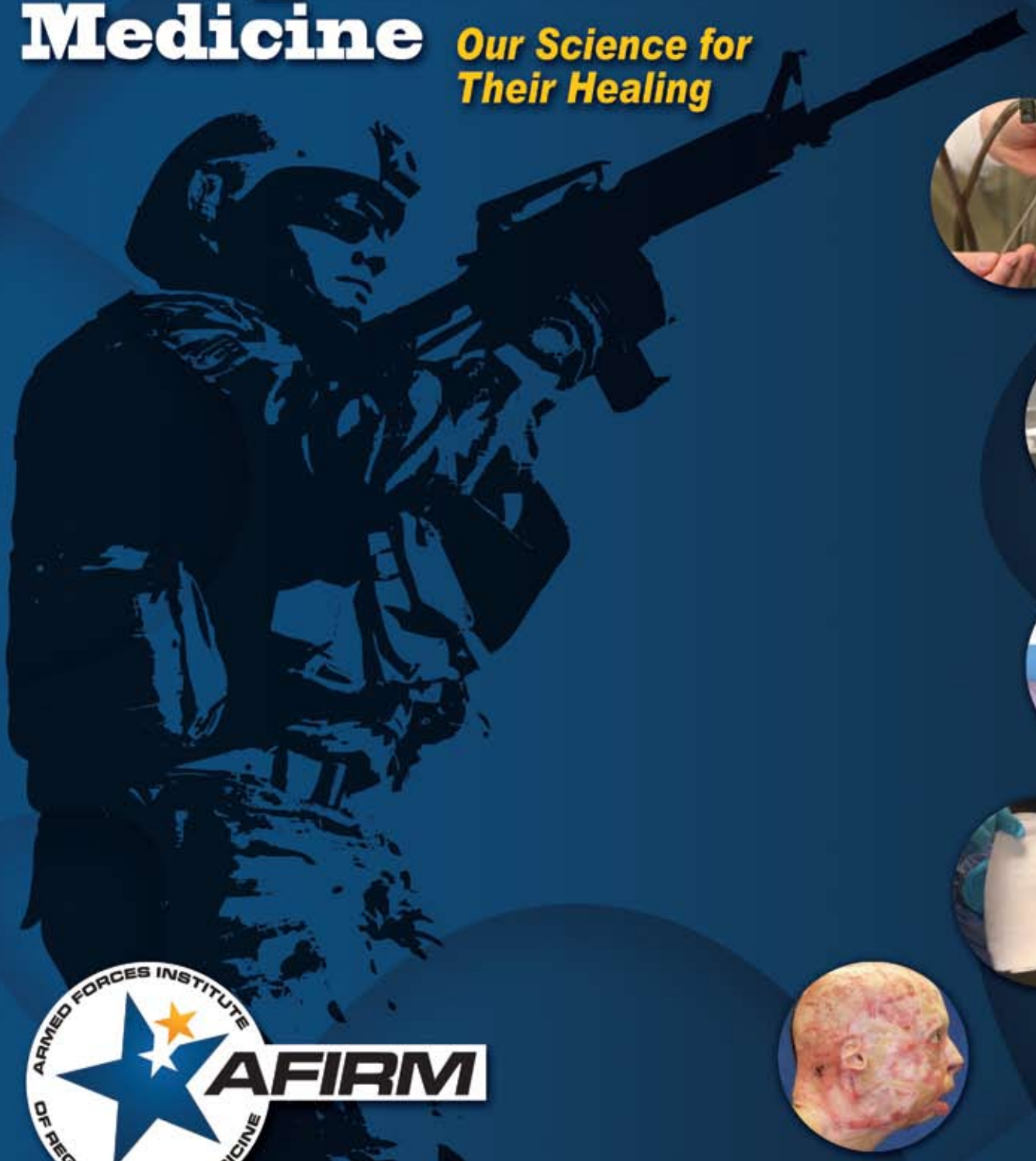
Annual Report 2009

Armed Forces Institute of Regenerative Medicine

Our Science for Their Healing



AFIRM

ARMED FORCES INSTITUTE OF REGENERATIVE MEDICINE

Armed Forces Institute of Regenerative Medicine

Annual Report 2009

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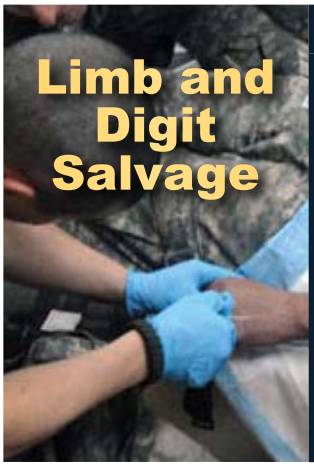
Executive Summary

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 usually 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 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, bone 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 with the mission of developing new products and therapies to treat severe injuries suffered by U.S. service members. This multi-institutional, interdisciplinary network of scientists has been designed to accelerate the delivery of regenerative medicine therapies for severely injured U.S. service members. Centered around well-established, proven research investigators, the AFIRM has been able to expand the rehabilitative medicine knowledge base, develop models of injury, and test advanced technology products.

Executive Summary



The Limb and Digit Salvage Program seeks to develop novel solutions using regenerative medicine that will allow victims of severe military or civilian trauma to recover more efficiently and reliably from their injuries and retain their limbs as they return to productive life.

A total of 19 projects were funded in Year 1. Projects span the following clinical challenge areas: Bone, Soft Tissue, and Nerve Repair/Regeneration; Composite Tissue Injury Repair; Transplantation; and Epimorphic Regeneration.

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. The AFIRM is a five-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. The AFIRM is composed of two independent research consortia working with the U.S. Army Institute of Surgical Research. One consortium is led by the Wake Forest Institute for Regenerative Medicine and the McGowan Institute for Regenerative Medicine in Pittsburgh (WFPC) while the other is led by Rutgers – the State University of New Jersey and the Cleveland Clinic (RCCC). Each consortium contains approximately 15 member organizations, which are mostly academic institutions.

Research activities are organized into five program areas: Limb and Digit Salvage, Craniofacial Reconstruction, Scarless Wound Healing, Burn Repair, and Compartment Syndrome. Nearly 60 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 among scientists working on different AFIRM projects is found in the Burn Repair Program. Dr. Arnold Caplan and colleagues at Case Western Reserve University are developing bone marrow-derived mesenchymal stem cells. They are testing the therapeutic effects of these cells in several AFIRM-funded models, including Dr. Richard Clark's rat hot comb model for burn injury progression at Stony Brook University, Dr. Steven Boyce's engineered skin substitute at the University of Cincinnati, and Dr. Thomas Mustoe's rabbit ear scarring model at Northwestern University.

First Year Research Highlights

Although the program has only recently entered its second year of funding, research efforts have already yielded a substantial number of noteworthy accomplishments. Dr. Maria Siemionow and colleagues at the Cleveland Clinic (Craniofacial Reconstruction Program) demonstrated the clinical feasibility of reconstructing tissue loss in the face following severe trauma by completing the first near-total face transplant in a civilian patient in the United States. Drs. William Wagner and Johnny Huard at the Stem Cell Research Center in Pittsburgh (Compartment Syndrome Program) created an abdominal wall defect model in the rat for the assessment of biodegradable scaffolds they are

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 11 projects were funded in Year 1. Projects span the following clinical challenge areas: Bone and Soft Tissue Regeneration, Skeletal Muscle and Nerve Replacement, Cartilage Regeneration (with a focus on the ear), and Virtual Modeling.

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 7 projects were funded in Year 1. Projects span the following clinical challenge areas: Control of Wound Environment Mechanics, Therapeutic Molecular/Gene Delivery to Wounds, Attenuation of Wound Inflammatory Response, and Scar Mitigation.

developing for treating compartment syndrome. Dr. W.P. Andrew Lee and colleagues at the University of Pittsburgh (Limb and Digit Salvage Program) performed hand transplantation on a former Marine who lost his hand in a training accident while on active duty. The patient is now, at 4 months after the transplant, on a minimal immunosuppression regimen without any adverse side effects.

Engineered skin substitutes, which consist of various types of skin cells attached to a collagen-based matrix, are being developed and tested clinically as an adjunctive treatment for burn repair. Drs. Steven Boyce and Dorothy Supp at the University of Cincinnati (Burn Repair Program) established advanced engineered skin substitute models with skin pigmentation and a supply of blood vessels. Drs. Geoffrey Gurtner, Michael Longaker, and Anthony Oro at Stanford University (Scarless Wound Healing Program) capitalized on the ability of wounded fetal tissue to regenerate with minimal scarring by developing a regenerative bandage containing a fetal-like matrix and stem cells derived from human amniotic fluid. They are refining this bandage so that it will maintain an acute wound in a pro-regenerative state and prevent the onset of scarring, fibrosis, and infection.

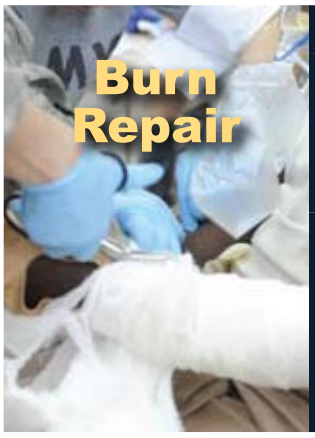
The projects highlighted in the preceding paragraphs are just a few examples of a long list of research developments and successes resulting from AFIRM-funded laboratories over the past year.

First Year Program Highlights

The AFIRM involves the efforts of nearly 300 individuals, including faculty members, postdoctoral fellows, graduate students, scientific and technical staff, and undergraduate students. AFIRM faculty members are highly accomplished scientists—over the first year of the program, 36 awards/honors were conferred upon AFIRM faculty, including awards from private foundations, selection to membership or leadership positions in professional societies, honorary degrees from academic institutions, and awards for distinguished careers from government agencies. Their AFIRM-related research efforts have substantially contributed to the scientific literature—over the first year of the program, they published 59 articles in peer-reviewed journals and produced 118 presentations and non-peer-reviewed publications. AFIRM scientists have also been making novel patentable discoveries in the field of regenerative medicine—over the first year of the program, they filed 27 invention disclosures, of which 6 have resulted in government patent applications filed.

From the Laboratory to the Battlefield

AFIRM-funded researchers share a strong commitment to developing commercial products and bringing therapies to wounded warriors and the civilian sector as quickly as possible. The ultimate goal of AFIRM-

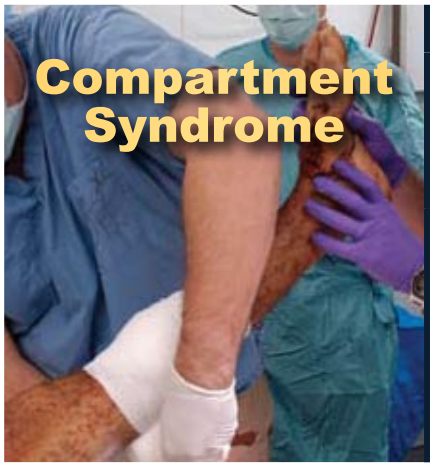


The Burn Repair Program seeks to design innovative regenerative medicine therapies for victims of severe military or civilian trauma so they can recover from their injuries with improved function and aesthetics.

A total of 15 projects were funded in Year 1. 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.

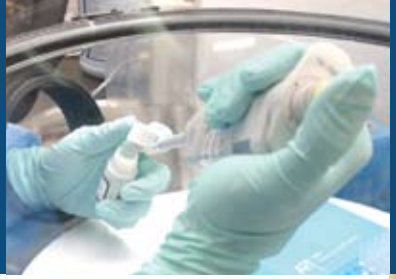
funded projects is the conduct of clinical trials; in fact, many AFIRM researchers anticipate the commencement of clinical trials within the next few years. For example, Dr. Richard A.F. Clark and colleagues of Stony Brook University have determined that fibronectin peptide P12 and curcumin (found in the spice turmeric) can significantly inhibit burn injury progression. They have scaled up the good manufacturing practice-compliant production of both P12 and curcumin. Once proof of principle is obtained, the researchers plan to file for orphan drug status with the U.S. Food and Drug Administration (FDA). They anticipate the initiation of Phase 1 clinical trials as soon as Year 3 in the AFIRM program.

Another example of a project headed toward a clinical trial is found in the Craniofacial Reconstruction Program. Dr. David Kaplan of Wake Forest University and Dr. J. Peter Rubin of the University of Pittsburgh have combined their expertise to develop four types of silk scaffolds that support the growth of soft tissue and have the potential to be used as either a stand-alone therapy or integrated with composite tissue regenerative medicine therapy of burns, craniofacial injuries, or extremity injuries. Drs. Kaplan and Rubin have recently obtained FDA approval for the silk-based biomaterial used in these studies. Continued effort on the project will focus on the rapid advancement toward clinical trials.



The Compartment Syndrome (CS) Program seeks to prevent or reverse secondary damages resulting from trauma so that repair and regeneration of wounded tissue are enhanced, and healing and return to functionality are improved.

A total of 7 projects were funded in Year 1. Projects span the following clinical challenge areas: Prevention/Early Treatment of CS, Cellular Therapy of CS, and Biological Scaffold-Based Treatment of CS.



Introduction

Background

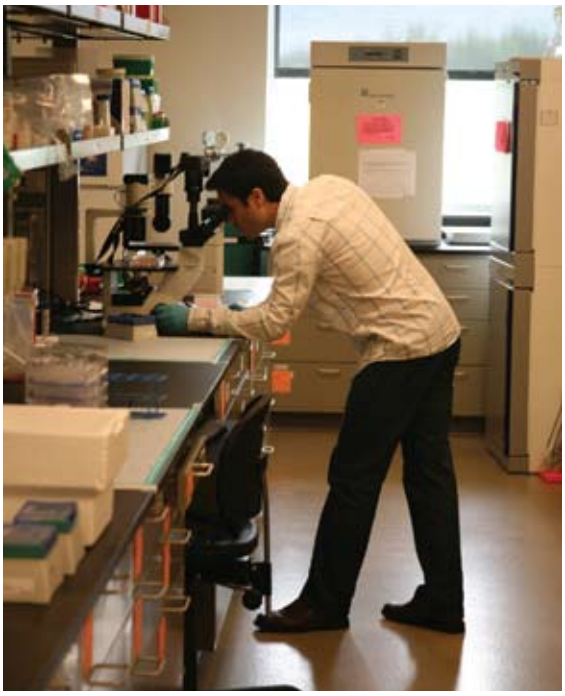
The wars in Iraq and Afghanistan have resulted in more than 5,000 U.S. military fatalities and more than 34,000 injuries.[1] Treatment of combat-related injury and trauma is particularly complex. Advances in body armor have substantially improved protection of the torso, which contains the vital organs. In addition, evacuations from the battlefield have become faster, and medical care has advanced. Due to all of these factors, survivability has increased. However, those who survive often have seriously debilitating injuries. Conventional weapons and the destructive force of improvised explosive devices ravage the face, neck, head, and limbs, causing massive trauma and tissue loss. According to the *Journal of Orthopaedic Trauma*, the use of improvised explosive devices in Operation Iraqi Freedom/ Operation Enduring Freedom has led to a substantial increase in severe blast trauma, which is now responsible for approximately 75% of all combat-related injuries.

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

[1] http://www.defenselink.mil/news/casualty.pdf (July 2009)

Research Goals

The Armed Forces Institute of Regenerative Medicine (AFIRM) is a multi-institutional, interdisciplinary network focused on developing advanced treatment options for severely wounded warfighters. The AFIRM is designed to speed the delivery of regenerative medicine therapies to treat the most severely injured U.S. service members from around the world. It is anticipated that the AFIRM will be able to translate many of its technologies to patients within the next 4 years.



Rutgers technician Jonathan Branch at the fluorescence microscope at the New Jersey Center for Biomaterials.

The AFIRM has five major research programs:

Limb and Digit Salvage

Saving the limb, also referred to as "limb salvage," at minimum requires (1) bridging large bony defects to reestablish a strong connection and mobility along the entire limb, (2) bridging soft tissues, such as muscle, nerves, tendons, and ligaments, to lend stability and enable movement, and (3) healthy skin to cover the injured area to provide a durable barrier to infection. This AFIRM program is dedicated to developing regenerative medicine therapies to help save and rebuild injured limbs. The program focuses on using new technologies in regenerative medicine and tissue engineering to provide surgeons with advanced tools and new options for repair and regeneration of these critical tissues. The goal is to allow victims of severe military or civilian trauma to be able to recover from their injuries more rapidly, more reliably, and also retain their limbs as they return to productive life.

Craniofacial Reconstruction

Massive bone and tissue loss to the face and head is a large problem of blast injuries for our warfighters. This complex area involves multiple levels and tissue types, which require different strategies for repair. The AFIRM Craniofacial Reconstruction Program designs, develops, and provides the treating surgeon with therapies for the wounded warfighter that will (1) regenerate bone form and function to calvarial-, upper- and mid-facial anatomies and the mandible, (2) restore sensate and motor competencies through muscle 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 drugs.

Scarless Wound Healing

Military trauma creates not only large wounds but also large scars. These scars are often very visible and can often draw unwanted attention to the wounded warrior. In some instances the scars become so thick that they can limit movement of joints and greatly restrict the patient's ability to move. This AFIRM program seeks to find new treatment options to prevent and manage scars.

Burn Repair

Burn injuries afflict both warfighters and civilians. Although recent advances in critical care and resuscitation have helped, there is still high morbidity and mortality associated with burns. Current treatment options include the administration of antibiotics and tissue excision for deeper burns, which are then replaced with skin grafts. The AFIRM Burn Repair Program is tackling the issues associated with these methods and using regenerative medicine to (1) prevent wound infection, (2) prevent burn inflammation and injury extension, (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. The overall goal of the program is to

allow victims of severe military or civilian trauma to be able to recover from their injuries more rapidly, more reliably, and with improved function and aesthetics.

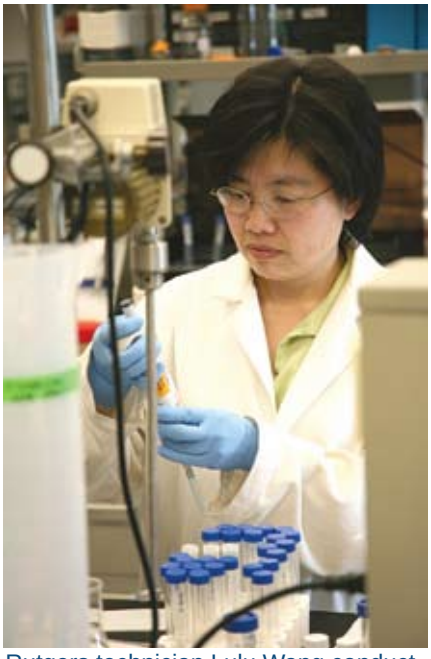
Compartment Syndrome
Compartment syndrome is often a secondary sequelae resultant from blast injuries, severe blunt or penetrating trauma, fractures, and vascular injuries. Muscles are encased in compartments of nonyielding tissue called fascia. Bleeding within a muscle compartment raises the pressure in the compartment which, if unchecked, can become high enough that blood flow into the compartment is reduced or completely stopped, which can destroy the nerves and muscles within the compartment. Once compartment syndrome is diagnosed, the only current treatment option is fasciotomy, where the pressure is released from the "compartment" by cutting open the surrounding fascia. This must be done within hours of onset to ensure effective treatment. Fasciotomy leaves an open wound, which is susceptible to infection and added complications. It is therefore imperative that new methods are developed for compartment syndrome detection and monitoring during triage of the critically wounded. This AFIRM program focuses on attacking the problem of compartment syndrome among military personnel and in instances of severely injured civilians through regenerative medicine therapies that can immediately improve patient outcomes. The goal is to prevent or reverse the secondary damages resulting from trauma so that repair and regeneration of wounded tissue are enhanced, and healing and return to functionality are improved.

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.



Rutgers technician Lulu Wang conducting drug delivery assays at the New Jersey Center for Biomaterials.

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 additional funding from the following five organizations:

- 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 $250M:

- $100M from U.S. Government funding (Army, Navy, Air Force, VA, and NIH).
- $68M 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.

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 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 the Wake Forest Institute for Regenerative Medicine and the McGowan Institute for Regenerative Medicine in Pittsburgh, and the other is led by Rutgers, the State University of New Jersey, and the Cleveland Clinic. Each of these civilian consortia is itself a multi-institutional network, as shown in the following.

Rutgers-Cleveland Clinic Consortium (RCCC)

The RCCC is directed by Joachim Kohn, PhD, Professor of Chemistry and Chemical Biology in Rutgers' School of Arts and Sciences, and co-directed by George Muschler, MD, an orthopedic surgeon at the Cleveland Clinic.

The RCCC consists of the following member institutions:

- Rutgers/New Jersey Center for Biomaterials
- Cleveland Clinic Foundation
- Carnegie Mellon University
- Case Western Reserve University
- Dartmouth Hitchcock Medical Center
- Massachusetts General Hospital/ Harvard Medical School
- Massachusetts Institute of Technology
- Mayo Clinic College of Medicine
- Northwestern University
- State University of New York at Stony Brook
- University of Cincinnati
- University of Medicine and Dentistry of New Jersey
- University of Pennsylvania
- University of Utah
- University of Virginia
- Vanderbilt University

Wake Forest-Pittsburgh Consortium (WFPC)

The 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 Alan Russell, PhD, Director of the McGowan Institute for Regenerative Medicine and Professor of Surgery at the University of Pittsburgh.

The WFPC consists of the following member institutions:

- The Wake Forest Institute for Regenerative Medicine
- The McGowan Institute for Regenerative medicine (University of Pittsburgh)

- Allegheny Singer Research Institute
- Carnegie Mellon University
- Georgia Tech University
- Institute for Collaborative Biotechnology (includes University of California, Santa Barbara; Massachusetts Institute of Technology; and California Institute of Technology)
- Oregon Medical Laser Center
- Stanford University
- Rice University
- Tufts University
- University of Texas Health Science Center at Houston
- Vanderbilt University

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.

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, the intramural researchers can serve as project leaders.

Management and Oversight

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 contains flag-level representatives from the Army, Navy, Air Force, NIH, VA, Office of the Assistant Secretary of Defense for Health Affairs, 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 Integrated Project Team and the Program Synergy Group.

Integrated Project Team
The AFIRM's IPT is chaired by the Director of the Clinical and Rehabilitative Medicine Research Program (CRMRP). IPT membership consists of a group of experts that 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.

Dr. Joerg Gerlach under the laminar flow hood spraying skin cells into dishes via the skin spray device in a WFPC laboratory.

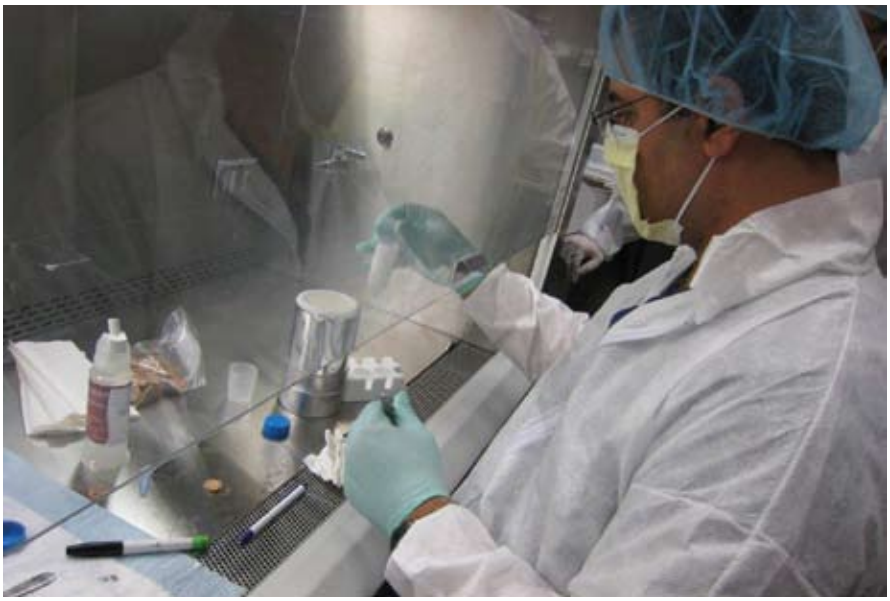
The specific responsibilities of the IPT are to:

- 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.
- 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 allocations and 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 contains 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 contains ex officio advisors from the Judge Advocate General, the DoD Human Use office, a commercialization expert, and a regulatory expert appointed by the CRMRP.



Dr. Burhan Gharaibeh of WFPC's Stem Cell Research Center freezing muscle tissue obtained from a compartment syndrome rat.

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.

Limb and Digit Salvage

Background

Injuries to arms and legs following blast injuries and severe civilian 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. Presently, when preservation, repair, or regeneration of these "bridging" tissues cannot be reliably achieved, an amputation of the arm or leg below the injured area becomes the best and only option to allow an individual to return to function. The AFIRM is dedicated to developing new regenerative medicine therapies for helping save and rebuild injured limbs and digits.

The AFIRM Limb and Digit Salvage Program focuses on using new technologies in regenerative medicine and tissue engineering to provide surgeons with advanced tools and new options for repair and regeneration of these critical bridging tissues. The researchers funded by this program are working toward increased limb salvage, or limb replacement when salvage is not possible, to improve the outcomes for warriors facing these overwhelming injuries. The goal is to increase the return to duty, or at least the return to functional, independent lives.

Rutgers-Cleveland Clinic Consortium (RCCC) researchers are pursuing tissue-engineering solutions for bone, nerve, vessels, fascia, meniscus, and skeletal muscle. In collaboration with RCCC researchers in the AFIRM's Craniofacial Reconstruction Program, they are working toward immunomodulatory solutions for composite tissue allografts. They are also collaborating with RCCC investigators in the AFIRM's Burn Repair Program to advance skin and soft tissue coverage solutions for these extensive wounds.

