to manipulate CCT-eta in wounds. They demonstrated that reduction of CCT-eta resulted in a decrease in alpha-smooth muscle actin (α-SMA), a major component of the force-generating complex in myofibroblasts. They also started to evaluate their siRNA constructs complexed with agarose in healing wounds with early promising results indicating diminished collagen content in treated healing wounds.

Research Progress – Year 3

siRNA Versus CCT-eta as an Antifibrotic Agent

The researchers had previously determined that siRNA versus CCT-eta complexed in agarose appeared to be more effective in inhibiting CCT-eta gene expression in vivo than other formulations (e.g., atelocollagen). They had also determined that repeated administration of CCT-eta siRNA ultimately generated a longer duration of effect than a single dose. They therefore began more intensive testing using these experimentally determined conditions in incisional wounds. Adult rabbits had multiple full-thickness integumentary wounds placed on their dorsa. Wounds were then treated with either control (vehicle alone), CCT-eta siRNA in the agarose vehicle, or scrambled siRNA in agarose vehicle as a further control. Wounds were protected with an occlusive dressing; at weeks 1 and 2 post-wounding another topical application of the treatment arms was performed. At 4–5 weeks post-injury, the healed wounds were re-excised as well as unwounded control skin. These tissues were then subjected to a series of molecular and histological examinations.

The researchers first sought to verify that they were in fact able to reduce the expression of their target gene, CCT-eta, in this in vivo setting. Total RNA was harvested from re-excised wounds and subjected to quantitative real-time reverse transcriptase-PCR assay for CCT-eta as well as another CCT subunit, CCT-beta. CCT-beta is the closest evolutionary homolog to CCT-eta, and the research team's previous studies had shown that CCT-beta expression remains invariant in healing wounds and is similar between fetal and adult fibroblasts. In addition, whereas reduction of CCT-eta can inhibit both fibroblast motility and contractility, and reverse the effects of profibrotic agents such as PDGF and epidermal growth factor, reduction of CCT-beta has no such effects.

The research team was able to effectively decrease the expression of CCT-eta in the wound and scar milieu by as much as 40% over a period of 4–5 weeks, a comparatively effective intervention given the overall difficulty many have observed in modulating gene

expression in healing wounds. The CCT-eta siRNA was specific—only CCT-eta and not CCT-beta messenger levels were affected. Also, scrambled siRNA control had no significant impact on CCT-eta siRNA levels, confirming the sequence specificity of the agent.

The researchers theorize that inhibition of CCT-eta can modulate scar formation by interfering with myofibroblast synthesis of α-SMA (among other proteins), which would in turn affect myofibroblast cellular function and, on a macroscopic scale, scar formation. They therefore also measured the expression of α-SMA following treatment of healing wounds with CCT-eta siRNA (or controls). They found that there was an increased accumulation of α-SMA mRNA at 4–5 weeks after wounding, and this was inhibited by CCT-eta siRNA. Control scrambled siRNA had no such effect. The researchers are currently in the process of confirming these results at the protein level by western blot assay.

The predominant histologic feature of scar contracture is organized bundles of collagen in a relatively hypocellular field. For this reason, the researchers examined the effect of CCT-eta siRNA on both collagen abundance and collagen organization in treated versus control wounds. Their working model predicates that successful scar mitigation will entail some reduction in the total amount of collagen deposited as well as a qualitative change in the organization that would occur in a typical scar pattern. To assay for this, siRNA-treated and control wounds were re-excised then subjected to both hydroxyproline assay and histological evaluation by MetaMorph® analysis. Assay for hydroxyproline is widely used to quantify the amount of collagen in a tissue/sample; MetaMorph is a computer program that analyzes the density and organization of collagen bundles in histological sections after Masson's trichrome staining. The MetaMorph algorithm summates its findings into a single numerical output that reflects both the quantity and the organization of collagen in the analyzed template.

The researchers found that treatment with CCT-eta siRNA markedly reduced the amount of collagen present in healed wounds at 4–5 weeks relative to untreated control or scrambled siRNA treated wounds. They further found that MetaMorph analysis showed that wounding significantly increased MetaMorph score compared to unwounded control and that CCT-eta siRNA reversed this increase but that scrambled control siRNA did not **(Figure IV-16** and **Figure IV-17)**.

The researchers noted the importance of ensuring that administration of their siRNA agent did not adversely affect wound healing in any way. Thus far they have encountered no problems with toxicity or infection, and CCT-eta siRNA-treated wounds heal with the same time course as control wounds. To further examine the effect of CCT-eta siRNA on wound integrity, they re-excised healed wounds at 4–5 weeks post-injury and tested

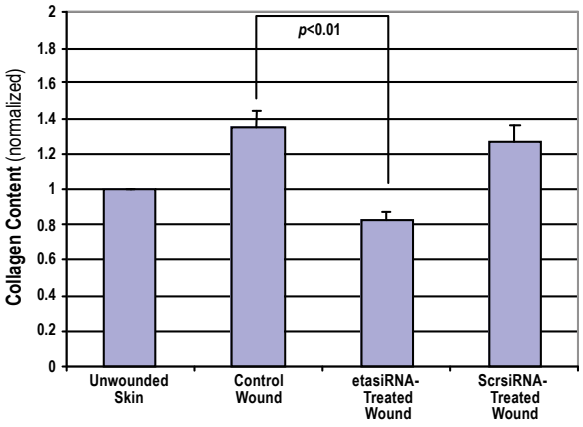

Figure IV-16. MetaMorph analysis of collagen content (and organization) in healing wounds. MetaMorph score for unwounded skin was normalized to one. Wounding increases the MetaMorph score; treatment with CCT-eta siRNA (but not scrambled siRNA control) reverses this increase.

the tensile strength by measuring the force required to disrupt the wound tissue in a tensiometer. They found that control wounds have a significantly lower tensile strength than unwounded control skin (as expected) but that CCT-eta siRNA administration actually increases the tensile strength of the wound at this time point **(Figure IV-18)**. This observation highlights the very attractive possibilities for this molecular intervention in that not only are the components of scar diminished, but the mechanical properties of the healing wound are simultaneously enhanced.

These results indicate that CCT-eta siRNA, alone or possibly in combination with other substances, can be a potent antifibrotic agent. The research team's approach is likely to be effective against multiple (perhaps all) molecular mediators of scar formation since the targeted effector cells are the myofibroblasts themselves and not just the individual inflammatory pathways that are typically targeted by competing agents.

Suppression of Scar in Infected Burn Wounds by Probiotics

Among the most problematic types of scar is the hypertrophic scar that frequently emerges after burn injury. Hypertrophic scar and contracture can afflict up to 60% of burn-injured patients, restricting movement and function and with obvious psychological and social costs. Therapy may require multiple surgical interventions as well as adjunctive measures, such as physical therapy and silicone sheeting. The best means of dealing with this issue would be to prevent it in the first place. Surgeons have long recognized that infected wounds of all sorts heal with greater scar and contracture than clean wounds, presumably due to the greater inflammatory stimulus elicited by pathogenic bacteria driving fibrosis. By suppressing the ability of pathogens to establish and persist in a burn wound, probiotic therapy, that is, the application of

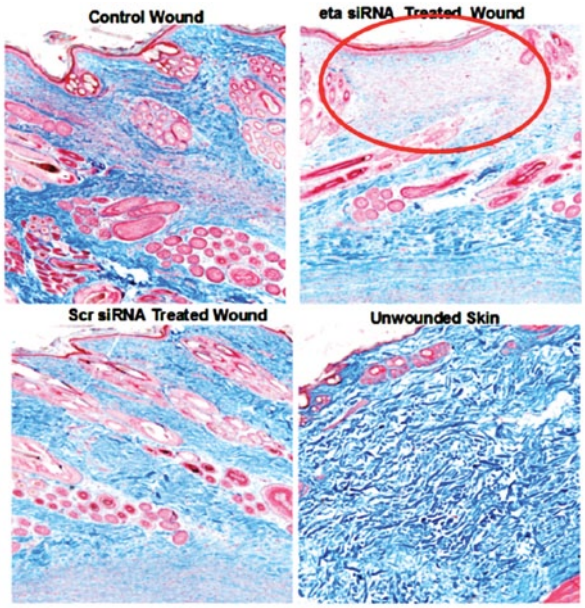

Figure IV-17. Histological examination of healing wounds after administration of CCT-eta siRNA (or control). Unwounded skin shows a reticular, "basket weave" pattern of collagen. Control wound and scrambled siRNA-treated wound both show denser accumulations of bundled collagen. The CCT-eta siRNA-treated wounds, however, show less abundant collagen deposition and an "airier" pattern of deposition (within the red oval).

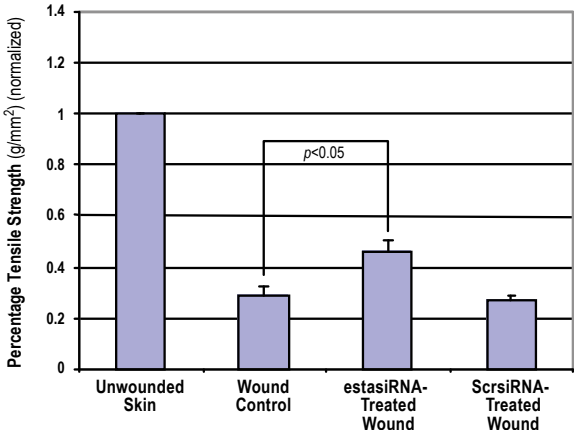

Figure IV-18. Effect of CCT-eta siRNA treatment on tensile strength of healing wounds. Control wounds display a significant loss of tensile strength compared to unwounded control skin, but CCT-eta siRNA actually increases the tensile strength of healed wounds. Scrambled control siRNA shows no such effect.

living bacteria that confer a health benefit to the patient, represents a novel means of potentially managing burn wound injuries to simultaneously reduce the risk of sepsis and scar. Instead of regarding every bacterium in the wound as a potential bad actor, probiotics seeks to reduce the population of recognized invasive pathogens by the application of non-pathogenic organisms. This approach would radically alter the calculus of wound healing biology in burn wounds (and has the promise of being potentially applicable to other types of wounds as well).

The researchers have obtained encouraging preliminary data from a rabbit model for the use of probiotics in burn wounds. Their experimental model is as follows: the dorsa of adult Dutch-belted rabbits were depilated and, under general anesthesia, full-thickness zones of burn injury were created by application for 30 seconds of a heated brass block (2.54 cm diameter) maintained at 100°C. Four wounds were placed on each rabbit; the affected skin was immediately noted to become firm and fixed (and full-thickness depth of injury was subsequently confirmed histologically). All wounds were left undisturbed for 5 days.

Each of the four wounds on a given animal were then assigned to an experimental condition: (1) control, where no further manipulation was performed; (2) inoculation with 10^9 CFUs of *L. plantarum* only; (3) inoculation with 10^8 CFUs of *Pseudomonas aeruginosa* variant Xen41 only; and (4) inoculation with 10^9 CFUs of *L. plantarum*, followed 6 hours later with inoculation of 10^8 CFUs of *P. aeruginosa.* Xen41 possesses a single copy of the *Photorhabdus luminescens luxCDABE* operon on its bacterial chromosome, affording it the physical property of bioluminescence.

Animals were then followed for approximately the next 5 weeks with periodic quantitation of biophotonic emissions. At approximately 5 weeks, all wounds were noted to have nearly completely re-epithelialized after sloughing the eschar with attendant scirrhous skin changes consistent with those seen in human patients. Animals were then sacrificed, and wound tissues (as well as native skin) were harvested for a variety of molecular and histological analyses.

The researchers previously first omitted *L. plantarum* treatment to simply establish the parameters of *P. aeruginosa* infection and to test whether infection in their model system exacerbated inflammation and scar. They found that burn wounds alone demonstrated much higher collagen levels than control skin and that infection with a bioluminescent variant of *P. aeruginosa* markedly increased the collagen accumulation and fibrosis even further. Histology showed a prominent increase in inflammatory cells/high-powered field in infected wounds compared to uninfected wounds. Masson's trichrome stain revealed a greater quantity of disordered collagen deposition in infected burn wounds compared to control uninfected.

Having established a protocol for *P. aeruginosa* infection and with evidence that infection does substantially contribute to the inflammation and scar that attends burn injury, the researchers next tested the ability of *L. plantarum* to counter the ability of *P. aeruginosa* to establish itself in the wound. Wounds treated with *L. plantarum* alone or with *L. plantarum* followed by *P. aeruginosa* challenge were added to the protocol as described previously. Wounds were followed by biophotonic imaging for approximately 5 weeks. The research team found that a single inoculum of *L. plantarum* pretreatment strongly inhibited the ability of *P. aeruginosa* to initiate and maintain an infection as evidenced by the relative paucity of bioluminescent signal observed whereas companion

wounds on the same animal inoculated with *P. aeruginosa* only consistently gave an active infectious profile **(Figure IV-19)**.

With evidence that *L. plantarum* can interfere with the development of a *P. aeruginosa* infection, the researchers then sought to characterize the resultant scar accumulation using molecular and histological assays. Specifically, they measured collagen deposition, both at the mRNA and protein level (with hydroxyproline), and collagen organization after histological evaluation with the MetaMorph program.

They found that type I collagen mRNA and protein were both increased after burn injury (compared to unwounded control) and increased further still by infection. Probiotic therapy, however, reversed this infection-related increase both at the mRNA and protein levels **(Figure IV-20)**.

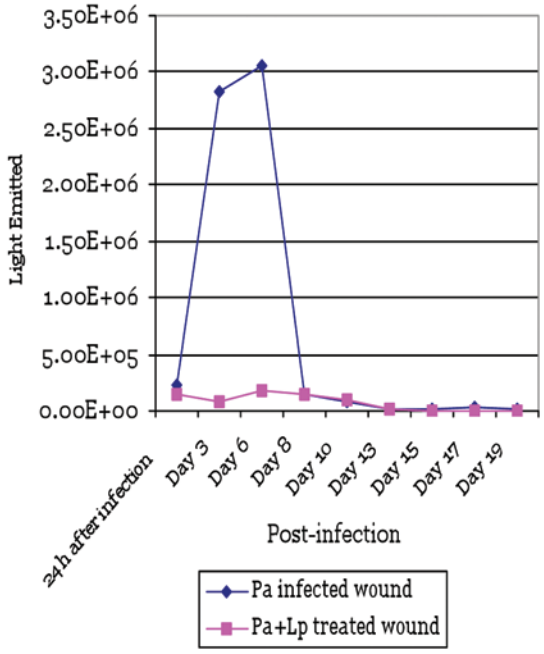

Figure IV-19. Biophotonic profile of infected burn wounds. Time course of quantitation of biophotonic signal from *Pseudomonas* only (blue) versus *Pseudomonas* pretreated with *Lactobacillus* (pink). Probiotic treatment with *L. plantarum* clearly inhibits *P. aeruginosa* growth compared to *P. aeruginosa* alone.

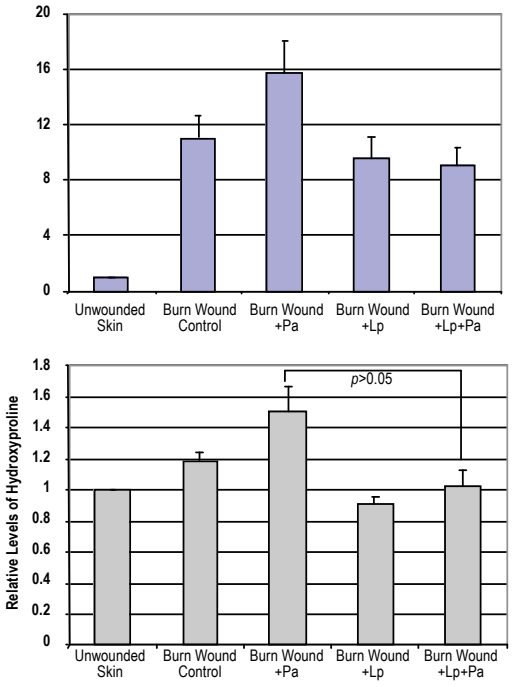

Figure IV-20. Effect of burn injury, pseudomonal infection, and probiotic therapy on burn wound collagen accumulation. (Top) Type I collagen mRNA levels in healed burn wounds; burn injury increases collagen message by approximately 10-fold compared to unwounded skin, and infection increases it by half again to some 15-fold. Probiotic therapy with *Lactobacillus* alone showed no increase above burn alone and reversed the infection-related increase completely. (Bottom) Collagen protein (as measured by hydroxyproline assay) was significantly increased after burn injury alone and increased further with infection. As with collagen mRNA, probiotic therapy completely reversed this infection-related increase.

The quantitative measurement of collagen in Figure IV-20 reinforced the results the researchers had obtained using MetaMorph analysis of histological sections of probiotic-treated versus control wounds. MetaMorph evaluation of the collagen architecture in the healed wounds showed that burn injury and *Pseudomonas* infection significantly increased the MetaMorph score whereas probiotic therapy with *Lactobacillus* actually significantly inhibited that elevation. These data, although previously reported, are presented again to consolidate these highly encouraging results in one location **(Table IV-3)**.

These results strongly suggest that probiotic therapy with *L. plantarum* can be an effective intervention in the management of burn wounds, serving to reduce pathogen bacterial burden and also markers of fibrosis.

Table IV-3. MetaMorph analysis of burn wounds. Note that the presence of *L. plantarum* significantly decreases the index of collagen deposition, rendering it more similar to unwounded skin.

	Unwounded Skin	Control Wound	Wound + *L. plantarum*	Wound + *P. aeruginosa*	Wound + *L. plantarum* and *P. aeruginosa*
Average	27.1	65.5	50.5	61.9	52.6
Standard Deviation	.015	3.6	6.9	1.4	0.3

Key Research Accomplishments

- Determined that sustained inhibition of CCT-eta by the research team's siRNA is specific and effective.
- Confirmed in a large number of animals that use of the research team's siRNA-complexed agent has no apparent toxicity to the animal and has no apparent negative effect on the rate of wound closure.
- Demonstrated that inhibition of CCT-eta can reduce the amount of α-SMA in a healing wound, which likely represents a decreased level of myofibroblast activity in the healing wound.
- Demonstrated that inhibition of CCT-eta results in an altered collagen architecture in the healing wound that more resembles unwounded skin.
- Demonstrated that inhibition of CCT-eta actually results in increased tensile strength in the healing wounds, a highly desirable outcome.
- Demonstrated that probiotic therapy of burn wounds favorably alters collagen architecture in healing burn wounds on challenge with *P. aeruginosa.*

Conclusions

The research team concludes that siRNA versus CCT-eta, delivered as a complexed nanoparticle in an agarose matrix, can effectively inhibit scar formation without any deleterious effects on wound healing. The researchers also conclude that probiotic therapy with *L. plantarum* can effectively abrogate pseudomonal (and possibly other) infections and significantly mitigate the scarring that can ensue after such infected burn injuries.

Research Plans for the Next 2 Years

With regard to the research team's siRNA-mediated antifibrotic therapy, the researchers

will complete characterization at the protein level of some markers for fibrosis in their animal models. They will continue to explore even more efficient means of delivering their siRNA agents with a longer duration, potentially through plasmid or minicircle vectors. In particular, they will explore whether ultrasound-mediated gene transfer, a highly promising technique well suited to the relatively accessible injuries they seek to address, can be used in conjunction with their technology to improve their results.

The researchers will also plan to either recapitulate their system in a porcine model (which more closely mimics human skin) or proceed to initial clinical trials in humans for safety, tolerability, and immunogenicity if their data thus far are strong enough.

With regard to burn injury and probiotics, the research team will test the ability of probiotics to reduce the local and systemic inflammation elicited by an infected burn wound. In an animal model, they will explore whether topical probiotic therapy can prevent septic translocation and death. They will also test the ability of probiotics to treat already infected burn wounds and determine how to counteract other burn wound pathogens with probiotics.

Planned Clinical Transitions

At this point, both interventional strategies have essentially demonstrated proof-of-concept benefit in animal models although some minor clarifying work still needs to be finished. The siRNA formulation is almost ready to proceed either to a porcine model (which more closely resembles human skin architecture) or may be a candidate to proceeding to initial Phase 1 studies in humans to evaluate for safety, toxicity, and immunogenicity. The research team will shortly explore with the AFIRM leadership exactly what more may be required to proceed to such clinical investigations.

The probiotic therapy has an even shorter route to clinical translation. The researchers have become aware of a recent report in which *Lactobacillus* as a probiotic intervention was used safely in burn patients with limited partial-thickness injuries although no evaluation of scarring as an end point was made. Probiotics already have a much more extensive history of clinical use in other scenarios (e.g., gastrointestinal and genitourinary infections), and this recent study gives promise to their efficacy in burn patients as well. The researchers will, in concert with their burn surgeon colleagues, plan to proceed with a clinical trial in humans, and discussions as to how best to select candidates for such a trial are under way.

Corrections/Changes Planned for Year 4

The biggest change anticipated by the researchers will be a more thorough investigation of ultrasound-mediated gene transfer as a means of delivering their agents of interest to healing wounds since preliminary data suggest that a high level and duration of transgene expression may be achieved in this manner.

In addition, as noted previously, they will specifically begin to focus on set tasks required so that they may be in a position to organize clinical trials to test some of their agents in humans.

Multi-Functional Bioscaffolds for Promoting Scarless Wound Healing

Project 4.5.3, WFPC

Team Leader(s): Newell Washburn, PhD (Carnegie Mellon University)

Project Team Members: Allison Elder, Emily Friedrich, and Mohamed Ramadan (Carnegie Mellon University)

Collaborator(s): Robert J. Christy, PhD (Institute for Surgical Research)

Therapy: Burn treatment.

Deliverable(s): Gels that control inflammation and promote burn healing.

TRL Progress: Start of Program, TRL 3; End Year 1, TRL 3; End Year 2, TRL 4; End Year 3, TRL 5

Key Accomplishments: The researchers identified and validated gel formulations that control inflammation and inhibit burn progression at burn sites. They tested the gels in a rat burn model and determined that conjugation of antitumor necrosis factor-alpha (anti-TNF-α) to HA provides a synergistic reduction in inflammatory responses at burn sites. The (anti-TNF-α)-HA conjugates were active in reducing local inflammation at the burn site in the rat model. The researchers are moving toward clinical trials.

Key Words: Burns, cytokines, inflammation, antibodies, gels

Introduction

The trajectory of burn wound healing is a complex process starting with necrosis due to thermal injury, followed by a two-stage inflammatory process, delayed cell death, formation of granulation tissue, and remodeling. The complications from partial- or full-thickness burns are broad ranging, including compromised protection by the epidermis and loss of resident leukocytes and lymphocytes, edema, reduced host defenses to bacterial colonization, multiple organ failure, and loss of connective tissue cells that would normally contribute to the repair response. Burned tissue has been modeled as having three concentric zones: (1) irreversibly damaged tissue in the zone of coagulation, (2) hypoperfused tissue in a zone of stasis, and (3) edematous tissue in a zone of hyperemia. The central necrotic zone often progresses into surrounding zones, which increases the likelihood of hypertrophic scarring and patient morbidity.

Deleterious physiological responses following thermal injuries are driven by inflammatory responses. Systemic immunosuppression, continued tissue necrosis, and scar formation result from intense inflammatory responses that are mediated by soluble signaling proteins, such as TNF-α, interleukin 1β (IL-1β), and interleukin 6 (IL-6). While inflammatory responses are necessary components of healing processes and are necessary for protecting the host from infections, they assume pathological levels in burn patients and counteract intrinsic regenerative responses.

The goals of treating burn patients are to promote healing while minimizing scarring and wound contracture. Current theories of burn progression suggest that the fundamental mechanism of continued tissue damage is driven by inflammatory responses due to the initial injury. Topical application of steroids, inhibitors of L-arginine uptake that reduce macrophage activities, and a ligand for peroxisome proliferation-activated receptor-γ have all shown some efficacy at inhibiting inflammatory processes and reducing burn progression. However, the broad-spectrum effects of these agents and high mobility in the tissue may make them unsuitable for clinical use. The approach in this research project is to topically deliver a gel therapeutic that locally modulates inflammation by selectively neutralizing TNF-α, a central mediator of deleterious physiological responses to thermal injuries.

Summary of Research Completed in Years 1 and 2

During the first year of this project, the researchers created uncross-linked HA gels by coupling monoclonal antibodies to HA. They also created cross-linked HA gels by covalently attaching the RGD peptide to HA-monoclonal antibody gels. They determined HA gels could modulate the macrophage phenotype without coupled antibodies. They demonstrated that neutralization of TNF-α alone may provide significant reduction of inflammatory signaling. Maximum reduction of inflammatory responses occurred when both TNF-α and IL-1β were neutralized. The researchers also identified material design parameters that optimized the activities of covalently attached monoclonal antibodies. During the second year of the project, the researchers identified formulations of cytokine-neutralizing gels

based on antibodies against TNF-α and IL-1β conjugated to HA that are effective at modulating acute inflammation. Additionally, they demonstrated that cytokine-neutralizing gels preserve viable tissue in a rat burn model.

Research Progress – Year 3

Figure IV-21 shows the rat burn model developed by the research team. Briefly, a 1 inch brass disk is heated to 85°C and pressed against the skin of an anesthetized rat for 10 seconds to create a deep partial-thickness burn. One day following burn injury, the eschar is removed and the treatments are applied to the site. The research team's goal was to test whether the remaining viable tissue beneath the primary injury can be rescued from inflammation-induced necrosis. The researchers tested the following treatments (n = 4 for each treatment and time point):

1. Saline
2. 1% HA solution
3. 100 µg/mL anti-TNF-α solution
4. 1% (anti-TNF-α)-HA solution (100 µg/mL anti-TNF-α)

Tissue samples were removed from the first group one day following application of the first treatment while the rest of the rats received a second treatment on day 2. Half of these rats were sacrificed on day 4 and tissue was recovered while the other half of the group received a third treatment. This last cohort was sacrificed on day 7, the final time point in the study.

Trichrome staining of tissue sections was used to provide gross histological assessment of tissue responses **(Figure IV-22)**. Tissue sections looked similar at day 1, indicating the

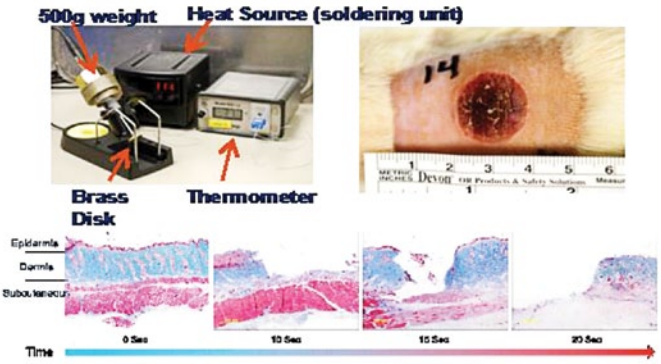

Figure IV-21. The rat partial-thickness burn model developed in this study.

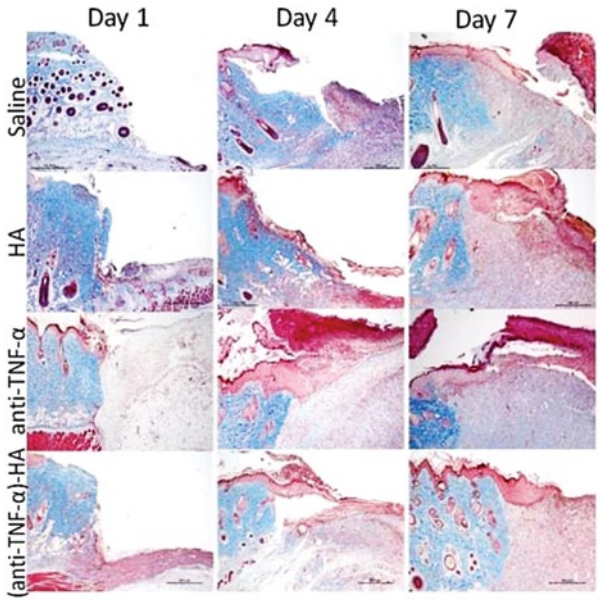

Figure IV-22. Trichrome-stained histology images showing representative burn sites as a function of time and treatment.

burn procedure was reproducible. Most sites were forming granulation tissue by day 4, but by day 7 many sites had formed a thick layer of intensely stained tissue at the outer layer of the site. This layer appeared to be thinnest in the sites treated with (anti-TNF-α)-HA.

Vimentin immunostaining was used to identify necrotic tissue. Results of vimentin immunostaining indicate that the layer observed in the trichrome sections was necrotic tissue **(Figure IV-23)**.

The thickness of the nonstained layer on blinded samples was quantified **(Figure IV-24)**. Sites treated with saline lost 700 μm of viable tissue by day 7, but this amount was reduced by 70% under treatment with (anti-TNF-α)-HA conjugates. HA treatment alone provided nearly a 40% reduction in burn progression, suggesting that HA has an anti-inflammatory effect. Furthermore, direct application of nonconjugated anti-TNF-α did little to inhibit burn progression, having no statistically significant difference with saline treatment at any time point. These data indicate that conjugation of anti-TNF-α to HA provides a synergistic reduction in inflammatory responses at burn sites (Figure IV-24).

To assess the overall state of inflammation at the burn site, extracts from homogenized burn tissue were recovered, and the concentration of IL-1β protein was measured using ELISAs. The production of IL-1β is tightly linked to TNF-α so reductions in the concentration of active TNF α should result in a decrease in the concentration of IL-1β. Interestingly, at day 1 delivery of nonconjugated anti-TNF-α resulted in the most significant decrease in IL-1β concentrations as shown in **Figure IV-25**. However, by day 4 and day 7 only treatment with (anti-TNF-α)-HA conjugates showed any measureable decrease in IL-1β concentrations. These results suggest that at day 1 vascular permeability is low and even rapidly diffusing anti-TNF-α is retained at the site, providing significant reductions in TNF-α activity.

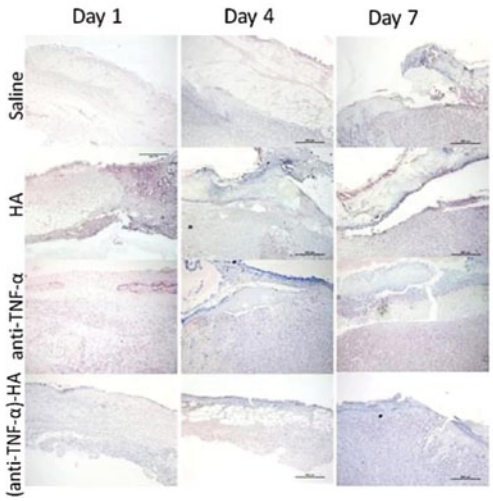

Figure IV-23. Vimentin-stained tissue sections showing burn progression at representative burn sites as a function of time and treatment.

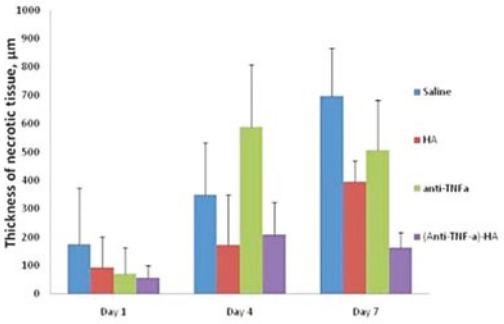

Figure IV-24. Quantification of vimentin immunostaining images showing the amount of necrotic tissue formed as a function of time and treatment.

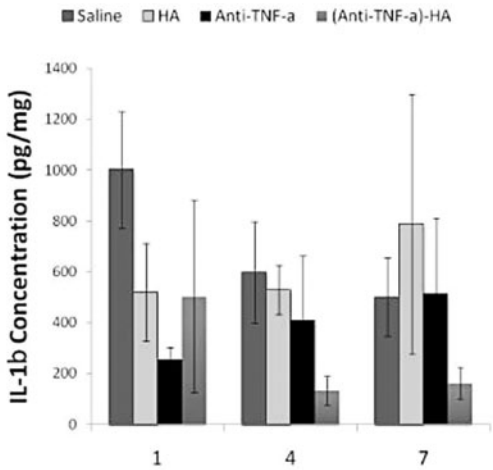

Figure IV-25. Results from ELISA analysis of IL-1β concentrations at burn sites.

However, later in burn progression, increases in vascular permeability could significantly decrease the residence time of nonconjugated anti-TNF-α, and only HA-conjugated anti-TNF-α will be effectively retained and active in reducing local inflammation.

Key Research Accomplishments

- Validated (anti-TNF-α)-HA gels in the rat burn model, demonstrating that 70% less tissue was lost in partial thickness burns with (anti-TNF-α)-HA conjugates compared to saline treatment.
- Determined that (anti-TNF-α)-HA conjugates were active in reducing local inflammation at the burn site in the rat model.

Conclusions

The researchers' results indicate that (anti-TNF-α)-HA conjugates are highly effective at inhibiting burn progression in a rat model. Due to fundamental similarities in early inflammatory responses across species, they expect a similar response in pigs and humans.

Research Plans for the Next 2 Years

The research plan for the following year will focus on determining the minimal number of doses necessary to achieve reductions in burn progression in the rat model as well as developing an improved understanding of the mechanism of (anti-TNF-α)-HA conjugates in reducing burn progression.

Planned Clinical Transitions

The planned clinical transition will involve Washburn Therapeutics. The company has an exclusive license for patents filed by Carnegie Mellon University and has formed a partnership with a manufacturer of anti-TNF-α that can be used in humans. The first step in this partnership is validating the new conjugates in a pig burn model, the gold standard in the regulatory approval process for burns.


our science for their healing
Nikon JAPAN
4× 0.10
Nikon JAPAN
Plan
10×/0.25
WD 10.5

Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration

Project 4.5.4, WFPC

Team Leader(s): Patricia A. Hebda, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)

Project Team Members: Joseph E. Dohar, MD and Tianbing Yang, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)

Collaborator(s): None

Therapy: Attenuate local inflammatory responses to reduce scarring and promote healing.

Deliverable(s): Combinatorial anti-inflammatory topical therapy to reduce scar formation.

TRL Progress: Start of Program, TRL 3; End Year 1, TRL 3; End Year 2, TRL 3; End Year 3, TRL 3

Key Accomplishments: The researchers established and characterized donor cells of different phenotypes, including cell types with regenerative healing capability, for continuing work with cell therapy. They also demonstrated that early, one-time topical treatment with isogenic ASCs, fetal skin fibroblasts (embryonic day 15), and even adult skin fibroblasts leads to reduced scarring and increased healing.

Key Words: Scarless healing, inflammation, fibrosis, cell therapy

Introduction

The Hebda group is focusing on two related processes highly relevant to scar formation: inflammation and fibroblast activity. The overriding hypothesis is that the development of fibrosis can be prevented by blunting early wound healing processes leading to fibroblast recruitment and activation of synthetic properties. To achieve regeneration, it is first essential to regulate the inflammatory response and the influx of host fibroblasts. Control of these two fibrogenic processes will serve to establish an optimal foundation for therapies and interventions leading to regenerative healing. The early inflammatory phase of tissue repair has been shown to be important for the long-term outcome of wound healing.

This project has three specific aims:

- To determine the potential of combinatorial anti-inflammatory therapy in decreasing subsequent fibroblast activity in the wound bed;
- To precisely characterize the contribution of the fibroblast phenotype to the overall degree of tissue fibrosis; and
- To design interventions based on the results of the first two aims that provide a wound environment for rapid, regenerative healing.

The research team proposes to use a novel method—transplantation of fetal fibroblasts into an adult dermal wound bed—to precisely characterize the impact of inflammatory and

other soluble mediators on the fibroblast phenotype. This approach will allow the researchers to determine if the fibroblast phenotype is a dynamic one that is largely influenced by the wound environment. Should this be the case, then prevention of fibrosis/scarring could be primarily a matter of reducing profibrotic signals in the wound bed. Alternatively, if donor fibroblast phenotype persists within the wound after transplantation, therapeutic efforts will be directed toward enhancing the wound healing contribution of fibroblasts with a regenerative phenotype. While there are a number of studies that have tested the effect of cell-based therapy for improved wound healing, the research team is aware of no studies investigating the combinatorial approach it is taking.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and PGE2 can attenuate the wound inflammatory response following skin incisional wounds in the rat, leading to a reduced amount of scarring and the promotion of healing. This determination was based on clinical assessment of healing, wound histology, tissue levels of ECM components, tissue biomechanics, and collagen organization. During the second year of the project, the researchers characterized the effects of therapeutic treatment on collagen production and organization in the healing wounds. They also established donor cell strains of different phenotypes, including cells strains with regenerative healing capability, for continuing work with cell therapy.

Research Progress – Year 3

Aim 1. To Determine the Potential of Combinatorial Anti-Inflammatory Therapy in Decreasing Subsequent Fibrotic Fibroblast Activity in the Wound Bed

The researchers found that the combination of nimesulide and PGE2 significantly increased the regain of tensile strength at 2 weeks. However, either reagent alone appeared to have no benefit for this measure. Additional animals were allowed to heal for 3 or 4 weeks, and it was found that wound tensile strength tended to be similar among vehicle and treatment groups; that is, the vehicle "caught up" to the treatments for this outcome measure **(Figure IV-26)**.

Aim 2. To Precisely Characterize the Contribution of the Fibroblast Phenotype to the Overall Degree of Tissue Fibrosis

From a small breeding colony of transgenic rats established for this aim, cell strains expressing GFP have been generated to serve as donor cells. GFP transgenic rats are of the same genetic background as those used for wound-healing studies so the risk of a host immune response is avoided. It has been well documented that in the mouse model of fetal wound healing, embryonic day 15 (E15) represents a time point at which scarless healing takes place, and embryonic

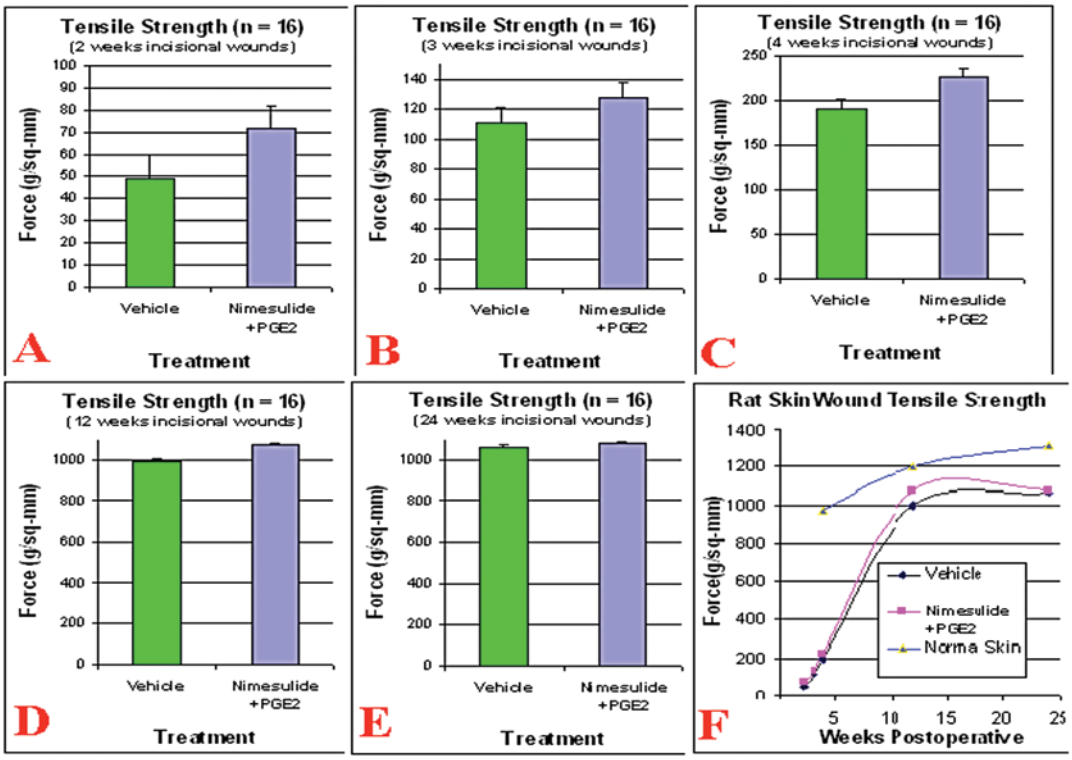

Figure IV-26. The combination of nimesulide and PGE2 significantly increased the regain of tensile strength at 2 weeks (A, $p = 0.03$), but the vehicle "caught up" to the treatment at 3 weeks (B, $p = 0.121$), 4 weeks (C, $p = 0.156$), 12 weeks (D, $p = 0.185$), and 24 weeks (E, $p = 0.739$). The vehicle group and the nimesulide plus PGE2 group reached a tensile strength closest to unwounded skin at 10 weeks post-treatment (F).

day 18 (E18) represents a time point at which fibrotic healing takes place. Therefore, four phenotypes of GFP-fibroblasts have been generated: (1) fetal skin fibroblasts (E15, scarless phenotype; E18, reparative phenotype), (2) adult skin fibroblasts (reparative phenotype), (3) bone marrow-derived MSCs, and (4) ASCs. These cells have been used for this aim and will be used together with the optimized therapies from Aim 1 to determine effects on the viability and responses of the transplanted cells and the quality of healing.

The researchers have adapted protocols from the literature for cell isolation and preparation. To avoid the possibility of stem cell differentiation and fibroblast instability, the optimal passage of all the cell strains selected for therapy was passage 5. The researchers observed that unlike E18 and adult rat skin fibroblasts, E15 skin fibroblasts show more stem cell colonies during primary culture. E15 fibroblasts were purified to delete these stem cells by limited dilution, and the purity was found to be adequate at passage 5. Extensive phenotypic study of cells prepared from GFP transgenic rats is under way. The initial data are encouraging. Vimentin is the most frequently found intermediate filament in fibroblasts. Thus, it is a reliable fibroblast marker. However, vimentin is also expressed on the cell surface of activated macrophages, platelets, and apoptotic T cells and neutrophils. Direct vimentin staining for flow cytometry indicated different cell surface expression among the four cell strains **(Figure IV-27)**.

CD54 is known as one of the most important cell surface markers in the regulation, recruitment, and retention of inflammatory cells. CD54 expression was detected by flow cytometry in the four cell strains **(Figure IV-28)**, and the results indicated that CD54 is expressed on 97% of adult skin fibroblasts (with mean fluorescence intensity [MFI] 2,873), 82% of ASCs (with MFI 2,237),

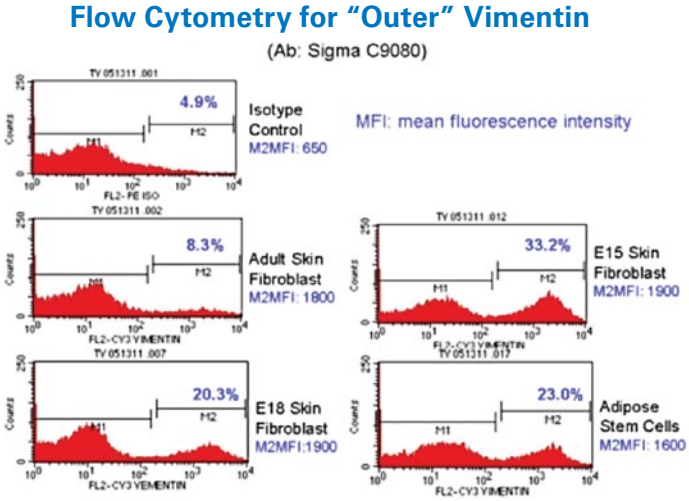

Figure IV-27. Flow cytometry for cell surface vimentin.

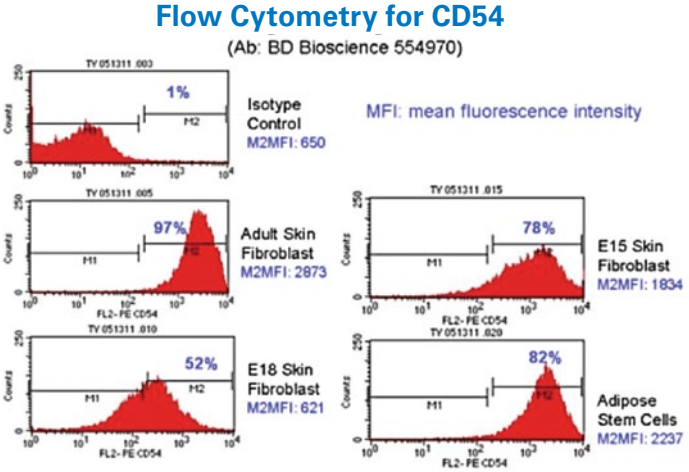

Figure IV-28. Flow cytometry for CD54.

78% of E15 fetal skin fibroblasts (with MFI 1,834), and 52% of E18 fetal skin fibroblasts (with MFI 621). The researchers are analyzing more flow cytometry results from different cell types with the same phenotypes and trying to compare MFI statistically.

The researchers have performed three animal experiments using the previously named cells as a therapeutic tool in the rat incisional wound model. Wound tissue analysis was performed using standard procedures to trace GFP-positive cells with fluorescence microscopy. With the assistance of a commercial antibody, the researchers were able to successfully confirm the presence of GFP-positive donor cells in the wound bed of the treated group but not in the vehicle group in 2 weeks' healing **(Figure IV-29)**. The procedure they used was to enhance the donor cells' fluorescence signal by fluorescein isothiocyanate (FITC)-labeled, anti-GFP antibody (Abcam) and meanwhile reduce endogenous skin fluorescence by counterstaining with 0.05% pontamine sky blue.

Tensiometry was conducted to evaluate the healing of incisional wounds treated with the cells after 2 weeks. The result indicated that the amount of force required to break apart ASC-treated wounds was significantly greater than that for vehicle control wounds in 2 weeks (p <0.001; analysis of variance [ANOVA]), and this increase in wound strength was followed by wounds treated with E15 fetal skin fibroblasts (p=0.007; ANOVA), demonstrating that ASCs and E15 fetal skin fibroblasts significantly increased the tensile strength. However, adult skin fibroblasts were marginally better (p=0.05) while, most interestingly, E18 skin fibroblasts and bone marrow stem cells had no benefit for this aspect of healing (p=0.51 and p=0.17, respectively).

Due to the different times required for obtaining these cell types (fetal skin fibroblast phenotypes took longer to generate), current data are not yet complete for every cell type. For example, the researchers tested total collagen deposition and collagen organization of the newly formed tissue in the wound bed of the groups without fetal skin fibroblasts, and the results indicate no difference in total collagen levels among groups by assessment with a colorimetric assay of

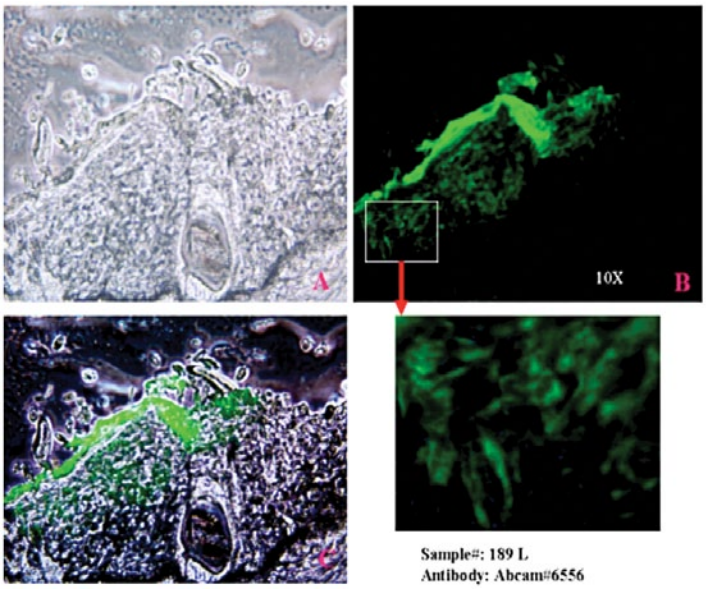

Figure IV-29. The presence of GFP-positive cells in the wound bed identified by cell morphology (an example of ASC treatment): (A) Differential interference contrast picture of wound bed under a light microscope showing the gross tissue outline. (B) Corresponding image with GFP-positive. (C) Overlays A and B to locate the green cells.

hydroxyproline. However, when polarized light microscopy of picrosirius staining was used for the assessment of collagen fibril architecture in the wound bed and normalized against unwounded normal skin, the threshold area (%) was calculated as 8.6±1.3 in the ASC-treated group, higher than other groups (p=0.04), but there was no difference among vehicle control (4.7±0.8), adult fibroblasts (6.7±3.7), and bone marrow stem cells (5.1±0.4) **(Figure IV-30)**. This result suggests that ASC treatment achieved better collagen matrix organization (wound maturation) without increased collagen deposition (fibrosis) and has important implications for the clinical reduction of scarring.

The researchers have been conducting research on total fibroblast (donor cells and host cells) infiltration into the wound area with hematoxylin and eosin staining and microscopy **(Figure IV-31)**, and they have further evaluated the contribution of the cells to wound healing. The MetaMorph imaging system was used for cell counting, and fibroblasts were identified based on cell size and morphological factors. These data show no significant changes in fibroblast infiltration (Figure IV-31). The researchers are now working on determining the percentage of the cell phenotypes in the wound bed.

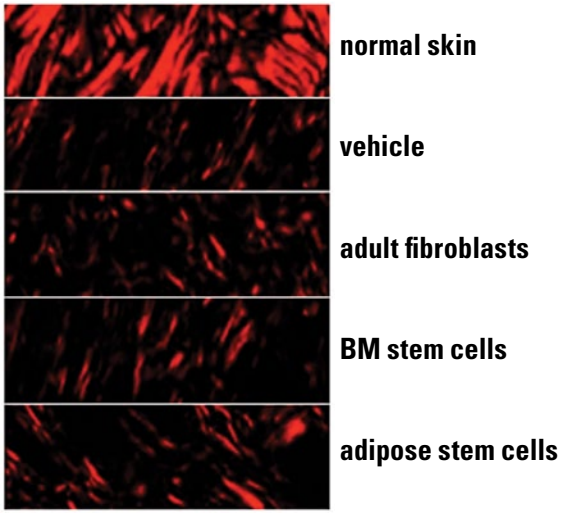

Figure IV-30. Collagen Organization: Polarized light microscopy of picrosirius staining was made for the assessment of collagen fibril architecture in the wound bed. Normalized against unwounded normal skin, the threshold area (%) was calculated as 8.6±1.3 in the ASC-treated group, higher than other groups (p=0.04), but there was no difference among vehicle control (4.7±0.8), adult fibroblasts (6.7±3.7), and bone marrow stem cells (5.1±0.4).

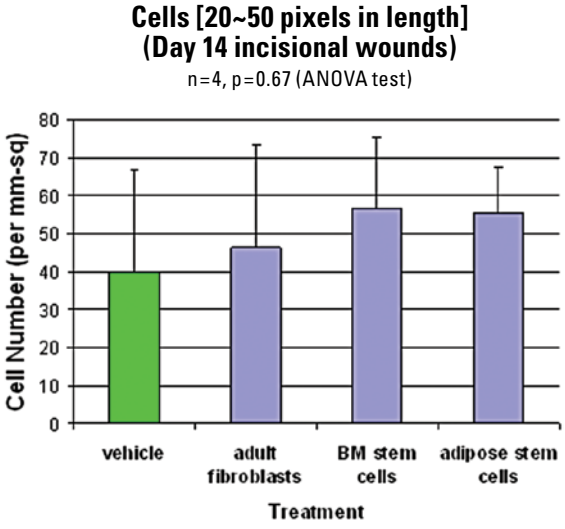

Figure IV-31. Fibroblasts in wound bed. Fibroblasts were defined according to the size and shape (length between 20 to 50 pixels). After hematoxylin and eosin staining by using the MetaMorph system, cell counting indicated no difference among the groups.

Key Research Accomplishments

- Established and characterized donor cells of different phenotypes, including cell types with regenerative healing capability, for continuing work with cell therapy.
- Demonstrated that early, one-time topical treatment with isogenic (same strain) ASCs, fetal skin fibroblasts (E15), and even adult skin fibroblasts leads to reduced scarring and increased healing.

Conclusions

The results show that early, one-time topical treatment with isogenic ASCs, fetal skin fibroblasts (E15), and even adult skin fibroblasts leads to reduced scarring and increased healing. In addition to the previous conclusion that early, short-term treatment with anti-inflammatory agents nimesulide and PGE2 combination can attenuate the wound inflammatory response with downstream effects on healing, a cell therapeutic strategy that will be used under anti-inflammatory conditions is ready to test in the next year of the project. These results are very encouraging as they suggest that the experimental plan is feasible and the milestones are achievable. The work will continue with additional studies to verify and optimize results to date.

Research Plans for the Next 2 Years

During the next 2 years, the researchers will address Aim 3 (the design of interventions) based on results of the first two aims to provide a wound environment for rapid, regenerative healing. They will combine anti-inflammatory treatments with donor fibroblast delivery to see whether donor fibroblasts (of favorable phenotypes) have a greater impact on healing outcomes. The focus of these studies will be the basis for developing a wound treatment regimen for a future clinical trial.

Planned Clinical Transitions

The researchers have selected therapeutic agents that have the advantage of already being approved for use in humans. Meanwhile, clinical trials using autologous stem cell therapies have begun in the United States within the past few years. This should facilitate the pathway toward a Phase 1 clinical trial. The optimal treatment will first be tested in an animal model analogous to the clinical target (still to be determined but possibly a burn injury). The results will determine whether the treatment is ready for clinical testing or whether additional refinement in the animal model is necessary. These clinical studies, however, will likely commence after year 5 of this project.

Corrections/Changes Planned for Year 4

This project has not had substantial changes made to it. However, the researchers intend to expand upon the original plan by including the testing of systemic treatment (in addition to topical delivery) to maximize the clinical applications for which this approach can be used.

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Peptide-Mediated Delivery of Therapeutic Compounds into Injured Tissues During Secondary Intervention

Project 4.5.6, WFPC

Team Leader(s): Erkki Ruoslahti, MD, PhD (Sanford-Burnham Medical Research Institute at University of California, Santa Barbara [UCSB])

Project Team Members: Tero Järvinen, MD, PhD (Sanford-Burnham Medical Research Institute at UCSB and University of Tampere, Finland) and Eunhye Lee, PhD, Sajid Hussain, PhD, and Chris Brunquell (Sanford-Burnham Medical Research Institute at UCSB and Institute for Collaborative Biotechnologies)

Collaborator(s): None

Therapy: Drug targeting to injured tissues/ preventing scarring and enhancing tissue regeneration.

Deliverable(s): Systemic and local wound targeting with peptides that penetrate into wound and scar tissue.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 3; End Year 3, TRL 5

Key Accomplishments: The researchers have made substantial progress in identifying peptides that home to wounds and can deliver a therapeutic payload to the wounds and other injured tissues. They determined that treatment of mice with skin wounds with the wound-homing CAR peptide promotes wound healing. The researchers generated a variant of the CAR peptide that is biologically more active than CAR. They also showed that fusion with the CAR peptide renders the antifibrotic protein decorin selective against the scar-inducing isoforms of TGF-β.

Key Words: Wound angiogenesis, homing peptides, anti-scarring, TGF-β

Introduction

The researchers previously published data concerning two wound-homing peptides that recognize wound blood vessels at different stages of healing. One of these peptides appears to recognize a wound-specific form of heparan sulfate; the target molecule for the other is not known.

The researchers have recently discovered and defined a pathway for coordinated cell and tissue penetration and delivery of pharmaceuticals to the desired location in a tissue-specific manner. Most recently, they found that this can be accomplished without physically coupling the pharmaceutical to the targeting peptide **(Figure IV-32)**. This system, which they have given the name CendR, is described in detail as follows together with their current efforts on establishing the CendR pathway for the targeted delivery of therapeutics to tissue injuries (Figure IV-32).

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers produced and purified a target-seeking antifibrotic agent (recombinant decorin fusion protein) in mammalian expression vectors and baculovirus. They established the in vitro biological activity of the decorin fusion protein. They achieved targeted delivery of the decorin fusion protein into regenerating

tissue following intravenous injection in mice. They demonstrated that decorin could inhibit TGF-β-dependent scar-associated processes. The researchers showed that decorin fusion protein could inhibit scar formation in mice during wound healing. They also found that wound-targeted decorin was more effective in suppressing various indicators of subsequent scarring than nontargeted decorin.

Research Progress – Year 3

Specific Aim 1

For wound-healing purposes, the researchers generated a truncated form from their wound-targeting peptide CAR (tCAR; CARSKNK) by making the second lysine the C-terminal residue to mimic the CendR peptides, which have an arginine or lysine residue at the C-terminus. They used Chinese hamster ovary (CHO) cells (CHO-K) and the pgsA-745 mutant CHO line that is defective in glycosaminoglycan biosynthesis to test the binding of CAR and tCAR to cell surface glycosaminoglycans. CAR phage bound to the CHO-K cells 55-fold more than nonrecombinant control phage ($p < 0.0001$), and there was no specific binding to the glycosaminoglycan-deficient cells, which is in agreement with previous data. The tCAR phage bound significantly better to the CHO-K cells than CAR. The difference was more than 150-fold over nonrecombinant phage, 3 times more than CAR phage. Almost 80% of the tCAR binding was lost when the pgsA-745 cells were used.

As tCAR displays a motif similar to the R/KXXR/K CendR motif and CAR penetrates in wound tissue, the researchers tested whether oligovalent complexes of the CendR peptide RPARPAR would interfere with CAR and tCAR binding to the CHO-K cells. The RPARPAR complexes inhibited the binding and internalization of RPARPAR phage

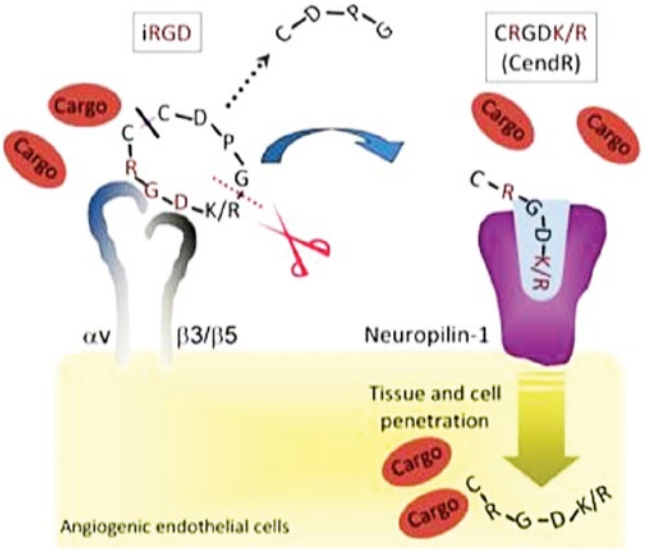

Figure IV-32. Multistep binding and penetration mechanism of CendR peptides. The principle of the CendR system as it is applied to a peptide dubbed iRGD (sequence: CRGDKGPDC). The two motifs in iRGD are the RGD motif, which mediates the binding of the peptide to αv integrins and a cryptic CendR sequence RGDK. The RGD homing sequence directs the peptide to angiogenic vasculature (expresses αv integrins) where the peptide is proteolytically processed by an endogenous protease such that the CendR motif (R/KXXR/K) becomes C-terminal and active. The activated CendR motif then binds to neuropilin-1, which mediates extravasation, tissue penetration, and cell entry of the C-terminally truncated peptide and can drive delivery of any pharmaceutical agent into cells and tissue without physically attaching the molecule to the iRGD peptide.

(positive control) to the cells but had no effect on the binding and internalization of the CAR or tCAR phages. These results suggest that CAR and tCAR enter into cells and tissues by a mechanism that is distinct from the CendR pathway and is heparin sulfate dependent.

Synthetic, fluorescamine-conjugated peptides were tested for their tissue distribution after intravenous injection into mice bearing either orthotopic 4T1 breast tumors or skin wounds. CAR and tCAR peptides produced strong fluorescent signals in the tumors, but the tCAR peptide showed substantially better spreading to the tumor parenchyma (not shown). Both CAR and tCAR also produced a high signal from the granulation tissue in wounds, especially where most of the angiogenesis takes place, whereas the control peptide, mutant CAR (mCAR; CAQSKNNDC), did not home to wound tissue **(Figure IV-33)**. There was a striking difference in the wound accumulation pattern of CAR and tCAR; tCAR peptide spread deeply into the granulation tissue whereas CAR peptide mainly stained the hypervascular bottom layer of the granulation tissue. The fluorescent signal produced by CAR and tCAR in nontumor tissues (i.e., liver, kidney, lung, heart, brain, pancreas, and spleen) did not differ from that of control peptides (not shown). CAR and tCAR did not accumulate in normal skin (Figure IV-33).

The researchers also explored the temporal extravasation pattern of tCAR peptide by coating nanoparticles with it. The tCAR nanoparticles were initially found in and around the blood vessels but over time extravasated and spread into the surrounding tissue (not shown).

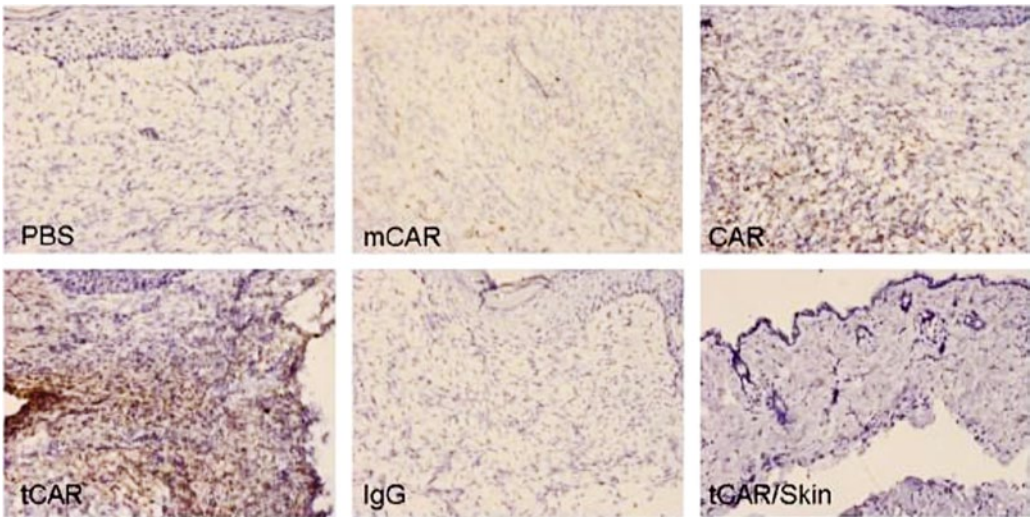

Figure IV-33. CAR peptide homing in wound tissue. Mice with full-thickness skin wounds received an intravenous injection of fluorescein-conjugated peptides on day 5 after wounding. After 2 hours, the location of peptides was determined with anti-FITC antibodies (brown). These antibodies were used to detect fluorescein because a high autofluorescent background in skin wounds makes it difficult to interpret the fluorescent signal. Two different antifluorescein antibodies gave a similar signal, confirming the specificity of the detection. The wounds of mice injected with CAR and tCAR provide strong staining whereas mCAR-injected wounds were weakly positive. The penetration of tCAR deep into the wounds is substantially greater than that of CAR, which seems to stay in the hypervascular area in the bottom of the dermis. No staining was observed in normal skin far from the wounds (shown for tCAR; tCAR/Skin) or in the normal skeletal muscle underlying the skin wounds of mice treated with any of the peptides. No staining was seen in wound tissue when class-matched mouse IgG was substituted for the antihistidine tag antibody (IgG). Representative fields are shown for each peptide from three wound-bearing mice with four different wounds.

A notable discovery is that treatment of mice with skin wounds with the CAR peptide promotes wound healing. CAR penetrates into cells and tissues in a manner similar to the recently identified CendR peptides. Based on these results, the researchers hypothesize that CAR may enhance wound healing by improving the availability of natural growth factors from the blood and serum to the regenerating tissue and that because of the wound specificity of CAR, this effect would be specific to wounds. In essence, the researchers would be pharmacologically manipulating a previously described plasma → serum → plasma transition that takes place during normal tissue repair and controls tissue regeneration **(Figure IV-34)**.

In the researchers' treatment experiments, intravenous administration of CAR or a control peptide was started 24 hours after wounding. The treatment was continued for 4, 6, or 9 days in two independent treatment experiments with 15 mice in each treatment group (n=54). CAR was administered in much higher doses than the dose used previously in targeting CAR-decorin fusion protein to skin wounds. The daily dose of 75 µg was chosen on the basis of previous results on CendR peptide treatments. The closure of wounds was significantly accelerated in CAR-treated mice than in controls (**Figure IV-35**, $p < 0.0001$ CAR versus control/mCAR for all time points from day 5 on).

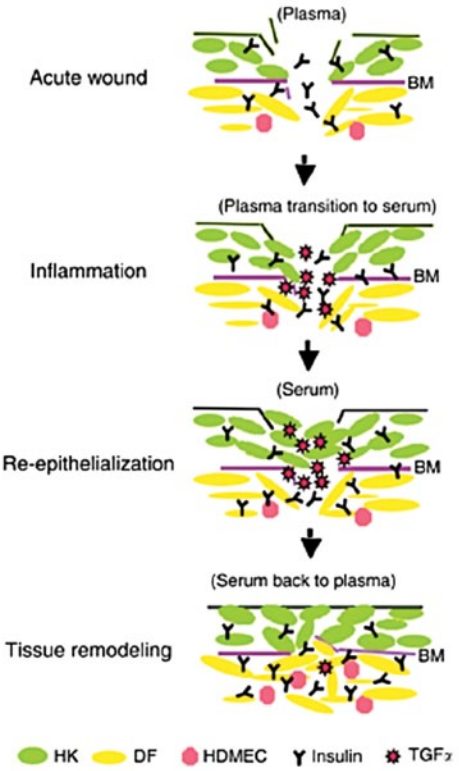

Figure IV-34. A schematic representation of how serum promotes re-epithelialization during wound healing. The three classical and sequential events of wound healing, inflammation, re-epithelialization, and tissue remodeling, are schematically depicted. The three major types of cells involved in wound repair, keratinocytes, dermal fibroblasts, and human dermal microvascular endothelial cells, are shown. The serum-derived TGF-α levels are dramatically increased in the wound fluid following the transition from plasma to serum in the wound bed. After the wound is closed and after transition of serum back to plasma, the levels of TGF-α go back to those in unwounded skin.

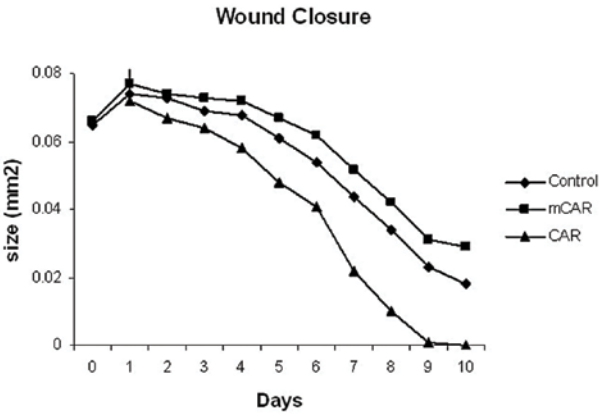

Figure IV-35. Wound closure in CAR-treated animals. Mice with full-thickness skin wounds received intravenous injections of 75 µg of CAR twice a day from day 1 after the wounding until sacrifice. The wounds were examined and photographed daily. Wound closure was recorded and expressed as the size of unclosed wound. The image shows accelerated wound closure in wounds treated with CAR compared with the control groups (PBS and mCAR peptide). Statistical significance was examined using the $\chi 2$ test (CAR versus control/mCAR for all time points from day 5 on $p < 0.0001$). n=72 on days 0–5, 48 on days 6–7, and 24 on days 8–10.

The accelerated wound healing in the CAR-treated mice was also evident when wound closure and re-epithelialization were analyzed by assessing the number of wounds that had completely closed/re-epithelialized **(Figure IV-36)**.

To confirm that the accelerated wound closure was due to faster re-epithelialization of the wounds in the CAR-treated animals, the researchers also examined histological sections. Significantly shorter distance between the tips of the epithelial tongues was measured for the CAR-treated wounds than in controls at all time points analyzed (p <0.001, ANOVA, **Figure IV-37**).

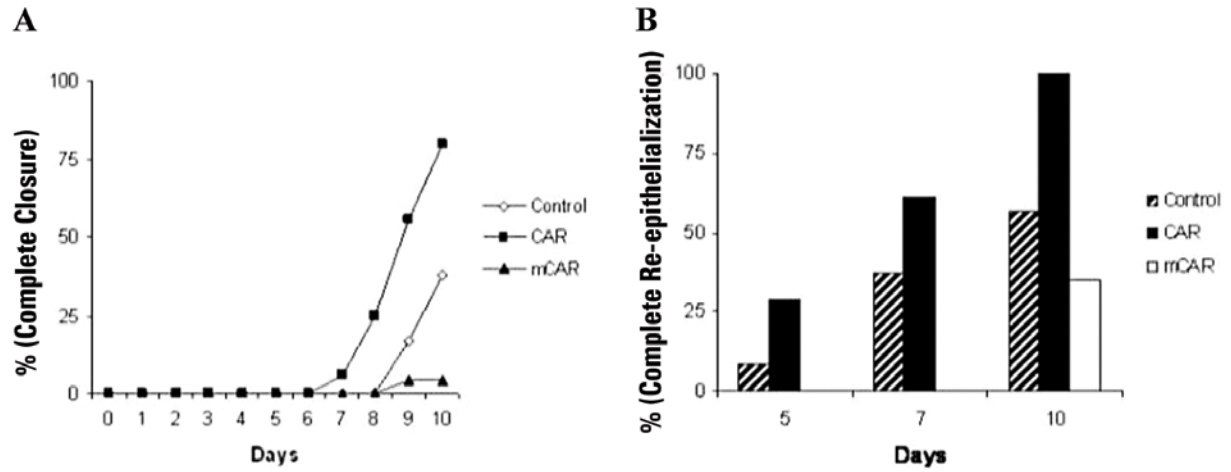

Figure IV-36. Complete wound closure/re-epithelialization in CAR peptide-treated animals. (A) The wounds of animals treated as in Figure IV-35 were examined and photographed daily. Wound closure was recorded and expressed as the percentage of wounds that had completely closed. (B) Re-epithelialization of the epidermis was quantified on days 5, 7, and 10 by examining two microscopic sections from each wound.

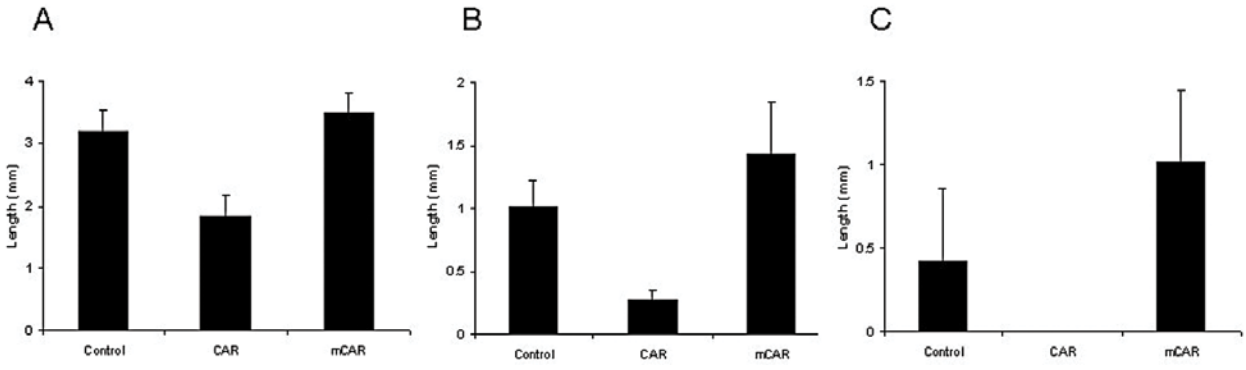

Figure IV-37. Wound re-epithelialization in peptide-treated mice. (A) The wounds of mice treated as in Figure IV-35 were harvested and analyzed microscopically. The gap in the epidermis was quantified on days (A) 5, (B) 7, and (C) 10 by examining two microscopic sections from each wound and expressed as the average of the two values. p <0.001 CAR versus control/mCAR at all three time points; statistical significance was examined using ANOVA. The results are expressed as mean ± SEM, n=24 at all three time points.

Specific Aim 2

Wound-Targeted Decorin

During the past year, the researchers have completed and published the scar suppression study. One of the key pieces of data is the unique selectivity of CAR-decorin against the different TGF-βs. CAR-decorin was significantly more active than decorin against TGF-β1 and TGF-β2 but, remarkably, had no effect on TGF-β3. The differential inhibitory activity of CAR-decorin against the TGF-β isoforms could have important implications for clinical medicine; TGF-β1 is the isoform responsible for scar formation, and TGF-β2 augments the profibrotic activity of TGF-β1. In contrast, TGF-β3 inhibits scarring. The researchers propose that CAR-decorin inhibits scar formation induced by TGF-β1- and TGF-β2 while leaving the beneficial TGF-β3 untouched.

Decorin is a proteoglycan, and the heterogeneity of its single glycosaminoglycan chain makes recombinant decorin produced in mammalian cells heterogeneous, which could cause regulatory problems. Previous data indicate that the protein core is responsible for the binding of decorin to TGF-β and that the chondroitin sulfate side chain can actually hinder this interaction. Thus, the chondroitin sulfate chain may not be needed for the inhibition of TGF-β1 activity by decorin, and it may be possible to use homogeneous, GAG-free CAR-decorin that is actually more active than the native decorin as an anti-scarring agent.

The researchers have introduced the previously used serine to alanine mutation at the chondroitin sulfate attachment site to produce decorin core protein with no side chain. They have also cloned decorin and CAR-decorin into a baculovirus expression system, which is not capable of incorporating glycosaminoglycans to proteins. For the protein expression of recombinant decorin by baculovirus, they needed to substitute the decorin native secretion signal with that from honeybee melittin gene to get protein expression using the virus. They have generated high titer viruses for both the decorin and CAR-decorin and produced high yields of decorins devoid of the chondroitin sulfate chain using baculovirus. Next, they will test these decorins for the anti-scarring activity.

Key Research Accomplishments

- Determined that treatment of mice with skin wounds with the CAR peptide promotes wound healing.
- Generated a variant of the CAR peptide that is biologically more active than CAR.
- Showed that fusion with wound-homing peptide CAR renders decorin selective against the scar-inducing isoforms of TGF-β.

Conclusions

The development of systemic delivery of therapeutic agents to injured tissues is progressing as planned. The new tissue-penetration technology offers particular promise. Scar tissue is dense and particularly impermeable to drugs. Preliminary data from this project suggest that it may be possible to enhance drug delivery to already established scar tissue. These data also show that the activity of the anti-scarring agent, decorin, can be greatly enhanced by homing peptide delivery. Further work to improve the properties of the targeted decorin to make the regulatory path easier is in progress in parallel with efforts to find a corporate partner, which have brought two companies to licensing discussion so far.

Research Plans for the Next 2 Years

The work on the peptide delivery systems will focus on the cell and tissue-penetrating properties of the CAR peptide and its use in enhancing wound and scar penetration of co-administered compounds. Characterization of the CAR variant tCAR, which appears to be more potent in wound homing than the original CAR, will be another focus area. The decorin project will focus on improving the properties of CAR-decorin to facilitate commercial production and regulatory approvals. Comparisons of CAR-decorin and tCAR-decorin in wound-homing and scar prevention experiments will also be carried out. The inherent biological activity of CAR in promoting wound healing suggested by initial experiments will be confirmed and its molecular basis explored. Possible synergies of the CAR-decorin and CAR peptide treatments will also be studied. Finally, the molecular basis of the wound-healing promoting activity of the CAR peptide will be studied to facilitate the transition of this treatment into the clinic.

Planned Clinical Transitions

The hope is that the improved efficacy demonstrated for the targeted decorin will encourage clinical trials. The advantages are that less of the recombinant protein needs to be manufactured and that patent coverage will extend into the late 2020s. The research team's publication at the end of 2010 has generated substantial interest among academia. Several promising collaborations have been initiated with prominent scientists in the field of scar and fibrosis prevention. The aim is to prove the effectiveness of the targeted decorin in other experimental models of fibrosis to generate additional interest from the biotechnology and pharmaceutical industries. Discussions with potential commercial partners and public funding agencies are under way to advance the project.

Corrections/Changes Planned for Year 4

The main new element that was not foreseen when the original application was submitted is that it is possible to use tissue-penetrating homing peptides to deliver drugs to a target tissue without coupling the drug to the peptide. The peptide activates a transport system in the specific target tissue that sweeps along any compound in the blood. Major improvements in the delivery of drugs to injured tissues may ensue.

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Scar Mitigation via Matrix Metalloproteinase-1 Tertiary Therapy

Project 4.5.7, WFPC

Team Leader(s): Alan Russell, PhD and Richard Koepsel, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)

Project Team Members: Johnny Huard, PhD, Harry Blair, MD, and Yong Li, MD, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)

Collaborator(s): None

Therapy: Scar mitigation by MMP-1 injection.

Deliverable(s): A method to reduce scarring using a readily available biotherapeutic enzyme.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 2; End Year 3, TRL 3

Key Accomplishments: The researchers developed a mathematical model for the residence time and potential activity of a bolus injection of MMP-1 at a scar site based on the kinetic behavior of the enzyme derived from in-house biochemical characterization of the enzyme and literature values of the enzyme's activity. The model is allowing the research team to bypass a planned initial preclinical study and proceed to an advanced preclinical trial in rats.

Key Words: Collagenase, matrix metalloproteinase, scar remediation

Introduction

The enzyme MMP-1 is capable of degrading the collagen present in scar tissue and is a candidate for the mitigation of muscle scars. Preliminary studies in mice have shown that a single dose of the enzyme can reduce the amount of collagen in a scar by up to 15% with a concomitant increase in the amount of muscle fibrils. In vitro studies of the enzyme have shown that the activity of the enzyme is dependent upon its ability to bind to the collagen substrate. The reduction in fibrillar collagen in a scar will depend on the amount of enzyme delivered to the site of the scar and the length of time that the enzyme remains at the scar site. Thus, the therapeutic dose for a given defect size can be determined by modeling the behavior of the enzyme in scar tissue.

Summary of Research Completed in Years 1 and 2

During the first year of this project, the researchers developed a method to produce pure homologous MMP-1 enzyme for use in preclinical trials, modified MMP-1 with a collagen-binding peptide, developed the dual-polarization interferometer as a platform for determining real-time MMP activity, and demonstrated differences in the activity of MMP-1, PEG-MMP-1, and peptide MMP-1. During the second year, the researchers showed that modification of MMP-1 to improve stability or binding results in a significant reduction of the enzyme's activity

against collagen. Modification with PEG did enhance stability; however, the enhancement was negligible under physiological conditions. Modification with a collagen-binding peptide enhanced collagen binding but reduced enzyme activity. Therefore, unmodified MMP-1 was selected for animal trials.

Research Progress – Year 3

During the past year, the researchers sought to develop a mathematical model of the enzyme MMP-1 within a scar that can: (1) predict the dosage of MMP-1 and the amount of time required to degrade the collagen in scar tissue and (2) provide guidelines for animal experimentation.

To model the diffusion of MMP-1 from a scar site, boundary conditions must be established. The scar is represented by the circle and the injection site by the dotted circle **(Figure IV-38)**. The boundary conditions are as follows: (1) the edge of the scar is modeled as an impermeable membrane allowing the entire site to act as a confined bioreactor and (2) the enzyme is freely diffusible away from the injection site. The model will thus give the limits to MMP-1 activity in extreme cases that can then be combined to predict real-world behavior.

The model is broken down into two conditions. The first condition involves no diffusion of the enzyme from the site, which allows for a determination of the maximum activity that could be expected. The Matlab calculations use the enzyme kinetic constants to calculate the amount of collagen degradation that will be degraded in the scar volume by a known amount of enzyme in a certain time.

The diffusionless half of the model was run with two assumptions. The first assumed that the K_{cat} of the enzyme was 49 sec^{-1}, a fast reaction time kinetic value derived for

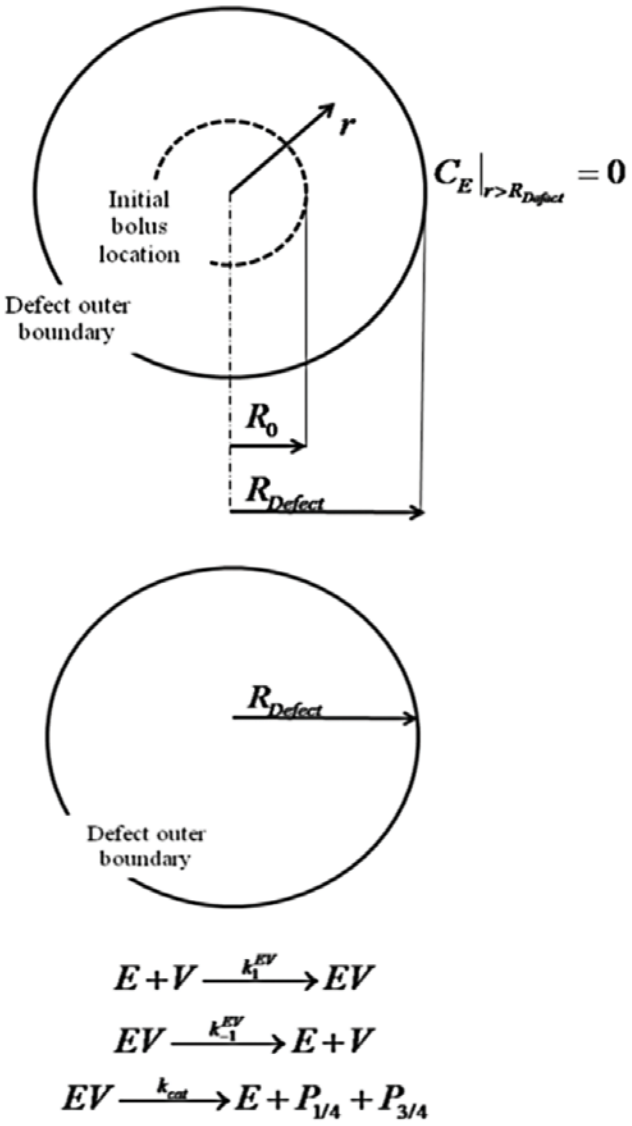

Figure IV-38. Mathematical model for the residence time and potential activity of a bolus injection of MMP-1 at a scar site. Assumptions include: (1) enzyme does not leave the defect site and (2) uniform enzyme concentration throughout defect (stirred tank reactor). E = enzyme (MMP-1), V = vulnerable collagen site.

the reaction of MMP-1 with a small molecular weight peptide substrate. The second assumption used a K_{cat} of 0.004 sec^{-1}, which was derived from experimental data using fibrillar collagen as the substrate. The results gave the limits of the time required for a given concentration of enzyme to degrade a given amount of collagen **(Figure IV-39)**. The results indicate that all concentrations of the enzyme would degrade the collagen in under 30 minutes if the K_{cat} was actually that reported for the small-molecule substrate but would require up to 16 hours at a turnover rate reflective of the natural enzyme substrate.

The second aspect of the model introduces diffusion away from the site. The diffusion of protein in tissue when it does not encounter any specific binding entity is close enough to that in water that the assumption of free diffusion is adequate to determine behavior. Binding of MMP-1 to collagen was measured and found not to be a factor in diffusion through a column containing solidified agarose as a tissue stimulant **(Figure IV-40)**. While the columns with collagen showed some retention of MMP-1, the concentrations in the initial slices from columns with and without collagen were not statistically different and total recovery if the initial MMP-1 activity was low in all of the experiments.

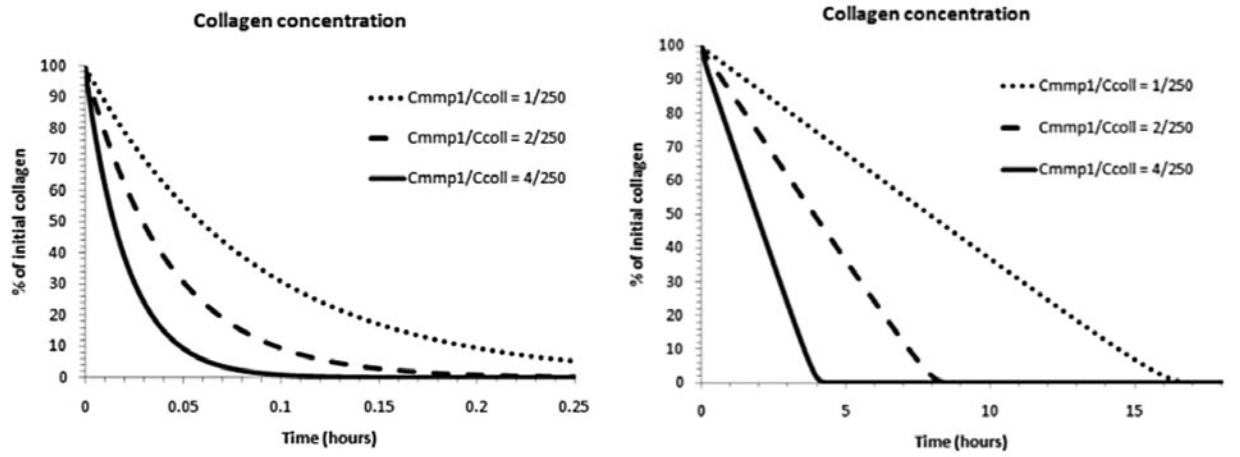

Figure IV-39. Fast reaction (left side); slow reaction (right side).