The Limb and Digit Salvage Program at the Wake Forest-Pittsburgh Consortium (WFPC) consists of seven independent but conceptually related projects involving an interdisciplinary, multipronged approach to the reconstruction/replacement of functional limb and digit tissue. Approaches being pursued

include transplantation, epimorphic (blastema-like) regeneration (i.e., causing the limb to regrow similarly to how amphibians regenerate severed limbs), and a component approach that involves the development of methods to reconstruct blood vessels, nerves, and muscle tissue separately in a common animal (rat) model with the eventual convergence of the projects. Notably, many of the studies in WFPC's Limb and Digit Salvage Program have substantial synergistic overlap with the studies pursued in WFPC's AFIRM-funded Compartment Syndrome Program.

Unmet Needs

Due to changes in the nature of the present wars and in modern battlefield medicine, many wounded warfighters now survive to face the challenge of overcoming severe limb, craniofacial, and burn injuries that in the past would have led to battlefield mortality. These injuries often involve a massive loss of tissue, including large defects in the continuity of bone, nerve, muscle, tendon, ligament, soft tissue, and skin. Musculoskeletal injuries represent 70% of all combat injuries and extremities are involved in 55% of them. Fractures account for 26% of all injuries and are frequently comminuted, complicated by extensive soft tissue loss, and even segmental bone loss of up to 20 centimeters.

These severe wounds are also frequently associated with compartment syndrome, burns, local infection, vascular or neural compromise, and scarring. Extensive tissue loss, whether of bone, peripheral nerve, or soft tissue, often results in the loss of functional kinetic control of the extremity and a higher risk of amputation. The limb may be salvageable but with the loss of one type of tissue, and current surgical techniques and materials for reconstruction are not perfectly reliable. Better techniques for providing interpositional tissue for large segment tissue loss is a critical unmet need for injuries involving bone and soft tissue (including arteries and nerves).

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. A critical need therefore exists to improve immunomodulation techniques to reduce the obstacles to composite tissue transplantation.

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 six "clinical challenge" topic areas: Bone Repair and Regeneration, Soft Tissue Repair and Regeneration (excluding nerve), Nerve Repair and Regeneration, Composite Tissue Injury Repair, Transplantation, 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.

Asa Vaughan, PhD, a postdoctoral fellow in the Kohn laboratory (RCCC), holding an extruded polycarbonate bone pin.

Bone Repair and Regeneration

Studies at RCCC

Overview: RCCC researchers aim to provide injured warriors with one or more new clinical methods that significantly improve the rate and reliability of regeneration of large traumatic bone defects. The RCCC strategy in bone regeneration is being implemented in a single accelerated and integrated collaborative program that is composed of four parallel, synergistic, and interdependent projects. The combined Bone Team representing laboratories at the Mayo Clinic, Massachusetts Institute of Technology (MIT), Rutgers, Cleveland Clinic, Vanderbilt University, Carnegie Mellon University, and collaborators at Therics, Inc., meets monthly in a formal teleconference and webinar session to review data, resolve questions, develop consensus, and integrate collaborative activities.

In Project 4.2.1, the researchers seek to identify and develop the most promising available bone-producing three-dimensional bone scaffolds made from degradable polymers. In Project 4.2.2, the researchers are optimizing methods for selecting and concentrating bone marrow cells capable of forming new bone. In Project 4.2.3, the research team is seeking to enhance the performance of bone-generating

Table II-1. AFIRM-funded projects per clinical challenge topic area.

Clinical Challenge Topic Area	Consortium	Project Number	Project Title
Bone Repair and Regeneration	RCCC	4.2.1	Optimizing Scaffolds for Repair of Bone Defects
		4.2.2	Optimizing Cell Sources for Repair of Bone Defects
		4.2.3	Advancing Bone Repair Using Molecular Surface Design
		4.2.4	Clinical Assessment of Ongoing Strategies for Treatment of Bone Defects
Soft Tissue Repair and Regeneration (excluding nerve)	RCCC	4.3.2	Development of Tissue-Lined Bioabsorbable Stent Graft for Treatment of Arterial Trauma
		4.4.3a	Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents
		4.4.3b	Functional Scaffolds for Soft Tissue Repair
	WFPC	4.4.6	Oxygen-Generating Biomaterials for Engineering Large Tissue Survival
Nerve Repair and Regeneration	RCCC	4.4.1/4.4.2	Optimizing Nerve Conduit Scaffolds for the Repair of Segmental Nerve Defects/Cell and Bioactive Molecular Delivery to Enhance the Repair of Segmental Nerve Defects
		4.4.1a	Developing Biodegradable Polyester "Biorubber" Nerve Regeneration Conduits for Repair of Segmental Nerve Defects
		4.4.1b	Developing Biodegradable Entubulation Grafts for Repair of Segmental Nerve Defects
		4.4.2a	Developing Epineural Conduits Filled with Bone Marrow Mesenchymal Stem Cells for Repair of Segmental Nerve Defects
	WFPC	4.4.4	Peripheral Nerve Repair
		4.4.5	Modular, Switchable, Synthetic Extracellular Matrices for Regenerative Medicine
Composite Tissue Injury Repair	WFPC	4.4.3	Engineered Delivery of Cues for Composite Tissue Injury Repair
Transplantation	WFPC	4.4.2	Hand Transplantation for Reconstruction of Disabling Upper Limb Battlefield Trauma – Translational and Clinical Trials
Epimorphic Regeneration (and associated methods)	WFPC	4.4.1	Blastemal Approach to Digit Reconstruction
		4.4.7	High-Throughput Technologies to Study Limb Regeneration
		4.4.8	Magnetophoretic Cell Sorting for Transplant Therapies

scaffolds developed in Project 4.2.1 using a technique known as molecular surface design (MSD), which involves linking bioactive molecules to the surface of a scaffold as a means of improving control over the cell and tissue response following implantation. Project 4.2.4 represents the proactive organizational arm of the RCCC Bone Program. A major focus of this project is the development of a clinical trial network in collaboration with other institutions, including the National Institutes of Health (NIH), U.S. Army Institute of Surgical Research (USAISR), and Orthopaedic Extremity Trauma Research Program (OETRP).

Status at End of Year 1: The research team of Project 4.2.1 identified and created four distinct families of biodegradable, copolymer-based bone-generating scaffolds for testing in animal models. The researchers found their polyester and tyrosine-based copolymer scaffolds to be superior to the others in the canine thigh defect model. The research team of Project 4.2.2 defined methods to increase the surgical yield of bone marrow harvest procedures without an increase in morbidity. The researchers made substantial progress in characterizing three alternative, practical methods to allow the processing of human or canine bone marrow samples: density separation, selective retention, and magnetic separation. In Project 4.2.3, the researchers used MSD to generate two-dimensional scaffolds containing proteins that enhance new bone tissue formation. Project 4.2.4's organizational team collaborated with Drs. Andy Polack and Michael Bosse to develop the Trauma Clinical Trial Network. The researchers also presented research results at a variety of symposia.

Research Plans for the Next 4 Years: In Year 2, the integrated RCCC Bone Regeneration Projects will complete the evaluation and comparison of highly promising scaffolds in animal models. The researchers anticipate advancing at least one scaffold from this initial assessment toward clinical trials. The merger of Projects 4.2.1 and 4.2.2 is on an accelerated track, and the researchers feel that the Year 3 goals initially proposed for these projects may be achieved by the end of Year 2. In Year 2, the researchers of Project 4.2.3 will modify scaffolds from Project 4.2.1 to present MSDs and begin testing the scaffolds in two-dimensional and three-dimensional constructs. They will advance selected surfaces to testing in animal models in Year 3, merging with Projects 4.2.1 and 4.2.2. Optimal scaffold materials and optimized cell-sourcing options will be advanced into more challenging bone defect models in Year 3, which is a year ahead of the proposed schedule. By Year 4, highly effective combinations of scaffolds with or without cells or bioactive surfaces will be evaluated in the most rigorous and appropriate preclinical model.

Planned Clinical Transitions: The researchers plan to initiate discussions in Year 2 with the U.S. Food and Drug Administration (FDA) regarding the review pathway for the scaffolds under development. By Years 4–5, the researchers expect to have one or more bone regeneration therapeutic strategies that exceed the current preclinical performance of available materials ready to be advanced to clinical trials. Clinical trials will require the collaboration of a large clinical trial network of civilian and military trauma centers. The most likely setting in which a clinical trial will be performed will be in open tibia (shinbone) fractures having lost at least 50% of circumferential contact.

Soft Tissue Repair and Regeneration (excluding nerve)

Studies at RCCC

Overview: In Project 4.3.2, the researchers are seeking to develop a durable bioabsorbable stent graft with a tissue lining for the minimally invasive treatment of arterial and venous trauma. They anticipate that the bioabsorbable stent grafts will minimize surgical morbidity and eliminate the long-term risk of fatigue fracture and secondary complications associated with contem-

Jenny Raynor, PhD, a postdoctoral associate in Dr. Kohn's laboratory (RCCC), using the lyophilizer to form bone regeneration scaffolds.

porary metal stents. The research team of Project 4.4.3a is focused on fascia, which is a thin, fibrous, and strong connective tissue that surrounds, protects, and supports the body's muscles. The researchers are developing a fascia scaffold using a biodegradable polymer that provides a natural, strong, and mechanically robust platform for bridging tendon or muscle defects. In Project 4.4.3b, the researchers are developing a clinically relevant, tissue-engineered scaffold that can be implanted in tissue such as the meniscus (internal cartilage) of the knee to prevent the onset of degenerative osteoarthritis associated with removal of the meniscus following injury.

Status at End of Year 1: In Project 4.3.2, the researchers identified a bioabsorbable material, polydioxanone, which fulfills the essential requirements for fabrication of a bioabsorbable tissue-lined stent to treat traumatic arterial and venous injuries. In Project 4.4.3a, the researchers developed a mechanically robust, reinforced fascia using a customized, biodegradable polymer that significantly increased its suture retention strength to physiologically relevant loads. In Project 4.4.3b, the research team developed a novel meniscus scaffold consisting of high-strength resorbable tyrosine-derived polymeric fibers arranged within a collagen matrix and determined that it promotes the synthesis of new, organized tissue when implanted as a total meniscal replacement in sheep.

Research Plans for the Next 4 Years: The researchers of Project 4.3.2 will focus on constructing a bioabsorbable, self-expanding, tissue-lined stent. They will test the feasibility of various designs and fabrication methods. They will then focus on canine implants in iliac arteries. The goal is to test the newly developed bioabsorbable tissue-lined stent and compare it to a bare metal, self-expanding tissue-lined stent. In Project 4.4.3a, the researchers will seek to identify a preferred fiber to use in reinforcing their fascia scaffold. They will then undertake scaffold efficacy studies in rat and canine models and human cadaver model systems. During the next 4 years, they plan to concurrently develop their scaffold for military trauma applications, such as bridging large muscle/tendon deficits to bone, and for the repair of abdominal wall fascia that has been herniated or released in compartment syndromes. In Project 4.4.3b, the researchers plan to fabricate second-generation meniscus scaffolds by optimizing the reinforcing fiber network and modifying the collagen sponge component of the scaffold and to evaluate the scaffolds in sheep.

Planned Clinical Transitions: The researchers of Project 4.3.2 anticipate a shift to clinical trials by Years 4–5. They note that industry support will be required for the clinical trials; however, recent FDA decisions allowing historical controls will make these trials less burdensome than in the past. In Project 4.4.3a, the researchers plan to use their animal data and manufacturing test results to apply for FDA clearance through the 510(k) mechanism. They anticipate completing the efficacy studies in animals and submitting an application for FDA clearance by the end of Year 3. The project team anticipates that its scaffold will be advanced into clinical trials for rotator cuff repair by Years 4–5. The research team of Project 4.4.3b plans to consult with the FDA in a pre-investigational device exemption (IDE) meeting. Pending success of large animal studies, the project will transition to clinical trials.

As part of the development process, the effects of a novel oxygen-generating material are assessed in a controlled environment by WFPC researcher Catherine L. Ward.

Studies at WFPC

Overview: Lack of oxygen due to a disrupted blood supply is a key factor that limits tissue salvage, wound healing, and viability of large engineered tissue constructs. The researchers of Project 4.4.6 are creating an injectable oxygen-generating biomaterial that can provide a sustained release of oxygen to wounds while supporting blood vessel networks are established or repaired.

Status at End of Year 1: The researchers produced an injectable gel solution capable of oxygen generation. They determined that their oxygen-generating gel is nontoxic to cells. In addition, they demonstrated that oxygen production can be sustained for up to 3 days post-injection.

Research Plans for the Next 4 Years: During Year 2, the researchers will examine the effects of their oxygen-generating gels on cell survival and growth. They will also look for signs of angiogenesis (i.e., the ingrowth of blood vessels). The researchers will further optimize their oxygen-generating gels based on the results of these studies. During Years 3–5, in vivo testing of the oxygen-generating gels will be undertaken in rodents.

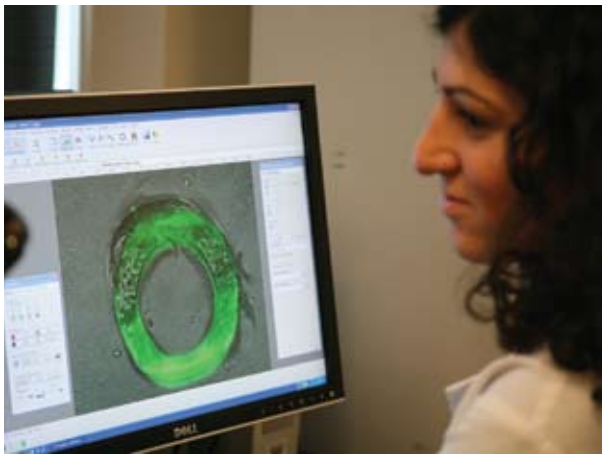
Nerve Repair and Regeneration

Studies at RCCC

Overview: RCCC will provide injured warriors with one or more new clinical methods that significantly improve the rate and reliability of regeneration of large traumatic nerve defects. The RCCC strategy in nerve regeneration represents one accelerated and integrated collaborative program that encompasses Projects 4.4.1/4.4.2, 4.4.1a, 4.4.1b, and 4.4.2a and builds on the synergistic collaboration of laboratories at the Cleveland Clinic (Siemionow), Mayo Clinic (Windebank and Yaszemski), MIT (Anderson and Langer), and Rutgers (Kohn). Novel scaffold materials are being developed and tested in three laboratories (Mayo Clinic, MIT, and Rutgers), cell-free allografts are being tested at the Cleveland Clinic, and mesenchymal stem cell (MSC) therapies are being developed at the Mayo Clinic. Each group has a history of materials development and testing and broad expertise and experience from polymer design through product deployment into clinical application. This expertise is now directed toward scaffold materials and design for peripheral nerve repair.

Status at End of Year 1: The research teams have made considerable progress over the past year toward their goals. The researchers of Project 4.4.1/4.4.2 effectively encapsulated and released proteins that stimulate the growth of neurons from polymer scaffolds and showed that the growth factors could enhance the regeneration of peripheral nerves. They also showed that MSCs provide a potential cell-based platform for growth factor delivery. In Project 4.4.1a, the researchers synthesized a family of new, biodegradable polyesters and fabricated a series of synthetic conduits for guiding nerve growth. In Project 4.4.1b, the researchers have designed a biodegradable nerve-guiding conduit with a filler containing collagen embedded with peptide growth factors. They demonstrated that this "functionalized" collagen conduit tube could improve recovery after large gap (5 mm) injuries to the femoral nerve in mice. In Project 4.4.2a, the research team implanted nerve-guiding conduits containing either bone marrow-derived mesenchymal stem cells or saline (control) into 2 cm nerve defects in the rat and determined that bone marrow-derived mesenchymal stem cell-filled conduits had faster functional recovery and increased myelin area, axon area, myelin thickness, and mean fiber diameter compared with the saline-filled conduits.

Research Plans for the Next 4 Years: Early in Year 2, the researchers of Projects 4.4.1/4.4.2, 4.4.1a, 4.4.1b, and 4.4.2a will meet to define a stage-gate process for selecting the best candidate poly-



Rutgers graduate student, Mindy Ezra, analyzing pore structure of nerve conduits and collagen gel filler located inside the inner lumen.

mers for nerve repair. This will involve establishing a single animal model for nerve conduit polymer testing that can be applicable within the consortium. A "screening" model will be the rat 1 cm sciatic nerve defect model. The evaluation time will be 8 weeks for neuromorphometry and 16 weeks for functional/behavioral tests. A secondary model for testing prime candidates will be the rat 2 cm sciatic nerve defect model. In Year 2, the researchers will extend the development of a complex wound model, which will include muscle ischemia/fibrosis, skin/muscle necrosis, segmental bone defect, and possibly infection. They have initiated the first phase, which involves the development of an ischemia/fibrotic nerve injury model.

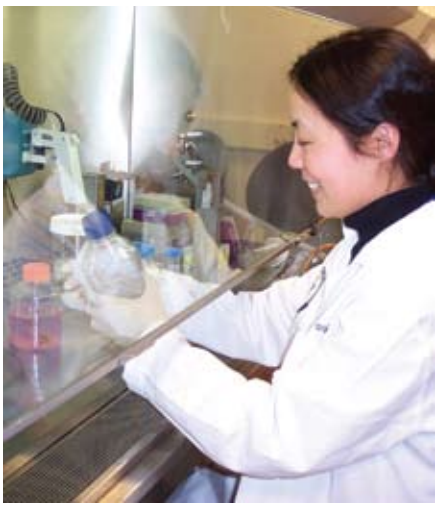
Planned Clinical Transitions: The research teams are pursuing two strategies to repair large segmental peripheral nerve defects: a transplantation strategy (human cadaver allograft nerve sheath with and without supplementation with isogenic or allogenic bone marrow stromal cells) and a polymer scaffold-based tissue engineering strategy. The transplantation strategy will be tested in the same model allowing objective comparisons to be made between materials. Clinical translation of each material may vary with respect to FDA interactions and requirements. If the allograft sheath plus saline or isogenic cell strategies are successful in a small number of animals in the tiered model systems, they will be immediately brought to clinical transition by the end of Year 2 using an adaptive human trial.

Studies at WFPC

Overview: Project 4.4.4 is a highly collaborative project among three laboratories at the forefront of peripheral nerve repair. All three have focused on a tissue-engineering approach for nerve repair over long gaps and now are combining their efforts in a synergistic manner. The researchers seek to develop a proactive biodegradable nerve guide system for peripheral nerve regeneration. The researchers of Project 4.4.5 hypothesize that additional filler materials within nerve guides are necessary to promote recovery of nerve function after injury. They are developing a scaffold based on synthetic peptide amphiphiles (PAs) that contains molecules with the ability to promote cell adhesion, migration, and nerve regrowth.

Status at End of Year 1: Besides establishing a collaborative, focused effort among the University of Pittsburgh, Tufts University, and Wake Forest University, the researchers of Project 4.4.4 developed a novel biodegradable conduit for nerve repair. Their data demonstrate improved functional recovery when damaged nerves are treated with a keratin gel-filled collagen conduit. The researchers of Project 4.4.5 have synthesized several PAs with controlled bioactive components that allow neural stem cells to attach, proliferate, and differentiate.

Research Plans for the Next 4 Years: The researchers of Project 4.4.4 will continue to test their novel nerve guides in animal models. After their rat studies are complete, they will move to a larger animal model (e.g., rabbit)



Ying Tang performing collagen coating in the tissue culture hood (WFPC).

and will enhance their interdisciplinary collaboration by further combining individual approaches (e.g., long-term drug delivery, novel silk-based conduits, and keratin gel fillers). The laboratory data being generated in Project 4.4.5 are being investigated for applicability to a rat model of sciatic nerve defect regeneration. The researchers will integrate their findings with other team members within the Limb and Digit Salvage Program to determine optimal ways in which to stimulate new tissue growth, resulting in eventual complete digit regeneration.

Planned Clinical Transitions: Clinical studies are expected to begin in Year 4 or 5 of Project 4.4.4. The researchers of Project 4.4.5 feel they are moving closer to a common animal model and eventual clinical application.

Composite Tissue Injury Repair

Studies at WFPC

Overview: In Project 4.4.3, the researchers seek to develop and test technologies that will enable the

restoration of limb function following composite tissue trauma. To meet this goal, they are developing animal models as well as testing the spatial and temporal delivery of cues that direct nerve, blood vessel, and bone growth in a synchronized manner.

Status at End of Year 1: The researchers of Project 4.4.3 have established promising regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and the sustained delivery of a protein known to induce bone growth. They completed a pilot surgery to create combined bone and nerve defects in a new composite injury rat model.

Research Plans for the Next 4 Years: The researchers of Project 4.4.3 aim to establish composite bone and vascular defect models and characterize functional regeneration. They will quantitatively evaluate the ability of a variety of agents known to promote nerve, blood vessel, or bone growth to restore function to limbs sustaining composite injuries. The researchers also plan to use their composite injury models to test promising limb regenerative strategies established by other researchers on the AFIRM team. The best of these approaches will then be transferred for evaluation in large animal models.

Planned Clinical Transitions: In Project 4.4.3, the researchers plan to pursue intellectual property protection to facilitate technology transfer to a biotechnology company interested in developing a clinical product for treating severe limb trauma.

Transplantation

Studies at WFPC

Overview: Composite tissue allografts (e.g., hand transplants) are now a clinical reality and have been performed in multiple centers worldwide. Apart from excellent and highly encouraging functional results, the procedure has not reached widespread clinical use because recipients require lifelong high-dose multidrug immunosuppression to prevent graft rejection. The research team of Project 4.4.2 is developing a protocol for hand transplantation using donor bone marrow stem cells in combination with novel fusion proteins (the Pittsburgh Protocol) that will minimize maintenance immunosuppression.

Status at End of Year 1: The researchers of Project 4.4.2 established a preclinical hindlimb transplant model in miniature Yucatan swine. They also performed hand transplantation using the Pittsburgh Protocol on a former Marine who lost his hand in a training accident while on active duty. At 4 months post-transplant, the patient was on low-dose, steroid-free monotherapy immunosuppression without any adverse side effects.

Research Plans for the Next 4 Years: The researchers of Project 4.4.2 plan to prolong limb allograft survival by using targeted skin immunotherapy with substances that inhibit the migration of white blood cells in combination with optimized protocols from Year 2 of the project. They feel that such targeted immunomodulatory protocols together with cell-based strategies could establish tolerance and ultimately eliminate the need for prolonged immunosuppression to maintain graft survival.

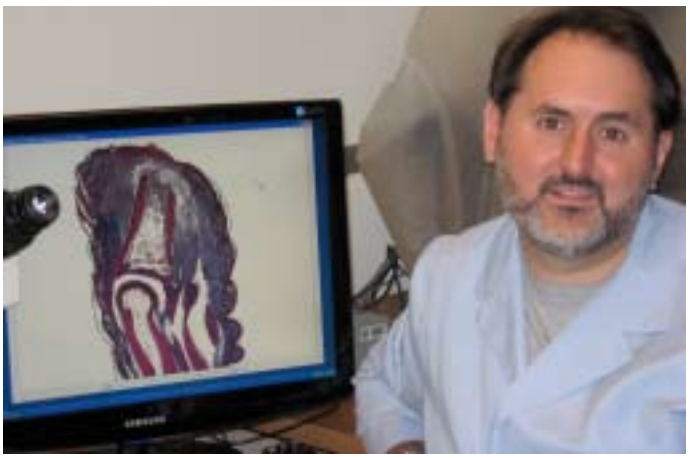
Planned Clinical Transitions: In Project 4.4.2, additional hand transplantations will be performed using the Pittsburgh Protocol. The researchers will design an optimized strategy combining targeted immunomodulation (utilizing bone marrow stem cells and fusion proteins) with topical migratory inhibitors to further reduce maintenance immunosuppression and allow weaning of systemic drug therapy in hand transplantation. They note that this will overcome side effects related to high-dose immunosuppression, which will enable widespread clinical application of hand transplantation.

Epimorphic Regeneration (and associated methods)

Studies at WFPC

Overview: The regeneration of lost limbs and digits as a result of trauma is being investigated by several laboratories through the concept of a "blastema-based approach." A blastema is the accumulation of preprogrammed stem cells that accumulate at the site of limb amputation in regenerative species such as the amphibian urodele (e.g., salamander). Such structures exist in human fetal development and can replace lost tissues and organs, but this ability disappears at approximately gestational Weeks 16–18 and is replaced by the reparative processes of inflammation and scar tissue formation.

The researchers of Project 4.4.1 are investigating mechanisms for recruiting large populations of



Scott Johnson, a staff scientist in Dr. Stephen F. Badylak's laboratory (WFPC), working with ECM in limb regeneration.

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 of Project 4.4.7 are studying tissue regeneration using high-throughput technologies (microarrays and next-generation sequencing). In collaboration with Project 4.4.1, they are analyzing amputated mouse digit tips both from untreated mouse digits and those treated with extracellular matrix (ECM) factors designed to enhance regenerative capabilities and to identify genes and gene networks that are either activated or repressed during treatment. The researchers of Project 4.4.8 are developing devices that can sort and isolate cells from complex mixtures of cells. They are collaborating with Project 4.4.1 to adopt their technology to isolate cells expressing markers associated with stem/progenitor cells.

Status at End of Year 1: Using a mouse digit amputation model, the researchers of Project 4.4.1 established a method for recruiting stem cells residing within the animal to the site of amputation. They identified numerous markers in the recruited cells indicative of stem/progenitor cells. Though the potential significance of this finding is important, the clinical utility will depend upon the ability to "instruct" these stem cells to form functional tissue. In Project 4.4.7, the researchers completed a pilot microarray study of mouse digit tips, which showed that treatment with regeneration-enhancing ECM factors led to the expression of ECM-remodeling genes and genes indicative of stem cell activity. The researchers of Project 4.4.8 developed a unique device that can achieve the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput. They also developed a device that allows for the isolation and purification of extremely rare cells from complex mixtures of cells with unprecedented cell recovery.

Research Plans for the Next 4 Years: In Project 4.4.1, the researchers will identify bioactive molecules that can instruct, facilitate, or promote the formation of a blastema-like structure following injury and test the therapeutic efficacy of the identified molecules in the mouse digit amputation model. They will also develop a "biodome" to facilitate spatial and temporal organization of the recruited stem cells at the injury site for the purpose of forming functional tissue. In Project 4.4.7, the researchers will continue to probe gene expression differences between ECM-treated mouse digits and controls. They will use this information in conjunction with existing literature on genes expressed in developing or regenerating limbs and digits to (1) characterize cellular features/changes related to treatment with ECM factors and (2) identify genes and gene networks that likely need to be activated or repressed to augment mouse digit regeneration. The researchers of Project 4.4.8 plan to develop a portable benchtop version of their cell isolation system so that collaborators within the AFIRM consortium can have access to this unique and powerful capability. They also plan to develop a two-stage device that incorporates a combination of the cell-sorting and isolation technologies to achieve even better performance in regard to purity.

Planned Clinical Transitions: The researchers of Project 4.4.1 plan by Year 4 to begin discussions with the FDA on evaluating their therapeutic molecules in humans. They hope to conduct a digit reconstruction pilot study on a human in 7–10 years based on successful completion of the specific aims. The researchers of Project 4.4.8 expect to scale up their unique cell isolation system so that it can process larger volumes of samples for human applications.

Project 4.2.1, RCCC

Optimizing Scaffolds for Repair of Bone Defects

Team Leader(s): *George F. Muschler, MD (Cleveland Clinic)*

Project Team: *Kentaro Shinohara, MD, Viviane Luangphakdy, MS, Carynne Fox, BS, Cynthia Boehm, BS (Cleveland Clinic); Joachim Kohn, PhD, Aniq Darr, PhD (Rutgers – The State University of New Jersey, New Jersey Center for Biomaterials); Linda Griffith, PhD, Linda Stockdale, MEd (MIT); Michael J. Yaszemski, MD, PhD, Brig. Gen. USAF, Suzanne Segovis, MS (Mayo Clinic)*

Collaborator(s): *Sunil Saini, Therics, LLC, Morrisville, Pennsylvania*

Therapy: *Bone graft substitutes to treat large bone deficits*

Deliverable(s): *Osteoconductive three-dimensional bone scaffolds made from biodegradable polymers*

Key Accomplishment(s): *The research team identified, designed, and fabricated four distinct families of copolymer-based osteoconductive scaffolds for in vivo testing and found the tyrosine-based copolymer and poly(alkyl ester) composites to be superior. Data obtained during Year 1 have enabled the selection of a preferred scaffold to advance research to the in vivo assessment of the cell-sourcing method being defined in Project 4.2.2.*

Introduction

Military fractures are frequently comminuted, complicated by extensive soft tissue loss, and even segmental bone loss of 5–20 cm. Defects lacking the capacity for spontaneous healing are common, and no single existing treatment option is appropriate for all defects. Limitations of the autograft and allograft for treating large bone defects have necessitated the pursuit of alternatives such as the development of bone graft substitutes.

An effective tissue-engineered bone graft requires four factors:

- Osteoconduction, which is the ability of a three-dimensional structural scaffold to allow for the ingrowth of capillaries and perivascular tissue.
- Osteoinduction, a chemical process that implies the recruitment of MSCs from the surrounding bed.
- Osteogenic cells in bone marrow, a subset of all connective tissue progenitors (CTPs), which express bone phenotypes and are capable of proliferating.
- Scaffold degradation properties, which are important for long-term biological function. Once bone forms within the porous structure of a scaffold, scaffold degradation must occur in a manner that preserves the local bone and allows functional bone remodeling.

An analysis of competitive technologies reveals that current large bone defect graft therapies produce variable results. While success is not uncommon, no existing treatment option provides over 90% success, even in relatively common settings and particularly in large complex defects.

The researchers of this project seek to screen the most promising available osteoconductive three-dimensional bone scaffolds made from degradable polymers. Four novel families of biodegradable, biocompatible copolymers are being developed and compared to autologous cancellous bone, which is the current benchmark material. The collective biomaterials and fabrication technologies expertise of scientists at Rutgers, Mayo Clinic, MIT, and Therics contribute to this project. The researchers anticipate that at least one scaffold will be advanced into clinical trials by Year 5. In addition, a durable strategy and infrastructure for the evaluation of future scaffolds will be established. A working group within the project team is addressing the development of general methods for sterilizing polymeric scaffold materials.

Research Progress – Year 1

The researchers have established an integrated collaboration team composed of laboratories with substantial expertise in bone reconstruction. They note that communication among partners has been important and has proven to be a powerful tool to save time and push the programs forward. They also note that monthly phone conferences and extensive file sharing and referencing through the

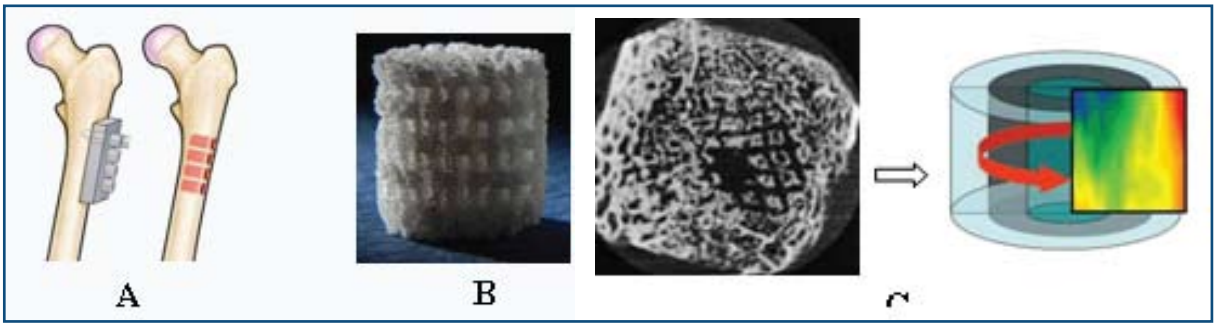


Figure II-1. Canine Femoral Multidefect Model. (A) Graft site preparation. (B) 3-Dimensional Printing™ (3DP) fabricated scaffold. (C) Percentage of bone volume (%BV) is calculated from micro-computed tomography (micro-CT) data and projected in a two-dimensional plot illustrating the pattern and density of bone formation in each defect.

WorkZone electronic collaborative environment have guaranteed good program review and documentation that complies with Department of Defense requirements.

The researchers have chosen a "tournament design" to enable rapid scaffold selection and advancement to the clinic. This approach is not designed nor intended to optimize all possible scaffold variations. Rather this is an "opportunistic" approach to identify the most effective of the available materials and methods and accelerate the rate that a promising scaffold can advance into prospective clinical trials. A series of 10 experiments will be conducted; two scaffold options will be compared in each experiment. In each comparison, the superior scaffold will move forward to subsequent comparisons. An integrated system of objective assessment and performance standards for biological scaffolds in the canine femoral multidefect model (**Figure II-1**) will enable a quantitative basis for the rapid assessment of scaffolds, thereby accelerating their pathway into clinical trials and effective clinical use.

In Year 1, the researchers successfully completed the first tier of the "tournament" test matrix, including scaffolds prepared by three-dimensional printing and porogen leaching, and four series of in vivo canine femoral multidefect model experiments. Scaffolds were fabricated by Therics using the 3DP rapid prototyping technology developed at MIT. Scaffolds were also prepared by stereolithography. Micro-CT scanning was used to obtain bone volume data from the in vivo experiments. These data were translated into contour plots to analyze the pattern and density of bone formation in each defect (Figure II-1C). Data analyses for these experiments, including histological evaluations, are under way.

Preliminary findings indicate significant advantages for tyrosine-based copolymer/inorganic composites and poly(alkyl ester) copolymer resin/inorganic composites. The researchers have characterized the differences in critical performance properties of the scaffolds in vitro, including their compressive strengths, flexibilities, cell attachment, cell proliferation, and osteogenesis, as a function of specific composition variables (monomer ratios and molecular weights). Sterilization methods evaluated by Therics to determine residual ethylene oxide levels, molecular weight loss, and percentage of volume shrinkage have not yet identified an obvious choice of an optimum sterilization protocol.

Key Research Accomplishments

- Identified, designed, and fabricated four distinct families of copolymer-based osteoconductive scaffolds for in vivo testing.
- Synthesized new, second-generation polyester and tyrosine-based copolymers and their composites with osteogenic inorganic particles.
- Fabricated complex three-dimensional printed scaffolds with controlled porosities from several of the copolymers and found them to be easily sterilizable.
- Completed all animal surgeries planned for Year 1 along with micro-CT scanning for statistical analysis of bone volumes and preparation of histology samples

for the initial evaluation of copolymer and composite scaffolds.

- Achieved the best in vivo performance to date using tyrosine-based copolymer and poly(alkyl ester) composites.
- Using stereolithography, prepared complex composite scaffold architectures and assessed sterilization methods for the scaffolds including gamma irradiation and ethylene oxide.

Conclusions

Objectives proposed in Year 1 of the original proposal have progressed in a timely, efficient, and effective manner across the broad range of laboratories involved. The research team created four distinct families of copolymer-based osteoconductive scaffolds for in vivo testing and determined that the tyrosine-based copolymer and poly(alkyl ester) composites provided the best in vivo performance. Data obtained during Year 1 have enabled the selection of a preferred scaffold to advance research to the in vivo assessment of the cell-sourcing method being defined in Project 4.2.2.

Research Plans for the Next 4 Years

In Year 2, the integrated RCCC Bone Regeneration Projects will complete the "opportunistic" series of in vivo evaluation/comparison of highly promising scaffolds, which was initiated in Year 1. The researchers anticipate advancing at least one scaffold from this initial assessment toward clinical trials. Rapid progress in Project 4.2.2 (cell sourcing) during Year 1 has created the opportunity to accelerate the combination of scaffold materials with cell-sourcing options in Year 2, in both in vitro and in vivo assessment models.

The merger of Projects 4.2.1 and 4.2.2 is on an accelerated track, and the researchers feel that the Year 3 goals initially proposed for these projects may be achieved by the end of Year 2. In Year 2, researchers in Project 4.2.3 (MSD) will modify scaffolds from Project 4.2.1 to present MSDs and begin in vitro testing in two- and three-dimensional constructs. They will advance selected surfaces to in vivo testing in Year 3, merging with Projects 4.2.1 and 4.2.2.

In Year 3, assuming that options for combination of cells and scaffolds will be advanced into the Year 2 activities, optimal scaffold materials and optimized cell-sourcing options will be advanced into more challenging defect models in Year 3, beginning with the 2.5 cm canine bilateral ulna defect model, a full year ahead of the schedule identified in the original AFIRM proposal. Scaffolds may be used alone but will most likely have optimal effects when used in combination with optimized methods for transplantation of autogenous osteogenic cells.

By Year 4, highly effective combinations of scaffolds with or without cells or bioactive surfaces will be evaluated in the most rigorous and appropriate preclinical model. This is currently defined as a 5 cm canine femur defect. However, a parallel collaboration to AFIRM funding among the Cleveland Clinic, USAISR, and the University of Minnesota is actively seeking to define a more rigorous model in the goat that includes bone tissue loss as well as features of vascular compromise, scarring, and muscle/soft tissue loss. A proposal to develop this more rigorous model was submitted as an NIH Challenge Grant in May 2009.

Planned Clinical Transitions

The researchers plan to initiate discussions in Year 2 with the FDA with regard to the pathway for regulatory review for the scaffolds under development. They will begin to plan the experimental design for new therapies for bone defects after the completion of the pending Request for Applications of their OETRP, which will establish a U.S. clinical trial network for trauma. OETRP and AFIRM leadership have agreed to work together and establish a group to proactively design and prioritize opportunities for the strategic assessment of new and high-impact clinical therapy options.

By Years 4–5, the researchers expect to have one or more bone regeneration therapeutic strategies that exceed the current preclinical performance of available materials ready to be advanced into clinical trials. Clinical trials are most likely to be funded by one or more industrial partners depending on the final scaffold, cell source, or molecularly designed surface that is involved. Clinical trials will require the collaboration of a large clinical trial network of civilian and military trauma centers. The most likely setting in which a clinical trial will

be performed will be in open tibia fractures having lost at least 50% of circumferential contact. The clinical trial(s) coming out of the AFIRM will not only establish the relationships needed for assessment of current grafting methods but will also seek to establish a durable strategy and infrastructure for the evaluation of future scaffolds.

Corrections/Changes Planned for Year 2

The work of the integrated bone projects has proceeded at or ahead of schedule. In addition to this acceleration in plans, the integrated bone team has recognized the need for collaborative work to standardize and optimize means for scaffold sterilization. As noted earlier, the team has also recognized the desirability to move from the established canine femoral defect model system into a more rigorous model in the goat tibia. These latter two programmatic changes are not yet funded within the AFIRM budget, but active measures are being taken to identify appropriate funding to support these synergistic activities. Also note that Projects 4.2.1 and 4.2.2 plan to merge over the next year, as described in "Research Plans for the Next 4 Years."

Project 4.2.2, RCCC

Optimizing Cell Sources for Repair of Bone Defects

Team Leader(s): *George F. Muschler, MD (Cleveland Clinic)*

Project Team: *Tanya Caralla, Viviane Luangphakdy, MS, Thomas Patterson, PhD, Cynthia Boehm, Macej Zborowski, PhD, Vincent Hascall, PhD (Cleveland Clinic)*

Collaborator(s): *Sunil Saini, Therics, LLC, Morrisville, Pennsylvania*

Therapy: *Stem/progenitor cells for the treatment of bone defects*

Deliverable(s): *Optimized methods for selection and concentration of osteogenic CTP cells*

Key Accomplishment(s): *The researchers have defined methods to increase the surgical yield of bone marrow harvest procedures without an increase in morbidity. Three practical methods have been characterized to enable the processing of human or canine bone marrow: density separation, selective retention, and magnetic separation.*

Introduction

An effective tissue-engineered bone graft requires consideration of several factors: an osteoconductive matrix, osteoinductive stimuli, osteogenic cells, and the appropriate biophysical environment. This environment provides suitable mechanical and electrical stimuli and mass transport properties that maximize the survival of transplanted cells.

Researchers at the Cleveland Clinic are devoting resources and expertise to refining the clinical harvest and intraoperative processing and assaying of bone marrow cells capable of forming new bone (referred to as osteogenic connective tissue progenitors [CTP-Os or CTPs]). Specific activities include: (1) systematically evaluating clinical methods for harvesting autogenous CTPs, (2) comparing available methods for the intraoperative concentration and/or selection of CTPs (density separation, selective retention, and magnetic separation), and (3) combining optimized cell-sourcing with optimized scaffolds.

Osteogenic cells are available from a variety of sources. However, bone marrow aspirates provide the inherent advantages of being available from everyone and being easily accessible with little morbidity and no immunogenic risk to the patient. A continual challenge in the bone repair field is the preferential selection of bone marrow cells capable of forming new bone (referred to as CTP-Os) from the heterogeneous mix of cells in a fresh bone marrow aspirate. A system for positive selection of CTPs would allow for not only an increased concentration of CTPs in a graft but also the elimination of the more numerous, nonosteogenic cells that do not contribute to new bone formation and may actually hamper new bone growth by competing with CTPs for the limited oxygen and nutrients available at the graft site. The researchers will investigate CD45 (a marker used to select hematopoietic cells) and hyaluronan (HA) as markers for depletion and enrichment, respectively, of CTPs in a fresh bone marrow aspirate.

Competing technologies in the field include density gradient separation systems that can concentrate CTPs in a small-volume sample and magnetic separation systems that tag cells of interest with magnetic beads and separate them in a magnetic field. Each system has its drawbacks, however. Density gradient separation can be inefficient, due to variability in the density between samples, and ends up harvesting all nucleated cells, necessitating an additional step to isolate CTPs. Magnetic systems are highly selective and easy to use but are limited by the availability of nonhumanized antibodies and scale-up expenses. Many different cell surface markers have been evaluated for CTP selection, but no marker to date has been found to be both effective and selective enough to isolate all CTPs and only CTPs.

Researchers at the Cleveland Clinic are devoting resources and expertise to refining the clinical harvest and intraoperative processing and assaying of CTPs. Specific

activities include: (1) systematically evaluating clinical methods for harvesting autogenous CTPs, (2) comparing available methods for the intraoperative concentration and/or selection of CTPs (density separation, selective retention, and magnetic separation), and (3) combining optimized cell sourcing with optimized scaffolds.

Research Progress – Year 1

Aim 1: *Characterize CTPs with bone marrow aspiration (BMA) and bone marrow excavation (BME).*

The overall effort to improve methods for harvest and yield of bone and marrow-derived cells and CTPs has been leveraged by funding through the Cleveland Clinic Foundation's Clinical Tissue Engineering Center Biomedical Research and Technology Transfer Partnership Program (State of Ohio Department of Development Contract TECH 05-065) and the Telemedicine and Advanced Technology Research Center (USAMRMC Proposal 04084002.02 entitled "Image-Guided Bone Marrow Harvester"). The research team has compared BMA and BME in 23 canine subjects.

Aim 2: *Test density separation system processing of CTPs.*

Density separation (e.g., centrifuge) devices are already used to process blood to prepare platelet-rich plasma gels, and preliminary data suggest that it can also be used to increase the concentration of CTPs using methods that separate a buffy coat from red blood cells and plasma fractions. Recently, several density separation devices have been in early-stage development for BMA processing and bone repair (**Figure II-2**). Density separation processing systems are available through the use or modification of devices designed for the clinical preparation of platelet-rich plasma. These systems have the advantage of quantifiable and programmable intraoperative processing and can concentrate CTPs in a small volume.

Aim 3: *Evaluate selective retention of CTPs in scaffolds (from Project 4.2.1).*

Scaffold fabrication in Project 4.2.1 has progressed sufficiently to justify and enable the assessment of the selective retention method using three-dimensional tyrosine-derived polycarbonate or PPF and beta-tri-calcium phosphate (β-TCP) scaffolds. This work was initially projected to begin in Year 3 but is expected to be accelerated by 1 full year from initial projections.

To avoid delaying objectives of Aim 3 due to any limitation in AFIRM funding, an RC1 proposal was submitted on April 27, 2009. If successful, this grant will remain affiliated and integrated with the RCCC AFIRM Bone Team, and it will continue to report on progress related to Aim 3.

Aim 4a/b: *Magnetic separation processing of bone marrow aspirates.*

Bone marrow was aspirated from the iliac crest in 2 mL aliquots according to approved Institutional Review Board protocol. Of note, all human work was done through funding from another grant (Ohio Department of Development BRCP 05-065). AFIRM funds were not used for the following experimental results. Bone marrow mononuclear cells were selected by density gradient separation ("buffy coated") and processed through the Easy-Sep® Magnetic Separation system (Stem Cell Technologies) on the basis of CD45 or HA expression in accordance with the manufacturer's recommendations. Two protocols were evaluated: the traditional depletion scheme and a simplified, operating room-friendly, single-pass protocol.

Figure II-2. Density separation devices.

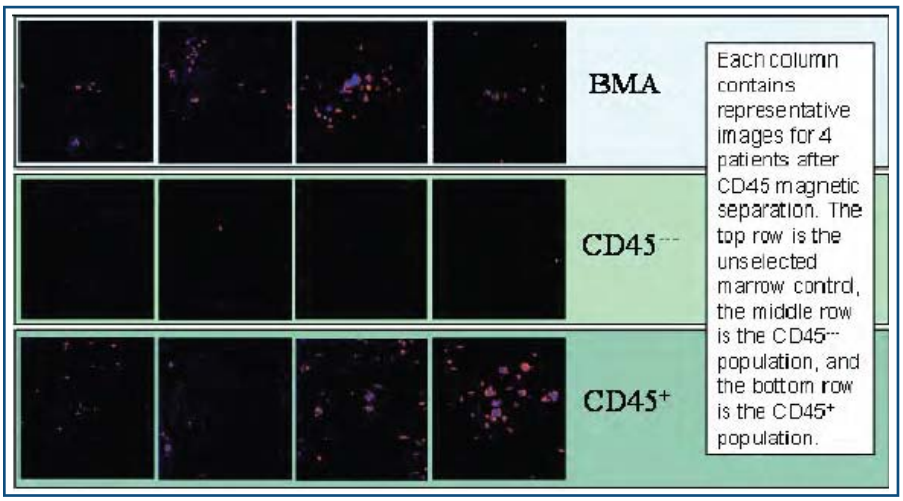


Figure II-3. The images show colony formation after 6 days of culture for four of the six patients. Here the nuclei (DAPI) staining is shown in blue, overlayed by alkaline phosphatase staining in red. The colony interior is also shown in pink. The BMA control illustrates the baseline prevalence for each patient. The second row is the CD45--- fraction, which is depleted in CTPs. The CD45+ fraction in the bottom row shows a similar prevalence to the BMA control.

- Selection for CD45 showed that CTPs partition to the CD45+ population with no significant enrichment of progenitors demonstrated. Because of the abundance of CD45 expression in the bone marrow mononuclear cell fraction, CD45 has not proved to be a useful selection tool.
- CTP-Os can be rapidly enriched based on the presence of HA at the time of collection and confirm that HA is a component of the matrix niche of at least one important population of osteogenic cells in bone marrow. These HA+++ CTPs were also significantly more proliferative and more rapidly differentiated to an osteoblastic phenotype, which may be important in optimizing in vivo performance.
- Magnetic separation using canine marrow for CD45 and HA resulted in similar enrichment trends observed in human marrow. This demonstrates that canine marrow provides a useful model for predicting performance of human marrow.

Key Research Accomplishments

- Designed and fabricated a new needle using a 5 mm diameter and curved tip with obturator to enable excavation without occlusion.
- Developed Computer-Assisted Navigation methods in prototype form to enable guided clinical excavation.
- Using the CD45 magnetic separation protocols, completed experimentation, staining, image acquisition, and image review on six patients; demonstrated that CTP-Os partition to the CD45+ population (**Figure II-3**).
- Obtained preliminary data showing the performance of canine marrow is comparable to human marrow with respect to CD45.
- Evaluated 11 patients using HA selection with the EasySep magnetic separation system and observed an enrichment in CTPs in every patient in the HA+++ fraction.
- Evaluated six patients with the multipass purification protocol as well as a simplified, one-pass protocol that is optimized for intraoperative processing; determined that one-pass processing is feasible but requires optimization to minimize nonspecific cell loss.

Conclusions

The team defined methods to increase the surgical yield of bone-marrow-harvest procedures without an increase in morbidity. They made substantial progress in characterizing three alternative, practical methods to allow the processing of human or canine bone marrow samples: density separation, selective retention, and magnetic separation. The team will competitively evaluate these three methods in vivo in Year 2 in combination with optimized scaffolds from Project 4.2.1.

Research Plans for the Next 4 Years

To avoid delaying objectives of Aim 2 due to any limitation in AFIRM funding, a study proposal was submitted to Arteriocyte, Inc. on February 23, 2009. If successful, this grant will remain affiliated and integrated with the AFIRM team, and it will continue to report on progress related to Aim 2. To avoid delaying objectives of Aim 3 due to any limitation in AFIRM funding, an RC1 proposal was

submitted on April 27, 2009. If successful, this grant will remain affiliated and integrated with the AFIRM team, and it will continue to report on progress related to Aim 3. See "Research Plans for the Next 4 Years" under Project 4.2.1 for additional information.

Planned Clinical Transitions

The researchers will initiate discussions with the FDA during Year 2 regarding the cell-sourcing strategies they are developing. Density separation methods are already available as 510(k) devices for "marrow processing" but not for bone-graft preparation. Selective retention strategies and magnetic separation strategies will each require separate consideration by the bone team, most likely as combination products. See "Planned Clinical Transitions" under Project 4.2.1 for additional information.

Corrections/Changes Planned for Year 2

The work of the integrated bone projects has proceeded at or ahead of schedule. Due to the rapid advance in Year 1 regarding the characterization of cell sourcing, processing, and magnetic separation methods, the in vivo assessment of cell-sourcing options has advanced from a Year 3 activity to a Year 2 activity. The merger of Projects 4.2.1 and 4.2.2 is on an accelerated track, and the team may achieve the Year 3 goals initially proposed for these projects by the end of Year 2.

Project 4.2.3, RCCC

Advancing Bone Repair Using Molecular Surface Design

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

Project Team: *Linda Stockdale, MEd, Luis Alvarez, PhD, James Serdy (MIT); Vivek Raut, Chris Heylman (Cleveland Clinic); Richard Clark, MD, (Stony Brook University); Joachim Kohn, PhD (Rutgers – The State University of New Jersey, New Jersey Center for Biomaterials)*

Collaborator(s): *Sunil Saini, Therics, LLC, Morrisville, Pennsylvania*

Therapy: *Regeneration of bone in large bone defects*

Deliverable(s): *Improved control over the cell and tissue response to an implant material using MSD*

Key Accomplishment(s): *The researchers fabricated two-dimensional scaffolds presenting tethered epidermal growth factor (tEGF) and a fibronectin-derived peptide P12 that enhance new bone tissue formation.*

Introduction

Project 4.2.3 specifically seeks 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, which involves the tethering of specific bioactive ligands (growth factors and small peptides) on a scaffold surface. MSD features systematic, designed interventions to link bioactive molecules to the surface of a biomaterial as a means of improving control over the cell and tissue response to an implant material. Modifications may be covalent or involve very high-affinity, tight-binding noncovalent interactions, or a combination of the two.

A workhorse technology is based on "comb" polymer technology developed at MIT by Dr. Linda Griffith. These polymers have a hydrophobic (water insoluble) backbone with water soluble "tethers" extending off the backbone like a comb; growth factors and adhesion molecules can be covalently linked to the ends of the tethers. These polymers are used to fabricate two-dimensional substrates representative of implantable three-dimensional bone scaffold materials initially using EGF and/or a bioactive fibronectin-derived peptide P12 provided by Dr. Richard Clark (Stony Brook University). MSD surfaces based on tethered protein growth factors for bone repair have been evaluated and found to have promising biological effects in vitro using human CTP cells, including protection against pro-death signals and promotion of CTP cell growth and differentiation. Based on initial evaluation, P12 is expected to have osteotropic bioactivity, but it has not yet been evaluated in a bone environment.