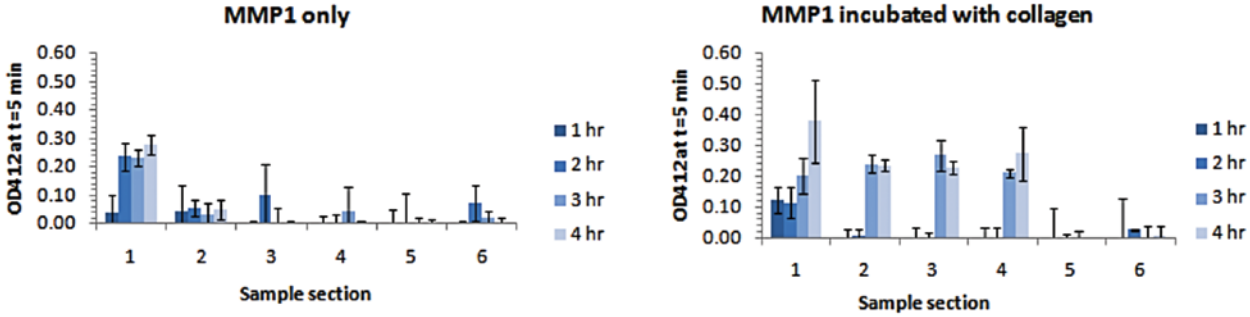

Figure IV-40. MMP-1 was loaded onto agarose columns with and without collagen inclusion. The columns were cut into sections and crushed for MMP-1 assay.

These results imply that the binding of MMP-1 to collagen does little to limit diffusion through tissue.

The model accepts the possibility of a binding effect on diffusion, and the extent of that effect can be varied.

A set of equations shown in **Figure IV-41** was used to determine diffusion rate.

Running these equations in the Matlab, the model resulted in a residence time of MMP-1 in the scar site. It was determined that the $T_{1/2}$ for a bolus of MMP-1 in a scar was about 20 minutes.

When the two halves of the model were combined, the results for the different substrate turnover numbers were dramatically different **(Figure IV-42)**. The slow K_{cat}, which best represents the real MMP-1/collagen interaction, shows that at 2 hours (a time when less than 10% of the enzyme remains on site) the highest concentration of enzyme results in about 15% of the collagen being degraded. Interestingly, the pilot study in mice showed that a single MMP-1 injection at a concentration equivalent to the one modeled resulted in an approximately 10%–15% reduction in collagen at the scar site. These results help to confirm the validity of the model.

Additionally, the modeling results suggest that a single dose of MMP-1 will not be sufficient to remove a scar and that multiple doses will be required.

$$\frac{\partial C}{\partial t} = D\left[\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C}{\partial r}\right)\right]$$

$$i.c.:\ C\big|_{t=0,r<R_0} = C_0$$

$$b.c.:\ C\big|_{t>0,r=R_{Defect}} = 0,\quad \left.\frac{\partial C}{\partial r}\right|_{r=0} = 0$$

Figure IV-41. Equations used to determine diffusion rate.

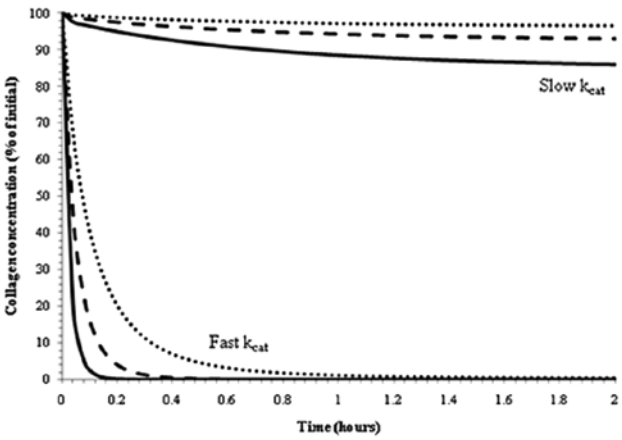

Figure IV-42. Calculation of the complete model.

Preclinical Trials

As a result of the modeling, the planned dose escalation trial in rats was canceled and replaced with a multi-dose trial. The trial is examining the effect of 1, 2, 3, and 4 doses of MMP-1 given at intervals of 2, 3, and 5 days. This will allow a determination of the

potential for damage from the multiple injections and an assesment of the contribution of the natural remodeling process in the progress of scar remediation. This trial is under way with a total of 16 of the planned 48 rats already enrolled or completed. Histological analysis is ongoing.

Key Research Accomplishments

- Developed and validated a mathematical model for MMP-1 administration. The model is applicable to any enzyme that will be used as a local therapuetic agent.

Conclusions

A mathematical model of MMP-1 within a scar was developed. The model accounts for the diffusive transport of MMP-1 and the full kinetics of the degradation of collagen by MMP-1. The model provides a rational method for predicting the amount of collagen that can be degraded by a given dose of enzyme. Further, the model gives an indication of the length of time a given dose of enzyme will maintain significant activity at the scar site. The results predicted by the model are in good agreement with the pilot study data. The model allowed the researchers to bypass the proposed dose-ranging preclinical trial protocol in rats and proceed directly to the multiple-dosing phase of the preclinical plan. Additionally, results suggest that a single dose of MMP-1 will not be sufficient to remove a scar and that multiple doses will be required.

Research Plans for the Next 2 Years

This project will conclude in December 2011.


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Neodyne's Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation – Clinical Trial

Project 4.5.9, WFPC

Team Leader(s): Bill Beasley (Neodyne Biosciences, Inc.) and Geoffrey C. Gurtner, MD (Stanford University)

Project Team Members: John Zepeda, Jasper Jackson, Rich Caligaris, Peggy McLaughlin, and Christy Cowley (Neodyne Biosciences, Inc.)

Collaborator(s): Michael T. Longaker, MD, MBA, Reinhold Dauskardt, PhD, and Paul Yock, MD (Stanford University)

Therapy: Control of wound environment to minimize scarring.

Deliverable(s): Commercially available devices capable of stress shielding mechanical forces to minimize scar formation.

TRL Progress: Start of Program, TRL 5; End Year 2, TRL 6; End Year 3, TRL 7

Key Accomplishments: Neodyne has finalized the design of a third-generation device capable of stress shielding wounds and off-loading pathologic mechanical forces to prevent fibrosis in a user-friendly design. The researchers have received approval from the HRPO to conduct a clinical trial to test the new design under the 2011 Clinical Trial grant from the AFIRM and are in the late stages of recruiting clinical sites to participate in the trial.

Key Words: Hypertrophic scarring, mechanobiology, wound device

Introduction

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. Multiple factors are known to influence wound repair (e.g., inflammation, oxygen tension, and ischemia), but therapeutic modalities aimed at these targets have been largely unsuccessful. Mechanical force has long been recognized to influence cellular behavior in vitro, and clinical observations based on Langer's lines and hypertrophic scarring corroborate this phenomenon in vivo.

Recently, the Gurtner laboratory published the first murine model of hypertrophic scarring based on increasing the skin stress of healing wounds. Dr. Geoffrey C. Gurtner's laboratory found that intrinsic skin mechanics correlated with scarring phenotype following wounding as low mechanical stress fetal wounds exhibit minimal fibrosis, and stiffer human skin displays robust scarring. These findings prompted the initial studies to examine the role of mechanical stress in scar formation and to develop a novel device to actively control wound environment mechanics to mitigate fibrosis.

Today, there are no commercially available wound care products that specifically address the mechanical stress state of healing wounds to reduce scarring. Elastic bandages and pressure dressings provide a widely variable range of compressive forces and are generally used for hemostatic purposes, not directly for scar attenuation. Negative

pressure wound sponge devices (WoundVac) are used on large, open exudative wounds but require elaborate components and an electrical energy source. In contrast to existing wound care options, Neodyne's technology enables precision stress shielding of area-specific wound forces through a portable, ready-to-use, simple, pressure adhesive dressing that can be readily employed following injury or surgery.

The Neodyne technology consists of a load-bearing biopolymer that is stretched by means of an applicator and then applied to the skin with a goal of optimizing a regenerative wound environment for minimal scar formation. The objective of the project is to complete clinical trial(s) utilizing a market-ready device to provide expanded data for de novo surgical incisions as well as explore the potential to improve scar appearance after a scar revision procedure. These data will ultimately be used to support the commercial launch of the product and to make the technology available to both military and civilian patients.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, Neodyne, in collaboration with the Stanford University group (Project 4.5.1), completed a Phase 1 first-in-man clinical trial with the stress-shielding device. The researchers found a dramatic reduction in hypertrophic scar formation in treated wounds compared to untreated wounds within patient controls. They also began a second human clinical trial with a second-generation stress-shielding device. The second trial was aimed at evaluating the performance of the Neodyne device when used for post-surgical incision care. Outcomes/results of the second (pilot) trial are presented as follows.

Research Progress – Year 3

Neodyne has completed work in the past year in two primary areas: (1) clinical study preparation activities and (2) product manufacturing design readiness to support the clinical study and to prepare for larger scale production and eventual commercialization.

Summary of Pilot Clinical Trial with a Second-Generation Device

A pilot clinical study with a second-generation device was conducted on 61 patients after abdominoplasty, scar revision, breast lift, and/or breast reduction procedures. The different anatomical locations were selected to test adherence in areas of varying skin contours and sensitivity (breast) and to gather data on lower tension incisions (scar revisions). The device was designed to deliver precise stress shielding at several different stress levels to determine the impact on efficacy and patient tolerance. Treatments were conducted from 8–12 weeks with dressing changes occurring weekly or biweekly, and manual measurements of skin strain (the amount of skin deformation due to the stress) were taken periodically to determine the accuracy of stress delivery. Finally, physician and patient feedback was collected on comfort, smoothness, and visual results.

The 61-patient pilot trial yielded a number of valuable results. First, the researchers found a clear correlation between delivery of higher stress and skin irritation. All patients who were treated with the highest stress experienced some irritation while none of the patients treated with the lowest stress reported irritation. Second, data collected on skin strain measurement indicated that the application mechanism was able to deliver precise stress for the full wearing period. Third, dressings continued to adhere well in all anatomical areas although general skin sensitivity to the treatment was found to vary. Finally, visual

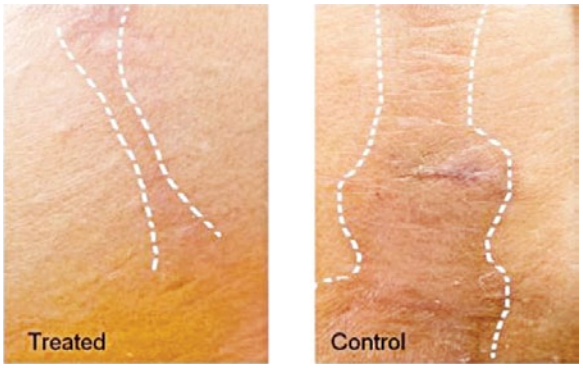

Figure IV-43. Pilot study treated and nontreated incisions (same patient) after 6 months.

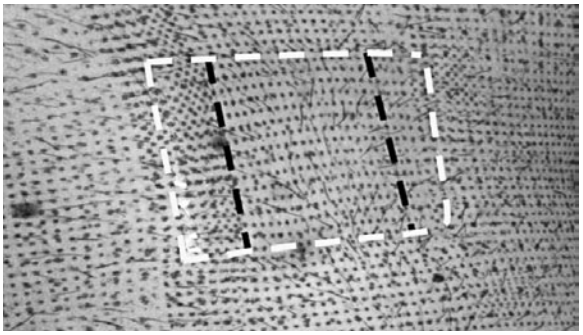

Figure IV-44. Deformed speckle pattern after dressing application.

results and positive feedback continued to be reported at 6- and 12-month follow-ups with 100% of patients and 83% of investigators indicating that the treated area of the incision was either "better" or "much better" than the control and 95% of responses indicating that the dressing was comfortable to wear. The treated side was rated as "smoother" in 70% of cases. **Figure IV-43** shows treated and control incisions from the pilot trial.

Skin Strain Analysis

Outside of the clinical trial in Neodyne's laboratories, devices were evaluated for adherence and proper skin straining using a DIC system. A noncontact, optical, three-dimensional, deformation-measuring system equipped with high-resolution digital cameras (Dantec Dynamics, Denmark) was used to measure in vivo three-dimensional deformation of the skin. A temporary speckle pattern was stamped on the patients prior to application to provide a reference for the DIC system. After application of the device, the deformation of the speckle pattern is recognized by the DIC system **(Figure IV-44)**. The reference data and the deformed condition data are processed to measure movement of the patterned skin surface relative to its reference position. Finally, Lagrangian strains were measured by finding the change in the displacement of the speckles with respect to their original position **(Figure IV-45)**.

Development of a Third-Generation Device

Several concepts and designs that had been prototyped prior to the clinical trial award were further evaluated and tested for both ease of use and likely market acceptance, and a final design was selected. Key features of the third-generation design include a unique and intuitive method (book concept) for delivering the appropriate stress to the dressing before application, a light and flexible

applicator that contours to the anatomy for ease of application, and a simple release mechanism for separating the applicator from the dressing upon delivery of the device. **Figure IV-46** shows the device that will be used in the 2011 clinical trial.

The research team completed initial design validation of the product via in-depth user interviews. They also purchased an imaging system to examine in vivo skin strains and conducted initial stress testing.

Third-Generation Clinical Trial

Based on results of the pilot trial, the biomechanical skin stress research, finalization of the third-generation product design, and feedback collected in market research studies, the third-generation trial design has been accomplished. The researchers have received approval from the HRPO and a private IRB. They are completing manufacturing and quality system steps, and are currently enrolling clinical sites in anticipation of beginning the trial in the summer of 2011. They have selected clinical staff for the study.

This trial will be a prospective, open-label, randomized study of up to 100 subjects to measure scar formation as a primary end point in surgical abdominoplasty procedures where patients will serve as their own control. Up to one-half of the incision will be treated by the Neodyne device, and the contralateral half will be treated with a physician-preferred standard of care. Ease of use, pain amelioration, comfort, and scar smoothness will be among outcome measurements. Follow-up will occur at 6 months and 1 year post-surgery.

In parallel, the team has been collaborating with the USAISR as a potential investigational site in the AFIRM-sponsored trial as well as in one or more future clinical trials designed to treat military injuries or reconstructive surgeries. The researchers have also completed a

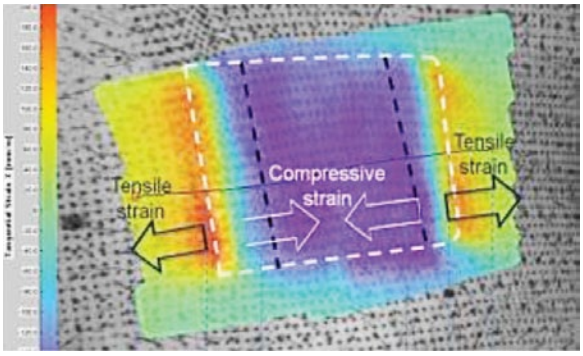

Figure IV-45. Lagrangian strains in the x-direction after application shows uniform compressive strain on skin.

Figure IV-46. Third-generation Neodyne device to be used in the 2011 AFIRM clinical trial.

quality system audit of their product in preparation for manufacturing.

Manufacturing Readiness

After completing the product design process, the Engineering and Manufacturing team has focused on developing the final specifications for the Neodyne third-generation device. Various mechanisms for optimizing the delivery of the scar minimization dressing have been evaluated by conducting a series of bench studies and measuring the stresses and forces delivered. Additionally, the team has engaged consultant resources to assist with the evaluation of current and potential vendors and suppliers to provide the raw materials, sub-assemblies, and infrastructure to support the growth in manufacturing that is required to deliver clinical product for the upcoming trial(s).

The manufacturing process flow has been finalized for the clinical trial **(Figure IV-47)** with shared responsibility between external suppliers and Neodyne for the assembly of the clinical product. Neodyne will be responsible for the final assembly, packaging, and delivery of devices to the participating clinical sites.

Key Research Accomplishments

- Completed 6-month follow-up of subjects for the pilot clinical study with a second-generation stress-shielding device, which yielded the following results:
  - Found a clear correlation between delivery of higher stresses and skin irritation.
  - Determined that the application mechanism was able to deliver precise stress for the full wearing period.
  - Found that dressings continued to adhere well in all anatomical areas.

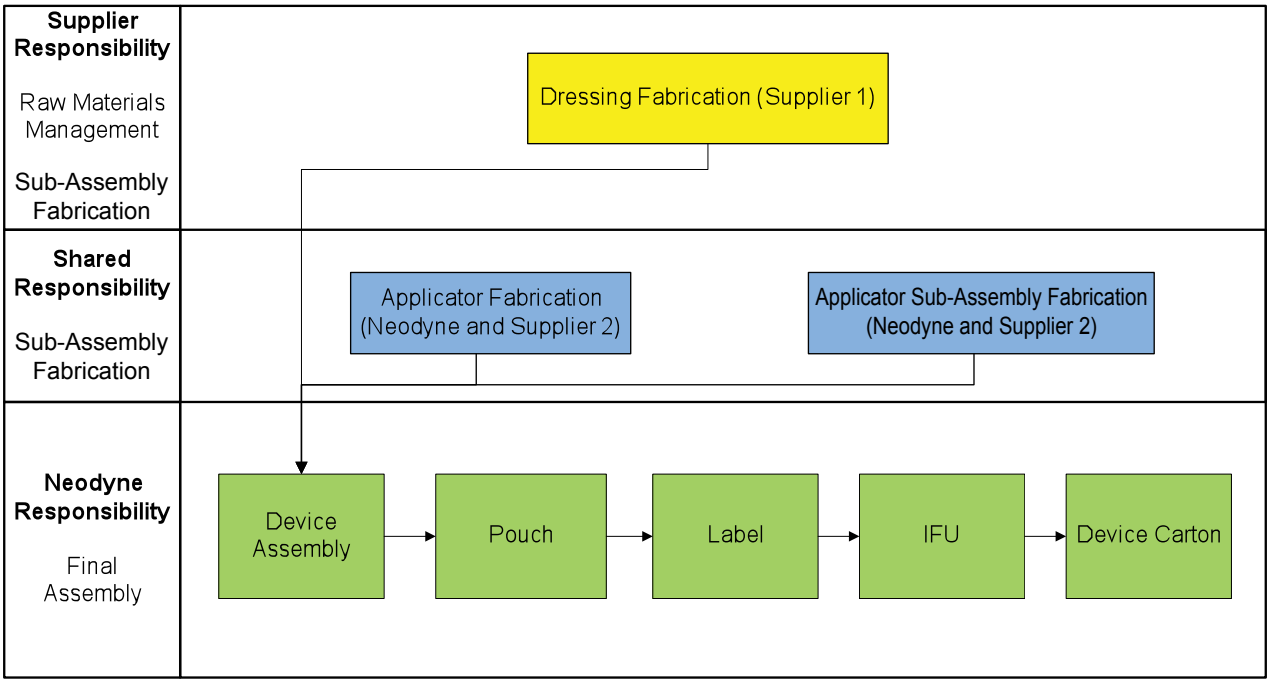

Figure IV-47. Manufacturing flow for 2011 clinical product.

- Received feedback with 100% of patients and 83% of investigators indicating that the treated area of the incision was either "better" or "much better" than the control and 95% of responses indicating that the dressing was comfortable to wear.

- Finalized the design of a third-generation stress-shielding device.
  - Identified product design improvements and preferred stress shielding.
  - Completed design of an applicator that contours to the body to facilitate dressing application.
  - Completed initial design validation of the product via in-depth user interviews.
  - Completed a quality system audit of the product in preparation for manufacturing.
  - Wrote the third-generation trial protocol and related study documents, and obtained HRPO and local IRB approvals for the third-generation trial.

Conclusions

In summary, Neodyne has extended the previous work at Stanford University and has utilized a novel stress-shielding device to safely and effectively modulate the mechanical wound environment in post-surgical human subjects to markedly reduce cutaneous scarring. This innovative device demonstrates the capability to precisely regulate skin fibrosis post-injury and is a promising translational approach to minimizing the biomedical burden of hypertrophic scar formation.

The first-in-man study set a strong foundation for the promise of the Neodyne technology in a limited number of patients. The pilot trial with a second-generation device further supported the need for precise control of skin strain and an intuitive user design, and suggests that there is a narrow range of stress levels that provide optimal scar reduction. With these understandings, the Neodyne team has developed a third-generation product and will shortly begin to treat both military personnel and civilians in clinical studies.

The current Neodyne device is able to create an optimal mechanical environment for linear incisions. With the use of DIC technology, the company has the capability to accurately measure the compressive and tensile forces in the skin within the treated area and surrounding tissue. This information will enable Neodyne to further improve the precision and efficacy of its incisional wound treatment.

The most recent product design has dramatically improved the usability and intuitiveness of the treatment and has potential uses beyond the application of Neodyne's stress-shielding device. The researchers are very interested in partnering with the AFIRM and USAISR to conduct additional clinical trials to test the expanded capabilities of the technology to maximize the benefit for the wounded warrior.

Research Plans for the Next 2 Years

In the next few years, Neodyne has several goals related to the commercialization of its technology using advanced versions of the polymeric device. Neodyne plans to continue with clinical studies in both military and civilian populations to validate the full range of potential uses for the product and to test hypotheses for use on incisional wounds as well as scar revision procedures. Neodyne will continue to collaborate with Stanford

University to conduct human trials with advanced versions of the technology that are further developed in the Gurtner laboratory.

Planned Clinical Transitions

Neodyne is in the process of enrolling its third-generation device trial, which aims to recruit a larger and ethnically broader patient population to test the efficacy and market readiness of its latest product design. In addition to the AFIRM-funded trial, Neodyne plans to enroll patients in a scar revision study to evaluate the efficacy of its product on lower tension incisions. In conjunction with the Materials Science and Engineering department at Stanford University, Neodyne will further refine the polymeric device to custom-design treatments for various size wounds and tension states. This will allow for body-specific, regional stress-shielding to address a wide variety of surgical wounds.


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Autologous Fat Transfer for Scar Prevention and Remodeling (AFT-SPAR) Clinical Trial

Project 4.7.3, RCCC

Team Leader(s): Adam J. Katz, MD (University of Virginia)

Project Team Members: Catherine Ratliff, PhD (University of Virginia); Hila Berger and Lauren Macri, PhD (Rutgers University); and Jane Reese and Emily Collins (Case Western Reserve University)

Collaborator(s): COL Robert Hale, DDS, Rodney Chan, MD, and Gale Mankoff, RN (USAISR/BAMC)

Therapy: Scar Prevention and Management

Deliverable(s): AFT-SPAR pilot clinical trial: A new indication for an existing surgical procedure.

TRL Progress: 2011, TRL 6

Key Accomplishments: To date, the researchers have treated eight patients within the study protocol and enrolled three additional patients for treatment. They have completed numerous revisions to the study protocol as needed and their Manual of Operations is nearly finalized.

Key Words: Adipose tissue, fat grafting, scar

Introduction

Severe blast and burn injuries are associated with extensive cutaneous scar formation even with the implementation of the most advanced reconstructive techniques. Such scarring can result in significant deformity and/or functional loss. Currently available therapies, such as semi-occlusive dressings (e.g., silicone), pressure (garment) therapy, and/or local steroid injections, are limited in their use and/or impact. There is clearly a need for more effective methods and therapeutics for scar prevention and management. Recent reports suggest that adipose tissue and the MSCs it contains may not only enhance the healing of difficult wounds but can beneficially affect the scarring process as well. AFT represents the simplest and most fundamental approach to exploring this concept with the potential to provide immediate positive impact.

AFT is a single-stage procedure that involves the removal of adipose tissue from one site of a patient followed by the immediate transplantation/infiltration of this tissue into a different site (of the same patient). AFT is a routine surgical procedure—albeit with the clinical intent of bulking and/or filling contour deformities—and has been proven to be safe (more than 50,000 procedures were performed last year in the United States alone). With evidence-based delineation of efficacious and reproducible treatment parameters and techniques, AFT-SPAR could emerge as a simple yet effective approach to improving the lives of wounded soldiers. The

results of this trial may change the manner in which burned (and other scarred) patients are routinely cared for, supporting the use of a relatively simple procedure to decrease the cosmetic, functional, and/or psychological consequences patients experience in association with scarring.

From a tissue-level perspective, many scar beds are devoid of a subcutaneous layer (hypodermis). This deficit is often overlooked or minimized as part of a patient's reconstruction and recovery, yet it presents a significant and important challenge and therapeutic target. The "creation" of a hypodermis layer beneath a tight, immobile scar bed (whether through AFT or by the de novo regeneration/engineered replacement of such tissue) has the potential to affect both the appearance and quality of such a scar and, therefore, to improve a patient's quality of life. In short, there is an emerging body of literature that suggests that AFT can enhance the quality and appearance of overlying scar tissue and skin in addition to promoting the healing of complex, challenging wounds. However, this approach has yet to be evaluated within the setting of controlled studies; evidence-based treatment parameters need to be established.

AFT is an established approach to the "filling" of soft-tissue contour defects. Recently, however, several publications have drawn attention to the potential effect that AFT has on overlying tissues, including the quality and appearance of skin and scar tissue. The biological mechanisms underlying this putative effect are not clear, but available information suggests that ASCs may play an important role. In this regard, ASCs have angiogenic, antiapoptotic, immunomodulatory, and matrix remodeling properties, any of which may impact or alter the outcome of the fibrotic response. Perhaps the most exciting work related to the potential benefits of this proposal is that of Sardesai and Moore (Sardesai MG and Moore CC. Quantitative and qualitative dermal change with microfat grafting of facial scars. *Otolaryngol Head Neck Surg.* 2007; 137(6):868-72). In this publication, they evaluate the impact of subdermal fat grafting on the maturation of facial scars for up to 1 year. Using subjective and objective scar assessment tools they found that fat grafting improved dermal elasticity, patient and observer perception of scar thickness, patient perception of stiffness, and observer perception of scar height and pliability. This work provides strong evidence in support of this proposed clinical trial that will test AFT-SPAR in the setting of suboptimal and/or dysfunctional scarring.

Clinical Trial Status and Results

- The study opened for enrollment in July 2010.
- A total of 24 candidate patients have been formally screened to date.
- A total of 10 patients have been enrolled in the study to date.
- Eight patients have been treated to date with 1-year follow-up of the first patient to occur in July 2011.
- All data acquisition has proceeded smoothly, but it is still too early to draw any conclusions from the results.
- Clinical trial protocol modifications have been completed, including:
  - Increase size of and broaden inclusion criteria related to treatable scars/wounds.
  - Increase laxity of use of traditional scar treatments, such as pressure garments, as long as both trial sites are treated in a similar manner.

  - Additional biopsy of scar/wound at time of initial treatment (baseline histology).

- USAISR/BAMC is nearing readiness for initiation of an AFT trial as a second site. Logistical meeting and coordination took place at the AFIRM All Hands Meeting in January 2011. Additional protocol modifications and the Manual of Operations necessary for this step are nearing completion.
- Active issues pertain primarily to continued efforts to augment the pool of screened and enrolled patients for the study. Multiple efforts are under way to address this bottleneck—both through Department of Defense channels and through local–regional efforts and advertisements–including reaching out to plastic surgery teams at military hospitals in the Washington DC, Virginia Tidewater, and Baltimore areas.

Key Research Accomplishments

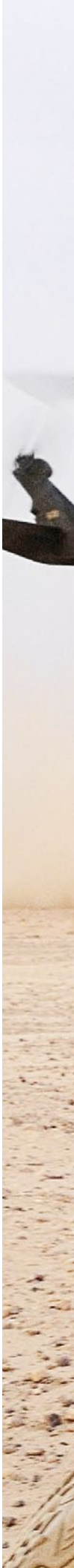
- Treated eight patients within the study protocol, with continued follow-up analysis of study patients per protocol, with no major difficulties.
  - Enrolled three additional patients for treatment.
- Modified the clinical trial protocol as needed.

Conclusions

The University of Virginia team has made excellent progress over the past year. Final IRB and HRPO approval was obtained after extended efforts, and the AFT-SPAR trial officially opened for enrollment at the University of Virginia in July 2010. Although enrollment has been slower than expected, eight patients have been treated to date with another three verbally committed to enrollment. There have been no adverse events, and all patient follow-up and analysis have proceeded smoothly. It is still too early to draw any conclusions from data obtained to date. Current efforts are focused on increasing enrollment through local advertisements and regional networking, and by establishing USAISR/BAMC as a second treatment site.

Research Plans for the Next 2 Years

In the upcoming year, the University of Virginia team plans to expand the study protocol to the USAISR/BAMC site and anticipates that enrollment in the study will accelerate. As long-term data accumulates for statistically meaningful numbers of treated patients, early analysis will be performed to explore the possibility of pertinent trends.

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V: Burn Repair

Background

Burn injuries inflict tremendous human costs in morbidity, mortality, and the losses of function, independence, and identity. Severe burns result in prolonged hospitalizations, substantial soft tissue loss, and dramatic scars, which affect appearance and limit function. Nearly 1 million U.S. civilians suffer burn injuries each year, accounting for 900,000 hospital stays, 4,500 deaths, and more than $1 billion in costs for treatment and lost productivity. The frequency of military burn injuries rose significantly during the course of Operation Iraqi Freedom and Operation Enduring Freedom as insurgent weaponry changed.

Current treatments for burn injuries, which primarily rely on early excision of damaged tissue and grafting healthy skin from another body region to cover the wound, are unsatisfactory and have been unchanged for decades. One of the most vexing problems with burn injuries, the tendency of the wound to enlarge in the first 24–48 hours, has no U.S. Food and Drug Administration (FDA)-approved therapy. Healthy skin is the first line of defense against bacteria, and battling infection is a constant challenge in treating burn patients. Current topical treatments are fraught with side effects, poor absorption, or toxicity at high doses. Finding solutions to the challenges of burn injuries that intervene at different points along the treatment time line to limit injury progression, prevent infection, minimize long-term sequelae such as severe scarring, and spare burn victims from large donor site wounds is essential.

Unmet Needs

Although respiratory distress is often the critical issue immediately after burn injury, skin loss becomes the major problem within the next 24 hours. Disruption of the skin barrier results in fluid and heat loss, and a predisposition to infection. Progressive inflammation and extension of burns during the first few days after injury compound these problems. Acutely, deep second-degree burns often extend to become full-thickness, third-degree burns with resultant increased tissue loss, longer healing times, and excess morbidity and mortality. Over the long term, burn progression results in increased scarring, wound contractures, and poor quality of life. Therapies such as nonsteroidal anti-inflammatory drugs (NSAIDs) and antioxidants have not shown substantial benefit in preventing injury extension. Hence, a critical unmet need is prevention of burn inflammation and injury progression.

Nonviable tissue within the burn favors bacteria colonization and infection. Despite a reduced incidence of invasive infection, this complication remains the most common cause of morbidity and mortality in patients with extensive burns. Although Sulfamylon®, Silvadene®, and silver nitrate are frequently used to prevent burn infections, each has disadvantages or adverse side effects. Sulfamylon, a suspension of mafenide acetate in a hydrophilic cream, is bacteriostatic for both gram-positive and -negative organisms, and penetrates the eschar (scab that forms over a burn injury) extremely well. However, it is painful when applied to partial-thickness burns, and it inhibits carbonic anhydrase, which can lead to metabolic acidosis if applied over an extensive surface. Silvadene, a suspension of silver sulfadiazine in a hydrophilic base, does not induce pain or disturb acid-base balance but fails to penetrate the eschar well and often does not protect against *Enterobacter* and *Pseudomonas.* Furthermore, it may induce neutropenia (a disorder characterized by an abnormally low number of neutrophils, a type of white blood cell that fights infection) or even pancytopenia (a medical condition characterized by reduced levels of white and red blood cells and platelets). Silver nitrate 0.5% solution is active against a broad spectrum of bacteria but cannot penetrate the eschar and is caustic, damaging otherwise viable tissue. Clearly new topical antibiotics are needed for prevention of infection in burn patients.

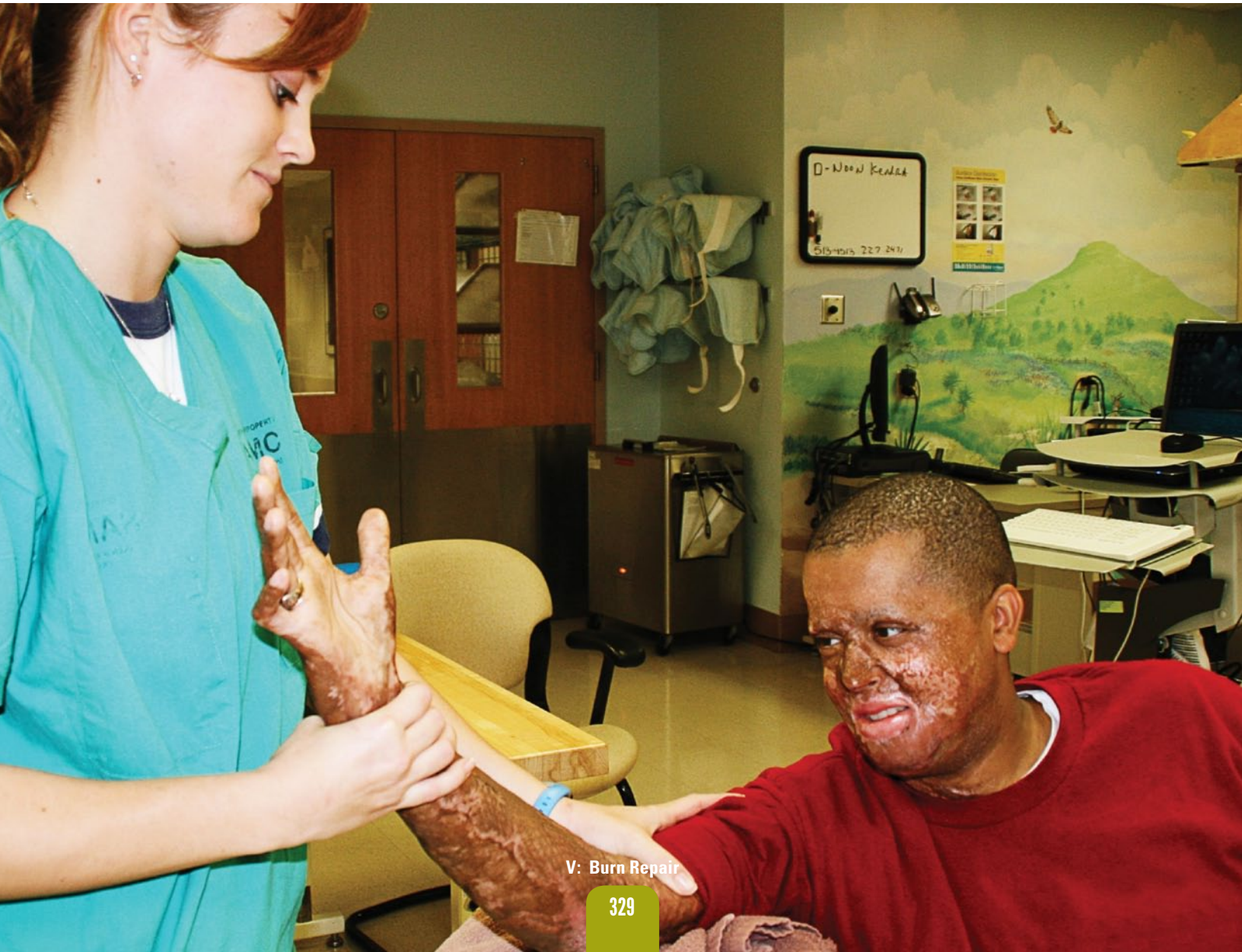
After burn wound excision, cutaneous autografts (skin grafts taken from an unburned part of the same patient) are optimal for closure; however, this mandates a viable wound bed and available donor sites. In patients with extensive burns, the area in need of grafting may outsize available donor sites. Thus, donor site reharvesting would be necessary as soon as possible; therefore, optimal, ongoing care of the site(s) becomes critical. Maximum rate of re-epithelialization and minimum trauma to the donor site are key goals of good care. A critical need therefore exists to speed re-epithelialization of autograft donor sites to reduce reharvest time.

Insufficient normal skin availability can limit the burn area covered even with meshed autografts and donor site reharvesting. If a temporary covering is indicated for the excised wound site, fresh cadaver skin allograft is currently favored although silver dressings, composite hydrogels, and cultured epithelial autografts can be useful. Frozen cutaneous allografts (tissue grafted from one individual to a genetically nonidentical member of the same species) and porcine cutaneous xenografts (tissue grafted from one species to an unlike species) are the two most readily available skin substitutes, but they are less adherent to the wound bed than fresh autografts, less able to control the

bacterial population of the underlying wound, and usually do not become well vascularized (infiltrated with blood vessels) from the underlying wound bed. Another alternative is cultured autologous keratinocyte sheets, but these are limited by a 3–4-week preparation time, sheet fragility, and susceptibility to infection.

Synthetic skin substitutes have also been used to treat burn injury with limited success. An effective synthetic skin substitute should be compatible with a patient's own tissue, have no antigenicity or toxicity, have water vapor permeability similar to that of skin, be impermeable to microorganisms, adhere to the wound, be readily vascularized, and have an indefinite shelf life. The available skin substitutes need to be modified to increase their clinical usefulness by enhancing both their resistance to infection and their ability to accelerate the formation of either neodermis or granulation tissue (the fibrous connective tissue that replaces a clot in healing wounds).

The therapies and innovations proposed by AFIRM researchers and described in this chapter should reduce wound scarring and contractures as well as prevent burn injury progression, reduce inflammation, and induce healing following burn injury.



Areas of Emphasis

Rutgers-Cleveland Clinic Consortium (RCCC), Wake Forest-Pittsburgh Consortium (WFPC), and U.S. Army Institute of Surgical Research (USAISR) researchers are pursuing a complementary mix of research projects focused on various aspects of burn injury. Projects can be grouped into four "clinical challenge" topic areas: Intravenous Treatment of Burn Injury, Topical Treatment of Burn Injury, Wound Healing and Scar Prevention, and Skin Products/Substitutes. Additional details on projects in each of these topic areas can be found in **Table V-1** and subsequent sections of this chapter.

Table V-1. Projects funded by RCCC, WFPC, and USAISR per clinical challenge topic area.

Clinical Challenge	Consortium/ Institution	Project No.	Project Title
Intravenous Treatment of Burn Injury	RCCC	4.6.1	Therapy to Limit Injury Progression, Attenuate Inflammation, Prevent Infection, and Promote Non-Scar Healing After Burns and Battle Trauma
Topical Treatment of Burn Injury	WFPC	4.2.3	Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues
	RCCC	4.6.2	Mesenchymal Stem Cells for Burn and Wound Healing
		4.6.4	Polymeric, Antimicrobial Wound Dressing Providing Sustained Release of Iodine
		4.6.5	Topical P12 Treatment of Burns Using Fiber Mats
Wound Healing and Scar Prevention	WFPC	4.2.2	Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device
		4.2.4	Artificial Extracellular Matrix Proteins for Regenerative Medicine
		4.2.5	In Situ Bioprinting of Skin for Battlefield Burn Injuries
Skin Products/ Substitutes	WFPC	4.2.1	Tissue Engineered Skin Products – ICX-SKN
		4.2.1a	Tissue-Engineered Skin Substitute for Burns at Organogenesis
		4.2.6	Amniotic Fluid-Derived and Placenta-Derived Stem Cells for Burn
		4.2.8	In Vitro Expanded Living Skin for Reparative Procedures
	RCCC	4.7.2	Engineered Skin Substitutes
	USAISR	4.6.8	Autologous Human Debrided Adipose-Derived Stem Cells for Wound Repair in Traumatic Burn Injuries

Intravenous Treatment of Burn Injury

Studies at RCCC

The **Lin/Clark group (Project 4.6.1)** at Stony Brook University conducted a focused screening investigation for agents that may inhibit the progression of burn injury. Agents were selected for their reported ability to inhibit oxidative stress, cytokine stress, or apoptosis. The researchers found that a single intravenous fibronectin P12 and curcumin infusion could significantly inhibit burn injury progression in the rat hot comb model. But only P12 significantly limited burn injury progression in a swine hot comb model. They completed a dose range study for intravenous P12 infusion in the swine hot comb model and determined that the most effective dose was 1 mg/kg. After conducting some pharmacokinetic studies, the research team found that P12 concentration decreased very quickly in the blood post-infusion. Over the next 1–2 years, P12 stability and pharmacokinetic studies will be completed, and tissue distribution of P12 will be studied using fluorescently labeled P12. The researchers hope to file an Investigational New Drug (IND) application for P12 infusion to limit burn injury progression with the FDA within the next 1–2 years. Once approved, Phase 1 and Phase 2 clinical trials will commence.

Topical Treatment of Burn Injury

Studies at WFPC

Keratins are tough, fibrous structural proteins found in structures that grow from the skin (e.g., hair and nails). The **Van Dyke group (Project 4.2.3)** at Wake Forest University School of Medicine is exploiting the thermoprotective properties of keratins to try and ameliorate the progression of injury immediately following a burn. The researchers are aiming to deliver a topical therapy that would limit the progression of the "zone of coagulation" following a burn thus limiting the overall size and severity of the injury. They have developed a cell culture heat shock model using mouse skin cells. They have used this model to identify a keratin subtype, gamma-keratose, that promotes cell survival following thermal injury.

RCCC researcher Dr. Nava Shpaisman synthesizes curcumin-containing nanospheres to promote scarless wound healing.

Initial gene expression experiments suggest that gamma-keratose causes a decrease in the expression of inflammatory genes in the surviving cell population and induces the expression of a "repairing" fibroblast genotype. The researchers have also successfully developed and optimized a burn model in swine and completed the in-animal portion of a pivotal trial. They transferred their keratin biomaterial manufacturing technology to a partner company, KeraNetics LLC, which scaled up and validated it. Over the next 2 years, the research team will complete its pivotal swine burn study and heat shock mechanism study analyses. Preclinical data for the swine study have been reviewed by the FDA, and initial approval for a clinical trial has been obtained.

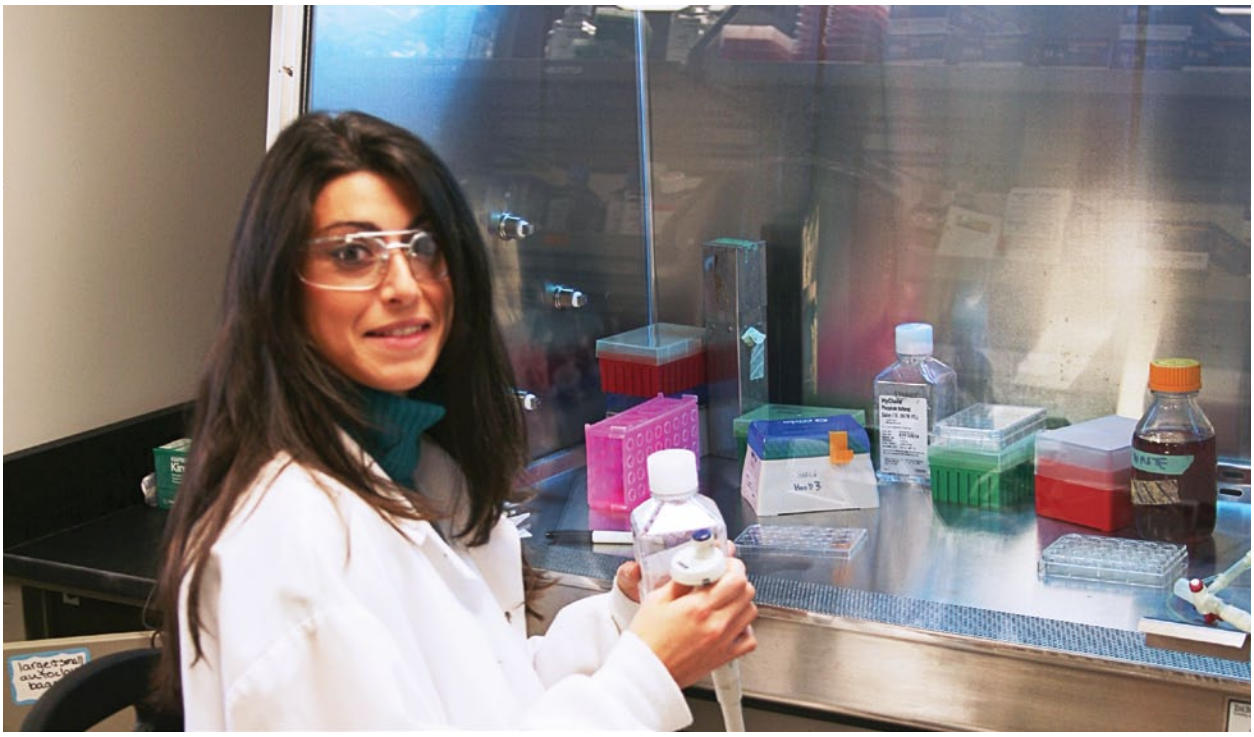
Studies at RCCC

In three related projects, researchers at RCCC are testing the topical application of adult human bone marrow-derived mesenchymal stem cells (MSCs) and three therapeutic agents (i.e., iodine, peptide P12, and curcumin) for the treatment of burns.

The **Caplan/Sorrell group (Project 4.6.2)** at Case Western Reserve University has been developing an off-the-shelf, cell-based therapeutic product (MSCs) that can be applied to the surfaces of extensive human skin wounds and/or burns to enhance wound repair and reduce scar formation. The researchers demonstrated that MSCs that were expanded in cell culture and fluorescently labeled for cell tracking could be successfully introduced into large, full-thickness skin wounds in mice. Topical delivery of MSCs to full-thickness mouse skin wounds on day 3 post-wounding resulted in an enhanced yield of cells to the wound bed and a more even distribution of cells throughout the wound bed. After initial experiments in the rat hot comb model showed minimal activity of MSCs, the researchers modified their protocol and repeated the experiments. Since the repeat experiments confirmed a negative response, this project was terminated in June 2011.

In **Project 4.6.4**, the **Iovine/Ramachandran group** at Rutgers University and the **Clark group** at Stony Brook University initially identified a polymer system that could release molecular iodine from a wound dressing potentially preventing infection. However, various technical and toxicity-related issues limited the usefulness and applicability of this system. The research team abandoned research efforts on the initial polymer system and instead created a tunable iodine delivery system in an absorbent wound dressing that releases iodine on demand and does not require frequent dressing changes, resulting in better patient comfort. This wound dressing technology is highly suited to battlefield conditions and the treatment of wounded warfighters. The researchers demonstrated significantly longer on-demand release of iodine than any other known or tested iodophor system. They have demonstrated basic principles of patentability, and the prototype product and process are now ready for pilot plant scale-up. The research team will test the product in relevant preclinical models. It will manufacture the product under Good Manufacturing Practice (GMP) conditions and will file a 510(k) application. After year 4, it was agreed that this project would be terminated from the RCCC-AFIRM portfolio and transitioned to a more appropriate funding source.

The **Macri/Clark group (Project 4.6.5)** at Stony Brook University is engineering a drug delivery scaffold for the topical therapy of large, acute burn injuries that are unable to close. The researchers are exploring the effects of sustained release of the fibronectin-derived peptide P12 in this scaffold model. They produced fibro-porous mats containing varying amounts of P12 and determined



RCCC researcher Dr. Lauren Macri prepares to evaluate the effects of biodegradable, drug delivery vehicles on the proliferation of human dermal fibroblasts.

that the mats could release P12 in a rate-controlled manner. They demonstrated the biocompatibility of the fibro-porous mats in a porcine model. At 28 days, treatment sites demonstrated decreased wound contraction and no evidence of inflammation. In a larger study, macroscopic evaluation suggested that granulation tissue accumulated in the wounds so that most wounds were completely filled in with new tissue on day 7. Recently, the researchers developed tyrosine-derived poly-carbonate (TyrPC) terpolymers as fiber-based drug delivery matrices for releasing P12 at ultrafast (8 hours) and fast (4 days) rates. They determined that aseptic ultraviolet (UV) radiation is a suitable sterilization technique for the P12-loaded TyrPC matrices. During the upcoming year, the research team will confirm the efficacy of topical P12 therapy in porcine models. In year 5, the researchers will perform definitive animal studies. They will also conduct safety and efficacy studies in Good Laboratory Practice (GLP) guidance. Finally, in year 5, the project team will submit an IND application and initiate the design of a clinical trial.

Wound Healing and Scar Prevention

Studies at WFPC

The **Gerlach group (Project 4.2.2)** at the University of Pittsburgh is finding that skin progenitor cells derived from human fetal skin tissue may provide an interesting new cell source for regenerative cell-based therapy for acute and chronic skin disease and burn patient treatment. The researchers are expanding on successful work conducted previously in Germany in which fetal skin

cells are sprayed onto excised burn wounds using a precision device (also known as a "Skin Gun"). They have established methods for isolating, growing, and freezing fetal skin cells. They demonstrated that these fetal skin cells can be used as off-the-shelf products for second- or third-degree burn healing. The researchers designed a cell spray model that works with various types of fetal skin cells and performed spray parameter tests to achieve maximum viability of the cells after spraying. Overall, they propose that frozen, preserved fetal skin cells can be used as an immediately available, "out-of-freezer" therapy for burn injury.

The **Tirrell group (Project 4.2.4)** at the California Institute of Technology aims to develop and produce artificial extracellular matrix (aECM) proteins that can be utilized in tissue repair. The researchers found that rates of wound healing on aECM proteins can be greater than those on fibronectin, in part owing to increased rates of cell proliferation. They developed a mechanistic model to explain the origin of the increase in wound-healing rates. They determined—both experimentally and by modeling—that the presence of aECM proteins, myosin activity, and wound geometry, but not wound size, are primary determinants of the amount of wound closure in their model of wound healing on aECM proteins. Most recently, they prepared a set of 12 aECM films for implantation in the pig burn model. They have also begun to conduct animal trials in collaboration with the Van Dyke laboratory (Project 4.2.3).

The **Yoo group (Project 4.2.5)** at Wake Forest University is using inkjet technology to achieve the "printing" of skin onto an excised burn wound. Since bioprinting has been successfully utilized to fabricate other tissues, skin should be similarly amenable to this approach especially given its minimally three-dimensional, linear spatial orientation. The researchers developed a portable skin printing device for direct in situ applications for a large animal study. This process involved design, construction, laser scanner integration, software development, and validation. The researchers achieved delivery of skin cells directly onto skin defects in a mouse model using the device. More recently, they performed experiments in a porcine model using their in situ skin bioprinting technology. They were successful in isolating and expanding dermal fibroblast and keratinocytes from porcine skin. The cells remained viable when delivered through the printer nozzles. The printed cells participated in the skin tissue formation and full-thickness wound repair. Over the next 2 years, the researchers plan to refine their large animal bioprinter. They will also design a third-generation bioprinter suitable for clinical application.

Skin Products/Substitutes

Studies at WFPC

The need for a skin replacement that is instantly available and alleviates the need to take a split- or full-thickness skin graft has long been sought. Two industry partners, **Healthpoint Biotherapeutics, Ltd. (Healthpoint),** and **Organogenesis, Inc.**, are rapidly developing separate and distinct products that will be tested in a clinical trial. The **Rolland/Kemp/Ronfard group (Project 4.2.1)** at Healthpoint is developing a permanent dermal skin graft replacement (ICX-SKN), which can be integrated and remodeled by the host. Over the past year, the researchers further characterized the ICX-SKN matrix during maturation. Examination of their manufactured constructs revealed a unique structure and strength. Novel methods of evaluation provided the researchers with a deeper understanding of the matrix maturation process. They developed assays that will aid in the evaluation of future constructs thus

ensuring uniformity. The **Bates/Wang/Segal group (Project 4.2.1a)** at Organogenesis, Inc., has made considerable progress toward the development of an advanced human cell-based therapy for the treatment of full-thickness or deep partial-thickness burn wounds. The researchers have developed the final embodiment of their therapy, which acts as a skin substitute and is intended to be used in one-step skin grafting procedures. Results from preclinical studies in a mouse burn wound model showed superior ingrowth of host tissue and blood vessels compared with currently available technologies. The team is on schedule to test this final embodiment in a porcine burn wound model to evaluate efficacy. The researchers plan to eventually evaluate the therapy in human patients.

The **Furth group (Project 4.2.6)** at the Wake Forest Institute for Regenerative Medicine (WFIRM) is developing an improved off-the-shelf bioengineered skin product for the treatment of extensive burns that utilizes amniotic fluid-derived stem (AFS) cells. The researchers are capitalizing on the capability of AFS cells to differentiate into skin progenitor (stem) cells without forming tumors thus facilitating the healing of a burn wound following the introduction of cells. During the past year, the researchers broadened their scope to include placenta-derived cells. They began systematic experiments to improve cell culture conditions to better permit the in vitro expansion of rare stem cells while maintaining their "stemness." They established a cell culture system that mimics physiological conditions by providing vulnerable cells with levels of oxygen normally experienced by humans rather than a hyperoxic (high oxygen) environment. They also confirmed equations that will aid in data extraction and analysis. Over the next 2 years, the researchers plan to test AFS cells for promotion of wound healing in mice (and eventually pigs) using the skin bioprinter that is being developed by the Yoo group (Project 4.2.5). They will test AFS cells in combination with human skin cells to determine if this will accelerate wound healing. They will also bioprint AFS cells with various types of cells that modulate the immune system, which they feel could accelerate the clinical translation of this project.

In the clean room, RCCC researcher Dr. Thomas Morrow, postdoctoral fellow, prepares to fabricate an electrospun, drug-loaded fiber mat used to treat burn injury.

USAISR researchers Sandra Becerra and Jennifer Wehmeyer isolate stem cells obtained from discarded tissue samples of severely burned patients.

The **Lee/Yoo/Holmes group (Project 4.2.8)** at Wake Forest University has developed an in vitro tissue expander system that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. This system will allow for an approximately 40 cm^2 split-thickness piece of skin to be harvested at the initial operation from a burn patient who is anticipated to require multiple acute operations. The skin is then expanded in the bioreactor to approximately 100 cm^2 over 2 weeks. The skin is subsequently "grafted" back onto the patient in the standard manner, with or without meshing, at the next operation. This technology is noteworthy in that it will produce an alternative for generating "more skin" for grafting when faced with limited donor sites. The researchers recently developed a microneedle tissue gripper system, which they feel will decrease tissue damage and reduce localized stress, resulting in fewer tears. They are working toward obtaining FDA approval for a prospective, multicenter, nonrandomized, uncontrolled pilot clinical study.

Studies at RCCC

Engineered skin substitutes (ESSs) have been developed and tested clinically as an adjunctive treatment for burn repair. Although ESSs reduce the requirements for harvesting skin autografts (i.e., skin grafted from one part of the body to another), there are two major deficiencies: (1) incomplete pigmentation, which does not resolve with time, and (2) the absence of a network of blood vessels, which limits the thickness and rate of engraftment of ESSs. The **Boyce/Clark group (Project 4.7.2)** of the University of Cincinnati and Stony Brook University, respectively, is designing and testing new prototypes of ESSs that restore skin color and develop vascular networks thereby resulting in improved outcomes in recovery from life-threatening burns. Skin color has been restored in an animal model of ESS. Most recently, the researchers demonstrated that the addition of human melanocytes to an ESS resulted in full pigmentation. They found that sufficient numbers of cells could be expanded in culture to seed in ESS in a time frame sufficiently rapid to meet the surgical schedules

for burn victims. The researchers tested for tumorigenicity of transplanted human melanocytes in immune-deficient mice and found that the cells had no tumor-forming potential in the animals. During the next 2 years, the research team plans to complete preclinical studies and initiate plans for clinical studies.

Studies at USAISR

Adipose-derived stem cells (ASCs) have the potential to grow into endothelial or epithelial cell lineages. The **Christy group (Project 4.6.8)** at USAISR hypothesized that a person's own ASCs could be used to produce a clinically relevant tissue engineered skin equivalent. The researchers used ASCs and a polyethylene glycol (PEG)-based biomaterial that mimicked ECM as a strategy to regrow blood vessels into traumatized tissue. They found that cells began to form vascular tube-like networks in the biomatrix in the absence of additional soluble cytokines. ASCs inside matrices were not only viable but proliferated and formed microvessels. Further analysis using specific markers identified ASCs that had differentiated into vascular cells. This ability to form capillaries is central to developing new therapies for wound healing and tissue engineering. The researchers characterized the ASC phenotype from various patients and initiated preclinical animal wound-healing studies. They delivered ASCs in a gel to a full-thickness excision wound in the rat and found enhanced growth of blood vessels compared to control gels lacking ASCs. The researchers plan to initiate studies in a more clinically relevant porcine model and will use this model for the development of a large total body surface area (TBSA) burn, which they predict will provide a stringent test for their and other AFIRM-related skin equivalent products.

Clinical Trials

Several AFIRM Burn Repair projects have technologies that are advancing to the human clinical trial stage. As shown in **Table V-2**, two projects have clinical trials that are open and enrolling patients, and a third project expects to begin a clinical trial in the near future. Additional details on these trials are provided as follows.

The **Holmes group (Project 4.2.7)** at Wake Forest University is conducting a multicenter FDA approval trial for ReCell®. ReCell is a technique whereby a small (approximately 4 cm^2) split-thickness skin graft/biopsy is harvested from a burn patient and prepared in the operating room so that cells from the junction between the superficial and deeper layers of skin are harvested and immediately applied

Table V-2. AFIRM-funded Burn Repair projects with pending, active, or completed clinical trials.

Project Title	Consortium	Project No.	Trial Phase	Current Status
A Multicenter Comparative Study of the ReCell® Device and Autologous Split-Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries	WFPC	4.2.7	Phase 3	Open
An Open-Label, Multicenter, Proof of Concept Study of the Safety and Efficacy of Stratagraft® Skin Substitute as an Alternative to Autografting in Promoting the Healing of Excised, Deep Partial-Thickness Burns	WFPC and USAISR	4.2.9	Phase 2	Open
Expedited Availability of Autologous Engineered Human Skin for Treatment of Burned Soldiers	RCCC	4.7.4	Phase 1	Awaiting IND Approval

to an excised burn wound via a syringe at an expansion ratio of 80:1. From approximately 4 cm^2 of skin, approximately 320 cm^2 of burn can be "grafted" using a patient's own cells without the need for any culture techniques. This technology has the potential to radically alter modern burn surgery. Patient enrollment began in May 2010. The researchers enrolled and treated 35 subjects at 6 clinical sites to date. They followed one subject all the way to 1-year post-treatment and obtained FDA approval for enrollment at six additional sites. They hope to achieve their trial enrollment goal (106 subjects) within the next 6 to 9 months.

In **Project 4.2.9**, **Dr. James Holmes** at Wake Forest University and **Dr. Booker King** at USAISR are conducting a multicenter, Phase 2 trial aimed at assessing the safety and efficacy of Stratgraft skin substitute when utilized as an autograft substitute in excised, deep partial-thickness burn injuries. Patient enrollment is nearly completed, and the trial is entering the terminal phases of final follow-up. Note: A progress report is not yet available for this project/clinical trial.

Researchers at **Lonza Walkersville, Inc. (LWI)** in **Project 4.7.4** have developed autologous ESSs to promptly and effectively close wounds resulting from extensive, deep burns. LWI has licensed the technology and is completing the technology transfer and product development. During the past year, LWI has qualified a clinical source of the matrix, conducted manufacturing technical transfer and training, produced non-GMP and current Good Manufacturing Practice (cGMP) lots of the matrix, and initiated planning of studies comparing ESSs with split-thickness autografts in massive burns in human subjects. LWI anticipates that it will enroll subjects at the USAISR upon approval of an IND by the FDA. Note: A progress report is not yet available for this project/clinical trial.


our science for their healing

Therapy to Limit Injury Progression, Attenuate Inflammation, Prevent Infection, and Promote Non-Scar Healing After Burns and Battle Trauma

Project 4.6.1, RCCC

Team Leader(s): Fubao Lin, PhD and Richard Clark, MD (Stony Brook University)

Project Team Members: Adam Singer, MD (Stony Brook University)

Collaborator(s): Molly D. Frame, PhD and Marcia G. Tonnesen, MD (Stony Brook University)

Therapy: Therapy for burn injury progression.

Deliverable(s): Intravenous delivery of P12.

TRL Progress: 2009, TRL 1; 2010, TRL 4; 2011, TRL 5; Target, TRL 5

Key Accomplishments: The researchers obtained the full range of P12 dose response on burn injury progression using a swine hot comb model. They determined that the optimal dose of P12 was 1 mg/kg. They developed a method to determine P12 levels in blood. They obtained preliminary PK data for P12 showing that P12 concentration decreased very quickly in the blood post-infusion.

Key Words: Burn, injury progression, curcumin, P12

Introduction

Battlefield polytrauma secondary to blasts and explosions is common, affects multiple sites, and is complex. Many of these injuries, but particularly burns, are subject to progressive tissue damage over subsequent days and can have a devastating effect. Over the course of a few days to 1 week, deep partial-thickness burns can become full-thickness burns, which in the short term, leads to increased tissue loss, longer healing time, and excess morbidity and mortality. In the long term, increased scarring, wound contractures, and poor quality of life are the major issues. Therapies to improve blood flow, such as NSAIDS and anticoagulants (heparin), have not shown substantial benefit in preventing burn injury progression.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers tested five therapeutic agents with low-risk profiles using a rat hot comb model: (1) human bone marrow-derived MSCs; (2) pentoxifylline, which inhibits tumor necrosis factor-alpha production; (3) curcumin, a potent antioxidant; (4) desferrioxamine, a potent iron chelator that blocks

free radical chain reactions; and (5) a 14-mer peptide (P12) from fibronectin with remarkable antiapoptotic properties (Lin and Clark, unpublished data). These agents target three of the major sequelae of reperfusion injury: (1) cytokine release, (2) generation of reactive oxygen species, and (3) markedly increased programmed cell death (apoptosis). These studies demonstrated that only P12 and curcumin showed significant inhibition on burn injury progression in the rat hot comb model. In contrast, bone marrow-derived MSCs, pentoxifylline, and desferrioxamine showed little effect. During the second year of the project, the swine hot comb model was developed to determine the effects of P12 and curcumin on limiting burn injury progression. The researchers found that P12 effectively limited burn injury progression at 1.0–3.0 mg/kg in the hot comb model. Surprisingly, curcumin showed no protective effect on limiting burn injury progression in the hot comb model.

Research Progress – Year 3

Year 3 studies focused on P12 in the swine hot comb model. In addition, Microconstants, Inc., San Diego, California, validated a method to detect P12 in biologic fluids, including blood, and studied P12 stability in vitro. The overall goal was to further determine P12 efficacy and establish dosing details for the reduction of burn injury progression.

Dose Response of P12 on Limiting Burn Injury Progression in a Swine Hot Comb Model

To establish a dose-response curve for P12, a series of experiments was performed with P12 doses from 0.01–10 mg/kg administered by intravenous infusion 1 hour after burn injury in the swine hot comb model. As shown in **Figure V-1,** burn injury progression in the swine hot comb model was reduced with one P12 dose infused 1 hour after burn. Effective doses of P12 that limited burn injury were from 0.01–3.0 mg/kg, and an apparent optimal dose occurred at 1 mg/kg. **Figure V-2** shows the photomicrographs of interspaces from a control necrotic burn (A) and P12-treated viable burn (B).

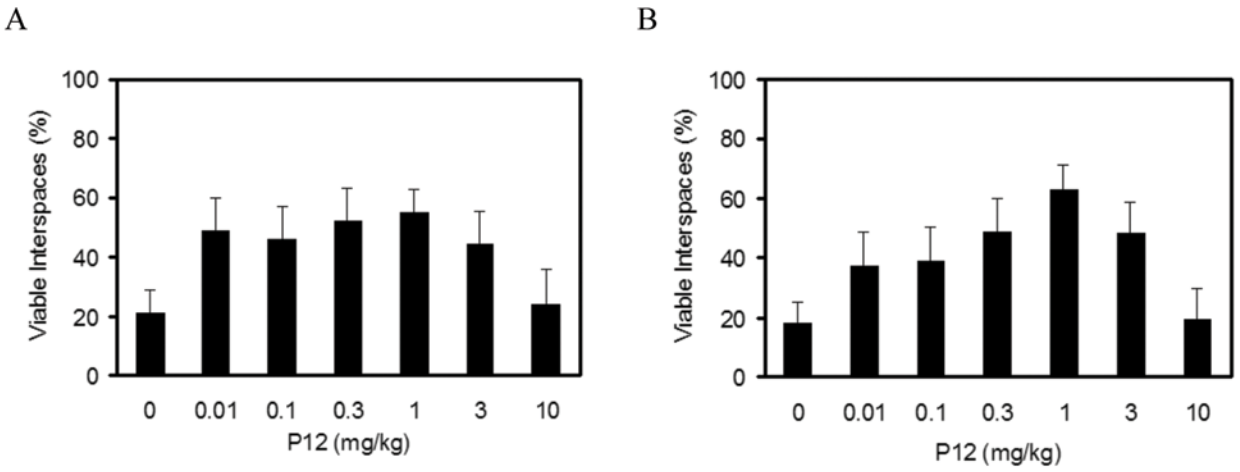


Figure V-1. Dose response of P12 (one infusion) on burn injury in a swine hot comb model. Buffer or P12 was administered intravenously in an ear vein at 1 hour after burn. Viable interspaces were evaluated 7 days after burn: (A) macroevaluation and (B) histological evaluation. The results are the combination of five experiments.

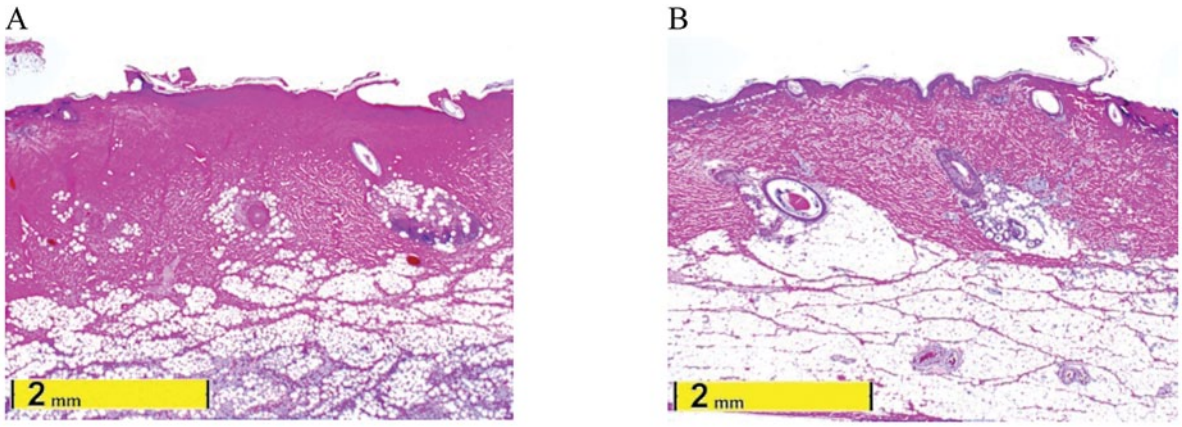


Figure V-2. P12 infusion limited burn injury progression in a swine hot comb model. Buffer or P12 was administrated intravenously in an ear vein at 1 hour after burn. Full-thickness biopsies from the interspaces 7 days after injury were stained with hematoxylin and eosin (H&E). Photomicrographs of a (A) necrotic interspace from a control animal and (B) viable interspace from a P12-treated animal.

P12 Concentration in Swine Blood and Pharmacokinetics After Infusion

To study the level of P12 in blood and its pharmacokinetics after infusion, a method to detect P12 in blood was developed by Microconstants, Inc. The method involves P12 derivatization, extraction, and mass spectroscopy analysis. The detection limit of the method was measured at 10 ng/mL. The results demonstrated that P12 concentration decreased very quickly in blood **(Figure V-3)**. For 1 mg/kg P12 infusion, the P12 level in blood after infusion was approximately 2% of the amount of P12 that had been infused and was undetectable 15 minutes after infusion. For 0.1 mg/kg P12 infusion, the P12 was not detectable immediately after infusion. The rapid decrease of P12 in blood may be due to degradation of the peptide or binding to endothelial cells since P12 is very cationic and the surface of endothelial cells is extremely anionic.

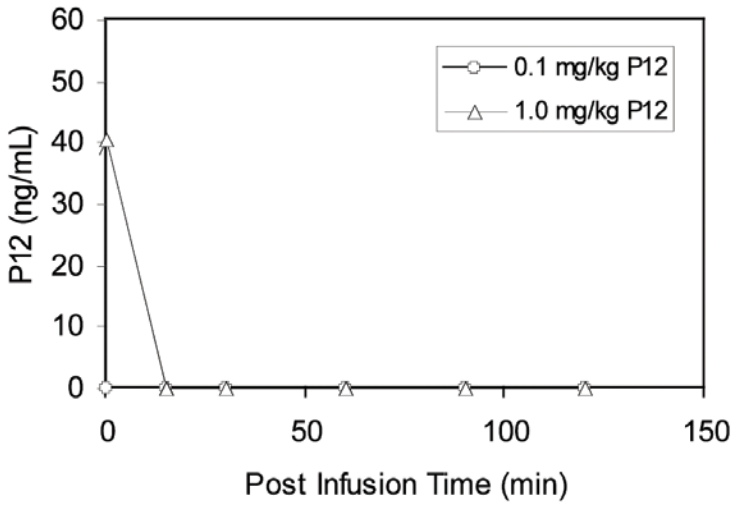


Figure V-3. P12 level in blood after infusion. Blood samples were collected at different time points after infusion. P12 concentration was determined by using mass spectrum, and ^{15}N-labeled P12 was used as an internal standard.

Microvascular Vasoactive Responses with Fibronectin and Fibronectin Fragments

To better understand the mechanism of P12 on the limitation of burn injury progression, the effect of P12; its parent protein, full-length fibronectin; and P12-derived peptides (P7, P18, and P39) on microvascular vasoactive responses was studied. P7, P18, and P39 are 7-mer, 8-mer, and 6-mer peptides, respectively, that are derived from the C-terminal end of P12. All data were obtained using the hamster cheek pouch (buccal) tissue model. Test agents were applied to individual arteriolar segments for 60 seconds using fine glass micropipettes. The diameter change is reported as the peak response over 60 seconds minus the baseline diameter divided by the baseline diameter (approximately 10 µm). This level of the microcirculation is relevant and important because these arterioles control erythrocyte flow (oxygen delivery) either to the capillaries or through non-nutritive pathways.

Figure V-4 shows the response to fibronectin and to four progressively shorter synthetic peptide fragments contained within fibronectin—P12, P18, P7, and P39. While fibronectin showed only a dilation response, P12, P18, and P7 showed a dilation peak at nanomolar concentrations followed by constriction. The shortest fragment, P39, showed only the constrictor response. The fragment with the widest dose-response range for dilation was P7. All peptides tested induce constriction at higher concentrations. **Figure V-5** shows the vasoactive response to P12 and P7 in the absence or presence of adrenergic blockade (P12 ab or P7 ab). Scrambled P12 and scrambled P7 were used as controls.

These data suggested that the dilation component to these fibronectin peptides was mediated by the β-adrenergic receptors, but the constriction component was not mediated by the β-adrenergic receptors. Scrambled peptides, P12s and P7s, were vasoconstrictive only, suggesting that the highly cationic nature of the peptides may be vasoconstrictive.

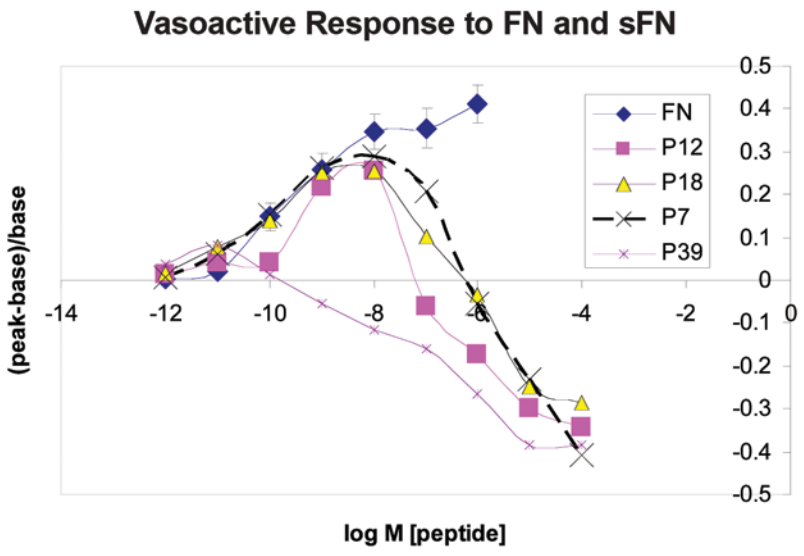


Figure V-4. The vasoactive response to full-length fibronectin and to synthetic peptide fragments of fibronectin (n=6 per group, mean ± SEM).

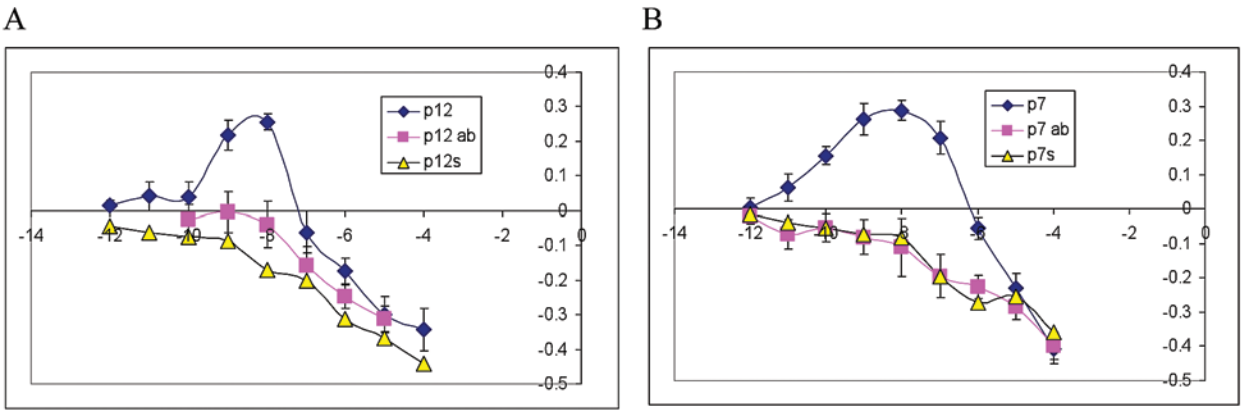


Figure V-5. The vasoactive dilatory response to P12 (A) or P7 (B) was abolished by adrenergic blockade (P12 ab or P7 ab) when β-adrenergic antagonist, propranolol 10^{-5} M, plus β-adrenergic antagonist, phentolamine 10^{-5} M were added to the water bath. The scrambled versions of P12 or P7 gave only vasoconstriction in the presence or absence of adrenergic antagonists (n=6 per group, mean ± SEM).

Key Research Accomplishments

- Obtained a full range of P12 dose response on burn injury progression in a swine hot comb model.
  - Determined that the optimal dose was 1 mg/kg.
- Developed a method to determine P12 levels in blood.
- Obtained preliminary pharmacokinetics data for P12 after infusion.
  - Determined that P12 concentration decreased very quickly in the blood post-infusion.

Conclusions

A single P12 infusion effectively limits burn injury progression from 0.01–3.0 mg/kg. The optimal dose occurs at 1.0 mg/kg. Pharmacokinetic results demonstrated that P12 concentration decreases rapidly in blood and becomes undetectable 15 minutes after infusion of 1.0 mg/kg P12. The ability of P12 to limit burn injury progression is consistent with the multifunctions of P12 to dilate terminal arterioles, protect cells from oxidative stress-mediated death, inhibit apoptosis, and enhance cell survival. These findings may lead to the development of a novel therapy to limit burn injury progression with a single P12 infusion.

On the other hand, curcumin was confirmed to have no effect on limiting burn injury progression at picomolar to nanomolar concentrations in the swine hot comb model although it inhibited burn injury progression in the rat hot comb model. These results indicated the differential response of large and small animals to curcumin.

Research Plans for the Next 2 Years

Depending on funding levels, over the next 1–2 years P12 stability and pharmacokinetic studies will be completed, and tissue distribution of P12 will be studied using fluorescently labeled P12. In addition, the effects of P12 on limiting burn injury progression will be evaluated in a swine vertical burn model. Optimism exists that the FDA will approve an Orphan Drug Designation for P12 to

limit burn injury progression within the next few months. Depending on funding levels, an IND application for P12 infusion to limit burn injury progression will be filed with the FDA within the next 1–2 years, and once approved, Phase 1 and Phase 2 clinical trials will commence.

Planned Clinical Transitions

Jules Mitchel, PhD, President of Target Health, has submitted a request for Orphan Drug Designation of P12. Microconstants, Inc., has been selected to initiate P12 stability and pharmacokinetic studies. American Peptide, Inc., has been instructed to move P12 peptide synthesis to its GMP facility. It will use the same manufacturing procedure as currently in use, and no further scale-up will be needed at this time. Hyaluron, Inc., has been selected to produce the final product, that is, finish and fill. NeoMatrix Formulations, Inc., founded by Dr. Richard Clark, has been activated, having selected a Board of Directors and Lan Hu, PhD, as CEO. The company has negotiated an option to license all P12 intellectual property from Stony Brook University and has submitted a fast-track Phase 1/2 Small Business Innovation Research grant application to the National Institutes of Health. Jules Mitchel, in collaboration with Stan Gerson, Richard Clark, Adam Singer, and Jimmy Holmes, will be responsible for developing a Phase 1 and Phase 2 clinical trial protocol and then submitting an IND to the FDA in year 4 or year 5.

Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues

Project 4.2.3, WFPC

Team Leader(s): Mark Van Dyke, PhD (Wake Forest School of Medicine)

Project Team Members: Deepika Poranki, MS (Wake Forest School of Medicine), Carmen Gaines, PhD (WFIRM), and Olga Roberts, PhD (Wake Forest University)

Collaborator(s): Jimmy Holmes, MD, Joseph Molnar, MD, PhD, Justin Saul, PhD, Mark Lively, PhD, Roche de Guzman, Jillian Richter, and Mary Ellenburg (Wake Forest School of Medicine); David Tirrell, PhD (California Institute of Technology); Luke Burnett, PhD (KeraNetics, LLC); and Michelle Merrill and Rawad Hamzi (Wake Forest University)

Therapy: Burn therapy.

Deliverable(s): Keratin biomaterial-based burn treatment development and preclinical testing.

TRL Progress: Start of Program, TRL 3; End Year 1, TRL 3; End Year 2, TRL 3; End Year 3, TRL 4

Key Accomplishments: The researchers have developed and optimized a model of burn injury in swine. They transferred their keratin biomaterial manufacturing technology to a partner company, KeraNetics LLC, which scaled-up and validated it. They initiated a pivotal animal study (second degree burns in swine) and completed the in-animal portion of the study.

Key Words: Burn, keratin, biomaterial, hydrogel, swine, pig, gel, dressing, wound, skin, TBSA, tissue salvage

Introduction

For burns requiring hospitalization, the standard of care often involves a period of wound care and observation until wound demarcation is complete and the burn surgeon can make a determination as to the need for excision of dead skin and grafting. This "wait and see" period is based on the process of conversion, where damaged cells "convert" from thermally stressed to dead tissue, and has been characterized according to Jackson's thermal wound theory, which states that burn injuries have three zones: (1) zone of coagulation, a necrotic zone where the original injury occurred; (2) zone of stasis, the area that surrounds the necrotic zone and has potentially viable cells that will survive if proper treatment is given; and (3) zone of hyperemia, where the tissue recovers within 7–10 days if further infection does not occur.

In many patients the zone of stasis converts to dead tissue and must be removed both because it is a necessary step in closing the wound and because the apoptotic cells produce a number of biochemical factors that elicit untoward systemic effects that can lead to death of a patient days after the initial burn injury (e.g., multiple organ failure). A high correlation between TBSA burned and mortality has been firmly established with some authors reporting that every 1% increase in TBSA leads to a 5%–10% increase in mortality. Importantly, there are currently no available treatments that act primarily in the zone of stasis and decrease conversion thereby reducing

TBSA. The standard of care in burn treatment is to cover the wound to avoid infection, and there are numerous products on the market that do so quite acceptably. Silver-containing dressings and creams, such as those containing silver sulfadiazine, are highly utilized. However, since TBSA correlates so strongly with mortality, it follows that any treatment that effectively reduces TBSA will save lives.

Keratin biomaterials have typically been described in the literature in terms of the procedures and processes used to extract them. Extracts of hair, wool, and feathers were often characterized as heterogeneous mixtures with estimates of more than 100 homologs being potentially present in wool extracts, for example. More recently, human hair fibers have been described as consisting of 17 type I and type II alpha keratins that exist as obligate heterodimers as well as 85 matrix proteins termed keratin-associated proteins. Dr. Van Dyke's group at the Wake Forest School of Medicine has spent the past 6 years developing and patenting methods to isolate and purify these keratins to the level of subtypes that possess structural properties common to each. In so doing, several characteristics of the materials emerge that are not manifest in the more heterogeneous fractions. For example, alpha keratins can be separated using techniques such as dialysis and chromatography to the level of K81/K31 and K81/K33 dimers. These dimers have been shown to have the capability to form stable tetramers in solution, a property that likely contributes to the strong self-assembly characteristics of these samples. Importantly, the type I keratins K31 and K33 possess peptide-binding domains such as leucine-aspartic acid-valine, which is recognized by the β1 integrin subtype expressed by many cells. Based on an investigation of these materials, these binding domains appear to mediate strong cell adhesion to certain keratin substrates.

While the specific structures and cellular interactions remain to be fully understood, keratin biomaterials offer a platform for biomedical applications wherein materials can be tuned to elicit behaviors of interest (e.g., protection after thermal stress and wound healing). Control over this platform exists in the ability to reproducibly manufacture keratins on a large scale under GLP conditions and in the intrinsic ability of keratins to self-assemble into predictable secondary and tertiary structures. For the first time, novel keratin biomaterials can be produced in large quantities that self-assemble into matrices that can promote specific cell behaviors such as survival after thermal injury.

Development of a keratin biomaterial hydrogel-based wound dressing, KeraHeal™, has been performed over the past 7 years at the Wake Forest School of Medicine by Dr. Mark Van Dyke in collaboration over the past 3 years with KeraNetics, LLC. Prior investigations by Dr. Van Dyke have included the general biocompatibility of keratins, cell growth experiments in various culture systems, resuscitation fluids in small and large animals, bone regeneration in rodents, and both chemical and thermal burns in rodents and swine, respectively. Much of this research has demonstrated the cell and tissue compatibility of the oxidized form of keratin, keratose, which is the base material of KeraHeal. Sierpinski, et al. (*Biomaterials* 2008; 29(1):118-28) showed good adhesion and growth in a model cell culture system using keratoses and biocompatibility of keratose scaffolds implanted into mice. Similar findings have been published by numerous other investigators.

Keratose has also been shown to be biocompatible by ISO 10993 safety tests conducted at Toxikon Corporation, a Contract Research Organization. These tests included methyl tetrazolium cytotoxicity, intracutaneous injection,

Kligman maximization, systemic injection, reverse mutation assay, rabbit pyrogen, and total bioburden. In addition, Dr. Van Dyke's laboratory has conducted two animal studies to demonstrate the feasibility of using keratin biomaterials for burn treatment: a mouse chemical burn model and thermal burns in swine. Pilot data generated from these studies demonstrate the feasibility of using a keratose hydrogel dressing to provide coverage to tissue and stabilize wound size (i.e., stop burn conversion and suppress an increase in TBSA). These pilot data also suggest that KeraHeal is able to facilitate the survival of tissue in Jackson's zone of stasis following burn injury.

The goal of this AFIRM project is to translate and commercialize the KeraHeal product so that it can be used for the treatment of burned soldiers and civilians. To that end, the researchers are: (1) investigating the thermoprotective characteristics of keratin biomaterials in vitro, (2) testing the thermoprotective characteristics of keratin biomaterials in a pig burn injury model, and (3) conducting the first clinical investigation of a keratin biomaterial treatment for burn injury.

Summary of Research Completed in Years 1 and 2

In the first 2 years of the project, the research team developed a cell culture heat shock model that was used for mechanistic studies and to determine biomaterial dosing formulations for burn studies in swine. The researchers identified a key component of the keratin biomaterial, gamma-keratose, and developed new formulations using this material that are being tested in a pivotal animal study. They also established manufacturing capabilities at a spin-off company and submitted an investigational device exemption (IDE) application to the FDA to obtain approval for a clinical study.

Research Progress – Year 3

During the past year, the researchers developed and optimized a model of burn injury in swine; transferred the keratin biomaterial manufacturing technology to a partner company, KeraNetics, LLC, where it has been scaled-up and validated; filed a pre-IDE data package with the FDA; held a pre-IDE meeting with the FDA to finalize the regulatory pathway; gained preliminary FDA and Institutional Review Board (IRB) approval for a proposed clinical trial design; drafted a formal IDE application; and completed the in-animal portion of a pivotal animal study (second degree burns in swine), which is now awaiting histological results and final data analysis. The research experiments related to the swine burn model development and pivotal animal study are more fully described as follows.