CTP cells are defined collectively as the heterogeneous population of tissue resident stem and progenitor cells in human bone marrow that are capable of proliferation and differentiation into one or more connective tissue phenotypes. Bone repair specifically requires a subset of CTP cells that is capable of forming mature osteoblasts, CTP-Os. Since bone defects are deficient in CTP-Os, the treatment of these defects cannot be optimized without the transplantation of CTP-Os from another remote source, such as bone marrow. However, transplantation is currently inefficient. Many transplanted cells do not survive or respond in a manner that contributes to new tissue formation. Surface modification using MSD is a potentially powerful means of optimizing the attachment, survival, proliferation, and migration of transplanted CTP-Os. The potential use of tethered growth factors in CTP-O performance optimization is explored here.

The research plan for Project 4.2.3 is built around four aims in which personnel from MIT, the Cleveland Clinic, and Stony Brook University participate.

Research Progress – Year 1

Aim 1: *Optimize methods for fabrication of surface coatings presenting one or more peptides with consideration of manufacturing, storage, and regulatory constraints.*

Comb polymer surfaces were prepared with tEGF or P12 and then successfully seeded with human telomerase reverse transcriptase MSCs (hTERT MSCs). The cells were imaged with a Zeiss Axiovert 100 inverted microscope to observe cell attachment, colony formation, and cell proliferation. Polyester copolymer/β-TCP surfaces were prepared by spin coating on coverslips with various sizes of β-TCP particles (**Figure II-4**). Scanning electron microscope images did not reveal if the β-TCP was exposed on the polymer surfaces. Results from the first β-TCP binding assay were inconclusive because the surface coatings did not adhere to the unsilanized coverslips in the aqueous environment used in the assay. Further optimization of the copolymer/β-TCP system is therefore necessary. All copolymer/β-TCP surfaces with or without comb polymers were stable in phosphate-buffered saline for 7 days. Cells adhered to and spread out on all scaffold, scaffold/β-TCP, and scaffold/β-TCP/comb surfaces.

Aim 2: *Optimize methods for in vitro surrogate assays of clinical efficacy of tEGF and fibronectin-derived P12 peptide in bone environment in terms of its effects on bone marrow CTP-Os using both model substrates and clinically relevant substrates.*

Protocols for comb copolymer synthesis and purification, surface preparation, and EGF and P12 tethering have been successfully developed. For tethering P12 peptides on a two-dimensional surface, an MSD experimental strategy has been evaluated. Methods for the quantitative measurement of in vitro cell response (attachment, survival, proliferation, and migration) have been established. Ongoing experiments focus on surface characterization of tethered P12 surfaces using radiolabeling studies, optimizing the protocols for surface peptide density, co-tethering of a combination of peptides, cell-seeding density, and in vitro culture conditions including hypoxic and hyperoxic conditions. Preliminary experiments demonstrate that tethered P12 surfaces induce an increase in colony formation and proliferation even in serum-starved conditions (**Figure II-5**).

Aim 3: *Develop rapid in vitro assays for quality control of the bioactivity of MSD surfaces and extend surrogate in vitro assays of osteogenic potential to clinically relevant three-dimensional scaffolds.*

An optimized construct was identified that has multiple β-TCP binding peptide (TCPBP) repeats. Detailed observation showed uniform surface coverage, and the treated β-TCP remained uniformly coated over a long period of time. A cell-based assay was used to determine the effect of prolonged culture under resource-limited conditions on CTP cells seeded on pure β-TCP scaffolds with and without surface treatment with TCPBP-EGF. Nuclear staining revealed significant differences in cell proliferation and survival under these conditions. The untreated control showed no cell survival after 23 days, whereas EGF-treated scaffolds showed robust cell growth, survival, and invasion of the scaffold. Histological sections revealed deep penetration and robust cell growth throughout the scaffold. The presence of tEGF appeared to allow CTP cells under osteogenic conditions to continue proliferating. No surface treatment resulted in reduction of cell numbers over 7 days. The ability to increase proliferation and maintain elevated levels of alkaline phosphatase activity indicated that surface tethering EGF on β-TCP may be a viable method of increasing the available pool of CTP cells in the days following seeding and implantation in a wound.

Aim 4: *Preventing hypoxic effects in avascular cellular microenvironments.*

CTP cell colony-forming efficiency was not significantly affected by glucose concentration under either normoxic (3% O_2) or hypoxic (0.1% O_2) conditions, confirming that cell death was not a result of

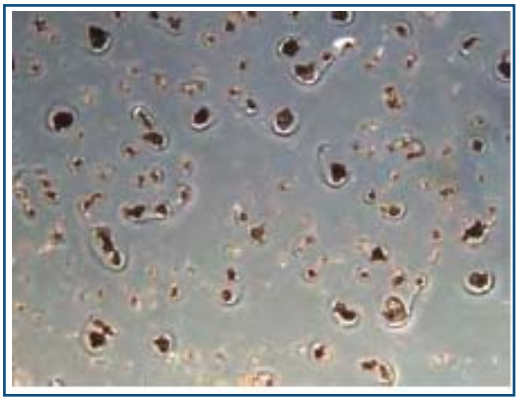


Figure II-4. Two-dimensional polymer/β-TCP surface spin coated 40 mg/mL in toluene. (Zeiss Axiovert 100 inverted microscope).

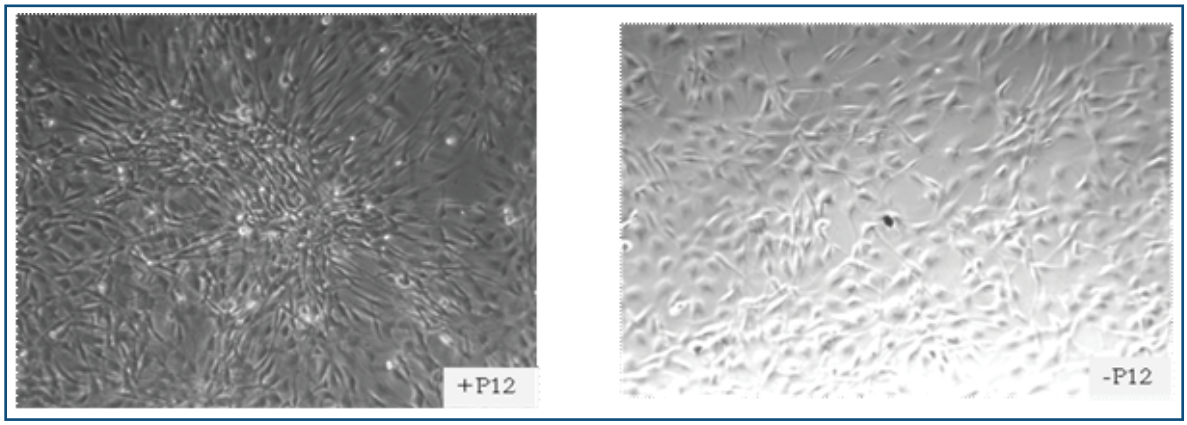


Figure II-5. Tethered P12 improves colony formation and proliferation of hTERT cells. Day 6 in serum-free culture medium.

glucose deprivation in a hypoxic environment. The delivery of oxygen via polymeric beads was effective but lacked the proper time and magnitude scales of oxygen delivery for optimal CTP cell survival and performance (**Figure II-6**). Further work to optimize bead formulation is scheduled for Year 2 with the aim of providing a relatively more constant and extended generation of oxygen. The sum of these experiments will yield an optimized combination of salt to polymer ration, polymer formulation, and geometry that provides an oxygen delivery profile that meets the needs of CTP cells in a hypoxic environment.

Key Research Accomplishments

Aim 1: *Optimize methods for fabrication of surface coatings presenting one or more peptides with consideration of manufacturing, storage, and regulatory constraints.*

- Successfully developed and/or implemented protocols for comb copolymer synthesis, purification, characterization, surface preparation, EGF, and P12 tethering and seeding of hTERT-MSCs.
- Successfully developed and implemented a protocol training program.
- Successfully developed and implemented a protocol for spin coating two-dimensional scaffold surfaces with and without comb coating to which hTERT MSCs attached and proliferated.
- Successfully detected β-TCP on scaffold surfaces.

Aim 2: *Optimize methods for in vitro surrogate assays of clinical efficacy of tEGF and fibronectin-derived peptide P12 in bone environment in terms of its effects on bone marrow CTP-Os using both model substrates (to parse effects quantitatively) and clinically relevant substrates.*

- Established methods for quantitative measurement of in vitro cell response (attachment, survival, proliferation, and migration).
- Conducted preliminary experiments to evaluate the in vitro response of tethered P12 peptide.
- Developed methods and protocols to characterize the two-dimensional scaffold design using radiolabeling and analytic chemistry techniques.
- Established a strong channel of communication for information

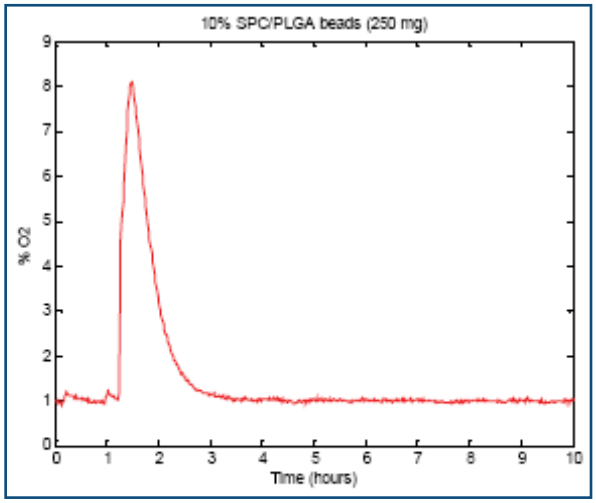


Figure II-6. Oxygen delivery kinetics of 10% sodium percarbonate (SPC) microbeads (1 mg SPC/9 mg poly(lactic-co-glycolic) acid [PLGA]). Data shown for 250 mg of beads placed in 2 mL of media.

and material exchange between various laboratories involved in Project 4.2.3.

Aim 3: *Develop rapid in vitro assays for quality control of the bioactivity of MSD surfaces and extend surrogate in vitro assays of osteogenic potential to clinically relevant three-dimensional scaffolds.*

- Developed an optimized β-TCP binding peptide that incorporates multiple β-TCP binding sites in fusion with EGF.
- Started in vitro studies of molecular surface characterization using CTP cells.
- Determined that tEGF can exert a strong proliferative response in CTP cells seeded on three-dimensional β-TCP scaffolds even while cultured under osteogenic conditions.
- Determined that tEGF does not reduce the early differentiation potential of CTP cells as evidenced by sustained alkaline phosphate levels at day 7 versus controls.

Aim 4: *Prevent hypoxic effects in avascular cellular microenvironments.*

- Demonstrated that glucose has a negligible effect under hypoxic conditions.
- Determined that oxygen deprivation (as opposed to glucose deprivation) is the cause of CTP cell death in a hypoxic cell environment.
- Successfully fabricated oxygen-generating microbeads (SPC-loaded PLGA beads).
- Confirmed oxygen delivery from oxygen-generating microbeads.

Conclusions

Tethered peptide P12 surfaces show improved colony formation and proliferation of hTERT progeny.

Experiments are presently being conducted to characterize comb polymer surface with tethered P12, and further experiments are needed to determine the optimal surface compositions. Methods must be developed to tether EGF and/or P12 to comb polymer in solution to prepare optimal three-dimensional scaffolds. Exposed β-TCP has been detected on two-dimensional scaffold surfaces, and hTERT MSCs adhere to and proliferate on two-dimensional scaffold surfaces with and without comb polymer. Tethered EGF can exert a strong proliferative response in CTP cells seeded on three-dimensional β-TCP scaffolds even while cultured under osteogenic conditions. Tethered EGF does not reduce the early differentiation potential of CTP cells as evidenced by sustained alkaline phosphatase levels at day 7 versus controls. Oxygen is the limiting factor leading to cell death in a hypoxic environment, and co-polymer beads have been shown to produce oxygen, but further experimentation is necessary to tune the delivery of oxygen to enact optimal CTP cell survival under hypoxia. Integration of oxygen-generating polymers into scaffolds will become necessary as optimal formulations are achieved.

Research Plans for the Next 4 Years

In Year 2, the MIT team will modify scaffolds from Project 4.2.1 to present MSD surfaces and begin in vitro testing in two- and three-dimensional constructs. The researchers will advance selected surfaces to in vivo testing in Year 3, merging with Projects 4.2.1 and 4.2.2. See "Research Plans for the Next 4 Years" under Project 4.2.1 for additional information.

Planned Clinical Transitions

See "Planned Clinical Transitions" under Project 4.2.1.

Corrections/Changes Planned for Year 2

None.

Project 4.2.4, RCCC

Clinical Assessment of Ongoing Strategies for Treatment of Bone Defects

Team Leader(s): *George F. Muschler, MD (Cleveland Clinic), Michael J. Yaszemski, MD, PhD, Brig. Gen. USAF (Mayo Clinic)*

Collaborator(s):

Societies: Society of Military Surgeons, Orthopaedic Trauma Association, American Association of Orthopaedic Surgeons, Orthopaedic Research Society

Companies: Synthes USA, Medtronic, Stryker, Depuy, Musculoskeletal Transplant Foundation

Key Accomplishment(s): *The organizational team collaborated with Drs. Andy Polack and Michael Bosse to develop the Trauma Clinical Trial Network. They also presented research results at a variety of symposia.*

Introduction

The ongoing clinical experience with innovative "off-label," "out-of-the-box" grafting strategies in both the military and civilian trauma communities has tremendous but as yet untapped potential value for both informing individuals about potentially useful and potentially flawed practices. The rate at which this information (both positive and negative) is collected and distributed is currently a limiting factor in clinical progress and safety. Other limiting factors include: authority to collect and distribute some types of information and the lack of appropriate forums for its objective evaluation and discussion.

It is widely recognized that a large fraction of current use of bone-grafting materials for the treatment of fracture non-union and bone defects represents "off-label" use, based on relatively unregulated personal clinical practice decisions, often based on word of mouth and assumptions of efficacy. Generally, this use is not subjected to rigorous evaluation and is not a current target for prospective clinical trials due to lack of sufficient financial incentive, regulatory barriers, and/or the availability of nonconflicted leadership.

This project represents the proactive organizational arm of RCCC's Bone Program. The broad aim of the project is to accelerate the rate at which objective information regarding current use of available products for both "on-label" and "off-label" clinical strategies is made available to the general community in a manner that may be responsibly integrated into clinical practice. A major focus of this effort is the development of a clinical trial network in collaboration with other institutions, including the NIH, USAISR, and OETRP. Proactive interactions to define the FDA regulatory pathways will become increasingly important in Year 2.

The specific aims of Project 4.2.4 are to (1) develop a network of participants who are able and willing to share information and experience related to current clinical practices and outcomes related to treatment of post-traumatic segmental bone defects, (2) develop and provide forum opportunities for public dissemination and open discussion of current trends, and (3) make periodic recommendations related to appropriate opportunities for prospective clinical trials.

Research Progress – Year 1

In collaboration with USAISR staff, the Joint Theater Trauma Registry and Military Orthopaedic Trauma Registry, Society of Military Surgeons, Orthopaedic Trauma Association, members of industry responsible for monitoring clinical utilization, and in consultation with the NIH, FDA, and Agency for Healthcare Research and Quality, an AFIRM task force is being established to: (1) appropriately solicit and review available data regarding both "on-label" and "off-label" therapies; (2) plan forums for public presentation and discus-

sion of "Therapies Repair of Bone Defects" at the annual AFIRM Symposium; (3) prepare a report on the results of this symposium, which will be submitted for presentation at the American Association of Orthopaedic Surgeons and/or Orthopaedic Trauma Association meetings and published; and (4) make periodic recommendations for the design of prospective clinical trials that will objectively assess and (where appropriate) compare the efficacy of available therapy options.

During the past year, the organizational team participated in the Peer Reviewed Orthopaedic Research Program Stakeholders Meeting and collaborated with Drs. Andy Pollack and Michael Bosse to develop the Trauma Clinical Trial Network. They presented research results at various symposia, including:

- A talk on *The AFIRM initiative* at the Orthopaedic Research Society Symposium in February 2009.
- A presentation on *Orthopaedic Challenges and Stem Cell Therapies for Bone Regeneration* at the Tissue Engineering and Regenerative Medicine International Society-North America Conference in San Diego, California, in December 2008.
- A presentation on *Bone Defects – Cell Therapies* at the Extremity War Injuries Symposium in Washington, DC, in January 2009.
- A talk on *Collaborative Efforts in Research, Host Nation Care, & Disaster Preparedness* at the Extremity War Injuries IV Conference in Washington, DC, in January 2009.

Research Plans for the Next 4 Years

In Year 2, the organizational team will increasingly focus on proactive interactions to define the FDA regulatory pathways. See "Research Plans for the Next 4 Years" under Projects 4.2.1, 4.2.2, and 4.2.3 for additional information.

Planned Clinical Transitions

Drs. Yaszemski and Muschler will remain actively engaged throughout Years 2–5 in the process of building the networks and relationships needed to move forward with a successful clinical trial, including close collaboration with the OETRP. See "Planned Clinical Transitions" under Project 4.2.1 for additional information.

Project 4.3.2, RCCC

Development of a Tissue-Lined Bioabsorbable Stent Graft for Treatment of Arterial Trauma

Team Leader(s): *Timur P. Sarac, MD (Cleveland Clinic)*

Project Team: *M. Bannazadeh, MD (Cleveland Clinic)*

Collaborator(s): *B. Cho, PhD, M. Poole (PeriTec Biosciences); P. Gingras, P. Mulroney (Proxy Biomedical); C. Bonsignore (Nitinol Development Corporation)*

Therapy: *Treatment of arterial and venous trauma*

Deliverable(s): *Bioabsorbable stent graft*

Key Accomplishment(s): *The researchers found that the bioabsorbable material polydioxanone fulfilled the essential requirements for fabrication of a bioabsorbable tissue-lined stent to treat traumatic arterial and venous injuries. They also discovered that mineral oil will serve as an effective storage emollient.*

Introduction

Soldiers who suffer penetrating vascular injuries can benefit from minimally invasive vascular stent graft repairs that will help minimize the risks, time, and expense of open vascular surgery and induce faster healing. Traditional, bare metal stents are a poor treatment for arterial injuries since the interstices between stent struts allow for continued exsanguination, and the stents can mechanically fatigue or fracture in the long term, especially when placed in areas of high arterial movement. The durability of traditional prosthetic-lined stent grafts is well known to be suboptimal for long-term potency. Bioabsorbable stent designs solve the problem of fatigue but still have interstices that allow blood to flow through and hemorrhage. Attempts to line stents with artificial materials have resulted in inflammation and other technical problems.

The goal of this project is to develop a durable bioabsorbable stent graft with a tissue lining for the minimally invasive treatment of arterial and venous trauma. The hybrid technology is designed to allow the stent and tissue to absorb into the vessel wall and seal the injury with an optimum time frame of 3 to 6 months. The stent graft must possess enough mechanical strength to cover the injury and to prevent exsanguination in the short term. This design eliminates the long-term fatigue issues of traditional metal stent designs and the bleed-through issues of earlier bioresorbable designs. Combining a bioabsorbable stent with a tissue is an optimal hybrid technology that will ultimately aid both the military and civilian populations.

Research Progress – Year 1

The research team is developing a bioabsorbable self-expanding tissue-lined stent (**Figure II-7**). This summary report briefly describes progress in material selection, tensile testing, solute testing, prototype modeling, prototype generation, physical handling, and stent construction in Year 1 and plans for continued research in Year 2.

After tensile testing, researchers chose polydioxanone as an appropriate resorbable polymer, with poliglecaprone as second choice, and polyglactin as the third choice. Polydioxanone is a suitable material for use in a peripheral vascular stent graft based on its biocompatibility and its inherent memory properties. Physical analysis has shown that orientation of the polymer followed by tubular formation increases its radial strength in comparison to nonorientated polymer. Researchers have modeled and fabricated various stent designs; testing is ongoing to evaluate factors such as stent cell size, radial strength, and radial stiffness. The bioresorbable stent design must contain radio-opaque material to be visible on imaging. A separate, nested stent design is being evaluated to accommodate the specific properties the tissue adds to the stent and to account for the sewing pattern and proximal radial force needed to prevent turbulent eddies.

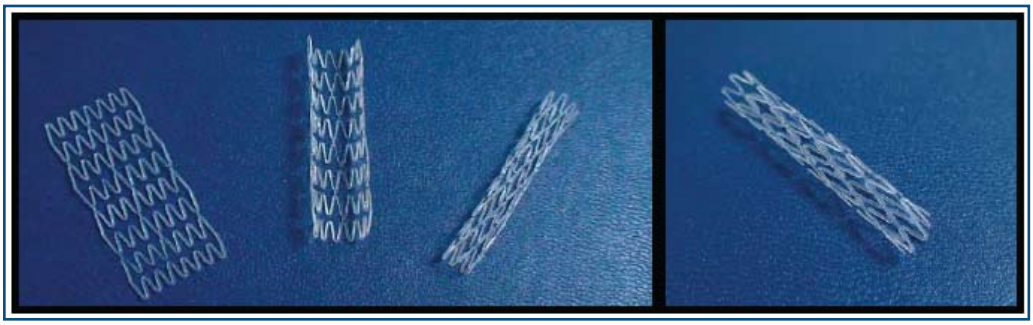


Figure II-7. Stent prototypes.

Finally, the team is evaluating the tensile strength of the tissue (bovine peritoneum) after incubation in mineral oil (the preservative identified from the first year's work). The tissue lining in animal and human studies appears to be resistant to infection and to intimal hyperplasia and allows re-endothelization. It is also thin (150 microns) so it should be easy to construct into a low-profile stent graft.

In summary, basic research, data collection, and analysis were initiated over the past year as the team tested hypotheses, explored alternative concepts, and identified and evaluated component technologies.

Partners. After the Cleveland Clinic research team and the initially identified corporate partner were unable to reach agreement, the team began developing a bioabsorbable stent in partnership with Proxy Biomedical. The researchers continue to work closely with PeriTec Bioscience on the tissue lining. In conjunction with PeriTec and Nitinol Development Corporation, the team has designed a nested self-expanding stent structure to accommodate the tissue. This allowed the researchers to simultaneously evaluate the degradation patterns of various bioabsorbable substances in several different mediums and to construct several different prototypes.

Key Research Accomplishments

- Identified a 3–6 month window for the degradation time line of polymers.
- Evaluated the tensile strength of several polymers; chose polydioxanone as an appropriate polymer, with poliglecaprone as second choice, and polyglactin a third choice.
- Identified mineral oil as the best substance for anhydrous solution for the stent graft.
- Completed a first-generation prototype and design of a bioabsorbable stent.

Conclusions

The development of a tissue-lined bioabsorbable stent graft for treatment of arterial and venous injuries is an important advancement for young and older soldiers, both in the field and immediately afterward for delayed injuries. The researchers have identified a polymer and storage solution that will allow them to continue to develop the preliminary stent graft prototypes that they have designed and built. They are now modifying construction of the stent to maintain radial force and proper mechanical nested design to accommodate the tissue. The researchers will develop a suture attachment pattern, perform tensile testing of the stent/tissue combination, analyze combined storage properties, and rework the delivery system. They will demonstrate the effectiveness of the technology via implantation in animal models. Once animal studies are complete, the researchers will conduct human clinical trials; this will require funding at a major industry-level scale to reach the ultimate goal of treating military injuries with the tissue-lined stent graft.

Research Plans for the Next 4 Years

In Year 2, work will concentrate on construction of a bioabsorbable self-expanding tissue-lined stent. The feasibility of various designs and fabrication methods will be tested. As a fail-safe mechanism cannot be defined, a balloon expandable stent will be carried forward. Once a design is finalized, work will progress to define the specific suture attachment patterns needed for the stent. Concurrently, work will test the tensile strength of the allograft tissue-lining material after incubation in optimized storage medium.

After completion of Year 2 milestones, Year 3 will focus on canine implants in iliac arteries. The goal

will be to test the bioabsorbable tissue-lined stent and compare it to a bare metal, self-expanding tissue-lined stent. This will involve explant post-processing and histological analyses beginning at 30 days and progressing to 180 days. These represent accepted preclinical end points. Mechanical testing will also be performed.

Work in Years 4 and 5 will concentrate on design control validation history, along with sterilization and delivery system modification, in preparation for design freeze and shift to clinical trials. While predicate devices may be available at that time, it is anticipated that the regulatory pathway will continue to be a pre-market approval. Industry support will be required for the clinical trials; however, recent FDA decisions allowing historical controls will make these trials less burdensome than in the past.

Planned Clinical Transitions

The FDA Center for Devices and Radiologic Health published on January 13, 2005 a "Guidance Document" that establishes guidelines (including ISO standards) for the preclinical evaluation of devices prior to clinical trials. To date, there is one approved stent graft (Viabahn-Gore Inc.) and one approved bare metal stent (Life Stent-Baird/Edwards Life Sciences) for use in blood vessels. However, since approval, the FDA announced it will allow historical controls, which decreases the number of patients required to enter by 30%–50%. There is precedent for the tissue processing in a 510(k), but preliminary discussions with the FDA indicate it likely will need an IDE. This takes into account the fact that there is no bioabsorbable stent approved on the market today. Additionally, pre-IDE meetings will be held. While approval is garnered for the use of the stent graft for blockages, a parallel application for a Humanitarian Device Exemption for trauma will allow quicker approval. Precedent has been set for covered stents for the coronary arteries.