Swine Burn Model Development Study

Porcine models are preferred animal models to study the effects of burns on dermal wound healing. However, many studies have been published in which little emphasis was placed on minimizing burn variability and inconsistency in porcine burn models. For treatment studies, inconsistent burns make it hard to quantify the healing capacity. To overcome these drawbacks, the researchers developed a novel method to create deep partial-thickness burns with greatly improved consistency and reproducibility.

A custom-made burn instrument **(Figure V-6)** was fabricated to control the pressure applied on the swine skin during burn creation, allowing the preservation of lateral

wound size and depth consistency when used by various operators. Cylindrical brass blocks, measuring 3 cm in diameter, are used to create the burns. A stainless steel post extends from the block for insertion into the instrument holder. The instrument holder is cylindrical in shape and made of insulative plastic for safe handling. An adjustable spring is loaded and set within the holder for block pressure control purposes. In the contact model of burn injury, researchers have typically used boiling water to heat metal blocks for burn wound creation on pigs. The research team's initial studies have shown that its brass blocks heated up to only 88°C–92°C when boiling water was used. This created superficial burns even with a contact time of 20 seconds. The use of an azeotropic mixture of boiling 80:20 PEG:deionized (DI) water, which raised the temperature of the blocks to 99°C–103°C, alleviated this problem **(Figure V-7)**.

In this study, three female Yorkshire swine were used under a protocol approved by the Wake Forest Animal Care and Use Committee (ACUC) and the Animal Care and Use Review Office (ACURO). Under general anesthesia, 10 burn wounds were created with heated cylindrical brass blocks, 5 on each side of the dorsal midline between the shoulder and hip. The brass blocks were heated in an 80:20 boiling PEG:DI water solution. Once the boiling liquid was heated to 105°C–115°C, the brass blocks were used to create burns on the pig. Different contact times ranging from 12 to 20 seconds were used to create burns on each swine, and each contact time was duplicated once (i.e., n=2). The position of different burn contact times on the swine was randomized and the wounds were labeled from 1 to 10. Post-burn, each wound was covered with saline-soaked, non-adherent gauze (Telfa™), and the entire back of the animal was covered with an occlusive dressing (adhesive Ioban™

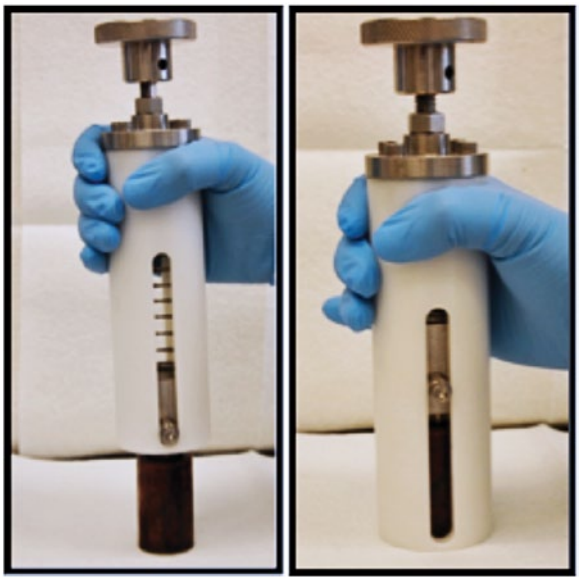


Figure V-6. Custom-made burn instrument. A brass block affixes to the cylindrical holder and insulates the heat from the user (left). The spring-loaded chamber creates constant pressure when the block is pressed against a surface (right). A retaining pin holds the brass blocks in place and allows them to be changed quickly thereby allowing blocks to be rotated so that maximum heating can be achieved each time the device is used.

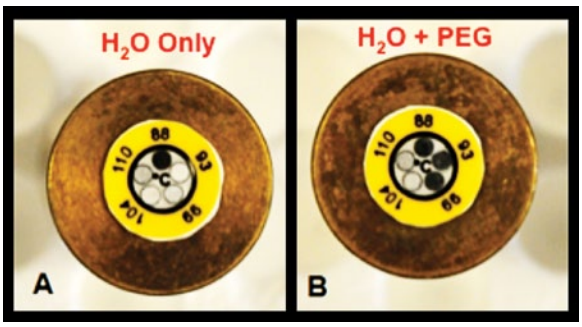


Figure V-7. Heating in a PEG:water solution results in higher block temperature. Many published studies rely on boiling water to heat the burn instrument (i.e., brass block). It was determined that boiling water heating resulted in a block surface temperature of 88°C (left) while the PEG:water azeotrope heated the block to 99°C (right).

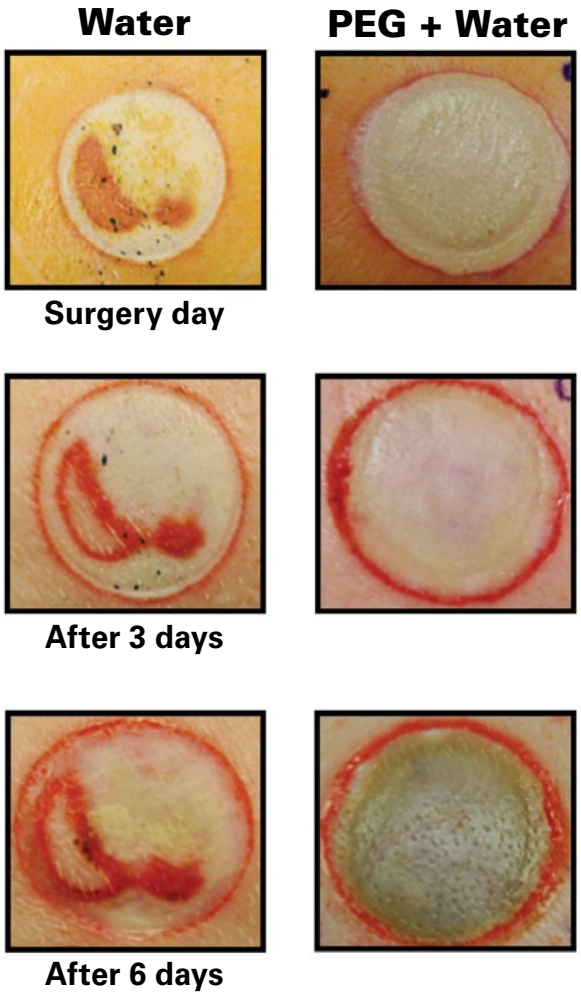


Figure V-8. Boiling water used for heating burn instrument forms "islands" of viable skin. The PEG:water azeotrope has lower surface tension and prevents vapor bubbles from producing cold regions on the surface of the brass blocks, resulting in even heating and more consistent burns.

dressing), a protective plastic shield, and a nylon jacket to keep the dressings in place. Every 3 days, the dressings were removed and the wounds cleaned and debrided with saline-soaked gauze. On days 1, 4, and 7 post-surgery, an animal was euthanized and tissue was collected for further histological analysis.

The skin tissue sections were further analyzed histologically using Gomori trichrome and H&E stains. The Gomori trichrome stain was used to assess the burn depth and H&E to assess cell infiltration into the damaged tissue. Typically all of the necrotic tissue is debrided or otherwise sloughed within a week. Histological sections showing the area of the skin that was burned after 24 hours, 4 days, and 7 days were measured to determine the amount of tissue loss. These data showed a 50%–60% loss of the skin tissue by 7 days when the porcine skin was contacted by a heated brass block for 20 seconds. This is considered a deep partial-thickness burn. Conversely, a 12-second contact time generated a very superficial burn. Importantly, the use of the PEG:water azeotrope generated a consistent burn free of unburned "islands" of viable skin **(Figure V-8)**, presumably formed by cold regions on the surface of the blocks as vapor bubbles are trapped during emersion in the boiling fluid. The PEG:water azeotrope has a lower surface tension and does not allow vapor bubbles to adhere to the bottom surface of the brass blocks.

Pivotal Swine Burn Study

The researchers used 28 female Yorkshire swine under a protocol approved by the Wake Forest ACUC and the ACURO. The animals were randomly divided into the two arms of the study, one in which treatment would be administered within 60 minutes of burning

and another in which treatment would be delayed for 10 hours. In each arm, 14 animals were randomized and 12 burn wounds were created with heated cylindrical brass blocks, 6 on each side of the dorsal midline between the shoulder and hip under general anesthesia. The brass blocks were heated in an 80:20 boiling PEG:DI water solution. Once the boiling liquid was heated to 105°C–115°C, the brass blocks were used to create burns on the pig by contact with the skin for 20 seconds as described in the method development study. The wounds were randomized into one of four treatment groups: saline-soaked gauze, Coloplast (a collagen-based wound gel), keratin treatment 1, and keratin treatment 2. Keratin treatment 1 represents the form of keratose hydrogel used in previous pilot studies and keratin treatment 2 represents the KeraHeal formulation. For each treatment, at each time point in both arms of the study, six replicate wounds were created using two animals. A total of 3 cc of keratin or Coloplast was used on each wound, and all wounds were covered with a Telfa pad, Ioban dressing, a protective plastic shield, and a nylon jacket as described previously. Every 3 days the dressings were removed and the wounds cleaned and debrided with saline-soaked gauze. Digital photos were taken with a color wheel and rule in view for digital image processing. On days 1, 3, 6, 9, 12, 15, and 30 post-surgery, two animals were euthanized and tissue was collected for histological analysis.

The skin tissue sections will be analyzed histologically using Gomori trichrome, H&E, von Willebrand factor (blood vessels), caspase 3a (apoptosis), and high-mobility group box protein 1 (necrosis) staining and quantified using typically morphometric techniques. Digital photos will be measured for wound size and degree of re-epithelialization and scored by two blinded clinical reviewers. These analyses are currently ongoing.

Heat Shock Mechanism Study

Additional heat shock experiments have been conducted using the in vitro model previously reported. Briefly, mouse dermal fibroblasts were isolated from postnatal pups and grown to near confluence. Cultures were treated at 44°C for 150 minutes to induce necrosis and stress. After 6 hours under normal culture conditions, nonadherent cells were removed and treatments applied. Cells were counted at 24, 48, and 72 hours. Treatments include the gamma-keratose fraction (ingredient in KeraHeal) at 0.001 mg/mL and normal fibroblast growth media. RNA was extracted at 0, 6, and 24 hours. Array analysis will be performed using an assortment of software and programs. Differentially expressed genes will be detected between groups and time points using a t-test adjusted with the Benjamini and Hochberg false discovery rate method. Between-group comparisons will be used to determine shared pathways and gene categories. Highly enriched and significant genes will be filtered setting specific criteria, including p values ≤ 0.01 and p values ≤ 0.05 and log ratios ± 1 and ± 0.6. Heat maps will be generated using Cluster 3.0 and TreeView, allowing for visualization of differentiation-specific expression profiles between groups. Venn diagrams will be generated from Venny and gene annotation and ontology searched using The Database for Annotation, Visualization, and Integrated Discovery (DAVID) as well as SOURCE software. Pathway and gene networks will be visualized using KEGG pathway analysis. Time points will be compared and contrasted using STEM software. Individual gene intensities will be compared across groups and time points using GraphPad Prism 5. These analyses are currently ongoing.

Key Research Accomplishments

- Developed and optimized a swine burn model.
- Completed the in-animal portion of a pivotal animal study.
- Completed the initial gene array experiments for the mechanistic study.
- Transferred the keratin biomaterial manufacturing technology to a partner company, KeraNetics, LLC, which scaled up and validated it.

Conclusions

The researchers have successfully developed and optimized a burn model in swine and completed the in-animal portion of a pivotal trial. Their mechanistic study has also progressed with completion of the initial gene array experiments. These data are being processed and additional experiments are planned. Commercialization of the KeraHeal product is progressing rapidly. KeraNetics, LLC has completed the setup and validation of its production facility and is in the process of securing funding to support the human clinical trial.

Research Plans for the Next 2 Years/Planned Clinical Transitions

During the next 2 years, the researchers will complete their pivotal swine burn study and heat shock mechanism study analyses. The preclinical data for the swine study have been reviewed by the FDA, and initial approval for a clinical trial has been obtained. The researchers have prepared a formal IDE application, and they are awaiting data from the pivotal study and validation data from KeraNetics, LLC. This application is expected to be submitted to the FDA in the second quarter of 2011.


our science for their healing

Mesenchymal Stem Cells for Burn and Wound Healing

Project 4.6.2, RCCC

Team Leader(s): Arnold Caplan, PhD and Michael Sorrell, PhD (Case Western Reserve University)

Project Team Members: Marilyn Baber, BA and Randall Young, VMD (Case Western Reserve University)

Collaborator(s): Glenn Prestwich, PhD (University of Utah), Fidia Advanced Biopolymers, and Richard Clark, MD (Stony Brook University)

Therapy: Topical delivery of adult human bone marrow-derived MSCs.

Deliverable(s): Adult human MSCs (hMSCs), in a therapeutic matrix.

TRL Progress: 2009, TRL 1; 2010, TRL 2; 2011, TRL 4

Key Accomplishments: The researchers modified the topical delivery of hMSCs to full-thickness mouse skin wounds to apply these cells to day 3 post-wounding. This resulted in an enhanced yield of cells to the wound bed and a more even distribution of cells throughout the wound bed. This approach facilitated the use of laser capture to analyze MSC function in the wound bed.

Key Words: Mesenchymal stem cells, wound healing, skin, hyaluronan

Introduction

Novel approaches are being developed to promote healing in extensive, acute skin wounds and in chronic nonhealing skin wounds. Wound studies have shown the importance for growth factor production to direct the overlapping series of events that characterize the healing process. Factors such as platelet-derived growth factor and vascular endothelial growth factor (VEGF) have critical roles in the healing process in that they regulate cellular migration and angiogenesis, both of which are critical for the formation of granulation tissue. Unfortunately, application of factors alone or in combination have proven to be ineffective approaches.

An alternate approach is to employ cells to deliver growth factors. Therapeutic cells, such as adult bone marrow-derived MSCs can be defined as a population of adult cells that release growth factors, cytokines, and chemokines that promote inflammation and granulation tissue formation. In this manner, cells are capable of releasing complex mixtures of bioactive factors that are more advantageous to wound healing. There is now an expanding body of evidence in both preclinical studies and clinical studies that validates this approach. In most studies, therapeutic cells were derived either from the bone marrow or from adipose tissue; however, such cells have been identified in many adult organs. The adult bone marrow-derived cells have been defined as MSCs for their ability to differentiate.

Tissue engineering technologies have been introduced to wound healing. One such tissue-engineered approach is to apply acellular scaffolds to wounds as a means of attracting wound cells and promoting the formation of granulation tissue. Granulation tissue formation is one of the earlier events in the wound repair process in which a neovasculature (new blood vessels) forms in the wound bed. This poorly organized vasculature provides a conduit for inflammatory cells and nutrients to the previously avascular wound bed. One type of scaffold material that has been employed in wound healing is composed of hyaluronan (HA). HA is a natural nonsulfated glycosaminoglycan that is not covalently bound to proteins unlike other glycosaminoglycans. Instead, HA binds selected proteins using noncovalent interactions. These interactions occur in both the ECM and on cell surfaces. Through these interactions, HA influences various tissue dynamics that are necessary for the formation of mature tissue. HA is highly expressed and active during the embryonic and fetal development of most tissues. These events are reprised during the early stages of wound repair.

It has been proposed that tissue-engineered HA scaffolds will enhance the repair process. Preclinical and clinical data support this notion. Native HA is unstable in wounds due to the presence of hyaluronidases (enzymes that degrade HA). Consequently, it is necessary to introduce chemical modification of the HA polymer. These modifications increase stability without interference of function. HA scaffolds are typically delivered to wounds in an acellular configuration that promotes interactions with wound cells. Smaller and intermediate-sized HA fragments that are released during the controlled digestion of this material bind to cell surface receptors and promote critical proangiogenic and proinflammatory events. In short, HA scaffolds have been successfully employed as wound-healing agents.

The application of therapeutic cells to wounds is a nontrivial issue. There are essentially two approaches: systemic delivery or topical delivery. The systemic delivery approach requires cells to survive their passage through the circulatory system and to home to a wound or ischemic site in sufficient numbers to modulate wound repair. The topical application of cells delivers them directly to the affected site, but cellular migration through connective tissue is often necessary for these cells to situate themselves in appropriate locations to affect wound repair. In the current study, concentration of cells is achieved through their application to HA carriers. This means that both therapeutic matrices and cells are delivered topically to wound sites. In this study, the wound is a full-thickness skin wound on normal, immunocompetent mice. This provides the means to assess the effects of the matrices and cells on wound repair and to assess any rejection responses through the use of xenogeneic therapeutic cells.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers developed a mouse excisional skin defect wound model that allowed quantitative fluorescent imaging of the localization of infused adult MSCs. They received approval on all animal protocols and documentation, and completed pilot experiments. During the second year of the project, the researchers demonstrated proof of principle for therapeutic cell delivery to full-thickness skin wounds in mice. They noted effective refinements in the procedures and began to implement them.

Research Progress – Year 3

In the original wound model that was developed for this study, hMSCs were delivered to the wounds using a Glycosil™ (provided by Dr. Glenn Prestwich) carrier at the time of surgery. These hMSCs typically migrated to the periphery of the wound bed prior to migrating to the subdermal vascular plexus that feeds the granulation tissue of the healing wound. This presented two problems. First, the hMSCs were not concentrated during their intermediate migration phase. Second, this was not a good model for the clinical treatment of skin wounds. It is much more effective to treat wounds after the event when patients are in a controlled medical setting.

During the past year, the researchers delivered hMSCs to mouse skin wounds 3 days post-injury using the following procedure: (1) removed bandages applied at the time of surgery, (2) gently removed scab, if it existed, and (3) placed the carrier-containing cells onto the wound bed. Previous studies demonstrated that a cellular but nonvascularized wound bed was present by 2 days post-surgery. The hMSCs applied to the wound bed on day 3 entered the wound bed and migrated as a cohort through the wound to the subdermal vascular plexus. The kinetics of this migration were similar to that when cells were delivered to an empty wound. However, the tight concentration of cells makes it possible to better quantify cellular delivery and to isolate human cells for RNA extraction.

RNA extraction is accomplished through laser capture of frozen tissue sections. Labeled hMSCs can be identified in sections, and this region can be selectively cut from the sections. At the same time, regions of the wound that do not contain human cells can also be cut from the wound for RNA extraction. This provides sources of RNA that can be used to help understand the function of hMSCs as they are migrating into the wound bed. The next step in this process is to develop methods to most effectively analyze the RNA pool obtained from these tissues. SABiosciences has recently introduced a new RNA array process that can handle very small amounts of RNA (down to 1 nanogram of RNA). This new application was developed for laser capture studies. RNA captured from wounds is now being evaluated using this method.

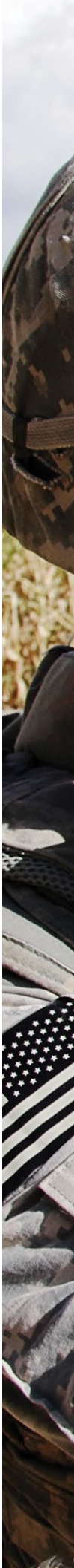
Key Research Accomplishments

- Modified delivery of hMSCs to day 3 post-surgery.
- Improved tracking of hMSCs in wounds.
- Initiated laser capture studies designed to understand the function of hMSCs as they migrate into the wounds and to better understand the response of mouse cells to the influence of hMSCs.

Conclusions

The penetration and migration of hMSCs is enhanced by their delivery on day 3 post-wounding. These cells migrate in a tight cohort, which makes it feasible to use laser capture technology to capture these cells during their migration to interrogate their RNA profiles. This will provide the basis for a better understanding of how hMSCs function in the context of a wound.

The original goal of this project was to treat large burns using hMSCs. In initial experiments, hMSCs provided by the Caplan laboratory, but cultured in the Clark laboratory, demonstrated minimal activity in the rat hot comb model. Since repeat experiments confirmed a negative response, Project 4.6.2 was terminated on June 30, 2011.



our science for their healing

Polymeric, Antimicrobial Wound Dressing Providing Sustained Release of Iodine

Project 4.6.4, RCCC

Team Leader(s): Carmine Iovine, MS and Niraj Ramachandran, PhD (Rutgers University) and Richard Clark, MD (Stony Brook University)

Project Team Members: Adam Singer, MD (Stony Brook University) and Joachim Kohn, PhD (Rutgers University)

Collaborator(s): None

Therapy: Treatment of infected traumatic wounds of soldiers.

Deliverable(s): Absorbent, conformable, nonadherent, self-supporting, foam-based wound dressing containing bound molecular iodine (2%–15% by weight), which is released in contact with a wound in an "on-demand," sustained manner over a period of several days, to minimize required dressing changes.

TRL Progress: 2009, TRL 2; 2010, TRL 3; 2011, TRL 4; Target, TRL 6

Key Accomplishments: The researchers have created a tunable iodine delivery system in an absorbent wound dressing that releases iodine on demand and does not require frequent dressing changes, resulting in better patient comfort. They developed and implemented a reproducible small-scale fabrication process and reduced it to practice. They developed and validated standard operating procedures (SOPs) for in vitro iodine loading and release. They demonstrated in vitro biocompatibility based on extraction analysis. Their iodine delivery system showed significantly longer on-demand release of iodine compared to any known or tested iodophor system.

Key Words: Iodine, polymeric iodophor, wound dressing, antimicrobial

Introduction

Although water-soluble polymeric iodophors (complexes of iodine in ionic or molecular form or both with a polymeric carrier) have an advantage over "tincture"-based iodine, they still release to the wound site too quickly because of their solubility. Often times, this quick-release characteristic provides a much higher dose of iodine than is required for the intended antimicrobial action, and the iodine is used up by side reactions with body fluids thus depleting the reservoir prematurely and allowing for bacterial re-colonization of the wound site. From a clinical point of view, wound dressings based on water-soluble polymeric iodophors can only contain low levels of iodine, and the dressings need to be changed very frequently in use.

By utilizing a water-insoluble polymeric iodophor as the carrier for iodine, the concentration of free iodine in the solution can be maintained at a low level thus avoiding the issues associated with water-soluble iodophors including the premature release of the iodine. Many approaches to insoluble polymer iodophors have been reported. These include various iodine-impregnated nylon fiber structures as wound dressings. These materials are not very adsorbent; they release iodine in minutes and their iodine-loading capacity is low. Broad-spectrum, antimicrobial dressing materials based on chemically modified polyvinyl alcohol (PVA) have also been reported and tested. These materials can typically carry up to 5% iodine by weight, which is released slowly over a period of hours.

However, the material is unstable in contact with heavily exuding wounds, releasing toxic byproducts slowly over time and thus increasing the water solubility of the iodophor and accelerating the release of iodine. Insoluble polyurethane antimicrobial foams and films have also been described in the literature. These materials are stable but can only release iodine over a period of several hours. The iodine is weakly bound to the polymer carrier as a charge transfer complex, and this accounts for the relatively short-duration release characteristics.

There is a need, therefore, for an insoluble, antimicrobial polymeric iodophor that is stable, highly adsorbent, can be loaded with high levels of strongly bound iodine, and can release the iodine in a controlled and sustained manner over a period of several days.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the research team identified a polymer system that could release molecular iodine from a wound dressing, potentially preventing infection. The system, called the I-Plex Absorbent Antimicrobial Wound Dressing, is a nonadherent, moist, formalin-treated PVA sponge that releases molecular iodine into wounds as exudates are absorbed by the polymer. The researchers completed feasibility studies for the preliminary evaluation of the device, including fabrication of the PVA sponge and complexing iodine with the sponge. They estimated the iodine loading and release rates from the PVA sponge and established a quality control procedure in which samples made from each batch were tested for the weight of iodine loaded.

Although the formalized PVA-based system failed 1.5 years into the program due to toxicity concerns, the project was brought back on track in year 2 by the development of a novel polymeric system for infection control. The researchers completed proof-of-concept testing of this novel wound dressing in pigs.

Research Progress – Year 3

At the start of year 3, a key decision was made to use a completely new dressing material, which has delayed the progress of this project. During the past year, the researchers found that a broad spectrum known antimicrobial agent (molecular iodine) can be incorporated at very high loading levels (10%–20%) into an absorbent and wound-conformable, unsupported wound dressing material. This material contains a chemically linked novel iodophor and a biodegradable synthetic polymer in a flexible, nontoxic insoluble polymer network. The novel iodophor has much stronger binding capacity with iodine compared to any other iodophor, making it ideal for use in on-demand, sustained release applications. The researchers can control the level of bound iodine in the network structure by varying the ratio of the novel iodophor and the biodegradable synthetic polymer used to constitute the network. They can control the rate, sustainability, and release time of the iodine antimicrobial by varying the type of novel iodophor utilized in the structure. The wound dressing is made as a wound-conformable, hydrophilic (but insoluble) absorbent foam structure by reaction of selected synthetic polymer compositions with the novel iodophor and various cross-linking agents.

The researchers' flexible and absorbent polymer iodophor showed promising results in a preliminary porcine infected burn model. The polymer demonstrated good antimicrobial

activity and minimal biological reactivity. Furthermore, dressing changes incurred no trauma to the epithelializing wound bed. The researchers have developed and implemented a reproducible and simplified small-scale laboratory manufacturing process. They demonstrated in vitro biocompatibility of the antimicrobial polymer using extraction and cell toxicity testing recommended by an FDA consultant. They developed and validated SOPs for in vitro iodine loading and release. They found on-demand release of iodine to be significantly longer than previous polymer iodophors. The researchers defined elements of patentability and completed a Rutgers invention disclosure and patent disclosure. The research team plans to file a provisional patent in the summer of 2011. The patentability elements underpin the proposed marketing and commercial advantages of the device.

Key Research Accomplishments

- Established a reproducible, small-scale laboratory batch processing method and reduced it to practice.
- Demonstrated biocompatibility of the antimicrobial polymer based on extraction analysis.
- Demonstrated significantly longer in vitro on-demand release of iodine than any other known or tested iodophor system.
- Established the formula for a final prototype of the iodine delivery system.

Conclusions

The researchers have created a tunable iodine delivery system in an absorbent wound dressing that releases iodine on demand and does not require frequent dressing changes, resulting in better patient comfort. This wound dressing technology is highly suited to battlefield conditions and the treatment of wounded warfighters. The researchers' prototype product and process is now ready for pilot plant scale-up. Basic principles of patentability have been demonstrated.

Research Plans for the Next 2 Years/Planned Clinical Transitions

Over the next year, the researchers will plan the pilot plant scale-up of their prototype. They will test the product in relevant preclinical models. They will manufacture their product under GMP conditions and will file a 510(k) application. After year 4, it was agreed that this project would be terminated from the RCCC-AFIRM portfolio and transitioned to a more appropriate funding source.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Topical P12 Treatment of Burns Using Fiber Mats

Project 4.6.5, RCCC

Team Leader(s): Lauren Macri, PhD and Richard Clark, MD (Stony Brook University)

Project Team Members: Adam Singer, MD (Stony Brook University) and Larisa Sheihet, PhD and Joachim Kohn, PhD (Rutgers University)

Collaborator(s): None

Therapy: Topical therapy to limit burn injury progression.

Deliverable(s): A controlled release formulation for topical therapy of burns.

TRL Progress: 2009, TRL 2; 2010, TRL 3; 2011, TRL 3; Target, TRL 5

Key Accomplishments: The researchers established sterilization and storage conditions for P12 fiber mats, validated a porcine burn model, and obtained in vivo proof of concept that P12 fiber mats limit burn injury progression.

Key Words: Burns, burn progression, biodegradable polymers, drug delivery, peptide, electrospinning, wound healing

Introduction

Burn injuries can be extremely painful, debilitating, and complex to treat. One reason for this is that burn injuries are not localized to the initial site of trauma; rather, they progress (or extend) in size in horizontal and/or vertical directions. Often, the tissue surrounding the burn, which initially looks like a healing partial-thickness burn, will convert into a nonhealing, full-thickness burn. Although burn wound care has advanced over the years (via resuscitation, emergency care, and transportation), there is still a critical need to develop treatments that limit burn injury progression and specifically (1) minimize tissue loss and need for grafting, (2) shorten healing and hospitalization time, (3) lower rates of morbidity and mortality, and (4) decrease scarring and contracture. Although many therapeutic agents have shown promise in small animal models, these beneficial effects have not been translated into FDA-approved therapies. Thus, a major clinical challenge exists to develop novel therapies that can reduce or prevent burn progression.

P12, a peptide derived from fibronectin (an ECM glycoprotein), has recently been identified by the Clark laboratory and shows significant promise in the treatment of burns. This peptide enhanced the survival of adult human dermal fibroblasts, which play a critical role in wound healing, when cells were exposed to stressful conditions. In addition, P12 reduced burn injury progression in both rat and porcine hot comb burn models. Therefore, the goal of this project is to develop biodegradable TyrPC

fiber mats that produce topical, localized, controlled, and sustained P12 delivery, addressing the requirements of an efficient treatment to limit injury progression of localized burns.

Summary of Research Completed in Years 1 and 2

During the first year of the study, the researchers identified polymer compositions that can degrade in the appropriate time frame relevant to the most crucial stages of cutaneous wound repair after third-degree burns. They achieved fabrication and characterization of the engineered P12 delivery scaffold—P12-containing fibroporous mats were successfully produced using the electrospinning technique. They began evaluating the P12 release kinetics from electrospun fibroporous mats. During the second year of the project, the researchers determined that the fibroporous mats could release P12 in a rate-controlled manner. They demonstrated the biocompatibility of the mats in vivo in a porcine model.

Research Progress – Year 3

Fabrication and Characterization of P12-Loaded TyrPC Fiber Mats

The researchers used electrospinning to fabricate TyrPC fiber mats that erode at ultrafast (8 hours) and fast (4 days) rates. They incorporated P12 into these fiber mats to assess the drug-release efficiency. Both TyrPC terpolymers were fast degrading and retained less than 20% of their initial molecular weight after 7 days. However, polymer erosion and P12 release were dependent on polymer composition. P12 release was predominantly controlled by polymer erosion of ultrafast fibers whereas the mechanism of release from fast fibers was governed by P12 diffusion. In vivo data confirmed the biocompatibility of both chosen polymer compositions. These results suggest that P12-loaded electrospun tyrosine-derived fibers offer the potential for topical clinical therapies that require ultrafast or fast delivery of the therapeutic agent, P12.

Evaluation of Sterilization Methods on P12-Loaded Fiber Mats

Sterilization of the medical device and/or pharmaceutical agent is mandatory for in vivo application. A suitable sterilization method is one that does not alter the physicochemical characteristics of the device and/or affect the biological/therapeutic performance of the payload. The project team electrospun TyrPC polymers into fiber mats containing P12 and sterilized them with ethylene oxide gas, irradiation (gamma or electron beam), or UV light radiation. Then, the researchers used a systematic approach to evaluate the effects of sterilization on fiber morphology, polymer molecular weight, P12 content, and bioactivity.

The exposure to ethylene oxide gas in the tested conditions affected the molecular structure of P12, which makes these ethylene oxide sterilization methods unsuitable for P12-containing devices. The tests showed that irradiation had a substantial impact on polymer molecular weight, which was likely due to oxygen radicals (i.e., hydroxy radicals and superoxide anion). Fortunately, sterilization of P12-containing ultrafast and fast TyrPC fiber mats by UV radiation provided the desired outcome with no measured effect on fiber morphology, polymer molecular weight, P12 content, or bioactivity. Hence, all fiber mats tested in preclinical studies will be sterilized with UV radiation. Future studies are recommended to optimize irradiation sterilization of P12-loaded fiber mats.

Investigation of the Shelf Life of P12-Loaded Fiber Mats

Shelf life is defined as the duration of time that P12-loaded fiber mats can be stored at specified conditions without deviating from their initial specifications. Unloaded and P12-loaded fiber mats were stored at -20°C, 4°C, and 25°C. Changes in fiber morphology, polymer molecular weight, and P12 content were determined for up to 12 months. These findings provide insight on the shelf life of P12-loaded fiber mats. The P12 molecule seems stable after storage in the fiber mat for 1 year at -20°C, 4°C, and 25°C; however, the stability of the polymer is temperature dependent. Therefore, these results suggest that both ultrafast and fast TyrPC fiber mats should be packaged under anhydrous conditions and preferably stored at -20°C. Furthermore, packaging under a controlled, anhydrous atmosphere is recommended, especially if storage at 4°C or 25°C is required.

Validation of the Animal Model

The Clark and Singer laboratory at Stony Brook University currently uses the validated porcine hot comb burn model to evaluate the effects of the intravenous administration of P12 on the limitation of burn injury progression. However, a modification to the protocol is required to evaluate the topical administration of P12. As a first step toward this goal, the project team used immediate burn excision to allow direct contact between the P12-loaded fiber mats and the uninjured interspaces. Therefore, the aim of this study was to determine the effects of immediate burn excision on injury progression in the porcine hot comb burn model.

The project team demonstrated, via the porcine model, that unburned interspaces of the excised burns were fully necrotic 7 days post-injury (as confirmed by both histology and gross analyses). Thus, the animal models suggest that surgical excision of the zone of coagulative necrosis (in humans) is likely not sufficient to effectively reduce or prevent burn injury progression. Furthermore, in the porcine model, excisional wounds with dimensions identical to the comb burn did not result in necrosis of the interspaces. The latter results demonstrate that excision did not devascularize the interspaces and was not responsible for burn progression. Therefore, the researchers believe that the effective clinical approach should focus on the combination of a suitable therapeutic(s) to prevent vessel occlusion or the proinflammatory role of cytokines and thus reduce both burn injury progression and burn excision to reduce scar formation and contracture. In conclusion, the porcine excised hot comb burn model is a validated model for the evaluation of topical therapies on the limitation of burn injury progression.

Pilot Evaluation of in Vivo Efficacy of P12-Loaded Fiber Mats on Burn Injury Progression

Using the porcine excised hot comb burn model validated and described previously, burns were treated immediately after excision with TyrPC ultrafast fiber mats containing P12 (1.5, 15, and 150 μM per excised burn) or fiber mats alone. The doses proposed for this pilot experiment were based on estimated concentrations in the extracellular fluid that parallel intravenous doses of P12 that were previously shown to be efficacious in the porcine hot comb model (Project 4.6.1). As shown in **Figure V-9**, the unburned interspaces of untreated comb burns had undergone progressive ischemia and were 33% (4/12) viable 7 days post-injury (based on histological specimens). Similarly, excised burns treated with unloaded fiber mats were 22% (2/9) viable 7 days post-injury. However, unburned interspaces of excised burns treated with P12 showed a reduction of burn

injury progression at the lowest dose of 3 µg per wound. Interestingly, decreased injury progression of 11% (1/9), 22% (2/9), and 56% (5/9) was observed with a decreased dose of P12 (150, 15, and 1.5 µM, respectively) (Figure V-9). In conclusion, these preliminary data support that P12-loaded (1.5 µM) TyrPC ultrafast fiber mats reduced burn injury progression into the zone of stasis (viable tissue that may or may not progress to necrosis). More studies are currently ongoing to confirm this result.

Key Research Accomplishments

- Developed TyrPC terpolymers as fiber-based drug delivery matrices for releasing P12 at ultrafast (8 hours) and fast (4 days) rates.
- Found that UV radiation was a suitable sterilization technique for P12-loaded TyrPC ultrafast and fast fiber mats.
- Developed and validated a porcine burn model suitable for evaluating topical therapies that may limit burn injury progression.
- In vivo results of a pilot study suggested that the topical delivery of P12-loaded fiber mats limited burn injury progression.

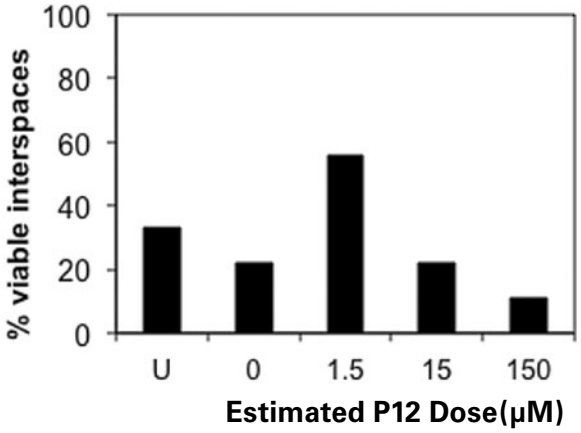

Figure V-9. Histological evaluation using H&E-stained specimens of unburned interspaces at 7 days post-injury. The y-axis reports the percentage of total interspaces that were viable on day 7. The abbreviation "U" represents untreated excised comb burns.

Conclusions

In year 3, the team focused on evaluating the stability of P12-loaded fiber mats during the storage and sterilization processes. They found that aseptic UV radiation was a more suitable sterilization technique than terminal sterilization with ethylene oxide gas, gamma irradiation, or electron beam irradiation. Although P12-loaded fiber mats failed to retain their initial specifications, a suitable sterilization method, using irradiation, may be possible with further optimization of the method parameters, particularly packaging

under hypoxic conditions and incorporating a stabilizer (i.e., antioxidant). Furthermore, non-sterilized, P12-loaded fiber mats are stable for at least 9 months when stored at -20°C.

The project team also developed and validated a porcine burn model that was suitable for evaluating topical therapies that may limit burn injury progression. The key aspect to the model is that surgical excision of the zone of coagulative necrosis (in pigs, corresponding to the edge of the visible burn) is not sufficient to effectively prevent burn injury progression. The researchers used this animal model to evaluate the efficacy of P12-loaded fiber mats in reducing the progression of burn injuries. The in vivo results of a pilot study suggested that the topical delivery of P12-loaded fiber mats limited burn injury progression. In conclusion, electrospun, tyrosine-derived fiber mats offer the potential for topical clinical therapies that require ultrafast or fast delivery of the therapeutic agent (P12).

Research Plans for the Next 2 Years

In year 4, the research team will confirm the efficacy of topical P12 therapy in porcine models, advancing the technology to TRL 4. The outcome of these studies will provide feedback for further optimizing these technologies to achieve the desired therapeutic effect. In year 5, the project team will perform definitive animal studies.

Planned Clinical Transitions

Pending the results of the animal studies in year 5, the researchers will initiate discussions with the FDA and the Center for Devices and Radiological Health for pre-IND review. Also in year 5, they will conduct safety and efficacy studies (TRL 5) in GLP guidance. Finally, in year 5, the project team will submit an IND and initiate the design of a clinical trial.

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Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device

Project 4.2.2, WFPC

Team Leader(s): Jörg C. Gerlach, MD, PhD (University of Pittsburgh)

Project Team Members: Patrick Over, Matthew Young, and Roger Esteban, PhD (University of Pittsburgh)

Collaborator(s): James Holmes, MD (Wake Forest University) and Steven Wolf, MD (USAISR) (year 5)

Therapy: Skin stem cell delivery for cell-based treatment of burn wounds.

Deliverable(s): (1) Optimized cell isolation and spraying methodologies and (2) next-generation skin gun, FDA-approved spray device that can deposit fetal skin stem cells onto wound surfaces.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 2; End Year 3, TRL 3

Key Accomplishments: The researchers have established methods for isolating, expanding, and freezing fetal epidermal basal progenitor cells and fetal dermal MSC-like cells from fetal human skin. They demonstrated the basal keratinocyte characteristics of fetal epidermal cells to use as an off-the-shelf product for second-degree burn healing. They devised a method to use fetal dermal fibroblasts jointly with fetal epidermal basal keratinocytes as an off-the-shelf product in third-burn degree healing. They designed an in vitro cell spray model that works with epidermal progenitors and dermal stem cells, and performed spray parameter tests to achieve maximum viability after spraying.

Key Words: Skin stem cells, burn wounds, human fetal tissue, progenitor cells, cell spray method

Introduction

The surgical treatments available to burn patients have had a dramatic effect on survivability of burn injury yet the outcome for a severely burned patient is still not ideal. The human body has an innate tendency to respond to injury by producing scar tissue and fibrosis, which often result in functional limitations and aesthetically unsatisfactory results. Currently, tissue autografting techniques, such as mesh grafting, are the gold standard for the treatment of severe burns. However, in cases in which there is insufficient healthy skin for use in grafting, other materials have been used. A host of synthetic and biologic materials are available to treat full-thickness burns. Although these therapies have improved patient outcomes, they all result in severe scarring and are a poor means by which to induce wound-healing responses. Regenerative medicine research may provide novel opportunities for burn treatment by introducing novel cell-based therapies and/or by accelerating innate regenerative processes that essentially recapitulate fetal-like wound healing in the adult.

In a landmark study by Hohlfeld, et al. (*Lancet* 2005; 366(9488):840-2), fetal-derived skin fibroblasts were banked and then used as a cell source for cell grafts that were transplanted onto pediatric patients. Although these fetal-derived constructs produced a more fetal-like healing response (i.e., rapid wound closure with no hypertrophic granulation tissue), the approach still delivered the cells in a simple and uncontrollable manner.

Interestingly, however, Hohlfeld and colleagues also demonstrated that the fetal fibroblasts were not detectable after several weeks at the transplant site; therefore, the cells appear to have established an appropriate environment for autologous healing in the wound bed. Thus, skin progenitor cells derived from human fetal skin tissue have the potential to serve as a regenerative, cell-based therapy for acute and chronic skin disease and burn injuries. The focus of this project is to develop methods of isolating and characterizing fetal skin cell populations, particularly the epidermal basal keratinocyte progenitor cells and dermal fibroblast MSC-like cells.

In addition, a method to deliver the fetal cells to a burn wound is required. The Gerlach group has developed a cell spray deposition device that can "seed" the burn site with any given cell type. The first clinical tests in Berlin, Germany, used expanded keratinocyte cultures that were cultured ex vivo and then delivered to wound sites on burned patients. In Berlin and now in Pittsburgh, the Gerlach group developed a new method in which cells are isolated and immediately sprayed onto a wound in a single, on-site session. The Gerlach group has also proposed to combine its clinical work with the work of Hohlfeld, et al. (2005) by exploring the use of the skin spray device with banked fetal-derived epidermal skin progenitor cells. Over the past year, the group was also able to isolate and culture adult dermal progenitor cells. The results of isolation, culture, characterization, and spray testing these adult dermal MSC-like cells suggested that these cells should also be included in the program.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers established an in vitro cell spray model and an in vitro wound capillary membrane model of an active wound dressing for the support of skin cells. These models work with epidermal progenitor cells and dermal stem cells. The researchers also established laboratory methods for the isolation and cell culture of fetal epidermal basal progenitor cells and fetal dermal MSC cells.

Research Progress – Year 3

The long-term clinical goal of the group is to refine and control the deposition of skin progenitor cells in a burn wound and develop the ability to control their differentiation into basal keratinocytes and other terminally differentiated skin cells. Previous clinical results from this group indicate that spray delivery of highly proliferative skin progenitor cells improves patient outcomes. During the last year, the Gerlach group developed an isolation protocol for adult and fetal epidermal and dermal progenitor cells. They have successfully established cell-sourcing logistics and collaborations. The group also finished SOPs for the isolation and cell culture methods for fetal skin progenitor cells, including basal keratinocytes, fibroblasts, and MSC-like cells, as well as for adult skin progenitor cells and dermal MSC-like cells. The milestone provision of a clone for July 2011 is described and illustrated in detail as follows.

A cell spray model has been adapted and used for in vitro studies, and the device has been used with epidermal progenitors and dermal MSC-like cells. In addition, this research has allowed the group to make a milestone decision to move forward with

adult autologous cells rather than fetal cells in the AFIRM 1 extension clinical study plans. This decision was made because research indicates that inclusion of the MSCs into the spray system would allow the project to extend from development of second-degree burn therapy to third-degree burn therapy. Finally, the 510(k) regulatory process for the skin gun device was initiated.

Fetal Skin Progenitor Cells

During the third year of AFIRM funding, the Gerlach group focused its efforts on improving skin cell progenitor culture and the characterization of several cell types to demonstrate the "stemness" of each as well as to obtain sufficient data on the suitability of fetal cells to be prepared as an off-the-shelf product. The appropriate immunological markers for this type of skin cell characterization, as well as a functional assay for stemness, were decided on after a thorough literature review.

The development of methods for isolation and expansion of fetal keratinocytes and fibroblasts, together with cryopreservation techniques, enable a system for cell banking and lay the foundation for both fetal cell types as an off-the-shelf product.

Fetal Epidermal Keratinocyte Expansion and Characterization

Methods for fetal keratinocyte proliferation have been improved by comparing and selecting various culture media. Studies using immunofluorescense analysis reveal the presence of cytokeratin 15 (CK15), a stemness marker that is only expressed by the epidermal stem cells on the basal membrane layer **(Figure V-10)**. The presence of ki-67, a proliferation marker, also confirms cell division activity even in the latest passages (Figure V-10). This confirms the feasibility of those cells for cell banking. The cytokeratin 5 marker, CK5, was used as a keratinocyte marker control.

Fetal Dermal Fibroblast Expansion and Characterization

Fetal dermal fibroblasts were highly expandable over several passages (up to passage 10). The isolated fibroblast population was investigated using the markers described previously to confirm these cells as MSCs according to the International Society for Cellular Therapy (ISCT) definition during 10 expanding generations. MSCs expressed CD105, CD90, and CD73 and did not express CD34, CD45, CD14 (or CD79a), and HLA-DR **(Figure V-11)**.

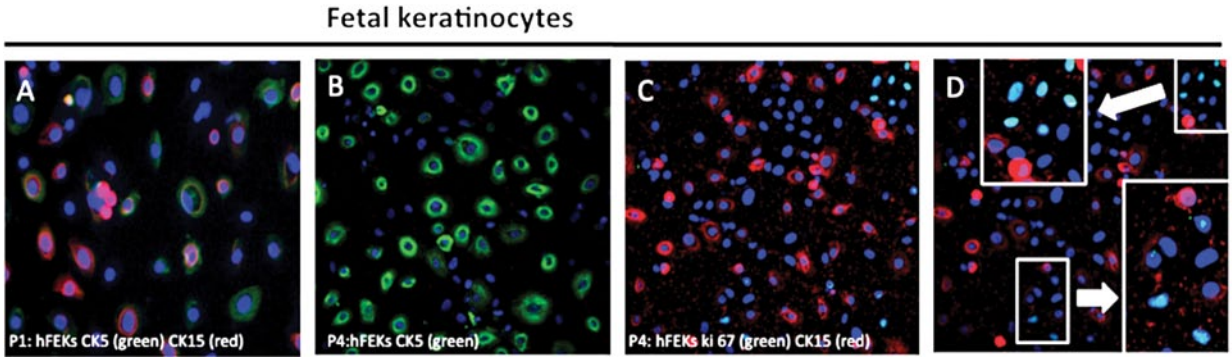

Figure V-10. Immunological staining of fetal epidermal cells. (A) Keratinocytes at early passage 1, showing keratinocyte antibody CK5 (green) and basal keratinocyte marker CK15 (red). (B) Keratinocytes at passage 4 showing the CK5 marker. (C) CK15 and proliferation marker ki-67 at passage 4. (D) Magnification details from panel C.

FACS markers comparison of fetal dermal fibroblasts at different passages.

	Fetal p3		Fetal p6		Adult p3	Adult p6
	#1	#2	#1	#2	#1	#1
IgG	0.1	0.1	0.1	0.2	0.2	0.1
CD34	7.4	0.1	0.9	0	8.3	1.1
CD45	0	0.1	1.6	1.1	2.6	2.6
CD14	5.2	0.1	0.1	0	3	0
HLA DR	0.1	0.1	1.2	1.3	0.5	1.8
HLA I Class	89	91	88.3	91.1	98	86.4
CD105	98	98.1	96.7	95.2	97	85.3
CD73	74	91.6	99.6	99.4	94	91.2
CD90	96	97	87.7	97.1	98	96.5
CD44	97	98	96.5	97.9	95	91.4
CD166	91	58	93.8	96.7	95	92.1
NESTIN	92.5	93	80	75.6	81	70.3
hTERT	94	97	89.4	98	92	83.2
VIMENTIN	73.5	97	99.4	98.1	66.9	98.2
Ki67	20	56	31.1	24.2	4.2	11.1
CD54	18.5	13.4	28	44.4	10.2	36.1
CD90/CD105	98.9	79.2	98.8	73	97.6	97.2
CD90/TERT	66	87.3	67	80	94.9	91

Main markers for mesenchymal stem cells (MSC) according to ISCT definition.

Negative marker	Positive marker	Control

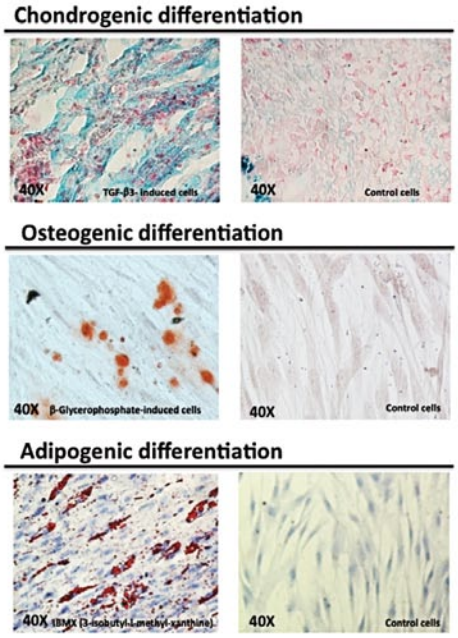

Figure V-11. Left: MSCs marker panel comparing fetal and adult fibroblasts. Right: Fetal dermal fibroblast under chondrogenic, osteogenic, and adipogenic differentiation medium comparing with their controls.

According to the ISCT definition, to classify a cell type as a stem cell, it must be confirmed that the cell population is able to differentiate into osteogenic, chondrogenic, and adipogenic cells after certain specified induction medium protocols. The group's results show that fetal dermal fibroblasts exhibit the capability to differentiate into these three cell types (**Figure V-12**, right panel). These results indicate that fetal dermal fibroblasts are a good candidate for anticipated use in third-degree burn wound therapy and that they could be used jointly with fetal keratinocytes for enhanced wound recovery. The raw dermal cell fraction that was isolated in this study contains at least 60% MSC-like cells, which appears to be sufficient as a starting point for cell clone generation and cell banking work.

Initial clonal growth of dermal MSC-like cells and epidermal progenitors has been completed and compared to adult skin cells; cell stability was tested up to 10 passages. The MSCs could be taken into culture, expanded, and then frozen/thawed to establish frozen off-the-shelf cell populations. Fetal fibroblasts showed high viability after thawing and follow-up culture. The results indicate that the C6295 cell-freezing medium from Sigma is the best freezing medium for this purpose, resulting in viabilities higher than 95% in most cases.

Cell Clone Generation

For cell clone generation, the researchers developed an innovative technique that benefits from their experience in embryonic cell culture methods (Figure V-12 and **Figure V-13**). Cells from the primary culture were isolated by trypsinization, and a few cells were placed in a Petri dish. After allowing some time for cell attachment, all cells except for one were removed mechanically from each plate using a stem cell knife. The success of this procedure could be controlled microscopically. The one remaining cell was then cultured, and clones were grown for several weeks until they expanded to sufficient quantities for freezing.

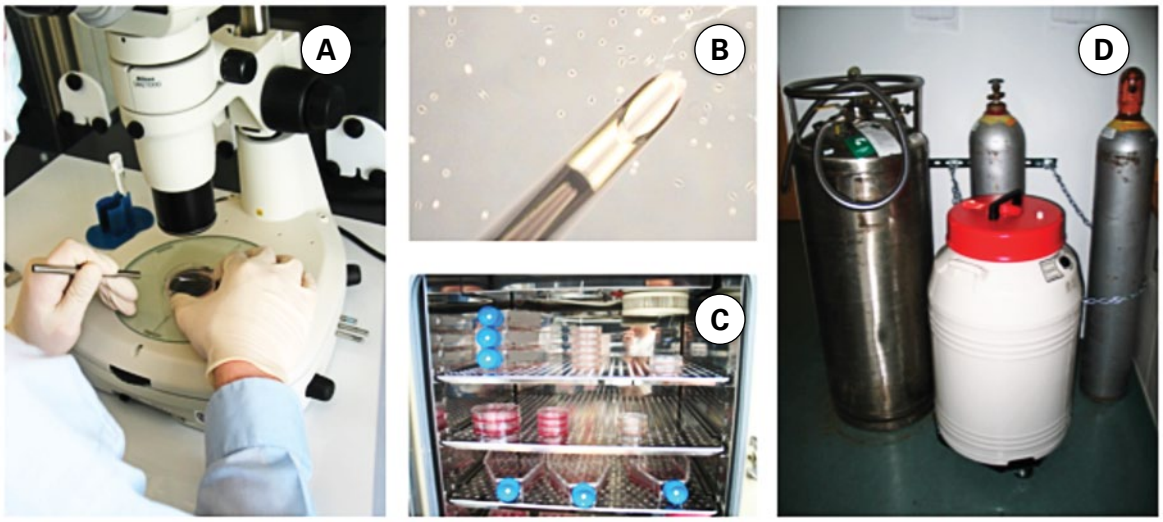

Figure V-12. Cell clone generation procedure. (A) Single-cell isolation under sterile conditions using equipment originally developed for embryonic stem cell techniques. (B) Stem cell knife used for removal of a single cell. Detail: 5x magnification. (C)Incubation process at 37°C with 5% CO_2. (D) Liquid nitrogen tank that contains frozen cells at -180°C.

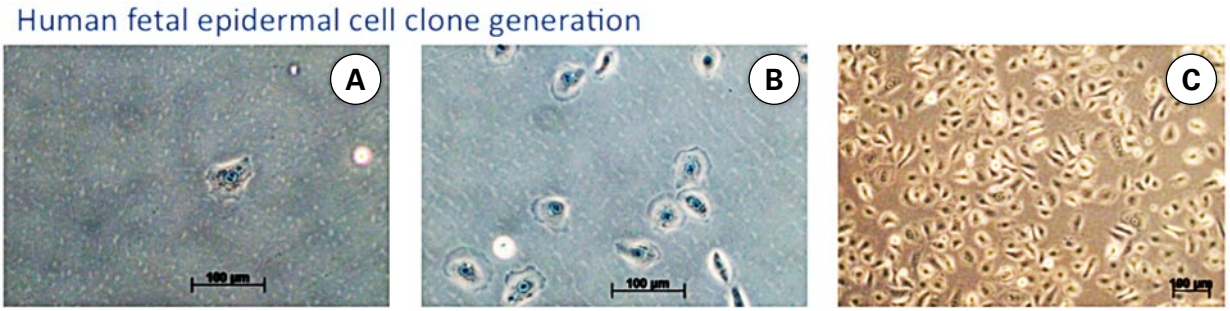

Figure V-13. Human fetal keratinocyte clone generation progenitor procedure. (A) Single fetal keratinocyte for clone generation (20x magnification). (B) Fetal keratinocyte clone expansion after 1 week (20x magnification). (C) Follow-up keratinocyte expansion example (10x magnification).

Additional Studies on Adult Skin Progenitor Cells for Autologous Therapy

Adult skin cultures were included in the program as well. Using autologous epidermal cells, the group explored the possibility of using off-the-shelf allogeneic dermal fetal cells combined with autologous epidermal cells for therapy. The current hypothesis is that using allogeneic dermal cells in combination with autologous epidermal cells could increase the engraftment rate in burn-injured patients when autologous keratinocytes are to be used during an on-site isolation procedure. Using both cell populations in combination could be advantageous in terms of immunologic tolerance and could also help to avoid engraftment rejection in third-degree burn patients.

Adult Epidermal Keratinocytes Characterization

The previous studies from the Gerlach group used adult keratinocytes for second-degree burn wound recovery. The adult cell isolation SOP revealed that adult keratinocytes show the same stemness markers as fetal keratinocytes (see **Figure V-14** and compare with Figure V-10). These studies also confirm that

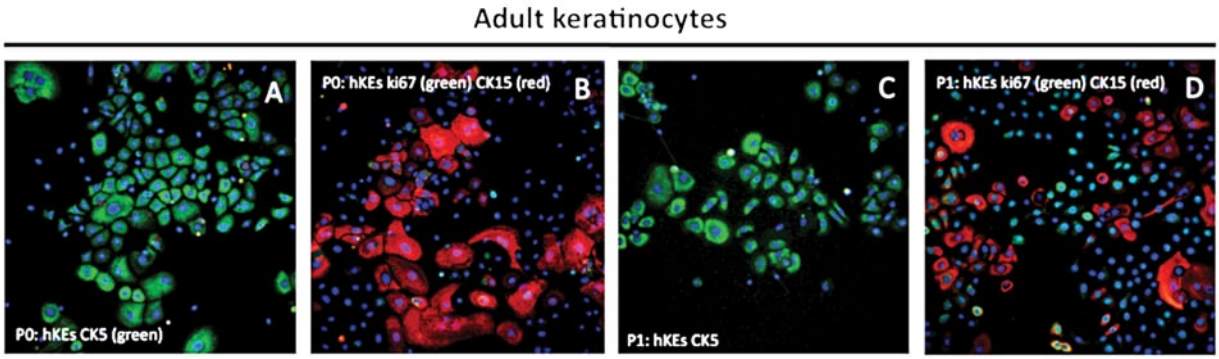

Figure V-14. Immunofluorescense staining on adult keratinocytes comparing passage 0 and passage 1. (A) Adult epidermal keratinocytes after isolation showing the keratinocyte marker CK5 in green and nuclear staining (DAPI) in blue. (B) The same cells express the proliferation marker ki-67 in green and the stemness marker CK15 in red. (C) Adult epidermal keratinocytes at passage 1 expressing CK5. (D) The same cells expressing ki-67 and CK15.

after isolation of epidermal keratinocytes, these cells showed regular proliferation rates, meaning the cells have a capacity for growth within a wound. The studies resulted in specific SOPs for the isolation of large numbers of epidermal progenitor cells from the basal membrane.

Characterization of Adult Dermal Fibroblasts

Working on enzymatic techniques led to methods for adult MSC-like cell isolation. These cells express the MSC markers as proposed by the ISCT **(Figure V-15)**. After culture and under differentiation conditions, the adult dermal cells showed adipogenic and chondrogenic differentiation. Osteogenic differentiation tests are also planned.

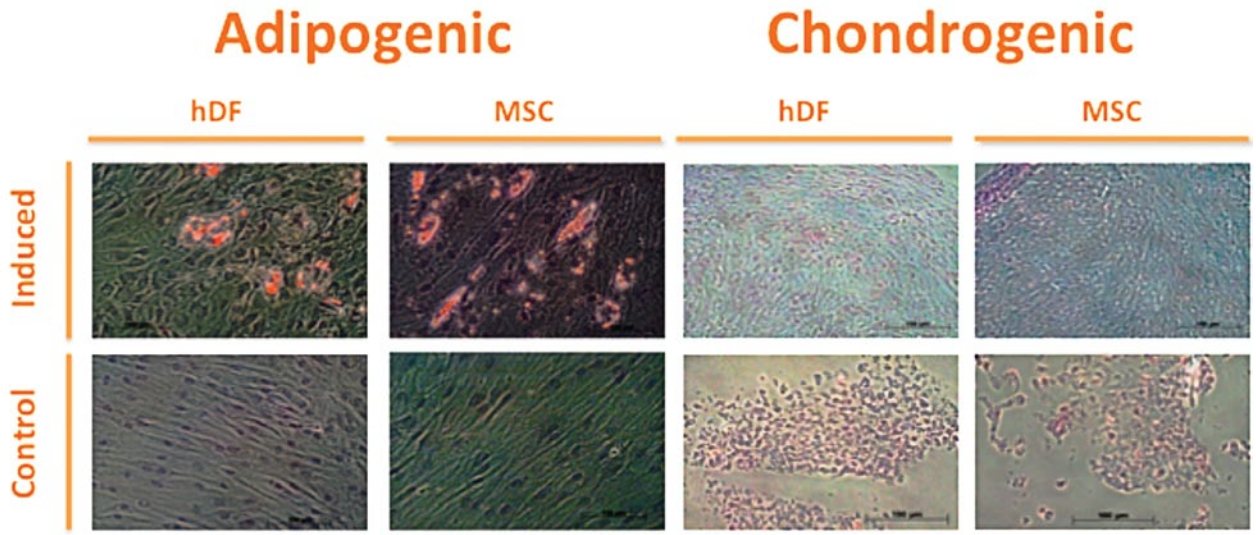

Figure V-15. Adipogenic and chondrogenic differentiation studies with adult fibroblasts at passage 2 and MSC at passage 1 as a control (32x magnification).

New Skin Gun Device Prototype
In collaboration with Stem Cell Systems, Berlin, Germany, a new prototype of the skin gun was evaluated and approved **(Figure V-16)**. Earlier results of the experimental in vitro cell spray deposition method were reevaluated. This is of importance for the cell deposition aspects of the project. These experiments were performed on test paper using the same amount of trypan blue dye on comparable surface areas to determine the relative number of cells that survived the spraying process. In addition, the group finalized in vitro evaluations of cell spraying with the skin gun using the developed experimental "test wound" in Petri dishes, and the results showed that there was no significant difference in cell survival between cells dripped with a pipette or deposited by the skin gun. These positive results confirm the group's earlier work on adult keratinocytes and suggest that the skin gun does not expose the cells to greater injury than conventional and routine laboratory pipetting.

The Gerlach group (collaborating with Stem Cell Systems, which designed the new skin gun prototype) initiated the 510(k) regulatory FDA approval process based on a device with disposable parts and an improved design.

Milestone Decision
In the previous project year, the group proposed to make a milestone decision on whether to move forward with either homologous progenitor cells or autologous progenitor cells for the first AFIRM 1 clinical study. It has been decided that the research with adult epidermal and dermal skin cells will be moved forward into the planned AFIRM 1 clinical study. However, the group is prepared to work on fetal dermal and epidermal cells in the anticipated AFIRM 2 project.

Key Research Accomplishments

- Established methods for isolating, expanding, and freezing fetal epidermal basal progenitor cells and fetal dermal MSC-like cells from fetal human skin.
- Established methods for freezing fetal epidermal basal progenitor cells and fetal dermal MSC-like cells from human skin.
- Demonstrated basal keratinocyte characteristics of fetal epidermal cells to use as an off-the-shelf product for second-degree burn healing.
- Devised a method to use fetal dermal fibroblasts jointly with fetal epidermal basal keratinocytes as an off-the-shelf product in third-burn degree healing.
- Designed an in vitro cell spray model that works with epidermal progenitors and dermal stem cells, and performed spray parameter tests to achieve maximum viability after spraying.

Figure V-16. Prototype of the new skin gun generation. (A) Skin gun device. (B) Detail of the spraying system where a needle tip meets an air tube. (C) Complete spraying system including the air compressor.

Conclusions

The researchers' results demonstrate that skin progenitor cells derived from human fetal skin tissue may provide an interesting new cell source for regenerative cell-based therapies for acute and chronic skin disease and the treatment of burn patients. These studies describe the preliminary isolation, characterization, and application methods for both fetal dermal fibroblasts and fetal epidermal keratinocyte progenitor cells.

Overall, development of the isolation techniques for the key cells of this project plan has been accomplished. In addition, an in vitro cell spray model has been established and cell spray parameters have been optimized.

Research Plans for the Next 2 Years

Further cell characterization work and investigations to optimize the methods for freezing and cell banking are ongoing.

The group proposes that cryopreserved, cell-banked fetal cells could be used as an immediately available, out-of-freezer therapy for burn injury. The group also recommends further studies on the human leukocyte antigen immunological aspects of fetal cells.

Planned Clinical Transitions

The group is ready to move the project into clinical trial preparations using autologous cells with a cell spray device to deliver progenitor cells onto burn wounds. The group entered into collaboration with the University of Pittsburgh Medical Center Mercy Hospital burn unit, which will assist with the development of the clinical trial concept. Clinical translation will be performed in collaboration with Dr. James Holmes at Wake Forest University. The knowledge gained in these studies will contribute to the field of skin stem cell research.

Artificial Extracellular Matrix Proteins for Regenerative Medicine

Project 4.2.4, WFPC

Team Leader(s): David Tirrell, PhD (California Institute of Technology)

Project Team Members: Phoebe Tzou, PhD and Eileen Fong, BS (California Institute of Technology)

Collaborator(s): Mark Van Dyke, PhD (Wake Forest University School of Medicine)

Therapy: Burn therapy.

Deliverable(s): Optimized aECM proteins for clinical burn repair.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 2; End Year 3, TRL 2

Key Accomplishments: The researchers have been working toward developing a thorough understanding of the mechanisms of wound healing on aECM proteins. They determined—both experimentally and by modeling—that the presence of aECM proteins, myosin activity, and wound geometry, but not wound size, are primary determinants of the spacing between adjacent leader-cell groups in their model of wound healing on aECM proteins. The research team prepared a set of 12 aECM films for implantation in the pig burn model. Animal trials, performed in collaboration with the Van Dyke laboratory, have begun ahead of schedule.

Key Words: Burn repair, tissue scaffolds, wound healing, extracellular matrix proteins, protein engineering

Introduction

aECM proteins are designed by combining elements drawn from natural ECM, including proteins such as fibronectin, collagen, laminin, keratin, and elastin. The needed elements are encoded into artificial genes, and the corresponding proteins are expressed in bacterial cells. The modularity of the gene design allows rapid and systematic variation in mechanical and biological properties and in the rate of protein degradation by proteolytic or hydrolytic processes. Matrices can therefore be optimized individually for regenerative therapies with distinct performance requirements. Under the auspices of the AFIRM, the Tirrell laboratory is exploring variations in ECM protein design to optimize matrices for burn repair and, ultimately, for other regenerative therapies. The specific aims for the project are as follows:

Specific Aim 1. Design and expression of optimized aECM proteins. Optimization parameters include density and identity of cell adhesion ligands and cross-link density.

Specific Aim 2. Preparation of matrix constructs for wound-healing studies in vitro. Constructs include adsorbed films, spin-coated films, and porous matrices.

Specific Aim 3. Determination of rates of wound healing on aECM matrices.

Specific Aim 4. Evaluation of aECM proteins in animal models of burn repair.

Studies conducted during the project's 5-year period are intended to position the laboratory for evaluation of aECM proteins for use in clinical burn repair.

Summary of Research Completed in Years 1 and 2

In year 1, the researchers focused on the expression of a new class of aECM proteins containing full-length cell adhesion domains derived from fibronectin. They also prepared the first generation of thin-film matrix constructs to be used for aECM protein evaluations. In year 2, the researchers discovered that this new class of proteins supports more rapid wound healing in vitro than any previous constructs. They developed a mechanistic model to explain the origin of the increase in wound-healing rates. The researchers' success in improving expression yields of epitope-free aECM proteins positioned their laboratory to begin animal trials.

Research Progress – Year 3

The primary effort during the past year was devoted to preparing aECM films for implantation in the pig burn model in collaboration with the Van Dyke laboratory at Wake Forest University. A set of 12 circular films, 30 cm in diameter, was prepared at the California Institute of Technology; a representative sample is shown in **Figure V-17**. These samples were transferred to Wake Forest University in January 2011. Implantation studies began in March 2011; analysis of the results is under way.

Figure V-17. aECM protein film prepared for implantation in the pig burn model.

A second effort was directed toward developing a more thorough understanding of the mechanisms of wound healing on aECM proteins. Two mechanisms have been observed—the "purse-string" and

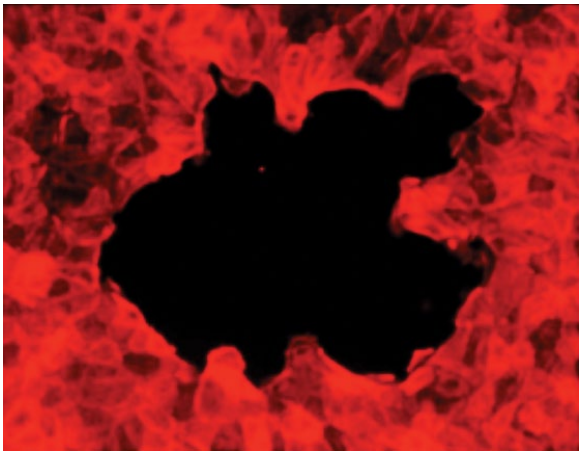

Figure V-18. Leader cells in MDCK cell monolayers cultured on aECM protein.

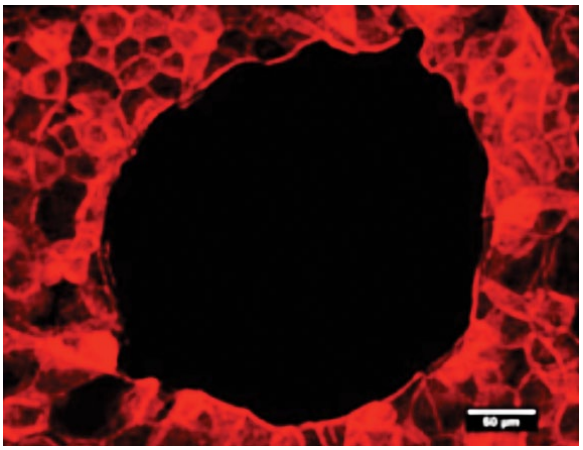

Figure V-19. MDCK cell monolayers cultured on glass control substrate. Note absence of leader cells.

"lamellipodial crawling" processes. **Figure V-18** shows the development of clearly defined "leader cells" in Madin-Darby canine kidney (MDCK) cell monolayers cultured on aECM proteins. The high adhesivity of the surface—a consequence of inclusion of fibronectin-derived cell adhesion ligands in the aECM protein sequence—promotes the formation of leader cells as the circular wound begins to close. In contrast, few leader cells are observed on the glass control surface, which presents no cell adhesion ligands **(Figure V-19)**. Wound healing on aECM proteins generates patterns characterized by periodic interspersing of leader-cell groups within purse-string groups. Careful study showed, both experimentally and by modeling, that the presence of aECM proteins, the myosin activity, and the wound geometry, but not the wound size, are primary determinants of the spacing between adjacent leader-cell groups. Inhibiting myosin activity or increasing the convexity of the wound edge reduces the spacing whereas the absence of aECM prohibits the formation of leader-cell groups.

In a related study of the wound-healing process, the Tirrell laboratory reported previously that epithelial monolayers heal approximately sixfold more rapidly on aECM surfaces that present high densities of arginine-glycine-aspartic acid (RGD) cell adhesion ligands than on surfaces of lower RGD density. It was proposed that the increase in wound-closure rate may be a consequence of an increased rate of boundary crossing when the leader cells in the monolayer encounter highly adhesive test surfaces. Tirrell and coworkers have now measured such boundary-crossing rates directly and confirmed that earlier conjecture. **Figure V-20** shows the results of measurements on five surfaces of increasing ligand density. In each case, the researchers counted the number of cells in the wound area that crossed the wound boundary during a 30-hour experiment. The number of

such cells increases roughly fivefold as the adhesivity of the surface increases. **Figure V-21** shows the results of a dynamic Monte Carlo simulation of the boundary-crossing process and illustrates the good agreement between simulation and experiment. Tirrell and colleagues also explored the possibility that faster wound healing may be a consequence of faster cell proliferation on more highly adhesive surfaces. The results showed conclusively that this is not the case; the rate of proliferation varies little from one surface to another and is too slow on all surfaces to account for a substantial fraction of the observed wound-healing response.

A third effort was focused on expression of keratin-based aECM proteins. Two sequences of interest to the Van Dyke laboratory were cloned and expressed in bacterial cells. Initial trials yielded low levels of expression. An alternative design was evaluated in which keratin sequences were flanked by elastin domains that are known to express well. The alternative design afforded improved expression, but yields have not yet reached the levels needed for preparation of film samples for implantation in the pig burn model.

Key Research Accomplishments

- Prepared a set of 12 aECM films for implantation in the pig burn model.
- Developed a mathematical model that describes the roles of aECM proteins, myosin activity, and wound geometry in determining the mechanism of wound healing in MDCK cell monolayers.

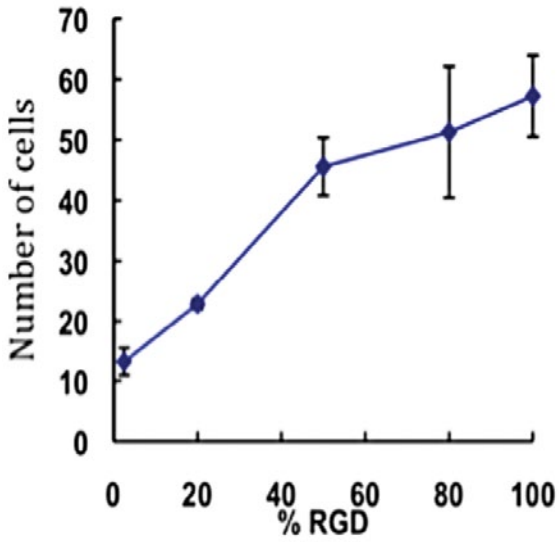

Figure V-20. Number of cells in the wound area that crossed the boundary during a 30-hour experiment plotted as a function of the percentage of adhesive protein in the aECM substrate film.

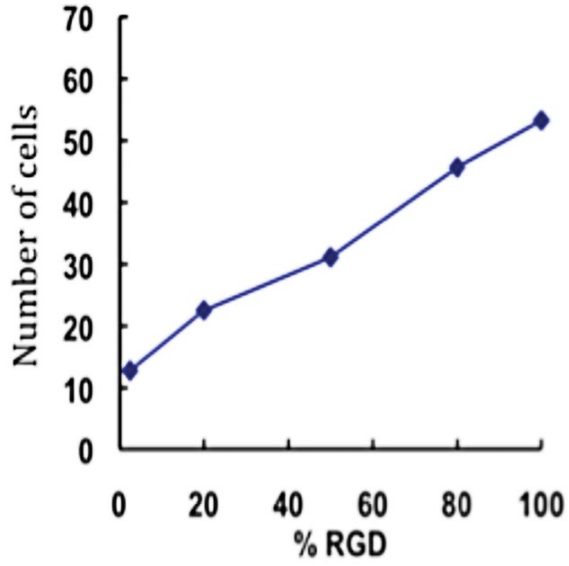

Figure V-21. Dynamic Monte Carlo simulation of the boundary-crossing process showing the number of cells in the wound area predicted to cross the boundary during a 30-hour experiment.

Conclusions

The researchers have been making progress toward elucidating the mechanisms of wound healing on aECM proteins. They determined, both experimentally and by modeling, that the presence of aECM proteins, myosin activity, and wound geometry, but not wound size, are primary determinants of the spacing between adjacent leader-cell groups in their model of wound healing on aECM proteins.

Research Plans for the Next 2 Years

This project as supported by AFIRM will conclude on December 31, 2011.


our science for their healing

In Situ Bioprinting of Skin for Battlefield Burn Injuries

Project 4.2.5, WFPC

Team Leader(s): James Yoo, MD, PhD (Wake Forest University)

Project Team Members: John Jackson, PhD, Kyle W. Binder, PhD, Weixin Zhao, MD, Dennis Dice, PhD, Josh Tan, MS, and Hyun-Wook Kang, PhD (Wake Forest University)

Collaborator(s): Lexmark, Inc. and Organogenesis, Inc.

Therapy: Burn repair.

Deliverable(s): Skin bioprinter.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 3; End Year 3, TRL 4

Key Accomplishments: In the past year, the researchers performed experiments in a large animal model (pig) using in situ skin bioprinting technology. They were successful in isolating and expanding dermal fibroblast and keratinocytes from porcine skin. The isolated and expanded cells remained viable when delivered through the printer nozzles. The printed cells participated in skin tissue formation and full-thickness wound repair.

Key Words: Wound, burn, skin, bioprinting, autologous cell transplantation, allogeneic transplantation

Introduction

Burns from combat explosions are increasing in size, frequency, and severity. The mortality rate increases significantly with increasing TBSA burned; therefore, soldiers with extensive burns must be treated immediately for an increased chance of survival. The current standard for replacement of skin defects is autologous skin grafts; however, donor tissue site availability is a limitation. Commercially available skin products are limited in size and some require a lengthy preparation time, making them unusable in severe cases that require prompt and aggressive measures to maintain the lives of wounded patients. Moreover, patient survival is inversely proportional to the amount of time required to cover and stabilize a wound. Therefore, a new approach that permits immediate burn wound stabilization with functional recovery is necessary. The researchers propose a novel treatment that would repair burn wounds in situ by using cartridge-based bioprinting to precisely deliver skin cells in a controlled manner to a wound.

The skin bioprinter uses a cartridge-based delivery system with a laser scanning system mounted on a portable XYZ plotting system. The cartridge system is similar to that used in traditional inkjet printing such that each cell type is loaded into an individual cartridge in the same way different colored inks would be contained in different cartridges. Data obtained from the laser scanner are pieced together to form a model of the wound surface. Together, these technologies print skin

that can match the skin that is missing from the wound.

This report describes the design and use of a novel delivery system for in situ bioprinting of the skin. The cartridge-based system can be easily transported from patient to patient and can rapidly print skin constructs consisting of any cell type or biomaterial that can be packaged into a compatible cartridge. The researchers successfully regenerated skin in a porcine wound model using fibroblasts and keratinocytes, demonstrating that the concept of in situ skin bioprinting is a viable technique. This work represents an important step forward in burn care for both the civilian and military populations.

Summary of Research Completed in Years 1 and 2

To demonstrate the feasibility of in situ skin printing, the skin bioprinter was used to bioprint human fibroblasts and keratinocytes directly in a nude mouse wound model. Wounds repaired using in situ skin cell bioprinting demonstrated that wounds treated with the printer closed up to 3 weeks faster than the negative controls. Printed skin cells required approximately 10–14 days to organize into skin, which is consistent with previous experiments using cell-spraying techniques. Complete closure of the wounds by 3 weeks was confirmed using several assays to indicate organization of the skin cells, organized dermal collagen, and a fully formed epidermis. Histological analysis demonstrated the presence of human skin cells in the dermis and epidermis of the new skin.

Based on the results of the murine experiment, the skin bioprinter was scaled up for preclinical studies in a porcine wound model. Full-thickness excisional wounds made on the dorsa were imaged with the laser scanning system, and the wound map was generated. This was followed by skin defect repair with the printer using autologous or allogeneic fibroblasts and keratinocytes. Bioprinted fibroblasts and keratinocytes were able to close the wound more quickly than the controls without cells. Autologous keratinocytes showed evidence of re-epithelialization in the wound center at 2 weeks post-printing. These areas of epithelialization progressively increased until the entire wound was covered. Fibroblasts and keratinocytes that had been labeled with fluorescent markers were visible in the center of the wound at 8 weeks after printing directly on the wound.

Research Progress – Year 3

Bioprinting of Full-Thickness Skin Using Skin Cells and Dermal Matrices

Animal Model

This experiment was performed in a porcine wound model because porcine skin is similar to human skin. The bioprinting procedure was divided into two stages. In the first stage, skin from the shoulder area was removed using a dermatome for preparation of fibroblasts and keratinocytes for bioprinting. After expanding these cells in culture, four full-thickness 10 × 10 cm^2 wounds were excised through the panniculus carnosus layer of the thoracic dorsa on the animals **(Figure V-22)**.

Each wound received a different treatment: no treatment, fibrin/type I collagen only, allogeneic fibroblasts and keratinocytes embedded in fibrin/type I collagen, or autologous fibroblasts and keratinocytes embedded in fibrin/type I collagen. Wounds were bandaged with nonadherent Telfa bandages, sterile gauze, Ioban™ adhesive wound dressing, and Coban™ elastic wrap. Wound size, contracture, and re-epithelialization were measured weekly using a digital camera at a

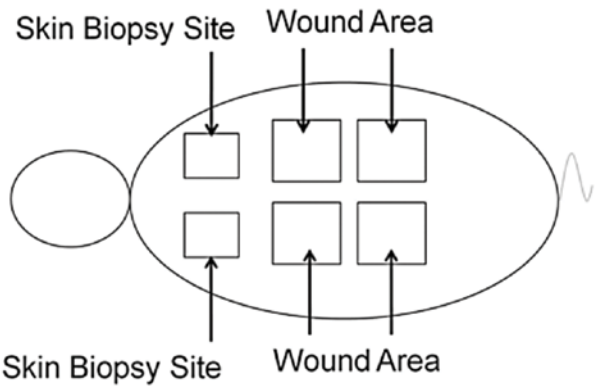

Figure V-22. Schematic representation of wound sites in the preclinical model. Skin was first removed from the area just above the shoulder using a dermatome (skin biopsy site). One site was used to gather cells with the second site remaining untouched in the event that more cells needed to be harvested. After skin cells were prepared for bioprinting, four areas of thoracic dorsal skin measuring 10 × 10 cm^2 were excised through the panniculus carnosus layer (wound area).

fixed focal length. Bandages were changed weekly, and animals were monitored daily for signs of infection and damage to the bandages.

Cell Preparation

Epidermal keratinocytes and fibroblasts obtained from enzyme-dissociated skin tissues were cultured and expanded to create a sufficient number of cells for the bioprinting process. Prior to bioprinting, cells were prelabeled with Molecular Probes® CM-DiI. DiI, CM-DiI, DiO, and DiD are lipophilic carbocyanine membrane intercalators. CM-DiI was chosen for this study because it does not photobleach and is stable in cell membranes through aldehyde fixation by incorporation of a chloromethyl substitute. Cell suspensions were centrifuged at 1,500 rpm for 5 minutes to obtain a cell pellet. Simultaneously, bovine plasma fibrinogen type I-S was prepared at 50 mg/mL in phosphate-buffered saline (PBS), and rat tail type I collagen was prepared at 2 mg/mL in PBS. Equal amounts of fibrinogen and type I collagen were combined to create a hydrogel solution with final concentrations of 25 mg/mL fibrinogen and 1 mg/mL type I collagen. Cell pellets for one cell type (e.g., autologous fibroblasts) were combined into one cartridge, and the final pellet was resuspended in 12 mL fibrinogen/type I collagen. Thrombin extracted from bovine plasma was prepared at 50 IU/mL in PBS.

Wound Creation and Bioprinting Procedure

For the creation of the defect, four skin wounds were created by removing 10 × 10 cm of full thickness skin in the central back along the thoracic and lumbar area. Subsequently, the wound area was placed under a portable bioprinter, and dermal cells and matrices were directly bioprinted onto the wound bed. One layer of 10 million fibroblasts (1.0 × 10^5 cells/cm^2) was bioprinted at

2 mm intervals between drops with thrombin sprayed from the atomizing nozzles simultaneously to form a fibrin/type I collagen hydrogel. Fifteen minutes was allotted to allow complete conversion of fibrinogen to fibrin. One layer of 10 million keratinocytes was bioprinted above the fibroblast layer. The wounds were covered with topical antibiotic cream, Telfa nonadherent bandages, loban, elastic bandages, and stockinettes.

Wound Healing Analysis

Wounds were imaged weekly at a fixed focal distance during bandage changes. Wound size, contracture, and re-epithelialization were evaluated using ImageJ software. Statistical analysis was performed in the S-Plus statistical analysis package using one-way analysis of variance (ANOVA) followed by Tukey's range test. Full-thickness skin wounds repaired by bioprinting of 10 million autologous fibroblasts and 10 million autologous keratinocytes showed complete re-epithelialization at 8 weeks. These skin constructs showed formation of epithelium in the center of the wound at 2 weeks **(Figure V-23)**. Keratinocytes in these skin buds proliferated

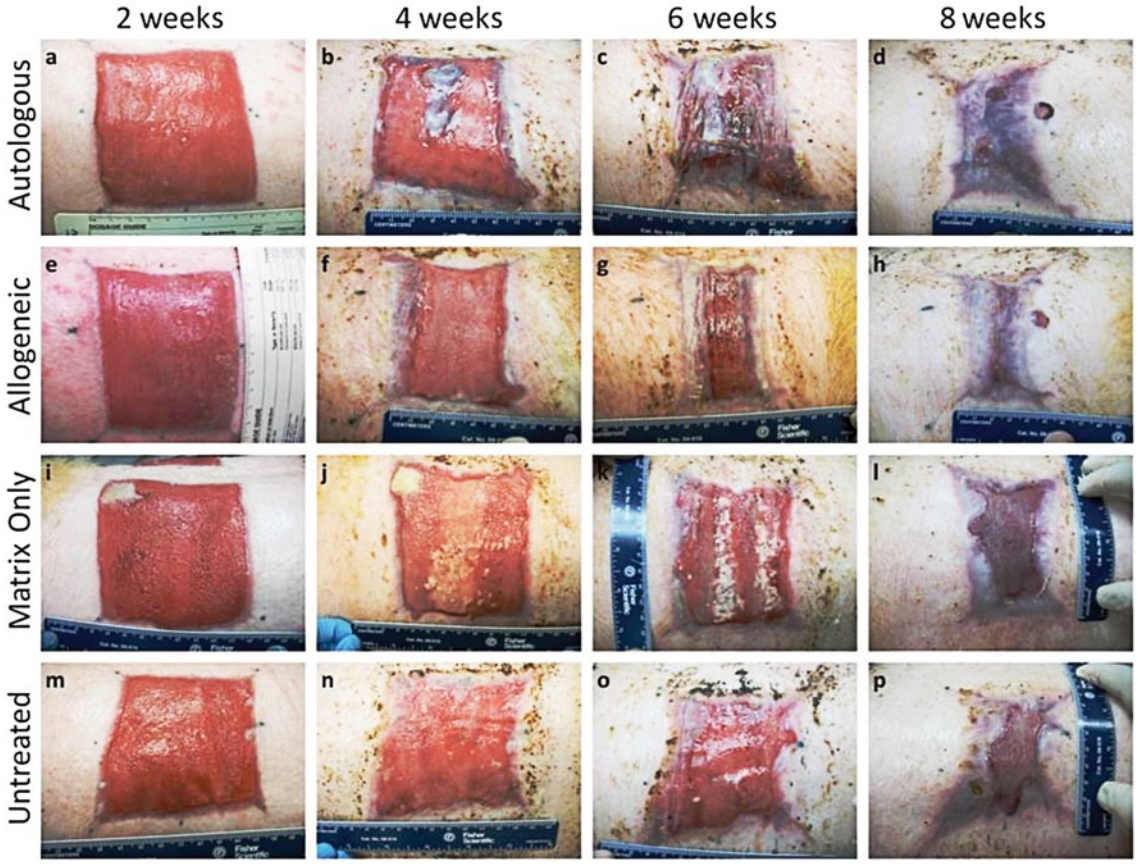

Figure V-23. Comparison of four different wound treatments over 8 weeks. Treatment groups: autologous fibroblasts and keratinocytes, allogeneic fibroblasts and keratinocytes, fibrin/type I collagen alone, and no treatment. (a-d) Circles of epithelialization are present in the centers of the wounds treated with autologous fibroblasts and keratinocytes. These areas increased in size over 8 weeks to cover the entire wound area. (e-h) Treatment with allogeneic cells showed no statistically significant difference in epithelialization over negative controls; however, prelabeled allogeneic fibroblasts and keratinocytes were present in the wound. (i-p) Wounds treated with fibrin/type I collagen alone and wounds that received no treatment did not achieve full epithelialization at 8 weeks.

over the course of 8 weeks to completely cover the wound. The formation of proliferative epithelium in the center of the wound on gross analysis demonstrates in a large animal model that bioprinted keratinocytes survive in a full-thickness skin wound and can form skin tissue. Furthermore, the epithelium from the bioprinted keratinocytes connected to the advancing wound edge. Future experiments will examine the effects of using additional fibroblasts and keratinocytes on the reduction of healing time.

However, similar results were not seen with bioprinted allogeneic fibroblasts and keratinocytes. New epithelium was not evident in the wound center nor did the wound edge advance faster than the negative controls. Neither the fibrin/type I collagen negative control nor the untreated control healed at 8 weeks. One fibrin/collagen control wound increased slightly in size at 1 week and one untreated wound greatly contracted at 4 weeks, resulting in wound size data that were skewed high for the fibrin/collagen group and skewed low for the untreated group. These effects are pronounced due to the low sample size. Wound size is a measure of the advancing tissue from the undamaged surrounding skin. No statistically significant differences were detected in the wound sizes with the exception of the 4-week data; however, the power of this study was not high enough to avoid type II errors **(Figure V-24)**. The increase in wound size in the one fibrin/collagen control wound is likely due to confounding effects resulting from the animal damaging the wound edge. Without that outlier, there are no statistically significant differences in the wound sizes at any time points.

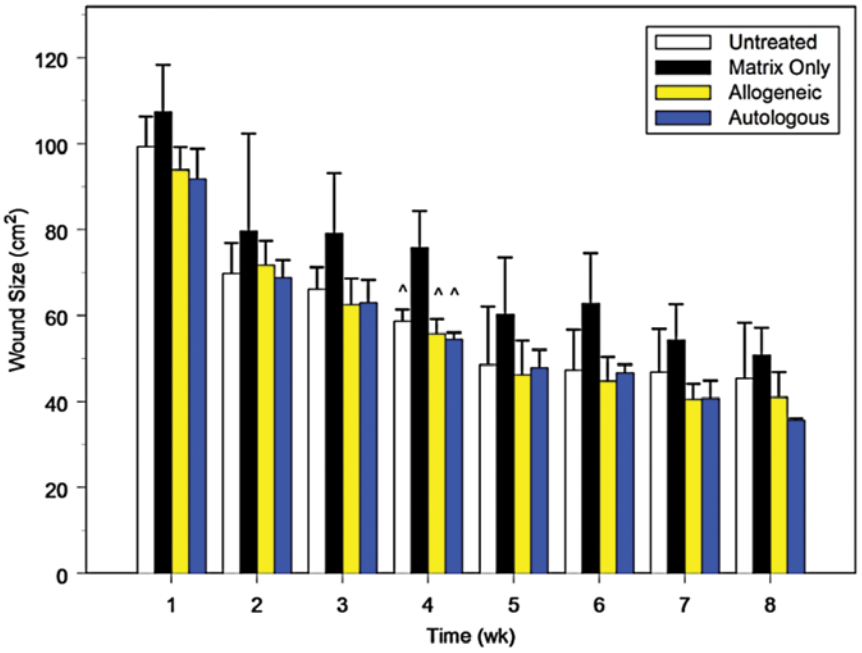

Figure V-24. Analysis of wound sizes over 8 weeks. Wound size was measured using ImageJ software to evaluate the total area encompassed by the wound edges including new epithelialization. The initial wound size was 100 cm^2. Data were analyzed by one-way ANOVA followed by Tukey's range test on statistically significant ANOVA tests. Significance was defined as $p < 0.05$, n = 3. ^: significant versus fibrin/type I collagen (matrix only) negative control. Wound size was not significantly different across treatment groups with the exception of the matrix-only group at 4 weeks. Overall, wound size was not significantly affected by treatment with skin cells. This expected result indicates that bioprinting 10 million fibroblasts and 10 million keratinocytes did not significantly affect advancement of the wound edge from the surrounding normal tissue. Data presented as mean + standard deviation.

This result indicates that bioprinting 10 million fibroblasts and 10 million keratinocytes do not affect healing from the surrounding normal skin on the wound edge. There are two possible explanations for this result. First, the bioprinted fibroblasts and keratinocytes may not affect proliferation of the wound edge. This is unlikely because of the wide array of growth factors and cytokines produced by skin cells in a wound bed. Second, the number of bioprinted cells may have been too low to affect the surrounding tissue. This explanation is most likely given the 6–8 weeks required to fully epithelialize the wound with autologous cells in this experiment. If the bioprinted cells healed the wounds faster, then the cells from the wound edge would not need to proliferate into the wound. Future experiments will use more fibroblasts and keratinocytes to examine the effect of cell number on proliferation of the wound edge.

Wound contracture was examined by measuring the area enclosed by the tattoo marks and expressing this value as a percentage of the initial area. This is a different measurement from the wound size since the normal tissue can proliferate into the wound, reducing the wound size while having no effect on scar contraction. Overall, no statistically significant differences were detected in wound contracture among the four wound treatments. This result was expected given the results of the gross analysis. Ideally, treatment with bioprinted fibroblasts and keratinocytes would heal the wound quickly enough that the wound edges would not contract. Future experiments will examine the effects of a higher number of bioprinted fibroblasts and keratinocytes on wound contracture.

In contrast to the wound size and contracture, wound epithelialization was significantly higher when treated with bioprinted autologous fibroblasts and keratinocytes. Epithelialization was examined by using ImageJ to measure the areas of epithelial tissue visible on gross images including the epithelium from the advancing wound edge. These results confirmed the gross analysis, showing that bioprinting of skin cells is a viable method of producing new skin for extensive wounds.

Histological Analysis

Histological samples taken from the centers of the wounds at 8 weeks showed fully formed dermis and epidermis in most cases with the exception of the fibrin/type I collagen-treated wounds **(Figure V-25)**. These findings are consistent with the gross analysis. Dermal papillae and rete ridges were visible, indicating that the bioprinted fibroblasts and keratinocytes were able to produce the proper skin structure for this area of the body. Prelabeled autologous fibroblasts and keratinocytes were visible by fluorescence analysis with DAPI counterstaining **(Figure V-26)**, indicating that the bioprinted skin cells were responsible for the re-epithelialization seen on gross analysis. Allogeneic keratinocytes were not visible although allogeneic fibroblasts could be seen. Again, this finding is consistent with the gross results, which showed that the wound treated with allogeneic fibroblasts and keratinocytes did not develop statistically significant re-epithelialization over negative controls. There are three possible explanations for this finding. First, the CM-DiI in the prelabeled allogeneic keratinocytes may have diluted to levels not visible by fluorescence imaging due to cell division. This is unlikely considering the relative lack of re-epithelialization and the visibility of prelabeled autologous keratinocytes. Second, the number of bioprinted skin cells may have been too low to create skin from allogeneic cells. Finally, cultures of keratinocytes may have been contaminated with antigen-presenting cells from the epidermis. Although allogeneic fibroblasts and keratinocytes can be used to repair skin wounds, the presence

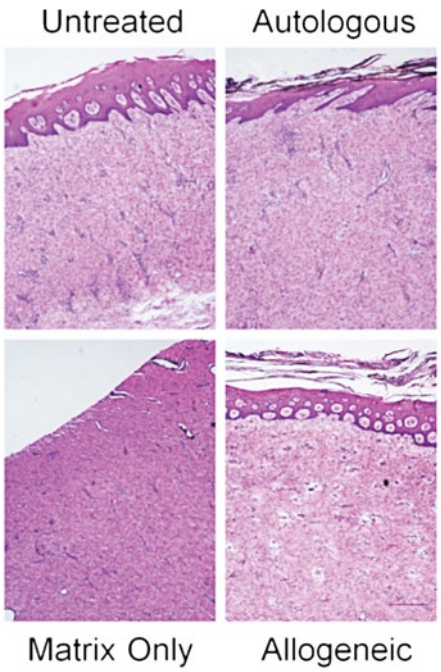

Figure V-25. H&E evaluation of four different wound treatments at 8 weeks. H&E stain of biopsies taken from the wound center show the presence of fully formed epithelial tissue in three of four treatments. The fibrin/type I collagen treatment (matrix-only) did not show epithelium formation, corroborating the gross analysis. Magnification: 50×. Scale bar: 50 μm. Untreated: normal skin.

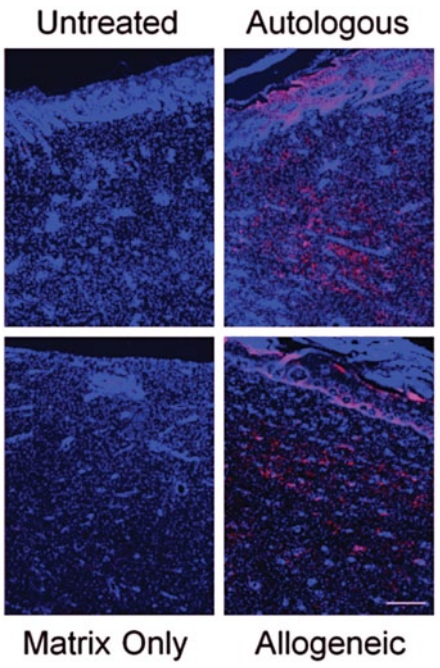

Figure V-26. Histological comparison of four different wound treatments at 8 weeks. Histological sections were counterstained with DAPI, a compound that intercalates in DNA and fluoresces blue. Bioprinted fibroblasts and keratinocytes were prelabeled with red fluorescent CM-DiI. These prelabeled cells are visible in the center of the wound for the autologous treatment. Only prelabeled fibroblasts are visible in the allogeneic treatment. Magnification: 50×. Scale bar: 50 μm. Untreated: normal skin.

of antigen-presenting cells would induce an immune response to these cells. Fibroblasts are weakly immunogenic due to the lack of CD28 ligand and would not be eliminated by the host immune response as quickly as the keratinocytes. Lack of re-epithelialization with bioprinted allogeneic fibroblasts and keratinocytes is likely due to a combination of too few cells and destruction of the cells that were present. However, treatment with autologous skin cells was able to close the wound with full re-epithelialization from bioprinted cells.

Summary

The goal of this project was to create a skin bioprinter capable of repairing wounds in a preclinical porcine model by using a scanning system to image the wound and then bioprint the missing skin cells based on this image. The skin bioprinter achieved most of the design goals established by the research team. It is a portable system capable of accommodating a wide range of body types and can precisely deliver up to eight different cell types in situ. The system can be easily sterilized, used repeatedly, and is easy to maintain in working condition. However, the imaging system did not perform to expectations during in vivo testing due to the breathing motion of the patient. This issue will be corrected by replacing the laser scanner with an imaging system that can take instantaneous snapshots of the wound and create the wound map. Wounds repaired

with autologous fibroblasts and keratinocytes demonstrated increased epithelialization over all other treatments although no statistically significant differences in wound size or contracture were detected. However, the power of this study is too low to avoid type II errors. Prelabeled autologous fibroblasts and keratinocytes as well as allogeneic fibroblasts were visible in the wound at 8 weeks, demonstrating that bioprinted skin cells can survive, proliferate, and form skin tissue in an immunocompetent large wound model. Although wounds were successfully repaired with bioprinted skin cells, significant optimization is needed before in situ skin bioprinting can be a viable technique for repair of human wounds.

Key Research Accomplishments

- Isolated and expanded dermal fibroblasts and keratinocytes from porcine skin.
- Delivered two different skin cell types onto a wound in a porcine model.
- Demonstrated that skin cells delivered through the printer nozzles remain viable.
- Demonstrated that skin tissue forms after bioprinting, and this leads to full-thickness wound repair.

Conclusions

The researchers have demonstrated that in situ skin bioprinting is a viable technique for repair of full-thickness skin wounds. They developed a skin bioprinter capable of delivering the contents of up to eight different cartridges directly into a wound and showed that fibroblasts and keratinocytes can be bioprinted in situ. These skin cells were able to regenerate the dermis and epidermis in a porcine full-thickness excisional wound model.

Research Plans for the Next 2 Years

Over the next 2 years, the researchers plan to refine the large animal bioprinter. They will deliver cells to a critical size burn defect in a porcine model. They will also design a third-generation bioprinter suitable for clinical application.

Planned Clinical Transitions

This basic research project is not slated for clinical trials during the next 2 years. The goal is to demonstrate the feasibility of developing a skin delivery system and test its applicability clinically.

Tissue Engineered Skin Products – ICX-SKN

Project 4.2.1, WFPC

Team Leader(s): Eric Rolland, PhD, Paul Kemp, PhD, and Vincent Ronfard, PhD (Healthpoint Biotherapeutics, Ltd.)

Project Team Members: Dennis L. Carson, PhD, DABT, Kathi Mujynya Ludunge, BS, MBA, and Sarah Ramsay, MS (Healthpoint Biotherapeutics, Ltd.)

Collaborator(s): Patricia A. Hebda, PhD (University of Pittsburgh) and Daniel Hartman (Rockefeller University/NOVOTEC)

Therapy: A permanent dermal skin graft replacement (ICX-SKN), which can be integrated and remodeled by the host for burns.

Deliverable(s): Initiate human clinical evaluation of ICX-SKN.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 2; End Year 3, TRL 2/3

Key Accomplishments: During the past 3 years, Intercytex and then Healthpoint developed the tissue-engineered skin product ICX-SKN. The researchers completed the extensive physical, biochemical, and biological characterization of the ICX-SKN matrix during maturation. They assessed improved methods of matrix production (e.g., ultrasound stimulation). They investigated new manufacturing processes (e.g., casting dish designs). They tested proof-of-concept on the pig burn model using four prototypes. This extensive characterization helps to define the product for manufacturing process control and final product specifications.

Key Words: Burn, matrix, skin graft replacement, human dermal fibroblasts, fibrin

Introduction

The need exists for an off-the-shelf skin replacement that is instantly available and alleviates the need to take full-thickness skin grafts. Several "living skin equivalents" and "living dermal equivalents" approved by the FDA are currently available. Although these materials, such as Apligraf®, Dermagraft®, and Orcel®, work as artificial skin grafts, in reality no current living product meets the rigorous requirements necessary to accomplish this function. Rather, the dermal component of these products is rapidly degraded in the wound environment, releasing the cells, which then contribute to wound healing by secondary intention.

This failure of these artificial skin grafts results from lack of a constant structural element due to the quantitative and qualitative nature of the ECM into which the cellular elements are deposited. The intended aim in these first-generational constructs was to obtain gradual implant remodeling while maintaining a structure sufficiently robust to resist degradation in the wound. With these first-generation materials, the implant rapidly degraded, producing a failed element within a wound that then slowly healed by secondary intent.

In contrast to these earlier living skin equivalents and living dermal equivalents that have used either a preformed collagen matrix or biodegradable synthetic mesh as the initial support system, the intent from the start of this project (ICX-SKN) was to develop a more biologically robust ECM by allowing the fibroblasts themselves to produce the material in vitro. Others have shown that fibroblasts allowed to grow to superconfluency in

vitro are able to synthesize a relatively strong ECM, but the resulting material is extremely thin and fragile.

The intent of this project, therefore, was to extend the findings of Neidert, et al. (*Biomaterials* 2002; 23:3717-3731), who have shown that cells grown within a fibrin scaffold gradually remodeled this scaffold into a cell-synthesized matrix. A freeze-drying process has been developed that consistently produces freeze-dried 10 cm x 10 cm dSKN intermediates to ICX-SKN product specifications, including the ability of the matrix to sustain viable human dermal fibroblasts (i.e., cell friendliness), the ability to withstand manual manipulation, adequate mechanical strength, and appropriate resistance to collagenase B digestion after storage. A regime of thermal treatment and primary drying steps with temperatures and hold times was created, which could ultimately be transferred to a contract manufacturer with GMP accreditation to successfully produce freeze-dried batches of 10 cm x 10 cm dSKN intermediates.

ICX-SKN has been shown to be a new generation of off-the-shelf dermal replacement. The intended use for burn injury treatment is the simultaneous application ICX-SKN and autograft in a single-step procedure.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers initiated the development of a casting dish for preclinical production of the ICX-SKN biological dermis. They also identified a suitable freeze-drying process for the constructs. They developed a burn pig protocol and characterized maturation of the ICX-SKN constructs by scanning electron microscopy and immunostaining. Finally, they identified ultrasound stimulation protocols that greatly increase the mechanical and biological handling characteristics of the constructs. During the second year of the project, the researchers characterized the ICX-SKN matrix during maturation, including composition (fibrin/collagen), density and pore size, cell distribution, and overall thickness. Asset transfer from Intercytex to Healthpoint was completed. Healthpoint's in vivo pig burn model was approved by the Institutional Animal Care and Use Committee, and model evaluation was completed.

Research Progress – Year 3

Macroscopic Evaluation

Over the course of maturation, the constructs go through a series of phases in which they transition from being thick and sponge-like to being thinner and more structurally sound as seen in **Figure V-27**. As the matrix matures, the metabolic needs of the constructs increase as indicated by a rapid decrease in media pH.

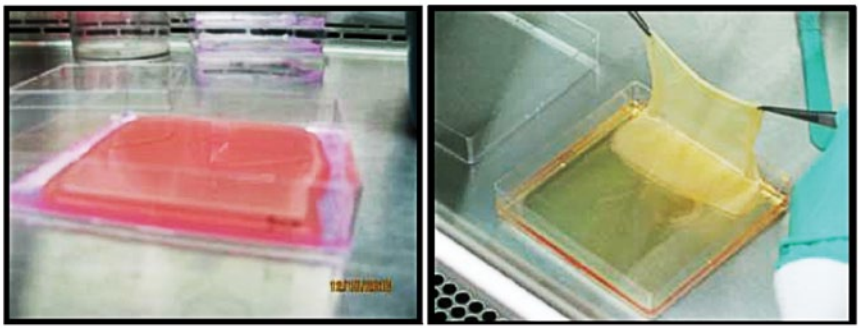

Figure V-27. Constructs immediately after casting (left) and at maturation (right).

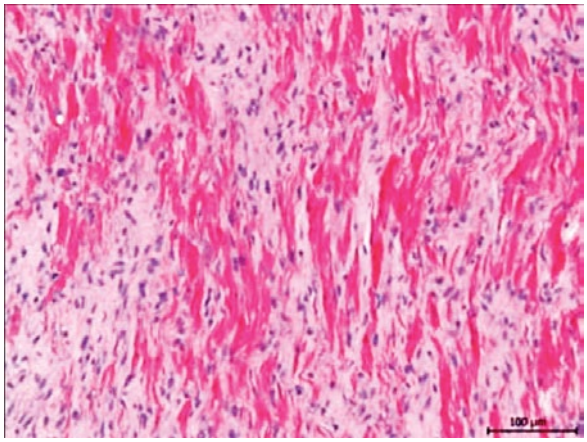

Figure V-28. HES staining.

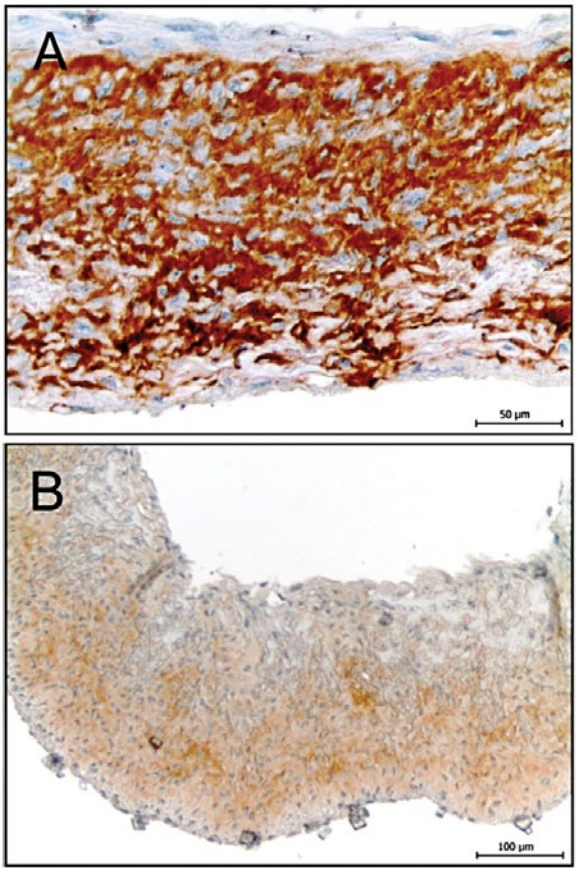

Figure V-29. (A) Collagen I staining, (B) Collagen III.

Histological Analysis

Histological analysis was carried out on the dermal equivalents to evaluate tissue structure and the evolution of matrix components over the maturation period. The influences of casting plate shape and the inclusion of vitamin C in the media were also evaluated. Three samples taken from the corners and middle of the gels were analyzed to evaluate possible positional differences.

Samples were processed, sectioned, and either stained or evaluated immunohistologically as follows:

- Hematoxylin-eosin-saffron (HES) staining: The cytoplasm is pink, the nucleus is violet, and the ECM is yellow.
- Immunohistological analysis using specific type I and III collagen antibodies.

HES Staining

The cells appeared spindle-shaped, and the cell number was constant between 5 and 7 weeks of culture **(Figure V-28)**. No abnormal cellular multiplication was noted. There were no significant differences between different areas of the constructs (corner versus middle).

Matrix Components

Type I and type III collagen distribution was evaluated by immunohistochemistry. Type I fibers are oriented vertically and horizontally **(Figure V-29)**. Few samples showed type III collagen staining. The type III fibers were often undulated (Figure V-29). Collagen I production was confirmed by western blotting.

Cytokine Release from Freeze-Dried Constructs

After maturation of the constructs, they are freeze-dried. To evaluate cytokine release from freeze-dried constructs, a human cytokine multiplex was performed. Of the

cytokines evaluated, epidermal growth factor, fibroblast growth factor-2, and VEGF were found in significant quantities.

Mechanical Properties

Two important properties needed for a usable dermal substitute are durability and mechanical stability. Several freeze-dried samples were tested for tensile strength, elastic modulus, elongation at break, and suture retention strength. Samples were tested both dry and after rehydration **(Table V-3)**.

Besides being mechanically stable, the ideal dermal substitute must also be able to integrate into the wound environment. The proteolytic environment of the wound was simulated with a mixture of collagenase and noncollagenase proteases. Samples were incubated in this simulated wound fluid, and the weights of lyophilized samples before and after digestion were used to evaluate percentage of degradation. As shown in **Figure V-30**, the constructs are highly degradable. This assay has been developed further and can be used to evaluate samples for uniformity in the future.

Pig Burn Wound Model

A porcine full-thickness (third degree) burn model was developed and refined to have an appropriate preclinical test system in place for ICX constructs. The full-thickness burns are created using metal burn rods heated to 100°C. The resulting wound develops an eschar resembling that formed in human third-degree burns. The wound bed is prepared by removal of the eschar. At the present time, the model is being used on a regular basis to evaluate debridement; thus, testing of the final ICX constructs is planned to be assessed using the researchers' expertise with this model.

Table V-3. Mechanical properties.

	Average	Standard Deviation
Peak Stress MPa (dry)	4.02	1.17
Peak Stress MPa (wet)	1.64	0.23
Elastic Modulus MPa (dry)	32.98	10.77
Elastic Modulus MPa (wet)	1.41	0.19
Elongation at Break % (dry)	17.79	5.83
Elongation at Break % (wet)	74.86	10.83
Suture Retention Strength	0.27	0.02

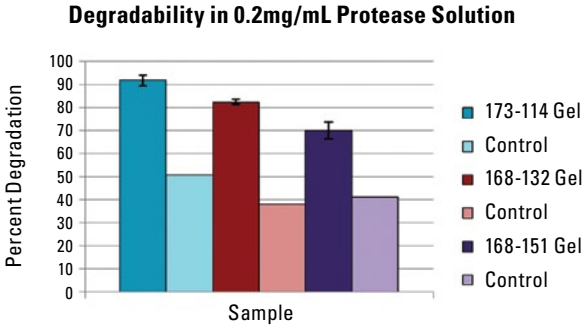

Figure V-30. Degradability of constructs.

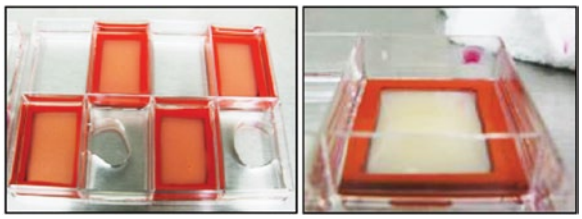

Figure V-31. Picture on the left is at seeding. Picture at right is after 49 days of maturation.

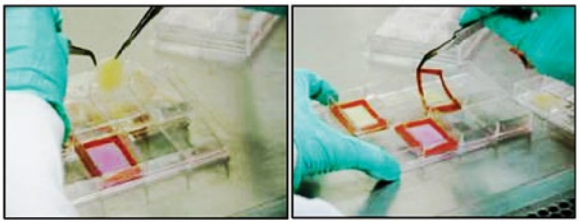

Figure V-32. At maturation the gaskets were removed. As can be seen in the picture on the right, the constructs appear uniform in shape.

Manufacturing

Refinement of the manufacturing process is ongoing in an attempt to decrease cost and time and increase uniformity of the constructs. One area in need of refinement was the fibrin rim needed for proper construct maturation. As a replacement, silicone rims were tested in an eight-well format. Results are depicted in **Figure V-31** and **Figure V-32**.

Augmentation of Research Capacity

To expand and advance the group's research capacity, in December 2010 several scientists at the Fort Worth, Texas, facility were trained in the manufacturing process of ICX-SKN. The future development involves manufacturing capabilities in both Fort Worth, Texas, and Lausanne, Switzerland.

Key Research Accomplishments

- Characterized ICX-SKN construct maturation.
- Evaluated cytokine release from freeze-dried constructs.
- Assessed the mechanical properties of constructs.
- Developed a reproducible enzymatic assay for testing degradation of constructs.
- Further refined the manufacturing process.
- Developed an in-house pig burn wound model for future construct testing.

Conclusions

During the past year, further characterization of the ICX-SKN matrix took place. Examination of the manufactured constructs revealed a unique structure and strength. Novel methods of evaluation provided the

researchers with a deeper understanding of the matrix maturation process. Several assays were developed that will aid in the evaluation of future constructs thus ensuring uniformity.

Research Plans for the Next 2 Years

Moving forward, Healthpoint will continue to produce ICX-SKN at two different sites. The immediate need is to pick the lead candidate and evaluate it in the pig burn model. The lead candidate from these studies will be selected for testing in humans. As this project moves forward, interactions with the FDA will begin as plans are finalized for an IND and the start of the clinical trial in 2013.

Planned Clinical Transitions

The project plans include evaluating the ICX-SKN in humans. An IND will be assembled and filed with the FDA. Once the lead candidate is selected, the development of the IND will begin. This will require the completion of an appropriate preclinical package along with a clinical protocol. In addition, the protocol will have to be approved through the appropriate IRB for the clinical site(s).

Corrections/Changes Planned for Year 4

Healthpoint purchased the ICX-SKN assets in February 2010 and has kept the program moving forward. Since that date, all research and collaborative efforts between Intercytex and Healthpoint have been performed without Department of Defense funding. The technology transfer and validation of the master cell banks have been accomplished. In May 2011, Healthpoint received the notification of the AFIRM grant agreement. This recent grant approval will provide the necessary resources to accelerate the development of ICX-SKN as a novel treatment for burn injuries incurred by those in the military and the public in general.

Tissue-Engineered Skin Substitute for Burns at Organogenesis

Project 4.2.1a, WFPC

Team Leader(s): Damien Bates, MD, PhD, Xianyan Wang, PhD, and Michael Segal (Organogenesis, Inc.)

Project Team Members: Lan Cao, PhD, Matthew Wong, MS, Katie Faria, Mark Tuden, Cecile Rousseau, PhD, Thomas Bollenbach, PhD, Parid Sava, Esin Yesilalan, MS, Lorraine Laham, PhD, Paige Sweeney, and Patrick Yu (Organogenesis, Inc.)

Collaborator(s): James Holmes, MD (Wake Forest University, United States) and Jean-Michel Rives, MD (Armand Trousseau Hospital, France)

Therapy: Burn therapy.

Deliverable(s): Develop an advanced dermal substitute for the treatment of full-thickness or deep partial-thickness burn wounds.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 2; End Year 2, TRL 2; End Year 3, TRL 4

Key Accomplishments: The Organogenesis project team developed the final product, which demonstrated superior vascularization and host tissue ingrowth in preclinical models compared with currently available technologies. The researchers established a complete manufacturing process, including cell banking, matrix production, packaging, and terminal sterilization. They initiated the transfer of technology from research and development to cGMP production.

Key Words: Burn, collagen, extracellular matrix, dermal regeneration template, human dermal fibroblast, animal model

Introduction

In this project the researchers aim to develop an advanced dermal substitute for the treatment of full-thickness (third degree) or deep partial-thickness burn wounds (second degree). Specifically, the objective is to leverage Organogenesis' VCT platform technology to construct an off-the-shelf product composed of a devitalized, de novo-generated human dermal fibroblast-derived ECM. The VCT-03 matrix is intended to act as a dermal substitute for the treatment of full-thickness or deep partial-thickness wounds when sufficient full-thickness autograft is either not available at the time of excision of burn wounds or not desirable due to the condition of the patient. The matrix is intended to allow simultaneous application with a split-thickness autograft in a one-step operation and provide improved cosmetic outcomes. The advantage of a one-step process is that it decreases the time to definitive closure of the wound, reducing the potential for infection. It is anticipated that the VCT-03 matrix will have improved biocompatibility and safety over existing xenogeneic or synthetic products.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers established a project management structure including the initiation of design control. They also established porcine fibroblast and keratinocyte cell banks. They

refined the porcine self-assembly dermal matrix. Finally, they began development of a porcine wound model and, in parallel, a human dermal matrix. During the second year of the project, the researchers completed the groundwork for the porcine preclinical model and developed three burn product embodiments leveraging Organogenesis' VCT platform technology and advanced biomaterials.

Research Progress – Year 3

Overview

After developing and testing more than 30 embodiments, the Organogenesis team selected a final embodiment that, in preclinical models, demonstrated superior vascularization and host tissue ingrowth compared with currently available technologies. They established a complete manufacturing process that includes cell banking, matrix production, packaging, and terminal sterilization. They initiated the transfer of technology from research and development to cGMP production. All of this was performed by a company-wide, cross-functional team operating under the design control process to ensure the successful and timely development of a safe and effective final burn therapy product.

Selection of Final VCT-03 Product

The Organogenesis team developed and tested more than 30 embodiments during the past year, including different permutations of de novo-generated human dermal fibroblast-derived ECMs (VCT-03) and silk protein matrices containing human dermal fibroblasts and ECM. The researchers evaluated these embodiments in preclinical screening studies. They used mouse models to assess the in vivo biocompatibility and efficacy of the embodiments in terms of host immune response, foreign body reaction, vascularization, fibroblast infiltration, construct remodeling, and interaction with a tissue-engineered, bilayered skin substitute. These screening studies helped to identify advantages, disadvantages, and questions that were addressed in follow-up studies. These studies led to the selection of the final VCT-03 embodiment, a devitalized, de novo-generated human dermal fibroblast-derived ECM, that met key criteria in the researchers' target product profile by demonstrating improved vascularization and host tissue ingrowth compared with currently available technologies.

Development of VCT-03 Product

The researchers developed a 10 cm × 10 cm prototype cell culture device to support the generation of VCT-03 matrices for large wound applications **(Figure V-33)**. Process development for manufacturing scale-up is currently under way to support the necessary capacity and requirements for production of matrices for clinical trials and, ultimately, commercial production.

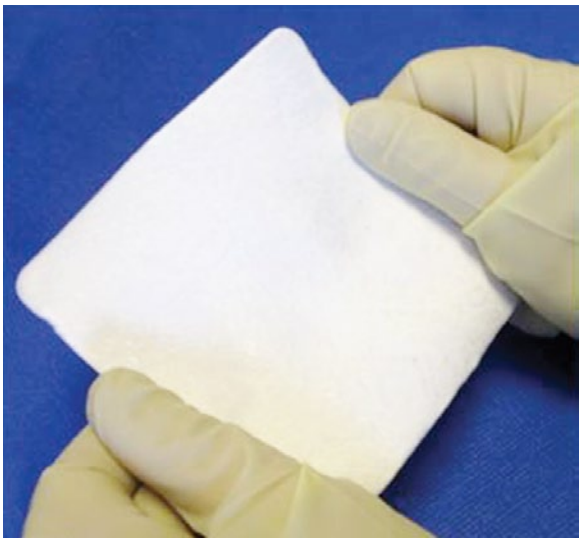

Figure V-33. Photograph of a 10 cm × 10 cm VCT-03 matrix.

Establishment of cGMP Facility

The researchers purchased new equipment and established new facilities to support cGMP pilot production of VCT-03. They initiated the first pre-engineering run with the purpose of transferring the VCT-03 technology from research and development to production. They initiated further process development work to scale up the manufacturing capacity for commercialization.

Key Research Accomplishments

- Designed and screened more than 30 embodiments in vivo using mouse subcutaneous and excisional full-thickness wound models.
  - ♦ Identified the final embodiment that demonstrated improved vascularization and tissue ingrowth compared with currently available technologies.
- Developed a 10 cm × 10 cm prototype cell culture device to support the treatment of larger wounds and initiated additional process development activities for manufacturing scale-up.
- Established a complete manufacturing process that includes cell banking, matrix production, packaging, and terminal sterilization.
- Acquired new equipment and established production facilities.
- Started pre-engineering run preparations for technology transfer to manufacturing and cGMP pilot production.

Conclusions

The Organogenesis team has developed its final embodiment for the treatment of full-thickness or deep partial-thickness burn wounds. Preclinical screening studies and development iterations were aimed at refining embodiments based on the researchers' previously established target product profile. The final embodiment leverages Organogenesis' VCT platform technology and is composed of a devitalized, de novo-generated human cell-derived ECM. This embodiment acts as a dermal substitute and is intended to be used in one-step skin grafting procedures. Results from preclinical studies in an immunocompromised mouse wound model showed superior vascularization and host tissue ingrowth compared with currently available technologies. The team is on schedule to test this final embodiment in an immunocompetent porcine model to evaluate efficacy. New equipment and cGMP facilities have been acquired and established, and the technology transfer process from research and development to production has been initiated to support the generation of product for clinical trials and commercial production. The project team is also consistently adhering to the design control process at every step to ensure the successful development of a safe and effective final burn product.

Research Plans for the Next 2 Years

Organogenesis will conduct efficacy assessments of the selected final embodiment in a current Good Laboratory Practice (cGLP) porcine full-thickness excisional model. A series of engineering runs for technology transfer have been planned. The cGLP biocompatibility testing per ISO 10993 will be started in the third quarter of 2011. Interactions with the FDA for regulatory classification and IDE/IND will continue. A funding proposal for the clinical trial will be submitted as requested.

Planned Clinical Transitions

The project plans include evaluating the VCT-03 matrix in human patients. An IDE/IND will be assembled and filed with the FDA. The development of the IDE/IND will begin once the cGLP preclinical study, biocompatibility testing results, and clinical protocols are available. In addition, the protocol will be submitted for approval through the appropriate IRB for the clinical site(s).

Amniotic Fluid-Derived and Placenta-Derived Stem Cells for Burn

Project 4.2.6, WFPC

Team Leader(s): Mark E. Furth, PhD (WFIRM)

Project Team Members: Chad D. Markert, PhD (WFIRM)

Collaborator(s): Shantaram Bharadwaj, PhD, Yuanyuan Zhang, MD, PhD, Aleksander Skardal, PhD, Emily Moorefield, MS, Colin Bishop, PhD, and James Yoo, MD, PhD (WFIRM) and Xinyi Guo, BS, Stephen Baker, BS, and Martin Guthold, PhD (Wake Forest University Department of Physics)

Therapy: Amniotic fluid-derived and placenta-derived stem cells for burn.

Deliverable(s): Characterization of the therapeutic potential of cells derived from perinatal sources (amniotic fluid and/or placental tissue) with a focus on the immunomodulatory properties of these cells.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 2; End Year 3, TRL 2

Key Accomplishments: During the past year, the researchers broadened their scope to include placenta-derived cells. They began systematic experiments to improve culture conditions to better permit in vitro expansion of rare stem cells while maintaining their stemness. They designed cell isolation experiments to enrich for cells with immunomodulatory properties. They established a cell culture system that mimics physiological conditions by providing vulnerable cells with a normoxic rather than hyperoxic environment. They perfected methods to perform indenting experiments on substrates covered with liquid to prevent dessication of samples. They also confirmed equations that will aid in data extraction and analysis.

Key Words: Stem cells, immunomodulation, regenerative medicine, living skin equivalents

Introduction

Directed differentiation of human AFS cells toward the epithelial lineage for creation of a bioengineered skin product remains a challenging yet promising potential means of wound healing in the treatment of extensive burns. AFS cells, obtained from amniocentesis samples, are isolated based on expression of the surface marker CD117 (c-kit). Due to their potential to differentiate toward multiple lineages, without the risk of teratoma formation, AFS cells are gaining increased attention in regenerative medicine.

Initial interest focused on the potential of AFS cells to yield multiple specialized cell types that may be applicable to a broad spectrum of diseases and injuries. However, the range of cell types outside of the mesenchymal lineages that can be generated efficiently from AFS cells remains to be determined. A new focus is on the immunomodulatory capacity of AFS cells. Coupled with their capacity for extensive expansion, this suggests that AFS cells could be banked to provide an off-the-shelf source of cells with anti-inflammatory and proregenerative activities that could be provided to unrelated recipients but with beneficial histocompatibility matching.

Efforts to direct differentiation of AFS cells to an epidermal fate over the initial 2 years of the project were limited by a lack of consistency in outcomes such as expression of two key epithelial markers, CK14 and ΔNp63, in multiple AFS cell lines (A1, H1, CB3, C3M6, and C1F8). Although some experiments were

promising, it became evident that variability in outcomes may have been due to problems intrinsic to the cell lines themselves, such as loss of stemness after maintenance in culture for many population doublings/passages; the literature suggests this phenomenon is common. Procurement of fresh amniotic fluid to isolate new AFS cell lines has been an ongoing challenge because the frequency of amniocentesis is decreasing in local medical practice. The fact that all of the above cell lines were passaged at least 15 times prior to use in the present experiments may explain their inconsistency in differentiation. Therefore, the researchers have initiated the collection of fresh cells from a more readily available source, the placenta, which they showed previously to contain a comparable population of cells that can be isolated by immunoselection for CD117 (c-kit). For simplicity in this report, the term "AFS cells" is used to denote stem cells obtained from a perinatal source, whether amniotic fluid or placenta.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers demonstrated that human AFS cells express CD146, a cell surface marker protein recognized as a marker of perivascular cells. They demonstrated enhanced skin wound healing by AFS cells in immune-deficient mice. They found initial evidence for the expression of stratified epithelial lineage markers p63 and CK14 by some AFS cell lines in response to in vitro differentiation conditions. During the second year of the project, the research team demonstrated the immunomodulatory activity of AFS cells against human T lymphocytes. The researchers optimized the expression of stem cell markers Oct4 and SOX2 by AFS cells under new culture conditions. They also induced CK14 in AFS cells cultured in inductive media.

Research Progress – Year 3

The researchers have identified growth conditions that may contribute to loss of the differentiation capacity of AFS cells. The goal is to maintain stemness in rare stem cells over multiple passages while they are expanded in a truly translational manner. The researchers identified four areas related to the stem cells' microenvironment ("niche") for experimental focus **(Figure V-34)**. A fifth area focuses on immunomodulation.

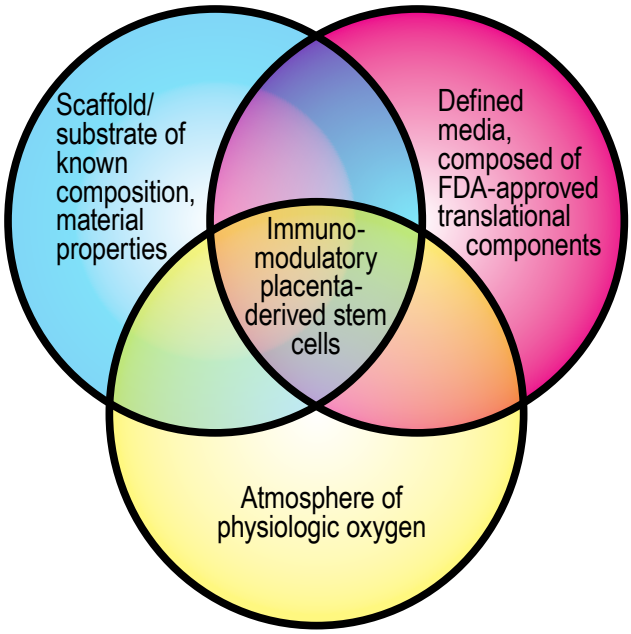

Figure V-34. Although progress in developing defined culture conditions for propagation of adult stem cells has resulted from elucidating the roles of soluble factors, there is still no cGMP-compliant protocol that supports large-scale expansion of these cells over multiple passages. Because these cells are relatively rare, expansion is required to achieve clinical utility. The research team's objective is to optimize the in vitro environment to permit this expansion while maintaining stemness and immunomodulatory properties. The physiochemical environment, growth factor supplements, and matrix substrata components will be considered both in terms of efficacy and potential for translation, using FDA and cGMP guidelines.

1. The composition of the matrix, or substrata, on which cultured cells are grown.
2. The elasticity of the matrix or substrata on which cultured cells are grown.
3. The oxygen tension in the cell culture environment.
4. A growth media recipe that is both physiologic and well suited for translation to expansion in compliance with cGMP.
5. Perfection of methods to isolate and characterize cells that are immunomodulatory.

Much of the research focus over the past year was on these areas to define conditions that begin to reflect the in vivo stem cell niche. Thus, fresh cells from amniotic fluid, placenta, or any tissue source can be systematically characterized under culture conditions that more closely mimic normal physiology. Anecdotal evidence suggested that in the past, when inconsistent results were obtained for the multiply-passaged yet widely available AFS lines listed in the Introduction, the researchers found fault with their culture media, culture conditions, or the cells themselves. The approach adopted by several researchers at the WFIRM was to manipulate culture conditions, most often media recipes or assay conditions. Alternatively, when a given set of conditions did not work for an established multiply-passaged stock AFS cell line, the same set of conditions may be tried again with more recently isolated but still multiply-passaged stock cell lines. The Furth/Markert team has broken this cycle and is systematically assessing the variables that contribute to the maintenance of both stemness and immunomodulatory activity.

With regard to areas 1 and 2, the researchers are writing a manuscript that will provide critical methods for engineering a niche for the putative immunomodulatory stem cells that they will isolate from term placenta. **Figure V-35** shows rheology pilot data. Obtained in collaboration with colleagues in the Wake Forest Department of Physics, these data were generated on agarose hydrogels as described in last year's report but using an atomic force microscope (AFM) rather than an Instron rheometer. The use of AFM considerably improves the precision of the

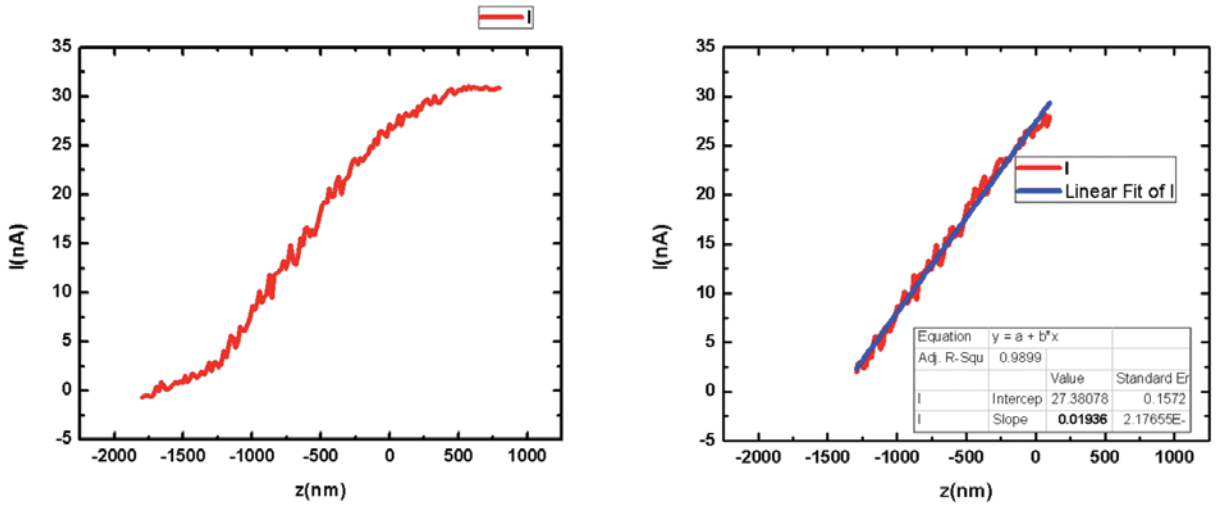

Figure V-35. Pilot AFM experiments on agarose gels. Shown is a set of sample loading curves. Force is plotted versus piezo movement. Using the equation shown in Figure V-37, data analysis on loading curves will provide precise measures of elasticity E of experimental biomaterials.

measurements. These data demonstrate that the researchers are well on their way to controlling two of the four critical determinants of improving the cell culture environment. First, they have provided defined, easy-to-follow recipes of various biomaterials thus providing control over the composition of the matrix or substrata on which cultured cells are grown. Second, AFM experiments are ongoing, and data generated will be the first to describe the elasticity of the same hydrogel recipes. Specific accomplishments include:

- Indented agarose substrates to compare conical to spherical tips as the indenters used on the AFM cantilever and determined that spherical tips provide valid, reproducible data to determine the Young's modulus E of the substrate **(Figure V-36)**. To date the researchers have generated more than 20 data curves on agarose gels.
- Obtained control measurements on hard surfaces.
- Perfected methods to perform indenting experiments on substrates covered with liquid (buffer), to prevent desiccation of samples.
- Determined a constant indentation velocity (2 µm/sec) provides valid, reproducible data to determine E.
- Confirmed equations that will aid in data extraction and analysis. These equations consider E, force F, indentation δ, Poisson's ratio ν, and radius of the sphere tip R. The researchers are currently analyzing these data to extract the Young's modulus of agarose gels.
- An equation for calculating the sample loading curve is provided in **Figure V-37**. Graphically, force is plotted on the y-axis, and piezo movement (sample indentation δ + cantilever deflection Δd), Δz, is plotted on the y-axis.

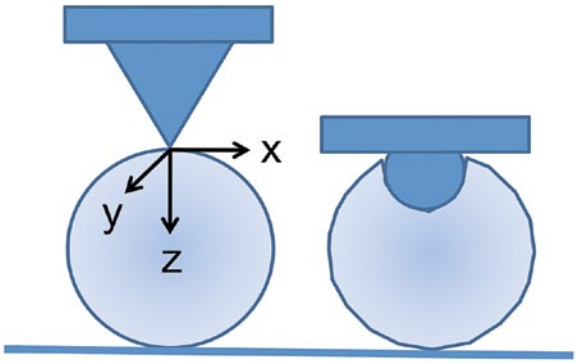

Figure V-36. Pilot AFM experiments on agarose gels with different geometries on the cantilever tip. These experiments concluded that a spherical tip was ideal for elasticity measurements and provided data for derivation of the data analysis equation shown in Figure V-37.

$$\frac{F}{k_c} + \gamma^{3/2} F^{2/3} = \Delta z$$

where

$$\gamma = \frac{4E\sqrt{R}}{3(1-\upsilon^2)}$$

Figure V-37. Sample loading curve equation. F – force, Δz – piezo movement, R – radius of indenter, υ – Poisson ratio ~ 0.5, and E – Young's modulus of the material.

With regard to area 3, the researchers have collected data covering the influence of oxygen tension in the cell culture environment. The rationale is that physiological levels of oxygen in tissues are much lower than in the atmosphere and that stem cells grow and maintain stemness better under conditions of reduced oxygen tension. One of the researchers' goals was to determine the actual oxygen utilization of their cells of interest. Although precise measurement of respiration at the cellular level in cell culture historically has been notoriously difficult, their pilot experiments with new oxygen-sensing coatings in microplates appear to provide an accurate estimate of cellular oxygen requirements. Using adult stem cells, they observed that cell proliferation increased inversely to oxygen concentration in a dose-response relationship regardless of seeding density **(Figure V-38)**. In the experiment, cells were plated at various densities in preblanked wells of an oxygen biosensor dish. Data were collected 24, 48, and 72 hours post-seeding. The researchers observed that cells incubated with oxygen levels closer to those in tissue (2% or 5%) exhibited enhanced proliferation at all time points versus cells grown in standard atmospheres (ambient 21% oxygen). This experiment is consistent with previous suggestions that most stem cell researchers culture their cells at toxic oxygen levels. Thus, the researchers have established a cell culture system that mimics physiological conditions by providing vulnerable cells with a normoxic rather than hyperoxic environment.

With regard to area 4, Dr. Chad D. Markert has met with Professor Lola M. Reid, Department of Cell and Molecular Physiology, the University of North Carolina at Chapel

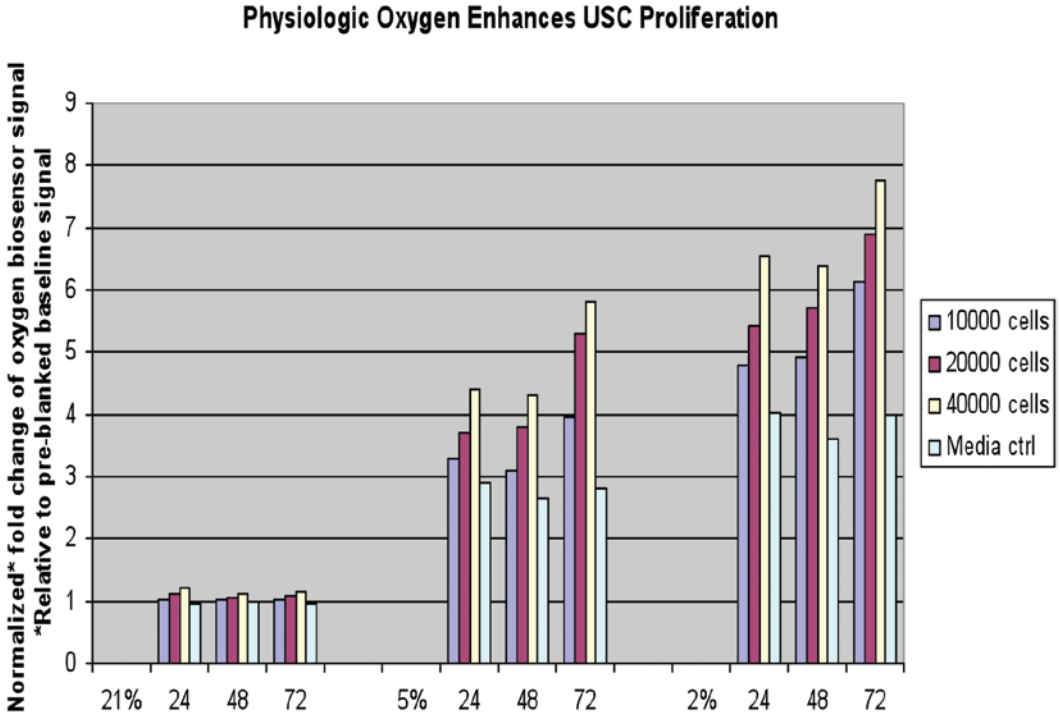

Figure V-38. Oxygen levels of the cell culture environment impact cell proliferation. Normoxia, the oxygenation experienced by cells physiologically, varies from 2%–9%. Standard cell culture conditions provide cells with an atmosphere of 21% oxygen, which is defined as hyperoxia. The oxidative stress of hyperoxia in typical cell culture conditions appears to play a role in limiting cellular proliferation while physiologic oxygen enhances cell proliferation.

Hill, and members of her laboratory to begin formulating a serum-free, hormonally defined cell culture medium that permits expansion of AFS cells without loss of stemness. Importantly, serum-free media has advantages that address both scientific and regulatory concerns. Dr. Mark E. Furth has previously collaborated with Dr. Reid on the isolation and expansion of hepatic stem cells from human liver in hormonally defined, serum-free medium. The Reid laboratory has subsequently adapted the same basal medium, with additional growth factors, for serum-free expansion of various mesenchymal cell populations from the liver. Because AFS cells share multiple surface markers and other phenotypic similarities with the mesenchymal cells utilized in Dr. Reid's studies, her work provides a good starting point for the goal of serum-free growth of AFS cells both to maintain the key properties of plasticity and immunomodulation and to facilitate the transition to cGMP-compliant cell production.

With regard to area 5, characterization studies provided clear evidence for immunomodulatory activity of AFS cells against human T lymphocytes. Dr. Markert initiated a collaboration with Wake Forest University's Department of Microbiology and Immunology to further investigate the immunomodulatory phenomenon in fresh or low-passage, placenta-derived cells. These studies will speak to the usefulness of AFS cell-like, placenta-derived cells both as potentially contributing structurally to engineered skin and also as potential regulatory mediators of the immune response to engineered skin.

Key Research Accomplishments

- Began systematic experiments to improve culture conditions to better permit in vitro expansion of rare stem cells while maintaining their stemness.
- Established a cell culture system that mimics physiological conditions by providing vulnerable cells with a normoxic rather than hyperoxic environment.
- Determined that spherical tips on the AFM cantilever provide valid, reproducible data to determine the Young's modulus E of the substrate.
  - Generated more than 20 data curves on agarose gels to date.
  - Obtained control measurements on hard surfaces.
- Perfected methods to perform indenting experiments on substrates covered with liquid (buffer) to prevent desiccation of samples.
- Confirmed equations that will aid in data extraction and analysis.
- Designed cell isolation experiments to enrich for cells with immunomodulatory properties.

Conclusions

The researchers' experimental framework has matured to the point of considering the interaction of the right cells with the right environment to promote the desired outcome. The goal is to isolate cells with immunomodulatory properties from renewable sources such as human term placenta. The first four areas described previously focus on factors that the researchers consider critical to establishing a permissive cell culture microenvironment. When incorporated into vastly improved cell culture conditions that mimic key components of human physiology, the immunomodulatory cells promise to retain their properties despite long-term expansion.

Research Plans for the Next 2 Years

Once promising cells are isolated, the researchers plan to grow them in the improved cell culture conditions described previously as needed for expansion. Reverse transcriptase-polymerase chain reaction and immunohistochemical screening of cells for mRNA and protein signatures characteristic of stemness and immunomodulation will occur routinely to confirm maintenance of these properties in cells that have undergone multiple passages. Immunomodulatory bioactivity will be tested against activated human T lymphocytes essentially as the researchers have previously reported. Long-term expansion capacity to at least 40 population doublings, corresponding to the potential for 1 trillion-fold expansion in cell number, will be assessed under optimized growth conditions. This work will be carried out in a "pre-cGMP" mode, in close collaboration with scientists in WFIRM's new cell manufacturing facility, to facilitate eventual translation to a product for clinical trials.

Cells will then be tested for promotion of wound healing in vivo using the skin bioprinter being developed in a separate AFIRM project. The initial approach will be to spray the cells on the backs of immunodeficient nude *(nu/nu)* mice with skin wounds and assess the rate of healing. The research team will also test the AFS cells in combination with human dermal fibroblasts and keratinocytes to determine if they accelerate wound healing. They will then plan to collaborate with the skin bioprinting project team to assess whether AFS cells accelerate healing in rodent burn models and, subsequently, in a large animal model (porcine). For the porcine studies, which will be critical to evaluate the potential for clinical translation in burn, the human cells may function xenogeneically; there is some precedent for this with hMSCs in animal models. However, it may be necessary to utilize porcine AFS cells isolated and expanded under essentially identical conditions to those the researchers will develop for human cells. They have already identified a commercially available monoclonal antibody with good binding affinity for porcine CD117 (c-kit) and have shown that it can be used for immunoselection of porcine AFS cells. Therefore, they anticipate that a large animal study in a clinically relevant burn model will be entirely feasible. They plan to use cell printing to apply the allogeneic (or xenogeneic if human cells prove active in the pig model) AFS cells at different stages after the burn injury alone or in combination with autologous dermal and epidermal cells.

Planned Clinical Transitions

Although it is too early to plan translation to clinical trials at this time, the in vitro and materials science work described in this progress report presents many opportunities for hypothesis-testing experiments in animal models of wound healing. These models are in place at WFIRM and are readily available. Transition of this 5-year project to clinical studies is anticipated to occur 2 years after the project ends. Success in bioprinting (as described previously) with undifferentiated stem cells and immunomodulatory cells could potentially accelerate clinical translation.

Corrections/Changes Planned for Year 4

The study will include the characterization of stem cells derived from amniotic fluid and/or placental tissue with a focus on the potential immunomodulatory properties of these cells.


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In Vitro Expanded Living Skin for Reparative Procedures

Project 4.2.8, WFPC

Team Leader(s): Sang Jin Lee, PhD, James Yoo, MD, PhD, and James Holmes, MD (Wake Forest University)

Project Team Members: John Jackson, PhD, Hyun-Wook Kang, PhD, Joshua Choi, BS, Abner Mhashilkar, PhD, Kevin Johnson, PhD, and Paul Scarpinato, BS (Wake Forest University)

Collaborator(s): None

Therapy: Treatment of burn injuries.

Deliverable(s): Autologous skin grafts.

TRL Progress: Start of Program, TRL 4; End Year 1, TRL 4; End Year 2, TRL 5; End Year 3, TRL 5

Key Accomplishments: The researchers are developing an in vitro tissue expander system that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. They completed a bioreactor design for use in clinical trials, and construction of a prototype is under way. They continued evaluating expanded skin grafts and developing protocols for optimizing the expansion parameters for the grafts. They also developed an effective microneedle tissue gripper system that critically affects the expansion of skin grafts.

Key Words: Autologous skin grafts, in vitro skin expander, bioreactor, burn repair

Introduction

Many reparative procedures due to battlefield trauma and burn may require additional skin for coverage. The standard of care for skin defect replacement is the use of autologous skin grafts. However, donor-site tissue availability is a major obstacle to the successful replacement of skin defects. Because of this limitation, other approaches are commonly employed to cover skin defects. These include commercially available skin products based on biomaterials and tissue engineering, allografts, and xenografts. However, these approaches also have limitations, such as the need for concomitant autograft, insufficient mechanical properties, high cost, lack of permanence, potential for infectious disease transmission, and inadequate biocompatibility. Nevertheless, many commercial skin products are being used as acceptable skin substitutes when autologous donor tissue is unavailable.

Alternatively, subcutaneous tissue expanders or meshed split-thickness skin grafts are used clinically to generate larger segments of autologous skin when donor-site tissue is limited. Subcutaneous tissue expanders are balloon implants that are sequentially filled with incremental volumes of saline to increase the amount of overlying skin. The physicomechanical stress of the tissue expander results in biologic creep, greater mitotic activity of cells, and increased vascularity, which ultimately leads to expanded skin. Subsequently, the expanded skin can be used as a tissue flap or harvested for use as a skin

graft. However, the use of a subcutaneous tissue expander is associated with an additional surgical procedure(s), which increases donor site and overall morbidity. In addition, this technique requires a lengthy wait time (on the order of months) to obtain sufficient tissue for intervention. Moreover, the discomfort associated with the increasing expander volume and the frequent tissue fibrosis remain as major limitations. Alternatively, meshed split-thickness skin grafts are obtained using a graft mesher that cuts the skin into a mesh pattern, which results in greater surface dimensions before application on the wound bed. However, meshed split-thickness skin grafts are not considered ideal for many applications because they leave large gaps of the open wound, which requires a longer healing time and results in a cross-hatched or cobblestone pattern of healed skin as scar tissue fills the gaps.

The overall goal of this project is to provide wounded soldiers with large dimensions of autologous skin for reparative procedures. The researchers have the following three aims:

- Optimize expansion parameters for maximizing surface dimensions of human skin.
- Establish SOPs for skin expansion parameters and delivery.
- Determine the applicability in wounded soldiers through a clinical trial.

Summary of Research Completed in Year 1 (funded in 2009)

The research group developed an in vitro tissue expander system that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. The expander system utilizes a computer-controlled bioreactor capable of providing an accurate expansion rate for yielding target skin dimensions over a defined time period. This system was successfully tested and validated on human skin samples. The group has been able to consistently double the surface area of donor skin within 2 weeks while maintaining cell viability. The expanded skin, grafted in small (mice) and large (pig) animal models, showed viable graft take when implanted on a recipient dermal bed. The researchers recently designed and built a clinically applicable bioreactor system.

Research Progress – Year 2

Skin Preparation

Discarded human skin samples were used. An 8 × 8 cm^2 skin graft (0.01 inch in thickness) was taken from discarded skin samples using a Padgett Electric Dermatome. Skin samples were rinsed thoroughly with PBS. Skin samples were placed in 70% ethanol for 1 minute for disinfection and then promptly rinsed in PBS prior to insertion into the skin bioreactor.

Skin Tissue Culture

To optimize culture conditions for skin grafts within the bioreactor, skin samples were cultivated at an air–liquid interphase in different culture media. Tissue sections taken from the cultured skin grafts at 7 and 14 days of culture were stained with H&E. H&E-stained sections showed that the epidermis and dermis were intact and that the general structure of the skin was maintained in both DMEM and RPMI culture medium with 10% fetal bovine serum (FBS). However, both DMEM and RPMI without FBS resulted in separation of the epidermal and dermal layers **(Figure V-39)**.

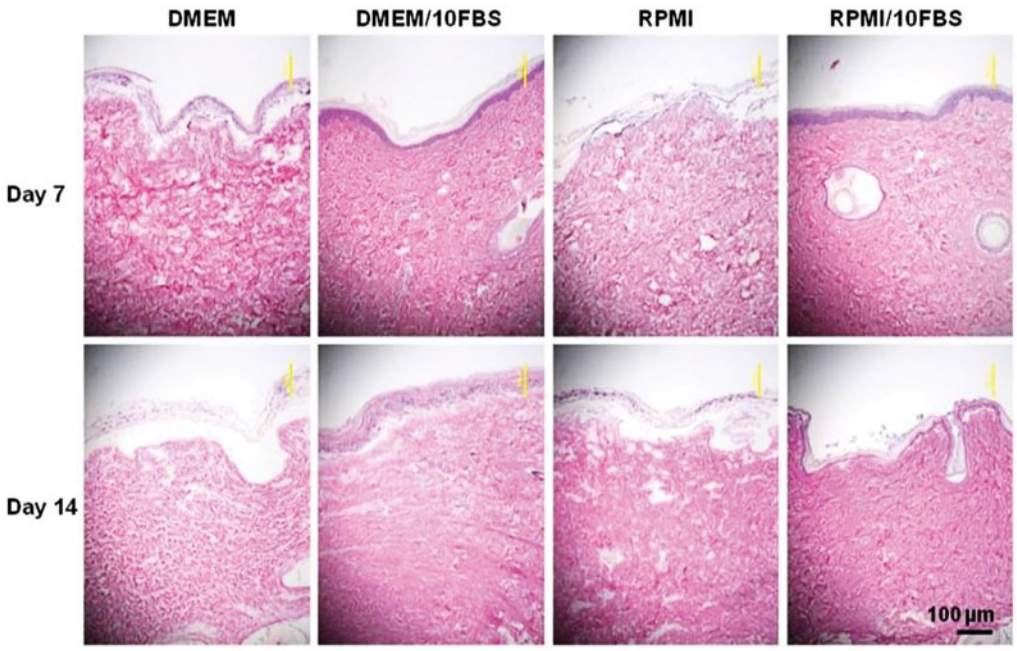

Figure V-39. H&E staining of the skin grafts at 7 and 14 days culture with different culture medium conditions.

The skin samples cultured in the presence of serum stained positively for proliferating cell nuclear antigen (PCNA) in the epidermal and dermis regions, indicating that the cells within the skin tissue were viable and maintained their ability to proliferate **(Figure V-40)**.

Skin Expansion Bioreactor System

Skin matrices were placed in a sterile bioreactor for expansion. The edges of samples were clamped at multiple areas in the biaxial bioreactor. After placement, the initial dimensions were measured using a sterile ruler to obtain a baseline value. Subsequently, 300 mL of DMEM with 10% FBS was added. Continuous flow of medium into the expansion chamber was maintained with a peristaltic pump. The bioreactor was monitored and kept at 37°C with a heated water jacket, and 5% CO_2 was circulated through the unit for the entire duration of expansion. Computer software was used to monitor the temperature, position, and load of the skin throughout stretching. The researchers have developed various protocols to maximize the surface dimensions of skin grafts. After stretching, the final dimensions of the expanded skin were measured. The skin sample was removed from the bioreactor in preparation for skin grafting.

Design Considerations

Various features were considered in the process of designing a bioreactor for clinical application. For example, the skin bioreactor system must be operated as a closed system to prevent potential contamination/infection and to minimize the frequency of manipulation. A skin bioreactor system has been designed and built for a clinical trial. Medical-grade materials such as stainless steel and Teflon® were used. Two stepper motors, a positioning sensor, an electric thermometer, and a force sensor were used for the construction of this fully automatic system **(Figure V-41)**. In addition, two circulating pumps were implemented for automatic

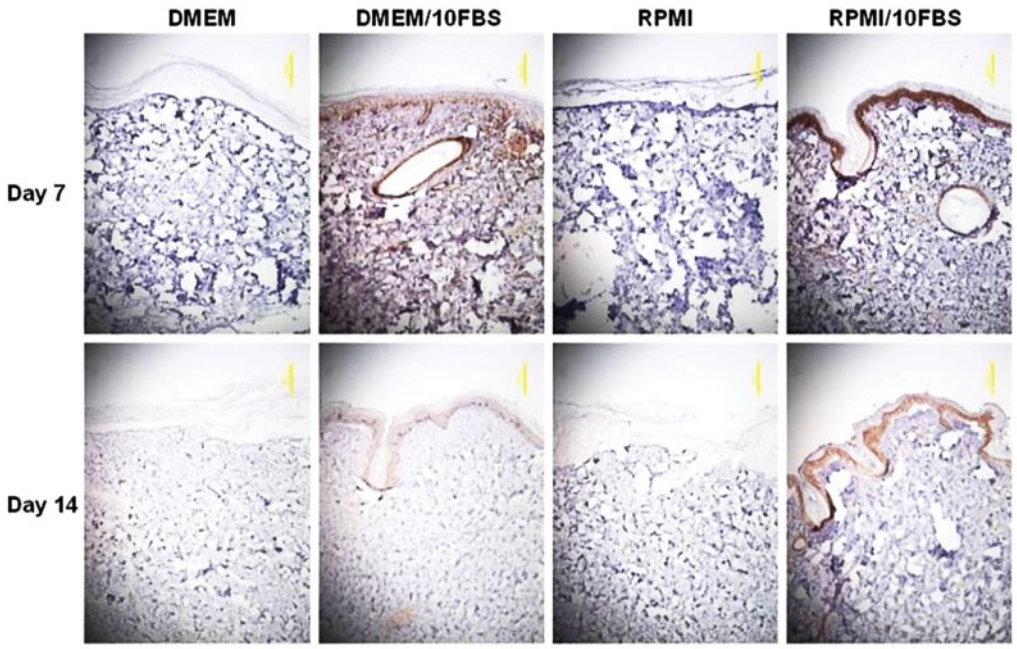

Figure V-40. PCNA immunohistochemical staining of the skin grafts at 7 and 14 days culture with different culture medium conditions.

medium exchange and temperature control. The expansion site is completely isolated from the outside environment to minimize the possibility of contamination.

A skin sample can be expanded to 150% of its original surface area within 2 weeks in the bioreactor. The amount of expansion depends on the properties of the initial skin graft. The research team will develop working parameters for the skin bioreactor expander system that will maximize skin expansion while maintaining tissue viability. These parameters include duration of expansion, frequency of incremental stretching, rate of expansion, and percentage of expansion. Tissue viability and structural changes will be assessed using histo- and immunohistochemistry and mechanical testing.

Figure V-41. Photographs of constructed hardware (left) and software system (right).

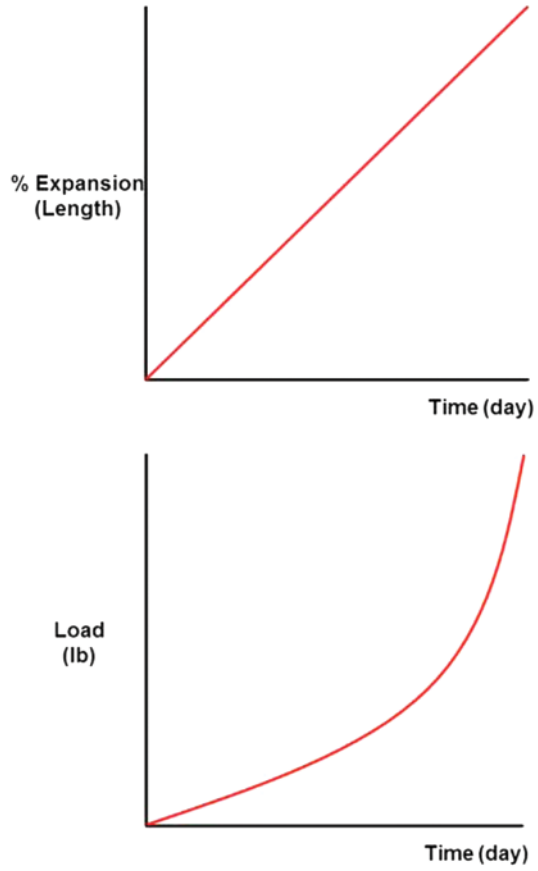

Figure V-42. Schematic diagram of bioreactor protocol 1 set-up for displacement (top) and load curve (versus time, bottom).

Optimization of Stretching Parameters (ongoing)

While skin expansion has been demonstrated to be a viable option for wounded soldiers who require large pieces of skin for reparative procedures, it is important to optimize the stretching protocol so that it does not result in tears, contracture, or other types of graft failure. The extent of expansion is presumed to be related to the pore size of the underlying matrix fibers, which may influence the levels of diffusion. However, consideration needs to be given to situations where excessive amounts of expansion may induce thinning of the skin, which may compromise the quality of the graft. For these reasons, it is essential to determine the quality of graft and the amount of graft take based on various degrees of skin expansion. In this task, the researchers aimed to optimize expansion parameters (described as follows) and determine whether in vitro expanded living skin tissues are able to maintain their surface dimensions and mechanical properties (strength and elasticity). This is necessary to maximize the utility of this technology.

Recently, the researchers focused on developing an effective tissue gripper system that critically affects the expansion of skin grafts. They identified a microneedle gripper system as a solution, and incorporation of this system is being designed and examined. Successful development of the microneedle gripper system would decrease tissue damage and reduce localized stress associated with the grippers, resulting in fewer tears.

a. Protocol 1: Displacement (Figure V-42) – Stretching speed: 0.005 mm/min, duration of skin expansion: 10–14 days, percentage of target expansion: 150% of original surface area, initial dimension: $8 \times 8\ cm^2 = 64\ cm^2 \rightarrow 160\ cm^2$ (approximately 12.7 cm in length).

b. Protocol 2: Incremental stretching with resting period (Figure V-43) – Skin expansion method: stretching for 1 day and resting for 1 day, stretching speed: 0.005 mm/min, duration of skin expansion: 12–14 days, percentage of target expansion: 150% of original surface area (8 × 8 cm^2 = 64 cm^2 → 160 cm^2), approximately 67% of length needs to be expanded (7 cm → approximately 11.7 cm).

C. Protocol 3: Gradual decaying stretching with resting period (Figure V-44) – Skin expansion method: stretching for 1 day and resting for 1 day with different stretching ratio, stretching speed: 0.005 mm/min, duration of skin expansion: 12–14 days, percentage of target expansion: 150% of original surface area (8 × 8 cm^2 = 64 cm^2 → 160 cm^2), approximately 67% of length needs to be expanded (7 cm → approximately 11.7 cm).

d. Protocol 4, Cyclic stretching (Figure V-45) – Skin expansion method: stretching-relaxation, stretching speed: 0.005 mm/min for stretching and 0.01 mm/min for relaxation, duration of skin expansion: 12–14 days, percentage of target expansion: 150% of original surface area (8 × 8 cm^2 = 64 cm^2 → 160 cm^2), approximately 67% of length needs to be expanded (7 cm → approximately 11.7 cm).

Evaluations of the Expanded Skin Grafts (ongoing)

Specimens of expanded skin were fixed in 10% phosphate-buffered formalin for 24 hours, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Serial 5 µm sections were obtained and stained with H&E and Masson's trichrome. Immunohistochemistry for PCNA and TUNEL staining for apoptosis were performed. Surface and cross-sectional morphologies of all skin matrices were examined by scanning

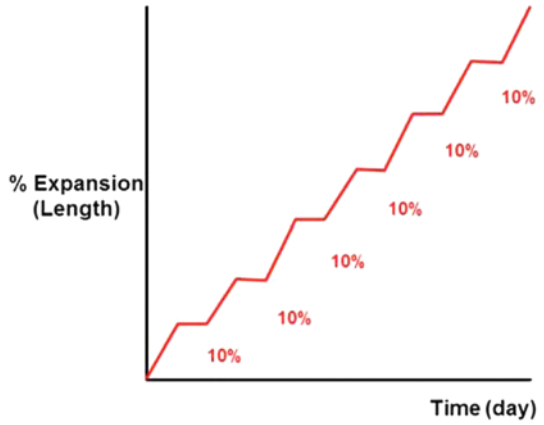

Figure V-43. Schematic diagram of bioreactor protocol 2 set-up for incremental stretching with resting period.

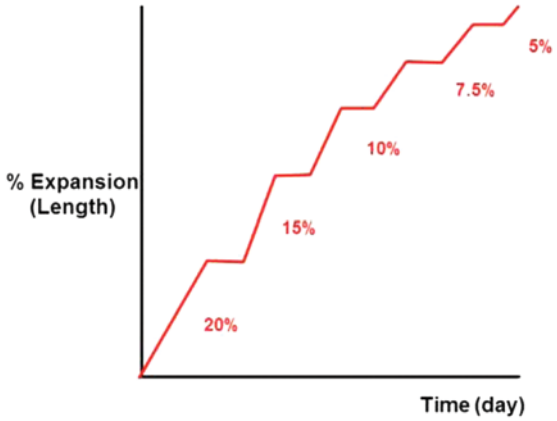

Figure V-44. Schematic diagram of bioreactor protocol 3 set-up for gradual decaying stretching with resting period.

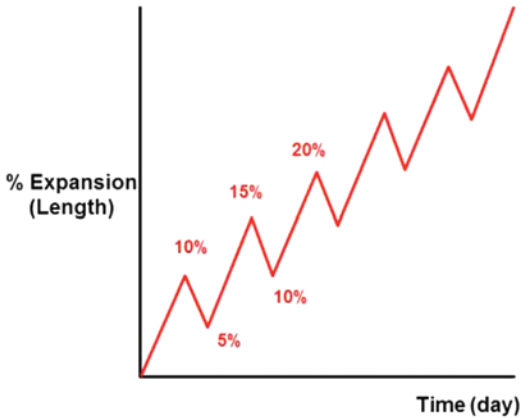

Figure V-45. Schematic diagram of bioreactor protocol 4.

electron microscopy. Tensile testing was also performed on all skin samples using an Instron 5544. The samples were kept moist with PBS during the entire period of mechanical testing.

Clinical Trial

Based on data obtained to date and the researchers' prior experience, they are confident that the proposed clinical trial will be successfully completed. However, should the expanded autografts fail to "take," the patient will not have suffered serious harm as the expanded autografts essentially would serve as autologous biologic dressings that can then be removed for subsequent autografting in the standard manner at an eventual third operation. It is possible that this may occur in some cases owing to the size of the overall injuries in which this new technology is being assessed. To ensure safe and effective production of cellular components for clinical protocols, all procedures will be performed according to cGMP for cell and tissue processing, including tissue expansion.

Key Research Accomplishments

- Completed a bioreactor design for use in clinical trials with construction of a prototype under way.
- Continued evaluating expanded skin grafts (e.g., immunohistochemistry and tensile testing) and developing protocols for optimizing the expansion parameters for human skin grafts.
- Developed a microneedle gripper system to hold skin within the bioreactor (ongoing).

Conclusions

An in vitro tissue expander system has been developed that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. The expander system utilizes a computer-controlled bioreactor capable of providing an accurate expansion rate for yielding target skin dimensions over a defined time period. A microneedle gripper system has been developed, and incorporation of this system is being designed and examined. The successful development of the microneedle gripper system would decrease tissue damage and reduce localized stress associated with the grippers, resulting in fewer tears.

Research Plans for the Next 2 Years

The researchers plan to conduct materials characterization and biocompatibility testing of the device and its components. They will perform a pilot study of human skin expansion in the prototype device. They will assemble study reports and information for submission to the Wake Forest University IRB. They will hold a pre-IDE/IND meeting with the FDA and prepare an IDE/IND submission. They will construct a clinically applicable skin expander system. Finally, they will seek IRB and FDA approval for a clinical trial and begin a Phase 1 clinical trial.

Planned Clinical Transitions

Because the skin expansion uses equipment without any cellular components, it is defined as a device and will be under regulation of the devices section of the FDA. The research team is working toward obtaining FDA approval for a prospective, multicenter, nonrandomized, uncontrolled pilot study (Feasibility/Phase 1). The researchers are currently seeking IRB approval.


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Engineered Skin Substitutes

Project 4.7.2, RCCC

Team Leader(s): Steven Boyce, PhD (University of Cincinnati) and Richard Clark, MD (Stony Brook University)

Project Team Members: Dorothy Supp, PhD (University of Cincinnati)

Collaborator(s): None

Therapy: Autologous ESSs.

Deliverable(s): Advanced therapy for extensive, deep burns.

TRL Progress: 2009, TRL 3/4; 2010, TRL 3/4; 2011, TRL 4; Target, TRL 4

Key Accomplishments: The researchers adapted prior protocols to increase the efficiency of melanocyte transplantation. They obtained uniform skin color with melanocyte densities that are clinically relevant. Tumorigenicity testing showed no detectable risk of tumor formation.

Key Words: Burns, engineered skin, pigmentation

Introduction

Mortality and morbidity from burns, trauma, and other skin loss injuries remain significant medical and socioeconomic problems estimated to cost more than $1 billion annually in treatment costs and lost productivity. Burns in the civilian population cause more than 900,000 hospital days in the United States annually, and full-thickness burns require treatment by excisional debridement and split-thickness skin grafting. Autografting donor skin is the procedure of choice to treat these burns, but victims of large burns do not have sufficient donor skin to complete grafting without multiple reharvesting of donor sites at 7- to 10-day intervals. With each harvest, healing time increases as epithelial sources (i.e., glands and follicles) are removed, leaving wounds and donor sites susceptible to microbial contamination. Sepsis, which develops in part from microbial contamination and invasion in wounds, accounts for 75% of deaths from burn injuries and is often associated with multiple organ failure. Other major aspects of recovery from burns, including immune function, positive nitrogen balance, and physical therapy, all depend on completion of wound closure. A significant source of long-term morbidity is scar development at both the donor sites of skin grafts and in wounds grafted with meshed and widely expanded skin grafts. Conversely, it is well known that grafting of wounds with sheet grafts suppresses scar formation.

To circumvent these difficulties, preclinical and clinical studies over 20 years have led to the development of autologous ESSs.

Classified as a medical device, an ESS currently consists of a lyophilized sponge of collagen and chondroitin sulfate populated with cultured dermal fibroblasts and epidermal keratinocytes that organize into an analog of skin tissue **(Figure V-46)**. The device develops an epidermal barrier and a basement membrane similar to functional skin. ESSs release high levels of angiogenic growth factors, including but not limited to VEGF, basic fibroblast growth factor, and transforming growth factor-β1. The secretion of these factors is instrumental in the integration of the transplanted ESS with the host tissue and vascularization. In addition, both keratinocytes and fibroblasts in culture are known to release inflammatory mediators that promote transient development of fibrovascular tissue.

Transplantation of human melanocytes for restoration of skin color has been demonstrated previously in the Boyce laboratory and by other investigators. The Boyce team's model is to graft ESSs to full-thickness, surgical wounds in athymic or severe combined immunodeficient mice. After healing for 4–8 weeks, ESSs with human melanocytes express pigment that remains with the grafted skin indefinitely (Figure V-46). This model has been validated and is highly reliable for testing the tumorigenicity of human melanocytes or other cell types in ESSs.

Summary of Research Completed in Years 1 and 2

During the first year of the project, the researchers established advanced models of ESSs with pigmentation and vascular analogs. They also demonstrated feasibility for advancement to animal studies. During the second year of the project, the research team calibrated human melanocyte densities in cocultures of human melanocytes and human keratinocytes for ESSs using flow cytometry. They completed animal studies of human melanocyte transplantation with restoration of skin color. They developed an enzyme-linked immunosorbent assay for CD31 to track human dermal microvascular endothelial cells (HDMECs) in ESSs. They transplanted human fibroblasts-HDMEC cocultures to athymic mice. Finally, they used CAD-CAM engineering of microperforations in biopolymer substrates to develop vascular channels in ESSs.

Research Progress – Year 3

During the past year, the research approach for the project was changed by the addition of human melanocytes to ESSs, resulting in more predictable efficiency and greater procedural reliability. In Figure V-46, the left image shows a human ESS containing no melanocytes. The right image shows an ESS

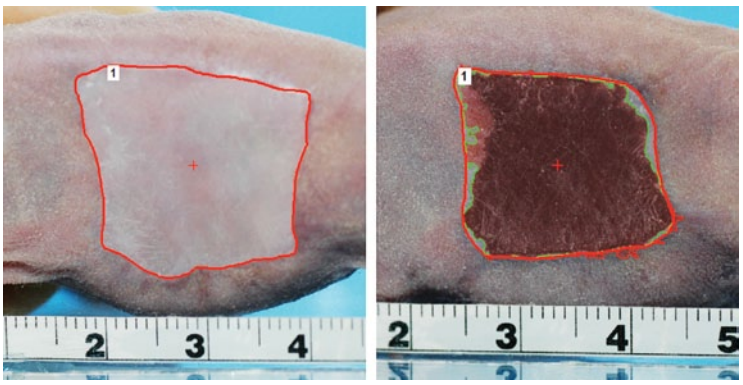

Figure V-46. Addition of natural color from melanocytes to ESS. Red contour is the edge of the human skin. Green contours are pigmented fields of human skin. (Left) No added melanocytes resulted in light skin. (Right) Simultaneous addition of melanocytes and epidermal keratinocytes generates virtually complete pigmentation. Scale in centimeters.

that was co-inoculated with human keratinocytes prior to implantation and was assessed for the development of skin color. The inclusion of human melanocytes created a uniform pigmentation in the graft area.

Key Research Accomplishments

- Completed restoration of skin color in an autologous ESS.
- Developed protocols to translate research procedures into a testable therapy.
- Developed intellectual property and technology transfer to a commercial partner.

Conclusions

In year 3, the Boyce group demonstrated that the addition of human melanocytes to an ESS resulted in full pigmentation. Protocols were then established to propagate autologously harvested human melanocytes. The research team found that it could expand sufficient numbers of cells in culture to seed in ESS in a time frame sufficiently rapid to meet the surgical schedules for burn victims. They performed tests to assess the tumorigenicity of transplanted human melanocytes. Results indicated that these cells do not have tumor-forming potential in immunodeficient mice. These studies have set the stage for review by the FDA and for planning clinical trials.

Research Plans for the Next 2 Years

During years 4 and 5 of the project, the researchers plan to complete preclinical studies and initiate plans for clinical studies. These plans will require advice from the FDA regarding the regulatory designation of this therapy, identification of a sponsor for the clinical study, and a commitment of support for the study.


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Autologous Human Debrided Adipose-Derived Stem Cells for Wound Repair in Traumatic Burn Injuries

Project 4.6.8, USAISR

Team Leader(s): Robert J. Christy, PhD (USAISR)

Project Team Members: Shanmugasundaram Natesan, PhD, Nicole Wrice, and Sharanda Hardy (USAISR)

Collaborator(s): Laura Suggs, PhD (University of Texas at Austin)

Therapy: Burn injury.