Corrections/Changes Planned for Year 2 and Rationale for Changes

When the initially planned industry partnership did not materialize, this project adapted and identified an exceptional partner in Proxy Biomedical. In this partnership, a bioabsorbable stent has been defined and advanced. This project continues to work closely with PeriTec Bioscience on the tissue lining and has designed a nested self-expanding stent structure to accommodate the tissue-lined constructs in conjunction with PeriTec and Nitinol Development Corporation.

Project 4.4.3a, RCCC

Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents

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

Project Team: *Joseph Iannotti, MD, PhD, Jesse McCarron, MD, PhD, Amit Aurora, MS, Ryan Milks, MS (Cleveland Clinic)*

Collaborator(s): *Musculoskeletal Transplant Foundation*

Therapy: *Repair of large tendon and muscle defects with good suture retention and enhanced wound healing*

Deliverable(s): *A polymeric fiber-reinforced fascia lata device for effective musculoskeletal repair*

Key Accomplishment(s): *The research team developed mechanically robust, reinforced fascia using customized, biodegradable, polylactic acid (PLA)/polyglycolic acid (PGA) braided fibers that significantly increased fascia suture retention strength to physiologically relevant loads relevant to bridging large muscle/tendon defects.*

Introduction

Warfighters who suffer musculoskeletal trauma often have defects in the limbs with profound loss of soft tissue, tendon, and muscle. In particular, scaffolds aimed at bridging massive tendon and muscle defects to bone would restore some degree of limb function in salvage procedures. For large tendon and muscle defects, there is no current tissue repair technique that results in a natural, strong, mechanically robust scaffold, has good suture retention, and provides enhanced wound healing. Engineered human fascia lata potentially offers all of these advantages. Fascia is a thin, fibrous, and strong connective tissue that surrounds, protects, and supports the body's muscles. Fascia lata are the long bands of fascia found in the legs of the human body (**Figure II-8**).

This research team has previously established the regional variability, processing methods, and mechanical, biochemical, and cellular properties of human fascia lata. Despite the favorable mechanical properties of allograft fascia and its established track record in orthopaedics, its suture retention properties are poor and currently limit the clinical utility of this biomaterial for load-bearing applications.

Research Progress – Year 1

The objective of this project is to engineer a fascia lata device for effective musculoskeletal repair by incorporating polymer into the scaffold in a unique, controlled manner to impart targeted mechanical strength and retain biocompatibility. Engineered fascia may provide a natural, strong, and mechanically robust scaffold for bridging large muscle/tendon deficits to bone, repairing rotator cuff tendons, or repairing abdominal wall fascia that has been herniated or released in compartment syndromes. The specific research aims of this work to date have been: (1) fascia scaffold development and (2) fascia scaffold assessment in a human cadaver model.

Fascia Scaffold Development. The scaffold development work first consisted of studies to incorporate

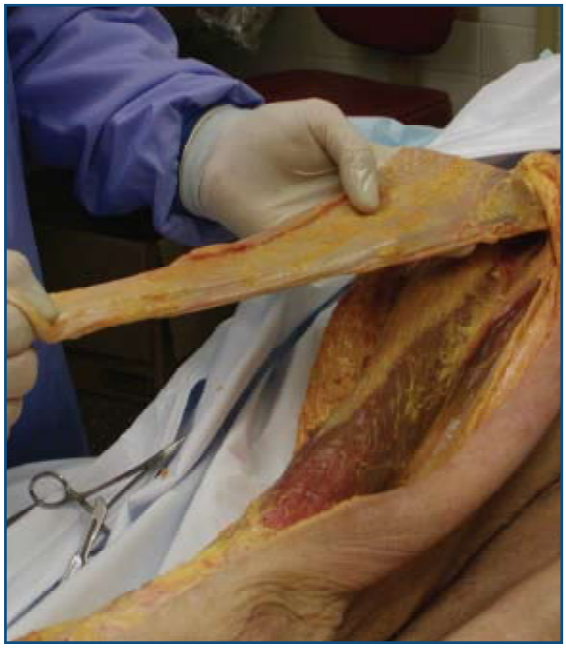


Figure II-8. Dissection of donor fascia lata.

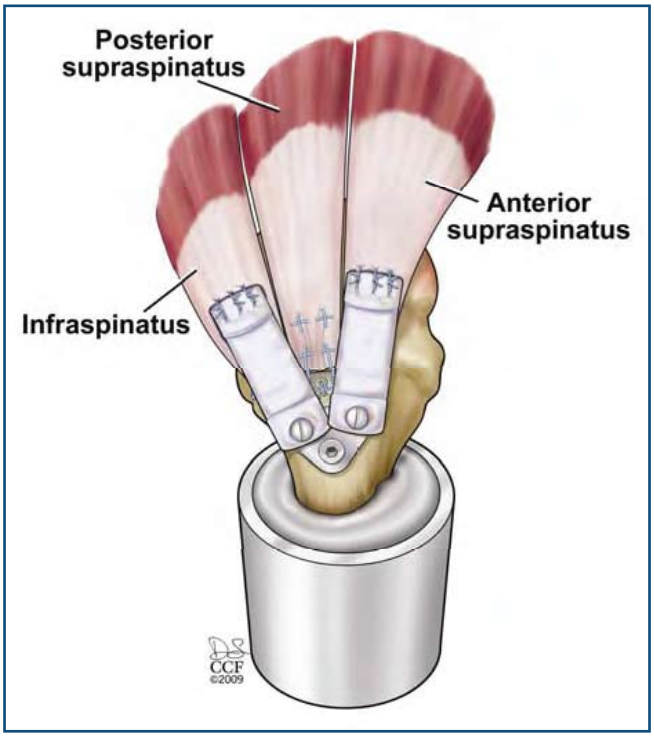


Figure II-9. Human cadaver rotator cuff injury and repair model to investigate the biomechanical properties of repairs performed with or without augmentation with reinforced fascia ECM.

various polymer fibers into fascia scaffolds to improve suture retention and tests to evaluate fiber type and configuration in vitro. 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 determined that reinforcing fascia ECM with resorbable polymer fibers significantly increased its suture retention strength and resistance to cyclic fatigue. A custom resorbable PLA/PGA braided fiber from Concordia Fiber, in Coventry, Rhode Island, emerged as the fiber of choice. However, to expedite FDA clearance for its reinforced fascia device, the team is trying to identify an FDA-approved fiber to use. The researchers have identified the best means to incorporate fiber into fascia and the optimum methods for affixing the engineered fascia scaffolds to bone. 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.

Fascia Scaffold Assessment in a Human Cadaver Model. The objectives of this research are to assess the extent to which augmentation with engineered fascia improves the biomechanical outcomes (repair gapping, stiffness, failure load) of rotator cuff repairs in human cadaver shoulders. This required the researchers to develop a method to monitor tendon repair gap formation during cyclic testing and to refine their human cadaver rotator cuff repair model (**Figure II-9**). Both of these objectives have been met, and cadaver studies are commencing. Magnetic bead testing was conducted to monitor tendon repair gap formation during cyclic testing and for real-time monitoring of the human cadaver rotator cuff repair model.

Key Research Accomplishments

- 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.
- 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.
- Established a magnetic displacement tracking system and a multiactuated mechanical test system for human cadaver studies.
- 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.

Conclusions

The researchers developed a means to reinforce fascia lata with a biodegradable polymer that significantly increases its suture retention strength to physiologically relevant loads. They demonstrated a way to fix the reinforced fascia scaffold to both bone and human rotator cuff tendon and were able to craft large patches or strips of the reinforced fascia ECM. Reinforced fascia may provide a natural, strong, and mechanically robust scaffold for rotator cuff tendon repair, bridging large muscle/tendon deficits

to bone and repair of fascia that has been released in compartment syndromes.

Research Plans for the Next 4 Years

In Years 2 and 3, the researchers will continue discussions with their regulatory consultant, Howard Schrayer, and the Musculoskeletal Transplant Foundation (MTF) to identify a preferred fiber to use in reinforcing the fascia scaffold. They will then undertake device efficacy studies in three model systems: (1) rat subcutaneous implantation, (2) human cadaver rotator cuff injury and repair, and (3) canine in vivo rotator cuff injury and repair. The researchers will use animal data and manufacturing tests to apply for FDA clearance through the 510(k) mechanism. The efficacy studies in animals will be completed, and an application for FDA clearance will be submitted by the end of Year 3.

By Years 4–5, the project team expects that its device will be advanced into clinical trials for rotator cuff repair. While rotator cuff studies are recognized to be of secondary military importance, efficacy studies for this model are most likely to first reach clinical trials and commercialization with industry partners because of the large civilian patient population that is available. Furthermore, improved outcomes for rotator cuff repair would have a positive impact on the combat readiness of soldiers who experience these injuries. During the next 4 years, the researchers plan to concurrently develop this device for military trauma applications, such as bridging large muscle/tendon deficits to bone, and for the repair of abdominal wall fascia that has been herniated or released in compartment syndromes.

Planned Clinical Transitions

The researchers have outlined necessary steps to seek relatively rapid approval from the FDA of the engineered fascia construct with their regulatory consultant, Howard Schrayer. They identified a commercial partner for the technology, the MTF, and signed both an Option to License Agreement and a Sponsored Research Agreement in June 2009. As they complete the human cadaver efficacy study and animal studies (including rat in vivo degradation study) over the next 2 years, the researchers expect that MTF will perform a number of the necessary production and regulatory steps required for FDA clearance of the device (identifying packaging components, preparing a design history file, performing a shelf-life/stability study, etc.). The researchers will conduct the canine rotator cuff injury and repair study, and by Years 4–5, they expect to have a device that is ready to be advanced into clinical trials for rotator cuff repair.

Corrections/Changes Planned for Year 2

The research team's rat abdominal wall experiments were delayed due to required work with the regulatory consultant and commercial partner to identify a preferred fiber (i.e., resorbable, braided, and already FDA cleared) for this application. Hence, some of the proposed Year 1 work has been rescheduled for Year 2. Further, a Sponsored Research Agreement with the MTF was executed on June 10, 2009. This will allow researchers to perform the human cadaver studies as part of the MTF contract instead of the AFIRM contract, as originally proposed.

Project 4.4.3b, RCCC

Functional Scaffolds for Soft Tissue Repair

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

Project Team: *Eric A. Balint, Asa Vaughan, PhD, (Rutgers – The State University of New Jersey)*

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*

Key Accomplishment(s): *The researchers have 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.*

Introduction

The menisci of the knee joint are two C-shaped discs of fibrocartilage found between the long bones of the leg. They play a critical role in the load transmission and shock absorption of the knee and aid in joint stability and lubrication. Despite the recognized importance of the tissue, arthroscopic removal of a torn meniscus is commonly performed in the United States even though poor long-term outcomes include loss of meniscal function and onset of degenerative osteoarthritis of the knee. Since meniscal tissue does not heal well, there are few treatments for significant meniscal deficiency. Some researchers employ natural (collagen-based) or synthetic polymer sponges as tissue engineering scaffolds to rebuild the meniscus. But both collagen and polymer implants have limited mechanical strength and organization when compared to natural cartilage tissues. Because the infiltrating cells are not given the proper mechanical environment, it is unlikely neotissue will form and remodel into a fibrous cartilage structure.

The goal of this study is to develop a clinically relevant, tissue-engineered scaffold that can be implanted in tissue such as the knee meniscus to prevent the onset of degenerative osteoarthritis associated with meniscectomy. The fiber scaffold will be made of hybrid collagen and a custom polymer (**Figure II-10**) to help regenerate cartilage tissue. If successful, the end product would be a device ready for use in service members suffering from moderate to severe meniscal damage. The technology could ultimately be developed for spine, craniofacial, and other extremity and scarring management applications.

Research Progress – Year 1

This project had three phases during Year 1:

(1) Evaluate the biological incorporation of a collagen/fiber scaffold under load-bearing conditions using an in vivo plug implantation model in sheep. In this experiment, portions of the fiber-reinforced scaffold were implanted into a surgically created circular defect in the sheep meniscus to determine the incorporation of and overall biological

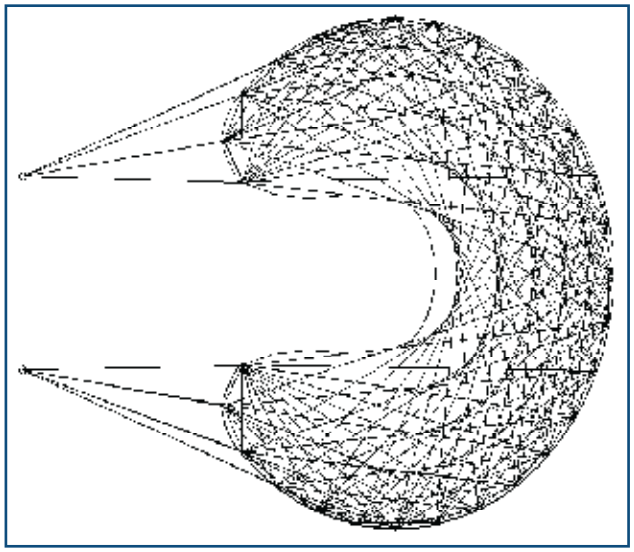


Figure II-10. Schematic of biodegradable polymeric fiber-reinforced collagen sponge meniscus replacement.

response to the scaffold material when implanted in a fibrocartilaginous tissue (**Figure II-11**). The researchers found that the meniscus failed in the animals at 8 weeks, which makes it a nonfunctional biocompatibility model. However, since previous in vivo experimentation had shown that the scaffold materials supported cellular and tissue ingrowth and would incorporate into synovial tissues, the team decided to continue with its evaluation of the scaffold as an implant to replace tissue after a full meniscectomy.

(2) Optimize the surgical procedure for implantation of a full meniscus scaffold at the site of a surgically resected meniscus. Mock surgeries were performed on three harvested sheep legs to optimize the surgical implantation procedure for full meniscal scaffolds. Due to the novelty of this type of scaffold, an implantation protocol had to be developed. The surgical and basic science/engineering team members worked to establish an efficient method for implanting the scaffold into the site of a total meniscectomy. Due to the size of the ovine knee, an open arthrotomy method was chosen. The surgeons removed the medial meniscus, fed the horns of the meniscal scaffold into bone tunnels made in the tibial plateau at the anterior/posterior horns, and anchored the implant with suture. After the mock surgeries, the team made a design modification to add a reinforcing peripheral stitch along the outside of the scaffold. They felt that this would help maintain the position of the fibers, and thus the shape of the implant, under handling and repeated loading.

(3) Short-term, full functional evaluation of meniscus scaffold implanted at the site of total meniscectomy in an ovine model. The team evaluated short-term implantations of the meniscal scaffold in sheep (**Figure II-12**) and measured the scaffold's performance by gross observations of joint tissues, as well as biomechanical, histological, and immunohistochemical analyses. By 8 weeks post-implantation, the researchers observed a significant amount of new tissue being laid down within the scaffolds. Newly synthesized collagen was seen to organize itself along the longitudinal axis of the polymer fibers. Some degeneration of cartilage was seen, especially when the posterior bone channel was placed too far forward, leading to excessive shear stress. The surgeon made modifications as needed to ensure proper placement of scaffold anchor attachments in future surgeries.

Overall, the research showed that the hybrid scaffold promotes the synthesis of organized, new tissue when implanted as a meniscal replacement. The team has demonstrated that the mechanical strength in hybrid scaffolds is dependent on both fiber density and fiber pattern and that its design can achieve mechanical properties comparable to those of the normal meniscus. Moreover, the tests of scaffold implantation in a meniscal reconstruction model demonstrated intact mechanical function and both tissue integration and biocompatibility in the synovial environment. Tests will continue with the sheep model in Year 2.

Key Research Accomplishments

- Developed a modified surgical procedure for meniscal scaffold implantation in an animal model

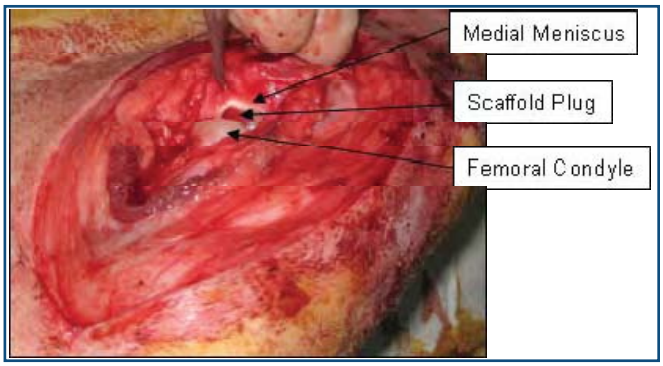


Figure II-11. Scaffold plug inserted into 6 mm punch biopsy taken from right leg, medial meniscus of ovine. Scaffold plugs were soaked in autologous fibrin glue and then press-fit into the meniscus.

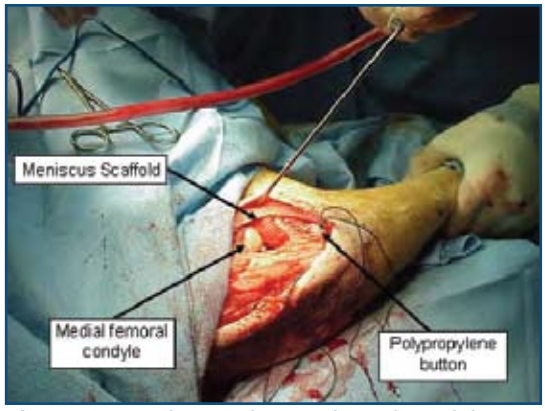


Figure II-12. Picture of open sheep knee joint after surgical implantation of a meniscus scaffold. The scaffold, femoral condyle, and fixation button are noted.

based on mock surgeries and initial results from full scaffold implantations.

- Completed full meniscal scaffold implantation surgeries. 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.
- 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.

Conclusions

The research team obtained very promising results from the first-generation meniscus scaffolds tested in animals. The scaffolds have been shown to be safe when implanted in a synovial environment in animal models. The matrix portion of the scaffold degrades significantly by 8 weeks and is replaced with new collagen organized along the longitudinal axis of reinforcing fibers. The degenerative changes observed in the articular cartilage in the animal tests prompted several design modifications. First, the surgical procedure was modified to ensure more precise and accurate placement of the scaffold anchors, which are necessary for proper biomechanical function of the implant. Second, the matrix portion of the scaffold will be modified to contain and promote the synthesis of proteoglycans (glycoproteins essential for the compressive resilience of scaffolds). The team will fabricate second-generation scaffolds and test them at longer time periods to determine their performance with regard to tissue incorporation, neotissue formation, and protection of the articular surfaces, advancing this new technology one step further toward human trials.

Research Plans for the Next 4 Years

In Year 1, the researchers showed that a novel meniscus scaffold—a hybrid of high-strength resorbable polymer fibers arranged within a collagen matrix—promotes the synthesis of new, organized tissue when implanted as a meniscal replacement. In Years 2–5, the team plans to fabricate second-generation meniscus scaffolds by optimizing the reinforcing fiber network and modifying the collagen sponge component of the scaffold. The goal is to improve the mechanical and compressive properties of the scaffold and encourage the ingrowth and maturation of fibrocartilagenous tissue following implantation. Researchers will evaluate the second-generation meniscus scaffolds in vivo in sheep, comparing two surgical techniques—the "meniscus plug" (partial meniscectomy) and total meniscectomy—to determine the amount and type of tissue ingrowth at 3, 6, and 12 months post-surgery. In addition to evaluating the "neomeniscus" tissue, the researchers will evaluate the knee cartilage to determine whether the cartilage structure is maintained in this mode.

Planned Clinical Transitions

The team plans to consult with the FDA in a pre-IDE meeting; the timing depends in part on the results of the large animal studies. Pending success of the large animal studies, the project will translate to clinical trials. It is likely that the clinical trials will initially be conducted through the RCCC, and the researchers expect the trials to be funded partly by the company that licenses the technology. It is also possible that the project leaders may obtain separate funding to create a start-up company to foster development of this technology and to accelerate preclinical and clinical trials. Specific details of the planned FDA meetings, industrial interactions, and Institutional Review Board strategies will become clearer in Year 2 as the team proceeds with large animal studies using the second-generation meniscus replacement devices.

Corrections/Changes Planned for Year 2

None.

Project 4.4.6, WFPC

Oxygen-Generating Biomaterials for Large Tissue Salvage

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

Therapy: *Supply temporary oxygen to tissue during vasculature establishment or repair*

Deliverable(s): *Injectable oxygen-generating biomaterial*

Key Accomplishment(s): *The researchers have prepared a controllable, injectable oxygen-generating biomaterial that could produce oxygen for up to 3 days.*

Introduction

Lack of oxygen, caused by a disrupted or nonexistent vasculature, is a key factor that limits tissue salvage, wound healing, and viability of large engineered tissue constructs. Clinically, elevated levels of oxygen have been used in wounds with reduced vascularity, irradiated tissue, and infected dermal wounds to improve wound healing. Such improvements in the healing and success rates of full-thickness skin grafts and flaps have been demonstrated using hyperbaric oxygen treatments. Lack of oxygen is also a common problem for tissue engineering. Implanted engineered tissue constructs that exceed the diffusion limits often become necrotic due to insufficient oxygen and nutrient delivery to cells. Thus, supplying oxygen appears to be a feasible method for improving success in all of these affected areas.

The researchers previously reported the synthesis of oxygen-generating biomaterials (Harrison et al., *Biomaterials* 28:4628-34). The technology involved a controlled chemical decomposition of solid sodium percarbonate to produce oxygen over a period of hours. Upon contact with water, solid sodium percarbonate decomposes producing oxygen as well as sodium and bicarbonate ions as biocompatible byproducts. The researchers have shown that oxygen-generating biomaterials are able to maintain cell viability under hypoxic conditions and that this material preserves tissue survivability and prevents necrosis during an ischemic event (**Figure II-13**).

The current project is focused on preparing an injectable form of a particulate oxygen-generating (POG) system (**Figure II-14**) that provides an in situ and sustained release of oxygen. Such material could create a favorable three-dimensional environment for maintaining cells in a hypoxic environment until other means of supplying oxygen to the tissue could take place.

Research Progress – Year 1

To overcome the issue of oxygen availability throughout a tissue, the researchers employed several strategies to facilitate the delivery of oxygen and improve the survival of implanted cells. Angiogenic growth factors such as vascular endothelial growth factor, which is a potent endothelial cell-specific mitogen, have been used to promote neovascularization. Although the effectiveness of an enhanced angiogenic response has been demonstrated in many tissue systems, the rate of angiogenesis could not be accelerated, thus limiting the size of implantable tissue masses. Another approach to maintain tissue viability in vivo is to place the engineered tissue adjacent to a heavily vascularized tissue such as omentum to achieve adequate vascularization. However, this approach may not always be feasible because the target implantation site may not be in close proximity to a heavily vascularized tissue. Other studies have examined the use of synthetic oxygen carriers such as perfluorocarbons and crosslinked hemoglobin.

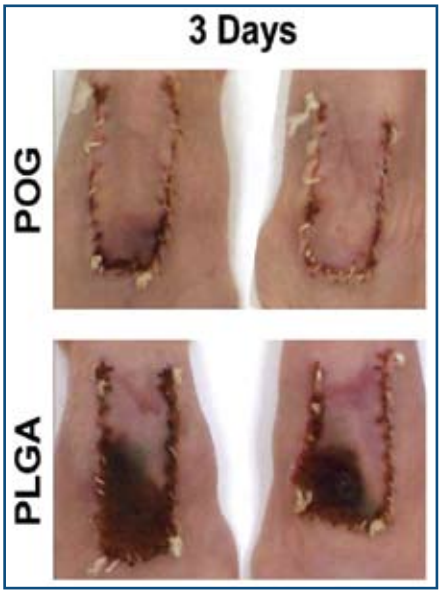


Figure II-13. Flap necrosis. Representative images of oxygen-producing (POG) and control (PLGA) groups at day 3 demonstrating a survival benefit for the polymeric oxygen-generating films group in the early time point.

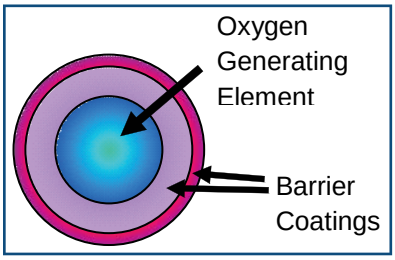


Figure II-14. Schematic of a POG.

Based on their preliminary results, the researchers expect that sustained generation of oxygen will enhance cell viability to improve the salvageability of large tissue masses and aid in the regeneration of engineered tissue.

Improvements in Injectable POGs

Reduction in particle size was accomplished by sequential sifting of smaller particles or rapid precipitation of the oxygen-generating element from its chemical precursors. Calcium peroxide-based POGs were obtained through sequential sifting. Particles were approximately 2–3 microns in diameter. The second method of rapid mixing of reactants resulting in precipitation also produces small particles because of the creation of a high density of nucleation sites. This method also has been attempted and found to be successful for creating particles even smaller than 1 micron.

To examine the effects on tissue, excised skeletal muscles from a recently sacrificed rat were injected with the injectable POG compositions. The muscles were kept at room temperature for 2 hours in phosphate-buffered saline followed by fixation, sectioning, and hematoxylin and eosin staining. Qualitatively, as shown in **Figure II-15**, the oxygen-generating gels (SPO) appeared to be less degraded than the control gel or untreated muscle (data not shown). However, it appears that significant optimization still needs to occur to maintain the 0 hour time point characteristics of normal skeletal muscle. This will be a focus of work in Year 2 of the project.

Key Research Accomplishments

- Produced an injectable solution capable of in situ oxygen generation.
- Demonstrated that oxygen production can be sustained for up to 3 days.
- Determined that the injectable oxygen-generating material is nontoxic to cells.