Deliverable(s): Dermal equivalent using autologous stem cells.

TRL Progress: 2009, TRL 3; 2010, TRL 3; 2011, TRL 4; Target, TRL 5

Key Accomplishments: The researchers are using a PEG-fibrin biomaterial and ASCs as a strategy for the revascularization of traumatized tissue. They isolated viable human ASCs from debrided burn patient tissue and evaluated viability and confirmed the multilineage differentiation potential of the cells. They also demonstrated the in vivo vascularization potential of ASC-PEGylated fibrin gel using a full-thickness excision wound in a rat model. They determined that human ASC-PEG-fibrin constructs have the ability to enhance the vascularization of a healing wound better than PEG-fibrin hydrogels alone.

Key Words: Debrided human adipose stem cells, burn therapy, revascularization, epithelialization

Introduction

Thermal injury accounts for approximately 5% of combat casualties, involving large TBSAs, and continues to be a significant source of morbidity. While a wide variety of dermal matrices have been developed, the lack of a host cell source and the time involved for cell expansion have limited their clinical application. The overall objective of this project is to develop a laminar skin substitute for military personnel using a dermal equivalent containing hydrogel-based biomatrices and ASCs that eventually differentiate into both epidermal and vascularized dermal layers. The biomatrices will be engineered to control cell differentiation in the absence of growth factor supplementation of culture media. An epidermal cell sheet derived from ASCs will be layered over the dermal equivalent to get a laminar skin substitute.

The discovery of multipotent stem cells within the stromal fraction of adipose tissue prompted their use for the healing and reconstruction of many tissues. ASCs differentiate into multiple phenotypes, including adipose, muscle, bone, neuronal, endothelial, hepatocyte, and epithelial-like cells. ASCs are easily isolated from the stromal vasculature of subcutaneous adipose tissue by liposuction with a minimally invasive procedure, and the excised adipose contains 100 to 1,000 times more pluripotent cells per cubic centimeter than bone marrow. The wound-healing

effects of ASCs were also verified with an in vivo animal study, demonstrating that ASCs significantly reduced wound size and accelerated re-epithelialization. This makes adipose tissue an attractive in vivo cellular source of autologous stem cells for regenerative therapies. The Christy laboratory has therefore hypothesized that autologous ASCs can be used to produce a clinically relevant tissue-engineered skin equivalent.

Summary of Research Completed in Year 1 (funded in 2009)

During the first year of the project, the researchers evaluated viability and confirmed the multilineage differentiation potential of ASCs. They characterized ASCs in PEGylated fibrin hydrogels with regard to viability and differentiation into an endothelial cell phenotype. They designed a bilayered dermal equivalent using chitosan microspheres loaded with ASCs in collagen and PEGylated fibrin matrices. They initiated preclinical animal wound-healing studies.

Research Progress – Year 2

After severe burn injury, the source(s) of adipose tissue is limited by the amount of uninjured viable tissue as well as by limited clinical access to normal sources of adipose tissue (e.g., subcutaneous lipoaspirate). In particular, combat injuries are massive and often result in mortalities due to injuries involving loss of viable skin extending beyond 40%–60% TBSA. Often these wounds involve contiguous areas of open soft tissues, and wounds are subjected to serial debridement until definitive coverage or closure can be performed. These burn wounds are the most challenging pathological situation, requiring extensive reconstructive approaches. A standard practice during burn wound treatment is the surgical debridement of necrotic tissue associated with burn eschar to acquire a vital wound bed. Debridement often involves the removal of subcutaneous layers and associated tissue structures, including cells, microvessels, and portions of intact subcutaneous adipose tissue. This intact hypodermis associated with the debrided skin tissue is an affluent repository of viable autologous stem cells that can potentially be isolated and expanded in vitro.

The researchers hypothesized that the intrinsic property of subcutaneous adipose tissue to function as a key thermoregulator may also insulate, conserve, and protect the resident stem cells associated within the hypodermis even after severe burn injury. This hypodermal region of discarded human skin, after burn wound debridement, can be a source of autologous stem cells of perivascular origin. These cells can be easily isolated and can be culture expanded as a pure population of stem cells retaining their multilineage differentiation abilities.

Debrided human skin samples after burn injury that are usually discarded were collected from a total of 21 patients containing portions of viable hypodermis, and the first 4 consecutive samples of the series (male patients/donors) of varying ages (i.e., BH1–24, BH2–26, BH3–49, and BH4–24) were chosen for further analysis. The tissues were obtained from the USAISR Burn Unit (Fort Sam Houston, Texas) under an IRB-approved protocol. Initial isolation involved the physical separation of dermal–hypodermal junctions and removal of thrombus and necrotic cellular components from the hypodermis. During cell isolation, all of the debrided burn samples were separated into two fractions: a floating fraction, comprising most of the viable fat tissue, and a pellet fraction composed of mostly extracellular components associated with some hypodermis and remnant dermal debris

(Figure V-47). The floating hypodermal fraction yielded between 1.5 x 10^5–2.5 x 10^5 stem cells/mL irrespective of the skin tissue viability. These cells when subjected to serial passaging showed a four- to fivefold increase in cell number every 48 hours up to 4 passages (initial seeding density of 1.5 × 10^5 cells/25 cm^2 culture surface) after which the proliferation rate marginally decreased to 3–4 population doublings up to passage 8 (data not shown). The research team evaluated the viability and confirmed the multilineage differentiation potential of the ASCs **(Figure V-48)**.

The researchers initiated animal studies of ASC-PEG-fibrin constructs to determine the impact of vascularization in vivo. They demonstrated the in vivo vascularization potential of ASC-PEGylated fibrin gel using a full-thickness excision wound in a rat model **(Figure V-49)**. The research team demonstrated that human ASC-PEG-fibrin constructs have the ability to enhance the vascularization of a healing wound better than PEG-fibrin hydrogels alone. The capacity to which ASCs do this remains unknown. Studies to determine whether human ASCs differentiate into a vascular cell phenotype and whether the cells integrate with the host's vasculature are currently under way. However, ASCs have been shown to secrete growth factors that support neovascularization. Indeed, separate studies have revealed that ASCs upregulate VEGF A expression when seeded in PEG-fibrin hydrogels, and this observation may account for the more mature vascular network that is seen in vivo. The inoculation of autologous grafts or bioengineered composites with the host's vasculature is critical, and the use of autologous ASCs in these procedures may improve this process and be used therapeutically for other applications besides burn injury.

Key Research Accomplishments

- Isolated viable human ASCs from debrided burn patient tissue.
  - ♦ Evaluated viability and confirmed the multilineage differentiation potential of the ASCs.

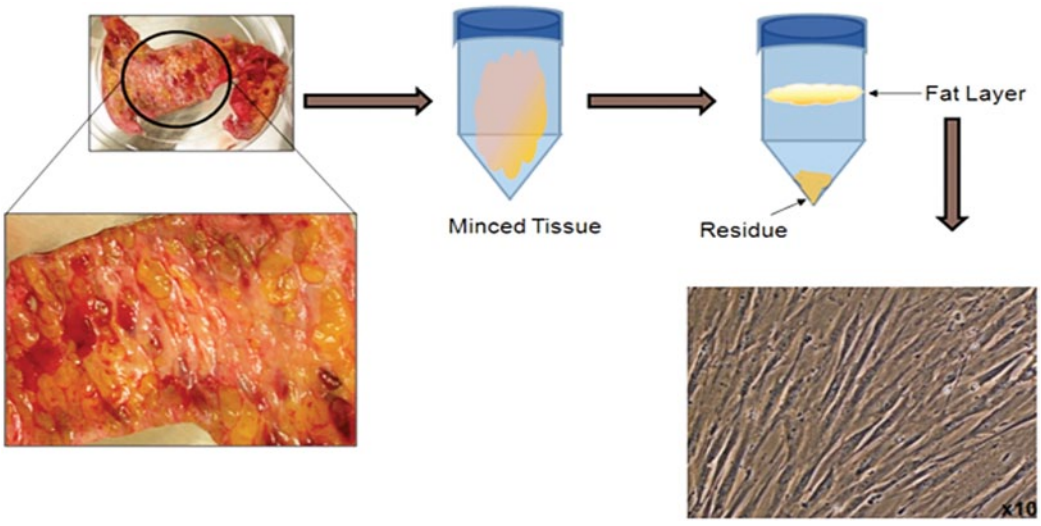


Figure V-47. Photograph of discarded human skin after burn wound debridement and procedure for the isolation of ASCs from debrided burn tissue.

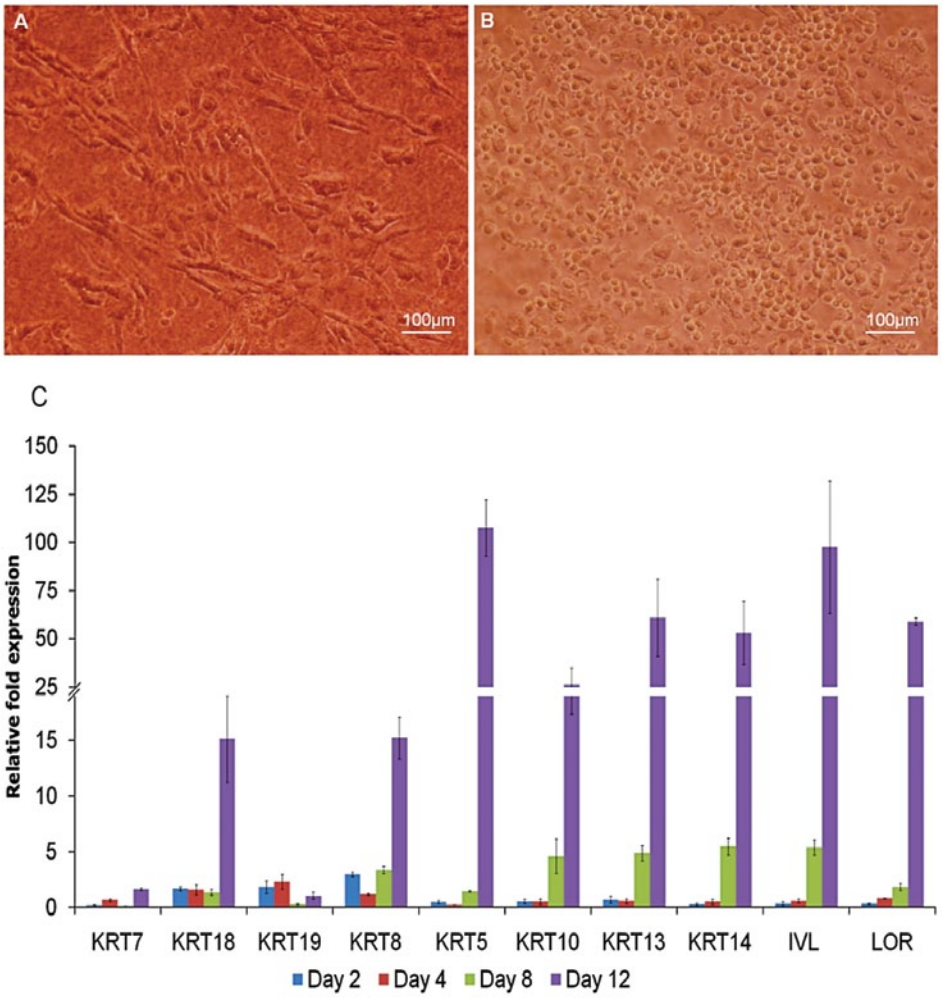


Figure V-48. Debrided skin ASC induction with differentiation factors. (A) Day 4 shows a squamous cell-like morphology. (B) Day 12 shows a stratified epithelial-like layer with a cuboidal cell morphology. (C) Reverse transcriptase polymerase chain reaction analysis of ASC for early and late markers of epithelial differentiation (days 2, 4, 8, and 12).

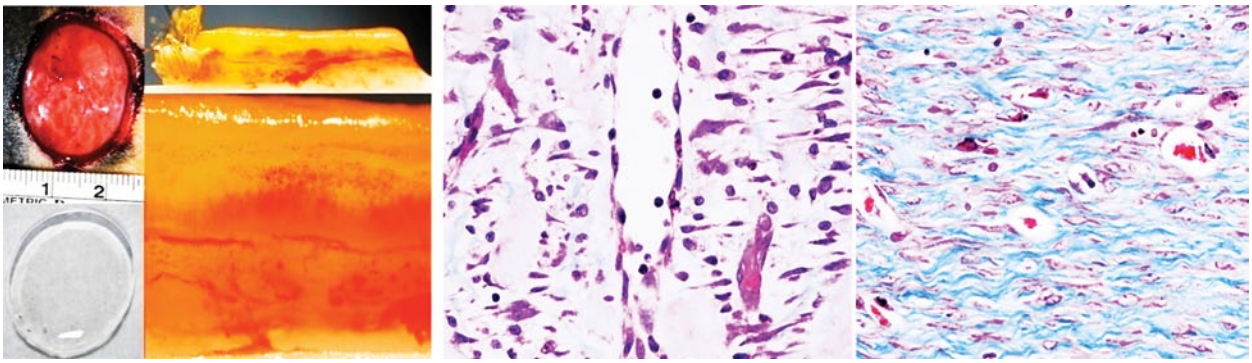


Figure V-49. Determination of the in vivo vascularization potential of ASC-PEGylated fibrin gel using a full-thickness excision wound in a rat model. (A) Full-thickness excision wound, PEGylated fibrin gel with human ASCs and its integration into the wound bed. Histological sections using Masson's trichrome staining of vascularized wound bed observed on day 8 (B) and day 12 (C).

- Demonstrated the in vivo vascularization potential of ASC-PEGylated fibrin gel using a full-thickness excision wound in a rat model.
    - Determined that human ASC-PEG-fibrin constructs have the ability to enhance the vascularization of a healing wound better than PEG-fibrin hydrogels alone.

Conclusions

Adult adipose tissues from various anatomical locations are an established repository of MSC populations. Yet, major physiological insults, such as extensive burn injuries, impose limitations on their availability for clinical therapeutic applications. The researchers are finding that the microenvironmental niche of subcutaneous adipose tissue protects the resident stem cell population, sustaining its ability to self-renew even after severe burn trauma.

Research Plans for the Next 2 Years

The researchers will continue to develop dermal equivalents that induce ASCs to differentiate toward vascular and dermal cells via properties of the scaffolds and through the integration of an epithelial cell sheet that is developed from ASCs. The present strategy is expected to provide an environment for the formation of blood vessels within the matrices that will increase the viability and survival of the graft thus facilitating its integration into both the burn and/or donor-site wound beds. This should lead to improved healing with less scar tissue formation.

Planned Clinical Transitions

Through a Cooperative Research and Development Agreement, the researchers plan to obtain a point-of-care device for the isolation of ASCs. This device will be used for the isolation of tissue from the debrided burn patient as well as from surgically isolated adipose tissue obtained from a newly approved IRB protocol. They will be initiating studies in a more clinically relevant porcine model and will use this model for the development of a large TBSA burn, which will provide a stringent test for their and other AFIRM-related skin equivalent products. They are continuing to address and use GMP products that are commercially available.

our science for their healing

A Multicenter Comparative Study of the ReCell® Device and Autologous Split-Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries

Project 4.2.7, WFPC

Team Leader(s): James Holmes, MD (Wake Forest University Baptist Medical Center Burn Center, Wake Forest University School of Medicine)

Project Team Members: Joseph Molnar, MD (Wake Forest University School of Medicine); Rajiv Sood, MD (University of Indiana); William Hickerson, MD (The University of Tennessee Health Science Center); David Mozingo, MD (University of Florida); Bruce Cairns, MD (University of North Carolina at Chapel Hill); Booker King, MD (USAISR); Kevin Foster, MD (Maricopa Integrated Health Systems); Marion Jordan, MD (Washington Hospital Center, DC); David Heimbach (University of Washington); Richard L. Gamelli, MD (Loyola University Medical Center); David Smith, MD (Tampa General/ University of South Florida); Michael Feldman, MD (Virginia Commonwealth University); Howard Smith, MD (Orlando Regional Medical Center); Tina Palmieri, MD (University of California, Davis); and John Griswold, MD (Texas Tech University)

Collaborator(s): Fiona Wood, MD (Royal Perth Hospital); William Dolphin (Avita Medical Limited); Andrew Quick (Avita Medical Americas, LLC); Annette Fagnant (MedDRA Assistance Inc.); and Susanne Panzera and Maureen Lyden (BioStat International, Inc.)

Therapy: Transplantation of autologous epidermal cells for treatment of second-degree burn injuries.

Deliverable(s): FDA-approved ReCell Autologous Cell Harvesting Device.

TRL Progress: Start of Program, N/A; End Year 1, TRL 7; End Year 2, TRL 7; End Year 3, TRL 7

Key Accomplishments: Significant accomplishments for this year include treatment of 35 subjects at 6 clinical sites, including 1 subject followed to 1-year post-treatment and FDA approval of enrollment at an additional 6 sites (over the 12 initially approved).

Key Words: ReCell system, cell spray, skin grafting, burns

Introduction

The skin, as the largest organ in the body, performs a range of vital protective, immunologic, neurosensory, thermoregulatory, and homeostatic functions. Therefore, any wound involving thermal, electrical, or chemical burn; trauma; abrasion; or laceration may seriously compromise the participation, performance, health, and, ultimately, the life of a patient. In addition to the acute, short-term effects of inadequate wound management, the long-term effects of wounds and wound scars include pain, restriction of movement, occupational limitations, disfigurement, and potential psychological impairment leading to lifelong disabilities, under-employment, and failure to fully reintegrate into society. The rapid and effective management of wounds of an injured warfighter is, therefore, a critical factor

in the determination of wound outcome and consequential morbidity and mortality.

The ReCell Device is based on previous work of Wood and Stoner and the recognition that autologous transplantation of epidermal cells could offer long-term wound closure in a clinically advantageous time frame while optimizing a patient's outcome. The device is designed to provide a simple, safe technique for the harvesting of epidermal cells for enhancement of epidermal repair. The initial step involves harvesting a split-thickness skin biopsy followed by separation of the dermis from the epidermis to harvest the cells of the epidermal-dermal junction. The separated cells are combined in suspension consisting of a mixed population of live keratinocytes, melanocytes, and papillary fibroblasts. The suspension is then sprayed onto the prepared wound bed. The cells migrate over the surface providing epidermal reconstruction with site-matched characteristics of color and texture. The applied cells are incorporated into the developing epidermis. The speed of re-epithelialization is very important as the "sealing" of the skin surface limits the inflammation that has been implicated as the pivotal factor in hypertrophic scar formation. By providing a source of viable and metabolically responsive epithelial cells on the wound surface, the ReCell Device technology may facilitate rapid wound healing while minimizing donor site morbidity and potentially eliminating or minimizing scar formation.

The aim of this research project is to collect clinical data to demonstrate the safety and effectiveness of the ReCell Device compared with the standard of care, split-thickness meshed grafts (STMGs), for the treatment of second-degree burn wounds. The results from this study will be used to support a premarket application to the FDA for the ReCell Device. For the regulatory application, the effectiveness hypotheses to be supported are: (1) noninferiority with the primary efficacy end point defined as recipient site wound closure at the week 4 follow-up visit of the ReCell-treated area as compared to that of the STMG-treated area and (2) superiority in the healing of the ReCell donor site as compared to the STMG donor site at week 1. Safety will be established via review of adverse events through week 16. However, in accordance with the AFIRM grant, subjects will be followed for up to 52 weeks following randomization to collect additional data pertaining to wound healing appearance/scar formation. The target enrollment in this study for evaluation of the regulatory hypotheses is 106 subjects (adjusted upward by 15% to account for potential withdrawals or nonevaluable subjects). This number of accrued subjects is also sufficient to assess the longer-term outcomes of scar formation consistent with the AFIRM grant objectives.

Since the ReCell Device is currently an unapproved technology in the United States, initiation of the clinical trial was contingent on obtaining approval from FDA/Center for Biologics Evaluation and Research (CBER) for an IDE application. Avita Medical Limited submitted the AFIRM study protocol to the FDA in April 2009, and through successful negotiations with the agency, obtained final approval for the IDE in December 2009. In parallel with the IDE process, the program collaborators identified and engaged with potential investigational sites and set up the infrastructure (i.e., clinical monitoring, clinical management, data management, and central reading facility) necessary to execute the trial.

Analysis of Competitive Technologies

The main competitive technology for ReCell for treating burn patients is the split-thickness skin graft. This has been the standard of care for more than 80 years and is used as either a

full sheet or meshed graft depending on the size of the area to be covered and the availability of donor sites. The cultured epithelial autograft also is a technology that has been used to treat burn patients since the early 1990s. This is a laboratory-based procedure that takes skin cells from the patient, isolates the keratincytes, and uses them to grow sheets of new skin. The process requires special equipment and personnel and takes up to 14–21 days to develop the autograft sheets. They are typically used for large burns that are greater than 30% TBSA and where there are limited donor sites. There are still questions about final patient outcome and scar quality associated with cultured epithelial autografts.

Leveraged Funding

Avita Medical Limited has funded the participation in the project of several key employees and its senior medical advisor. They have participated in discussions with the FDA, developed the study protocol and clinical implementation plan, evaluated and trained clinical study sites, and developed and negotiated the contracts with each study site.

Summary of Research Completed in Year 1 (funded in 2009)

The researchers obtained both FDA and U.S. Army Medical Research and Materiel Command Office of Research Protections/ Human Research Protection Office (HRPO) approval of the study protocol, executed contracts with key vendors for support of the clinical trial (i.e., regulatory affairs, clinical management, biostatistics, data management, and the independent reading facility for primary end point adjudication), and initiated subject enrollment during the first 2 years of the study.

Research Progress – Year 2

On-site study initiation training visits were successfully conducted at seven sites, bringing the total number of sites trained and cleared to enroll to nine. Drs. Holmes and/or Quick attended the first ReCell case at the University of Tennessee, University of North Carolina, Maricopa Medical Center, and Washington Hospital Center. Including Drs. Rajiv Sood and Joseph Molnar, who had participated in an earlier pretrial study, ReCell subjects (totaling 35 as of June 18, 2011) were enrolled and treated at six clinical sites. At an operational level, refinements were made to the protocol documentation, instructions for use, and training/ reference materials. These have been brought forward via formal amendments in collaboration with HRPO and local IRBs. Enrollment levels are below expectation, in part because the protocol is a rigorous one, requiring significant commitment on the part of often overbooked investigators. This is being mitigated by the addition of five newly engaged clinical trial sites, which are anticipated to be cleared for enrollment in July 2011.

Target Product Profile

Product Description/Name

The ReCell Autologous Cell Harvesting Device is a stand-alone, battery operated, cell separation device containing enzymatic and carrier solutions, sterile surgical instruments, and spray applicators. The ReCell Device enables a thin split-thickness skin biopsy to be processed to separate cells in a suspension for immediate delivery onto a prepared skin surface. Instructions for use for the ReCell Device are provided as part of the draft labeling.

Regulatory Approval End Point

To be determined.

Anticipated Label Indication

The ReCell Autologous Cell Harvesting Device is intended to be used at point-of-care for the safe and rapid preparation of an epidermal cell suspension from a small sample of a patient's own skin. Under the supervision of a health care professional, the epidermal cell suspension produced by the ReCell System is suitable for the management of mechanically or surgically debrided wounds.

Competing Products

See "Analysis of Competitive Technologies."

Advantages over Competing Products

ReCell is a stand-alone, rapid, autologous cell harvesting, processing, and delivery technology that enables surgeons and clinicians to treat skin defects using a patient's own cells. Compared with conventional skin grafting methods, use of the ReCell technology minimizes the size of the donor site required to cover a wound site. For example, each ReCell Device can process a maximum biopsy of 2 cm^2. This will cover an area of approximately 320 cm^2. This is advantageous for reducing donor site morbidity and maximizing wound coverage when donor sites available for grafting are limited. Furthermore, the procedure is performed on-site, not requiring external laboratory facilities.

Key Research Accomplishments

- A total of 35 subjects have been enrolled.
- One subject has been followed through 1-year post-treatment.
- There are six actively enrolling sites.
- There are nine sites cleared to enroll subjects.
- Six additional sites are anticipated to be cleared to enroll by the end of July 2011.
- Informal review of the (non-blinded) site investigators' assessments of donor sites and burn wound healing (the study coprimary end points) for the first 23 subjects indicates that the results appear to be on track for demonstration of the effectiveness of ReCell.

Conclusions

The researchers have accomplished the enrollment and treatment of 35 subjects at six clinical sites, including following 1 subject all the way to 1-year post-treatment and obtaining FDA approval of enrollment at six additional sites.

Research Plans for the Next 2 Years

The researchers are now aiming to complete their trial enrollment goal, which is the accrual of 106 subjects.

Planned Clinical Transitions

The clinical program will be transitioning from an execution phase to a final reporting phase with completion of subject accrual anticipated over the course of the next 6 to 9 months. Avita Medical Limited continues to be an industry collaborator on this program.

Corrections/Changes Planned for Year 4

The program time line has been extended by approximately 1 year due to program delays as a result of the challenges of enrolling subjects in the rigorous protocol approved by FDA/CBER.

VI: Compartment Syndrome

Background

The muscles of the arms and legs are surrounded and bound together by thick layers of connective tissue called fascia. The fascia, a dense, fibrous tissue with limited ability to stretch or expand, divides muscle groups into confined spaces called compartments through which travel muscles, nerves, and blood vessels. Compartment syndrome (CS) is a potentially serious medical condition in which increased pressure or swelling within a compartment compromises the blood supply to the muscles located within that space. CS can result from fractures, blunt and penetrating trauma, blast trauma, injury to blood vessels, and the return of blood flow to compartment tissues following a period of limited blood supply (reperfusion), such as that which may occur with the use of a combat tourniquet in the field. The treatment of CS requires the surgical release of the fascia that encloses the muscle compartment as soon as CS is diagnosed. The fascia should be cut open (known as a fasciotomy) within the first 3 to 6 hours to prevent irreversible injury to the muscles, nerves, and blood vessels (vasculature).

Soldiers who develop CS have prolonged recovery times, rarely recover complete muscle or nerve function, and usually do not return to active duty at the same level of performance. Most CS injuries of the extremities result in permanent disability. A safe and effective new therapy to replace and regenerate cells and tissues damaged by CS

USAISR researcher Monica Jolomo performs magnetic cell sorting of bone marrow-derived stem cells for studying bone regeneration.

A WFPC researcher measures muscle force in injured muscle after treatment of experimental animals with anti-fibrotic medicines.

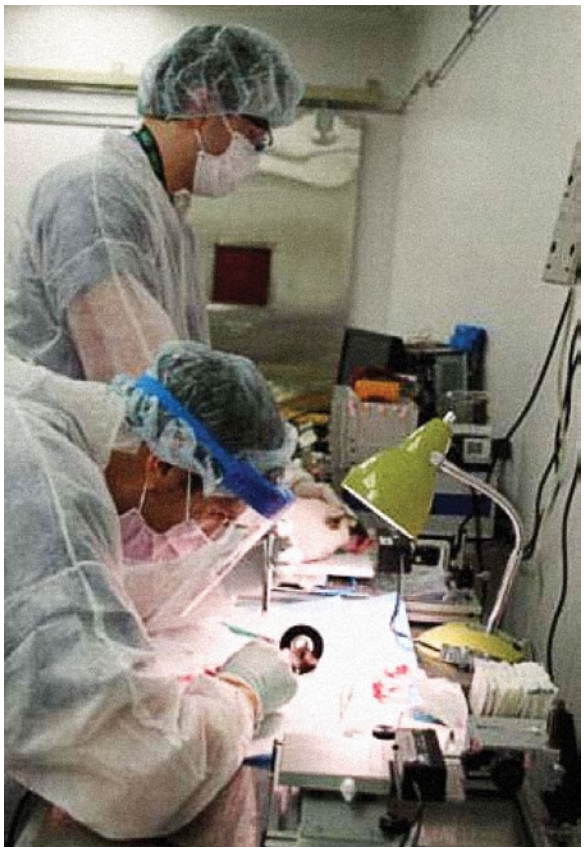
is urgently needed as there are no effective treatments available for military surgeons to overcome this important problem. The overall goal of the AFIRM CS Program is to reduce the impact of CS on wounded warriors and improve their functional recovery through the application of regenerative medicine. The projects seek to deploy a combination of stem cells and inductive biodegradable scaffolds for the reconstruction of functional compartment tissues with the aim of improving the functional outcome of limbs damaged by CS. The regenerative medicine technologies described herein have been used safely and effectively for civilian tissue injuries by AFIRM investigators and others and thus substantiate the rationale for using this approach to solve an important unmet need in the treatment of battlefield injuries.

Unmet Needs

While CS is well recognized as a limb-threatening injury that frequently results in chronic disability due to irreversible muscle and nerve damage, there has been almost no improvement in the treatment of this problem in more than 100 years since the advent of surgical fasciotomy. Research progress has been hindered by the failure, for decades, to develop a satisfactory animal model in which to study prospective treatments. The AFIRM is supporting the successful creation of platform animal models of CS and the instrumentation to quantitatively assess muscle and nerve regeneration in a precise, reproducible manner. These animal models have laid the foundation to test hypotheses that can advance regenerative medicine technologies designed to treat this important battlefield injury.

Untreated forearm CS can lead to Volkmann's contracture, a permanent shortening of musculature of the hand and forearm, which results in a claw-like deformity of the hand.

Failure of muscles and nerves to recover from advanced CS can lead to permanent paralysis of the affected limb. At that stage, amputation of the affected limb may be the patient's only remaining treatment option. Therefore, partial replacement of the dysfunctional tissue by living engineered muscle tissue is an attractive concept and an unmet need. AFIRM researchers are generating technologies that will provide an improved functional recovery for injured soldiers through the regeneration of muscle, nerve, and blood vessels lost to CS and other battlefield wounds.



WFPC researchers Adam Wright, MD, and Nick Oyster conduct animal surgery on a CS project.

Areas of Emphasis

AFIRM researchers are pursuing a complementary mix of research projects focused on various aspects of treatment of CS. Projects can be grouped into two "clinical challenge" topic areas: Cellular Therapy of CS and Biological Scaffold-Based Treatment of CS. Additional details on projects in each of these topic areas can be found in **Table VI-1** and subsequent sections of this chapter.

Table VI-1. Projects Funded by WFPC* and USAISR** per Clinical Challenge Topic Area

Clinical Challenge	Consortium/ Institution	Project No.	Project Title
Cellular Therapy of CS	WFPC	4.3.1	Cellular Therapy for the Treatment and Consequences of Compartment Syndrome
		4.3.2	Use of Bone Marrow-Derived Cells for Compartment Syndrome
	USAISR	4.3.6	Improving Cell-Based Approaches for Extremity Trauma
Biological Scaffold-Based Treatment of CS	WFPC	4.3.3	Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy
		4.3.4	Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome
		4.3.5	Material-Induced Host Cell Recruitment for Muscle Regeneration

**Wake Forest-Pittsburgh Consortium*
***U.S. Army Institute of Surgical Research*

Cellular Therapy of CS

Studies at WFPC

WFPC researchers are using human muscle-derived and bone marrow-derived stem and progenitor cells to reconstruct functional compartment tissues following the development of CS. In **Project 4.3.1**, the **Huard group** at the McGowan Institute for Regenerative Medicine (MIRM) and the **Soker group** at the Wake Forest Institute for Regenerative Medicine (WFIRM) are improving the prognosis following CS muscle injury by reducing fibrosis, improving the growth of blood vessels into affected tissue, and injecting autologous (a person's own) muscle-derived stem cells (MDSCs) into damaged tissue. The researchers have developed a novel small animal model of CS. They found that MDSCs can be implanted and tracked in the damaged muscle area and significantly reduce fibrosis in the injured area. They determined that the U.S. Food and Drug Administration (FDA)-approved drug losartan, which is used for hypertension and to prevent pathologic scarring after a heart attack, can serve as an effective treatment for CS by reducing fibrosis and increasing muscle function. Recently, the researchers developed a contusion model of skeletal muscle injury, which can be used to examine the effectiveness of various pressure reduction therapies. They have just received Institutional Review Board approval to collect biopsies from CS patients giving informed consent, which will allow for better characterization of the injury in human tissues under a variety of conditions. In collaboration with Dr. Kenton Gregory, the researchers plan to begin a losartan clinical trial to determine the effectiveness of this treatment in human CS patients.

The **Gregory group (Project 4.3.2)** at the Oregon Medical Laser Center (OMLC) has completed a pilot study to evaluate the effectiveness of autologous bone marrow mononuclear cell (BM-MNC) treatments in CS injuries in Sinclair mini-swine. The researchers developed an in vivo cell tracking technique that allows them to demonstrate extremely robust cell engraftment up to 3 months post-treatment. They have created and modified cell invasion assays and flow cytometry protocols that can assess the function, viability, and identification of cells used in their treatments. They have also developed a stem cell functionality assay. In the upcoming year, the research team will begin a 6-month safety and efficacy study with 8 control animals and 8 treatment animals. The team will also perform a cytotoxicity study in rodents to evaluate toxicology, biodistribution, tumorigenicity, and microbiological effects of its treatment protocol. In conjunction with these studies, the researchers will prepare an Investigational New Drug (IND) application with the hope of initiating a Phase 1 human clinical trial in year 5.

Studies at USAISR

Ischemia/reperfusion injury (I/R) can be caused by tourniquet application, vascular trauma, or acute CS. The **Rathbone/Walters group (Project 4.3.6)** at USAISR is developing cell-based regenerative medical approaches aimed at reducing the magnitude of injury, hastening healing, and improving the outcomes of wounded soldiers suffering from I/R-related muscle injuries. The researchers are focusing on autologous cell sources—specifically bone marrow stromal stem cells (BMSCs)—and methods of delivery that they expect will lead to the most rapid transition into clinical practice. They have established and validated an in vitro muscle system and software to determine functional outcomes following I/R injury. They have also completed preliminary studies aimed at determining the feasibility of injecting freshly isolated BMSCs into I/R-injured skeletal muscle. They plan to determine the most appropriate time point for injection and the influence of I/R injury on the regenerative potential of BMSCs. The researchers have recently established a collaboration with

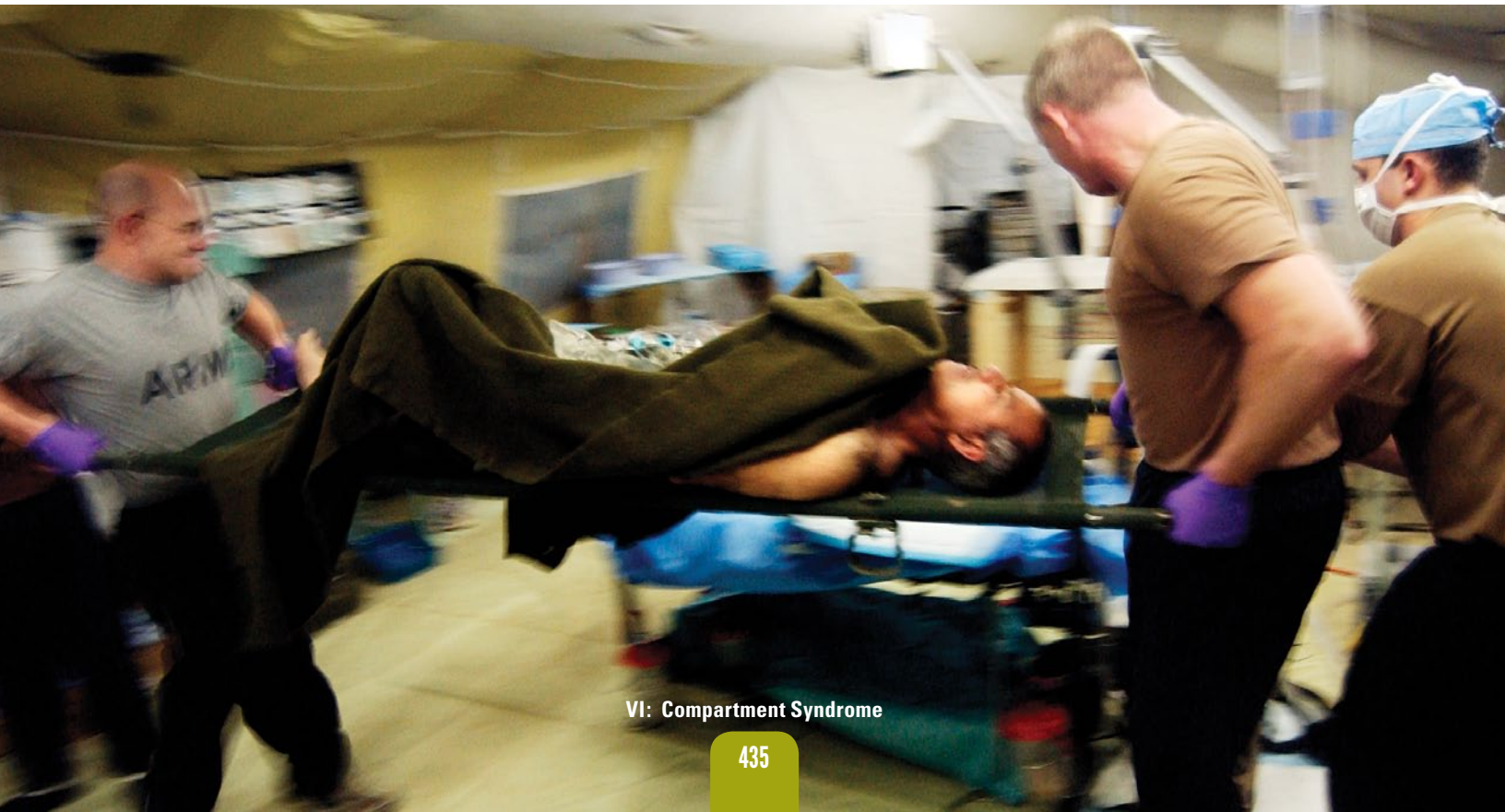
Arteriocyte to develop treatments for CS using a swine model of I/R injury.

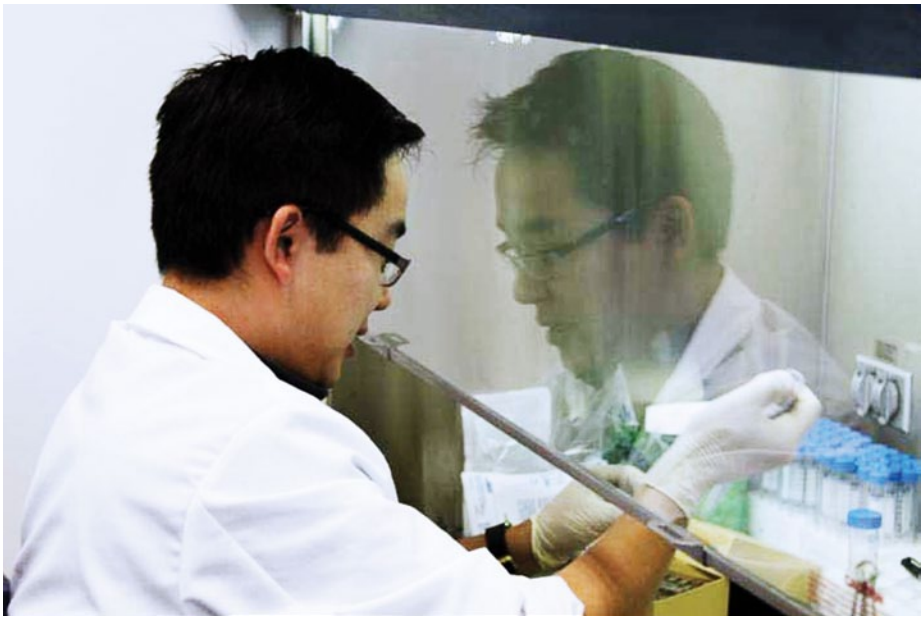
Biological Scaffold-Based Treatment of CS

Studies at WFPC

WFPC researchers are developing animal models of CS and implantable scaffolds that can be used to treat this potentially devastating condition. The **Wagner group (Project 4.3.3)** at the MIRM is focused on developing biodegradable scaffolds with elastic properties that can be integrated with autologous MDSCs to reconstruct fascia (thick, fibrous tissue that encloses and protects the organs) after abdominal CS injury. The researchers have created three scaffold designs that incorporate skin extracellular matrix (ECM) and an elastic polymer. They assessed the bioactivity and mechanical properties of these scaffolds in a full-thickness abdominal wall reconstruction model in the rat. The scaffold that performed the best in the rat model consisted of outer layers of elastic polymer surrounding an inner hybrid skin ECM-polymer layer. Eight weeks post-implantation, these "sandwich" scaffolds were visually and mechanically very similar to native tissue. The researchers incorporated fluorescently labeled MDSCs into the scaffold and observed abundant numbers of MDSCs within the scaffold 14 days post-implantation. They plan to initiate large animal trials with a porcine model in year 4 using cell-free constructs. Upon successful completion of the first set of porcine experiments, the researchers will engage the FDA in discussions to determine the preclinical data that would be required to justify filing for an Investigational Device Exemption and move toward clinical trials.

The **Badylak group (Project 4.3.4)** at the University of Pittsburgh is investigating a method for utilizing the inductive properties of ECM as a scaffold for the recruitment of endogenous (originating from within an organism) stem cells and the attachment, proliferation, and spatial organization of these cells into functional tissue. The researchers





WFPC researcher Dr. Hongshuai Li is growing populations of MDSCs that will be used in different projects in the laboratory.

are also developing methods of removing the cells from necrotic tissue (decellularization) while retaining native ECM. They found that treatment of necrotic tissue in a rabbit model of CS with a combination of saline, 0.1% peracetic acid (PAA), and 2% deoxycholate (DOC) led to complete decellularization of the affected tissue. Muscle fibers, nerves, fat cells, and connective tissue were found in the defect at 3 months post-treatment with saline/PAA/DOC. The researchers have recently expanded their study to include determination of the fate of injected autologous mesenchymal stem cells (MSCs) into the defect. In the upcoming year, they will move to a larger animal (porcine) model. If their large animal preclinical studies show positive results, they will prepare a human clinical trial for year 5.

The **Lee group (Project 4.3.5)** at Wake Forest University is focused on developing an approach to enhance the recruitment of endogenous stem and progenitor cells to the site of CS injury to increase the regenerative response. The researchers are using biomaterials containing myogenic (muscle cell)-inducing factors that can be implanted within the injured muscle compartment. They have demonstrated that host muscle progenitor cells can be mobilized into implanted biomaterials in situ and that these cells can be transformed into muscle cells using myogenic-inducing factors. The researchers also developed a novel combination of "systemic delivery" of substance P (SP) via intravenous injection and "local release" of stromal-derived factor-1α (SDF-1α) from an implanted scaffold. They found that the combined delivery system for both SP and SDF-1α further enhanced host stem cell recruitment into the implanted scaffolds. In the upcoming years, the Lee group will continue the long-term in vivo evaluation of its therapeutic scaffolds in a rat CS model. Overall, the study suggests that it may be possible to use the body's biologic and environmental resources for in situ muscle tissue regeneration.


our science for their healing

Cellular Therapy for the Treatment and Consequences of Compartment Syndrome

Project 4.3.1, WFPC

Team Leader(s): Johnny Huard, PhD (MIRM, University of Pittsburgh) and Shay Soker, PhD (WFIRM)

Project Team Members: Burhan Gharaibeh, PhD, Nick Oyster, BS, Michelle Witt, MS, Minakshi Poddar, MS, and Aaron Boyer, BS (University of Pittsburgh) and Tracy Criswell, PhD and Zhan Wang, PhD (Wake Forest University)

Collaborator(s): Kenton Gregory, MD and Rose Merten, BS, Bioengineering (OMLC); Robert Guldberg, PhD (Georgia Tech); and George Christ, PhD (Wake Forest University)

Therapy: Stem/progenitor cells and anti-fibrotics (losartan) for treatment of CS.

Deliverable(s): (1) Determine and compare the regenerative capacities of human MDSCs with that of human myoblasts after implantation in CS-injured skeletal muscle. (2) Investigate the effect of angiogenesis on the regenerative capacity of human MDSCs injected into CS-injured skeletal muscle. (3) Develop biological approaches to prevent and eliminate scar tissue and improve skeletal muscle healing after CS injury.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 3/4; End Year 3, TRL 4/5

Key Accomplishments: The Huard and Soker research teams have developed novel murine models of CS, allowing for a less expensive and more comprehensive study of the disease. Losartan has been used as an effective treatment for CS by reducing fibrosis and increasing muscle function. A method for fluorescently labeling a coculture of cells was developed allowing researchers to track the fate of the cells in vivo.

Key Words: Compartment syndrome, stem cells, pericytes, losartan, fibrosis, muscle

Introduction

Among the musculoskeletal injuries that result from battlefield trauma, the injury needing the most clinical implementation is CS. CS is a serious injury best described as an increase in pressure in an enclosed space leading to compromised circulation, tissue necrosis, rhabdomyolysis, renal failure, and even death. A variety of combat-related injuries, including fractures, contusions, burns, trauma, post-ischemic swelling, and gunshot wounds, have been found to be the initiating factors for CS. Diagnosis is primarily made by clinical observations to assess the six "Ps": pain, paresthesia, paresis (paralysis), pulse, pink (color), and pressure. Treatment for CS is aimed at eliminating or, at a minimum, reducing the elevated pressure in the compartment to minimize the damage to the tissues within the compartment. Clinically, fasciotomy is the most common, if not the sole, method of alleviating elevated compartment pressure but is less than ideal given the opportunity for infection and scarring at the incision site(s). Even when a fasciotomy is performed, the pressure may remain elevated to a point that damages underlying tissues, or the fasciotomy can be performed after tissue damage has already occurred (i.e., delayed diagnosis).

The aims of this research project are designed to improve prognosis following CS muscle injury by reducing fibrosis and improving angiogenesis through the injection of MDSCs into damaged tissue. A novel murine animal model of CS has been developed allowing for comprehensive in vivo studies

testing the efficacy of the project's proposed treatments. More recently, another skeletal muscle injury model, contusion, was examined for occurrences of increased compartment pressure. Early data indicate that the contusion model may be an ideal model to examine the effectiveness of alternative (to fasciotomy) pressure reduction therapies.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers created a model of CS in a rat leg muscle using a combination of a tourniquet and an external compression device (ECD). They performed a microscopic evaluation of the tourniquet model using a variety of stains. They isolated, characterized, and banked human and rodent MDSCs to use in stem cell repair strategies. They found that MDSCs can be implanted and tracked in the damaged muscle area and lead to a significant reduction in fibrosis in the injured area. In addition, the researchers showed that endothelial cells cocultured with myoblasts enhanced muscle fiber formation and tissue growth on collagen-based scaffolds in vivo. Finally, they showed that the angiotensin receptor II blocker losartan reduced fibrosis and improved muscle function in damaged muscle in humans.

Research Progress – Year 3 (Huard Group)

Statistics

All of the results in this report were expressed as the mean ± standard deviation. Significance was calculated using the Student's t-test and differences were significant with p values ≤0.05.

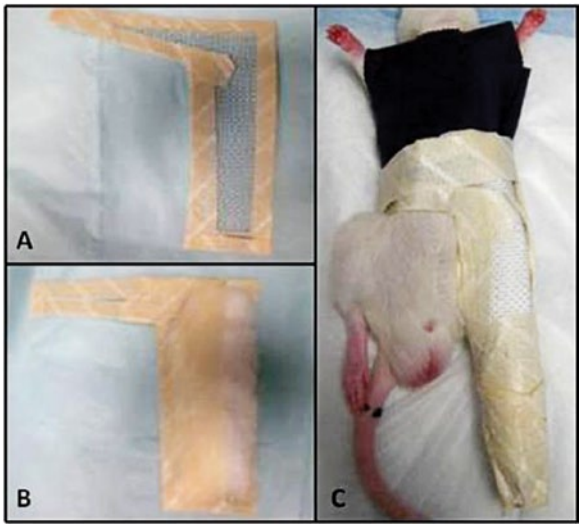


Figure VI-1. (A) The steel mesh wrap. (B) The steel mesh wrap lined with cotton balls and secured with adhesive tape. Two of these L-shaped components are used in the ECD. (C) The ECD secured on an animal during the procedure. The ECD consists of two cotton-lined mesh wraps and an elastic shirt to prevent the lower components from slipping out of position.

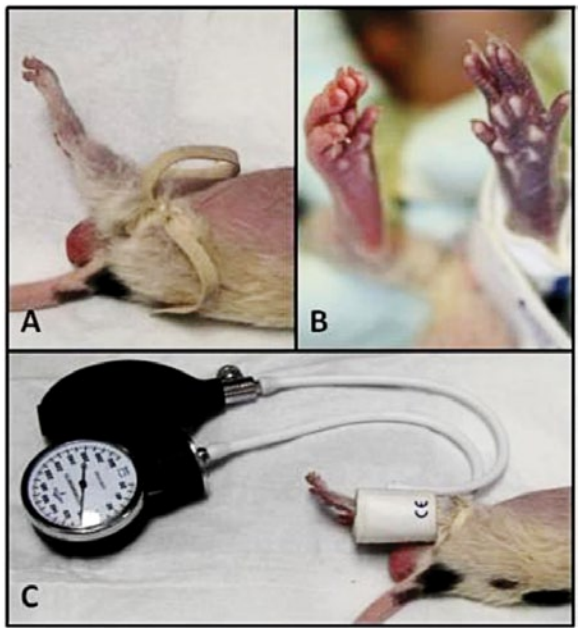


Figure VI-2. (A) A tourniquet is applied above the trochanter major creating ischemic conditions in the lower leg. (B) Once the cuff and tourniquet are applied, a lack of pink coloration in the sole of the affected foot indicates blood flow is restricted. (C) The cuff was inflated to a pressure between 300–320 mmHg using a bulb and gauge from a sphygmomanometer.

Animal Models

As reported last year, experiments examining CS used an ECD in conjunction with a tourniquet applied to just above the trochanter major to create CS in the hindlimb of Sprague Dawley rats **(Figure VI-1)**. The ECD was effective in creating an injury consistent with CS, but results were variable between users of the device, and manufacturing the devices was inconsistent. Differences in the size of the animals being injured and the size of each handmade ECD allowed for variances in the manner in which the ECD was secured over the anterior compartment. A less variable compression device (neonatal cuff) already being utilized by the Soker group (as a tourniquet) has replaced the ECD as the CS model's compression device **(Figure VI-2)**. Two methods of applying the cuff were tested (concurrent and consecutive application) to determine if compression during ischemia or reperfusion yields significantly different results. For the concurrent method, the tourniquet and cuff are applied at the same time. For the consecutive method, the tourniquet alone is applied and removed after 90 minutes; the moment the tourniquet is removed the cuff alone is applied for 90 minutes.

In parallel with the CS injury model described previously, the Huard laboratory studied the occurrence of CS following contusion injuries to determine if this model could be utilized as an early-stage CS animal model. The contusion model has been widely utilized by members of the laboratory for several years. To create this injury, the hindlimb was positioned by extending the knee and plantar flexing the ankle 90 degrees. A 16.2 g, 1.6 cm stainless steel ball was dropped from a height of 100 cm onto an impactor (a small rod on a spring) that hit the tibialis anterior (TA) muscle.

A pilot studying examining the occurrence of CS following contusion injury was conducted

using 4 C57BL/6J mice (8 weeks old). Prior to injury, the pressures in both posterior compartments were measured to confirm no significant differences. The posterior compartment was chosen as it is not the target of the contusion injury (anterior) and contains the gastrocnemius (GST) muscle, which is an ideal muscle for physiological testing. Compartment pressures were measured using a Stryker pressure gauge with a 27.5 gauge butterfly needle inserted into the posterior compartment perpendicular to the exterior surface. The compartment pressure was recorded after the pressure reading stabilized within 40 seconds of the start of the measurement. Twenty-four hours after a unilateral contusion injury, pressure measurements were recorded in the exact same manner in the injured limb and contralateral control limb. A statistically significant increase in pressure was seen in the posterior compartment in the contused limb compared to the contralateral control pressure post-injury. Pressures in the contralateral control posterior compartments showed no significant changes post-injury (pre: 5±2; post: 5±1; $p = 0.86$). The occurrences of swelling and edema were investigated by measuring the external diameter of the lower leg and differences in tissue weight between affected and control muscles. Twenty-four hours after contusion injury, the average diameter of injured limbs was 16% larger than contralateral control limbs. The contusion injury model does not utilize any external compression methods so increased compartment pressure will be caused by increased content within the compartment (i.e., edema). The additional compartment volume applies outward pressure on the surrounding fascia, which in turn applies an inward force on the contained tissues. While the fascia provides resistance to the increasing compartment volume, the fascia is pliable and it can expand to a certain extent. Due to the fascia's pliability, external compression of the fascia (ECD, neonatal cuff) was utilized in the CS injury model. An indirect measurement of edema was taken using the wet weights of injured and contralateral control TA and GST muscles. Injured TAs were an average of 29% heavier than controls (not significant). Injured GSTs were an average of 6% heavier than controls (not significant).

Loss of function was measured with an in situ muscle physiology apparatus that allowed for a direct calculation of the force generated by each muscle. TAs and GSTs from injured limbs both generated less force than contralateral controls.

These data reveal that the GST is being injured even though it is not the target of the contusion injury nor is it in the same compartment as the TA (posterior vs. anterior). Further experiments are being conducted to determine if the contusion model does in fact directly injure the GST or if the reduced function and edema are due to CS.

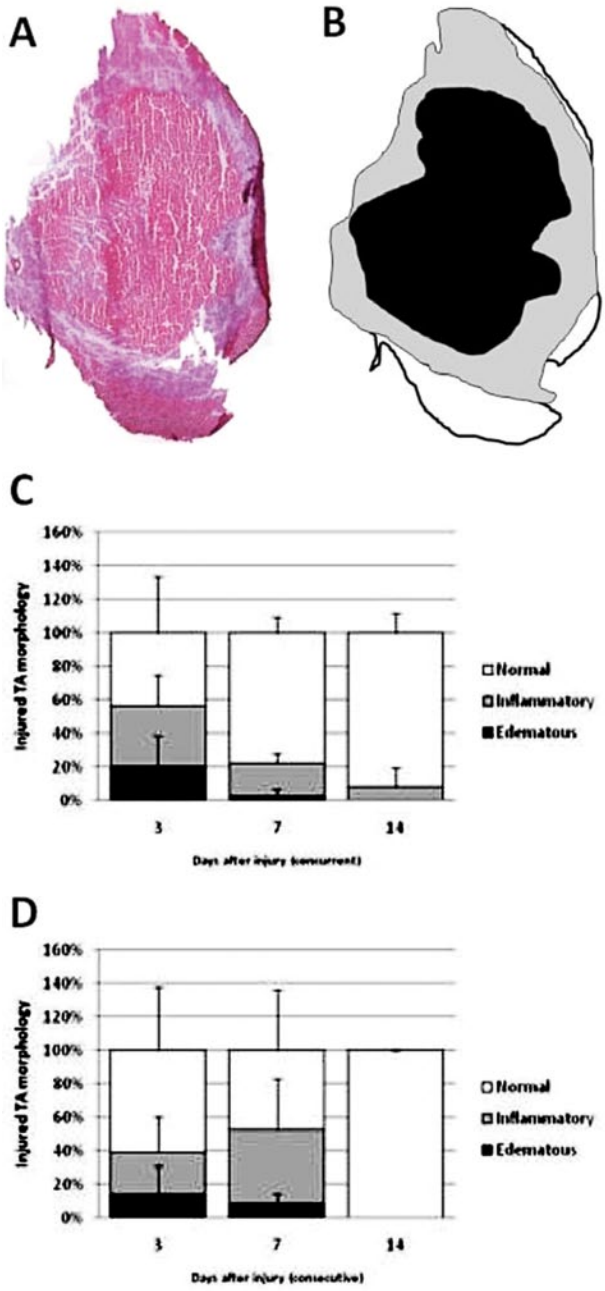


Figure VI-3. (A) Composite image of hematoxylin and eosin (H&E)-stained TA 3 days after CS injury. (B) Various shades represent the three tissue morphologies present in image A. (C) Analysis of composite images from TAs injured with concurrent application. (D) Analysis from TAs injured with consecutive application.

Characterization of CS Skeletal Muscle Injury

All data presented after this point use the tourniquet and cuff injury model.

H&E- and Masson's trichrome-stained TA muscle sections were quantifiably analyzed for statistically significant differences **(Figure VI-3)**. H&E-stained muscles were examined to identify areas of normal, inflammatory, or edematous tissue. Normal tissue was identified as consisting of non-centronucleated fibers with normal amounts of interstitial space between fibers. Inflammatory tissue was identified by having increased amounts of nucleated cells in the interstitial space. Immunostaining of tissue for the inflammatory cell marker CD68 was performed (data not shown) in parallel to confirm the nucleated cell infiltrate consisted of inflammatory cell types. Edematous tissue was characterized as having little to no H&E-positive cells or fibers as well as increased interstitial space. For both application methods (concurrent and consecutive), inflammatory and edematous tissue was seen at increased levels up to 7 days after injury (Figure VI-3). Between 7 and 14 days post-injury, the amount of normal tissue increased with edematous tissue not appearing in any tissue sections. There were no significant differences seen between application methods. Collagen deposition was examined in tissues by Masson's trichrome stain, and the images were analyzed with Northern Eclipse software to determine the level of collagen (blue) stained tissue. All injured animals showed increased collagen deposition in TA muscles within 72 hours after injury. The level of collagen trended toward an increase between 3 and 7 days but decreased to baseline levels by 14 days. As with H&E analysis, there were no significant differences between application methods.

Functional tests were conducted in the same manner described previously for contusion-injured animals. Only the TA muscles were isolated for physiological testing as these were analyzed histologically as well. Data displayed in **Figure VI-4** show significantly reduced muscle function 3 days following either injury method. Muscles injured with consecutive application show significantly reduced force 7 days after injury, but significance is lost by 14 days. Muscles injured with concurrent application did not show significantly reduced force after 3 days. Trends indicated increasing the n for each time point would be useful in achieving statistically significant differences. Not displayed in the plot, forces generated 28 days after injury using the concurrent application were 58%±12% of contralateral controls. These data indicate there is a level of endogenous healing and improvement of TA function without any intervention. With this in mind, assessment of therapies will be made within the first 7 days of injury so the benefits of treatment are not lost in the animals' natural improvement.

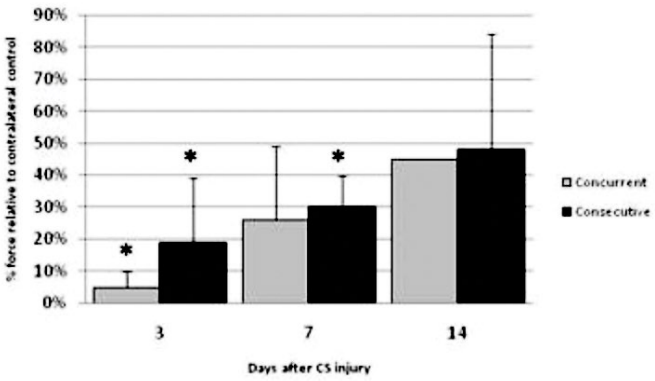


Figure VI-4. Relative (to contralateral controls) tetanic forces of TAs following CS injury using two cuff application methods. *$p < 0.005$

Losartan Treatment Improves Functional Outcome Following CS

Losartan was administered at a dose of 10 mg/kg/day, 72 hours after CS injury. The dose and time of administration were determined in another study conducted in the Huard laboratory. Functional testing was conducted 4 and 11 days after administration of losartan (7 and 14 days, respectively, after injury). Data presented in **Figure VI-5** show tetanic force as a percentage of contralateral control muscles under control (black) conditions and after losartan (gray) treatment (i.e., gray bars are the additional force generated from losartan treatment). Initial data show functional improvement following CS injury when losartan is administered 72 hours after injury at a dosage of 10 mg/kg/day.

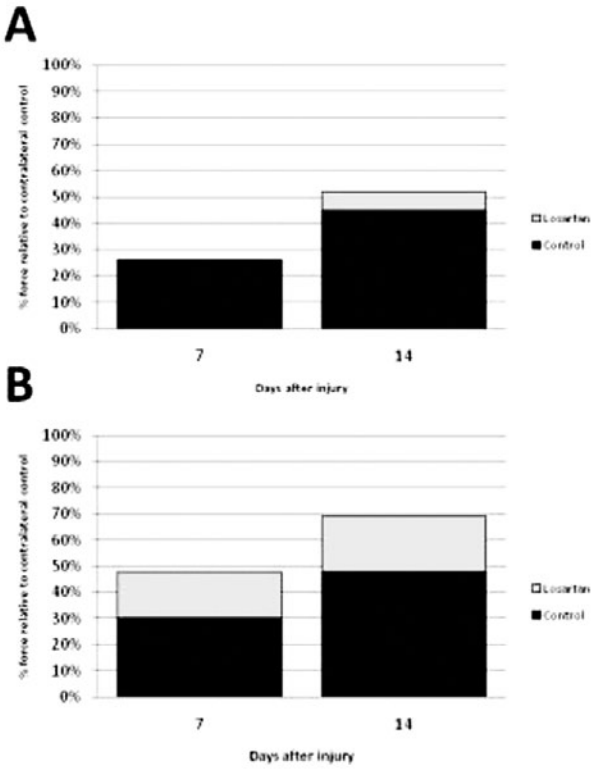


Figure VI-5. Tetanic force of CS- injured TAs as a percentage of the contralateral control TA. (A) Concurrent application. (B) Consecutive application.

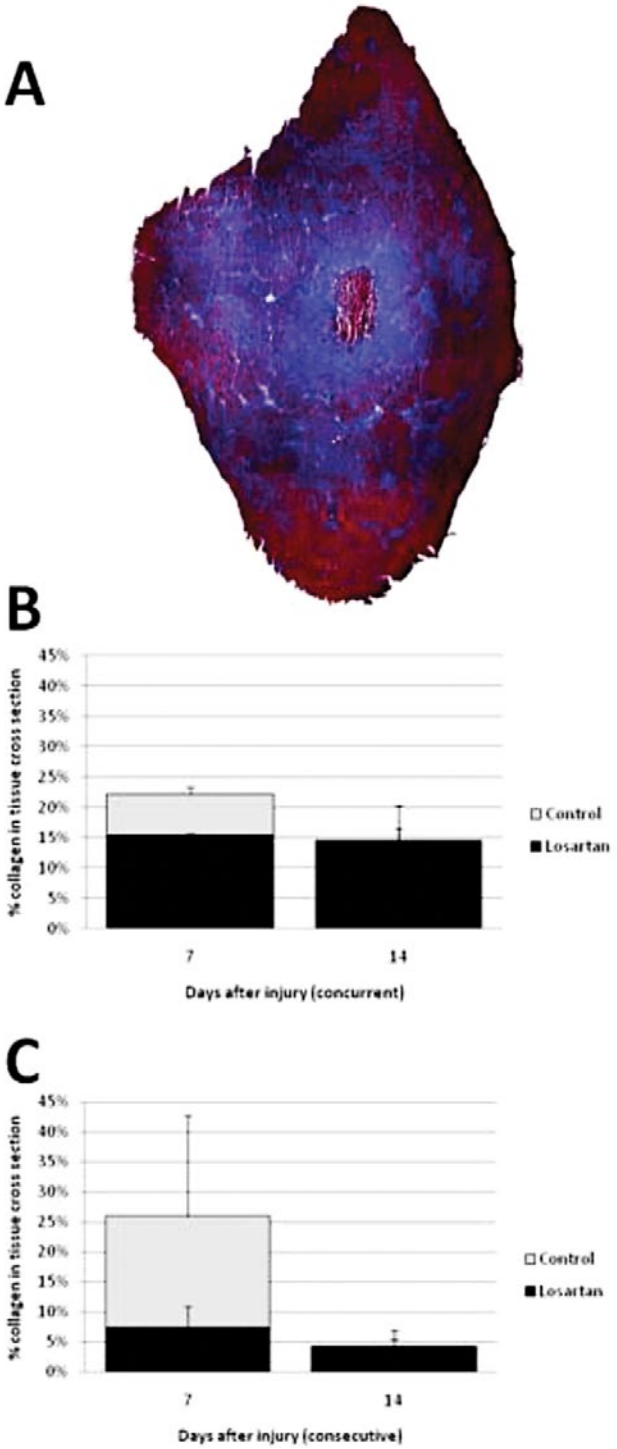


Figure VI-6. Analysis of fibrosis in TAs. (A) Representative image of Masson's trichrome-stained TA. (B) Collagen deposition seen after concurrent cuff application. (C) Collagen deposition seen after consecutive cuff application.

Losartan Treatment Reduces Fibrosis Following CS

As described previously, fibrosis was calculated by Masson's trichrome staining of 10 µm thick cross-sections of TAs and imaging (40x) the entire cross-section. The images were compiled to get an image similar to **Figure VI-6A**. Composite images were analyzed for the number of collagen-stained (blue) pixels relative to the total number of pixels stained. The trends for both cuff application methods are identical in that collagen-stained tissue is reduced in the presence of losartan after 4 days of treatment (7 days post-injury), but no difference is seen after 11 days of treatment (14 days post-injury) (Figure VI-6B, C). Differences were not significant due to the low n, but experiments are being repeated.

Key Research Accomplishments

- Finalized CS animal model using neonatal cuff and tourniquet.
- Determined that a contusion injury model may be a pre-CS model and could be used to test pressure-reducing therapies.
- Found that losartan effectively reduces fibrosis and improves skeletal muscle function after CS.

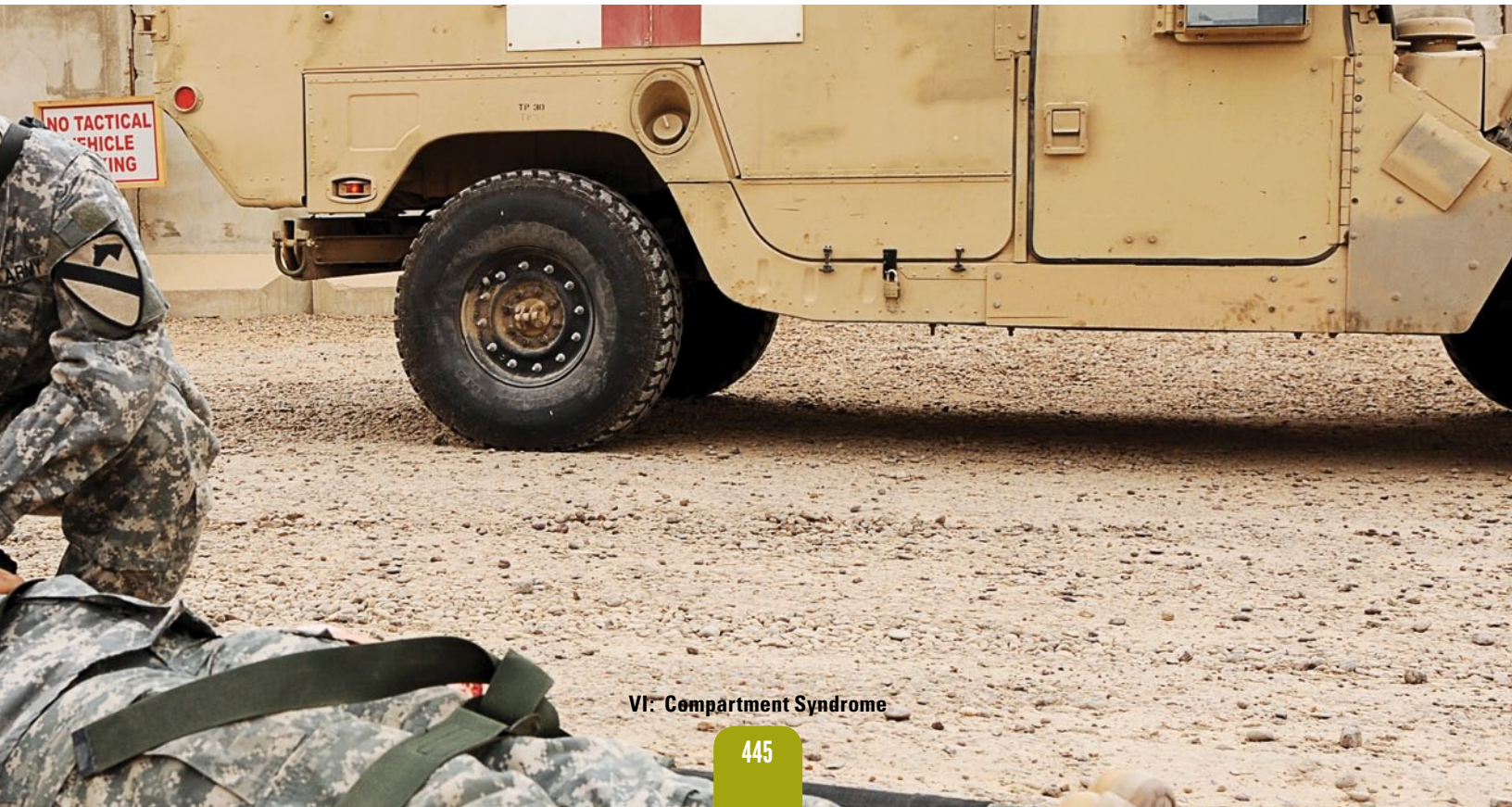
Conclusions

Data from the past year show that a consistent injury can be created with the neonatal cuff as a replacement for the previously utilized ECD. Compression during ischemia (concurrent) or reperfusion (consecutive) seems to have little effect on the outcome of the injury. Future studies will use the concurrent application technique because it is simpler from a logistic standpoint as both devices can be applied at one time, reducing the amount of time each animal is handled.

As mentioned, experiments need to be repeated to increase the n to achieve significant differences among treatment groups.

Research Plans for the Next 2 Years

Experiments using a specific subset of MDSCs as a therapy following CS injury have begun. This subset (ALDH high) shows increased survival under stressful (inflammatory, oxidative) in vitro and in vivo conditions. This subpopulation, along with unsorted MDSCs, will be transfected with angiogenic protagonistic (vascular endothelial growth factor [VEGF]) and antagonistic (SFLt-1) proteins to determine the effect of angiogenesis on the prognosis following CS. The researchers have just received Institutional Review Board approval to collect biopsies from CS patients giving informed consent. These data are very important for better characterization of the injury in human tissues under a variety of conditions. The results can be useful controls for future losartan-based clinical trials.



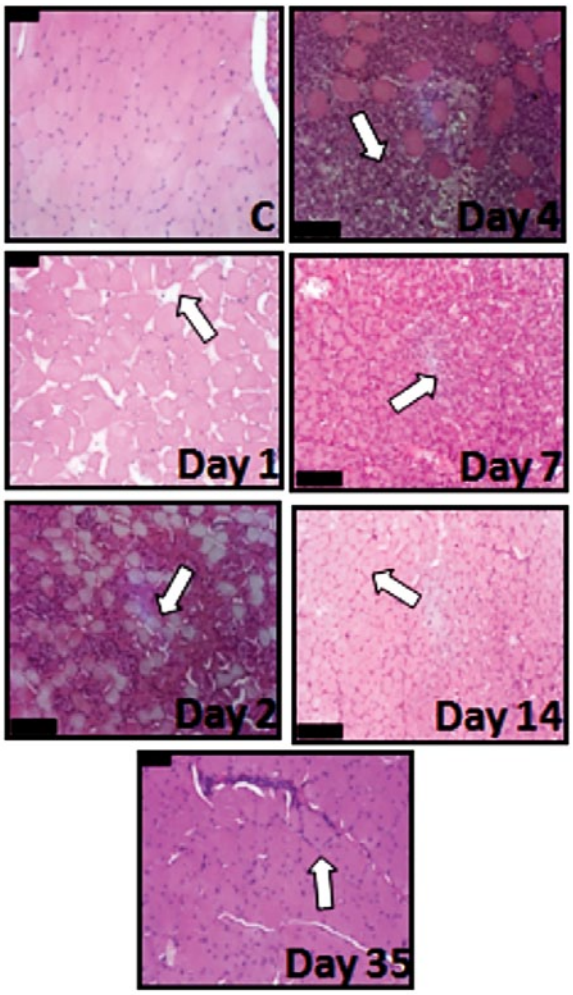


Figure VI-7. H&E analyses of damaged TA muscles after 120–140 mmHg applied for 3 hours. TA muscles were harvested and fixed in 10% NBF overnight then embedded in paraffin. H&E analyses of cross-sections at indicated time points after injury. Histological data are representative of three separate experiments each containing at least n=3. Magnification 200x.

Planned Clinical Transitions

The Huard group has applied for funding to begin a losartan clinical trial to determine the effectiveness of this treatment in human CS patients. If funded, the researchers anticipate to start recruiting in early 2012.

Research Progress – Year 3 (Soker Group)

I. CS Model Characterization

Histological Analyses of Muscle Damage

The researchers' model of CS utilizes neonatal blood pressure cuffs placed on the hindlimb of rats and held at 120–140 mmHg for 3 hours. The use of blood pressure cuffs allows for strict control of the amount of pressure given to each limb. In this way, each experiment is closely controlled, allowing for accurate data comparison between experimental groups. Damage to the TA muscle after CS induction was initially assessed by histology **(Figure VI-7)**. H&E analysis of explanted TA muscles was used to document degeneration and regeneration of the tissue. Edema and swelling are evident by the increased diameter and space between the individual muscle fibers 24 hours after injury (Day 1, arrow). Infiltration of immune cells was found 2 days after injury (Day 2, arrow), with maximal infiltration of neutrophils and monocytes occurring 4 days after injury (Day 4, arrow). Regenerating myofibers were detected beginning at 7 days (Day 7, arrow), and small but morphologically normal myofibers were observed by 14 days (Day 14, arrow). Healthy muscle tissue architecture was observed at 35 days although immature fibers, with centrally located nuclei, were still present (Day 35, arrow).

Analyses of Muscle Function
The researchers have concentrated their functional analyses on the damage of the TA muscle since it is well documented that many of the sequelae of blunt force trauma (i.e., CS) occur in the anterior muscle compartment. However, since the TA lacks a tendon at the distal end of the muscle it is not compatible with organ bath analysis. The extensor digitorum longus (EDL) muscle is located under the TA and receives a similar amount of pressure. The amount of damage observed in the EDL was correlated to the damage in the TA. Functional capacity of the anterior crural muscles (i.e., TA and EDL muscles) was determined using two methods: (1) indirectly, through organ bath studies on the EDL and (2) through direct measurement of TA function by recording the dorsiflexion force of the ankle after stimulation of the peroneal nerve. In vitro organ bath studies, soon after injury, showed both twitch and tetanic specific forces reduced by approximately 50% and 75%, respectively. Twitch forces were then elevated at 7 days post-injury before returning to values similar to control at 14 and 35 days post-injury. In contrast, peak tetanic force at 7 and 14 days post-injury remained reduced by approximately 30% but did produce forces similar to uninjured control values by 35 days post-injury.

In vivo functional assessment of the anterior crural muscles was determined via neural stimulation. The anterior crural muscle peak tetanic and twitch isometric torque was significantly reduced over the approximately 1-month period after injury. Specifically, peak isometric torque at 4, 14, and 35 days post-injury was approximately 84%, 55%, and 16% less than pre-injury values, respectively. While peak torque did not completely recover to pre-injury levels approximately 1 month after injury, there was a significant recovery of peak torque from 4 to 14 days and from 14 to 35 days. Conversely, twitch force did not exhibit significant recovery from 14 to 35 days post-injury. Notably, at 4 days post-injury neural stimulation at submaximal frequencies did not elicit a measurable torque response, and the magnitude of peak tetanic torque was comparable to twitch responses at other post-injury time points (e.g., 4 days tetanus versus 14 days twitch; 16.0 ± 2.6 vs. 14.0 ± 3.1 Nmm/kg).

Analysis of Neuromuscular Dysfunction
The discrepancy between the organ bath data and the in vivo function of the anterior crural muscles is suggestive of neural injury. To investigate neuronal damage, α-bungarotoxin (BTX) was used to label acetylcholine receptors at neuromuscular junctions (NMJs) **(Figure VI-8)**. Uninjured muscle showed normal staining of acetylcholine receptors within the NMJs as demonstrated by the typical folded morphology concentrated at the periphery of the myofibers. In contrast, 2 days after injury, injured myofibers showed a diffuse peripheral α-BTX staining pattern suggestive of denervated fibers.

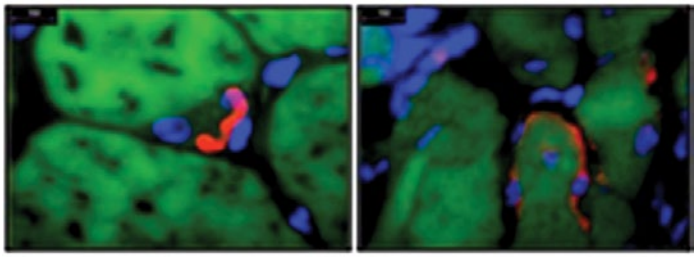


Figure VI-8. CS injury results in disruption of the NMJs. α-BTX staining of function acetylcholine receptors in a normal NMJ in a healthy myofiber (left panel) compared to the peripheral punctate staining found in a denervated myofiber (right panel) at 2 days after injury. Magnification 1,000x.

Analysis of Vasculature Disorganization
Histological data indicated a significant disruption of the vasculature after compression injury in the researchers' model. Since functional vasculature is required for efficient regeneration of skeletal muscle tissue, the researchers expected the vasculature to be restored during muscle tissue regeneration. They used fluorescent microscopy to observe vascular and muscle disruption and regeneration **(Figure VI-9)**. They took advantage of the autofluorescence of muscle fibers, which fluoresce in green, and of red blood cells, which fluoresce in green and red and, thus, are seen as yellow (Figure VI-9A). WGA-594 was used to delineate the plasma membranes of the myofibers. As early as 1 day after injury, gaps appeared within the muscle tissue (Day 1, arrow). Significant damage in the muscle tissue at 2 days was associated with blood vessel dilation and extravasation of red blood cells into the tissue (Day 2, arrow). By 4 days, large hemorrhagic areas were detected within the tissue (Day 4, arrow). Restoration of the vasculature began at 7 days, where vessel diameter decreased (Day 7, arrow), and was associated with the appearance of small muscle fibers with central nuclei. By 14 days, the vasculature was restored with small vessels associated with healthy myofibers (Day 14, arrow). Average blood vessel diameter was quantified during the 14-day period, showing increased vessel diameter at 2 days after injury with a peak increase in vessel diameter by 4 days and restoration of vessel diameter by 14 days.

Vascularization of Regenerating Skeletal Muscle

Using fluorescently labeled endothelial progenitor cells, pericytes, and myoblasts, the researchers have developed an in vitro culture system that will allow for the visualization of myoblast differentiation and the neovascularization of regenerated muscle tissue. Human umbilical vein endothelial cells (HUVECs), harvested from umbilical vein cord blood and sorted by fluorescence-activated cell sorting (FACS) for CD31 expression, were used as the source of endothelial progenitor cells. Murine 10T ½ cells were used as pericytes, and myoblasts were derived from single muscle fibers from green fluorescent protein (GFP)-positive mice.

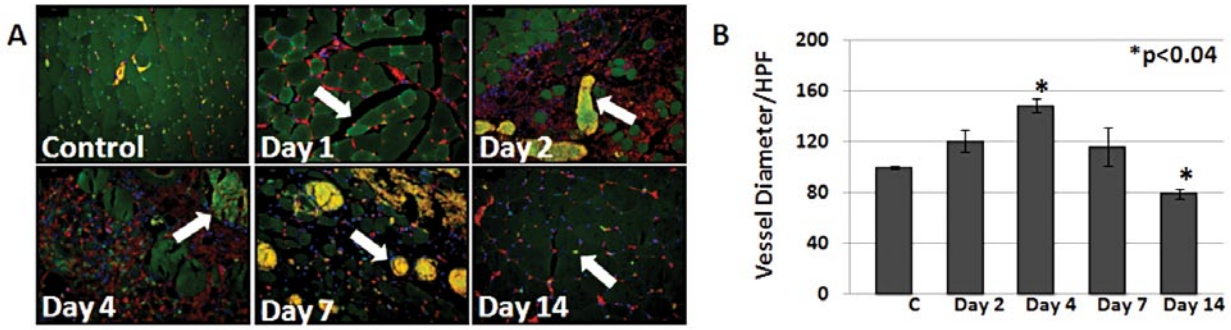


Figure VI-9. Muscle-associated vasculature injury and reformation accompanies muscle degeneration and regeneration. (A) WGA-594 was used to label cell membranes. The autofluorescence (green) of myofibers and the autofluorescence of red blood cells (yellow) were used to highlight the presence and disruption of vessels within the tissue. Day 1, enlargement of the myofibers and separation of the fascicles (arrow) indicate swelling and edema. Day 2, enlarged vessels are present (arrow). Day 4, enlarged vessels and hematoma formation were found within degenerating muscle tissue (arrow). Day 7, newly forming vessels were observed (arrow). Day 14, vessels are associated with newly regenerated myofibers (arrow). Magnification 400x. (B) ImageJ was used to quantitate the vessel diameter per high-power fields (HPF).

Cultures of fluorescently labeled myoblasts (green), HUVECs (red), and pericytes (faux blue), alone or together, were seeded on porcine bladder acellular matrix and implanted into the subcutaneous space of nude mice. Scaffolds were explanted after 8 weeks, embedded in paraffin blocks, and analyzed using standard histological methods.

Fluorescently labeled HUVECs were found to be integrated into mature vasculature on scaffolds after being grown in the subcutaneous space of nude mice for 8 weeks **(Figure VI-10A, B)**. Implanted myoblasts formed muscle tissue on the scaffolds as confirmed by immunohistochemistry with a GFP-specific antibody (Figure VI-10C, D). In addition, explanted scaffolds containing HUVECs and/or pericytes contained significantly more blood vessels than scaffolds seeded with myoblasts alone **(Figure VI-11)**.

Fluorescently labeled HUVECs and GFP-positive myoblasts were injected into the TA of uninjured rats to determine optimum cell number for cell therapy and the ability of these cells to survive in skeletal muscle tissue. The researchers were able to detect cells 24 hours after injection **(Figure VI-12)**.

These data suggest that the use of fluorescently labeled cells is a viable method of tracking stem cells being used in cell therapy for CS. Additionally, these data demonstrated that the use of endothelial cells in combination with myoblasts can improve vascularization and potentially improve the regenerative potential of the cellular therapy.

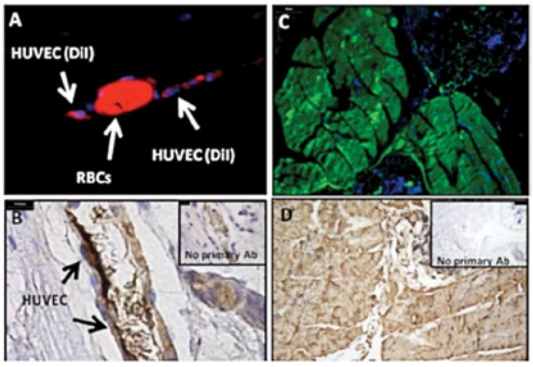


Figure VI-10. HUVECs integrate into vasculature, and myoblasts differentiate into muscle fibers when grown in the subcutaneous space of nude mice. HUVECs (A, B - red) and myoblasts (C, D - green).

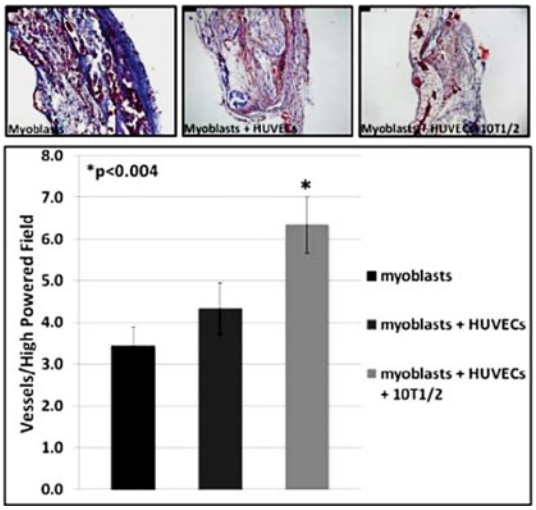


Figure VI-11. Scaffolds containing HUVECs and/or pericytes contained significantly more blood vessels than scaffolds seeded with myoblasts alone. Scaffolds seeded with combinations of cells were explanted from the subcutaneous space of nude mice. Vessels were counted on at least 15 HPF from at least 3 different experiments.

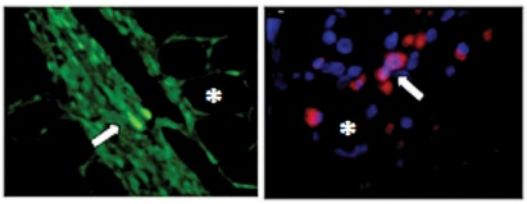


Figure VI-12. Fluorescently labeled cells can be detected and tracked after injection into muscle tissue. GFP-positive myoblasts (left panel, arrow) and HUVECs (right panel, arrow) were injected into the TA of a nude rat and imaged by fluorescent microscopy. Asterisk denotes host myofibers.

Growth of Myoblasts in Vitro

A significant number of myogenic precursor cells are required for muscle tissue engineering and cell therapy. The ex vivo expansion of myoblasts is problematic since they quickly lose their growth and differentiation potential. The researchers investigated different culture conditions to optimize the growth of these cells. They tested the growth and differentiation of myoblasts in MyoD media or complete media on either Matrigel or uncoated tissue culture plates. They found that MyoD culture media and Matrigel maintain the highest level of growth and differentiation potential of these cells **(Figure VI-13)**. This work provides a starting point for the development of a clinical protocol for the use of a defined culture media for the growth and expansion of human skeletal muscle precursor cells.