Conclusions

An injectable oxygen-generating biomaterial has been prepared. Oxygen generation from the nontoxic gel can be sustained for up to 3 days although further optimization is needed to meet the needs of the particular in vivo systems to be tested. A controllable oxygen-generating gel could serve as a supplement to treat ischemic tissue to help maintain the histomorphological and functional characteristics of the native tissue. This injectable oxygen-producing fluid could possibly prolong the viability of tissue that has been cut off from a blood supply or removed from the body altogether. Such a simple and portable approach could be used by first responders to treat damaged tissue until more permanent measures can be taken to recover the tissue as well as a tool for tissue engineers for improving three-dimensional tissue regeneration.

Research Plans for the Next 4 Years

During Year 2, the effects of POGs on cell viability and proliferation will be assessed in vitro. The researchers will also analyze for signs of angiogenesis in vitro. Ex vivo tests on tissue that has been recently excised will be injected with the oxygen-generating materials, and tissue morphology will be monitored over time. Oxygen-generating biomaterials will be further optimized based on these results. In vivo tests will begin in ischemic tissue models to determine the effect on tissue survival. During Years 3–5, additional effort on in vivo testing will be undertaken. This will include work in rodents and further assessing the effects

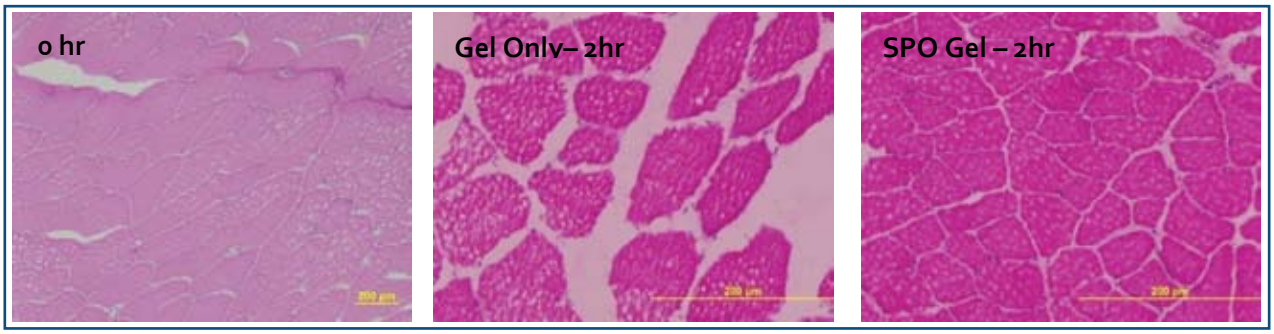


Figure II-15. Histological analysis of gels injected into ex vivo tissue.

on tissue regeneration. The results of the rodent studies will be used to determine the best large animal model to test the oxygen-generating material.

Planned Clinical Transitions

Not specified.

Corrections/Changes Planned for Year 2

Because the optimal application for oxygen-generating biomaterials has yet to be determined, the biocompatibility of these materials will not be limited to a single tissue type (e.g., wound healing of excisional grafts) since limb and digit are a composite tissue made of bone, muscle, skin, etc. In addition, sufficient progress in Year 1 was made to begin preliminary in vivo studies in rodents to accelerate translation and optimize the understanding of the oxygen-producing material.

Project 4.4.1/4.4.2, RCCC

4.4.1–Optimizing Nerve Conduit Scaffolds for the Repair of Segmental Nerve Defects

4.4.2–Cell and Bioactive Molecular Delivery to Enhance the Repair of Segmental Nerve Defects

Team Leader(s): *Michael J. Yaszemski, MD, PhD, Brig. Gen. USAF; Anthony Windebank, MD (Mayo Clinic)*

Project Team: *Raphael Walker-Santiago, Huan Wang, MD, PhD, Bingkun Chen, MD, PhD, Andrew Knight, PhD, Mahrokh Dadsetan, PhD, Diana Angius, MD, Gemma Rooney, PhD, Jewel Podratz, LouAnn Gross, Jarred Nesbitt (Mayo Clinic)*

Collaborator(s): *Ralph Carmichael, BonWrx, Inc., Phoenix, Arizona*

Therapy: *Treatment of peripheral nerve lacerations or segmental defects*

Deliverable(s): *Polymer and cell-based delivery systems for delivering neuronal growth factors to large segmental nerve defects in limbs*

Key Research Accomplishment(s): *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 MSCs provide a potential cell-based delivery platform for growth factor delivery.*

Introduction

From March 2003 to the present, American warfighters have incurred injuries that involve disruption of peripheral nerves in salvageable extremities. These injuries are frequently complicated by extensive regional loss of overlying soft tissue and muscle and often involve loss of entire segments of major nerves spanning 5–20 cm (**Figure II-16**). Restoring nerve function is essential for restoring limb function. A limb salvaged without nerve function is useless and usually painful. Nerve conduits are already in commercial use for the repair of small defects in sensory nerves; however, none are suitable for use with nerve gaps greater than 4 cm, and none are recommended for use in motor nerves.

Novel scaffold materials for nerve conduits are being developed and tested in three laboratories (Mayo Clinic, MIT, and Rutgers), MSC therapies are being developed at the Mayo Clinic, and decellularized allograft is being tested at the Cleveland Clinic. Projects 4.4.1 and 4.4.2 share the goals of enhancing the repair of large segmental nerve defects necessary for limb salvage procedures. These projects involve optimizing nerve conduit scaffolds and cell and bioactive molecular delivery of neuronal growth factors. They are reported together from the same group at the Mayo Clinic.

The contemporary standards of care for treatment of peripheral nerve lacerations or segmental defects include primary repair or nerve grafting. Direct coaptation of severed nerve ends provides the best opportunity for regeneration. When direct repair is not possible, the most effective method to reestablish functional nerve continuity is the use of an autologous nerve graft. However, autologous nerve grafts have several drawbacks. As an alternative to nerve autografts, a number of different natural materials have been explored for use in

Figure II-16. Upper limb injury in a warfighter. There is massive loss of tissue in the distal forearm with relative preservation of muscles, bone, blood vessels, and skin in the hand. Without repair of the large segmental nerve gap (~25 cm), there will be no restoration of hand function (Image: M.Yaszemski, 2006).

aiding nerve regeneration, including vein segments and nerve sheath segments of both autogenous and allograft origin.

The researchers seek to develop polymer nerve conduits capable of repairing large defects in mixed nerves necessary for limb salvage procedures or for the repair of small defects in motor nerves in the face. These conduits will have the ability to guide accurate regeneration across the defect, resulting in a higher degree of motor and sensory recovery distal to the nerve injury. Various polymers will be screened to identify the best candidate polymer for use as a nerve conduit. Upon polymer selection, single and multi-lumen conduits will be fabricated to investigate the effect of tube architecture on nerve regeneration. The conduit will also be conditioned with various growth factors and/or cells to augment the efficacy of the nerve conduit and increase functionality of the newly regenerated nerve. Upon completion of the research, an off-the-shelf nerve conduit enhancing nerve regeneration and functional restoration comparable to an autologous nerve graft will be available for clinical application.

To achieve the goals described earlier, the researchers have the following specific objectives: (1) development of a well-validated animal upper limb model to mimic single or multiple nerve injuries that can be evaluated quantitatively because upper limb injuries are generally more common than lower limb injuries in the civilian and warfighter populations, and there are no well-validated animal upper limb models; (2) in vitro and in vivo neuronal growth factor release studies of axonal growth including studies of size and viscosity of microspheres used to deliver the growth factor, as well as other factors affecting growth factor degradation; (3) comparison of different synthetic polymer conduits and study of decellularized allografts and biological scaffolds with various structural configurations; (4) a pilot proteomic analysis of nerve regeneration; and (5) stem cell studies including stem cell-enhanced polymer conduits and FDA approval for the manufacture of autologous human MSCs.

The studies in these projects share numerous methods. Construction of conduits includes using a vacuum solvent-extraction/molding technique. Candidate polymers include polycaprolactone fumarate (PCLF), PLGA, oligo-(polyethylene glycol) fumarate hydrogel (oligo-PEGF), and surface charge variants of oligo-PEGF. Biologically derived conduits include collagen tubes (commercially available and in-house) and decellularized nerve sheath (processed by Axogen). Nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) for the potential enhancement of the nerve conduits include the PC12 cell line (surrogate neurons) and primary dorsal root ganglion neurons. Animal models currently used for the in vivo studies are the rat sciatic nerve 1 cm gap model and the rat forelimb complex nerve injury (median, ulnar, radial nerve). To assess the functional recovery and nerve repair efficacy following in vivo experiments, various evaluation methods have been developed and used including compound muscle action potential measurement, somato-sensory evoked potential measurement, and two-dimensional video gait analysis.

Research Progress – Year 1

To reliably assess the results for the various conduits of interest, an upper limb model of nerve injury needed to be developed. The Yaszemski-Windebank team therefore developed a model that would mimic single or multiple nerve or root injuries in the rat forelimb. The researchers carried out validations of quantitative evaluation modalities in both upper and lower limb models of the rat. These evaluation modalities reflect nerve function and correlate with recovery following nerve repair.

In vitro and in vivo neuronal growth factor release studies. NGF and GDNF were tested for the potential enhancement of the nerve conduit in microspheres. Release of growth factors was biologically active and degraded appropriately. A short-term microsphere/growth factor study was completed and demonstrated a clear benefit on axonal growth of delivering GDNF at a dose of 50 µg/mL. Microsphere growth factor studies using both NGF and GDNF are more than 60% complete, and results indicate that these treatments have similar compound muscle action potentials to autologous grafts.

Comparison of synthetic polymer conduits is two-thirds complete. Four groups of surgeries were carried out to bridge a 1 cm sciatic nerve gap. These groups include an autologous nerve graft, PCLF tubulization, PLGA tubulization,

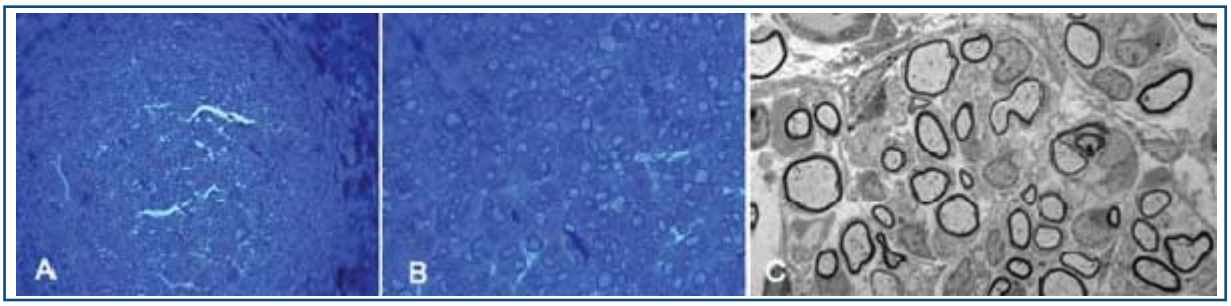


Figure II-17. Mid-tube section of rat sciatic nerve defect bridged by 1 cm in-house single-channel collagen tube harvested at 4 weeks following implantation. A core of regenerating axons is surrounded by a thick perineurium-like sheath. Toluidine blue staining showed myelinated fibers (dark blue circles) organized into microfascicles typical of a regenerating nerve (A 40x, B 100x). Transmission electron microscopy of the same sample showing myelinated axons with typical Schwann cell morphology including extracellular deposition of collagen and basal lamina (C).

and PCLF-oligo(polyethylene glycol) fumarate tubulization. Nerve conduction study, gait analysis, muscle weight, and isometric muscle force measurement were acquired from these groups. There was successful reestablishment of innervation of target muscles in all groups. Study of the decellularized allograft has just been completed; comparison of the data showed that the PCLF tube and the decellularized allograft were superior to other tube materials.

A study of biological scaffolds designed to test in-house collagen tubes with various structural configurations (e.g., single channel and various multiple channels) is under way. Preliminary results indicate that these tubes are strong enough to hold sutures. Microscopy showed early regeneration in single channel tubes 4 weeks following implantation (**Figure II-17**).

There are many changes occurring following nerve injury and repair that are complex and difficult to understand. Changes in protein profiles in scaffold-supported regeneration compared with those occurring in nerve regenerating under ideal conditions (nerve crush) or uninjured nerve may yield novel therapeutic targets. To better understand factors that regulate nerve growth, a pilot study of proteomic analysis has been carried out, and the proteomic data are being analyzed. Technical reproducibility is high. A longer term study of proteomic analysis has begun.

Stem cell studies. Project 4.4.2 seeks to determine whether MSCs will enhance scaffold-supported regeneration. MSCs may provide a "perineurium-like" structure that ensheaths the regenerating nerve and allows better contact between the regenerating nerve and the supporting scaffold. Enhancing contact between material and regenerating nerve is regarded as a key step in enhancing nerve repair. The second role for MSCs is to provide a source of growth factors to guide regenerating axons toward their target. The Yaszemski-Windebank team has developed lentiviral constructs to transduce MSCs to express NGF, GDNF, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and ciliary neurotrophic factor.

A study of stem cell-enhanced polymer conduits has just begun in vitro and in vivo. Adipose tissue-derived stem cells are injected into the PCLF conduit for repair of 1 cm sciatic nerve gap. The Mayo team has FDA approval for the manufacturing process for adipose-derived autologous MSCs. This cell manufacturing process is immediately transferable to any group with an accredited current good manufacturing practice (cGMP) cell-processing laboratory. The cells will be ready for bone and nerve AFIRM projects when all other components to the project are ready.

Key Research Accomplishments

- Optimized functional evaluation modalities, specifically those for motor functions, including computerized video motion analysis, noninvasive nerve conduction studies, isometric muscle force measurement, grasp task, and vertical rope climbing test. In addition to the rat sciatic nerve injury model, upper limb nerve injury and repair models have also been developed and validated. These models and functional evaluation modalities laid foundations for testing the

conduits under development in a more complex and clinically relevant context.

- Demonstrated that neuronal growth factors can be encapsulated and released from polymer scaffolds and polymer microspheres in a biologically active form and with a time course suitable to enhance peripheral nerve regeneration.
- Constructed four types of polymer scaffolds (i.e., PCLF, PLGA, oligo-PEGF, and positively charged oligo-PEGF) for peripheral nerve application and showed that these scaffolds are mechanically suitable and biologically compatible.
- Tested multiple polymer scaffolds in a rat 1 cm sciatic nerve defect model and demonstrated the ability of the synthetic conduits to guide nerve growth across the injury gap and support axonal regeneration.
- Formulated the concept of multichannel nerve conduits and showed its potential advantage of facilitating more accurate nerve regeneration and therefore better functional recovery.
- Initiated proteomic analysis for investigation of protein profile changes following nerve injury and repair and obtained preliminary data.
- Designed model of nerve injury accompanied with soft tissue assaults to investigate nerve growth in an ischemia/fibrotic soft tissue bed to facilitate studies of complex nerve injury and repair and further development of strategies to enhance nerve regeneration in a complex wound often seen in war injuries.
- Obtained FDA approval for manufacture of autologous human MSCs under cGMP.
- Agreement with an industrial partner (Bonwrx) to conduct preclinical toxicology and safety testing for nerve polymer candidates. Bonwrx has taken polymer products through the FDA and CE (Conformité Européene) marking processes and has a fully accredited cGMP-compliant site in Phoenix, Arizona.

Conclusions

The Mayo team demonstrated that NGFs and GDNFs may be encapsulated and released from polymer scaffolds and polymer microspheres in a biologically active form and with a time course suitable to enhance peripheral nerve regeneration. The team also showed that MSCs provide a potential cell-based delivery platform for growth factor delivery. The in-house polymer conduits are mechanically suitable for repairing nerve defect and support nerve regeneration across a 1 cm gap. Reestablishment of useful muscle innervation is shown by the recovery, although partial, of compound muscle action potentials and gait patterns. The best candidate polymer will be chosen once the tissue processing, neuromorphometric analysis, and statistical analysis are complete.

Lower and upper limb nerve injury and repair models have been established along with validated evaluation modalities. A solid foundation for screening, testing, and comparing various nerve tubes has also been developed. A standard procedure of stem cell preparation has been established. Once the appropriate polymer is selected and fabricated into a conduit, it can be combined with the proper growth factors and cells to further optimize the nerve tube construct and move toward preclinical and clinical application.

Research Plans for the Next 4 Years

Early in Year 2, the researchers will meet to define a stage-gate process for selecting the best candidate polymers for nerve repair. This will involve establishing a single animal model for nerve conduit polymer testing that can be applicable within the consortium. A "screening" model will be the rat 1 cm sciatic nerve defect model. The evaluation time will be 8 weeks for neuromorphometry and 16 weeks for functional/behavioral tests. A secondary model for testing prime candidates will be the rat 2 cm sciatic nerve defect model.

When a blast injury occurs in the limbs, it can result in loss of skin and muscles in addition to nerve and vessel damage. Nerve injury is rarely treated in a primary procedure. The priority and realistic initial treatment is management of the wound and restoration of blood circulation. This usually leaves a limb with extensive scarring, muscle defect, or fibrosis. Nerve repair and reconstruction in this fibrotic and ischemic environment present a challenging problem. In Year 2, the researchers will extend the development of a complex wound model, which will include muscle ischemia/fibrosis, skin/muscle necrosis, segmental bone defect, and pos-

sibly infection. They have initiated the first phase, which involves the development of an ischemia/fibrotic nerve injury model.

Planned Clinical Transitions

Two strategies are being pursued to repair large segmental peripheral nerve defects: a transplantation strategy (human cadaver allograft nerve sheath with and without supplementation with isogenic or allogenic bone marrow stromal cells) and a polymer scaffold-based tissue engineering strategy. The transplantation strategy will be tested in the same model allowing objective comparisons to be made between materials. Clinical translation of each material may vary with respect to FDA interactions and requirements. If the allograft sheath plus saline or isogenic cell strategies are successful in a small number of animals in the tiered model systems, they will be immediately brought to clinical transition by the end of Year 2 using an adaptive human trial.

Comprehensive tissue-sourcing and processing agreements are already in place with a leading tissue procurement agency (Lifebank). The use of sheath plus allogeneic cells will follow a clinical 510(k) approval strategy common to other polymer-based materials. Networks available for clinical trials in nerve repair, both within and outside the United States, are being developed through professional contacts among the integrated team members. Appropriate confidentiality and intellectual property agreements are in place between appropriate commercial partners and relevant institutions and the AFIRM.

Validation of sterilization procedures has begun in the nerve group for the tissue-engineered, polymer-based scaffolds. The goal is to develop and validate a sterilization method that will be generally applicable to polymer constructs developed through the AFIRM. As in the RCCC bone group, as candidate material scaffolds move from the intermediate to a more definitive preclinical large defect model, a business plan and economical feasibility analysis of scaling to mass production will be performed.

Corrections/Changes Planned for Year 2

None.

Project 4.4.1a, RCCC

Optimizing Nerve Conduit Scaffolds for the Repair of Segmental Nerve Defects Developing Biodegradable Polyester "Biorubber" Nerve Regeneration Conduits

Team Leader(s): *Daniel Anderson, PhD, Robert Langer, PhD (MIT)*

Project Team: *Hao Cheng, PhD, Nathaniel Vacanti (MIT)*

Therapy: *Treatment of nerve segmental defects*

Deliverable(s): *Biodegradable polyester "biorubber" nerve regeneration conduits*

Key Accomplishment(s): *A family of new, biodegradable sebacate- and xylitol-based polyesters has been synthesized and a series of various nerve conduits has been fabricated, showing mechanical properties that mimic peripheral nerve, biocompatibility, and a degradation rate superior to conventional nerve conduits.*

Introduction

The adult peripheral nervous system is capable of some functional repair. For a short segmental defect, direct coaptation of the two severed nerve ends provides the best opportunity for regeneration to occur. For a large defect, the use of autologous nerve graft is currently the most common clinical therapy. However, the drawbacks associated with autologous nerve grafts, including loss of function in the donor sensory nerve distribution and the limited supply of nerves, have motivated researchers to look for alternative methods to repair large defects. Synthetic conduits fabricated from polymers such as PGA or natural materials, such as bovine collagen, have proven successful in repairing small nerve gaps. Unfortunately, these conduits are still suboptimal because of suboptimal biocompatibility and poor mechanical properties and are not as effective as autologous nerve grafts in repairing nerve defects.

The goal of this project is to develop new synthetic nerve grafts that perform as well as or better than autologous nerve grafts. To this end, new elastic, degradable synthetic materials, termed "biorubbers," are being developed and fabricated into a variety of nerve conduits with potential use in peripheral nerve repair. Poly(glycerol-co-sebacate) (PGS) is one such polymer characterized by improved biocompatibility and mechanical properties similar to those of soft tissue. Small libraries of related materials, for example, poly(xylitol-co-sebacate) (PXS) and poly(glycerol-co-sebacate)-acrylate (PGSA), have also been synthesized with tunable degradation rates and mechanical properties. These materials are being developed in combination with natural materials to fabricate nerve guide conduits that promote nerve growth. Advanced manufacturing techniques including micropatterning and nanofiber electrospinning are being explored to manipulate the topography of conduit surface to enhance axonal growth.

Research Progress – Year 1

PGSA synthesis and fabrication of biorubber nerve conduits. One of the key parameters governing the mechanical properties of the biorubbers is their degree of crosslinking. The MIT team sought to optimize this property for the purposes of nerve repair. PGS pre-polymer is synthesized through polycondensation of glycerol and sebacic acid and then modified with varying degrees of acrylation. PGSA is then crosslinked by ultraviolet irradiation. A dip-coating method is used to fabricate PGSA conduits. To promote crosslinking, the team optimized the degree of acrylation of PGSA.

PXS biorubber conduit fabrication, characterization, in vivo biocompatibility, and degradation rate. Polymers with chemical structures similar to PGS, known

as PXS have also been developed. PXS is synthesized through polycondensation between xylitol and sebacic acid. The advantage of this material is that its mechanical properties and degradation rate can be easily controlled by the adjustment of the xylitol to sebacic acid ratio during synthesis. Currently, the MIT team is testing the degradation rate and biocompatibility of the PXS conduits in vivo. Histological studies of the conduit at this time revealed only minor inflammation caused by the conduit and no notable perivascular infiltration in the surrounding tissue (**Figure II-18**).

Multichannel PXS biorubber conduits. Multichannel conduits may prove superior in their ability to support nerve repair as compared to single-channel conduits. The MIT team developed multichannel conduits using an optimized PXS biorubber and a modified fabrication method developed by the Vacanti laboratory at Massachusetts General Hospital (**Figure II-19**).

Electrospun fiber alignment. Studies on electrospinning have increased greatly in recent years because of the ability to use this technique to generate biomaterial fibers that mimic the natural ECM. Aligned fibers have been reported to help guide the growth direction of neurons in vitro. The Langer group modified the interior surface of the polymer conduits with nano or submicron textured grooves to provide topographical cues for nerve growth. The density of the aligned fibers is critical for nerve regeneration; the MIT team is currently working on optimizing the density of the aligned fibers within the mat.

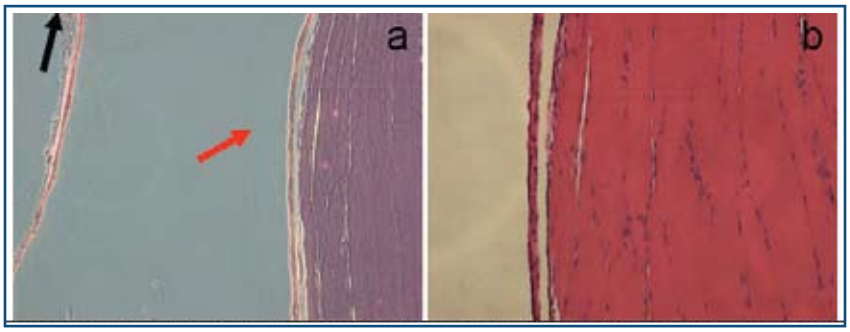


Figure II-18. Hematoxylin and eosin staining of the cross-section (along the long axis) of the PXS conduit 1 month after the conduit was implanted in the rat leg adjacent to the sciatic nerve. The inflammatory area caused by the suture in (a) is indicated by a black arrow. The red arrow indicates the boundary of conduit wall. (b) Surrounding tissue of PXS conduit.

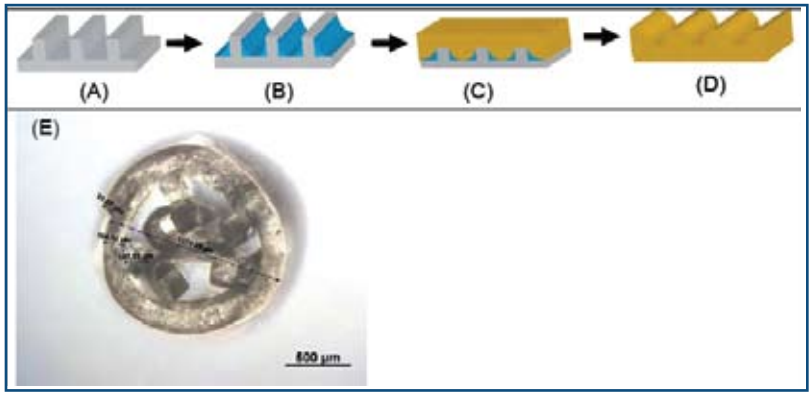


Figure II-19. Schematic diagram showing how to prepare patterned PXS films and the cross-section of a multichannel PXS conduit. (A) Place patterns on a silicon wafer. (B) Spin coat a thin sacrificial sucrose layer on top of the wafer for PXS film release. (C) Spin coat a PXS layer on top of the sucrose layer. (D) Dissolve the sucrose layer in water and release the PXS film after curing. (E) Cross-section of a multichannel PXS conduit.

Key Research Accomplishments

- Synthesized PGSA and fabricated PGSA biorubber nerve guide conduits.
- Synthesized PXS and fabricated single-channel PXS biorubber nerve guide conduits.
- Fabricated multichannel PXS nerve guide conduits.
- Fabricated PXS films with aligned electrospun fibers.

Conclusions

A family of new, biodegradable sebacate- and xylitol-based polyesters has been synthesized, and a series of various nerve conduits has been fabricated, showing mechanical properties that mimic peripheral nerve and biocompatibility and a degradation rate superior to conventional nerve conduits. The MIT team is systematically examining the performance of its novel conduit architectures in vivo.

The ultimate effectiveness of these synthetic nerve guides will be determined through in vivo testing. The in vivo surgery of the sciatic nerve with PXS conduits will commence in July. All the conduits will be tested in a rat sciatic nerve model. Future work will include further optimization of the polymer composition, the structure of the conduit, surface patterning, and the filling of the inner lumen of the conduits with various materials including growth factors to help enhance regeneration.

Research Plans for the Next 4 Years, Planned Clinical Transitions, and Corrections/ Changes Planned for Year 2

Information can be found under RCCC Project 4.4.1/4.4.2.

Project 4.4.1b, RCCC

Optimizing Nerve Conduit Scaffolds for the Repair of Segmental Nerve Defects

Team Leader(s): *Melitta Schachner, PhD, (Rutgers – The State University of New Jersey)*

Project Team: *Kathryn Uhrich, PhD, David Shreiber, PhD, Jian Chen, PhD, Shirley Masand, Mindy Ezra, Jeremy Griffin, Joachim Kohn, PhD (Rutgers – The State University of New Jersey); Sally Meiners, MD (University of Medicine and Dentistry of New Jersey/ Robert Wood Johnson Medical School)*

Therapy: *Repair of peripheral nerve critical size defects with biodegradable, bioactive conduits*

Deliverable(s): *A mechanically flexible, robust, biodegradable polymeric conduit tube with collagen hydrogel filler containing peptide growth factors to repair peripheral nerve defects*

Key Accomplishment(s): *In vivo rat femoral nerve injury studies comparing polyethylene and tyrosine-based copolymer tubes filled with bioactive peptides grafted to collagen demonstrated that (1) collagen grafted with peptides significantly improves functional nerve regeneration and (2) the degradable tyrosine-based conduits outperform the polyethylene tubes.*

Introduction

Note: This project is AFIRM research fully supported in Year 1 by leveraged funding (DoD Award No. W81XWH-04-2-0031, Center for Military Biomaterials Research).

This project aims to create biodegradable entubulation grafts for the reconstruction of peripheral nerves that have experienced significant trauma resulting in a large (>1 cm) gap that needs to be bridged.

Currently, autograft entubulation is the clinical gold standard for peripheral nerve reconstruction. Biodegradable nerve guidance conduits are promising alternatives to autologous nerve or vein autografts for tissue regeneration since they do not require secondary nerve damage and loss of function at the donor site and can provide a more specific size and functional match. The goal of Project 4.4.1b is to identify a polymer for use as an entubulating material and a type I collagen-based hydrogel material for use as a supporting scaffold within the entubulation graft to enhance functional recovery following peripheral nerve injury.

The following two polymer families are being tested as entubulation materials: (1) tyrosine-based copolymers and (2) polyester copolymers. For both families of copolymers, collagen with immobilized peptides known to enhance peripheral nerve regeneration is being used as the filler material. These peptides are known to accelerate axon growth, improve neuron survival, and induce preferential motor reinnervation. The Shreiber laboratory has covalently grafted these peptides to collagen. The collagen self-assembles into a fibrillar gel upon incubation at physiological temperature and pH, which immobilizes the peptide to provide the distinct benefit of sustained and well-controlled presentation of the peptide. Previous work done by Schachner et al. has determined the bioactivity of these hydrogels in vitro. The tyrosine-based copolymers are being tested in a mouse femoral nerve injury model, and the polyesters are being tested in a rat sciatic nerve injury model. Promising results from one model will be tested in the other model.

Research Progress – Year 1

Femoral nerve injury. The femoral nerve injury procedure has been completed for the set of experimental conditions (n=4–5 per condition). C57/B6 female mice were subjected to a femoral nerve transection in the left leg approximately 3 mm proximal to the bifurcation point. The proximal and distal ends of the transected nerve were inserted into polyethylene or polycarbonate tubes and sutured in place. The lumens of the tubes were filled with saline, native collagen, or peptide-grafted collagen. The animals were videotaped weekly for 15 weeks for functional assessment. After 15 weeks, a secondary procedure was performed in which the muscle and cutaneous branches of the femoral nerve were

severed approximately 5 mm distal to the bifurcation, and different fluorescent retrograde tracers were applied to each branch. To assess the accuracy of regeneration by evaluating the population of motor neurons in the ventral horn, animals were observed again after 1 more week, during which time the dyes were transported retrogradely to nuclei in the spinal cord. Spinal cords were harvested for immunohistochemical analysis after fixation.

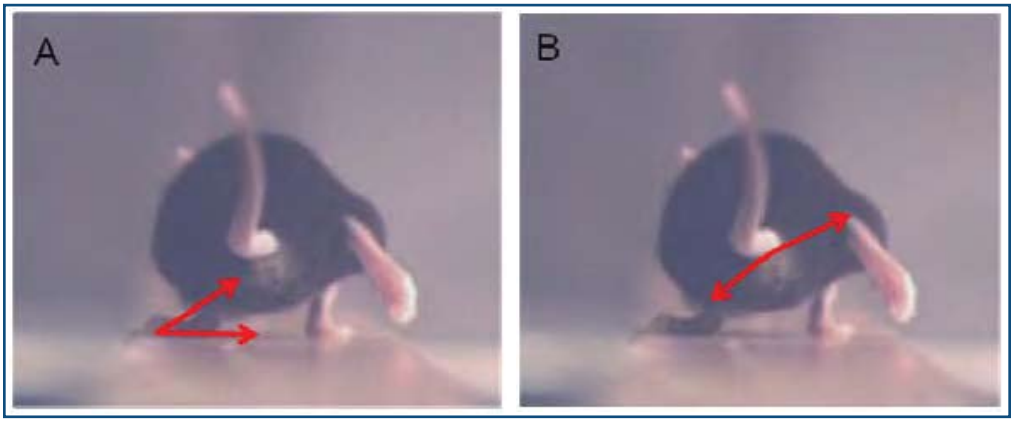


Figure II-20. Single frame extracted from video of beam walk. Frames are extracted when the contralateral limb is at peak vertical displacement. The foot-base angle (A) and heel-tail angle (B) are extracted and compared before, 1 week after, and at subsequent time points to assess functional recovery.

From the video recordings of mice pre- and post-injury, single-frame motion analysis was utilized to assess muscle function in mice after damage to the femoral nerve, which affects knee extension. Using SIMI Motion software, foot-base and heel-tail measurements were taken at the point where the uninjured foot was at its highest point (**Figure II-20**).

Polyethylene tubing and peptide-grafted collagen. For studies with polyethylene tubing and grafted collagen filler, analysis has been completed for Weeks 0, 1, and 12. The researchers noticed a clear trend for any treatment with collagen to be better than saline alone. Moreover, all of the bioactive peptide treatments were significantly different than saline alone.

Tyrosine-based copolymer tubing with grafted collagen. Preliminary analysis has been completed on animals treated with saline, native collagen, and peptide-grafted collagen filler in both polyethylene and tyrosine-based conduits. Foot-base angle (FBA) measurements were analyzed at 0, 1, and 15 weeks post-injury. The FBA measurements clearly demonstrate functional improvement in gait for all animals treated with native collagen filler or peptide-grafted collagen filler as compared to saline filler in a polyethylene conduit at the 15-week time point. In this set, no clear synergistic activity between the two different bioactive peptides was observed.

Degradation properties and polymer/conduit design. To examine the degradation properties of the polymer conduits being fabricated for the peripheral nerve application, three polymer compositions have been selected from the tyrosine-based copolymer library of the Kohn laboratory for an in vivo analysis via a subcutaneous implantation study, which is in progress.

In vivo immune response of poly(esters). As previously proposed, conduits were melt-extruded from two different polymer compositions. These conduits do not produce an adverse inflammatory reaction.

Key Research Accomplishments

- Completed the in vivo work for the initial femoral nerve injury study with polyethylene and tyrosine-based tubes filled with saline or peptide-grafted collagen.
  - Captured videos for the duration of the study.
  - Completed the secondary procedure to retrogradely label regenerating axons.
  - Sacrificed all animals and harvested all tissue.
- Determined through single-frame motion analysis of the videos that (1) collagen grafted with peptides significantly improves functional regeneration, and (2) degradable tyrosine-based tubes outperform polyethylene tubes.
- Determined that degradable poly(ester) tubes do not produce an adverse inflammatory reaction.

Conclusions

Initial analysis of recovery indicates that the functionalized collagen improves recovery after a large gap (5 mm) injury to the femoral nerve. This is a substantial improvement over use of the peptides in soluble form, which improved recovery only for small injuries (1–2 mm). Completion of the study and data analysis should indicate the importance of a degradable sheath as well as the two peptides presented in combination. Since only one concentration of grafted peptide was tested in the study, and results are promising, dose-response experiments, either in vitro or in vivo, are warranted to target the optimal concentrations.

Another exciting finding was the improvement of function in animals implanted with the degradable tyrosine-based copolymer versus polyethylene. The poly(ester)-degradable polymer was shown to only induce limited inflammation, and sciatic nerve injuries are in place using this material. Completion of the study and data analysis as originally proposed should indicate the importance of a degradable conduit as well as the two peptide mimics presented in combination. The Rutgers team is also actively pursuing an automated approach using image analysis of FBA measurements to extract the recovery parameters and potentially identify new metrics.

Research Plans for the Next 4 Years, Planned Clinical Transitions, and Corrections/ Changes Planned for Year 2

Information can be found under RCCC Project 4.4.1/4.4.2.

Project 4.4.2a, RCCC

Cell and Bioactive Molecule Delivery to Enhance the Repair of Segmental Nerve Defects

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

Project Team: *William Duggan, MD, Arkadiusz Jundzill, MD, Grzegorz Brzezicki, MD, Aleksandra Klimczak, PhD, Joanna Cwykiel, MSc (Cleveland Clinic)*

Therapy: *Treatment of segmental nerve defects*

Deliverable(s): *Method of cellular therapeutics for peripheral nerve defects by local administration of bone marrow-derived MSCs into transplanted epineural tubes*

Key Accomplishment(s): *Nerve regeneration was demonstrated in vivo for rat sciatic nerve 2 cm defects bridged with epineural conduits filled with bone marrow MSCs, as indicated by faster functional recovery, increased myelin area, axon area, myelin thickness, and mean fiber diameter, compared with saline-filled conduits.*

Introduction

In the quest to rebuild tissues lost to war injuries, the role of peripheral nerves is often overlooked. Unresolved peripheral nerve injury can significantly undermine the healing process, resulting in a significant burden for both the patient and the medical community as a whole. The resultant morbidities (i.e., poor function, reinjury due to loss of sensation, and poor patient perception of outcomes) are, more often than not, both permanent and significant. Effective nerve gap repair remains an elusive enemy to even the most accomplished surgeon.

The traditionally and most widely accepted standard of care for the surgical treatment of peripheral nerve lacerations or segmental defects include primary repair or nerve grafting. When direct repair is not possible, the most effective method to reestablish functional nerve continuity is the use of an autologous nerve graft (e.g., sural nerve). Autologous nerve grafts, however, have several drawbacks, including but not limited to loss of function in the donor sensory nerve distribution and size mismatches between the autologous donor nerve and the injured nerve. Moreover, the amount of tissue available from this source is limited. Finding solutions to the challenge of repair of segmental nerves will improve outcomes for wounded warfighters. This project addresses novel methods of nerve gap repair using epineural conduits filled with bone marrow-derived MSCs.

Clinical experience shows that the adult peripheral nervous system is capable of functional repair. However, regeneration is generally best over short distances and in younger individuals. Nerve grafting addresses factors required for good nerve recovery: minimal tension at the repair site, adequate coaptation of the nerve ends, preservation of neurotrophic factors, minimal fibrosis, and minimal foreign body reaction at the repair site. However, autologous nerve grafts, as stated before, present significant drawbacks, including the length and diameter of the tissue available.

As an alternative to nerve autografts, a number of different natural materials have been explored for use in aiding nerve regeneration, including vein segments and nerve sheath segments of both autogenous and allograft origin. These natural materials have inherent bioactivity and biocompatibility that may aid in nerve regeneration. However, synthetic scaffold conduits have proven to be successful in repairing small gaps (i.e., 2 cm or less) but have shown little to no efficacy to date for the repair of longer defects in either civilian or military applications. Previous studies on conduits highlight the importance of a favorable microenvironment to optimally enhance nerve growth. Moreover, conduits of varying materials have demonstrated varying degrees of success when supported by trophic factors. Because of previous work on the epineural sleeve technique,

the Cleveland Clinic team hypothesizes that supported by cellular therapy, the epineural tube and its incipient trophic factors provide the best tool for optimal nerve recovery.

The possibility of regenerating nervous tissue using adult MSCs has generated expectations with respect to the treatment of neurodegenerative diseases (e.g., multiple sclerosis) and brain damage previously assumed to be irreversible. In many studies, bone marrow-derived MSCs were applied as a supportive therapy for tissue regeneration because of their plasticity. Bone marrow-derived MSCs comprise a multipotential heterogeneous population of cells that contribute to the regeneration of different types of tissues, for example, bone, cartilage, fat, and muscle. In conjunction with a naturally occurring biological conduit, such as the epineural tube, the multipotency of the cells is augmented by the presence of neurotrophic factors in the tube itself. Recently, studies proved that bone marrow-derived MSCs are capable of producing many trophic and supporting substances that give direction to the cell lineages resulting in preferential nerve cell differentiation and ultimately optimal nerve regeneration. This project proposes to introduce a novel method of cellular therapeutics by local administration of bone marrow-derived MSC therapy into transplanted epineural tubes, which may facilitate the process of nerve regeneration.

Research Progress – Year 1

Methods

Seventy-two adult male Lewis rats were used in this study. All animals underwent 2 cm right sciatic nerve gap repair with isogenic epineural tubes and were distributed into three experimental groups:

Group 1 – Epineural tube filled with saline (control group)

Group 2 – Epineural tube filled with isogenic bone marrow-derived MSCs (Lewis RT1[i])

Group 3 – Epineural tube filled with allogenic bone marrow-derived MSCs (ACI RT1[a])

Surgical procedures and electrophysiological evaluations were performed while the rats were under subcutaneous ketamine cocktail anesthesia. The left limb sciatic nerves served as sham controls. The experimental protocol included the functional evaluation of sciatic nerve recovery at 6, 12, and 24 weeks. Methods of functional assessment, including clinical assessment by the pin-prick (**Figure II-21**) and toe-spread (**Figure II-22**) tests, were used for the evaluation of sensory recovery. Electrophysiological assessment was made by evaluating somatosensory-evoked potential. Evaluation of the effects of denervation on the gastrocnemius muscle was performed by the assessment of the gastrocnemius muscle index. Histomorphometric analyses of epineural tube section and proximal and distal stumps are also under way using the same timetable.

Results

This project is still in its early stages and data are only available from epineural tubes 6 weeks post-operation; all other animals are still under observation. However, bone marrow-derived MSC therapy groups demonstrate higher scores on toe-spread tests, indicating better sensory recovery than the control saline group. This correlates favorably with preliminary histological data indicating that there were significant increases in myelin area, axon area, myelin thickness, and mean fiber diameter on comparison of the isogenic bone marrow-derived MSC-filled epineural tube group (Group 2) to the saline-filled epineural tube group (Group 1) and allogenic bone marrow-derived MSC group (Group 3) (**Figure II-23**). Elec-

Figure II-21. Pin-prick test. Grades 1 to 3 (left to right).

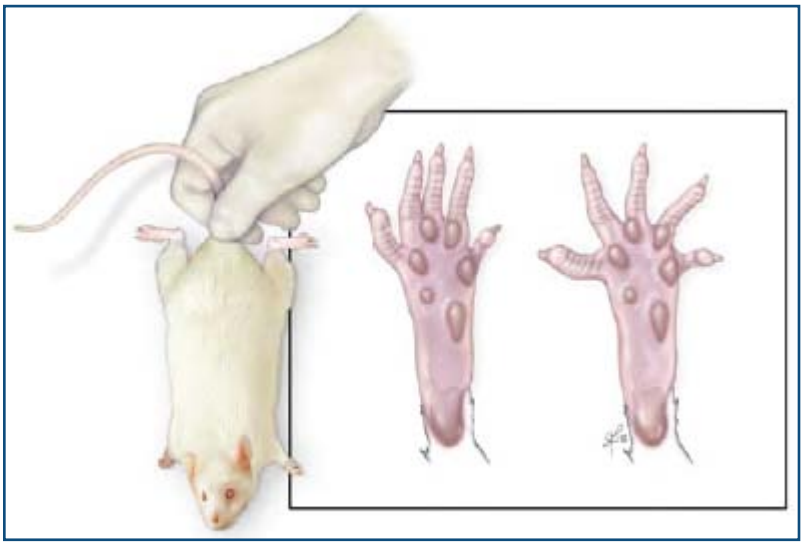


Figure II-22. Toe-spread test.

trophysiological data revealed no statistical differences in wave latencies or amplitudes among the three groups. No differences in gastrocnemius muscle index were observed among groups. Work will proceed as proposed to observe and assess experimental animals at 12 and 24 weeks.

Key Research Accomplishments

All data are reported only for the 6-week time point:

- Successfully performed the transplantation of the isogenic epineural tube and bone marrow-derived MSC delivery into transplanted tube.
- 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 support nerve regeneration in the presence of transplanted isogenic bone marrow-derived MSC therapy.
- Histomorphometric analyses indicate a positive effect of isogenic bone transplantation on nerve regeneration.

Conclusions

Preliminary outcomes from 6-week groups indicate successful nerve regeneration over a 2 cm defect bridged with an epineural conduit filled with either saline or bone marrow-derived MSCs. Functional evaluation (toe-spread, pin-prick) and histomorphometry (nerve counting) show beneficial effects—faster functional recovery, increased myelin area, axon area, myelin thickness, and mean fiber diameter of transplanted bone marrow-derived MSCs when compared to saline-filled conduits.

Research Plans for the Next 4 Years, Planned Clinical Transitions, and Corrections/ Changes Planned for Year 2

Information can be found under RCCC Project 4.4.1/4.4.2.

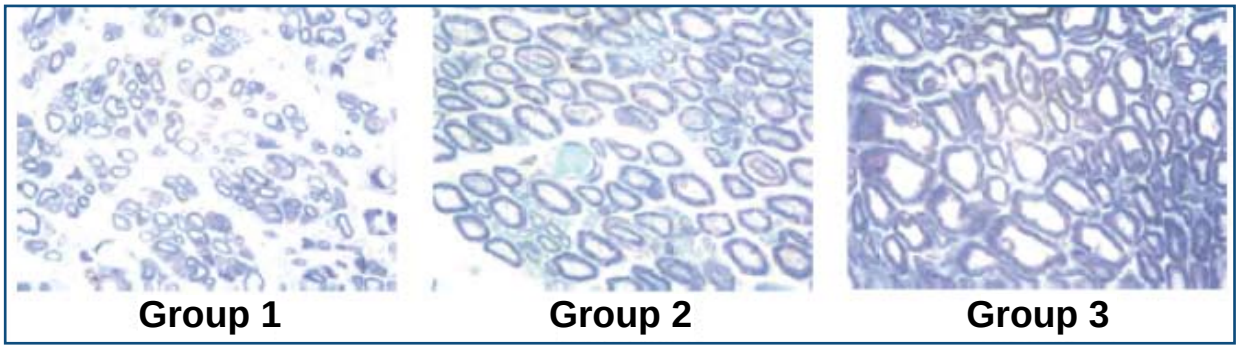


Figure II-23. Histomorphometric analysis (toluidine blue stain, 100x).

Project 4.4.4, WFPC

Peripheral Nerve Repair

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

Collaborator(s): *Georgia Tech/ Emory laboratory, Tirrell laboratory at University of California, Santa Barbara*

Therapy: *Combinatorial strategy for regeneration over long peripheral nerve gaps*

Deliverable(s): *Proactive biodegradable nerve guide system for peripheral nerve regeneration*

Key Accomplishment(s): *The researchers (1) established a collaborative, focused effort among the University of Pittsburgh, Tufts University, and Wake Forest University and (2) developed a novel biodegradable conduit for nerve repair.*

Introduction

This is a highly collaborative project among three laboratories at the forefront of peripheral nerve repair. All three laboratories have focused on a tissue-engineering approach for nerve repair over long gaps and now are combining their efforts in a synergistic manner. The Pittsburgh group will continue developing delivery systems for neurotrophic factors using a gradient concentration of microspheres in the nerve conduits. The Tufts group will utilize its expertise in silk scaffolds to develop tubular constructs. The Wake Forest group will incorporate keratin-based gels within the conduits to enhance axonal elongation. The combination of these approaches, all using FDA-approved materials, will lead to a therapeutic strategy that has the potential to be an off-the-shelf guide for long gap nerve repair.

Research Progress – Year 1

The first year of research has yielded exciting progress in developing novel nerve guides for long gap nerve repair with the short-term goal of developing an off-the-shelf guide. Both original objectives for Year 1 were met with the establishment of a focused, collaborative effort among the University of Pittsburgh, Tufts University, and Wake Forest University and the development of a novel biodegradable conduit for nerve repair.

Each of the laboratories in this collaboration had been examining promising approaches to long gap nerve repair, and the team is combining their most promising strategies. Specifically, the following approaches were examined in Year 1:

- Nerve guides consisting of FDA-approved materials were examined in a rat sciatic nerve defect (**Figures II-24** and **II-25**). These materials included collagen, silk, and polycaprolactone (PCL).
- Neurotrophic growth factors (e.g., glial cell line-derived neurotrophic factor) were encapsulated in double-walled polymer microspheres, resulting in an extended, long-term release (e.g., 90 days). These microspheres were embedded in the walls of both the PCL and silk nerve guides.
- Sophisticated functional analysis is being conducted on rats. Histological analysis of the guides includes axon counting and inflammatory response evaluation.
- Preliminary results from a rabbit study indicate that keratin-filled collagen nerve guides can bridge a 2 cm rat tibial defect in >50% of the animals tested.

The next step for the group is to identify the next large animal model study, in collaboration with the Georgia Tech/Emory laboratory, which is also working on this project.

Specific progress made on in vitro studies:

- Silk protein nerve guide tubes were prepared with suitable diameters for peripheral nerve studies. A versatile silk protein processing and tube prepara-

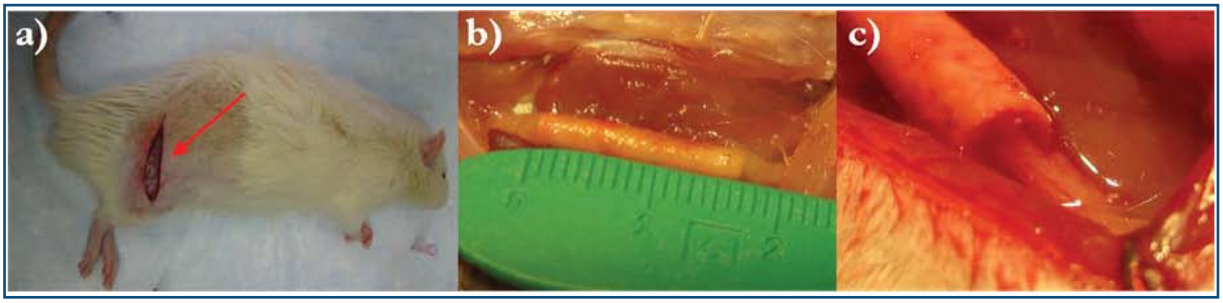


Figure II-24. (a) Sixteen PCL nerve guides were implanted across a 1.5 cm defect in the sciatic nerve (red arrow) of male Lewis rats. (b-c) To create the injury, the sciatic nerve was transected, allowed to retract, and then 0.5 cm of nerve was excised. The proximal and distal nerve stumps were sutured 1 mm into either side of a 1.7 cm nerve guide. Immediately prior to sacrifice, functional recovery and muscle reinnervation were measured through gastrocnemius contraction force. The animals were euthanized after 16 weeks, and the operated nerve was harvested.

tion process was developed to offer reproducible generation of the structures with control over structure and morphological features.

- Microspheres in the micron range were prepared from silk and embedded in the walls of the silk tubes, establishing a materials platform for functionalization of the silk tubes with delivery of nerve growth factors.
- Initial studies on silk biomaterial surface patterning are under way to optimize morphological features related to guided nerve growth. For neuron axon alignment, grooved silk films coated with laminin were developed. Design parameters for the patterned silk films included alternate grooves with widths 2, 5, 10, 15, and 30 μm. Various widths are being assessed to provide information on axon alignment on the silk films.
- Initial studies on screening key nerve growth factors were initiated to determine gradient effects. Two "layer-by-layer" drug release silk studies were conducted over 4 weeks, which involved assessing the amount of NGF released using methanol- and nonmethanol-treated silk films.
- Initial p19 neuron in vitro studies revealed metabolic activity increased with neurons plated onto laminin substrates with the greatest differences after 1 day of culture.
- Double-walled PLA and PLGA microspheres were prepared and proteins/neurotrophic factors were encapsulated. In vitro release data revealed release kinetics of active factors up to 90 days.
- Microspheres were incorporated into the walls of polymeric nerve guides for in vivo studies.
- Collaboration with the Tirrell laboratory has been initiated, and this laboratory will visit the Pittsburgh laboratory in August/September 2009.
- Keratin gels will be obtained from Wake Forest in June 2009, and rheological properties of the keratin and HA gels will be determined in June/July 2009 in preparation for in vivo studies in fall 2009.