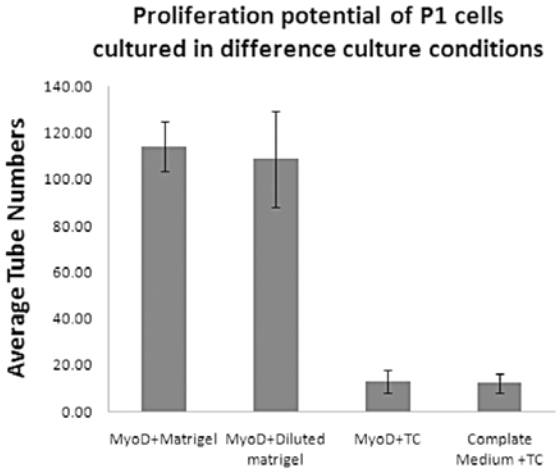


Figure VI-13. Myoblasts grown on Matrigel in MyoD media maintain their growth and differentiation potential. Myoblasts were disassociated from the skeletal muscle from GFP-positive mice and grown for 3 days before being plated on the indicated substrate in the indicated medium for 5 days. Myotubes were quantitated and compared between groups.

Key Research Accomplishments

- Characterized the anatomic changes in the muscular, vascular, and neural components of the hindlimb muscles following compression-induced damage in a reproducible rat model of CS that mimics CS seen in clinical patients.
- Documented muscle function after damage and during tissue regeneration using in vitro organ bath studies as well as an in vivo measure of dorsiflexion force of the ankle after stimulation of the peroneal nerve.
- Determined the optimal tissue culture conditions for maintaining the growth and differentiation potential of myoblasts in vitro.
- Developed a fluorescent cell-labeling system that allows for detection of stem cells at least 8 weeks after implantation.
- Developed a cell coculture system that allows for the growth of fluorescently labeled myoblasts, HUVECs, and pericytes in vivo.
  - Upon implantation into the subcutaneous space of nude mice, myoblasts differentiated into mature muscle fibers and HUVECs integrated into mature blood vessels.
- Determined that endothelial cells and/or pericytes cocultured with myoblasts enhance the vascularization of collagen-based scaffolds after implantation in vivo.

Conclusions

To better understand CS, the researchers have created an animal model of CS in rats by the use of a modified blood pressure cuff on the hindlimb that allows them to closely control the amount of pressure received by each animal. They characterized the damage induced to the muscle, vasculature, and neurons in this injury model. The process of degeneration and regeneration that occurs in their model closely mimics the sequelae of CS in humans. This model provides a unique opportunity to test the effects of human muscle cell therapy on tissue recovery after CS damage.

To follow muscle tissue regeneration after CS, the researchers developed a technique to grow and visualize fluorescently labeled HUVECs, pericytes, and myoblasts in a coculture system. They used this technique to follow muscle tissue regeneration, for up to 8 weeks, when the muscle constructs were implanted into the subcutaneous space of nude mice. They found that the presence of HUVECs and/or pericytes in the muscle construct increases the number of blood vessels in the regenerated tissue. These experiments demonstrate that the use of fluorescently labeled cells is a viable method of following cell fate during neovascularization and muscle tissue regeneration currently in vitro and in vivo.

Use of Bone Marrow-Derived Cells for Compartment Syndrome

Project 4.3.2, WFPC

Team Leader(s): Kenton Gregory, MD (OMLC)

Project Team Members: Bo Zheng, MD, Cynthia Gregory, PhD, Michael Rutten, PhD, Jeff Teach, RN, Hua Xie, MD, PhD, Ping-Cheng Wu, MS, Bioengineering, and Rose Merten, BS, Bioengineering (OMLC)

Collaborator(s): CV-Path Institute Inc., USAISR, Special Operations Medical Command-Fort Bragg, Biosafe-America, Biologics Consulting Group, and Torston Tonn, MD (Johann Wolfgang University, Frankfurt, Germany)

Therapy: Autologous bone marrow stem cell treatment for CS.

Deliverable(s): A large animal CS model to evaluate efficacy of multiple stem cell treatments to regenerate muscle and nerve damage in extremity wounds complicated by CS.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 3; End Year 3, TRL 3/4

Key Accomplishments: The researchers completed a large animal pilot study using Sinclair mini-swine to evaluate the effectiveness of autologous BM-MNC treatments in CS injuries. They have developed an in vivo cell tracking technique that has allowed them to demonstrate extremely robust cell engraftment up to 3 months post-treatment. In addition, they have created and modified cell invasion assays and flow cytometry protocols that can assess the function, viability, and identification of cells used in their treatments. The researchers have completed 90% of the control animals for their multi-treatment study as part of their pivotal cell dose study.

Key Words: Extremity compartment syndrome, autologous bone marrow mononuclear cells, porcine model, automated bone marrow stem cell separator, gait analysis, cell engraftment

Introduction

Tissue wounds to the extremities are the most common battlefield injuries sustained by soldiers during Operation Iraqi Freedom and Operation Enduring Freedom. Improvised explosive devices frequently result in blast injuries to extremities that lead to CS. Unless adequately treated, the related swelling and increased pressure within tissue compartments quickly lead to permanent muscle, nerve, and vascular cell death. Soldiers developing CS have prolonged recovery times and rarely recover complete function. The current technology gap in CS is regeneration of muscle, nerves, and vasculature to achieve full functional recovery and return to active duty. The Gregory team's product is an automated stem and progenitor separation technology that utilizes a disposable flow path to produce nonmanipulated autologous stem cells for local injection. The researchers have developed injection and cell retention techniques that safely produce robust, long-term treatment cell engraftment and differentiation into muscle, nerve, and vascular cells to accelerate healing and regenerate damaged tissue.

The goal of this project is to improve the endogenous cellular regeneration response through the use of autologous bone marrow stem and progenitor cell therapy. The aim of this bone marrow treatment is to shift the balance from cellular atrophy and fibrosis to the generation of physiologically active cells, resulting in improved muscle and nerve function. This approach to accelerating healing

and regenerating tissue lost to battlefield blast and other extremity trauma offers a unique, safe, practical, and significant opportunity to improve functional recovery for the injured soldier.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the researchers developed a large animal model of CS using Sinclair mini-swine that could be used to test whether the application of bone marrow progenitor cells can enhance the healing and function of an injured limb. They created cell invasion assays as well as flow cytometry protocols to help assess the function, viability, and identification of cells used in bone marrow treatments. They also developed a cell colony-forming unit assay protocol for adult porcine bone marrow to examine the effects of stem cell colony formation in response to bone marrow cells loaded with and without the cell trackers DiI or quantum dots. They completed a comparative flow cytometry analysis of BM-MNCs in uninjured and CS-injured pigs. Their preliminary results indicated upregulated expression levels of certain identifiable cell phenotypes in CS-injured pigs.

Research Progress – Year 3

The Gregory group completed a large animal pilot study in Sinclair mini-swine during year 3. The pilot study evaluated the use of normal saline infusion into the TA muscle compartment to induce CS. Unlike plasma infusion, the saline infusion was unable to produce a severe injury that would allow for long-term treatment evaluation. The Gregory group moved forward with its pivotal study using autologous plasma infusion to induce CS in swine. This study is designed to determine the autologous BM-MNC dose for treatment in a CS injury. The animals in this study will be used as the controls of the AFIRM multi-treatment study. More details on this work is presented as follows.

Sinclair Mini-Swine Feasibility Study

The researchers designed a pilot feasibility study to evaluate multiple parameters in the new Sinclair model before beginning an optimal cell dose study. CS was induced in two Sinclair mini-swine by infusing 0.9% saline into the left TA muscle compartment. Infusion rates were varied to hold the compartment pressure between 120–140 mmHg for 6 hours. After infusion, a fasciotomy was performed to relieve compartment pressure.

One week after injury, bone marrow was harvested from the tibial crest of the uninjured limb and from the sternum using the FDA-approved Vidacare OnControl® System. The cells were separated using the Sepax Cell Separation device. One treatment animal (P9227) received labeled BM-MNCs injected into the TA at a concentration of 5×10^6 cells/cm^3 of muscle tissue. The other animal (P9242) was the control and did not receive cells. Unfortunately, the treatment animal developed an infection in the muscle within a week after cell treatment. This animal was treated with antibiotics for the infection but was sacrificed 1 month post-injury. The animal was replaced with another (P9378) to ensure it was not the treatment application that caused the infection. The control animal (P9242) was sacrificed 2 months post-injury after which histology samples were evaluated to determine the percent of fibrosis. The replacement treatment animal (P9378) was sacrificed at 3 months post-injury to evaluate histology results as well as to locate engrafted labeled stem cells. No signs of infection were noticed in the second treatment animal. BM-MNCs

from all 3 Sinclair mini-swine were characterized for expression of cell surface markers and functional (i.e., migratory) capacity.

Animal gait was measured with the use of a pressure mat system (Tekscan, South Boston, Massachusetts) that captures and analyzes multiple sequential foot strikes of the animal. This system records force, pressure, timing, center of force, and area of contact. Muscle function was measured through the use of a biomechanical muscle tester custom-designed to accommodate large animals (Aurora Scientific, Ontario, Canada). This biomechanical tester measures isometric forces.

Muscle Function Analysis
Motor nerve conduction and dorsiflexion force measurements demonstrated that, following saline infusion, muscle and nerve function recovered by 2 months post-injury.

Gait Analysis
Gait analysis was completed prior to injury and showed symmetry between right and left sides. Analysis of gait symmetry for the control and treatment animals post-saline-infusion CS injury showed that the uninjured right rear leg was favored over the injured left rear leg. However, by week 4 both animals returned to normal gait symmetry.

BM-MNC Characterization
Bone marrow was harvested from the Sinclair mini-swine 1 week post-CS injury and processed using a SEPAX machine to isolate the BM-MNCs. Prior to administration to autologous recipients, aliquots of the BM-MNCs were reserved for cell function and phenotype analysis. The functional capacity of the BM-MNCs was assessed by their ability to migrate in response to an SDF-1 gradient. In all cases the BM-MNCs were functional as demonstrated by their increased invasion in response to SDF-1 versus no chemokine. Flow cytometric assessment of monoclonal antibody staining demonstrated that the adult Sinclair mini-swine BM-MNCs were heterogeneous for their expression of mesenchymal stromal cell markers CD29, CD90, and CD44; the pan-hematopoietic lineage marker CD45; endothelial cell marker CD31, as well as perivascular cell (pericyte) markers CD146 and CD105; and primitive stem cell and myogenic cell markers cKit, Sca-1, CXCR4, and CD56 **(Figure VI-14)**. These results suggest that adult bone marrow is a potential multipotent cell source for injured tissue repair.

BM-MNC CM-DiI-Labeling and In Vivo Tracking
SEPAX-purified BM-MNCs were labeled with the lipophilic membrane dye, CM-DiI (Molecular Probes). Aliquots of the labeled cells were assessed by fluorescence microscopy to determine the labeling efficiencies, which for P9227 and P9378 were 51.35% and 70.57%, respectively.

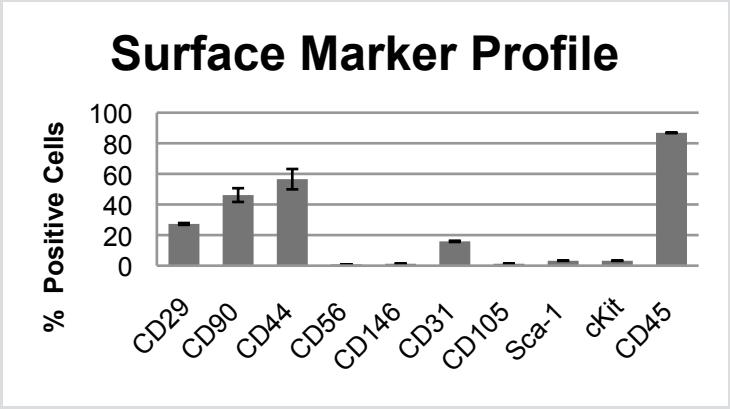


Figure VI-14. Adult mini-swine BM-MNCs are heterogeneous for their expression of various cell surface markers, including those for hematopoietic, mesenchymal, endothelial, myogenic, and stem cells. Flow cytometric analysis of cell surface marker expression was performed on BM-MNCs immediately following their isolation using a SEPAX machine. All data are shown as mean ± SD (P9242, P9378, and P9227).

CM-DiI-labeled autologous BM-MNCs were injected along with FluroSpheres into the TA muscles of autologous recipients 1 week post-induction of unilateral CS injury. The injured TA muscles were harvested at 3 weeks (P9227) and 11 weeks (P9378) post-cell treatment, and tissue sections were assessed by confocal microscopy for the engraftment of the injected CM-DiI-labeled autologous BM-MNCs.

Highly successful engraftment of autologous BM-MNCs was demonstrated by the large number of CM-DiI-labeled cells in the injected muscle tissue of both treatment pigs. Assessment of tissue sections from P9227 harvested 3 weeks post-treatment revealed a surprisingly large number of CM-DiI-labeled BM-MNCs distributed throughout the TA muscle, including areas well beyond the injection sites. Some of the BM-MNCs were found to be in close proximity to the capillaries and astrocytes within skeletal muscle. In P9378 the labeled autologous BM-MNCs also could be readily found in large numbers throughout the muscle at 11 weeks after cell injection. The cells appeared to distribute preferentially within regions of skeletal muscle fibers and reside near the capillaries and the newly regenerated myofibers **(Figure VI-15** and **Figure VI-16)**. These results suggest that transplantation of BM-MNCs into injured muscle may promote myogenesis, angiogenesis, and neurogenesis. The very large number of engrafted cells and widespread distribution throughout the injured and healing muscle far exceed that seen in published studies of skeletal or myocardial muscle injury treated with cell therapy regimens.

Histological Analysis

At 12 weeks post-injury, the TA muscle was removed and sectioned into four pieces. Each piece was sent out for pathological evaluation. All sections were examined by light microscopy to assess extent of injury, necrosis, inflammation, and tissue regeneration or degeneration.

The TA muscle of the control animal (P9242) showed viable skeletal muscle with minimal residual myocyte injury. There was limited dense collagenous tissue approximating 5% of the overall segments in the entire muscle. The collagenous tissue was covered by granulation tissue with abundant angiogenesis, minimal acute hemorrhage, and chronic inflammation. There were early necrotic myocytes, chronic inflammatory cells, and occasional regenerating muscle cells in the transitional region **(Figure VI-17)**.

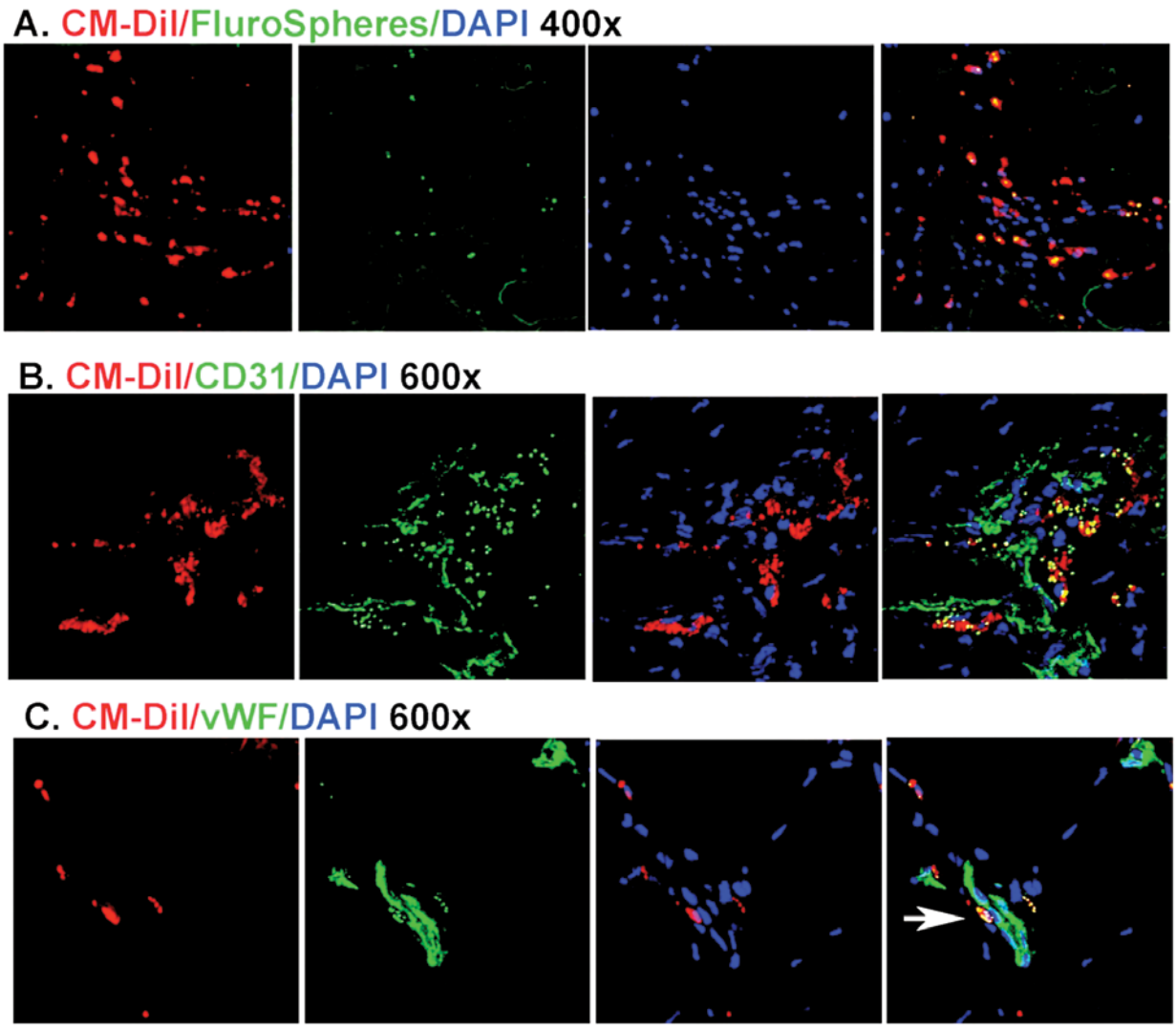


Figure VI-15. Confocal images of CM-DiI-labeled autologous BM-MNCs detected in TA muscle tissue 11 weeks after injection into the damaged muscle (P9378). CM-DiI-labeled BM-MNCs and co-injected FluroSpheres were detected within skeletal muscle tissue (A) and in proximity to the capillaries CD31 (B) CD31/green, and (C) vWF/green, arrow. Nuclei (blue) are stained with DAPI. Green-colored FluroSpheres (that were co-injected with the BM-MNCs) were detectable within and around injected cells. Magnification 600x.

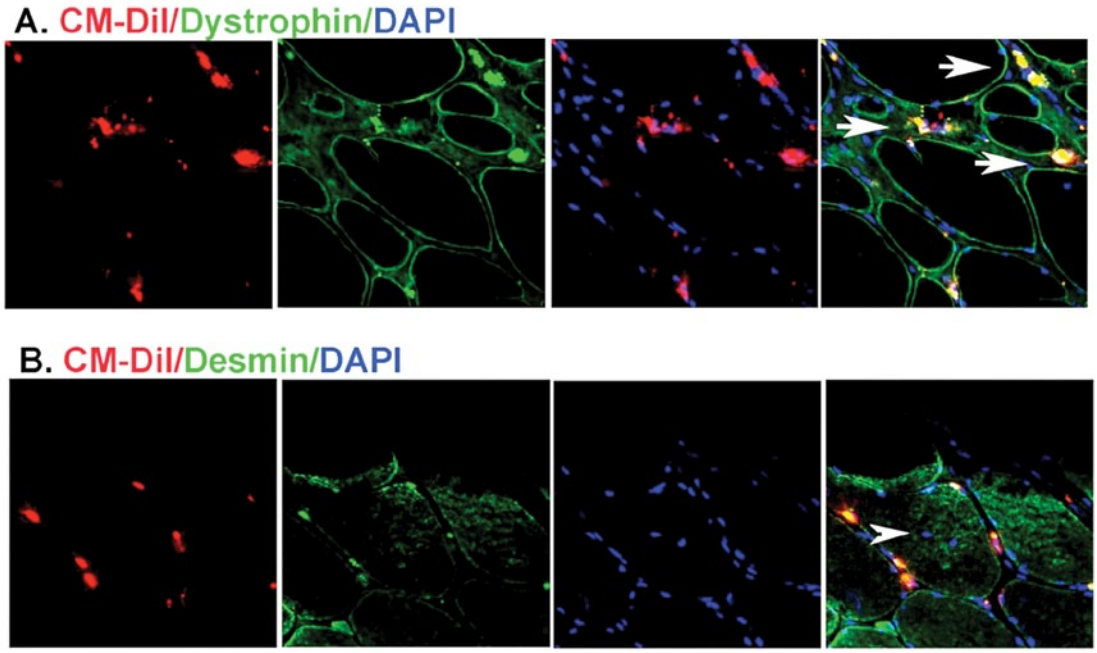


Figure VI-16. CM-DiI-labeled autologous BM-MNCs (arrows), detected within frozen sections of swine TA muscle tissue at 11 weeks after injection into the injured muscle (P9378), could be detected in proximity to regenerated skeletal myofibers (arrow heads) (A) Dystrophin/green and (B) Desmin/green. Nuclei (blue) are stained with DAPI. Green-colored FluroSpheres (that were co-injected with the BM-MNCs) were detectable within injected cells. Magnification 600x.

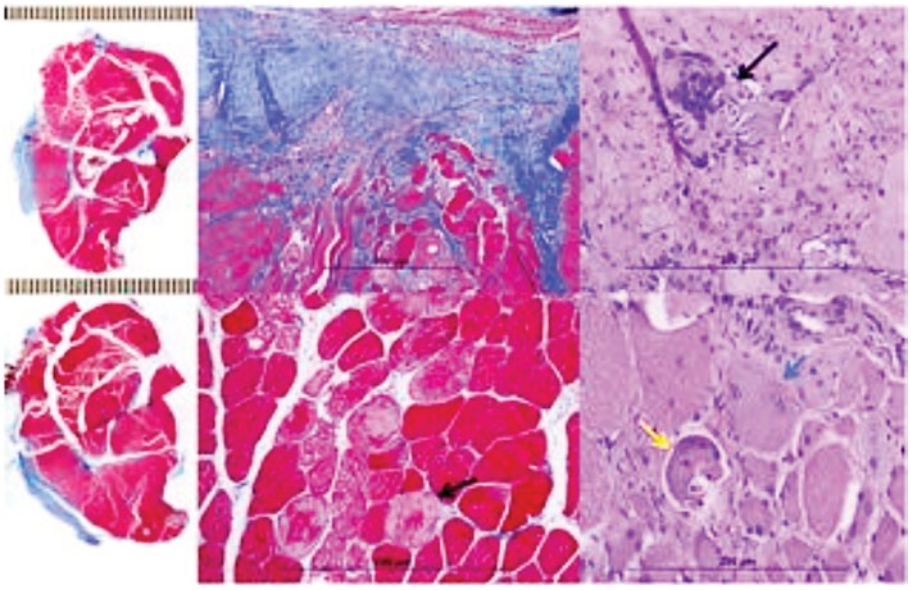


Figure VI-17. Animal P9242. Left images: Control animal Masson's trichrome-stained macro-images show viable myocytes (red-stained tissue) with a small rim of fibrous granulation tissue (blue-stained tissue) at one aspect. Middle images (Top, Masson's trichrome 4x): highlight transition from injured muscle to granulation tissue (blue-stained region); (Bottom, Masson's trichrome 10x): shows region of early myocyte necrosis (black arrow). Right images (H&E 40x) show phagocytosis (black arrow), necrotic myocyte (yellow arrow), and regenerating muscle fiber (blue arrow) in transition region.

The TA muscle of the BM-MNC-treated animal (P9378) demonstrated minimal fibrosis **(Figure VI-18)** with well-organized and orientated dense peripheral collagenous tissue and muscle bundles. The muscle bordering the fibrosis shows hypereosinophilic myocytes with occasional multinucleated cells consistent with regenerating myocytes. The fibrosis extended interstitially into adjacent viable muscle **(Figure VI-19)**. The nerve bundles were intact without any noticeable damage and pathological changes **(Figure VI-20** and **Figure VI-21)**.

Pivotal Cell Dose Study

In the pivotal randomized cell dose study, the researchers are evaluating 3 treatment groups with 10 animals in each group:

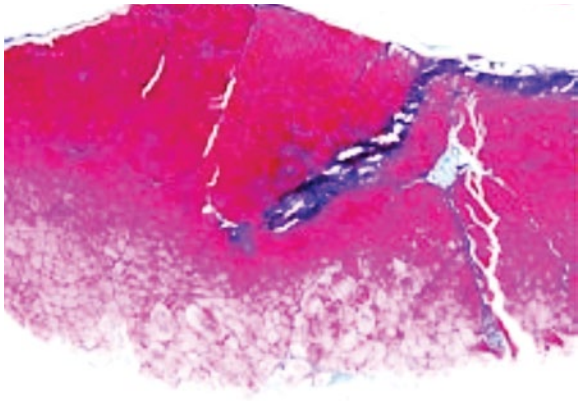


Figure VI-18. Animal P9378: Masson's trichrome stain of treatment animal TA muscle (4x). Animal was sacrificed 12 weeks after the CS injury.

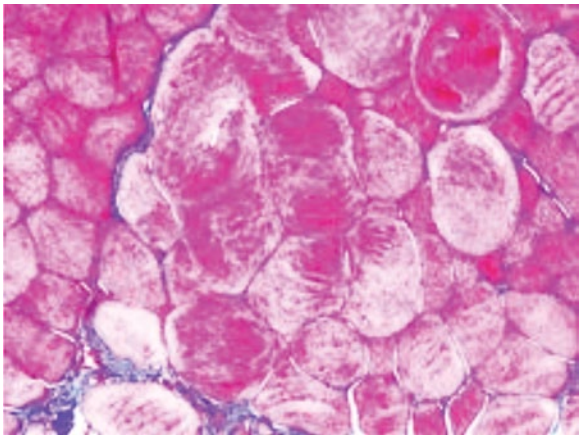


Figure VI-19. Animal P9378: Masson's trichrome stain of treatment animal TA muscle (20x). Animal was sacrificed 12 weeks after the CS injury.

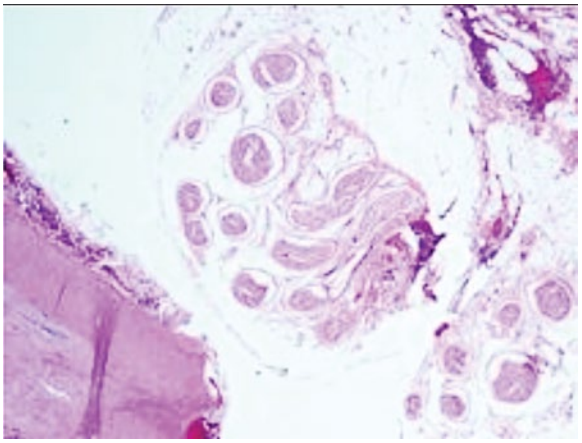


Figure VI-20. Animal P9378: Masson's trichrome stain of treatment animal TA muscle underlying nerve (4x). Animal was sacrificed 12 weeks after the CS injury.

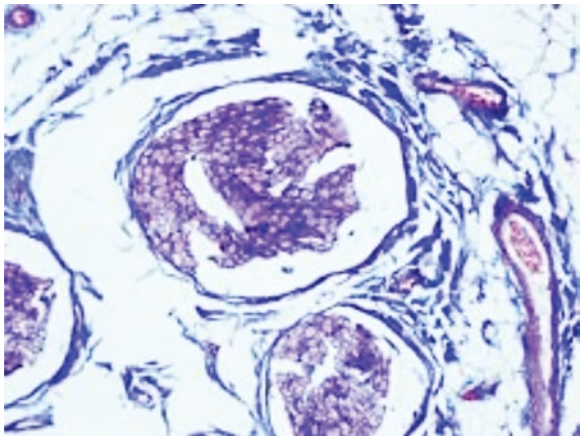


Figure VI-21. Animal P9378: Masson's trichrome stain of treatment TA muscle underlying nerve (20x). Animal was sacrificed 12 weeks after the CS injury.

control (no treatment with BM-MNCs), low dose (50 x 10^6 cells), and high dose (100 x 10^6 cells). On the day of the surgery to create the CS, each animal undergoes biomechanical testing and nerve conduction velocity studies prior to injury induction. CS is induced by a unilateral TA muscle injury. Specifically, autologous plasma is infused into the muscle of an anesthetized pig for 6 hours to maintain the compartment pressure above 140 mm Hg. After plasma infusion, biomechanical testing and nerve conduction velocity studies are repeated, and then a fasciotomy is performed to relieve compartmental pressure. Finally, the injured leg is wrapped and the swine allowed to recover from anesthesia.

One-week post-creation of the CS injury, each swine is returned to the surgical suite where biomechanical testing and nerve conduction velocity studies are repeated. Bone marrow is drawn from both the tibial crest and sternum of each animal using an FDA-approved, commercially available system (Vidacare®). Approximately 20 mL of bone marrow is aspirated from both sites and the bone marrow transported to the Cell Laboratory where it is processed to isolate the BM-MNCs. The injured TA muscle of each swine is then treated with either autologous BM-MNCs or, if it is a control animal, Hanks Balanced Salt Solution (HBSS) only. To maintain consistency between the experimental and control swine, the bone marrow is drawn from the control swine that are maintained on anesthesia for 4 hours until their injured TA muscle is injected with HBSS (no cells). After the treatment, the injured/injected leg is wrapped, and the swine are allowed to recover from anesthesia.

The swine are tested for gait analysis as well as nerve and muscle function at scheduled intervals throughout the study. The swine are also evaluated for lameness and pain during the post-operative period. Twenty-two swine have reached the 3-month study end point and have been euthanized. Their injured TA muscles were harvested and sent to Texas Veterinary Pathology for assessment. Seven more swine are to be euthanized in the next 2 months. One last swine still needs to be treated in the study.

The animals in this study will serve as the control and single-treatment (day 7) animals for the AFIRM multi-treatment study. The multi-treatment study will evaluate treatment time points of (a) 7 days, (b) 7 and 14 days, and (c) 7, 14, and 28 days post-injury. All animals will be studied for 3 months post-extremity CS injury with the results of the three study groups to be compared to each other as well as to untreated controls. There will be 8 Sinclair mini-swine per treatment groups (b) and (c) for a total of 36 swine evaluated. The experiment procedures will be complete by November 2011.

Key Research Accomplishments

- Completed a large animal pilot feasibility study for CS injury in swine.
- Showed that infusion of saline created an acute CS injury. Therefore, saline, unlike plasma, is not an appropriate infusion medium for a chronic model.
- Developed an in vivo cell tracking technique that allowed detection of treatment cells 11 weeks after injection.
- Developed cell delivery methods that result in extremely robust cell engraftment up to 3 months post-cell injection.
- Adapted automated commercial stem cell separation technology for use in animal and human studies.

- Developed a stem cell functionality assay.
- Completed 90% of the control animals in the multi-treatment study as part of the pivotal cell dosing study.

Conclusions

As a chronic model, saline is not an appropriate infusion medium. The saline infusion-induced injury was acute (normal function returned within the 3-month study period) and produced only 5% muscle infarction. It did not create an injury requiring treatment. The researchers will use plasma injury in their model due to its ability to create 30%–60% muscle infarction. They will continue to use Sinclair mini-swine in future studies since the older mini-swine replicate the young-adult age of soldiers and the animals are easy to manage.

The results of this project demonstrate that BM-MNCs from adult Sinclair mini-swine are heterogeneous for the expression of well-known mesenchymal, hematopoietic, endothelial, perivascular, myogenic, and stem cell markers. The results suggest that adult bone marrow is a potential multipotent cell source for injured tissue repair.

The pilot study demonstrated that autologous BM-MNCs labeled with CM-DiI were detectable at least through 11 weeks post-injection into damaged TA muscle. This indicates that CM-DiI labeling is a reliable method for long-term cell-fate tracking within the muscle tissue. The same results have been seen in the pivotal study.

BM-MNCs labeled with CM-DiI are detectable in proximity to newly regenerated myofibers and seen close to the capillary and peripheral nerve of skeletal muscle in vivo. These results suggest that transplantation of BM-MNCs into injured muscle may promote myogenesis, angiogenesis, and neurogenesis.

Enzymatic digestion of intact skeletal muscle can produce a single-cell population that can be used for cell sorting to generate a pure population of CM-DiI-labeled cells for phenotypic identification. The characterization of these purified CM-DiI-labeled cells indicates that the previously injected CM-DiI BM-MNCs are capable of multilineage differentiation desirable for muscle, nerve, and vascular regeneration.

Research Plans for the Next 2 Years

The Gregory team will be finishing its 3-month study by the end of 2011. In the first quarter of 2012, it will begin a 6-month safety and efficacy study with 8 control animals and 8 treatment animals. The treatment will be determined from the 3-month study results. The investigators, in collaboration with the Huard group from Pittsburgh, are researching the use of an angiotensin receptor blocker, losartan, to accelerate and improve the healing of severe muscle injuries. The Gregory group has applied for a grant to evaluate the use of losartan in its CS injury model with 8 Sinclair mini-swine. The results will be compared to the previous results of the cell dose study and the multi-treatment study.

Planned Clinical Transitions

Successful results from the multi-dose treatment study will be the basis for the researchers to begin the process of filing an IND with the FDA and initiating a Phase 1 human clinical trial in year 5. They will perform a cytotoxicity study in rodents to evaluate toxicology, biodistribution, tumorigenicity, and microbiological effects of their treatment protocol.

This will be done simultaneously with preparation of the IND. The rodent study will be paid for by non-AFIRM funds. The Gregory group will also begin closer work with its FDA consultant to make a smooth transition from preclinical animal trials into a Phase 1 human trial. The trial will include 20 patients and will be performed under the auspices of Col. John Holcomb at Brooke Army Medical Center.

Corrections/Changes Planned for Year 4

Due to housing limitations, it has taken longer than expected to complete the large animal study. The large animal studies must be completed before moving into clinical studies, thereby delaying the start date for the Phase 1 clinical trial.

Improving Cell-Based Approaches for Extremity Trauma

Project 4.3.6, USAISR

Team Leader(s): Chris Rathbone, PhD and Thomas J. Walters, PhD (USAISR)

Project Team Members: Joseph C. Wenke, PhD, Robert J. Christy, PhD, and Xiaowu Wu, MD (USAISR)

Collaborator(s): None

Therapy: Adult stem cell transplantation.

Deliverable(s): Strategies to improve the use of BMSCs for the treatment of I/R injury.

TRL Progress: End of Year 3, TRL 3; Target, TRL 3

Key Accomplishments: The researchers established and validated an in vitro muscle system and software to determine functional outcomes following I/R injury. They completed preliminary studies to determine the feasibility of injecting freshly isolated BMSCs into I/R-injured skeletal muscle. They also initiated studies to determine the effect of the timing of injection on cell engraftment.

Key Words: Stem cells, muscle, injury

Introduction

Historically, 70% of all battlefield injuries involve extremity injury. Most extremity injuries are caused by explosive munitions that can cause fractures, tissue loss, and vascular injury, all of which place extremities at risk for CS and skeletal muscle (I/R) injury. Currently, the clinical treatment of complex I/R is insufficient. Numerous intervention strategies have been proposed that are aimed at reducing I/R. Many of these strategies have shown some level of success in reducing the extent of I/R when applied prior to, during, or immediately after ischemia, but few have shown benefits when applied at delayed time points. This is an important distinction because in a trauma setting pretreatments are impractical, and treatments applied during or immediately after ischemia are rarely feasible. A treatment that could be applied after patient stabilization would be much more practical.

Developing cell-based therapies may provide such an option. Bone marrow aspirates represent attractive in vivo cellular sources of autologous stem cells for regenerative therapies for I/R, and BMSCs can be concentrated from bone marrow aspirates in the operating room using FDA-approved point-of-care devices. The current availability of point-of-care devices and the potential to obtain BMSCs using these devices mean that the time required for their clinical application for I/R is less. However, most studies directed toward cellular therapies for muscle injury rarely use freshly isolated cells, a scenario inconsistent with the use of point-of-care

devices. Therefore, in the current project freshly isolated BMSCs will be studied with the intent of facilitating their use for regenerative medicine approaches to improve muscle function following I/R.

One of the factors that should be taken into consideration is whether BMSCs should be harvested from the injured or non-injured limb. Currently, almost everything known about stem cells is based on cells that have been obtained from healthy uninjured animals and volunteers, and often from allogeneic sources. Unfortunately, warriors who have traumatic injuries are not like healthy volunteers. To date, little consideration has been given to the effect of I/R on the regenerative potential of these stem cells. Based on this discrepancy between experimental and clinical procedures, whether I/R affects the regenerative potential of BMSCs should be determined as this could impact future experimental designs and influence clinical practice.

Another important factor to consider is the mode in which stem cells are delivered. Given the relatively large volume of muscle that is affected by injury, it is difficult to effectively administer cells to treat the entire affected area. The most common method of cell delivery for skeletal muscle repair is that of intramuscular injection. Intuitively, it seems the injection of cells in close proximity to the area of damage would result in the most effective treatment; however, it is often not feasible to deliver a sufficient number of stem cells homogenously to all of the affected tissue. To this end, it is appropriate to explore the systemic delivery of BMSCs.

Something else that should be taken into consideration with regard to the administration of stem cells is the most appropriate time to deliver the stem cells. It remains to be determined if the delivery of BMSCs to injured skeletal muscle is best when the muscle is undergoing a period of increased inflammation and degeneration (approximately 2 days), when inflammation is starting to subside and muscle regeneration is initiated (7 days), when muscle regeneration and remodeling are predominant (2 weeks), or when both inflammation and muscle regeneration have significantly subsided (4 weeks). In summary, improvements in the harvesting and the mode and timing of delivery will aid in the application of BMSCs for repair following I/R.

Summary of Research Completed in Year 1 (funded in 2009)

During the first year of the project, the researchers began to demonstrate the benefits of cell-based therapy following I/R injury. In these studies, cells were transplanted via direct injection. Their preliminary findings indicated that early treatment of I/R with direct injection of mesenchymal progenitor cells could improve muscle function in the short term.

Research Progress – Year 2

Histological analysis is commonly used as a primary end point to assess the magnitude of muscle injury and treatment strategies aimed at improving muscle healing. However, it has been demonstrated that histology often correlates poorly with functional measurements. It therefore seems appropriate that the ultimate end point measurement for testing therapies for treating muscle injury must include functional measurements. To this end, the researchers established an in vitro system to assess muscle function **(Figure VI-22A)**. In addition, a LabView-based system (National Instruments) was developed to control the parameters and to collect and analyze data (Figure VI-22B). Using this

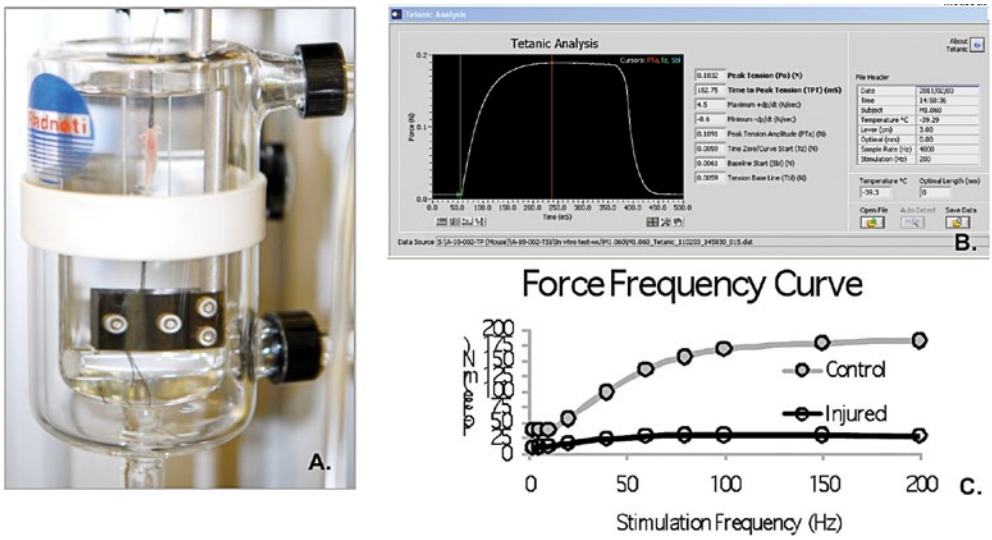


Figure VI-22. (A) A picture of the in vitro muscle bath containing a mouse EDL muscle. The proximal tendon is attached to a force transducer (not shown); this distal tendon is fixed in a clamp. Muscle is bathed in oxygenated Krebs solution and stimulated with field electrodes (parallel to muscle). (B) A screen capture of the signal produced by a tetanized muscle using the LabView-based software developed by the research team to control, capture, and analyze in vitro muscle function as part of this project. (C) Isolated EDL muscles were stimulated over a range of frequencies and the peak force was measured in vitro. Measurements were made 7 days post-injury.

system, a preliminary study was conducted to determine the impact of 2 hours of tourniquet-induced ischemia on in vitro isometric force. Muscles were tested 7 days following injury and the results are shown in Figure VI-22C. The researchers show in the mouse model that 2 hours of ischemia resulted in an 84% reduction in peak muscle force at 200 Hz. Note that muscles were stimulated directly with field electrodes; therefore, the loss of force is not attributable to nerve injury.

The majority of studies directed toward cellular therapy for muscle injury rarely use freshly isolated cells, a scenario inconsistent with the use of FDA-approved point-of-care devices that concentrate BMSCs at the bedside in the operating room. Preliminary studies were completed to determine the feasibility of administering freshly isolated BMSCs to animals with I/R early after injury (2 days). Stem cells were isolated from the tibia and femur of non-injured FVB-L2G85 transgenic mice using magnetic-activated cell sorting and a lineage depletion kit (Miltenyi Biotec) to emulate the cells concentrated from bone marrow using point-of-care devices. FVB-L2G85 transgenic mice (Jackson Laboratories) were used as donor mice because they express firefly luciferase and GFP under the control of the widely expressed β-actin promoter in all tissues. This allows for the use of freshly isolated cells and circumvents the need for transfection or infection. Approximately 250,000 freshly isolated BMSCs were injected intramuscularly or intravenously into syngeneic FVB mice that received I/R injury 2 days earlier. Bioluminescent imaging was performed 1, 3, 7, 14, 21, and 28 days after injection to estimate in vivo cell survival **(Figure VI-23)**. Preliminary results support the feasibility of using freshly isolated BMSCs in a scenario consistent with the use of point-of-care devices in conjunction with experimental models of I/R injury.

The researchers have initiated a subsequent study to determine the importance of timing in

Figure VI-23. To demonstrate the feasibility of using freshly isolated BMSCs for I/R injury, cells were isolated from FVB-L2G85 transgenic mice using magnetic-activated cell sorting and injected intramuscularly into mice injured with I/R 2 days earlier. Cell survival was verified using bioluminescent imaging.

the administration of freshly isolated BMSCs following injury. They also further optimized experimental parameters based on the initial results, which will be used in subsequent studies including bioluminescent imaging parameters (distance from camera to animal and animal positioning), tissue collection for the purpose of GFP detection, and imaging time points (the addition of imaging on the day of injection for baseline measurements).

Key Research Accomplishments

- Established and validated an in vitro muscle system and software to determine functional outcomes following I/R injury.
- Determined the feasibility of delivering freshly isolated BMSCs to measure cell survival following I/R injury.
- Initiated studies aimed at determining the importance of timing after injury in the delivery of BMSCs to I/R-injured muscle.

Conclusions

The feasibility of completing the proposed experiments was established, including the determination of muscle function and the use of freshly isolated BMSCs for measuring cell engraftment and survival following I/R injury. Studies to determine the importance of timing in the delivery of freshly isolated BMSCs following I/R injury have been initiated.

Research Plans for the Next 2 Years

The ongoing studies directed toward determining the most appropriate time point for injection and the influence of I/R on the regenerative potential of BMSCs will be completed. Future studies will incorporate the use of cultured stem cells, explore the ability of systemic delivery of BMSCs to treat I/R injury, and establish conditions that result in an improvement over the current parameters.

Planned Clinical Transitions

The proposed studies will provide information needed to complete large animal studies using suitable FDA-approved devices. A Cooperative Research and Development Agreement has been established with Arteriocyte.

Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy

Project 4.3.3, WFPC

Team Leader(s): William R. Wagner, PhD (MIRM, University of Pittsburgh)

Project Team Members: Keisuke Takanari, MD, PhD, Ryotaro Hashizume, MD, Yi Hong, PhD, Nicholas J. Amoroso, BSE, and Alex Huber, PhD (MIRM)

Collaborator(s): Stephen F. Badylak, MD, PhD, DVM and Johnny Huard, PhD (MIRM)

Therapy: Treatment of abdominal CS; development of fascial repair technology.

Deliverable(s): Biodegradable elastomeric scaffolds for fascial reconstruction.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 1; End Year 2, TRL 2/3; End Year 3, TRL 3

Key Accomplishments: The researchers created three different designs of a novel biohybrid scaffold composed of dermal extracellular matrix (dECM) and poly(ester urethane)urea (PEUU). They implanted these constructs in a rat full-thickness abdominal wall defect model and assessed their biocompatibility and mechanical properties. They found that separation of the processing streams of dECM and PEUU resulted in better scaffold bioactivity although mechanical properties in vivo were compromised. However, development of a "sandwich" fabrication technique overcame this drawback and achieved excellent mechanical properties that mimicked native abdominal muscle tissue. The regeneration of muscle was observed by Masson's trichrome staining in a sandwich sample at the 8-week time point, which was confirmed by immunostaining for alpha-sarcomeric actin. The researchers also created a tissue construct by combining GFP transgenic MDSCs and PEUU using a microintegration technique. In a pilot experiment, 14 days after implantation, GFP-positive cells were seen in abundance within the scaffold.

Key Words: Abdominal compartment syndrome, biodegradable elastomer, abdominal defect, extracellular matrix, mechanical property, rat model

Introduction

A severe abdominal wall defect due to laparotomy after abdominal CS or severe abdominal injury remains a challenging problem for surgeons. Primary closure is difficult because of the risk of abdominal hyper pressure or intra-abdominal infection. The standard treatment includes open treatment followed by secondary abdominal wall reconstruction.

Many techniques utilizing autologous tissue or prosthetic materials have been applied to reconstruct full abdominal wall defects. The most common disadvantages for autologous tissue transfer are donor site morbidity and secondary complications in the donor site. Disadvantages with prosthetic materials include the risks of intestinal fistula formation, prosthetic infection, adhesions, and recurrent hernias, which are related to the foreign body response and mechanical property mismatch.

This project is focused on creating a biohybrid composite material that offers both strength and bioactivity for optimal healing toward native tissue behavior. Such a material would be applicable in a variety of fascial tissue repair and replacement procedures. dECM gel (extracted from porcine dermal tissue) possesses attractive biocompatibility and bioactivity with weak mechanical properties and rapid degradation while conventionally electrospun biodegradable, elastomeric PEUU has strong mechanical properties with limited cellular infiltration and tissue integration. The Wagner group hypothesized that these two different materials could be combined in a manner that would leverage each material's advantages. In the past year, the Wagner group developed and characterized three different methods to create biohybrid materials and assessed their bioactivity as well as mechanical properties in a rat full-thickness abdominal wall defect model.

Summary of Research Completed in Years 1 and 2

During the first 2 years of the project, the Wagner group completed much groundwork for the study. The researchers began creating the biodegradable, elastic scaffolds for reconstruction of the abdominal wall after the development of CS. They produced abdominal wall patch materials, a series of novel dECM digests and electrospun PEUU blends, tissue constructs combining MDSCs and PEUU, and an abdominal wall defect model in the rat for the in vivo assessment of the biodegradable scaffolds. They completed some in vivo testing in which they compared: (1) expanded polytetrafluoroethylene as a control, (2) dry PEUU, (3) a new wet electrospinning technique where serum-based medium was electrosprayed concurrently with electrospinning of PEUU (wet PEUU), and (4) PEUU blended with digested porcine dECM in a full-thickness abdominal defect rat model. Results from this in vivo study showed that wet PEUU has suitable characteristics for further investigation given the mechanical properties observed, which mimic native abdominal wall tissue.

Research Progress – Year 3

PEUU Blended with dECM in a Single Processing Stream

A biodegradable elastomeric scaffold was created through electrospinning by combining PEUU with particulate porcine dECM. Electron micrographs and differential scanning calorimetry demonstrated partial miscibility between PEUU and dECM. With increasing dECM content, scaffolds were found to possess lower breaking strains and suture retention strength. However, the initial modulus increased with larger concentrations of dECM. The scaffolds containing 0% to 50% dECM had tensile strengths of 5–7 MPa, breaking strains of 138%–611%, initial moduli of 3–11 MPa, and suture retention strengths of 35–59 MPa.

In a rat full-thickness abdominal wall replacement model, there was no herniation, infection, or tissue adhesion observed after 4 and 8 weeks with a scaffold containing 25% dECM. Scaffolds incorporating dECM were significantly thicker at the time of explant with greater numbers of associated smooth muscle actin-positive staining cells compared with the PEUU alone, but minimal cellular infiltration and remodeling of the scaffold were detected regardless of dECM addition.

PEUU and dECM Formed into a Composite by Two Independent Processing Streams

To improve the performance of a biohybrid scaffold of dECM gel and PEUU, the Wagner group separated the two material types into independent processing streams thus avoiding the potential denaturation of the ECM components and preserving the structural integrity of the PEUU fibers.

Scaffold Synthesis and Evaluation

A concurrent electrohydrodynamic processing approach was utilized where PEUU was electrospun while a dECM gel was electrosprayed onto a mandrel **(Figure VI-24)**. The formed biohybrid constructs had high flexibility and elasticity. Cross-sectional images demonstrated integrated regions that were enriched in fibers or in dECM gel. Moreover, distinct lamellar structures (PEUU fiber rich and ECM rich) with interconnecting fibers in the dECM-rich regions were observed as schematically represented in **Figure VI-25**. Imaging with Masson's trichrome staining **(Figure VI-26)** also supported these findings.

The scaffold tensile properties were tunable by altering the PEUU/dECM mass ratios. Tensile strength increased with increasing PEUU wt% in the longitudinal axis (parallel to the collecting mandrel axis) and in the circumferential axis. Peak strains were demonstrated to be more than 600% for all scaffolds. Suture retention strength ranged from 7–9 MPa.

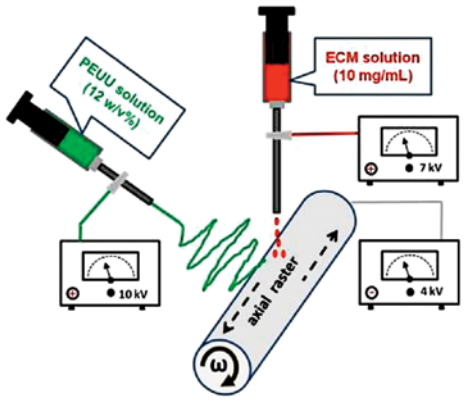


Figure VI-24. Combination technique of electrospinning with electrospraying.

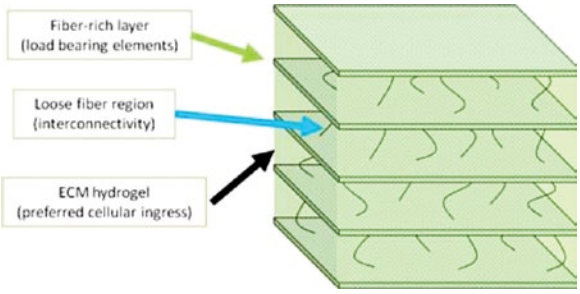


Figure VI-25. Simplified model of scaffold microstructure.

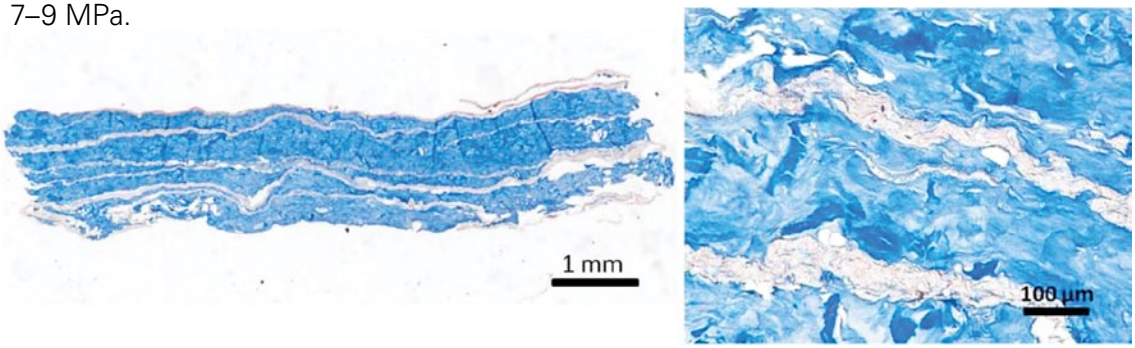


Figure VI-26. Cross-section staining with Masson's trichrome.

Animal Study
A defect (1 x 2.5 cm) involving all of the layers of the abdominal was created in the center abdominal wall of the Lewis rat. Subsequently, the created defect was repaired by either PEUU blended with dECM in a single-stream process (control) or PEUU blended with dECM by a two-stream process. For each group, implanted samples were retrieved at 4 and 8 weeks post-operatively (n=7 per group per time point).

Four weeks after implantation, there was no herniation, tissue adhesion, or infection observed at the surgical site for either group. However, 3 of 7 samples (43%) had abdominal hernia at 8 weeks for the two-stream group **(Figure VI-27)**. The thickness of the explanted construct of both groups at 4 weeks appeared to be similar to native tissue while at 8 weeks the two-stream processed samples had thinned by half **(Figure VI-28)**.

Minimal cellular infiltration and material degradation were found for the single processing stream scaffolds, as was evident from the Masson's trichrome-stained explanted cross-sections while the two-stream scaffold demonstrated extensive cellular infiltration and high material degradation **(Figure VI-29)**.

PEUU and dECM Processed by Dual-Stream "Sandwich" Technique

Based on the previous observations, a new design was developed to strengthen the two-stream processed scaffold by utilizing a sandwich fabrication technique.

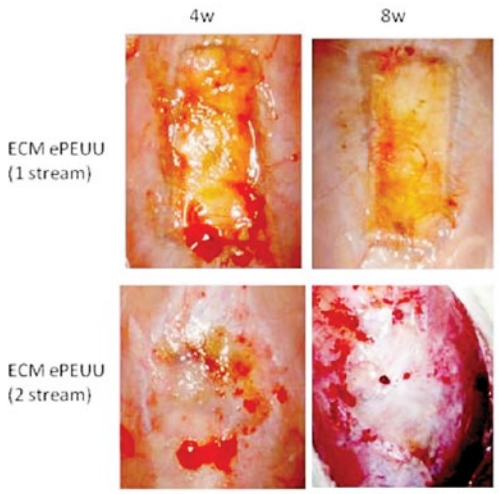


Figure VI-27. Gross observations of the materials.

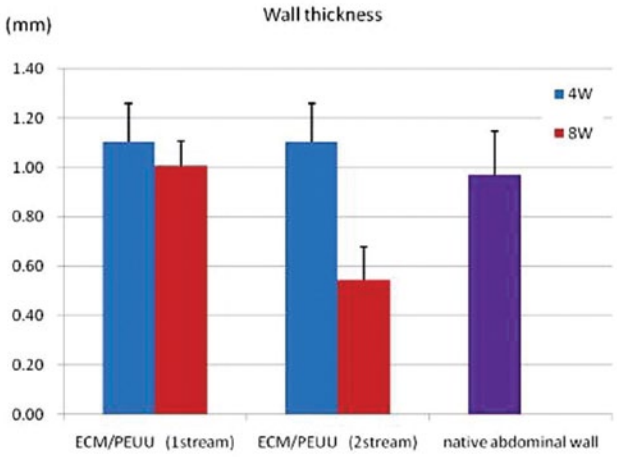


Figure VI-28. Wall thickness of the explanted materials.

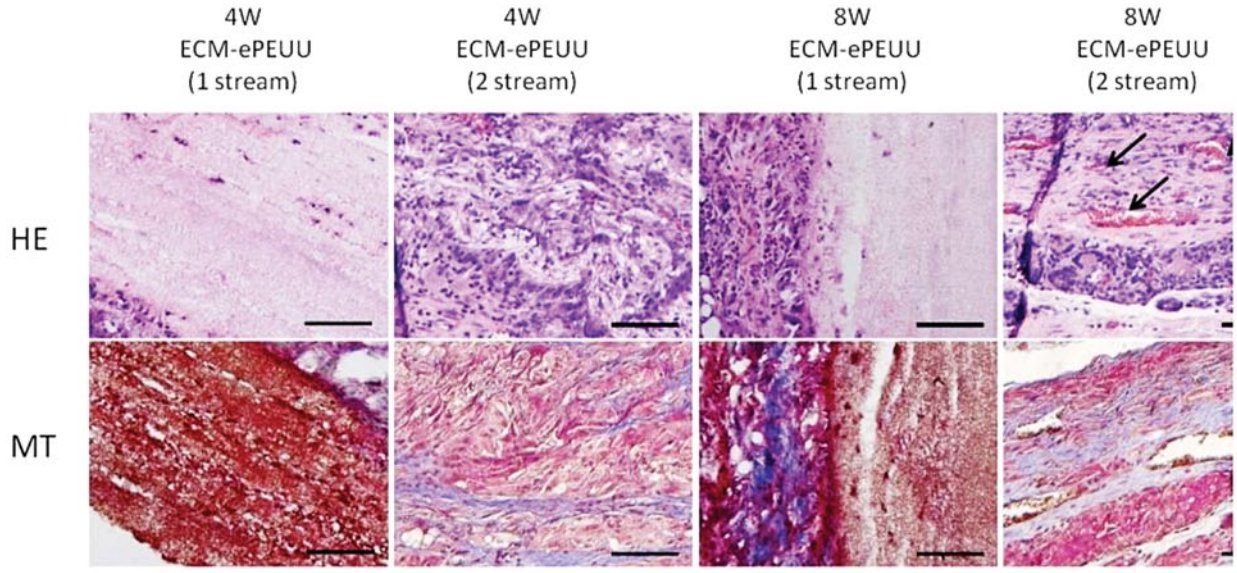


Figure VI-29. High-magnification images of implanted samples at two time points staining with H&E and Masson's trichrome. Arrows point to blood vessels. (Scale bar = 100 μm).

Scaffold Synthesis and Evaluation

The outer layers of the sandwich were formed by electrospraying saline concurrently with PEUU electrospinning at the beginning and end of the processing period. For the PEUU fiber/dECM gel hybrid layer, porcine dECM gel solution was electrosprayed instead of saline while PEUU was electrospun **(Figure VI-30)**. The sandwiched scaffold was transferred into a 37°C incubator for 45 minutes to allow complete dECM gelling. Various scaffolds were generated by altering the outer layer electrospinning time (10, 20, and 30 minutes). The resulting sandwich scaffolds appeared an opaque white in color. Electron micrographs of lyophilized scaffold cross-sections showed a distinct three-layer structure with outer regions of electrospun PEUU and central fiber/gel biohybrid layers **(Figure VI-31)**. There existed a trend of tensile strength increasing with electrospinning time for the outer layers. The peak tensile strengths in the longitudinal direction (along mandrel axis) were 273–416 kPa with peak strains of 520%–781%, and the peak tensile strengths in the circumferential direction were 54–103 kPa with peak strains of 443%–851%. Equibiaxial tensile testing demonstrated that the scaffolds exhibited strongly anisotropic behavior, mimicking the native rat abdominal wall.

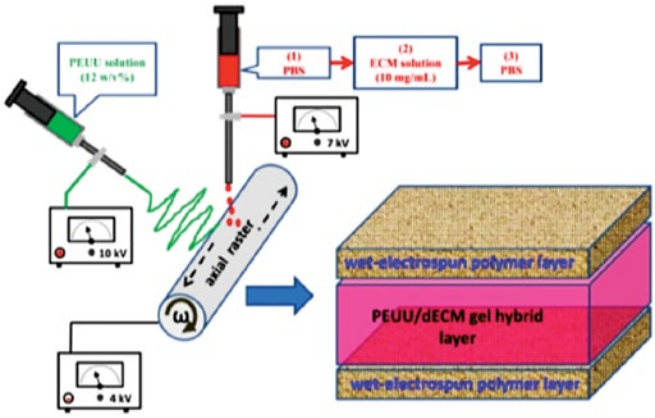


Figure VI-30. Sandwich fabrication technique.

Animal Study

A defect (1 x 2.5 cm) involving all of the layers of the abdominal wall was created in the lateral (right side) of the abdominal wall of Lewis rats. Subsequently, the created defect was repaired by either PEUU processed with dECM in the previously described two-stream process or with the sandwich processing technique. For each group, the implanted samples were retrieved at 4 and 8 weeks post-operatively (n=7 per group per time point). No herniation, tissue adhesion, or infection was observed 4 and 8 weeks after implantation at the surgical site **(Figure VI-32)**. The sandwich samples sustained the wall thickness at 8 weeks while, in contrast, the dual-stream processed samples again were found to thin markedly at the same time point **(Figure VI-33)**.

Histological assessments showed that both scaffold types had good cellular infiltration at both time points. Masson's trichrome staining revealed regenerating muscle in regions of the sandwich sample near the muscular limb at 8 weeks **(Figure VI-34)**. This observation was further supported by immunostaining for alpha-sarcomeric actin. Biaxial mechanical testing showed anisotropic behavior of the sandwich scaffold, mimicking native abdominal wall, whereas the two-stream hybrid construct did not have anisotropy at either time point **(Figure VI-35)**.

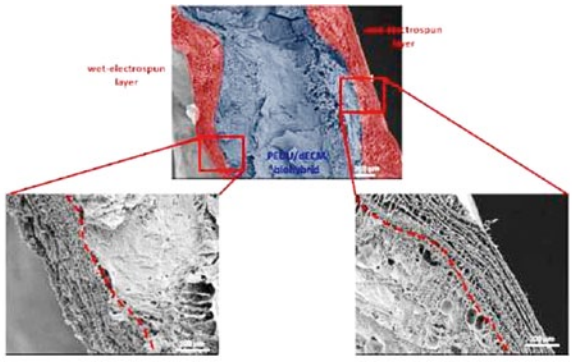


Figure VI-31. SEM cross-sectional image of sandwich sample.

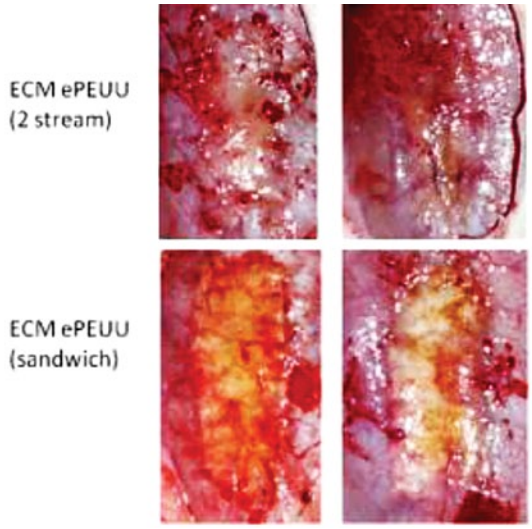


Figure VI-32. Gross observation of the scaffold after 4 and 8 weeks implantation.

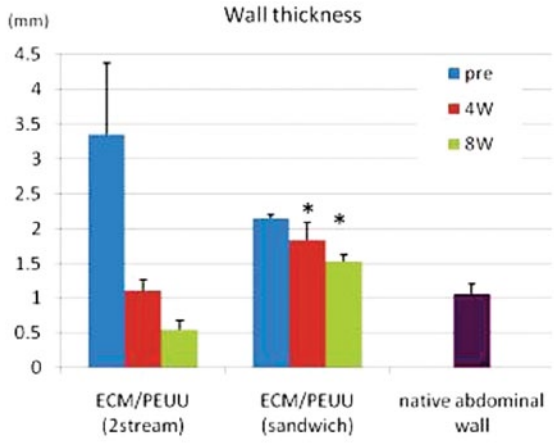


Figure VI-33. Wall thickness of the material. *$p < 0.01$ compared with dECM/PEUU (two stream) at same time point.

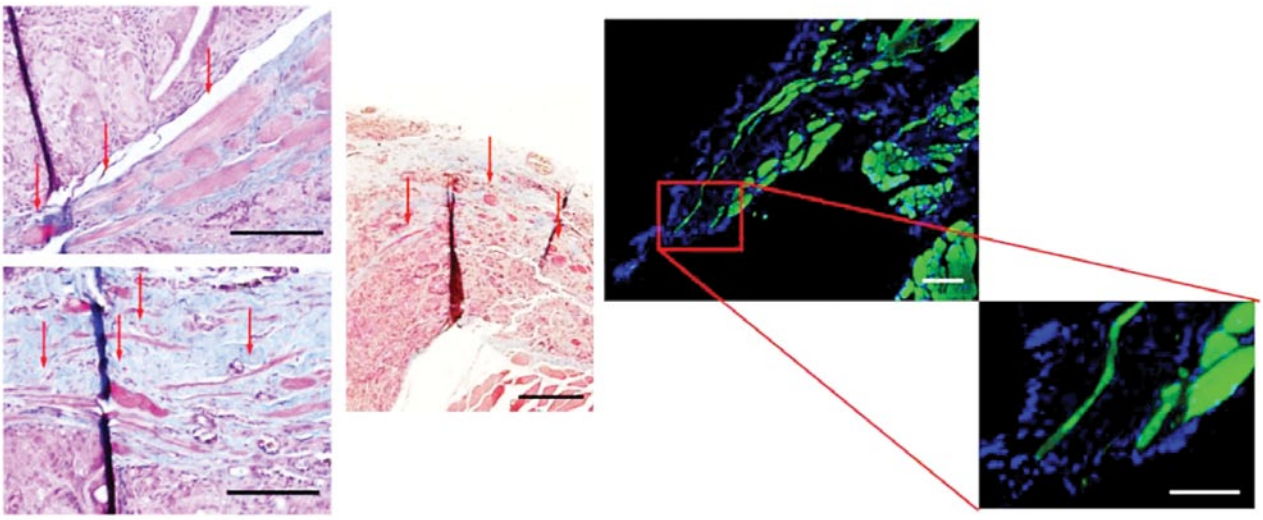


Figure VI-34. Masson's trichrome staining showed muscle regeneration in the scaffold (left three images). Immunostaining for alpha-sarcomeric actin backed up this finding (right two images). (Scale bar: 100 μm)

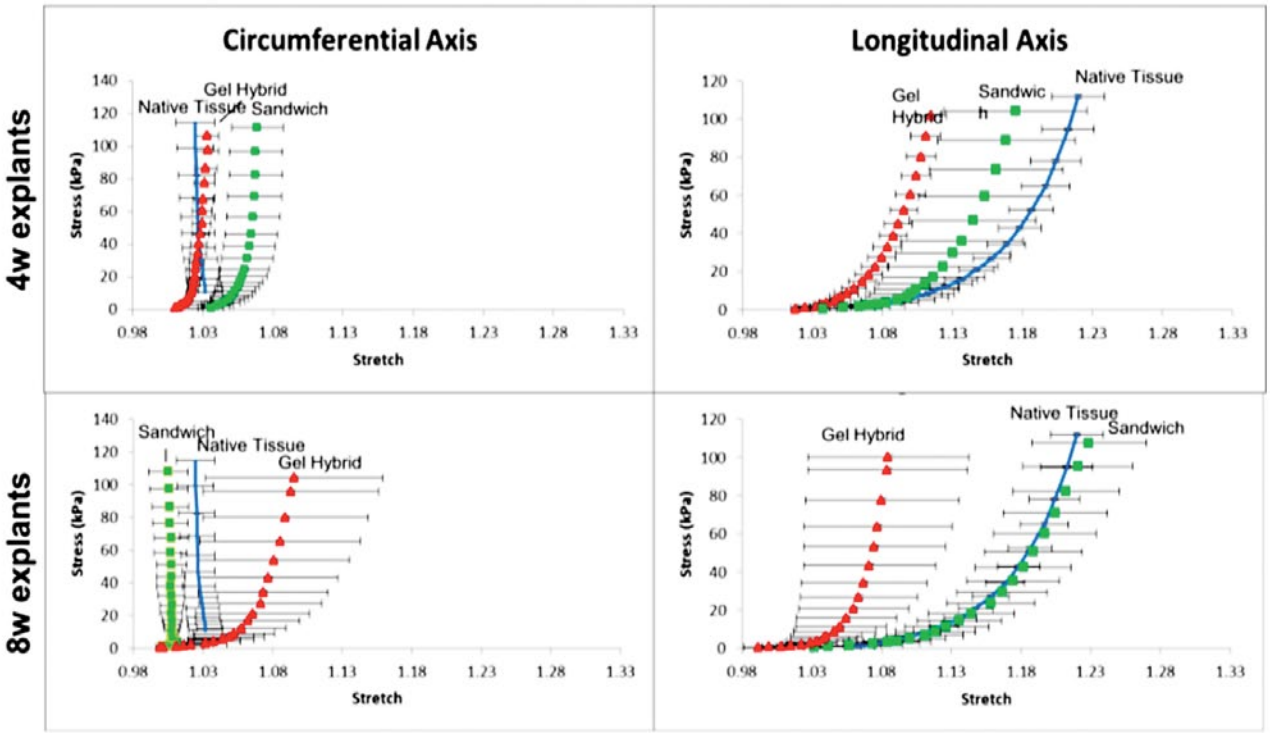


Figure VI-35. Biaxial mechanical properties of explanted samples at 4- and 8-week time points. Gel hybrid: two-stream group.

MDSC-Integrated Wet Electrospun PEUU

The Wagner group is currently investigating concurrent electrospinning/electrospraying to microintegrate GFP transgenic MDSCs into the scaffolds. While the MDSC microintegration process has previously been demonstrated to be viable, GFP cells isolated from a newly generated transgenic colony have enabled in vivo work to begin with cell tracking. Allogeneic transplantation was performed with this MDSC-integrated material. Although only a pilot study has been performed to date, 14 days after implantation, GFP-positive MDSCs appeared to have increased in number and spread throughout the scaffold **(Figure VI-36)**.

Key Research Accomplishments

- Created three different types of a biohybrid scaffold of dECM and PEUU using electrospinning alone, concurrent electrospinning and electrospraying, or concurrent electrospinning and electrospraying with a novel sandwich fabrication technique.
  - Implanted these constructs in a rat full-thickness abdominal wall defect model and assessed their biocompatibility and mechanical properties.
- Determined that separating the processing streams of the two materials resulted in better scaffold bioactivity although mechanical properties in vivo were compromised.
  - However, development of the sandwich fabrication technique overcame this drawback and achieved excellent mechanical properties that mimicked native abdominal muscle tissue.
- Observed the regeneration of muscle by Masson's trichrome staining in a sandwich sample at the 8-week time point and confirmed this result by immunostaining for alpha-sarcomeric actin.
- Created a tissue construct by combining GFP MDSCs and PEUU using a microintegration technique and confirmed the distribution of the stem cells throughout the scaffold.
  - In a pilot experiment, after 14 days implantation, GFP-positive cells were seen in abundance within the scaffold.

Conclusions

Three different fabrication techniques were developed to create biohybrid scaffolds containing dECM and an elastic polymer.

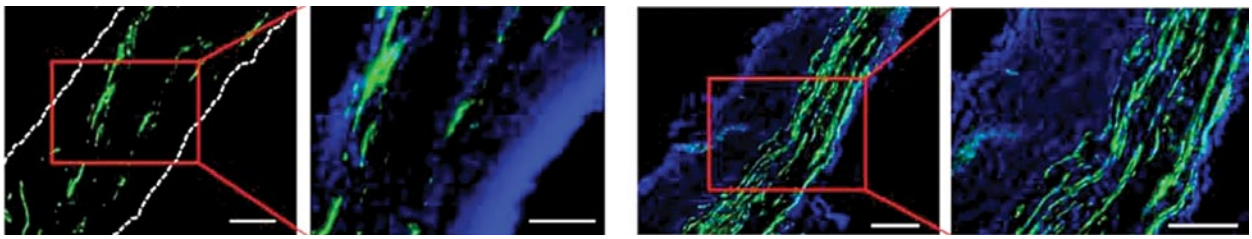


Figure VI-36. One and 14 days after implantation of an MDSC-integrated scaffold (left two images, 1 day; right two images, 14 days). Implanted cells express GFP (green); blue is Hoechst nuclear staining. Dotted line indicates the scaffold border. GFP-positive cells appear to have increased with time (Scale bar = 100 µm).

The bioactivity and mechanical properties of these scaffolds were successfully assessed in a rat full-thickness abdominal wall reconstruction model. This animal model will also be utilized for ongoing evaluations using MDSC-microintegrated scaffolds. Separation of polymer and dECM into different fabrication streams resulted in better bioactivity with higher cellular infiltration, and the mechanical properties could be manipulated by employing a novel sandwich technique. Biaxial mechanical assessments of sandwich-patched walls after 8 weeks were anisotropic with compliance curves similar to native tissue.

Research Plans for the Next 2 Years

Data collection is ongoing from explanted abdominal wall samples for the sandwich scaffolds and the control groups for that study. Constructs are being mechanically and histologically characterized over the implant period. Investigations have also been initiated with the GFP transgenic MDSC microintegrated scaffolds both in terms of in vitro characterization and in the small animal model. In the coming year, the Wagner group will determine the regenerative potential of the MDSC microintegrated scaffold approach from bioactivity and mechanical perspectives. If the MDSC approach continues to show promise, it can be combined with the described dECM sandwich scaffold approach or continued without dECM as is the current model.

With optimization of scaffold design ongoing in years 3 and 4, the researchers plan to initiate large animal trials with the porcine model later in year 4 using acellular constructs. Complexity will be built into the porcine model (in terms of construct design, evaluation methods and implant time) in years 4 and 5. In year 5, the researchers anticipate being in communication with the FDA regarding clinical trials. Applications of the developed materials will be considered for other applications as the technology matures. Potential applications include skin, craniofacial, and soft tissue reconstruction. When the large animal model is reached, it is anticipated that this technology may potentially be evaluated in concert with approaches developed in other AFIRM projects.

Planned Clinical Transitions

The Wagner group expects to continue exploring partnering opportunities with industry for the use of the developed materials in the coming years. There are ongoing discussions with a major manufacturer of abdominal wall and pelvic floor repair materials. Upon successful completion of the first set of porcine experiments, the researchers will engage the FDA in discussions to determine the preclinical data that would be required to justify filing for an Investigational Device Exemption. This work would occur in years 6 and 7. Once this milestone is met, clinical trials can commence, potentially in year 8.


our science for their healing

Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome

Project 4.3.4, WFPC

Team Leader(s): Stephen F. Badylak, MD, PhD, DVM (MIRM, University of Pittsburgh)

Project Team Members: Kerry Daly, BVSc (DVM equivalent), PhD and Scott Johnson, MS (MIRM, University of Pittsburgh) and Matt Wolf, BS (Department of Bioengineering, University of Pittsburgh)

Collaborator(s): None

Therapy: Treatment for peripheral CS.

Deliverable(s): Optimization of skeletal muscle extracellular matrix (smECM) and characterization of its properties. Reconstruction of functional compartmental tissue in animal models utilizing the inductive properties of biologic scaffolds and stem cells.

TRL Progress: Start of Program, TRL 1; End Year 1, TRL 2; End Year 2, TRL 3; End Year 3, TRL 5

Key Accomplishments: The researchers have optimized their smECM decellularization protocol to allow in situ decellularization of compartment tissues thus providing autologous ECM to the damaged area. They created autologous smECM by flushing compartment tissues with saline, 0.1% PAA, and 2% DOC. They characterized in situ autologous smECM with reference to growth factor content. They observed adipose tissue, muscle fibers, nerves, and connective tissue in the defect in the saline/PAA/DOC group at 3 months post-treatment.

Key Words: Peripheral compartment syndrome, extracellular matrix, mesenchymal stem cells

Introduction

Peripheral CS represents a serious complication of traumatic extremity injury, especially the type of trauma sustained by soldiers in combat. The fundamental problem is thought to be the severe swelling that occurs within a confined space (compartment), typically in the lower limb. The swelling and associated increased intracompartmental pressure severely compromise blood flow resulting in ischemic necrosis of all tissues within the compartment (e.g., muscle, nerves, and associated structures). The loss of functional tissue is frequently severe enough to require amputation of the affected limb. The standard of care for peripheral CS is fasciotomy with an attempt to salvage the viability of as much functional tissue as possible. Morbidity is high and includes severe aesthetic abnormalities (because of lost compartmental space).

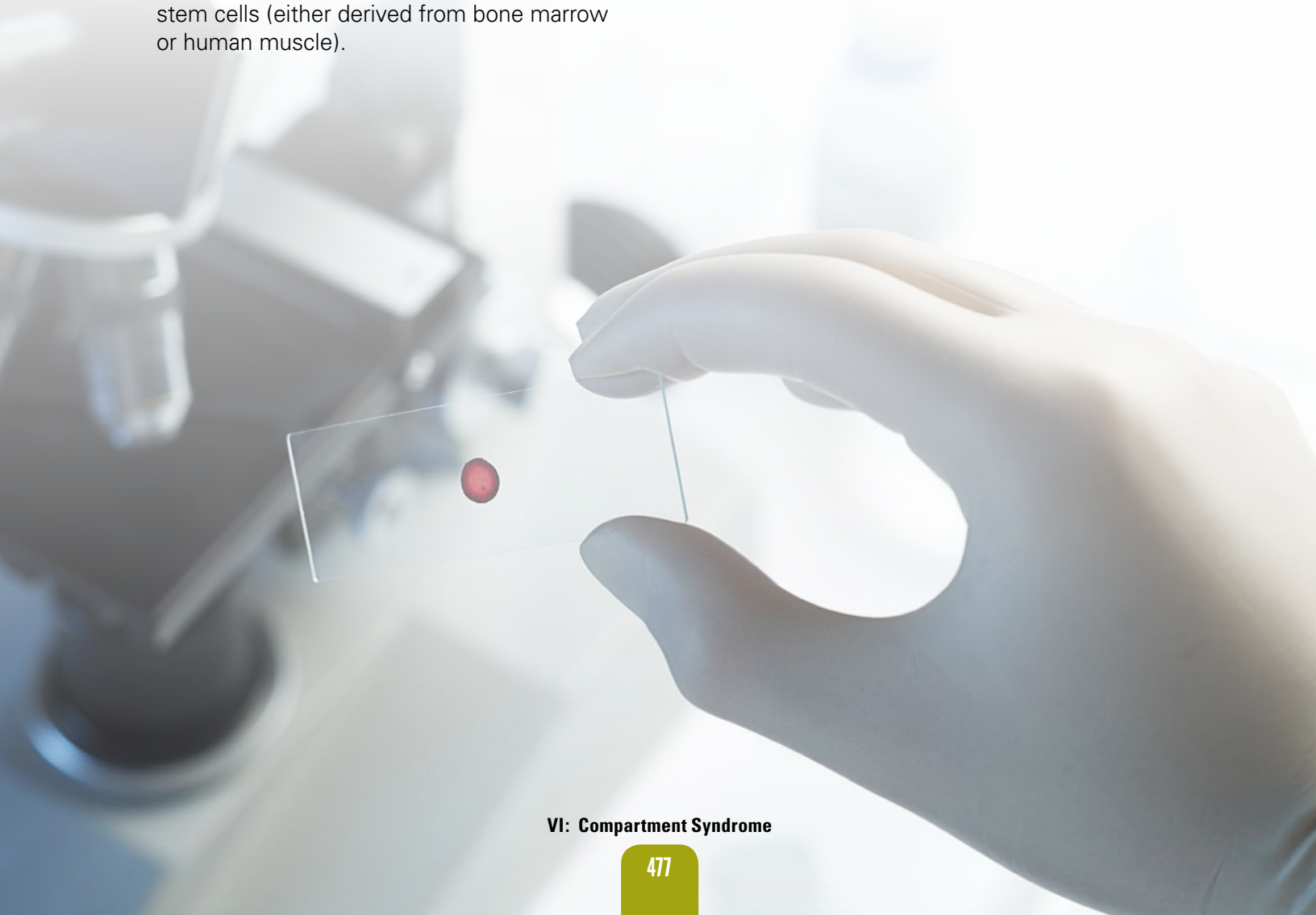
The Badylak group is investigating a method for utilizing the inductive properties of ECM as a scaffold for the recruitment of endogenous stem cells and the attachment, proliferation, and spatial organization of these cells into functional tissue. Previous work has shown that manufactured forms of ECM (e.g., porcine small intestinal submucosa, porcine urinary bladder, porcine and bovine dermis, pericardium, and others) have the potential to promote constructive remodeling of damaged or missing body parts in place of inflammation and scarring. The present work extends this concept by investigating

methods for the use of ECM scaffolds (with and without stem cells) in conjunction with traditional treatment methods to better facilitate regeneration of affected skeletal muscle compartments. In addition, the researchers are developing methods of in situ decellularization of the necrotic tissue while retaining the native ECM (autologous ECM). Stated differently, the ECM within the compartment is isolated from its original cell population (which has now become necrotic), and this matrix is then used as a template for tissue reconstruction.

The specific aims of this project are to (1) optimize the preparation/manufacturing of smECM and characterize its properties in vitro and (2) reconstruct functional compartmental tissue in animal models utilizing the inductive properties of biologic scaffolds and stem cells (either derived from bone marrow or human muscle).

Summary of Research Completed in Years 1 and 2

In the first 2 years of the project, the researchers established a reproducible model of peripheral CS in rabbit and dog. These models have now become the basis for preclinical animal studies. Previous work also included optimization of smECM decellularization and characterization of its properties considered to be important for biocompatibility in vitro and regeneration of functional skeletal muscle tissue in vivo. The researchers also conducted preclinical studies of the suitability of currently available biologic scaffolds and stem cells for treatment in their animal models.



Research Progress – Year 3

Optimization of Preparation/ Manufacturing of smECM

During the past year, the researchers further modified their smECM decellularization method to allow in situ decellularization of compartment tissues thus providing autologous smECM to the damaged area. Autologous smECM was created by flushing compartment tissues with saline, 0.1% PAA, and 2% DOC **(Figure VI-37A)**. These flushes, and in particular the combination of all three agents, resulted in a disruption of the normal skeletal muscle architecture, and led to a decrease in cell nuclei and DNA content (Figures VI-37B and C) when performed ex vivo.

The autologous smECM created by these methods also had decreased cellular proteins such as beta-actin, myosin, and heat shock protein 60 (HSP60) **(Figure VI-38A)**. However, growth factors, such as hepatocyte growth factor (HGF), VEGF, and basic fibroblast growth factor (bFGF) were retained in autologous smECM (Figures VI-38A and B). ECM that contains high amounts of DNA and cellular proteins may induce an increased pro-inflammatory response, which is associated with fibrosis and scarring rather than constructive remodeling. The retention of growth factors by ECM is important to provide inductive signals to the host tissue that facilitate constructive remodeling.

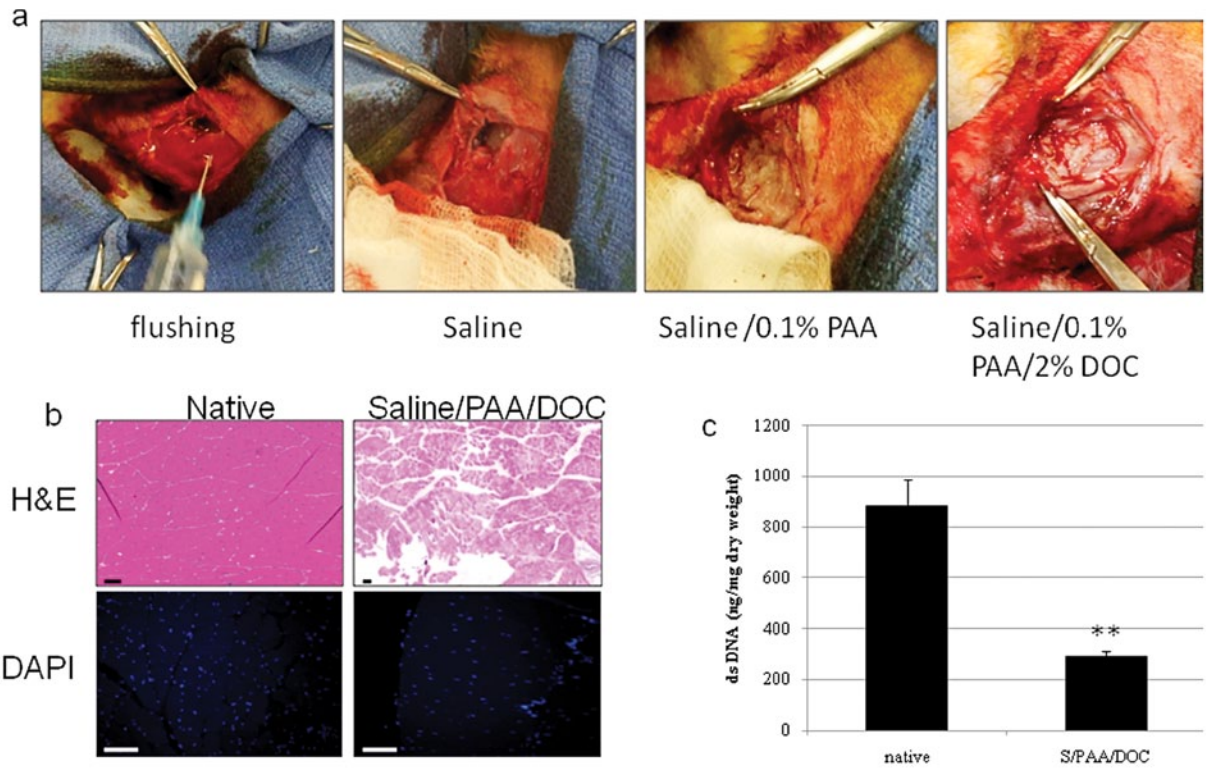


Figure VI-37. (A) Creation of autologous smECM by flushing of the compartment with saline, 0.1% PAA, and 2% DOC. (B) H&E and DAPI images following each treatment show an increased disruption of the tissue architecture and decrease in cell nuclei. (C) DNA content is decreased after full decellularization (saline/PAA/DOC) compared to the native tissue as demonstrated by picogreen assay.

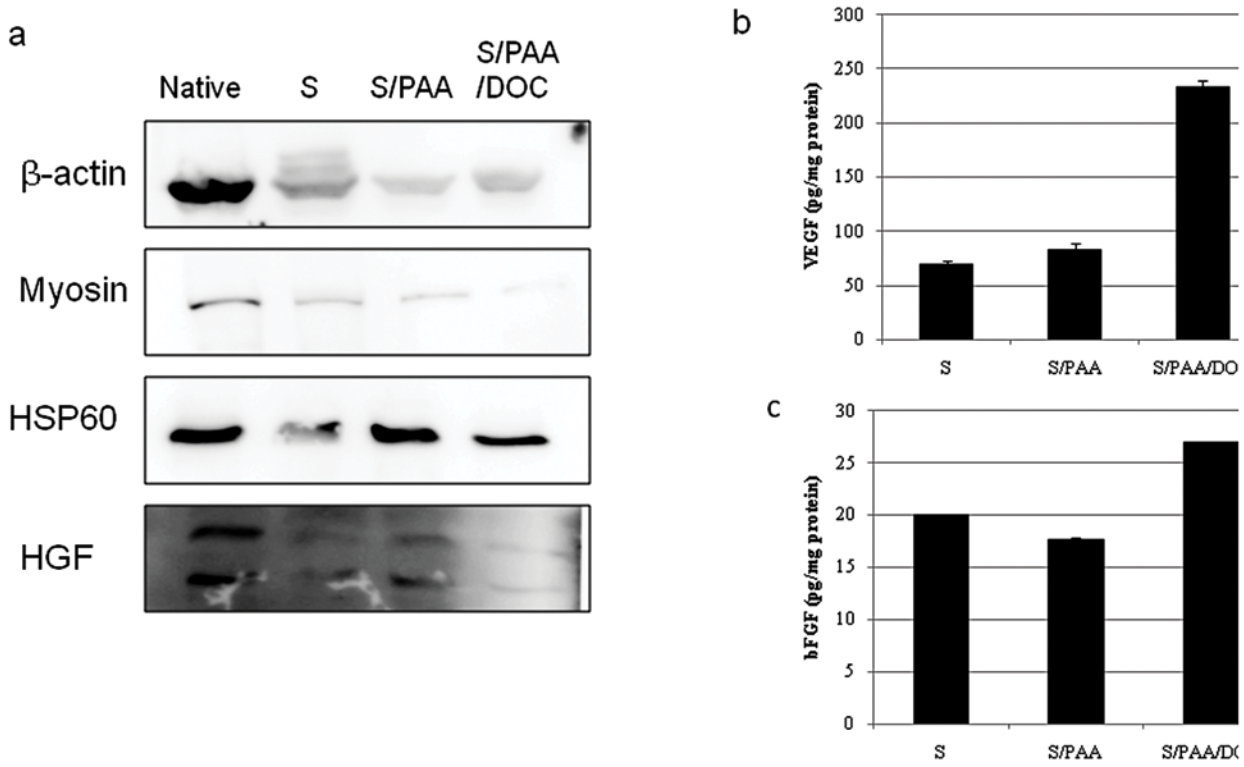


Figure VI-38. (A) Western blots of protein extracts from native tissue, saline (S), S/PAA, and S/PAA/DOC smECM indicate a decrease in cellular proteins β-actin, myosin, and HSP60, but HGF is retained. (B) Decellularized compartment tissue retains VEGF. (C) Decellularized compartment tissue retains bFGF.

Reconstruction of Functional Compartmental Tissue in Animal Models Utilizing the Inductive Properties of Biologic Scaffolds and Stem Cells

The reproducible model of peripheral CS in rabbit and dog that was established during the first 2 years of the project has become the basis for the researchers' current preclinical animal studies. The methods for creation of autologous smECM (detailed previously) were used for treatment of peripheral CS in the rabbit model. In addition, autologous MSCs were delivered to the affected compartment after decellularization of the compartment and isolation in situ of autologous ECM. This approach overcomes many of the current concerns about ECM in that both the cells and ECM are autologous thus avoiding concerns about xenogeneic sources.

At 1 month post-treatment, new muscle cells and inflammatory cells were found in the defect area only in the group treated with saline/PAA/DOC **(Figure VI-39)**. Many of the muscle fibers within the defect area have multiple, centrally located nuclei and stain positive for embryonic myosin (Figure VI-39), which suggests myogenesis. In the saline and saline/PAA, scar tissue is seen within the defect (Figure VI-39). At 3 months following surgery, fibrous connective tissue is seen within the defect area on both saline and saline/PAA groups **(Figure VI-40)**. In contrast, adipose tissue, muscle fibers, nerves, and connective tissue are found in the defect in the saline/PAA/DOC group at the

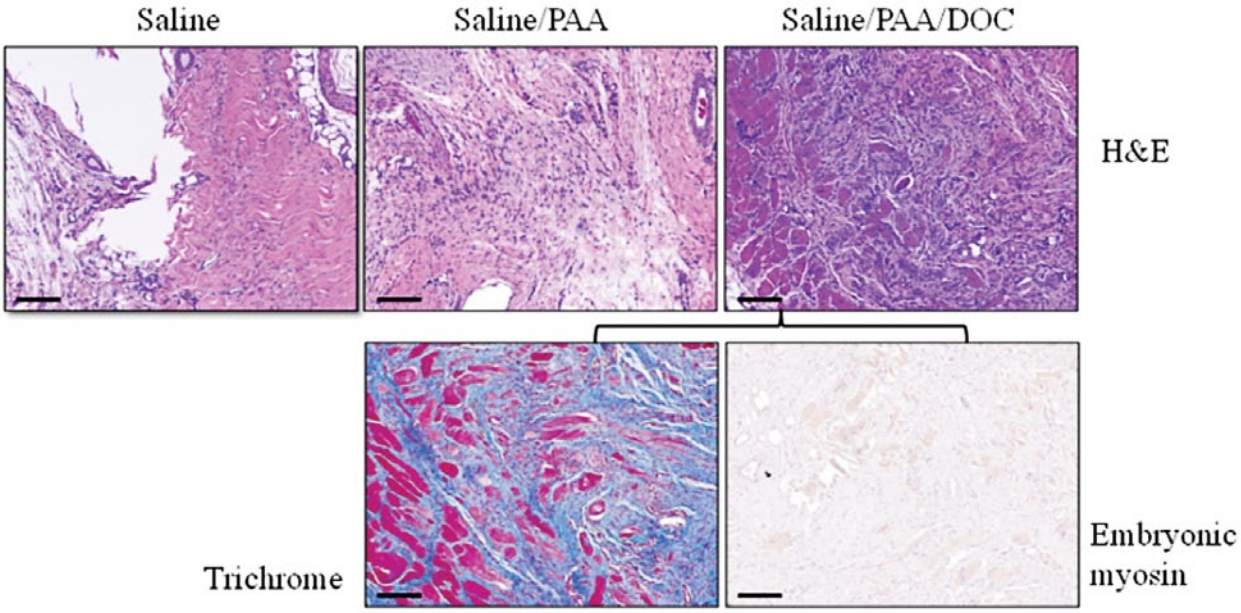


Figure VI-39. Fibrous connective tissue is found within the defect in saline and saline/PAA groups 1 month after surgery. Islands of muscle and inflammatory cells are seen in the saline/PAA/DOC group. Many of these muscle cells stain positive for embryonic myosin. Scale bar represents 100 μm.

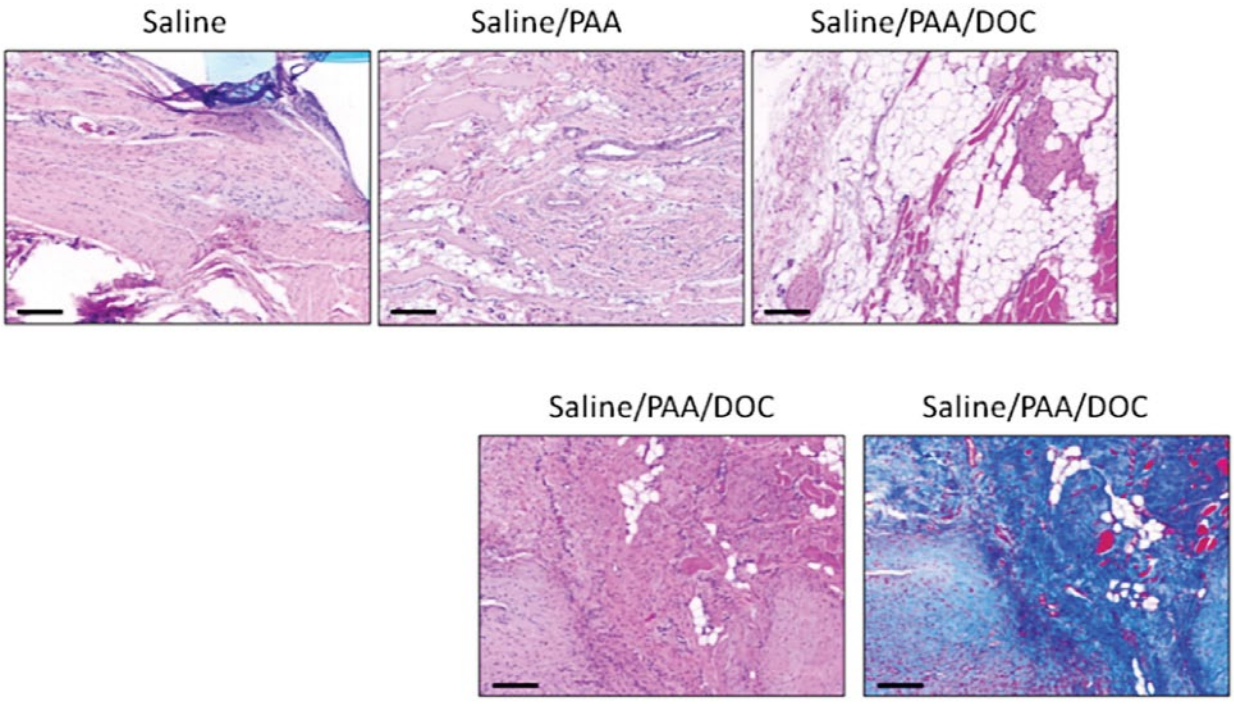


Figure VI-40. Fibrous connective tissue is found within the defect in saline and saline/PAA groups 3 months after surgery. Muscle fibers, nerves, adipose, and fibrous tissue are seen in the saline/PAA/DOC group. Scale bar represents 100 μm.

3-month time point (Figure VI-40). Therefore, complete decellularization with the saline/PAA/DOC resulted in a better outcome than those animals in which cells or cell remnants were left in situ by ineffective methods of decellularization.

To determine the potential role of the autologous MSCs in the myogenesis seen in the saline/PAA/DOC group, MSCs were labeled with Qdot® nanocrystals (Qtracker®-655, Invitrogen). One month following their implantation in saline/PAA/DOC-treated animals, Qdot-labeled MSCs were found surrounding muscle fibers **(Figure VI-41A)** and within the perimysium between muscle bundles (Figure VI-41B). The survival of these MSCs up to 3 months is currently being assessed.

Key Research Accomplishments

- Optimized the smECM decellularization method to allow in situ decellularization of compartment tissues thus providing autologous ECM to the damaged area.
  - ♦ Created autologous smECM by flushing compartment tissues with saline, 0.1% PAA, and 2% DOC.
- Characterized in situ autologous smECM with reference to growth factor content.
- Evaluated the host response to in situ autologous smECM at 1 and 3 months and found that at 1 month post-treatment, new muscle cells and inflammatory cells were located in the defect area only in the group treated with saline/PAA/DOC.
- Observed adipose tissue, muscle fibers, nerves, and connective tissue in the defect in the saline/PAA/DOC group at 3 months post-treatment.

  Therefore, complete decellularization with saline/PAA/DOC resulted in a better outcome than in animals in which cells or cell remnants were left in situ by ineffective methods of decellularization.

Conclusions

The researchers are finding that complete decellularization of smECM with saline, 0.1% PAA, and 2% DOC in a rabbit model can result in the growth of adipose tissue, muscle fibers, nerves, and connective tissue in the defect at 3 months post-treatment. They are elucidating the role of autologous MSCs

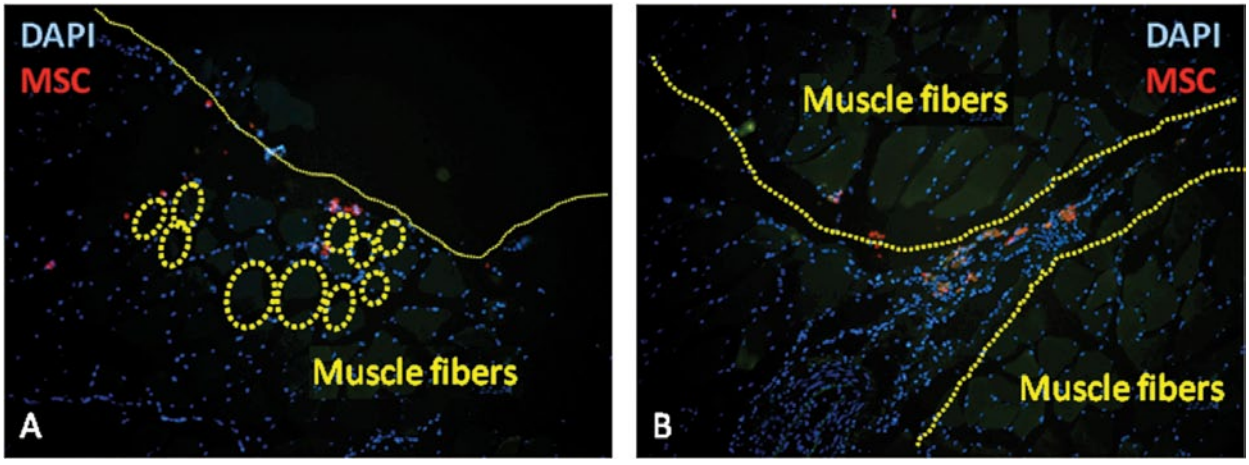


Figure VI-41. Saline/PAA/DOC group 1 month following treatment. MSCs (red) labeled with Qdot nanocrystals can be seen surrounding the muscle fiber (A) and within the perimysium (B). Sections are counterstained with DAPI (blue).

in the myogenesis that is observed in the saline/PAA/DOC-treated animals.

Research Plans for the Next 2 Years

The researchers plan to complete the rabbit animal study. Since this study involves a 6-month follow-up, the time to complete the study is not trivial. Samples are being collected, histology is being completed, and functional assays are being evaluated. As detailed previously, the researchers have expanded the study to include determination of the fate of injected autologous MSCs. They have used Qdot technology to show that injected cells with various forms of ECM do indeed remain at the site of remodeling. They aim to determine whether the MSCs have transformed into differentiated muscle cells. Following completion of the rabbit study, the researchers will use the most appropriate in situ autologous smECM treatment method in a larger animal (porcine) model.

Planned Clinical Transitions

There are few options for the treatment of advanced peripheral CS. Complete loss of compartmental functional musculature results in the need for prosthetic devices in most cases. Alternative approaches to reconstructing individual components of the compartment such as muscle, blood vessels, and nerves are in progress, but to current knowledge, there is no work currently being done to reconstruct the complex architecture of the innervated, vascularized skeletal muscle architecture of the compartment. Many approaches are investigating the use of stem cells, deposited at the site of damaged tissue, for their ability to reconstitute functional tissue, but this approach would combine such cells with the ECM bioscaffold to optimize outcome. If the researchers' large animal preclinical studies in year 4 show positive results, they will prepare a human clinical trial for year 5.

Material-Induced Host Cell Recruitment for Muscle Regeneration

Project 4.3.5, WFPC

Team Leader(s): Sang Jin Lee, PhD (Wake Forest University)

Project Team Members: James Yoo, MD, PhD, Benjamin Harrison, PhD, Young Min Ju, PhD, and In Kap Ko, PhD (Wake Forest University)

Collaborator(s): Shay Soker, PhD (WFIRM)

Therapy: Treatment of muscular injuries through in situ muscle tissue regeneration.

Deliverable(s): Demonstration of in situ muscle tissue regeneration using a target-specific scaffolding system.

TRL Progress: Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 3; End Year 3, TRL 3

Key Accomplishments: The researchers demonstrated that host muscle satellite/progenitor cells can be mobilized into implanted biomaterials in situ and that these cells can be differentiated into myogenic lineage using myogenic-inducing factors in vitro. They also developed scaffolds that incorporate myogenic-inducing factors for in vivo demonstration of host muscle satellite/progenitor cell differentiation. In addition, they developed a novel combination of "systemic delivery" SP via intravenous injection and "local release" of SDF-1α from an implanted scaffold to improve the efficiency of host stem cell mobilization.

Key Words: Biomaterials, myogenic-inducing factor, in situ tissue regeneration, host stem cell mobilization, compartment syndrome, muscle regeneration

Introduction

CS is a common traumatic injury that results in muscle, nerve, and vessel damage due to increased pressure within a confined space in the body. Although CS can affect any limb or muscle compartment, including the abdomen, it frequently occurs after trauma to the lower leg such as fracture. The standard treatment is fasciotomy, which is considered as the definitive and only treatment for acute CS. Although this procedure is able to relieve immediate concerns, muscle weakness and atrophy are continued sequelae. Various management approaches have been introduced, including physical therapy, muscle transplantation, and myoblast cell therapy. However, none has entirely addressed the problems associated with the long-term consequences of CS in wounded soldiers.

In this project, the researchers aim to use stem or progenitor cells residing in the host to regenerate muscle tissue through the use of a target-specific scaffolding system. This approach is based on the demonstration that almost every tissue in the body contains some type of stem or progenitor cells. The putative healing mechanisms and classic foreign body reaction to implanted biomaterials have also been characterized. However, these two mechanisms would seem to be in conflict with one another particularly with respect to functional outcome. While small, localized, day-to-day injuries are repaired by the body's stem and progenitor cell machinery, large traumatic injury overwhelms this system and survival mechanisms take over. This process often

creates a deficit of functional recovery. The specific aims of this project are to investigate this possibility using an animal model to initiate cell mobilization, recruitment, and differentiation in vivo and to demonstrate in situ muscle tissue regeneration using a target-specific scaffolding system.

Summary of Research Completed in Years 1 and 2

The researchers previously demonstrated that when biomaterial scaffolds were implanted in muscle, host muscle stem/progenitor cells that expressed the markers PAX3, PAX7, and MyoD became localized within the scaffolds. During the first 2 years of the study, they introduced a variety of myogenic-inducing factors into scaffolds and tested their ability to promote host muscle satellite/progenitor cell mobilization, including migration, proliferation, and differentiation. They found that cell proliferation was significantly increased by the addition of insulin-like growth factor I (IGF-I), IGF-II, or bFGF compared to controls. The researchers also measured the ability of these myogenic factors to induce cell migration in a Boyden chamber plate assembly with polycarbonate porous membrane inserts. They found that the presence of HGF, IGF-I, IGF-II, and bFGF significantly increased cell migration when compared to controls.

Research Progress – Year 3

In Vitro Evaluation of Myogenic-Inducing Factors

To evaluate the myogenic factors that affect muscle cell differentiation (myotube formation), C2C12 cells were placed in each well of a 6-well culture plate. The cells were grown in standard growth medium for up to 24 hours followed by incubation in differentiation medium containing each growth factor (100 ng/mL) for up to 10 days to induce the formation of myofibers. At day 10, the fused cells were characterized by immunofluorescent staining using an anti-MHC antibody (MF20) as the primary antibody. After immunofluorescent staining, the cells were viewed using a fluorescence microscope. Five HPF were analyzed to calculate the average number of fibers per HPF. Twenty fibers from each myogenic condition were analyzed to calculate the average number of nuclei per fiber. **Figure VI-42** indicates the number of myotubes per HPF and the number of nuclei per myotube for each condition and that myogenic-inducing factors including IGF stimulate muscle cell differentiation.

Heparin-Immobilized Gelatin Scaffold and Release Kinetics

To incorporate myogenic factors into the scaffold, heparin was immobilized onto gelatin scaffolds (Gelfoam®, Upjohn, Kalamazoo, Michigan). Heparin, a sulfated polysaccharide, has been widely used as a surface modifier to enhance the stability of biologically important proteins, such as growth factors and cytokines, via binding affinity. **Figure VI-43A** shows a schematic illustration that indicates how the myogenic factors were incorporated into the heparin-immobilized gelatin scaffolds.

For the protein release study, lysozyme was used as a model protein. The lysozyme was loaded onto both heparin-modified or unmodified gelatin scaffolds using a solution dropping method. Sixty microliters of PBS containing 120 μg of lysozyme was dropped onto the dried gelatin scaffolds ($5 \times 10 \times 4$ mm^3) and the samples were allowed to react at 4°C for 12 hours to allow protein loading. The protein-loaded scaffolds were then suspended in 1 mL phosphate-buffered saline (PBS) (containing 0.1% bovine serum albumin) at 37°C. At various time points the supernatants were collected, and the amount

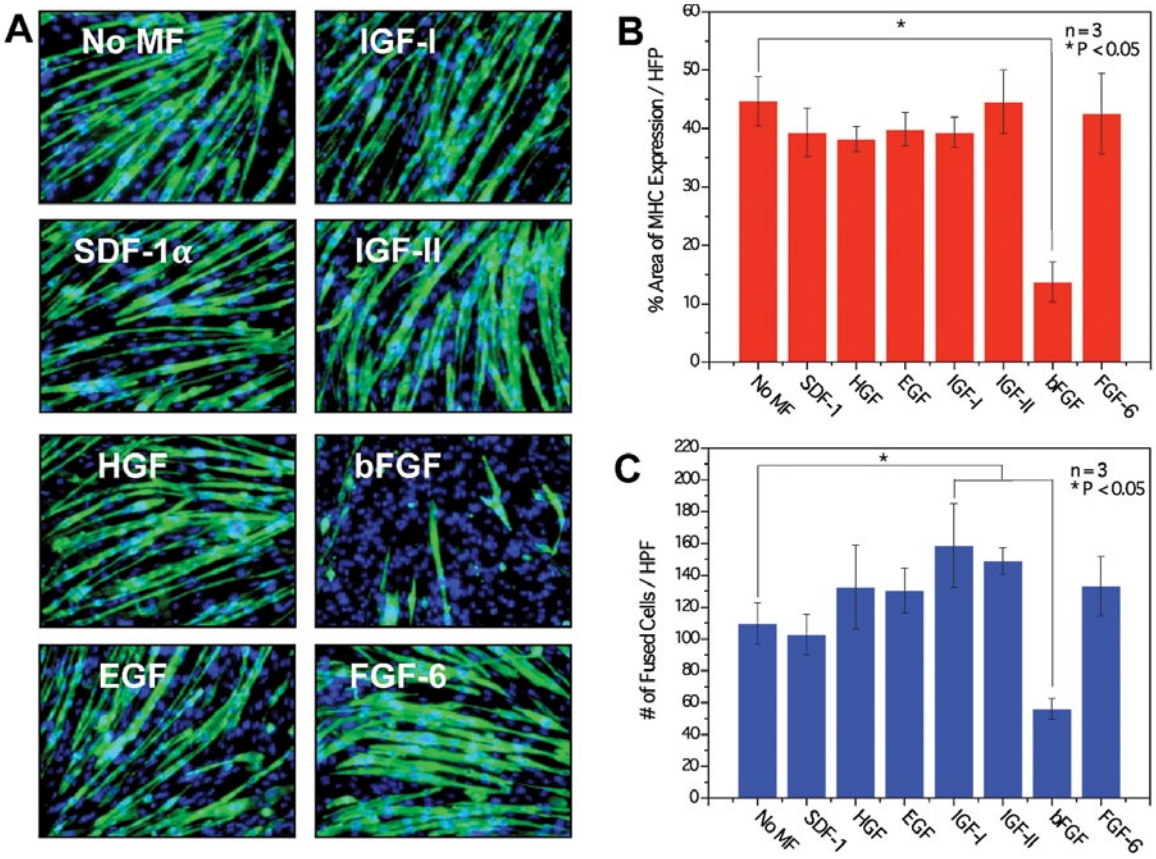


Figure VI-42. (A) Immunohistochemistry using an anti-MHC antibody. After 10 days in differentiation medium, C2C12 cells fused, formed myotubes, and aligned. Magnification = 100x. Histomorphometrical evaluation of (B) percentage area of MHC expression and (C) number of fused cells per HPF. Results are means ± S.D. of samples. * indicates that values are significantly different from the corresponding value of control cells ($p < 0.05$). (MF: Myogenic Factor)

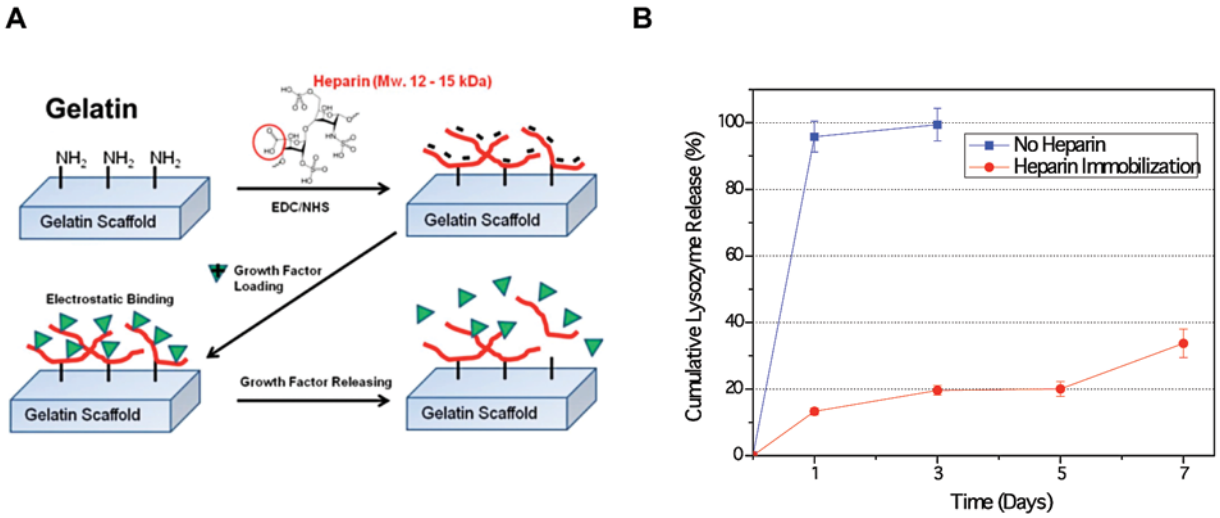


Figure VI-43. (A) Schematic illustration of incorporation of myogenic factors on the heparin-immobilized gelatin scaffold. (B) In vitro release profiles of lysozyme from the modified and unmodified gelatin scaffold for 7 days.

of released lysozyme was determined by direct absorbance using a spectrophotometer (405 nm). Figure VI-43B shows the controlled released profile of lysozyme obtained using a heparin-immobilized gelatin scaffold, and this is compared to the release profile of an unmodified scaffold.

Effects of Myogenic Factors in Vivo

To evaluate the myogenic factors that affect the recruitment of muscle progenitor cells in vivo, the researchers implanted heparin-immobilized gelatin scaffolds ($5 \times 10 \times 4$ mm^3) that were preloaded with various myogenic factors (500 ng/mL of SDF-1α, HGF, IGF-I, and bFGF) into the lower leg muscles in SD rats. The scaffolds were then retrieved at 1, 2, 8, and 12 weeks after implantation. At these set time intervals, the retrieved scaffolds were fixed in 10% buffered formalin and embedded in paraffin. Six μm thick sections were stained with H&E, Masson's trichrome, and various immunohistochemical markers.

Host cell infiltration into the scaffold increased up to 2 weeks post-implantation and was still maintained 12 weeks after implantation. By the 8th and 12th weeks, host cells had accumulated within each individual scaffold region, and abundant host vasculature was found within the scaffold. Particularly, a large area of fat tissue formation was observed in the bFGF-loaded gelatin scaffold at 12 weeks post- implantation. Masson's trichrome staining of representative sections after 1, 2, 8, and 12 weeks of implantation showed the gradual buildup of a neo-muscle fiber bundle structure around the IGF-I-loaded gelatin scaffold **(Figure VI-44)**.

To determine whether muscle progenitor cells were present in these scaffolds, the infiltrating cells were stained using an antibody to the muscle progenitor cell marker anti-Pax7. In **Figure VI-45A**, immunohistochemical assessment showed a dramatic increase in the population of Pax7-positive cells over time, and the numbers of these muscle

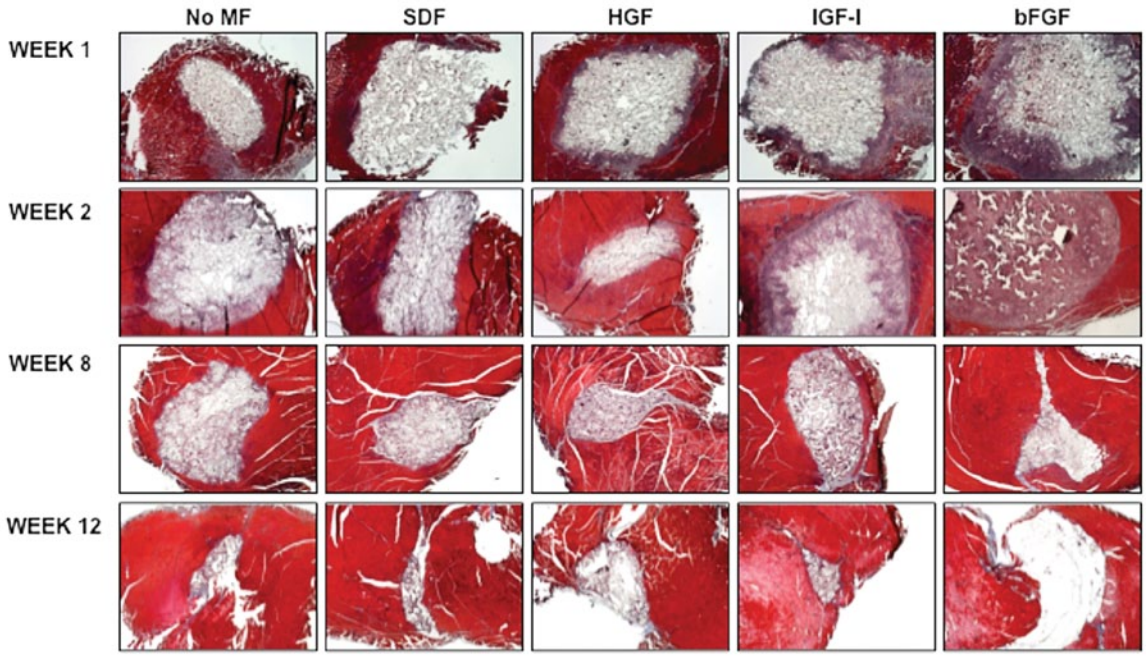


Figure VI-44. Histological evaluation of the implanted scaffolds. Masson's trichrome staining of the retrieved scaffolds at 1, 2, 8, and 12 weeks after implantation.

progenitor cells were significantly increased by the addition of myogenic factors to the scaffold prior to implantation. In particular, muscle progenitor cells were mobilized to the IGF-I and bFGF-loaded scaffolds at 1 and 2 weeks post-implantation (Figure VI-45B).

In this study, the researchers developed a new and innovative delivery method that combined systemic and local delivery of factors (SP and SDF-1α) that have been shown to enhance recruitment of endogenous stem cells, such as MSCs and hematopoietic stem cells. These two factors were chosen to attempt to induce balanced cell infiltration into implanted scaffolds. Specifically, the study was designed to (1) increase the number of host stem cells in the body's stem cell pool via systemic SP injection and (2) enhance the recruitment of these host stem cells into the implanted scaffolds by local release of SDF-1α by the scaffolds themselves. The schematic diagram describes the research team's proposed strategy for enhancement of host stem cell infiltration into the implanted scaffold in detail **(Figure VI-46)**. To investigate whether this combined "systemic and local delivery" of SP and SDF-1α could promote host stem cell recruitment in the implanted scaffolds, they implanted SDF-1α-loaded biomaterial scaffolds under the dorsal skin of mice and then injected SP systemically through the tail vein. They then characterized the cells that infiltrated into implanted scaffolds by performing immunohistochemistry and flow cytometric analysis of cells isolated from the retrieved scaffolds.

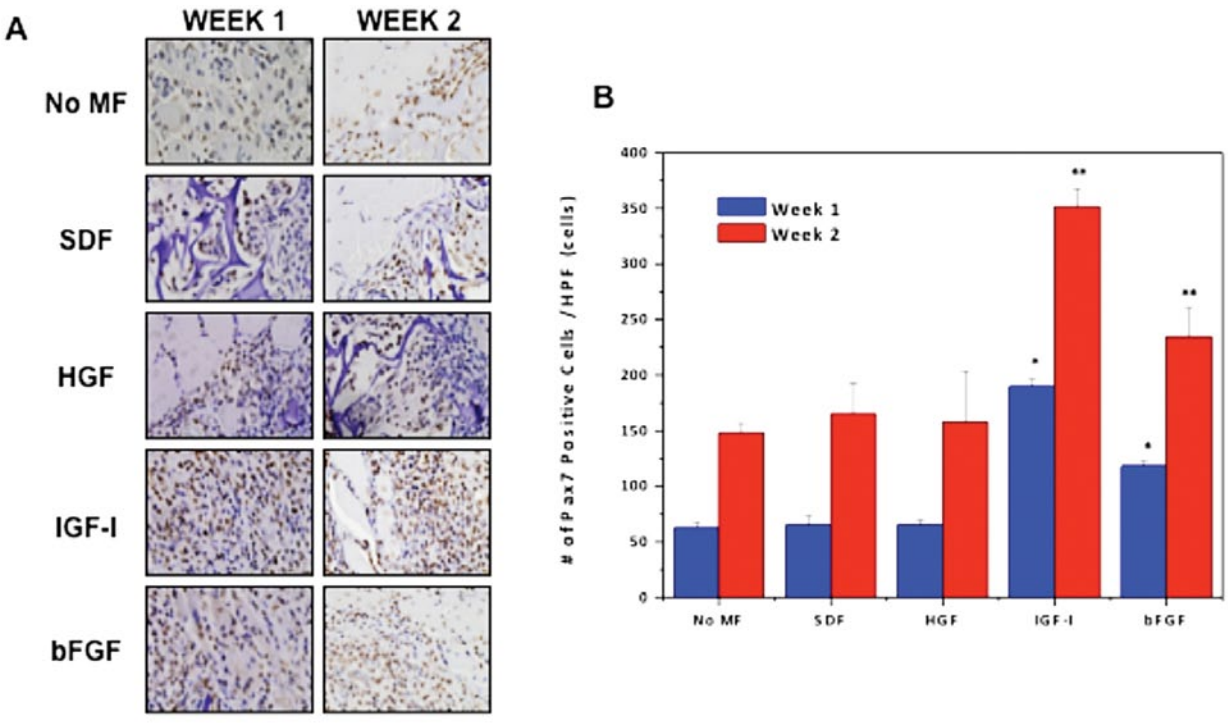


Figure VI-45. Immunohistochemistry images of muscle progenitor cell marker, (A) Pax7 expression of tissue sections retrieved at 1 and 2 weeks after implantation. (B) Histomorphometric analysis of number of anti-Pax7- positive cells. Results are means ± S.D. of three replicates. *, ** indicate that values are significantly different from the corresponding value of cells under no myogenic factor condition ($p < 0.05$). (MF: Myogenic Factor).

Figure VI-47A-C shows representative confocal images of sites within the interior of the implanted scaffolds in the control group (scaffold only) and SP(iv)/SDF group (combination delivery) (Figure VI-47B) that have been immunostained for CD29 and CD45. The control scaffold has only a few $CD29^+$ cells and larger numbers of $CD45^+$ cells, which may be inflammatory macrophages or dendritic cells. On the other hand, immunohistochemical evaluation showed that the retrieved scaffolds in the SP(iv)/SDF group contained more $CD29^+$ cells than the control group but almost no $CD45^+$ cells. To quantify these results, the numbers of $CD29^+CD45^-$ and $CD29^-CD45^+$ cells were counted in merged images. The combination delivery system produced the highest number of $CD29^+CD45^-$ cells within the implanted scaffolds, and this was especially noticeable within the interior of the scaffold where there was a 10-fold increase in cell number when compared to the control group (Figure VI-47C). This significant difference was also observed when the scaffolds from the combination delivery group were compared to groups that received only local delivery of single or dual factors (ANOVA analysis, $p < 0.001$ for interior and total and $p < 0.05$ for periphery site and Tukey post hoc test at $p < 0.05$).

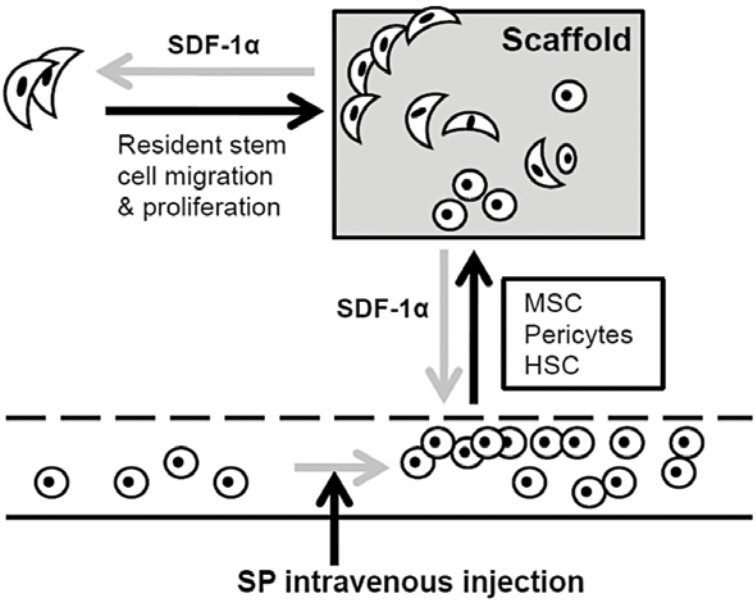


Figure VI-46. Proposed mechanism for efficient recruitment of host stem cells into an implanted scaffold using a novel combined delivery system composed of systemic injection of SP and local release of SDF-1α from the implanted scaffold. Systemically administrated SP will mobilize host stem cells, such as MSCs, from the bone marrow to the blood stream and induce the MSC enrichment. Locally released SDF-1α from the implanted scaffold will recruit the resident stem cells, which express the CXCR4 protein (SDF-1α receptor), into the scaffolds.

The researchers demonstrated that the novel combined system of systemic injection of SP and local release of SDF-1α can be a powerful tool for efficient tissue regeneration in situ. Their results show that delivery of both single factors (SP or SDF-1α) from poly L-lactic acid (PLA)/gelatin scaffolds leads to increased recruitment of host stem cells that may be able to contribute to in situ tissue regeneration. However, the combined delivery system for both SP and SDF-1α further enhanced host stem cell recruitment into the implanted scaffolds.

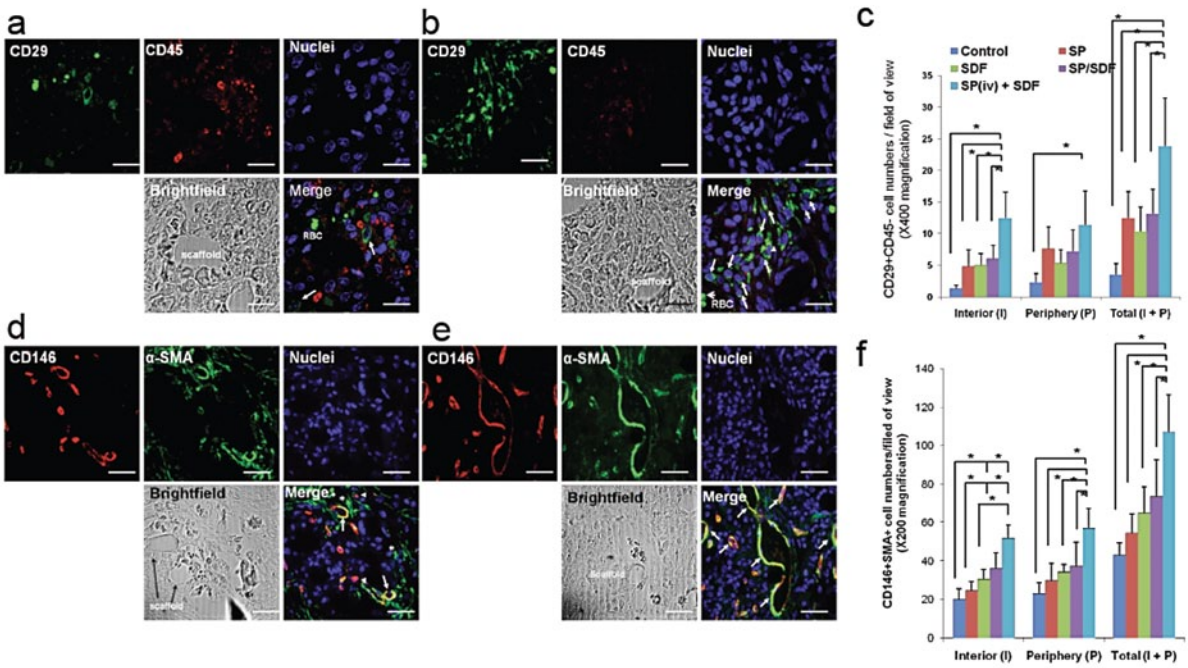


Figure VI-47. Immunostaining for MSC surface markers CD29/CD45 (a-c) and pericyte markers CD146/α-SMA (d-f). The retrieved scaffolds from control (no delivery) (a and d) and SP(iv)$^{+}$SDF (combined delivery groups) (b and e) were immunostained, and confocal images of the interior sites of the scaffolds are shown. The recruited CD29^{+}CD45^{-} and CD146^{+}α-SMA^{+} cells were counted in the confocal images (n=3 images per both interior and periphery sites) and plotted as a graph (c and f). This result strongly indicates that the combined delivery system recruited the highest number of CD29^{+}CD45^{-} cells into both the interior and the peripheral sites of the scaffolds (n=4–6, ANOVA at $p<0.001$ in interior and total and $p<0.05$ for periphery, *Tukey post hoc test at $p<0.05$). Results for the CD146^{+}α-SMA^{+} cell population were similar (n=6–8, ANOVA at $p<0.0001$ in interior, periphery, and total, *Tukey post hoc test at $p<0.05$). Scale bar indicates 25 μm (a and b) and 50 μm (d and e). RBC indicates red blood cells. Arrows in merged images indicate CD29^{+}CD45^{-} cells (a and b) and CD146^{+}α-SMA^{+} cells (d and e). Arrowhead and asterisk (*) indicate CD146^{+}α-SMA^{-} and CD146^{-}α-SMA^{+} (d and e) cells, respectively.

Key Research Accomplishments

- Evaluated a variety of myogenic-inducing factors for muscle satellite/progenitor cell mobilization in vitro.
- Incorporated myogenic-inducing factors within the heparin-immobilized biomaterial system.
- Demonstrated host muscle satellite/progenitor cell infiltration into myogenic-inducing factor incorporated scaffolds in vivo.
- Developed a novel combination delivery system for efficient host stem cell mobilization and demonstrated host stem cell mobilization in vivo using this system.
- Established a CS model in rats (in collaboration with Dr. Shay Soker).

Conclusions

The Lee group evaluated various myogenic-inducing factors for muscle cell migration, proliferation, and differentiation in vitro and investigated the possibility of using an appropriate biomaterial to initiate cell mobilization and recruitment in vivo. This study suggests that it may be possible to use the body's biologic and environmental resources for in situ muscle tissue regeneration. The researchers demonstrate that PAX7-expressing cells can be mobilized into an implanted biomaterial and that these cells are capable of differentiating into muscle cells.

The Lee group also demonstrated that a novel combined system of systemic injection of SP and local release of SDF-1α can be a powerful tool for efficient tissue regeneration in situ. Their results show that delivery of both single factors (SP or SDF-1α) from PLA/gelatin scaffolds leads to increased recruitment of host stem cells that may be able to contribute to in situ tissue regeneration. However, the combined delivery system for both SP and SDF-1α further enhanced host stem cell recruitment into the implanted scaffolds. The incorporation of multiple regulatory signals into a scaffolding system may be a promising approach for more efficient and effective tissue regeneration in situ.

Research Plans for the Next 2 Years

In the upcoming years, the Lee group will continue the long-term in vivo evaluation of the myogenic-inducing factor-incorporated biomaterials and continue the development of a smart scaffolding system for application. Additionally, they will perform in vivo studies investigating in situ muscle tissue regeneration in a rat CS model.

Planned Clinical Transitions

This basic research project is not slated for clinical trials during the first 5 years of the award.

VII: AFIRM Statistics

Introduction

The AFIRM is a large, complex biomedical research and development consortium of collaborating scientists, engineers, and medical product development experts. Across the AFIRM consortia, hundreds of basic and clinical researchers apply their expertise in regenerative medicine research and development to find medical solutions to treat our service members with severe combat wounds. AFIRM investigators and product developers represent more than 50 research universities and hospitals and 18 commercial partners when accounting for all core institutions and collaborating organizations.

While the previous chapters of this report demonstrate the depth of the AFIRM's research projects, this chapter displays the research consortium as a whole, rather than as many individual components, to provide a global perspective of the program's breadth. This chapter demonstrates the extent and quality of scientific and technical expertise being applied to the problems of regenerative medicine by displaying aggregated program data. This chapter also demonstrates tangible, scientific outcomes attributable to AFIRM-supported research: inventions disclosed, patent applications filed, research or review articles published, conference and meeting presentations and posters presented, and the advancement of products through research and development stages. The AFIRM data shown in this chapter cover the first 3 years of the program with particular emphasis placed on the AFIRM in program year 3 (PY3).

Personnel

A substantial workforce has been funded through the AFIRM to conduct research on regenerative biology and medicine, from faculty members to undergraduate students **(Figure VII-1)**. Notably, more than 100 research faculty were funded through the AFIRM in PY3.[1] Another 64 postdoctoral associates and fellows and 85 scientific and technical staff were funded through the AFIRM in PY3.[2] Finally, with more than 40 graduate students and 22 undergraduate students funded through AFIRM projects to conduct research, the program is substantially contributing to the training of the next generation of scientists to advance regenerative medicine research and development into the future.

In addition to the many scientists directly supported by the AFIRM, many personnel conducting research for the program were not directly supported with AFIRM funds (shown as the green bar and purple bar extensions in **Figure VII-2**). These scientists contribute complementary technical expertise needed to achieve the goals of the program. For example, 27 faculty contributed to Wake Forest-Pittsburgh Consortium (WFPC) and Rutgers-Cleveland Clinic Consortium (RCCC) research projects without being funded by the AFIRM. An additional 22 postdoctoral fellows, 20 graduate students, 26 undergraduate students, and 28 staff scientists and technicians contributed to AFIRM research projects in the third program year without being funded by the program (Figure VII-2).

Another highlight of the AFIRM is the substantial recruitment of young talent into the field of regenerative medicine. More than 100 students (64 graduate students and

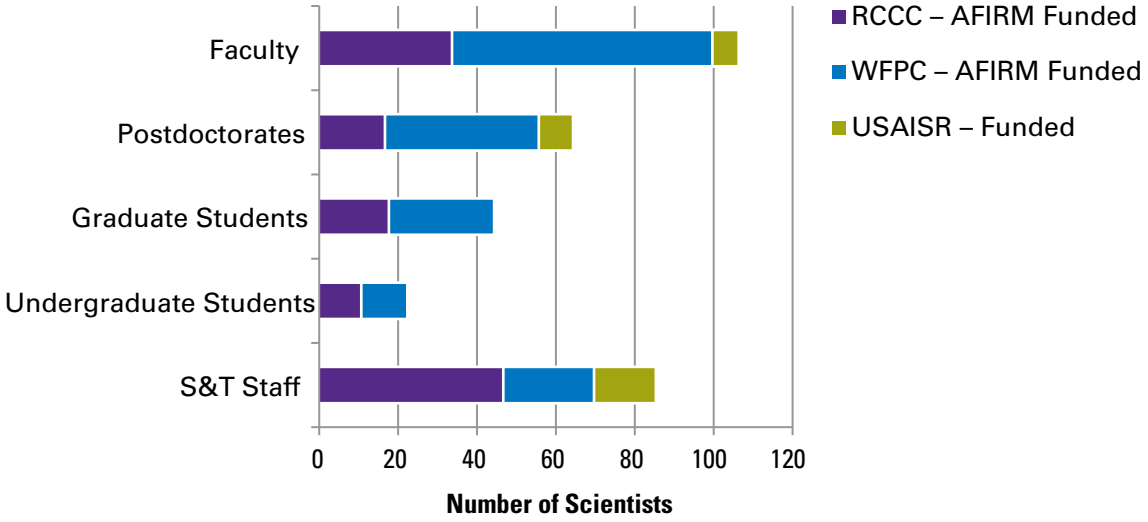


Figure VII-1. Numbers of scientists and students supported through the AFIRM program during PY3.[3]

[1] For the purpose of this annual report, PY3 is defined as the period from June 1, 2010 to May 31, 2011. PY2 spans June 1, 2009 to May 31, 2010, and PY1 spans the period from the initiation of research projects in May 2008 through the end of May 2009.

[2] The numbers may be slightly overestimated due to some individuals working on multiple projects. To minimize duplicate counts of the same individuals, the names of scientists and students provided by project investigators were cross-referenced. Anyone who contributed to more than one project was counted only once. However, not all individuals who worked on the AFIRM were named; thus, it is possible that some individuals working on two or more projects could have been included in the count for each separate project.

[3] This chart displays the number of unique individuals supported by the AFIRM program during any part of the third program year.

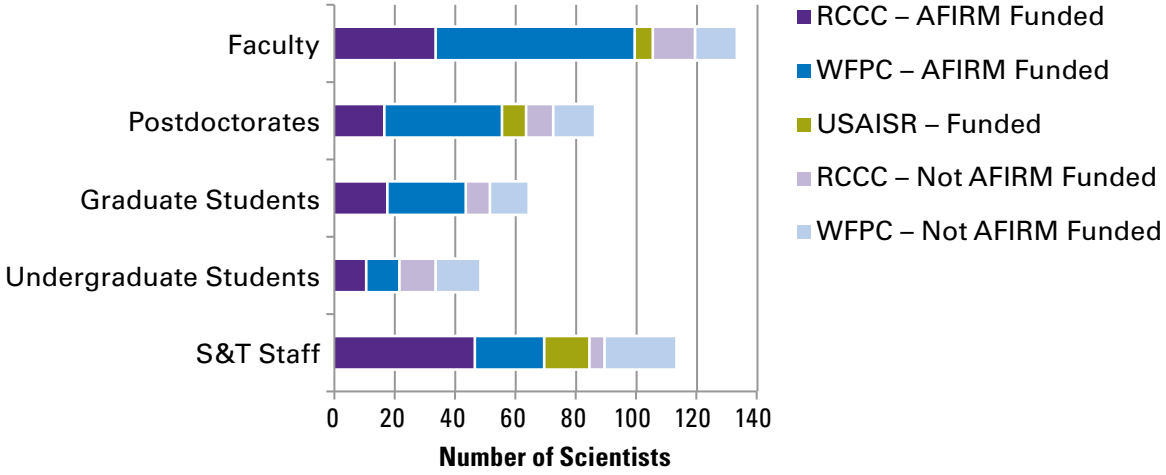


Figure VII-2. Numbers of scientists and students who conducted research for AFIRM projects during PY3.

48 undergraduate students) received valuable scientific training through AFIRM-sponsored research projects in PY3 (shown in Figure VII-2). The numbers of AFIRM-supported graduate students who completed their degree requirements each year of the program are shown in **Figure VII-3:** from 3 graduate degree recipients in PY1 to 12 in PY2 and 17 in PY3. From PY1 to PY3, the number of students who completed master's degrees increased from 1 to 3, and the number of students who completed PhD degrees increased from 2 to 14. For the 3-year span of the program, a total of 7 master's degree recipients and 25 PhD degree recipients received training through the AFIRM program.

The AFIRM program was organized into five research program areas: Limb and Digit Salvage, Craniofacial Reconstruction, Burn Repair, Scarless Wound Healing, and Compartment Syndrome.[4]

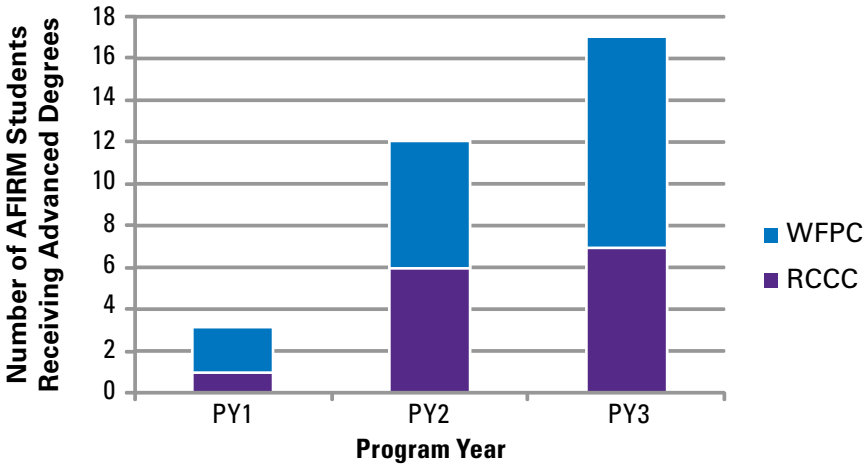


Figure VII-3. Number of graduate degrees awarded to students who received training through AFIRM projects.

[4] As the AFIRM program has matured, the overarching research program areas have been redefined by the consortia to more accurately describe the topic areas. To compare the consortia across different years, Figure VII-4 displays the original research program areas.

Figure VII-4 depicts the proportion of all core personnel, funded and unfunded, who worked on the different program areas in PY3.[5] Accounting for more than 30% of AFIRM personnel, the Limb and Digit Salvage program is the largest research program area.

Honors and Achievements

The AFIRM program's faculty are highly accomplished in their respective scientific fields. From June 2010 through May 2011, 52 honors and awards were conferred upon AFIRM faculty as self-reported by the researchers. These honors include selection to membership or leadership positions in professional societies, honorary degrees from research/academic institutions, awards from private foundations, honorary lectureships, and faculty teaching awards. The distribution of the honors received is displayed according to the type of conferring organization in **Figure VII-5**.[6] The complete lists of honors and awards received by AFIRM faculty during PY3 are shown in **Appendix A**.

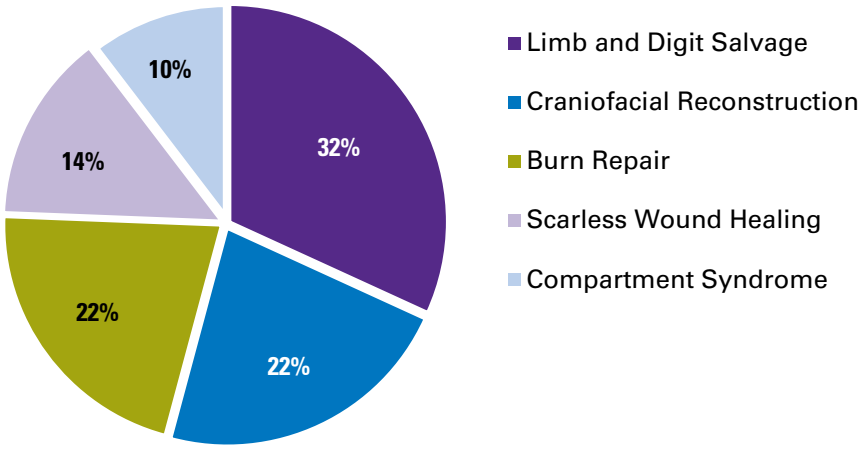


Figure VII-4. Percentage of personnel conducting research in the AFIRM program across the five program areas in PY3.

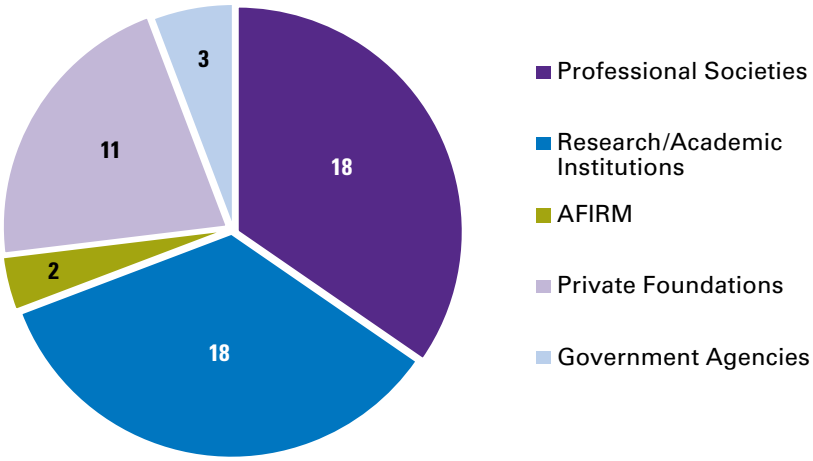


Figure VII-5. Distribution of honors and awards to AFIRM faculty by type of conferring organization in PY3.

[5] Figure VII-4 counts each person once only. The small number of researchers who worked on projects in two or more program areas are only represented once in the chart according to their principal research area.

[6] Awards to faculty exclude awards to postdoctorate fellows and students and also exclude the awarding of competed grants and contracts.

In addition to awards and honors received by AFIRM researchers, many AFIRM investigators have successfully competed for new research funds. AFIRM investigators reported the submission of 20 proposals in PY3, and 30 newly competed grants, contracts, or subcontracts began in PY3.[7]

Publications and Presentations

The presentation and publication of research findings are the most immediate output accomplishments of AFIRM-supported researchers.

For the purposes of this report, the following definitions have been applied for consistency:

Non-Peer-Reviewed Publications and Presentations
Meeting symposia, invited talks, oral presentations, and posters delivered or accepted are included in the program year numbers regardless of the review process for accepting a presentation or the eventual publication of an abstract in a scientific journal. Additionally, editorial comments, letters, non-peer-reviewed book chapters, and other types of non-peer-reviewed published works are included.

Peer-Reviewed Publications
Research or review articles accepted to, in press, or published in peer-reviewed journals or peer-reviewed edited books are included for each program year. Research or review manuscripts in preparation or submitted to a journal but not yet accepted are not included in this annual report.

The number of non-peer-reviewed publications and presentations resulting from AFIRM-sponsored research by WFPC and RCCC investigators increased from 118 in PY1 to 160 in PY3 **(Figure VII-6)**.[8] In addition,

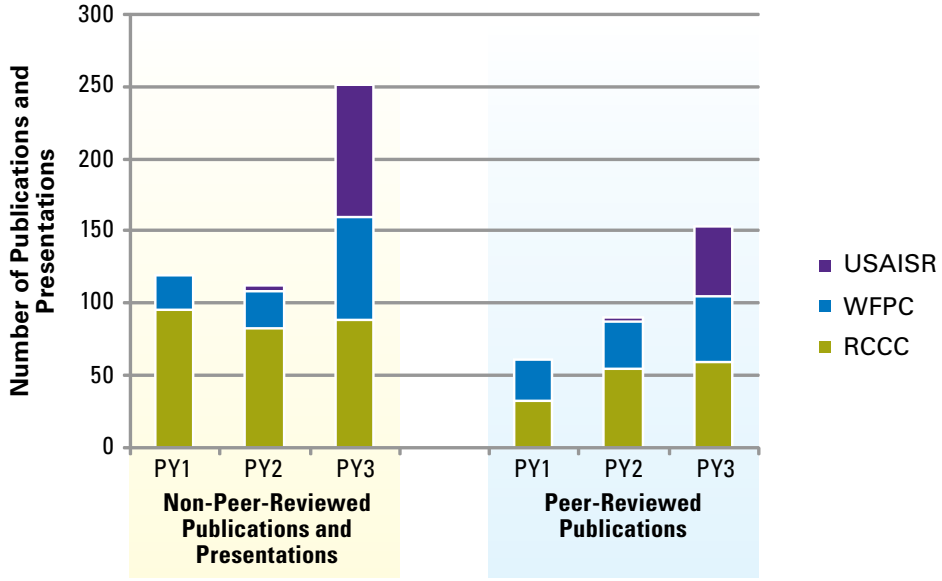


Figure VII-6. Dissemination of AFIRM-sponsored research findings to the scientific community through presentations and publications.

[7] The number of proposals submitted includes those with self-reported submission dates falling within PY3 as well as proposals that were reported without submission dates. The number of new grants or contracts includes those reported with funding start dates within PY3 and those that did not report funding dates.

[8] Articles published or accepted and presentations and posters delivered or accepted during the program years were included in the count for only 1 program year even if the acceptance and publication dates spanned 2 program years.

the number of peer-reviewed publications resulting from the WFPC and RCCC investigators' research increased from 59 in PY1 to 105 in PY3. Investigators from the U.S. Army Institute of Surgical Research delivered 89 presentations and published 47 peer reviewed articles in PY3. The complete lists of AFIRM researchers' publication and presentation citations from PY3 are shown in **Appendix B**.

Inventions, Patent Applications, and Patents

The successful development of tangible products or inventions can be tracked across three milestone phases: (1) an invention disclosure is filed by a researcher with his/her institutional technology licensing office, (2) a patent application is submitted to the government patent office (e.g., U.S. Patent and Trademark Office or USPTO), and (3) a patent is awarded by the USPTO for the intellectual property.

Many of the AFIRM program's principal investigators were already developing regenerative medicine-related research products at the time the program was initiated. Products developed before the AFIRM program existed are not recognized as AFIRM program outcome accomplishments.[9] However, products initially developed prior to AFIRM support but refined during the AFIRM program period are considered AFIRM program outcome accomplishments as are all newly disclosed intellectual property.

Through the first 2 years of the program, a combined 39 intellectual property filings (e.g., invention disclosures and/or patent applications) were made.[10] Also through the first 2 years of the program, 22 patent applications were filed with government patent offices. In PY3, 20 inventions were disclosed by AFIRM investigators, and 14 patent applications were filed **(Figure VII-7)**. While 4 patents were awarded to AFIRM researchers in PY3, the patent applications were filed

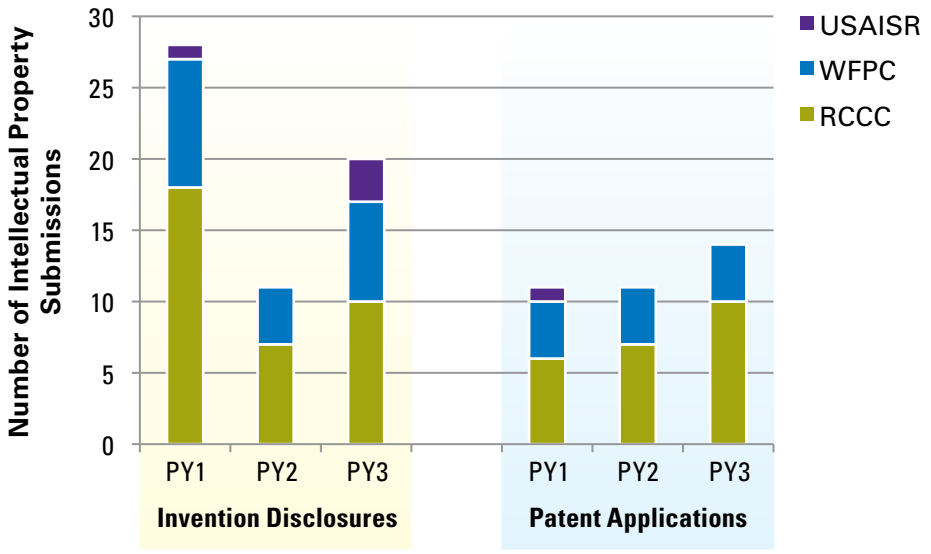


Figure VII-7. AFIRM-attributable invention disclosures and government patent applications filed.

[9] Definitions of AFIRM-attributable inventions, patent applications, and patents were developed to standardize the self-reported intellectual property and are described in Appendix C.

[10] The number of invention disclosures includes self-reports of invention disclosures to institutional technology offices and self-reported patent applications filed that were not previously reported as "invention disclosures."

prior to the formation of AFIRM and thus not considered to be a result of AFIRM support.[11] The complete lists of patent applications and inventions disclosed filed in PY3 that are attributable to AFIRM-sponsored research are shown in **Appendix C**.

Developmental Accomplishments and Milestones

Technology Readiness Levels of Products

Research progress can be measured in terms of the transition of products through research and development stages.[12] Biomedical research and development activities funded through the U.S. Army Medical Research and Materiel Command are categorized by Technology Readiness Levels (TRLs). TRLs identify a given product's research and development stage along a 9-point scale, which for therapeutic products extends from basic research at TRLs 1 and 2 to proof-of-concept studies (TRLs 3–4), preclinical (TRL 5) and clinical (TRLs 6–8) technology development stages, and post-marketing surveillance (TRL 9).

Figure VII-8 shows the AFIRM's overall research and development progression from predominantly basic research to

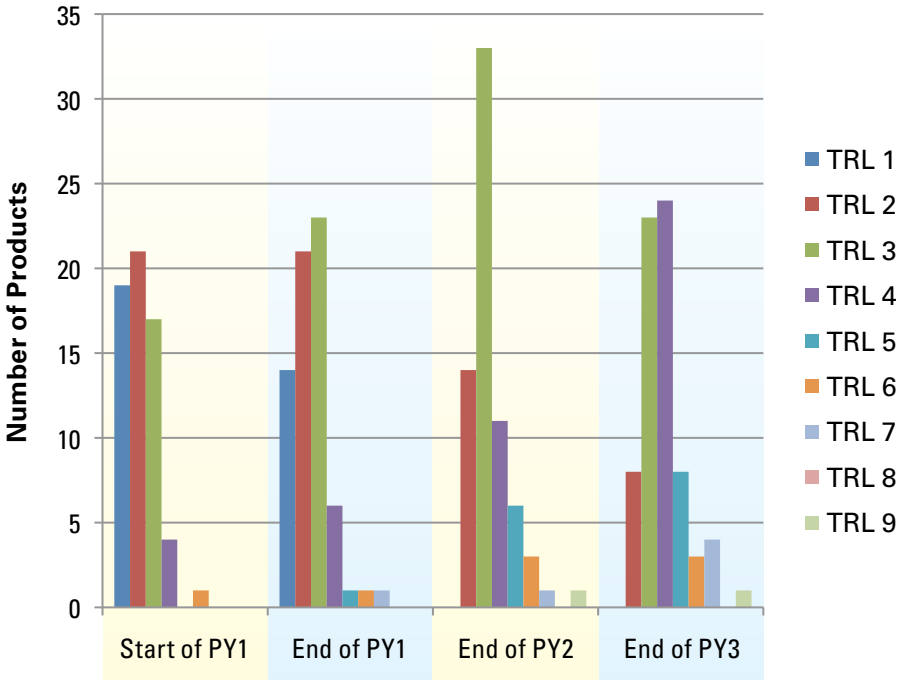


Figure VII-8. AFIRM research products' progression through research and development stages.

[11] Considering that the average time for the USPTO to render the final disposition on Biotechnology and Organic Chemistry applications is 35 months, according to the USPTO's Performance and Accountability Report Fiscal Year 2008, research sponsored by the 3-year-old AFIRM program is not likely to have resulted in a patent award at this time.

[12] A given research project can develop one or more products. Each developed product is separately assigned a TRL; therefore, a given project may have one or more products each of which would be designated a separate TRL. For the TRL analysis, the term product can describe either the project (if no specific product has resulted from basic research) or product (if a specific product is being further developed).

proof-of-concept and technology development stages.[13] At the start of the AFIRM program, 57 of 62 research and development products (92%) were nearly evenly distributed across TRLs 1, 2, and 3; the other 5 products were initially at TRLs 4 and 6.[14] By the end of PY2, no products remained at TRL 1, and only 14 products were at TRL 2. Most of the products (44 of 69) were at TRL 3 or 4, and the other 11 products were distributed at TRLs 5 and above. By the end of PY3, the number of products at TRL 4 had increased to 24 from 11 at the end of PY2. Additionally, the 8 products at TRL 5 and the 8 products at TRL 6 and above demonstrate the increasing translation of products into preclinical studies and human clinical studies, respectively.

While Figure VII-8 displays the status of all products' TRL stages at annual intervals, it does not track how each product transitioned since the beginning of the AFIRM program. **Figure VII-9** summarizes the transition of the 61 products that have continued from the start of the AFIRM program through PY3. During the first 3 years of the program, 23 products (38%) advanced 1 TRL and another 27 products (44%) advanced 2 or more TRLs.

Preclinical Models and Clinical Studies

Products at TRL 4 or 5 are being tested in in vivo animal models, some of which had to be newly developed or validated for the purpose of testing the products in an adequate injury model. In PY3, AFIRM researchers completed the development and/or validation of 12 experimental models for studying injury mechanisms, developing therapeutic approaches, and conducting preclinical studies to demonstrate the potential of therapeutic products.

Products at TRL 6 and above are being evaluated in human clinical studies that require federal regulatory approval and approval through local institutional review boards (IRBs). In PY3, AFIRM investigators advanced products through clinical study planning, approval, and execution stages. Six clinical

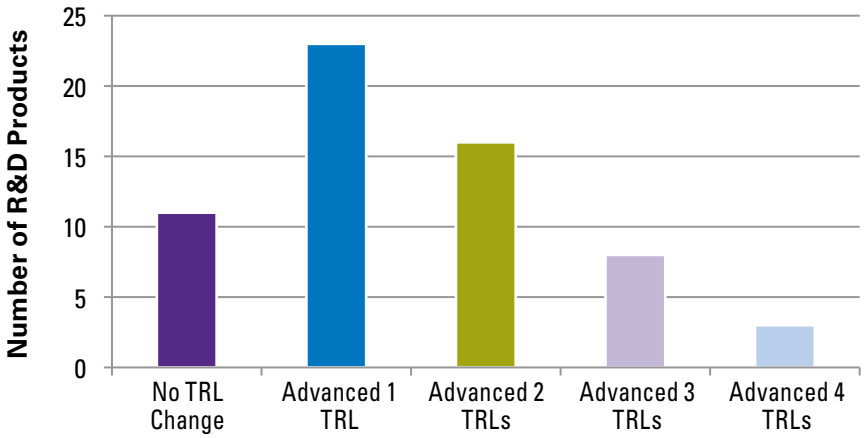


Figure VII-9. Advancement of AFIRM products through TRL stages since the start of the program.

[13] As the program progressed, AFIRM investigators better understood TRL definitions, and some previously self-reported TRL values have been revised. The TRL values used for Figure VII-8 reflect the most recent assessment of past and present TRL values and may not be consistent with previously reported TRL values.

[14] The AFIRM is a dynamic program, and investigators make programmatic decisions about the continuation or termination of projects or the initiation of new projects. As such, the portfolio of AFIRM research and development products will change from year to year.

trials were open to patient enrollment in PY3, including one Phase 3, two Phase 1/2, and three Phase 1 protocols. One Phase 1 study was completed in PY3. In addition, one Phase 2 study was completed, and the investigators received approval to begin the Phase 3 study. Another four clinical protocols were submitted to IRBs for Phase 1 studies of which one has received formal approval. **Figure VII-10** shows the number of unique products undergoing clinical evaluations by the most advanced stage of development.

Commercialization Plans

Commercial partnerships are important to the final development and fielding of medical materiel products. The collaboration of AFIRM investigators with commercial partners will enable clinical trials to be conducted as commercial and venture capital is leveraged with government funds. Furthermore, commercial partners can provide expertise and facilities for GMP-compliant product manufacturing, product testing and validation, clinical study design and execution, and filing regulatory submissions for approval to market the products. The formal agreement between an investigator and an industry partner is also a surrogate measure of the potential utility of the product being developed. In PY3, 36 commercial organizations were involved in the AFIRM program in a variety of capacities, 16 of which are AFIRM members or collaborating with the AFIRM, and the other 20 partners provided materials or services under contracts or agreements.[15] In addition, investigators have identified potential commercial partners or have initiated talks with potential partners to develop another 6 products.

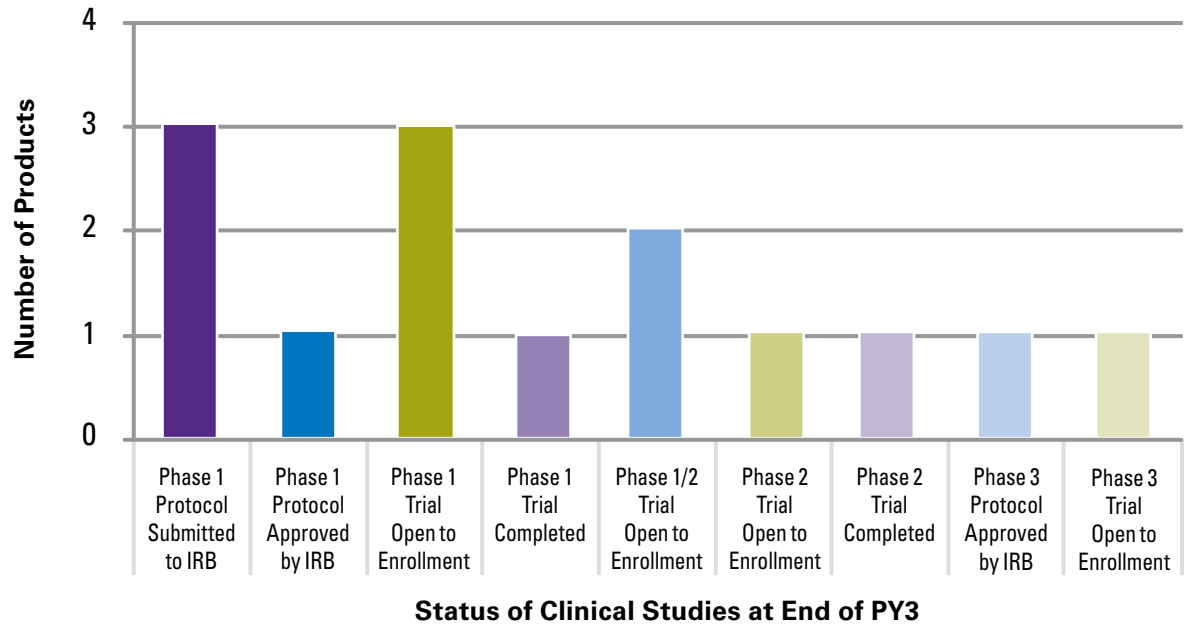


Figure VII-10. A snapshot of the clinical protocol stages of AFIRM products at the end of PY3.

[15] Some of the commercial partnerships preceded the start of the program, including some investigations of off-the-shelf products or products licensed by the partnering company.

Appendices

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Appendix A: Honors and Awards to AFIRM Faculty

During the reporting period from June 2010 through May 2011, 52 honors or awards were received by AFIRM faculty, as self-reported. The honors and awards are listed as follows by the recipient faculty member.

Rutgers-Cleveland Clinic Consortium

Boyce, S (University of Cincinnati): Charter Inductee, National Academy of Inventors, Cincinnati Chapter.

Caplan, A (Case Western Reserve University): Fellow, American Institute for Medical and Biological Engineering (AIMBE).

Griffith, L (Massachusetts Institute of Technology): Fellow, Biomedical Engineering Society, 2010.

Griffith, L (Massachusetts Institute of Technology): Member, National Academy of Engineering, 2011.

Katz, A (University of Virginia): Fellow, American Association of Plastic Surgeons.

Katz, A (University of Virginia): Visiting Professor for the 25th Annual S. Dawson Theogaraj Lectureship, Division of Plastic Surgery, Virginia Commonwealth University Medical Center, 2011.

Langer, R (Massachusetts Institute of Technology): Honorary Degree, Bates College.

Langer, R (Massachusetts Institute of Technology): Judah M. Folkman Lecturer, Children's Hospital, Boston.

Langer, R (Massachusetts Institute of Technology): College of Fellows, Controlled Release Society.

Langer, R (Massachusetts Institute of Technology): Academic Career Achievement Award, IEEE Engineering in Medicine and Biology Society.

Langer, R (Massachusetts Institute of Technology): Founders Award, National Academy of Engineering.

Langer, R (Massachusetts Institute of Technology): International Fellow, Royal Academy of Engineering.

Langer, R (Massachusetts Institute of Technology): Investiture Lecturer, Thayer School of Engineering, Dartmouth College.

Langer, R (Massachusetts Institute of Technology): Robert Fletcher Award, Thayer School of Engineering, Dartmouth College.

Langer, R (Massachusetts Institute of Technology): Storer Life Sciences Lecturer, University of California, Davis.

Langer, R (Massachusetts Institute of Technology): Allan S. Hoffman Lecturer, University of Washington.

Macri, L (Stony Brook University): 3rd Place Poster Presentations, AFIRM All Hands Meeting, AFIRM, 2011.

Sarac, T (Cleveland Clinic Foundation): Innovation Award, Cleveland Clinic, 2010.

Siemionow, M (Cleveland Clinic Foundation): Medal of Recognition Award, The Kosciuszko Foundation, 2010.

Siemionow, M (Cleveland Clinic Foundation): Top 150 "Movers and Shakers" in Northeastern Ohio, Crain's Cleveland Business Award, 2010.

Siemionow, M (Cleveland Clinic Foundation): Teraz Polska Diploma Award, Polish Promotional Emblem Foundation, 2011.

Siemionow, M (Cleveland Clinic Foundation): Outstanding Achievement in Clinical Research Award, Plastic Surgery Educational Foundation, 2010.

Siemionow, M (Cleveland Clinic Foundation): Medal of Recognition (Medal Polskiej Akademii Nauk), President of Polish Academy of Science, 2011.

Siemionow, M (Cleveland Clinic Foundation): Honorary Member, The Polish-American Medical Society, 2011.

Siemionow, M (Cleveland Clinic Foundation): Honored Guest, The Polish-American Medical Society's 61st Annual Physicians' Ball, 2011.

Siemionow, M (Cleveland Clinic Foundation): Tytus Chalubinski Medal Award, Warsaw Medical University, 2011.

Siemionow, M (Cleveland Clinic Foundation): Eugeniusz Kwiatkowski Medal, Council of the City of Gdynia, 2011.

Sundback, C (Massachusetts General Hospital): 1st Place Poster, 10th NJ Symposium on Biomaterial Science and Regenerative Medicine, 2010.

Wake Forest-Pittsburgh Consortium

Christ, G (Wake Forest University): Best Poster, North Carolina Tissue Engineering and Regenerative Medicine Conference, 2010.

Guldberg, R (Georgia Institute of Technology): Parker H. Petit Director's Chair for Engineering and Medicine, Georgia Institute of Technology, 2011.

Gurtner, G (Stanford University): Basic Science Researcher of the Year Award, Association of Plastic Surgeons, 2011.

Kasper FK, Kretlow JD, Mikos AG, Shi M, Spicer PP, Wong ME, and Young S (Rice University): Hershel M. Rich Invention Award, Rice University, 2011.

Kasper, FK (Rice University): Graduate Student Association Faculty Teaching and Mentoring Award, Rice University, 2011.

Koepsel, R (University of Pittsburgh): Carnegie Science Award for Advanced Materials, Carnegie Science Center, 2011.

Longaker, M (Stanford University): Frederick Birnberg Award for Excellence in Dental Research, Columbia University, 2011.

Longaker, M (Stanford University): Honorary Fellow, Society of Black Academic Surgeons, 2011.

Longaker, M (Stanford University): Flanc-Karl Award, American Surgical Association, 2011.

Mikos, AG (Rice University/University of Texas Health Science Center): Fellow, Controlled Release Society, 2011.

Mikos, AG (Rice University/University of Texas Health Science Center): Founders Award, Society for Biomaterials, 2011.

Ruoslahti, E (University of California, Santa Barbara): Visiting Professor – Sass Foundation for Medical Research, 2011.

Russell, A (University of Pittsburgh): Carnegie Science Award for Advanced Materials, Carnegie Science Center, 2011.

Soh, H-T (University of California, Santa Barbara): Fellow in Natural Sciences - Engineering, John Simon Guggenheim Memorial Foundation, 2010.

Soh, H-T (University of California, Santa Barbara): Edward C. Nagy New Investigator Award/Lectureship, National Institute of Biomedical Imaging and Bioengineering/ National Institutes of Health, 2011.

Thomson, J (University of Wisconsin): Albany Medical Center Prize in Medicine and Biomedical Research, Albany Medical Center, 2011.

Thomson, J (University of Wisconsin): King Faisal International Prize in Medicine, Kingdom of Saudi Arabia, 2011.

Thomson, J (University of Wisconsin): Alumni Award of Merit, Penn Veterinary Medicine, 2010.

VanDyke, M (Stanford University): Faculty Excellence Award, Wake Forest University Graduate School, 2011.

Wagner, WR (University of Pittsburgh): Clemson Award for Applied Research, Society for Biomaterials, 2011.

Wagner, WR (University of Pittsburgh): Pitt Innovator Award, University of Pittsburgh, 2010.

Wagner, WR (University of Pittsburgh): Chairman, 2013 Annual Meeting, Biomedical Engineering Society, 2010.

Washburn, N (Carnegie Mellon University): Coulter Translational Research Award, Wallace H. Coulter Foundation, 2011.

U.S. Army Institute of Surgical Research

Christy, RJ (U.S. Army Institute of Surgical Research): Best Poster Award, AFIRM All-Hands Meeting, 2011.

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Appendix B: Publications and Presentations

Peer-reviewed journal articles are defined as research articles and review articles "accepted" to, "in press," or published in scientific and technical journals from June 2010 through May 2011. Additionally, book chapters are included as peer-reviewed publications when indicated by investigators. The articles shown in **Tables B-1a, B-1b, and B-1c** were self-reported by the AFIRM investigators.

Table B-1a. Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium

Alghoul M, Mendiola A, Seth R, Rubin B, Zins JE, Calabro A, Siemionow M. Effect of hyaluronan on fat graft survival. *Plast Reconstr Surg.* 2011 May;127(5S): 98.
Alghoul MS, Gordon CR, Yetman R, Buncke GM, Siemionow M, Afifi AM, Moon WK. From simple interrupted to complex spiral: a systematic review of various suture techniques for microvascular anastomoses. *Microsurgery.* 2011 Jan;31(1):72-80.
Aurora A, Mesiha M, Tan CD, Walker E, Sahoo S, Iannotti JP, McCarron JA, Derwin KA. Mechanical characterization and biocompatibility of a novel reinforced fascia patch for rotator cuff repair. *J Biomed Mater Res A.* In Press.
Bailey AM, Kapur S, Katz AJ. Characterization of adipose-derived stem cells: an update. *Current Stem Cell Research and Therapy.* 2010;5:95-102.
Bassiri Gharb B, Rampazzo A, Madajka M, Altuntas SH, Cwykiel J, Siemionow M. Effectiveness of topical immunosuppressants in prevention and treatment of rejection in face allotransplantation. *Plast Reconstr Surg.* 2011 May;127(5S):13.
Brzezicki G, Jankowski R, Blok T, Klimczak A, Szymas J, Huber J, Szukala A, Siemionow M, Nowak S. Postlaminectomy osteopontin expression and associated neurophysiological findings in rat peridural scar model. *Spine.* 2010 Sep 10. [Epub ahead of print]
Chandra P, Lai K, Sung H-J, Murthy NS and Kohn J. UV laser-ablated surface textures as potential regulator of cellular response. *Biointerphases,* 2010, 5(2), 53-59.
Cheng H, Hill PS, Siegwart D, Vacanti N, Lytton-Jean A, Cho S, Ye A, Langer R, Anderson DG. A novel family of biodegradable poly(ester amide) elastomers. *Adv Mater.* 2011 Mar 25;23(12):H95-100. doi: 10.1002/adma.201003482. Epub 2011 Mar 10.
Cwykiel J, Klimczak A, Jundzill A, Siemionow M. Therapeutic potential of ex-vivo fused chimeric cells in prolonging vascularized skin allograft survival. *Plast Reconstr Surg.* 2011 May;127(5S):26.
de Boer R, Knight AM, Borntraeger A, Hébert-Blouin MN, Spinner RJ, Malessy MJ, Yaszemski MJ, Windebank AJ. Rat sciatic nerve repair with a poly-lactic-co-glycolic acid scaffold and nerve growth factor releasing microspheres. *Microsurgery.* 2011;31(4):293-302.
de Boer R, Knight AM, Spinner RJ, Malessy MJ, Yaszemski MJ, Windebank AJ. In vitro and in vivo release of nerve growth factor from biodegradable poly-lactic-co-glycolic-acid microspheres. *J Biomed Mater Res A.* 2010;95(4):1067-73.
Dewar A, Clark RAF, Singer AJ, Frame MD. Curcumin mediates both dilation and constriction of peripheral arterioles via adrenergic receptors. *J Invest Dermatol.* 2011 Aug;131(8):1754-60. doi: 10.1038/jid.2011.96. Epub 2011 Apr 28.
Feng G, Yang X, Shang H, Marks IW, Shen FH, Katz AJ, Arlet V, Laurencin CT, Li X. Multipotential differentiation of human anulus fibrosus cells: an in vitro study. *The Journal of Bone and Joint Surgery.* 2010;92:675-85.
Forouzandeh F, Jalili RB, Boyce ST, Supp DM, Ghahary A. Local expression of indoleamine 2, 3-dioxygenase (IDO) suppresses T-cell mediated rejection of an engineered bilayered skin substitute. *Wound Repair Regen.* 2010 Oct 18. [Epub ahead of print]

Table B-1a. Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Gordon C, Avery R, Abouhassan W, Siemionow M. Cytomegalovirus and other infectious issues related to face transplantation: specific considerations, lessons learned, and future recommendations. *Plast Reconstr Surg.* 2011 Apr;127(4):1515-1523.
Gordon C, Zor F, Siemionow M. Skin area quantification in preparation for concomitant upper extremity and face transplantation: a cadaver study and literature review. *Transplantation.* 2011 May;91(9):1050-6.
Gordon CR, Siemionow M, Eghtesad B, Fung J. A novel cadaver study quantifying the antigenic skin component accompanying concomitant face and upper extremity transplantation. *American Journal of Transplantation.* 2010;10S(1): 57.
Hogan MV, Bagayoko N, Roshan J, Starnes T, Katz AJ, Chhabra, B. Current tissue engineering solutions for tendon repair. *Journal of the American Academy of Orthopaedic Surgeons.* 2011;19:134-142.
Kempen DH, Lu L, Creemers L, Heijink A, Maran A, Dhert, Yaszemski MJ. Enhanced BMP-2 induced ectopic and orthotopic bone formation by intermittent PTH(1-34) administration. *Tissue Eng Part A.* 2010;16(12):3769-3777.
Kim J, Sharma A, Runge MB, Waters H, Doll B, McBride S, Alvarez P, Dadsetan M, Yaszemski MJ, Hollinger JO. Osteoblast growth and bone healing response to three-dimensional poly(e-caprolactone fumarate) scaffolds. *Journal of Tissue Engineering and Regenerative Medicine.* In Press.
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Seetharaman S, Natesan S, Stowers RS, Mullens C, Baer DG, Suggs LJ, Christy RJ. A PEGylated Fibrin-Based Wound Dressing with Antimicrobial and Angiogenic Activity. *Acta Biomaterialia.* ePub April 2011.
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Shah AR, Shah SR, Oh S, Ong JL, Wenke JC, Agrawal CM. Migration of co-cultured endothelial cells and osteoblasts in composite hydroxyapatite/polylactic acid scaffolds. *Ann Biomed Eng.* 2011 Jul 16. [Epub ahead of print]. PMID: 21769541
Son JS, Appleford M, Ong JL, Wenke JC, Kim JM, Choi SH, Oh DS. Porous hydroxyapatite scaffold with three-dimensional localized drug delivery system using biodegradable microspheres. *J Control Release.* 2011 Jul 30;153(2):133-40.
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Sun LT, Friedrich E, Heuslein JL, Pferdehirt RE, Dangelo NM, Natesan S, Christy RJ, Washburn NR. Reduction of burn progression with topical delivery of (anti-TNF-a)-hyaluronic acid conjugates. *Wound Repair and Regeneration* (Accepted).
Walters TJ, Mase V, Roe J, Dubick M, and Christy R. Effect of Poloxamer 188 on Skeletal Muscle Tourniquet Induced Ischemia-Reperfusion in Rats. *J Trauma.* 2011;70(5):1192-7.
Wenke JC, Milutinovich AB, Pollak AN. Congressionally Directed Research Will Improve Outcomes through Funding Opportunities for Orthopaedics. *J Am Acad Orthop Surgeons.* 2011;19(suppl 1):S40-S43.
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Tables B-2a, B-2b, and B-2c display non-peer-reviewed publications and all presentations self-reported by AFIRM investigators. The non-peer-reviewed publications are defined as editorials, letters, or opinion writings that have been "accepted" to, "in press," or published in scientific and technical journals from June 2010 through May 2011. Books and book chapters are included as non-peer-reviewed as indicated by the authors. Presentations include all invited talks, symposia, oral presentations, and posters presented at scientific research conferences and meetings regardless of the peer review process. All such presentations made and all presentations "accepted" from June 2010 through May 2011 are included. Presentations not specifically labeled as "accepted" in the researchers' progress reports were not assumed to be accepted and were not included in the following tables.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium

Alvarez-Urena P, Kim J, Magno MHR, Street R, Darr A, Olabarri-Santos CM, Kohn J, Hollinger JO. PDGF-BB and tyrosine-derived polycarbonate with calcium phosphate scaffolds induce osteogenic differentiation. TERMIS-NA Meeting; 2010; Orlando, FL.
Avery R, Gordon C, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 37, Infectious issues in face transplantation.
Bassett E. Minimizing distortion in engineered ear constructs with a bimodal scaffold. Poster session presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Bassiri Gharb B, Rampazzo A, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 36, Sensory recovery in face transplantation.
Boyce ST, Lynch KA, Zimmerman RL, Supp DM, Kagan RJ. Flow cytometry for regulation of melanocyte density in engineered skin substitutes. Proceedings of the Armed Forces Institute for Regenerative Medicine (AFIRM) All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Boyce ST, Zimmerman RL, Lynch KA, Supp DM, Kagan RJ. Regulation of skin pigmentation in engineered skin substitutes. Proceedings of the Armed Forces Institute for Regenerative Medicine (AFIRM) All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Boyce ST, Zimmerman RL, Supp DM, Kagan RJ. Tumorigenicity testing of human melanocytes for engineered skin substitutes. Proceedings of the Armed Forces Institute for Regenerative Medicine (AFIRM) All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Brzezicki G, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 21, Methods of sensory recovery of face transplantation.
Cheng H, Hill PS, Vacanti N, Ma M, Langer R, Anderson DG. Development of biocompatible elastomeric nerve conduit scaffolds for repair of peripheral nerve defects. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Cheng H, Hill PS, Vacanti N, Ma M, Langer R, Anderson DG. Development of biocompatible elastomeric nerve conduit scaffolds for repair of peripheral nerve defects. Presented at: American Institute of Chemical Engineers (AIChE) Annual Meeting; 2010 Nov 7-12; Salt Lake City, UT.
Clements, B. Design of a Biodegradable, Biocompatible and Flexible Nerve Conduit for Peripheral Nerve Regeneration. Presented at 10th NJ Symposium on Biomaterials Science and Regenerative Medicine, 2010 Oct; New Brunswick, NJ.
Coffman K, Gordon C, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 13, Psychological aspects of face transplantation.
Cwykiel J, Klimczak A, Jundziłł A, Matejuk A, Siemionow M. Therapeutic potential of ex vivo fused chimeric cells in prolonging vascularized skin allograft survival. Oral presentation at: Plastic Surgery Research Council; 2011 Apr; Louisville, KY.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Cwykiel J, Klimczak A, Jundziłł A, Matejuk A, Siemionow M. Therapeutic potential of ex vivo fused chimeric cells in prolonging vascularized skin allograft survival. Poster session presentation at: Translational Regenerative Medicine Forum; 2011 Apr; Washington, DC. Recipient of AFIRM Award (Wake Forest).
Cwykiel J, Klimczak A, Jundziłł A, Siemionow M. Therapeutic potential of ex vivo fused chimeric cells in prolonging vascularized skin allograft survival. Oral presentation at: AFIRM All Hands Meeting; 2011 Jan 17-19; Clearwater, FL.
Dewar A, Frame M, Singer AJ, Clark RAF. Curcumin and beta-adrenergic involvement in remotemicrovascular preconditioning. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Frame M, Halaibeh M, Khan A, Singer AJ, Clark RAF. Temperature and hematocrit dependence of erythrocyte clumping in flowing suspensions. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Gatherwright J, Siemionow M, Djohan R, Rose E, Gottlieb L, Papay F. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 29, Comparative cost analysis of conventional reconstruction versus the first face transplantation in the U.S.
Gatt CJ. Mechanically relevant scaffolds for soft tissue regeneration. Oral presentation at: 10th NJ Symposium on Biomaterials Science and Regenerative Medicine, 2010 Oct; New Brunswick, NJ.
Gordon C, Siemionow M. Plastic and reconstructive surgery (Springer Specialist Surgery Series). Springer-Verlag London Ltd.; 2010. Chapter 52, Clinical experience with hand transplantation.
Gordon C, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 23, The institutional review board approval process.
Gordon C, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011.Chapter 7, Timeline and evolution of face transplant cadaver models.
Gordon C, Zor F, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 40, Concomitant face and upper extremity transplantation.
Griffith LG. Integration of molecular and macroscopic cues in regenerating bone. Oral presentation at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Hill PS, Cheng H, Vacanti N, Ma M, Langer R, Anderson DG. Development of biodegradable biorubber nerve conduit scaffolds for repair of extremity nerves. Presented at: Advanced technology applications for combat casualty care (ATACCC); 2010 Aug 16-19; St. Pete Beach, FL.
Hill PS, Pritchard CD, O'Shea TM, Ye J, Luque B, Siegwart DJ, Calo E, Anderson DG, Reynolds FM, Thomas JA, Slotkin JR, Woodard EJ, Langer R. An injectable polyethylene glycol (PEG)-based hydrogel with sustained release of methylprednisolone succinate for neurosurgical applications. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Hill PS, Vacanti N, Cheng H, Tram Dang, Ma M, Langer R, Anderson DG. Encapsulation of anti-inflammatory drugs into electrospun scaffolds reduces the local host immune response at the site of tissue injury. Presented at: Tissue Engineering and Regenerative Medicine International Society (TERMIS) Conference; 2010 Dec 5-8; Orlando, FL.
Hirth DA, Singer AJ, Clark RAF, McClain SA. Hematoxylin-phloxine-saffron: a superior stain in histopathologic analysis of burn injury? Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Hirth DA, Singer AJ, McClain S, Lee S, Clark RAF. Endothelial cell necrosis plays a central role in the vertical progression of burn injury. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Jia S, Zhao Y, Singer A, Clark RAF, Hong SJ, Mustoe TA, et al. Systemically administrated curcumin promotes wound healing and reduces hypertrophic scar formation in the rabbit ear wound model [Abstract]. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Kandula S, Shirsat S, Clark RAF. Products for the care of chronic wounds. In: Wolverton SE, editor. *Comprehensive dermatologic drug therapy.* 3rd ed. Elsevier; In Press.
Katz AJ, Mericli AF. Stem cells derived from fat. In: Atala A, Lanza R, editors. *Principles of Regenerative Medicine.* 2nd ed. San Diego (CA): Elsevier; 2011.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Kim J, Magno MHR, Waters H, Doll B, McBride S, Alvarez P, Darr A, Vasanji A, Kohn J, Hollinger JO. Bone regeneration in rabbit calvaria using rhBMP-2 treated tyrosine-derived polycarbonate scaffolds. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Klimczak A, Siemionow M. *Plastic and reconstructive surgery* (Springer Specialist Surgery Series). Springer-Verlag London Ltd.; 2010. Chapter 2, Immunology of tissue transplantation.
Klimczak A, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 3, Immunological aspects of face transplantation.
Li M, Dickinson C, Finkelstein EF, Pomerantseva I, Neville CM, Sundback CA. Replacement vascularized skeletal muscle for craniomaxillofacial reconstruction. Poster presented at: 10th NJ Symposium on Biomaterial Science and Regenerative Medicine; 2010 Oct 27-28; Rutgers University, New Brunswick, NJ.
Lin F, Singer AJ, Crawford L, McClain SA, Clark RAF. A novel fibronectin derived peptide improves tissue viability in a porcine comb burn model. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Luangphakdy V, Shinohara K, Pan H, Griffith LG, Yaszemski MJ, Kohn J, Muschler GF. Competitive assessment of degradable osteoconductive bone scaffolds in the canine femoral multi-defect model. AFIRM All Hands Meeting; 2011 Jan 17-19; Clearwater, FL.
Luangphakdy V, Shinohara K, Pan H, Griffith LG, Yaszemski, MJ, Kohn J, Muschler GF. Competitive assessment of degradable osteoconductive bone scaffolds in the canine femoral multi-defect model. Association of Bone and Joint Surgeons Annual Meeting; 2011 Jun 9; Dublin, Ireland.
Luangphakdy V, Shinohara K, Pan H, Stockdale L, Darr A, Hefferan T, Runge T, Saini S, Griffith L, Kohn J, Yaszemski M, Muschler GF. Systematic evaluation of osteoconductive scaffolds for bone repair in the canine femoral multi defect model. TERMIS; 2010 Dec 5-8; Orlando, FL.
Macri LK, Clark RAF. Cutaneous wound pathobiology: raison d'etre for tissue engineering. In: Hollinger J, editor. *CRC manual on biomaterials*. In Press.
Madajka M, Cwykiel J, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 42, Cellular therapies in face transplantation.
Magno MHR, Darr A, Kim J, Hollinger JO, Kohn J. Tyrosine-derived polycarbonate/ calcium phosphate composite scaffold for bone tissue engineering. AFIRM All Hands Meeting, 2011 Jan 17-20; Clearwater, FL.
Magno MHR, Kim J, Waters H, Doll B, Sharma A, McBride S, Alvarez P, Srinivasan A, Darr A, Vasanji A, Hollinger JO, Kohn J. Tyrosine-derived polycarbonate and calcium phosphate composite scaffolds for bone regeneration. Presented at: TERMIS-NA Meeting; 2010; Orlando, FL.
Mendiola A, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 22, Methods of assessment of cortical plasticity in patients following amputation, replantation, and composite tissue allograft transplantation.
Morrow T, Jia S, Zhao Y, Vracar-Grabar M, Clark RAF, Kohn J, Mustoe T, Sheihet L. Topical curcumin-loaded TyroSpheresTM to enhance wound healing. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Morrow T, Macri LK, Singer A, Kohn J, Clark RAF, Sheihet L. Curcumin containing TyroMatsTM: controlled delivery and in vivo biocompatibility. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Neville CM. Engineered skeletal muscle. Presented at: TERMIS-NA Annual Conference; 2010 Dec 5-8.
Ortiz O, Magno H, LeGeros RZ, Darr A, Kohn J. Calcium phosphate-coated Tyr-PC scaffolds: optimization and characterization. Presented at: AFIRM All Hands Meeting; 2011; Tampa, FL.
Ortiz O, Magno H. LeGeros RZ, Darr A, Kohn J. Towards the enhancement of bone tissue regeneration: Tyr(PC)/ calcium phosphate composite scaffolds. Presented at: Society for Biomaterials Conference; 2011; Orlando, FL.
Pan H, Luangphakdy V, Shinohara K, Boehm C, Wenke J, Pluhar L, Bechtold J, Muschler GF. Assessment of bone regeneration strategies in chronic caprine tibial defect. AFIRM All Hands Meeting; 2011 Jan 17-19; Clearwater, FL.
Rampazzo A, Bassiri Gharb B, Siemionow M. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 28, Addressing cultural, religious, and philosophical differences in views on face transplantation.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Randolph MA. A novel three-dimensional collagen based scaffold for possible auricular reconstruction. Poster presented at: 10th New Jersey Symposium on Biomaterials Science and Regenerative Medicine; 2010 Oct 27-28; Rutgers University, New Brunswick, NJ.
Randolph MA. Advances in the development of engineered ear for the wounded warrior & chondrogenesis by bone marrow-derived stem cells in chondrocyte conditioned media. Oral presentation at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Raut V, Alvarez LM, Rivera J, Stockdale L, Boehm C, Patterson T, Griffith LG, Muschler G. Bone regeneration scaffolds with molecular surface modification presenting epidermal growth factor (EGF) tethered to β-tri-calcium phosphate using a bTCP binding peptide. Poster session presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Raut V, Alvarez LM, Rivera J, Stockdale L, Boehm C, Patterson T, Griffith LG, Muschler G. In vitro testing of beta-tri-calcium phosphate scaffolds coated with epidermal growth factor using a novel bTCP binding peptide sequence. Poster presented at: ORS Annual Meeting; 2011 Jan; Long Beach, CA.
Rui J, Wang H, Runge MB, Spinner RJ, Yaszemski MJ, Windebank AJ. Effects of exogenous VEGF on peripheral nerve regeneration – a preliminary report. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Rui J, Wang H, Yaszemski MJ, Spinner RJ, Windebank AJ. Controlled release of VEGF using PLGA microspheres. AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Shinohara K, Luangphakdy V, Pan H, Stockdale L, Darr A, Hefferan T, Runge T, Saini S, Griffith L, Kohn J, Yaszemski M, Muschler GF. Systematic evaluation from available promising three-dimensional osteoconductive bone scaffolds in canine femoral multi defect model. 57th Annual Meeting of the Orthopedic Research Society; 2011 Jan 13-16; Long Beach, CA.
Siemionow M, Bassiri Gharb B, Rampazzo A. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 2, The face as a sensory organ.
Siemionow M, Djohan R, Bernard S, Papay F. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 33, The Cleveland Clinic experience with the U.S. first face transplantation.
Siemionow M, Nasir S. *Plastic and reconstructive surgery* (Springer Specialist Surgery Series). Springer-Verlag London Ltd.; 2010. Chapter 51, Experimental composite tissue transplantation models.
Siemionow M, Ozturk C, Altuntas S. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 44: An update on face transplants performed between 2005 and 2010.
Siemionow M, Ozturk C. Cambridge University Press. Chapter 37, Face transplantation. In *Organ Transplantation: A Clinical Guide.* In Press.
Siemionow M, Sonmez E, Klimczak A. *Microsurgical reconstruction of the head and neck,* Quality Medical Publishing, Inc.; 2010. Chapter 42, Composite tissue allotransplantation.
Siemionow M, Sonmez E, Papay F. *Plastic surgery secrets plus.* 2nd ed. Mosby, Inc.; 2010. Chapter 112, Principles of facial transplantation.
Siemionow M, Sonmez E. *Plastic and reconstructive surgery* (Springer Specialist Surgery Series), Springer-Verlag London Ltd.; 2010. Chapter 37, Peripheral nerve injuries.
Siemionow M, Sonmez E. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 1, Face as a functional organ – the functional anatomy of the face.
Siemionow M, Zor F. *Gesichtsfremdtransplantation – fehlbildungen.* Vol 5. Springer-Verlag GmbH Berlin Heidelberg. Chapter 14, Die allogene gesichts transplantation, neue trends in der forschung. Forthcoming.
Siemionow M, Zor F. *Handbook of burns: reconstruction and rehabilitation.* Vol II. New York: Springer Verlag Wien. Chapter, Burn reconstruction – future perspectives: facial transplantation. In Press.
Siemionow M, Zor F. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 4, Experimental studies in face transplantation – rodents.
Siemionow M. Twarza w twarz: moja droga do pierwszego pelnego przeszczepu twarzy. Krakow: Wydawnictwo Znak; 2010.
Singer AJ, Hirth DA, McClain SA, Clark RAF. Lack of agreement between gross visual and histological assessment of burn reepithelialization. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Singer AJ, Hirth DA, McClain SA, Crawford L, Lin F, Clark RAF. Validation of a vertical progression porcine burn model. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Sundback CA. Engineered replacement ear. Product-line presentation at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Sundback CA. Product reviews: muscle regeneration for craniomaxillofacial applications. Product-line presentation at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Sundback CA. Replacement skeletal muscle for craniomaxillofacial reconstruction. Oral presentation at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Treiser M, Abramson S, Langer R and Kohn J. Bioresorbable and bioerodible materials, in: *Biomaterials Science: An Introductory Text,* 3rd edition, (B. Ratner, A. S. Hoffman, F. J. Schoen and J. E. Lemons, eds.), 2011, Academic Press, San Diego, in press.
Wang H, Runge MB, Rui J, Knight A, Spinner RJ, Windebank AJ, Yaszemski, MJ. Development of conductive polymer scaffolds as nerve conduits for bridging segmental nerve defect: an in vivo study. Presented at: AFIRM All Hands Meeting, 17-20 Jan 2011; Clearwater, FL.
Wang H, Runge MB, Rui J, Yaszemski MJ, Windebank AJ, Spinner RJ. Conductive olymer scaffolds for repair of rat sciatic nerve. Presented at: Annual Meeting of American Society for Peripheral Nerve; 2011 Jan 14-16; Cancun, Mexico.
Wang H. Animal models and evaluation modalities in peripheral nerve research. Presented at AFIRM Interim Meeting; 2010 Dec 8-9; Washington DC.
Windebank AJ, Yao L, de Ruiter GCW, Wang H, Knight AM, Spinner RJ, Yaszemski MJ, Pandit A. Development of a multichannel collagen nerve conduit and its effect on controlling dispersion of axonal regeneration. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Windebank AJ. Regeneration in the nervous system. Presented at: Frontiers in Biomedical Research; 2010 Nov 4; Karolinska Institute, Stockholm, Sweden.
Windebank AJ. Tissue engineering in the nervous system. Presented at: 10th New Jersey Symposium on Biomaterials Science; 2010 Oct 27-28; New Brunswick, NJ.
Yahav J, Maloney L, Kim D, Sperandeo M, Lin F, Clark RAF. Extracellular hydrogen peroxide is decreased by increased cell density. Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.
Yang TL, Yoo J, Siemionow M, Atala A. *The know-how of face transplantation.* London: Springer-Verlag London, Ltd.; 2011. Chapter 43, Tissue engineering for facial reconstruction.
Zeng Q, Macri L, Prasad A, Zeugolis DI, Hanley C, Garcia Y, Pandit, Clark RAF. Skin tissue engineering. In: *Comprehensive biomaterials.* Elsevier; In Press.
Zeng Q, Prasad A, Clark RAF. Skin stem cells and wound healing, In: Gurtner G, editor. *Yearbook of wound healing.* In Press.
Zhou L. Engineering ears with a combination fibrillar collagen-wire skeleton scaffold. Poster presented at: 10th New Jersey Symposium on Biomaterials Science and Regenerative Medicine; 2010 Oct 27-28; Rutgers University, New Brunswick, NJ.
Zhou W, Hirth DA, McClain SA, Singer AJ, Clark RAF. Vascular occlusion in burn injury: erythrocyte aggregation or thrombus formation? Presented at: AFIRM All Hands Meeting; 2011 Jan 17-20; Clearwater, FL.

Table B-2b. Presentations and Non-Peer-Reviewed Publications: Wake Forest-Pittsburgh Consortium

Allen, A., Matheny, R.G., Gazit, D., Gazit, Z., Su, S., Stevens, H.Y., Guldberg, R.E. Synthetic vs. Natural Membranes for Cell-Based Bone Tissue Engineering. The 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, January, 2011.
Beasley B, Longaker MT, Gurtner GC, Dauskardt GC, Dauskardt RH, Yock P. Stress-shielding device demonstrates dramatic decrease in scar formation in first-in-man study. ATACCC meeting August 2010. Abstract.
Binder K, Yoo JJ, Zhao W, Dice D, Josh T, Kang HW, Atala A. A Novel Device for In Situ Bioprinting of the Skin. Division of Surgical Sciences 18th Annual Residents' and Fellows' Research Day. Wake Forest University School of Medicine. Winston-Salem, NC November 11, 2010.
Binder K, Zhao W, Dice D, Tan J, Kang HW, Atala A, Yoo JJ. Development of a Novel Delivery Device For In Situ Bioprinting of the Skin. TERMIS-NA Annual Conference and Exposition. December 5-12, 2010, Orlando, Florida.
Binder KW, Zhao W, Dice D, Tan J, Kang HW, Atala A, Yoo JJ. A Novel Device for In Situ Bioprinting of the Skin. North Carolina Tissue and Engineering & Regenerative Medicine Society Conference. November 12, 2010, Durham, NC.
Boerckel, J.D., Kolambkar, Y.M., Dupont, K.M., Uhrig, B.A., Phelps, E.A., Garcia, A.J., Guldberg, R.E. BMP-Mediated Large Bone Defect Repair is Dependent on Growth Factor Dose and Delivery System Release Kinetics. The 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, January, 2011.
Boerckel, J.D., Lin, A., Kolambkar, Y.M., Dupont, K.M, Guldberg, R.E. Non-Destructive Finite Element Analysis Prediction of Biomechanical Properties in Mechanically Stimulated Large Bone Defects. The 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, January, 2011.
Brandacher G, Gorantla VS, Schneeberger S, Shores JT, Imbriglia J, Azari K, Fletcher D, Zeevi A, Demetris A, Starzl TE, Losee JE, Lee WPA. Hand/Forearm transplantation using a novel cell-based immunomodulatory protocol – experience with five patients. *Am J Transplant.* 2011; Vol.11, Suppl.2, 190.
Burnett L, Binder K, Yoo JJ, Zhao W, Dice D, Josh T, Kang HW, Atala A. Keratin-based treatment (KeraHeal™) for radiation wounds. A Novel Device for In Situ Bioprinting of the Skin. Division of Surgical Sciences 18th Annual Residents' and Fellows' Research Day. Wake Forest University School of Medicine. Winston-Salem, NC November 11, 2010.
Corona BT, Ward CL, Harrison BS, Christ GJ. Regenerative medicine: basic concepts, current status, and future applications. *J Investig Med.* 2010 Oct;58(7):849-58.
Dosier, C.R., Erdman, C.P., Schwartz, Z., Boyan, B.D., Guldberg, R.E. Resveratrol Effect on Osteogenic Differentiation of Adipose-Derived Stem Cells is Species Dependent. The 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, January, 2011.
Esteban R, Young M, Chinicci C, Plettig J, Johnen C, Triolo F, Turner M, Thompson R, Over P, Rubin P, Amico G, Gerlach J. Delivery of Epidermal and Dermal Stem Cells to a Burn Wound via a Spray Device - Exploring Human Fetal Skin Progenitor Cells for Regenerative Medicine Cell Based Therapy. 2011.
Gaines C.V., Van Dyke, M. Dermal cell-keratin biomaterial interactions to access efficacy in in vitro wound healing models. Tissue Engineering International & Regenerative Medicine Society – North America (TERMIS-NA) Annual Meeting, Orlando, FL. December 2010.
Gerlach J, Plettig J, Turner M, Thompson R, Chinicci C, Triolo F, Johnen C. In vitro studies on human fetal skin cell isolation – preliminary data on a comparison of seven methods. 2010.
Gharaibeh B, Ingram SM, Distefanno G, Sicari B, Oyster NM, Fu FH, Huard JH. A New Reliable Murine Model for Compartment Syndrome. XXIII International Congress of the Transplantation Society, Vancouver, Canada, August 15-19, 2010.
Gharaibeh B, Oyster NM, Boyer A, Blackwell T, Poddar M, Distefanno G, Kobayashi T, Al Hallak A, Viswanathan V, Ahani B, Lavasani M, Witt M, Huard JH. Histological Analysis of Muscle and Nerve Injury in a New Model of Compartment Syndrome. Armed Forces Institute of Regenerative Medicine All-Hands Meeting, Clearwater, FL, January 17-20, 2011.
Gharaibeh B, Oyster NM, Boyer A, Blackwell T, Poddar M, Distefanno G, Kobayashi T, Al Hallak A, Viswanathan V, Ahani B, Lavasani M, Witt M, Huard JH. Histological Analysis of Muscle and Nerve Injury in a New Model of Compartment Syndrome. Orthopaedic Research Society, Long Beach, CA, January 13-16, 2011.
Hong Y, Huber A, Hashizume R, Amoroso NJ, Badylak SF, Wagner WR. A biohybrid composite of extracellular matrix gel and elastomeric fibers for soft tissue repair. Biomedical Engineering Society Annual Fall Conference, 2010.

Table B-2b. Presentations and Non-Peer-Reviewed Publications: Wake Forest-Pittsburgh Consortium (cont.)

Hwang CM, Lee BK, Green D, Jeong SY, Atala A, Lee SJ, Yoo JJ, Cartilage covered alloplastic medical device improves implant stability, Tissue Engineering and Regenerative Medicine International Society (TERMIS)-North America, December 5-8, 2010, Hilton in the Walt Disney World Resort, Orlando, FL, USA.
Januszyk M, Rustad KC, Major MR, Wong VW, Glotzbach JP, Gurtner GC. Automated Detection of Wound Area in a Stented Excisional Model of Murine Wound Healing. Plastic Surgery Research Council 56th Annual Meeting, Louisville, KY. April 30, 2011.
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Ju YM, Atala A, Yoo JJ, Lee SJ. Biomaterial-Induced Host Stem Cell Recruitment for In Situ Muscle Tissue Regeneration. 3rd AFIRM "All Hands" Meeting, January 17-20, 2011 in Hilton Clearwater Beach Resort, Clearwater, FL, USA.
Ju YM, Yoo JJ, Atala A, Lee SJ. Biomaterial induced host stem cell recruitment for in situ muscle regeneration, Advanced Technology Applications for Combat Casualty Care (ATACCC), August 16-19, 2010, St. Pete Beach, FL, USA.
Ju YM, Yoo JJ, Atala A, Lee SJ. Biomaterial induced host stem cell recruitment for in situ muscle tissue regeneration, Society for Biomaterials 2011 Annual Meeting & Exposition, April 13-16, 2011, Disney's Contemporary Resort, Orlando, FL, USA.
Ju YM, Yoo JJ, Atala A, Lee SJ. Biomaterial induces host stem cell recruitment for in situ muscle tissue regeneration, 18th Annual Residents' and Fellows' Research Day, November 12, 2010, Wake Forest University School of Medicine, Winston-Salem, NC, USA.
Ju YM, Yoo JJ, Atala A, Lee SJ. Biomaterial induces host stem cell recruitment for in situ muscle tissue regeneration. North Carolina Tissue Engineering & Regenerative Medicine Conference, November 13, 2010 in NC Biotechnology Center, Triangle Park, North Carolina, USA.
Ju YM, Yoo JJ, Atala A, Lee SJ. Biomaterial induces host stem cell recruitment for in situ muscle tissue regeneration. Tissue Engineering and Regenerative Medicine International Society (TERMIS)-North America, December 5-8, 2010, Hilton in the Walt Disney World Resort, Orlando, FL, USA.
Kang H-W, Bae JH, Ladd M, Holms J, Atala A, Yoo JJ, Lee SJ. Development of in vitro skin expansion bioreactor system for burn injuries. Advanced Technology Applications for Combat Casualty Care (ATACCC), August 16-19, 2010, St. Pete Beach, FL, USA.
Kang H-W, Choi J, Green D, Atala A, Holmes J, Yoo JJ, Lee SJ. Optimized Parameters of In Vitro Skin Expansion Bioreactor System for Burn Injuries. 3rd AFIRM "All Hands" Meeting, January 17-20, 2011 in Hilton Clearwater Beach Resort, Clearwater, FL, USA.
Kang H-W, Ladd M, Atala A, Yoo JJ, Lee SJ. In vitro expanded living skin matrix for burn injuries. Tissue Engineering and Regenerative Medicine International Society (TERMIS)-North America, December 5-8, 2010, Hilton in the Walt Disney World Resort, Orlando, FL, USA.
Kang H-W, Scarpinato P, Lee SJ, Johnson K, Holmes J, Atala A, Yoo JJ. Design for the clinically applicable skin expansion bioreactor and regulatory pathway for clinical trial. Advanced Technology Applications for Combat Casualty Care (ATACCC), August 16-19, 2010, St. Pete Beach, FL, USA.
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Table B-2b. Presentations and Non-Peer-Reviewed Publications: Wake Forest-Pittsburgh Consortium (cont.)

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Lee BK, Hwang CM, Green D, Lee SJ, Atala A, Yoo JJ. Cartilage covered alloplastic medical device improves implant stability. Advanced Technology Applications for Combat Casualty Care (ATACCC), August 16-19, 2010, St. Pete Beach, FL, USA.
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Oyster NM, Gharaibeh B, Boyer A, Poddar M, Kobayashi T, Terada S, Peet E, Floh T, Huard JH. Gait Dynamics and Balance Testing in Rat Lower Limb Compartment Syndrome Model. Orthopaedic Research Society, Long Beach, CA, January 13-16, 2011.
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Roy A, Onishi S, Singh SS, Syed-Picard F, Li J, Wu H, Sfeir C, Kumta PN. Novel Synthetic Bone Cements for Orthopaedic and Craniofacial Regeneration. Advanced Technology Applications for Combat Care (ATACCC), August 16-19, 2010, Poster presentation.
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Soker S, et al. A Standardized Rat Model of Compartment Syndrome. Armed Forces Institute for Regenerative Medicine All-Hands Meeting, Clearwater, FL, January 2011.
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Soker S, et al. Endothelial Progenitor Cells and VEGF Increase Neovascularization and Volume Preservation of Engineered Tissues in vivo. Advanced Technology Applications for Combat Casualty Care (ATACCC) June 2010.
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Soker S, et al. Hypoxic conditions have differential effects on colony formation, proliferation and in vitroangiogenesis of endothelial progenitors. Tissue Engineering International & Regenerative Medicine Society (TERMIS)-NA, Orlando, FL, December 2010.

Table B-2b. Presentations and Non-Peer-Reviewed Publications: Wake Forest-Pittsburgh Consortium (cont.)

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Soker S, et al. Imaging Vasculogenesis in Regenerating Muscle Tissue In Vitro. Tissue Engineering International & Regenerative Medicine Society (TERMIS)-EU, Galway, Ireland, June 2010.
Soker S, et al. Imaging Vasculogenesis in Regenerating Skeletal Muscle. North Carolina Tissue Engineering and Regenerative Medicine Society, Raleigh, NC, November 2010.
Soker S, et al. Imaging Vasculogenesis in Regenerating Skeletal Muscle: From Science Fiction to Science. Wake Forest Institute for Regenerative Medicine Annual Retreat, Pinehurst, NC, March 2011.
Soker S, et al. Imaging Vasculogenesis Regenerating Skeletal Muscle. Armed Forces Institute for Regenerative Medicine All-Hands Meeting, Clearwater, FL, January 2011.
Soker S, et al. Vasculogenesis in Regenerating Skeletal Muscle. Tissue Engineering International & Regenerative Medicine Society (TERMIS)-NA, Orlando, FL, December 2010.
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Sorkin M, Wong VW, Longaker MT, Gurtner GC. Utilization of explantable microvascular networks from adipose tissue for organ-level tissue engineering. 56th Annual Meeting of the Plastic Surgery Research Council, Louisville, KY. April 27, 2011. Podium presentation.
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Takanari K, Hashizume R, Hong Y, Huber A, Amoroso NJ, Badylak SF, Wagner WR. A biohybrid composite of extracellular matrix gel and elastomeric fibers for soft tissue repair. McGowan Institute Scientific Retreat, 2011.
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Willett, N., Li, M.T.A., Uhrig, B.A., Warren, G., Guldberg, R.E. Functional and Structural Analysis of Limb Restoration Using a Novel Rat Model of Composite Bone and Muscle Injury. Armed Forces Institute for Regenerative Medicine(AFIRM) Annual Conference, January 19, 2011, Tampa, Florida.
Willett, N., Li, M.T.A., Uhrig, B.A., Warren, G., Guldberg, R.E., "Functional and Structural Analysis of Limb Restoration Using a Novel Rat Model of Composite Bone and Muscle Injury," The 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, January, 2011.
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Wimmers E, Wachtman G, Gorantla V, Lin C, Schneeberger S, Jindal R, Unadkat J, Zheng XX, Brandacher G, Lee WPA. Biologics and Donor Bone Marrow Cells for Targeted Immunomodulation in Composite Tissue Allotransplantation – A Large Animal Translational Trial. AFIRM All-Hands Meeting 2011, St. Petersburg, FL, January 2011.
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Yang TB, Cetin-Ferra S, Dohar JE, Hebda PA. Cell-based therapy to reduce scarring and improve healing in the wound bed. Symposium for Advanced Wound Care- Wound Healing Society Joint Meeting. Dallas, TX. April 14-17, 2011.

Table B-2c. Presentations and Non-Peer-Reviewed Publications: U.S. Army Institute of Surgical Research

Baer DG, Dubck MA, Wenke JC, McGhee LL, Convertino VA, Cancio LC, Wolf SE, Blackbourne LH. Advanced Product Development for Combat Casualty Care at the U.S. Army Institute of Surgical Research. NATO Symposium, 2010.
Baer DG, Dubick MA, Wenke JC, McGhee LL, Convertino VA, Cancio LC, Wolf SE, Blackbourne LH. Development of Advanced Technology and Innovative Procedures for Medical Field Operations. NATO Symposium, 2010.
Beltran MJ, Burns TC, Eckel TT, Potter BK, Wenke JC, Hsu JR. Peripheral Nerve Recovery Following Combat-Related Type III Open Tibia Fractures. SOMOS, 2010.
Beltran MJ, Burns TC, Eckel TT, Potter BK, Wenke JC, Hsu JR, STReC. Peripheral Nerve Recovery Following Combat-Related Type III Open Tibia Fractures. Limb Lengthening and Reconstruction Society, 2010.
Blair JA, Masini BD, Wenke JC, Stinner DJ. Characterization of Animal Research Models Published in Orthopaedic Trauma Literature. SOMOS, 2010.
Brown Baer PR, Silliman DT, Wenke JC, Guelcher SA, Dumas JE, Hale RG. Early Results of Injectable Synthetic Scaffold, rhBMP-2 and Bone Mineral in a Rabbit Critical Size Defect. TERMIS, 2010.
Brown KV, Wenke JC, Guda T, Li B, Perrien DS, Guelcher SA. Decreasing Complications in Open Fractures with a Dual-Delivery Bone Graft. AAOS, 2011.
Burns TC, Stinner DJ, Possley DR, Mack AW, Eckel TT, Poter BK, Wenke JC, Hsu JR, STReC. Does the Zone of Injury in Combat Related Type III Open Tibia Fractures Prelude the use of Local Soft Tissue Coverage? American Orthopaedic Association, 2010.
Chen XK, Roe, JL, Sanchez ME, Wu, X, Rathbone CR, Yoo, JJ, Walters TJ. Treatment of Tourniquet-Induced Ischemia/Reperfusion Injury with Muscle Progenitor Cells. Orthopaedic Research Society. January, 2011.
Chen XK, Yoo JJ, Christ GJ, Walters TJ. A Novel Critical Sized Defect Model of Latissimus Dorsi Muscle in Rat. AFIRM All Hands Meeting, January, 2011.
Christy R, Natesan S, Baer D. Adipose Derived Stem Cells for the Treatment of Burns. Boswick Burn and Wound Care Symposium, February, 2011.
Christy RJ. Growing New Skin from Burned Layers. *San Antonio Express News/Stars and Stripes.* April, 2011.
Christy RJ, Natesan S, Suggs L, Baer D. Stem Cells for the Treatment of Soft Tissue Trauma and Burns. San Antonio Stem Cell Conference. October, 2010.
Christy, R. Adipose Derived Stem Cells for the Treatment of Burns. University of Texas San Antonio, Dept. of Biology. April, 2011.
Cross JC, Rathbone CR, Wenke JC. Comparison of Osteogenic Potential of Different Bone Morphogenetic Proteins. AO North, 2010.
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Cross JD, Ficke JR, Hsu JR, Masini BD, Wenke JC. Battlefield Orthopaedic Injuries Cause the Majority of Long Term Disabilities. SOMOS, 2010.
Cross JD, Ficke JR, Hsu JR, Masini BD, Wenke JC. Battlefield Orthopaedic Injuries Cause the Majority of Long Term Disabilities. Southern Orthopaedic Association, 2010.
Cross JD, Ficke JR, Hsu JR, Masini BD, Wenke JC. Orthopaedic Impact on Disabling Conditions Resulting from Combat Wounds. Southern Orthopaedic Association, 2010.
Cross JD, Hsu JR, Wenke JC, Rathbone CR. Non-Steroidal Anti-Inflammatory Drug Effect on Osteoblast Viability In Vitro. AAOS, 2011.
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Cross JD, Johnson AE, Wenke JC, Ficke JR. Female Casualties of Operations Enduring Freedom and Iraqi Freedom. SOMOS, 2010.

Table B-2c. Presentations and Non-Peer-Reviewed Publications: U.S. Army Institute of Surgical Research (cont.)

Cross JD, Rathbone CR, Wenke JC. Bone Morphogenetic Proteins: Comparison of migratory, proliferative, angiogenic, and osteogenic potentials. SOMOS, 2010.
Cross JD, Rathbone CR, Wenke JC. Bone Morphogenetic Proteins: comparison of osteogenic, angiogenic and migratory potentials. OTA, 2010.
Cross JD, Rathbone CR, Wenke JC. Comparison of Osteogenic Potential of Different Been Morphogenetic Proteins. OTA, 2010.
Cross JD, Stinner DJ, Burns TC, Wenke JC, Hsu JR. Return to Duty after Type III Open Tibia Fracture. Limb Lengthening and Reconstruction Society, 2010.
Cross JD, Stinner DJ, Burns TC, Wenke JC, Hsu JR, STReC. Return to Activity Duty Following Type III Open Tibia Fracture. Roy Davis Research Competition, 2010.
Cross JD, Stinner DJ, Burns TC, Wenke JC, Hsu JR, STReC. Return to Duty after Type III Open Tibia Fracture. AAOS, 2011.
Cross JD, Stinner DJ, Burns TC, Wenke JC, Hsu JR, STReC. Return to Duty after Type III Open Tibia Fracture. ATACCC, 2010.
Cross JD, Stinner DJ, Burns TC, Wenke JC, Hsu JR, STReC. Return to Duty after Type III Open Tibia Fracture. SOMOS, 2010.
Cross JD, Wenke JC, Ficke JR, Johnson AE. Impact of Traumatic Arthritis on Combat Casualties. AAOS, 2011.
Cross JD, Wenke JC, Ficke JR, Johnson AE. Impact of Traumatic Arthritis on Combat Casualties. SOMOS, 2010.
Cross, JD, Rathbone CR, Wenke JC. Bone Morphogenetic Proteins: Comparison of Osteogenic, Angiogenic, and Migratory Potentials. American Academy of Orthopedic Surgeons, January, 2011.
Devore DI, Leung K. Penetration of biofilm defenses with nanocomplexes of cationic antimicrobial peptides. MIDRP Wound Healing Symposium, 2011.
Guda T, Hernandez JW, Singleton B, Appleford MR, Walker JA, Pilla M, Son JS, Oh S, Ong JL, Wenke JC. Histomorphometry of Varying Hydroxyapatite Scaffold Architectures in Vivo. Biomedical Engineering Society, 2010.
Guda T, Walker JA, Oh S, Appleford MR, Ong JL, Wenke JC. Bone Defect Regeneration In Vivo Using Hydroxyapatite Scaffolds: A Biomechanical Perspective. AFIRM, 2010.
Hardy SK, Seetharaman S, Natesan S, Stowers RS, Baer DG, Suggs LJ, Christy RJ. An Antibacterial Fibrin-Based Wound Dressing to Induce Angiogenesis in Wound Repair. ATACCC, August, 2010.
Johnson AE, Cross JD, Wenke JC, Ficke JR. Female Casualties of Operations Enduring and Iraqi Freedom. ATACCC, 2010.
Johnson AE, Wenke JC, Bosse MJ, Cross JD, Ficke JR. Female Casualties of Operation Enduring Freedom: Status and Future Directions. AAOS, 2011.
Johnson AE, Wenke JC, Bosse MJ, Cross JD, Ficke JR. Female Casualties of Operations Enduring and Iraqi Freedom: Status and Future Directions. ATACCC, 2010.
Johnson AE, Wenke JC, Bosse MJ, Cross JD, Ficke JR. Female Casualties of Operations Enduring and Iraqi Freedom: Status and Future Directions. OTA, 2010.
Krueger CA, Stinner DJ, Masini BD, Wenke JC, Hsu JR. What do I do now with drop-contaminated bone? SOMOS, 2010.
Lofgren AL, Wenke JC, Rathbone CR. The Effect of Lovastatin on Cell Migration. ATACCC, 2010.
Masini BD, Owens BD, Hsu JR, Wenke JC. Rehospitalization after Combat Trauma. SOMOS, 2010.
Masini BD, Owens BD, Wenke JC, Hsu JR. Combat Trauma: Resource Utilization Beyond Initial Hospitalization. AAOS, 2011.
Masini BD, Ritenour AE, Racusin AW, Wenke JC, Gerlinger TL, Hsu JR. Acute Compartment Syndrome of the Thigh in Combat Casualties. AAOS, 2011.
Masini BD, Ritenour AE, Racusin AW, Wenke JC, Gerlinger TL, Hsu JR. Acute Compartment Syndrome of the Thigh in Combat Casualties. ATACCC, 2010.
Masini BD, Stinner DJ, Waterman SM, Wenke JC. Bacterial Adherence to High Tensile-Strength Suture: A Bioluminescent In-Vitro Analysis. Arthroscopy Association of North America, 2010.
Masini BD, Stinner DJ, Waterman SM, Wenke JC, Gerlinger TL. Bacterial Adherence to Titanium versus Bioabsorbable Interference Screws. Arthroscopy Association of North America, 2010.

Table B-2c. Presentations and Non-Peer-Reviewed Publications: U.S. Army Institute of Surgical Research (cont.)

Natesan S, Christy RJ. Laminar Construct for Tissue-Engineering Skin Equivalent. University of Pittsburgh. December, 2010.
Natesan S, Seetharaman S, Wrice NL, Zamora D, Baer DG and Christy RJ. Human Adipose Derived Stem Cells Induce Vasculogenesis. ATACCC, August, 2010.
Natesan S, Zamora DO, Wrice NL, Suggs LJ, Christy RJ. A PEGylated Fibrin-Based Matrix Induces Vascularization of Human Adipose Derived Stem Cells. TERMIS, December, 2010.
Natesan S, Zamora DO, Zhang G, Suggs LJ, Christy RJ. Development of a Vascularized Dermal Equivalent using Adipose Derived Stem Cells. AFIRM All Hands Meeting, January, 2011.
Natesan S, Zhang G, Walters TJ, Christy RJ, Suggs LJ, Wrice NL. A Bilayer Construct Directs Adipose Derived Stem Cell Differentiation. BMES, October, 2010.
Patzkowski JC, Banck RV, Owens JG, Wilken JM, Kirk KL, Wenke JC, Hsu JR. Comparative Effect of Orthosis Design on Functional Performance. LLRS, 2011.
Patzkowski JC, Banck RV, Owens JG, Wilken JM, Kirk KL, Wenke JC, Hsu JR. Comparative Effect of Orthosis Design on Functional Performance. Omer Day, 2011.
Patzkowski JC, Blank RV, Owens JG, Wilken JM, Kirk KL, Wenke JC, Hsu JR. The Effect of Orthosis Type on Functional Performance in Lower Extremity Limb Salvage Soldiers. Extremity War Injury Symposium, 2011.
Patzkowski JC, Blank RV, Owens JG, Wilken JM, Kirk KL, Wenke JC, Hsu JR, STReC. Comparative Effect of Orthosis Design on Functional Performance. AOFAS, 2011.
Patzkowski JC, Cross JD, Ficke JR, Wenke JC. The changing face of Army Disability: The OIF/OEF Effect. SOMOS, 2010.
Patzkowski JC, Cross JD, Ficke JR, Wenke JC. The Changing Faces of Army Disability: The OIF/OEF Effect. Omer Day, 2011.
Patzkowski JC, Stinner DJ, Wenke JC, Masini BD. Animal Models of Orthopaedic Infections. AAOS, 2011.
Penn-Barwell J, Wenke JC. Determining Contamination Threshold for Infection in an Open Fracture Model. AAOS, 2011.
Rathbone CR, Appleford MR, Oh S, Guda T, Ong JL, Wenke JC. Improved Bone regeneration with Hydroxyapatite Scaffolds seeded with mesenchymal Stem Cells. OTA, 2010.
Rathbone CR, Cross J, Brown K, Murray CK, Wenke JC. Effect of Various Concentrations of Antibiotics on Osteogenic Cell Viability and Activity. Orthopaedic Research Society, January, 2011.
Rathbone CR, Guda T, Singleton B, Oh S, Appleford MR, Ong JL, Wenke JC. Effect of hydroxyapatite scaffolds seeded with cells on in vivo bone regeneration. AFIRM All Hands Meeting, January, 2011.
Rathbone CR, Lofgren A, Sanchez M, Wenke JC, Walters TJ. Survival of Freshly Isolated Stem Cells Following Ischemia-Reperfusion Injury. ATACCC, August, 2010.
Seetharaman S, Natesan S, Stowers RS, Mullens C, Baer DG, Suggs LJ, Christy RJ. An Antibacterial Fibrin-Based Wound Dressing to Induce Angiogenesis in Wound Repair. AFIRM All Hands meeting, January, 2011.
Seetharaman S, Stowers RS, Natesan S, Mullens C, Suggs LJ, Christy RJ. Fibrin Based Hydrogels for Infected Wound Therapy. TERMIS, December, 2010.
Seetharaman S, Stowers RS, Natesan S, Zamora DO, Christy RJ, Suggs LJ. Fibrin Based Hydrogels to Promote Healing and Limit Infection in Wound Therapy. Society for Biomaterials, April, 2011.
Shah AR, Wenke JC, Agrawal M. Effect of Endothelial Cell and Osteoblast Co-culture Ratios on Angiogenesis and Mineralization. Biomedical Engineering Society, 2010.
Shanmuganathan, S and RJ Christy. Treatments to Prevent Infection of Combat Related Injuries. University of Pittsburgh. December, 2010.
Siller-Jackson A, Bowman P, Wenke JC, Bynum JA, Hamamieh R. Temporal Gene Expression Profiling of Bone Repair in a Rat Calvarial Defect. Experimental Biology, 2010.
Siller-Jackson A, Bowman P, Wenke JC, Bynum JA, Hammamieh R. Temporal Gene Expression Associated with Healing and Non-Healing Bone During the Repair Process in Rat Calvarial Defects. ORS, 2010.
Silliman DT, Dumas JE, Guelcher SA, Wenke JC, Hale RG, Brown Baer PR. Dose and Delivery Kinetic Effects of rhBMP-2 on Post Operative Swelling in New Zealand White Rabbit Critical Size Defect Cranial Surgery. ATACCC, 2010.

Table B-2c. Presentations and Non-Peer-Reviewed Publications: U.S. Army Institute of Surgical Research (cont.)

Stinner DJ, Beltran MJ, Masini BB, Hsu JR, Wenke JC. Spatial Distribution of Bacteria on Contaminated External Fixators Before and After Surgical Prep. OTA, 2010.
Stinner DJ, Beltran MJ, Masini BD, Wenke JC, Hsu JR. Bacteria on External Fixators: Which Prep is best? SOMOS, 2010.
Stinner DJ, Hsu JR, Wenke JC. Negative Pressure Wound Therapy Reduces the Effectiveness of Antibiotic Beads. SOMOS, 2010.
Stinner DJ, Hsu JR, Wenke JC. NPWT Reduces Effectiveness of Antibiotic Beads. OTA, 2010.
Stinner DJ, Krueger C, Wenke JC, Masini BD. The time dependent effect of chlorhexidine surgical prep. SOMOS, 2010.
Stinner DJ, Waterman SM, Masini BD, Wenke JC. Effect of Silver Dressing on Gram Positive Organisms in a Contaminated Open Fracture Model Treated with Negative Pressure Wound Therapy. American Orthopaedic Association, 2011.
Stinner DJ, Waterman SM, Masini BD, Wenke JC. Effect of silver dressing on gram positive organisms in a contaminated open fracture model treated with negative pressure wound therapy. SOMOS, 2010.
Stinner DJ, Waterman SM, Wenke JC. Negative Pressure Wound Therapy in a Contaminated Musculoskeletal Wound Model: Is NPWT Effective on S. aureus? European Federation of National Associations of Orthopaedics and Traumatology Congress, 2010.
Stinner DJ, Waterman SM, Wenke JC. Negative Pressure Wound Therapy in a Contaminated Musculoskeletal Wound Model: Is NPWT effective on S. aureus? Mid-America Orthopaedic Association, 2010.
Tarte N, Archer C, Devore D. Nanoparticle drug delivery for the treatment of biofilm infections. PTEI Summer Interns Program, 2010.
Walters, TJ. Engineered Skeletal Muscle for the treatment of Volumetric Muscle Loss (VML): Testing Platforms and Functional Assessment. TERMIS. December, 2010.
Washburn N, Sun LT, Natesan S, Gilbert T, Badylak SF, Christy RJ. Effects of Cytokine-based Gels in Rat Incisional and Burn Models. Wound Healing Society, April, 2011.
Wenke JC, Guda T, Walker JA, Oh S, Appleford MR, Ong JL. Guiding Hydroxyapatite Scaffold Based Bone Regeneration in Vivo with Collagen Membranes. Society for Biomaterials, 2010.
Wu X, Walters TJ, Sanchez M, Rathbone C. Fiber-type specific alterations in skeletal muscle satellite cell activity in response to severe burn in rats. ATACCC, August, 2010.
Zamora DO, Natesan S, Suggs LJ, Christy RJ. Promoting Vasculogenesis of Human Derived Adipose Stem Cells. NAVBO, October, 2010.

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Appendix C: Patent Applications and Invention Disclosures

The attribution of inventions and patent applications to specific research support is subject to varying interpretations in the absence of a standard definition. Optimally, only those patents and patent applications displaying the AFIRM contract number in the Government Interest field in the U.S. Patent and Trademark Office (USPTO) patent application record should be included as directly attributable to the AFIRM program; however, this strict definition would exclude provisional patent applications left undisclosed to the public and recently filed applications not yet included in government databases. Rather than applying the more rigid validation approach outlined above, the following definitions were applied to self-reported intellectual property milestones:

A self-reported invention disclosure filed with the inventor's institutional technology licensing office during a given program year is attributed to the AFIRM program in that program year.

A self-reported patent application filed with a government patent office between October 2008 and May 2009 is attributed to the AFIRM program in program year 1 (PY1). Thereafter, a self-reported patent application filed with a government patent office during a 12-month program year period is attributed to the AFIRM program that program year (e.g., June 2010 – May 2011 for PY3).

A self-reported patent award will be attributed to the AFIRM program based on the patent application filing date meeting the minimal criteria for patent applications above (i.e., filed after September 2008).

All self-reported patent application numbers and inventors (i.e., principal investigators) were queried against the World Intellectual Property Organization (WIPO) patent application database **http://www.wipo.int/pctdb/en/** and the USPTO AppFT patent application database **http://patft.uspto.gov/**. The database queries were used to (1) identify patent applications filed for self-reported inventions and (2) identify and validate filing dates for patent applications.

AFIRM researchers self-reported 14 government-filed patent applications that included a filing date or year; a patent priority number, serial number, or other patent application number; and/or were identified on the USPTO or WIPO databases.

Patent Applications: Rutgers-Cleveland Clinic Consortium

Molecular Surface Design of Tyrosine-Derived Polycarbonates for Attachment of Biomolecules. Filed on January 17, 2011. Bushman J (Rutgers University).

Low Dose IV FN Vasoactive Peptides for Burns. Filed on February 1, 2011. Clark R (Stony Brook University).

Reinforced Tissue Graft. A Continuation-In-Part (PCT/US2009/038570). Filed on June 28, 2010. Derwin K, Aurora A, Iannotti JP, and McCarron JA (Cleveland Clinic Foundation).

Patent Application (Priority Number 61/419,946). Filed on December 6, 2010. Muschler, G (Cleveland Clinic Foundation).

Injectable Allograft PUR Composite Carrying rhBMP2 (Priority Number 61/406,098). Filed on October 22, 2010. Guelcher SA and Dumas JE (Vanderbilt University).

Isocyanate Manufacture (Serial Number 13/021626). Filed on February 4, 2011. Guelcher SA, Bhattacharyya S, Gopal D, and Burello M (Vanderbilt University).

Weight-Bearing Polyurethane Composites and Methods Thereof (Serial Number 12/783548). Filed on May 19, 2011. Guelcher SA, Dumas JE, and Boyce TM (Vanderbilt University).

Injectable Resorbable Allograft Bone Polyurethane Bone Cement (Priority Number 61/294,466). Filed on January 12, 2011. Guelcher SA, Prieto EM, Dumas JE, and Zienkiewicz KJ (Vanderbilt University).

Biocompatible Polycaprolactone Fumarate (PCLF) Formulations. Filed in 2010. Yaszemski, MJ (Mayo Clinic).

Calcium Phosphate Coatings on Porous 3-Dimensional Polypropylene Fumarate Scaffolds. Filed in 2010. Yaszemski, MJ (Mayo Clinic).

Patent Applications: Wake Forest-Pittsburgh Consortium

Bone Substitute Compositions, Methods of Preparations and Clinical Applications (PCT/US2010/048930). Filed on September 15, 2010. Kumta PN, Roy A, and Sfeir C (University of Pittsburgh).

Matricryptic ECM Peptides for Tissue Reconstruction (PCT/US2010/051947). Filed on April 14, 2011. Badylak SF, Agrawal V, Tottey S, and Johnson SA (McGowen Institute).

CAR Peptide for Homing, Diagnosis & Targeted Therapy for Pulmonary and Fibrotic Disorders (PCT/US2011/26535). Filed on February 28, 2011. Ruoslahti E, Komatsu M, and Mann D (University of California, Santa Barbara).

Combined Space Maintenance and Bone Regeneration System for the Reconstruction of Large Osseous Defects (PCT/US2011/021266). Filed on January 14, 2011. Mikos AG, Wong ME, Kasper FK, Young S, Kretlow JD, Shi M, and Spicer PP (Rice University).

Invention disclosures are not publicly reposed in standard databases; therefore, the AFIRM consortium reports are the only information source for inventions disclosed. The provided information did not indicate a date when the inventions were filed with the institutional technology licensing office, and most records did not indicate a case reference number assigned to the invention. Due to these limitations, all invention disclosures without a date or reference number were assumed, but not validated, to have been filed from June 2010 through May 2011. Also, self-reported patent applications that only listed an invention disclosure number, but not a patent application filing number or serial number, were considered invention disclosures and not patent applications. Eight invention disclosures were made by AFIRM faculty during this period.

Invention Disclosures: Rutgers-Cleveland Clinic Consortium

Notice of Invention Forms filed with the Rutgers Office of Technology Transfer. Iovine C (Rutgers University).

Invention Disclosures: Wake Forest-Pittsburgh Consortium

A Novel "Sandwich" Electrospun Composite of Polymer Fibers and ECM Gel. Wagner WR (University of Pittsburgh).

Skin Gun Device. Gerlach J (University of Pittsburgh).

Invention Disclosure. Gerlach J (University of Pittsburgh).

PCL/Microsphere Nerve Guide. Marra K and Kokai L (University of Pittsburgh).

Injectable Silk Scaffolds. Kaplan D (Tufts University).

Invention Disclosures: U.S. Army Institute of Surgical Research

Graft copolymer complexes for delivery of therapeutic cationic peptides (Invention Disclosure Docket ISR 11-34). Devore DI (U.S. Army Institute of Surgical Research).

Compositions comprising wound tissue progenitor cells and methods of their use. Natesan S, Baer DG, and Christy RJ (U.S. Army Institute of Surgical Research).

Dual Delivery of Growth Factor and Antibiotic. Wenke JC, Guelcher SA, Li B, and Brown KV (U.S. Army Institute of Surgical Research).

our science for their healing

Appendix D: Acronym List

α-SMA ..... alpha-smooth muscle actin
β-TCPBP ..... β-TCP binding peptide
2-D ..... two-dimensional
3-D ..... three-dimensional
ACUC ..... Animal Care and Use Committee
ACURO ..... Animal Care and Use Review Office
aECM ..... artificial extracellular matrix
AFIRM ..... Armed Forces Institute of Regenerative Medicine
AFM ..... atomic force microscope
AFS ..... amniotic fluid-derived stem
AFT ..... autologous fat transfer
AFT-SPAR ..... autologous fat transfer for scar prevention and remodeling
ANOVA ..... analysis of variance
APB ..... abductor pollicis brevis
ASC ..... adipose-derived stem cell
BAM ..... bladder acellular matrix
BAMC ..... Brooke Army Medical Center
bFGF ..... basic fibroblast growth factor
BIODOME ..... Biomechanical Interface for Optimized Delivery of MEMS Orchestrated Mammalian Epimorphosis
BM-MNC ..... bone marrow mononuclear cell
BME ..... bone marrow excavation
BMP-2 ..... bone morphogenetic protein 2
BMSC ..... bone marrow stromal stem cell
BOD ..... Board of Directors
BSA ..... bovine serum albumin
BTX ..... bungarotoxin
CaP ..... calcium phosphate
CAR ..... CARSKNKDC peptide
CBER ..... Center for Biologics Evaluation and Research
CCT ..... chaperonin-containing T-complex polypeptide
CCTD ..... chronic caprine tibial defect
CDHA ..... calcium-deficient hydroxyapatite
CFMD ..... canine femoral multi-defect
CFR ..... craniofacial reconstruction
cGLP ..... current Good Laboratory Practice
cGMP ..... current Good Manufacturing Practice
CHO ..... Chinese hamster ovary
CK15 ..... cytokeratin 15
CM ..... conditioned medium
CMF ..... craniomaxillofacial
COI ..... conflict of interest
CRMRP ..... Clinical and Rehabilitative Medicine Research Program
CS ..... compartment syndrome
CSD ..... critical-size defect
CT ..... computed tomography
CTA ..... composite tissue allograft
CTP-O ..... osteogenic connective tissue progenitor
DCBP ..... demineralized cortical bone powder
dECM ..... dermal extracellular matrix
DI ..... deionized
DIC ..... digital image correlation
DOC ..... deoxycholate
DoD ..... Department of Defense
DS ..... density separation
ECD ..... external compression device
ECM ..... extracellular matrix
EDL ..... extensor digitorum longus

e-film ..... electronic film
EGF ..... epidermal growth factor
EHL ..... extensor hallicus longus
ELISA ..... enzyme-linked immunosorbent assay
EMB ..... explanted microvascular bed
ePTFE ..... expanded polytetrafluroethylene
ESS ..... engineered skin substitute
EVG ..... Elastica Verhoeff-van Gieson
FACS ..... fluorescence-activated cell sorting
FAK ..... focal adhesion kinase
FBS ..... fetal bovine serum
FDA ..... U.S. Food and Drug Administration
FITC ..... fluorescein isothiocyanate
GAG ..... glycosaminoglycan
GDNF ..... glial-derived neurotrophic factor
GFP ..... green fluorescent protein
GLP ..... Good Laboratory Practice
GM ..... growth medium
GMI ..... gastrocnemius muscle index
GMP ..... Good Manufacturing Practice
GST ..... gastrocnemius
H&E ..... hematoxylin and eosin
HA ..... hyaluronan/hyaluronic acid
hASC ..... human adipose-derived stem cell
HBSS ..... Hanks balanced salt solution
HDE ..... Humanitarian Device Exemption
HDMEC ..... human dermal microvascular endothelial cell
HES ..... hematoxylin-eosin-saffron
HGF ..... hepatocyte growth factor
HHK ..... human hair keratin
HMG ..... high-mobility group
hMSC ..... human mesenchymal stem cell
hNSC ..... human neural stem cell
HPF ..... high-power fields
HPMC ..... hydroxypropyl methylcellulose
HRP ..... horseradish peroxidase
HRPO ..... Human Research Protection Office
HSP60 ..... heat shock protein 60
HUVEC ..... human umbilical vein endothelial cell
hVEGF ..... human vascular endothelial growth factor
I/R ..... ischemia/reperfusion
IDE ..... Investigational Device Exemption
IGF ..... insulin-like growth factor
IL ..... interleukin
IND ..... Investigational New Drug
IRB ..... Institutional Review Board
IPT ..... Integrated Project Team
ISCT ..... International Society for Cellular Therapy
ISO ..... International Organization for Standardization
Kd ..... dissociation constants
LD ..... latissimus dorsi
LWI ..... Lonza Walkersville, Inc.
M ..... million
MCA ..... mineralized cancellous allograft
mCAR ..... mutant CAR
MCP-1 ..... monocyte chemoattractant/chemotactic protein 1
MDCK ..... Madin-Darby canine kidney
MDSC ..... muscle-derived stem cell
MGH ..... Massachusetts General Hospital
MiPS ..... microfluidic phage selection
MIRM ..... McGowan Institute for Regenerative Medicine
MIT ..... Massachusetts Institute of Technology
MMP-1 ..... matrix metalloproteinase 1
mMSC ..... murine mesenchymal stem cell
MPC ..... muscle progenitor cell

MS ..... magnetic separation
MSC..... mesenchymal stem cell
MSD .....molecular surface design
MTT.....methyl tetrazolium
Na .....nanospheres dispersed in PBS
NanoCaP .....nanostructured calcium phosphate
NC.....nano-HPMC–nanospheres-gel
NCV ..... nerve conduction velocity
NHP .....nonhuman primate
NIH..... National Institutes of Health
NMJ .....neuromuscular junction
NRP-1 .....neuropilin 1
NSAID.....nonsteroidal anti-inflammatory drug
OEF .....Operation Enduring Freedom
OIF ..... Operation Iraqi Freedom
OMLC ..... Oregon Medical Laser Center
P3..... passage 3
PA ..... peptide amphiphile
PAA .....peracetic acid
PBS..... phosphate-buffered saline
PCL .....polycaprolactone
PCLF ..... poly(caprolactone fumarate)
PCNA ..... proliferating cell nuclear antigen
PCR..... polymerase chain reaction
PDGF .....platelet-derived growth factor
PDO ..... polydioxanone
PEG..... polyethylene glycol
PEO..... polyethylene oxide
PEUU..... poly(ester urethane)urea
PG .....propylene glycol
PGA ..... polyglycolic acid
PGE2..... prostaglandin E2
PLA.....poly L-actic acid
PLCL ..... (poly(65/35(85/15L/D)-lactide-ε-caprolactone
PLGA ..... poly(lactic-co-glycolic acid)
PMMA ..... poly(methyl methacrylate)
PMO ..... Project Management Office
POG .....particulate oxygen generator
PP ..... pinprick
PPF ..... poly(propylene fumarate)
PUR.....polyurethane
PVA..... polyvinyl alcohol
PXS..... poly(xylitol-co-sebacate)
PY3 ..... program year 3
QUT ..... Queensland University of Technology
RCCC .....Rutgers-Cleveland Clinic Consortium
RGD .....arginine-glycine-aspartic acid
rhBMP-2.....recombinant human bone morphogenetic protein 2
RT-PCR ..... reverse transcriptase-polymerase chain reaction
RWP .....Regenerative Wound Paste
S .....saline
SCEC .....stromal cell epineural construct
SDF-1α .....stromal-derived factor-1α
SEM.....scanning electron microscopy
siRNA..... small inhibitory RNA
smECM.....skeletal muscle extracellular matrix
SOP..... standard operating procedure
SOW ..... Statement of Work
SP ..... substance P
SR ..... selective retention
SSEP..... somatosensory evoked potentials
STMG.....split-thickness meshed graft
TA..... tibialis anterior
TBSA..... total body surface area
TCP ..... tricalcium phosphate
TCR..... T cell receptor
tEGF.....tethered epidermal growth factor

TEM ..... transmission electron microscopy
TEMR ..... tissue-engineered muscle repair
TGF-β1 ..... transforming growth factor-β1
TLI ..... therapy to limit injury
TNF-α ..... tumor necrosis factor-alpha
TRL ..... Technology Readiness Level
TS ..... toe spread
TyrPC ..... tyrosine-derived polycarbonate
UBM ..... urinary bladder membrane
UCSB ..... University of California, Santa Barbara
UHG-MACS ..... ultrahigh gradient magnetic activated cell sorter
USAISR ..... U.S. Army Institute of Surgical Research
USAMRMC ..... U.S. Army Medical Research and Materiel Command
USPTO ..... U.S. Patent and Trademark Office
UV ..... ultraviolet
VA ..... U.S. Department of Veterans Affairs
VEGF ..... vascular endothelial growth factor
VEGF-A ..... vascular endothelial growth factor-A
VML ..... volumetric muscle loss
WFIRM ..... Wake Forest Institute for Regenerative Medicine
WFPC ..... Wake Forest-Pittsburgh Consortium
WIPO ..... World Intellectual Property Organization
WRNMMC ..... Walter Reed National Military Medical Center
XRD ..... x-ray diffraction

Appendix E: Index of Team Leaders and Project Team Members

Agrawal, Vineet 26, 112, B-4, C-2
Alvarez, Luis 42, B-12
Alvarez, Pedro 176, B-2, B-11
Amoroso, Nicholas J. 466, B-4, B-14, B-17
Anderson, Daniel G. ..... ix, x, 96, 182, 232, B-1, B-2, B-3, B-9, B-10
Baber, Marilyn 354
Badylak, Stephen F. ..... x, 68, 112, 476, B-4, B-7, B-14, B-16, B-17, B-21, C-2
Barnwell, Jonathan 78
Bassett, Erik 226, B-4, B-9
Bates, Damien 396
Beasley, Bill 314, B-14
Beckowski, Samantha 78
Bellamkonda, Ravi 106, B-4, B-17
Bellas, Evangelia 150, 190, B-4
Beniash, Elia 164, B-16
Berger, Hila 322
Binder, Kyle W. 382, B-14
Blair, Harry 308
Boehm, Cynthia 36, B-11, B-12
Bolikal, Das 17, 30, 176, 186
Bolikal, Veena 186
Bollenbach, Thomas 396
Bothman, David 126
Boyan, Barbara 106, B-14
Boyce, Steven 416, A-2, B-1, B-2, B-3, B-9
Boyer, Aaron 438, B-14, B-16
Brandacher, Gerald 132, 137, B-4, B-6, B-14, B-17
Brogan, David 96
Brown-Baer, Pamela 172
Brunquell, Chris 300
Bushman, Jared 48, B-3, C-1
Cairns, Bruce 426
Caligaris, Rich 314
Cao, Lan 396
Caplan, Arnold 354, A-2, B-3
Caralla, Tonya 36
Carson, Dennis L. 390
Chagwedera, Danai 226
Chen, Jian 96
Cheney, Mack 226
Cheng, Hao 96, B-1, B-9, B-10
Choi, Joshua 408, B-4, B-15
Christ, George 200, A-3, B-5, B-14, B-16, B-18
Christy, Robert J. ..... xi, 420, 462, A-3, B-6, B-8, B-18, B-19, B-20, B-21, C-3
Clark, Richard 247, 266, 340, 345, 358, 362, 416, B-1, B-2, B-10, B-11, B-12, B-13, C-1
Clements, Basak 23, 96, B-9
Clements, Isaac 106, B-17
Collins, Emily 322
Cooney, Damon S. 132
Corona, Benjamin T. 200, B-5, B-14, B-16
Cowley, Christy 314
Criswell, Tracy 438
Cwykiel, Joanna 100, 216, B-1, B-2, B-9, B-10, B-11
Dadsetan, Mahrokh 30, 138, 176, B-2, B-3, B-4

Daly, Kerry....476, B-4
Darr, Aniq .... ix, 30, 148, 176, 186, B-2, B-9, B-11, B-12
Dauskardt, Reinhold.... 252, B-4, B-14, B-17
Demian, Nagi 156, B-5
Derwin, Kathleen.... 68, B-1, C-1
Desai, Seema....88
Dice, Dennis....382, B-14
Dickinson, Caitlyn....210, B-11
Dohar, Joseph E. 292, B-17
Dumas, Jerald 172, B-18, B-20, C-2
Dunn, Michael G. ....x, 72, B-3
Elder, Allison.... 286
Esteban, Roger....368, B-14
Evans, Glenda....96
Ezra, Mindy....96
Faria, Katie.... 396
Farias, Richard 48
Fatica, Richard 240
Feldman, Michael 426
Finkelstein, Eric....210, B-11
Flechner, Stuart 240
Fong, Eileen 376, B-4
Foster, Kevin.... 426
Friedrich, Emily....286, B-8
Furth, Mark E.....400, 405, B-16
Gaines, Carmen....346, B-14, B-16
Gamelli, Richard L.... 426
Gatt, Jr., Charles J....x, 72, B-3, B-10
Geringer, Mathew.... 260
Gerlach, Jörg C. 368, B-4, B-14, C-2, C-3
Gharaibeh, Burhan 10, 438, B-14, B-16
Gharb, Bahar Bassiri....216, B-1, B-3, B-9, B-11, B-12
Ghaznavi, Amir Mahan 78, B-4
Goldfarb, David 240
Gorantla, Vijay S. 132, B-4, B-6, B-14, B-17
Gregory, Cynthia....452, B-5
Gregory, Kenton....xi, 434, 452, B-5
Griffith, Linda..... ix, 30, 42, A-2, B-10, B-11, B-12
Griswold, John 426
Guelcher, Scott A. 172, B-6, B-18, B-20, C-2, C-3
Guldberg, Robert..... 106, 110, A-3, B-4, B-5, B-6, B-14, B-17
Gurtner, Geoffrey C. 60, 252, 272, 314, A-3, B-4, B-6, B-13, B-14, B-15, B-17
Hadlock, Tessa 210, 226
Hale, COL Robert.....143, 172, B-7, B-8, B-18, B-20
Hardy, Sharanda 420, B-19
Harrison, Benjamin.... 43, 54, 484, B-14
Hashizume, Ryotaro 466, B-4, B-14, B-17
Haulsee, Zachary....54
Hebda, Patricia A....292, B-17
Hefferan, Theresa 30, 32, B-11, B-12
Heimbach, David.... 426
Henderson, Tess 30
Henslee, Allan 146, 156
Henstrom, Douglas.... 210, 226
Hershey, Jack....96
Heylman, Chris 42
Hickerson, William 426
Hill, Paulina S..... 96, B-1, B-2, B-9, B-10
Hill, Tanner 220
Hirt, Dietmar 186
Hollinger, Jeffrey 176, B-2, B-9, B-11
Holmes, James..... 338, 375, 408, 426, 428, B-15
Hong, Seok Jong....249, 260, B-10
Hong, Yi..... 466, B-4, B-14, B-17
Hoyer, Rachel 190
Huard, Johnny 308, 438, B-14, B-16

Huber, Alex 112, 466, B-4, B-14, B-17
Hussain, Sajid 300
Hwang, Chang Mo 190, 220, B-15, B-16
Hwang, Nathaniel ..... ix, 182, 232
Iannotti, Joseph 68, B-1, C-1
Iovine, Carmine 186, 358, B-2, C-2
Jackson, Jasper 314
Jackson, John 220, 382, 408
Järvinen, Tero 300, B-5, B-6, B-15
Jia, Sheng-Xian 260, B-10, B-11
Johnson, Kevin 408, B-15
Johnson, Matt 226
Johnson, Scott 112, 476, B-4, C-2
Jordan, Marion 426
Joshi, Powrnima 36
Ju, Young Min 190, 220, 484, B-5, B-15, B-16
Kang, Hyun-Wook 382, 408, B-14, B-15
Kaplan, David 78, 109, 110, 190, 206, B-4, B-5, C-3
Kasper, F. Kurtis 156, A-3, B-5, C-2
Kathju, Sandeep ..... x, 278, B-4, B-5
Katz, Adam J. ..... xiv, 268, 322, A-2, B-1, B-2, B-10
Kemp, Paul 390
Kim, Jinku 176, B-2, B-9, B-11
Kinard, Lucas 156
King, Booker 338, 426
Knight, Andrew 96, 138, B-1, B-13
Ko, In Kap 484, B-5, B-15, B-16
Koepsel, Richard 308, A-3
Kohn, Joachim 5, 96, 210, 358, 362, B-1, B-2, B-3, B-9, B-11, B-12, B-13
Kokai, Lauren 78, B-4, B-5, C-3
Kolambkar, Yash 106, B-4, B-5, B-14
Kretlow, James D. 156, A-3, B-5, C-2
Krogstad, Dan 88, 94
Kumta, Prashant 164, B-16, C-2
Laham, Lorraine 396
Landazura, Natalia 106
Langer, Robert S. ..... ix, x, 60, 96, 182, 232, A-2, B-1, B-2, B-3, B-9, B-10, B-13
Laughland, Matthew 30, 176, 186
Lee, Bukyu 220, B-15, B-16
Lee, Eunhye 300
Lee, Sang Jin 190, 200, 220, 408, 484, B-5, B-15, B-16
Lee, W.P. Andrew ..... xiv, 132, 137, B-4, B-6, B-14, B-17
Lewis, Julia 138, 140
Li, Mei 210, B-11
Li, Yong 308, B-7
Li, Zhongyu John 78
Lin, Angela 106, B-14
Lin, Brian 88
Lin, Fubao 340, B-2, B-11, B-13
Lin, YenChih 78, B-5
Longaker, Michael T. ..... ix, 60, 252, 272, A-3, B-4, B-6, B-14, B-15, B-17
Losee, Joseph E. 132, B-14
Luangphakdy, Viviane 30, B-11, B-12
Ludunge, Kathi Mujynya 390
Machingal, Masood A. 200, B-5, B-16
Macri, Lauren 322, 333, 362, A-2, B-2, B-11, B-13
Madajka, Maria 100, 216, B-1, B-2, B-11
Magno, Hanshella 176, B-2, B-9, B-11
Marcus, Bob 186
Markert, Chad D. 400, 404, 405, B-16
Marra, Kacey 78, 190, B-4, B-5, C-3
Masand, Shirley 96
Matejuk, Agata 216, B-9, B-10
McAtee, Jedidiah 78, B-5
McBride, Sean 176, B-2, B-11

McFadden, Jake 210
McLaughlin, Peggy....314
Megley, Katie....88
Mendiola, Amanda 100, B-1, B-2, B-3, B-11
Merriam, Aaron....72
Merten, Rose....438, 452
Mhashilkar, Abner 408
Mikos, Antonios G. 156, A-2, B-5, C-2
Minteer, Danielle 78
Molnar, Joseph 346, 426, 428
Mozingo, David.... 426
Murthy, Sanjeeva 21, 210, B-3
Muschler, George ....ix, 30, 36, 42, 110, B-11, B-12, C-1
Mustoe, Thomas....249, 260, B-10, B-11
Mwizerwa, Olive....210
Myles, Jon 240
Natesan, Shanmugasundaram....420, B-8, B-18, B-19, B-20, B-21, C-3
Nectow, Alexander 78
Neville, Craig 210, 226, B-11
Nolan, Ryan....78
Noorani, Sabrina....164
Onishi, Shinsuke.... 164, B-16
Oro, Anthony.... 272
Ortiz, Ophir....30, 149, 176, B-11
Over, Patrick....368, B-14
Oyster, Nick 433, 438, B-14, B-16
Pace, Lauren....78
Palmieri, Tina.... 426
Pan, Hui 30, B-7, B-11, B-12
Paniliatis, Bruce....190
Park, Sang-Hyug....190
Poddar, Minakshi....438, B-14, B-16
Pomerantseva, Irina....226, 232, B-4, B-11
Poranki, Deepika....346, B-16
Prieto, Margarita 172, C-2
Probasco, Mitch....120
Ramachandran, Niraj 358
Ramadan, Mohamed 286
Ramadan, Mostafa 78, B-5
Rampazzo, Antonio.....216, B-1, B-3, B-9, B-11, B-12
Ramsay, Sarah.... 390
Randolph, Mark....226, B-4, B-12
Rask, Kenneth 210, 226, 232
Rathbone, Chris.....462, B-6, B-7, B-8, B-18, B-19, B-20, B-21
Ratliff, Catherine.... 322
Raut, Vivek.... 42, B-12
Reddy, Chandan 96
Reese, Jane 241, 322
Reing, Janet....20, 112, B-4
Rivera, Jaime.... 42, B-12
Roberts, Olga.... 346
Rolland, Eric.... 390
Ronfard, Vincent.... 390
Rousseau, Cecile 396
Roy, Abhijit.... 164, B-16, C-2
Rubin, Peter ....ix, 25, 190, B-14
Rui, Jing....96, B-12, B-13
Runge, Brett.....30, 96, 138, 176, B-2, B-3, B-4, B-12, B-13
Ruoslahti, Erkki ... 126, 300, A-3, B-5, B-6, B-15, C-2
Russell, Alan....308, A-3
Rutten, Michael....452, B-5
Sacks, Justin M. 132
Sahoo, Sambit 68, B-1
Saini, Shaili 186, B-11, B-12
Sarac, Timur Paul 64, A-2
Satirraju, Malika 64

Satish, Latha 278, B-4, B-5
Sava, Parid 396
Scarpinato, Paul 408, B-15
Schachner, Melitta 96
Schmalz, Deborah 186
Schneeberger, Stefan 132, B-14, B-17
Schrayer, Howard 186
Segal, Michael 396
Segovis, Suzanne 30, 96, 138
Sengupta, Srikumar 120
Sfeir, Charles 164, B-16, C-2
Shang, Hulan 268, B-1
Sheihet, Larisa 362, B-11
Shi, Meng 156, A-3, B-5, C-2
Shinohara, Kentaro 30, B-11, B-12
Shreiber, David 96
Siemionow, Maria 100, 216, 238, 240, A-2, A-3, B-1, B-2, B-3, B-9, B-10, B-11, B-12, B-13
Singer, Adam 340, 345, 358, 362, B-1, B-10, B-11, B-12, B-13
Sirvinsoot, Sirinrath 54
Sivak, Wesley 78, B-5
Smith, Cheryl 238, 240
Smith, David 426
Smith, Howard 426
Smith, Tom 78
Soh, H. Tom 126, A-3
Soker, Shay 438, 490, B-16, B-17
Sommerfeld, Sven 48
Sood, Rajiv 426, 428
Sorrell, Michael 354, B-3
Spicer, Patrick 156, A-3, B-5, C-2
Spinner, Robert 96, 138, B-1, B-2, B-12, B-13
Srinivas, Titte 240
Stewart, Ron 112, 120
Stockdale, Linda 30, 42, B-11, B-12
Suh, Won H. 88
Sundback, Cathryn ..... x, 210, 226, A-3, B-4, B-11, B-13
Supp, Dorothy 416, B-1, B-2, B-3, B-9
Sweeney, Paige 396
Szopinski, Jacek 216
Takanari, Keisuke 466, B-4, B-17
Tan, Josh 382, B-14
Taylor, Robert 106
Teach, Jeff 452
Thomson, James 120, A-3
Tirrell, David 376, B-4
Tirrell, Matthew 88
Tottey, Stephen 112, B-4, C-2
Tseng, Alan 226
Tuden, Mark 396
Tupaj, Marie 78, B-5
Turner, Neill 112
Tzou, Phoebe 376
Uhrig, Brent 25, 106, B-4, B-14, B-17
Vacanti, Joseph ..... x, 210, 226, B-4
Van Dyke, Mark 78, 346, 347, B-14, B-16
Viswanathan, Nickesh 88
Vracar-Grabar, Marina 260, B-11
Wagner, William R. 466, A-3, B-4, B-14, B-17, C-2
Walters, Thomas J. 202, 462, B-5, B-6, B-7, B-8, B-18, B-20, B-21
Wang, Huan 96, 138, B-2, B-12, B-13
Wang, Jinpeng 126, B-6
Wang, Xianyan 396
Wang, Zhan 438
Ward, Catherine 54, B-14
Ward, Donna 190
Washburn, Newell 286, A-3, B-6, B-8, B-21

Wenke, Joseph C. 39, 172, 462, B-6, B-7, B-8, B-18, B-19, B-20, B-21, C-3
Wiggins, Michal 64
Willett, Nick 106, B-17
Wimmers, Eric 132, B-17
Windebank, Anthony ..... xiv, 96, 138, B-1, B-2, B-3, B-12, B-13
Witt, Michelle 10, 438, B-14
Wolf, Matt 476
Wong, Mark E. 156, A-3, B-5, C-2
Wong, Matthew 396
Wrice, Nicole 3, 420, B-8, B-20
Wu, Ping-Cheng 452
Wu, Xiaowu 462, B-18, B-21
Xiao, Yi 126
Xie, Hua 452
Xu, Weijie 190, 220
Yang, Ning 268
Yang, Tianbing 292, B-17
Yaszemski, Michael ..... ix, xiv, 30, 96, 138, B-1, B-2, B-3, B-4, B-11, B-12, B-13, C-2
Yesilalan, Esin 396
Yoo, James ..... xi, 190, 200, 220, 382, 408, 484, B-5, B13, B-14, B-15, B-16, B-18
Young, Matthew 368, B-14
Young, Randall 354
Young, Simon 156, A-3, B-5, C-2
Yu, Patrick 396
Zborowski, Maciej 36
Zepeda, John 314
Zhang, Li 112
Zhang, Shuya 96
Zhao, Weixin 382, B-5, B-14, B-16
Zheng, Bo 452
Zheng, Xin Xiao 132, B-17
Zienkiewiczm, Kasia 172

Donald Lindorfer of Rutgers, The State University of New Jersey is acknowledged for his work with the people-based RCCC images.

Steven Galvan, Public Affairs Officer, U.S. Army Institute of Surgical Research, is acknowledged for his work with the people-based USAISR images.

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