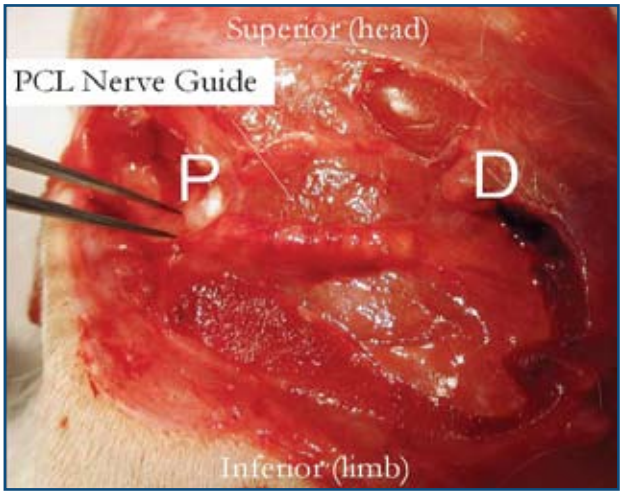


Figure II-25. Following 16 weeks in vivo, PCL nerve guides appeared well vascularized with a mild to moderate fibrous capsule. The guide material remained soft and pliable and could easily be removed from nerve tissue regenerating through the lumen of the guide. The proximal (P) and distal (D) ends of the guide were completely sealed with fibrous tissue. In a small percentage of animals, neuromas were evident at the proximal nerve stump. Muscle reinnervation was evident in 70% of the animals treated with PCL guides without any additional growth factors present.

Specific progress made on in vivo studies:

- Twelve silk guides were implanted in an 8 mm rat sciatic nerve defect. Animals will be sacrificed after 4 weeks (n=6) and 8 weeks (n=6). Histological and functional analyses will be conducted. Rats have been sacrificed and histomorphometric analysis is under way.
- Collagen nerve guides were received from the Wake Forest group, and the University of Pittsburgh group implanted 12 guides into 8 mm rat sciatic nerve defects. Animals will be sacrificed after 4 weeks (n=6) and 8 weeks (n=6). Histological and functional analyses are under way.
- GDNF-containing PCL nerve guides were implanted in a 1.5 cm sciatic nerve defect in the Lewis rat (pilot study with 9 rats). Initial histological results indicate that GDNF-containing guides show improvement in nerve repair (e.g., axonal regeneration) at 6 weeks. The researchers have analyzed axon counting from 6 of 9 animals and will finish the remaining 3 animals in 2009. Longer time points have been initiated, and these animals will be sacrificed in August 2009 for analysis.
- A pilot nerve regeneration study in rabbits utilizing a keratin hydrogel incorporated within a commercial collagen conduit has been completed with matching funding (Wake Forest). The study employed a 2 cm segmental defect in the tibial nerve and compared empty conduits and cable autografts (sural nerve) to keratin-filled conduits at 3 months by electrophysiological and histological outcomes. Functional recovery as measured by electrophysiology was present in 4 of 12 animals in the empty conduit group, 8 of 8 in the autograft group, and 4 of 10 in the keratin group. Electrophysiological measurements suggested that when functional regeneration was present, keratin-treated nerves produced better conduction delay and amplitude of the compound motor action potential. Histomorphometric analysis showed that keratin-treated nerves were larger and therefore contained a larger number of myelinated axons although average axon diameter was statistically indistinguishable from the other treatment groups.
- These data demonstrate improved functional recovery when damaged nerves are treated with a keratin gel-filled collagen conduit. However, consistency of repair was lacking in this study in the conduit treatment groups. Future work under the AFIRM program will focus on improving the keratin hydrogel to increase consistency of the repair.

Key Research Accomplishments

- Developed a novel biodegradable conduit for nerve repair and conducted several in vitro and in vivo studies.
- Established a collaborative, focused effort among the University of Pittsburgh, Tufts University, and Wake Forest University.

Conclusions

The researchers of this project reached first year milestones with the establishment of a collaboration involving three laboratories and the development of a novel conduit for nerve repair. They surpassed these milestones and expectations, establishing a new collaboration with Dr. Matthew Tirrell's laboratory at the University of California, Santa Barbara and conducting multiple in vivo studies. They feel that comparisons of fillers from Pittsburgh (e.g., HA fillers), Wake Forest (e.g., keratin-based fillers), and University of California, Santa Barbara (e.g., peptide-modified amphiphilic matrices) will result in a nerve guide with optimal axonal regeneration properties.

Research Plans for the Next 4 Years

The researchers will continue to test their novel nerve guides in animal models. Notably, their rat sciatic nerve defect studies are near completion. They will move to a larger animal model (e.g., the rabbit tibial nerve defect) and will enhance their interdisciplinary collaboration by further combining individual approaches (e.g., long-term drug delivery, novel silk-based conduits, and keratin gel fillers).

Planned Clinical Transitions

Clinical studies are expected to begin in Years 4 or 5 of this project.

Corrections/Changes Planned for Year 2

None.

Project 4.4.5, WFPC

Modular, Switchable, Synthetic, Extracellular Matrices for Regenerative Medicine

Team Leader(s): *Matthew Tirrell, PhD (University of California, Santa Barbara)*

Collaborator(s): *Kacey Marra, PhD (University of Pittsburgh Medical Center [UPMC]), Ravi Bellamkonda, PhD, and Robert Guldberg, PhD (Georgia Tech), David Schaffer, PhD and Kevin Healy, PhD (University of California, Berkeley)*

Therapy: *Induce peripheral nerve growth following traumatic amputation*

Deliverable(s): *Matrices that modulate components of the ECM and support growth and differentiation of neural stem cells (NSCs)*

Key Accomplishment(s): *The researchers synthesized several double-tailed PAs with controlled bioactive components that when incorporated into lipid bilayers allow NSCs to attach, proliferate, and differentiate.*

Introduction

The restoration of functional limb and digit tissue involves the orchestrated growth and differentiation of multiple tissue types in a spatially oriented and site-appropriate pattern. Regeneration of traumatic limb wounds is often limited, even in amphibians, by peripheral nerve damage. As part of a collaborative effort with other researchers in the Limb and Digit group of the AFIRM team, the researchers in Dr. Matthew Tirrell's laboratory are pursuing an approach to inducing peripheral nerve growth following traumatic amputation by modulating components of the naturally occurring ECM. Specifically, they use synthetic peptide amphiphiles (PAs; lipopeptides) that contain subsequences that are known to support growth and differentiation of NSCs to construct self-assembled two-dimensional surfaces and three-dimensional fibrous scaffolds. The regenerative potential of these matrices is assessed by studying the adhesion and neuronal differentiation of NSCs (**Figure II-26**).

First the researchers will utilize synthetic chemistry to construct PAs with controlled bioactive components and test their in vitro efficiencies in adhesion, proliferation, and differentiation of NSCs on surfaces. Next, they will identify conditions that transform a solution of PA micelles into a three-dimensional fibrous gel and test the ability of this peptide-based scaffold to control the behavior of NSCs in vitro. Further, this novel injectable gel will be assessed for its regenerative potential in a rat model of sciatic nerve injury in collaboration with Dr. Kacey Marra's team at UPMC. The researchers at the Tirrell laboratory are also collaborating with the Georgia Tech team headed by Dr. Ravi Bellamkonda and Dr. Robert Guldberg on testing these materials in animal models of composite injury.

Research Progress – Year 1

Aim 1: *PA synthesis and fibroblast experiments.*

The researchers synthesized several different PA variants (**Figure II-27**). They first confirmed the ability of PA diC16-(EO) n-GRGDSP (n=2, 4, 5) to promote adhesion of NIH 3T3 cells. The density of ligands on the surface required to promote maximal adhesion of the fibroblast cells was determined. This information was then applied to the design of surfaces for NSC adhesion.

Aim 2: *Murine NSC adhesion on lipid bilayers.*

In collaboration with Drs. David Schaffer and Kevin Healy (Department of Bioengineering, University of California, Berkeley), the researchers conducted cell adhesion experiments with murine NSCs. They tested the self-renewal and differentiation capacities of the murine NSCs on the peptide displaying lipid bilayers. Prior to this experiment, they investigated the dependence of NSC adhesion based on the mol% peptides displayed on the surfaces and concluded that the optimum mol% for bsp-RGD is 20% and GRGDSP is 40%.

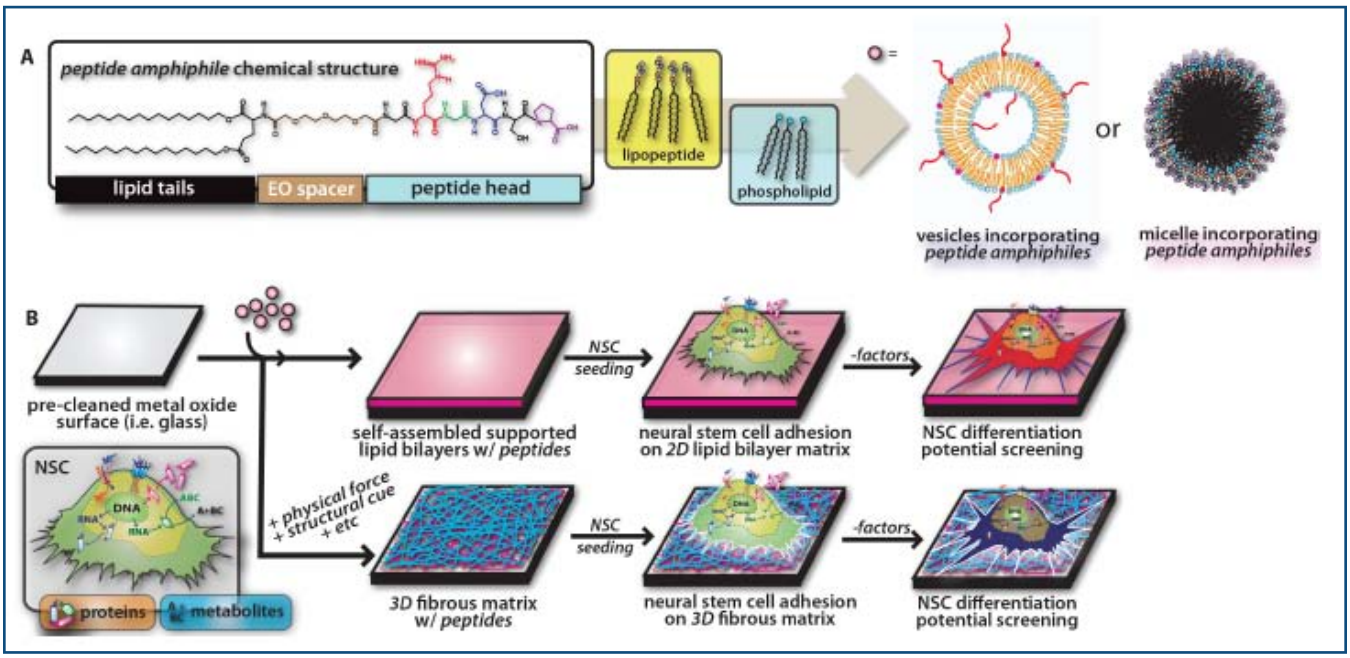


Figure II-26. Two- and three-dimensional synthetic ECMs from PAs. (A) Vesicle and micelle formation from phospholipids and PAs (lipopeptide). (B) Scheme to generate two- and three-dimensional matrices from vesicles formed in A and their application in NSC engineering. NSC adhesion and differentiation will be analyzed via immunocytochemistry and gene expression profiling. NSC cell machinery illustration adapted from Suh et al., Synthetic extracellular matrices and nanostructures for stem cell engineering, first Nano Today Conference, Singapore, August 2009.

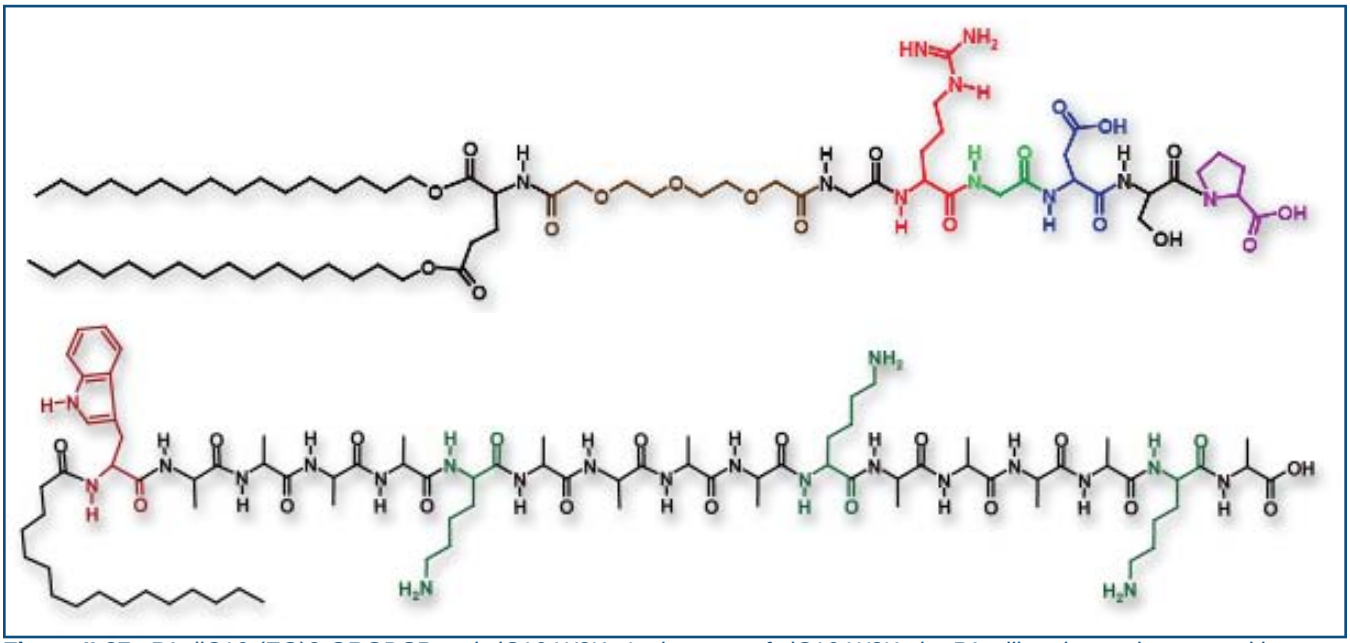


Figure II-27. PA diC16-(EO)2-GRGDSP and siC16-W3K. In the case of siC16-W3K, the PA will undergo phase transition from spherical micelles into cylindrical worm-like structures in aqueous conditions.

Further, NSCs grown on 20 mol% bsp-RGD surfaces proliferated, displayed morphology similar to that on control laminin surfaces, and exhibited significant expression of the neural progenitor marker nestin.

Aim 3: *Human NSC adhesion and selection for neurospheres.*

Human NSCs (ReNcell VM, Millipore, Billerica, Massachusetts) were also tested on peptide displaying lipid bilayers. The human NSCs chosen are normally cultured on laminin-coated plates using serum-free medium supplemented with growth factors bFGF and EGF (originally developed by ReNeuron Group plc). Cell attachment on peptide surfaces was evaluated at 1 hour after incubation in serum-free media.

ReNcell VM is a commercially available human neural progenitor cell line developed from the ventral mesencephalon region of the developing human brain (i.e., human fetal brain tissue) and immortalized by retroviral transduction with the v-myc oncogene. This cell line is reported to have a stable phenotype and genotype in culture that grows as a monolayer on laminin-coated surfaces in addition to its capacity to differentiate into all three neuronal lineages (neurons, astrocytes, and oligodendrocytes). Neuronal differentiation can be initiated by withdrawing bFGF and EGF.

Upon experimentation, it was found that human NSCs adhere to a limited extent, similar to the murine NSCs as mentioned earlier. However, in the human NSC case, the peptide experiments lead to a pre-selection of NSCs to form a population of neurospheres. The researchers hope to further investigate this methodology for its potential to facilitate and accelerate the integration of their fibrous matrices with NSCs. In this case, the neurospheres can be included in the suspension of PA micelles prior to in situ three-dimensional scaffolding and stay in direct contact with them. This approach will be complementary to work with adherent cells that usually require dissociation before implantation.

Aim 4: *Fibrous network of PAs.*

The researchers have determined the secondary structures that siC16-W3K PAs form at multiple temperatures via time-dependent circular dichroism. They found that it takes more than a week for the PAs to switch structures at room temperature (25°C), but when the temperature is doubled to 50°C or higher, the transition takes less than 1 hour.

For instance, at 60°C (20 mg/mL), the PA aqueous solution gels into a viscous material (**Figure II-28**), which would be ideal as a three-dimensional tissue culture matrix. The electron microscopy images show that the PA-based fibrous matrix has sub-100 nm features that will interface well with mammalian cells including NSCs. The researchers also synthesized a cell-adhesive variant of the fiber-forming PA (siC16-W3K-RGD) and confirmed that this PA is also capable of forming a self-standing gel with a nano-fibrous architecture.

Key Research Accomplishments

- Synthesized double-tailed PAs with controlled bioactive components that when incorporated into lipid bilayers allow NSCs to attach, proliferate, and differentiate.
- Discovered that single-tailed PAs lacking bioactive sequences form three-dimensional fibrous networks by standing at room temperature for several days.

Figure II-28. Gelled PA siC16-W3K. Photograph of PA siC16-W3K gelled (left) after 1 hour incubation at 60ºC (20 mg/mL). At lower concentrations, siC16-W3K (1 mg/mL) will remain an aqueous solution with gels (allows) forming slowly over a 24-hour period at 60ºC (middle). Microscope image of a siC16-W3K PA gel fixed with glutaraldehyde. Substructures are visible at the micron size range (right).

- Determined that this process can be accelerated, leading to shortening of the transition time (i.e., a few hours) when the temperature is increased to 60°C.

Conclusions

While the use of synthetic nerve guides has provided a viable alternative to autografts in long gap nerve repair, it is now believed that additional filler materials within the guides are necessary to promote recovery of nerve function after injury. The researchers made considerable progress in the development of a fibrous scaffold based on synthetic PAs that present ECM-derived peptide ligands that can bind to specific cellular receptors and subsequently promote cell adhesion, migration, and nerve regrowth.

Research Plans for the Next 4 Years

The researchers' in vitro findings are being further investigated for application to a rat model of sciatic nerve defect regeneration, which is being developed by Limb and Digit partner Dr. Kacey Marra's laboratory at UPMC. Using the model as described before, the researchers will investigate the most promising ligands in a logical, algorithmic fashion to determine the temporal-spatial effect upon peripheral innervation at the amputation (nerve gap) site. They will integrate findings with other team members within the Limb and Digit group to determine optimal ways in which to stimulate new tissue growth, resulting in eventual complete digit regeneration. To this end, the researchers have initiated discussions with the Georgia Tech team, led by Dr. Robert Guldberg and Dr. Ravi Bellamkonda, to explore possible synergies in the development and in vivo testing of peptide-based fibrous matrices in peripheral nerve repair. This collaborative effort will integrate efforts into a fuller broader objective of composite tissue regeneration (nerve + bone + vasculature).

Planned Clinical Transitions

The integrations and cooperation described are bringing results into a common animal model and closer to eventual clinical application.

Corrections/Changes Planned for Year 2

None.

Project 4.4.3, WFPC

Engineered Delivery of Spatial and Temporal Cues for Composite Tissue Injury Repair

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

Therapy: *Improve limb function following composite tissue trauma*

Deliverable(s): *Animal models of composite tissue trauma and insight into spatial and temporal delivery cues that direct nerve, vascular, and bone growth in limbs*

Key Accomplishment(s): *The researchers established promising regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of a clinically approved inductive protein, bone morphogenetic protein-2 (BMP-2). They completed a pilot surgery to create a combined bone and nerve defect in a new composite injury rat 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 CTP cells. The researchers seek to develop and test technologies that will enable the restoration of limb function following composite tissue trauma. To meet this goal, they are developing 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. They are also testing spatial and temporal delivery of cues that direct nerve, vascular, and bone growth in a synchronized manner. The rationale for this approach is based on recent observations that vascular and neural development occur in tandem and perhaps synergistically during fetal bone formation and growth.

Research Progress – Year 1

Study 1: *Bone Defect Repair*

Approaches for the regeneration of massive bone defects have typically focused on the use of structural allografts or three-dimensional scaffolds having adequate strength to support in vivo loading. However, allografts revascularize and remodel incompletely, and scaffolds do not provide an optimal environment for cellular function and suffer from slow resorption kinetics. Thin, two-dimensional membranes have been used to promote bone repair by placing them along the periosteal surface to demarcate the osseous region from the non-osseous region. Although this technique (called guided bone/tissue regeneration) has been used successfully in the dental field, it has not been quantitatively evaluated for segmental long bone defects. Electrospun nanofiber meshes have recently emerged as a new generation of scaffold membranes, possessing a number of features suitable for tissue regeneration.

Bilateral 8 mm segmental defects were created in the mid-femoral diaphyses of 13-week-old female Sprague-Dawley rats using a miniature oscillating saw and stabilized by custom fixation plates. Nanofiber tubes were placed around the adjacent bone so that the tube lumen contained the defect.

The researchers found that a spatial (nanofiber mesh) and temporal (alginate hydrogel) delivery strategy of a clinically approved osteoinductive factor (BMP-2) can restore function to massive bone defects in just 12 weeks. It is important to note that the 8 mm defect in the rat is 60% larger than the standard critical size (5 mm) for rat long bone defects and thus represents a highly challenging model. The researchers believe that the perforated nanofiber mesh design accelerated bone repair at 4 weeks by enhancing the ingrowth of vascularity and/or

osteoprogenitors from the surrounding soft tissues. This observation supports their hypothesis that composite tissue injuries will require a spatially and temporally coordinated treatment approach. Details on the experimental results of these studies can be found in **Figures II-29** and **II-30**.

Study 2: *Nerve Defect Repair*

Nanofiber-based nerve guidance channels were designed to stimulate peripheral nerve regeneration across critical length injury gaps (>10 mm). The scaffolds were fabricated by interposing a single thin-film of aligned poly(acrylontrile-co-methylacrylate, random copolymer, 4% methylacrylate) (PAN-MA) fibers within a polymeric (polysulfone) guidance channel. To create the aligned polymer fiber films, a 15% (w/v) PAN-MA solution was first prepared by dissolving PAN-MA into the organic solvent N, N-dimethyl formamide (DMF, Acros Organics) at 60ºC. Finally, 2.2 mm wide sheets of aligned thin-films were manually cut with a razor blade and separated from the collected polymer mass with fine forceps for use in channel construction.

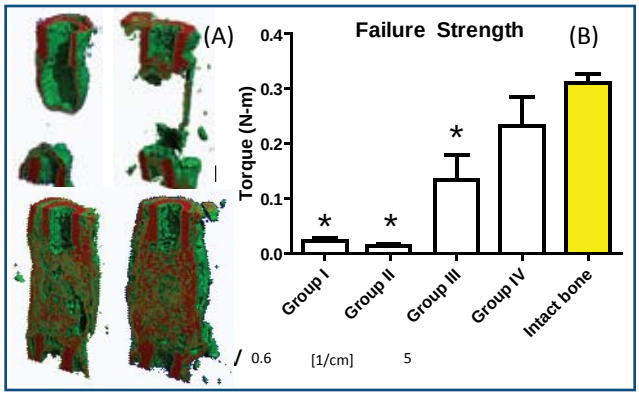


Figure II-29. (A) Cross sections of three-dimensional µ-CT images at 12 weeks with colors indicating linear attenuation of tissue. (B) Failure strength at 12 weeks as given by maximum torque during torsional testing. * Indicates significantly less than that of intact bone ($p<0.05$).

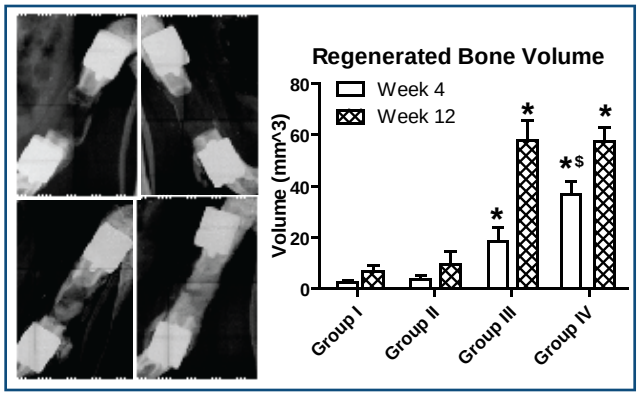


Figure II-30. (A) Representative radiographs at 4 weeks. (B) In vivo µ-CT volumes of generated bone at 4 and 12 weeks. * Indicates significantly greater than I and II at same time point; $ greater than III at same time point ($p<0.05$). (I) Tube alone, (II) Tube+alginate, (III) Tube+alginate+BMP-2, (IV) Perforated tube+alginate+BMP-2.

To assess the ability of these nanofiber-based conduits to promote peripheral nerve regeneration, the conduits were implanted across gaps of various lengths in rat tibial nerve. The researchers found that aligned nanofiber-based guidance channels promoted robust levels of axonal regeneration even across critical length nerve gaps. Significantly, this regeneration occurred in the absence of any exogenous ECM or trophic factors. The aligned topography of the interposed nanofiber thin-films stimulated endogenous repair processes, promoting a sequence of regenerative events that normally fail to occur over critical length nerve gaps.

Study 3: *Bone and Nerve Defect Pilot Study*

To investigate simultaneous in vivo tissue regeneration in composite bone and neural tissue trauma, an animal model was developed based on the previously described individual bone (8 mm) and nerve (15 mm) segmental defect models. The pilot study initiated in January 2009 incorporated the temporal and spatial guidance strategies successfully employed in the single injury studies. **Figure II-31** illustrates the surgery performed in this study.

The tissue regeneration observed for both the nerve and bone defects in this composite model are encour-

aging. However, caution must be exercised in drawing conclusions given the limited sample size of the study. Both the nerve and bone defects experienced regeneration comparable to that shown in the previously characterized single defect models. The most striking difference between the composite model and the single injury models is in relation to the restoration of gait function postoperatively. In bilateral bone segmental defect surgeries, normal gait is regained within 2–3 days after surgery. In unilateral nerve gap surgeries, normal gait is restored almost immediately. The composite bone-nerve defect appears to, at minimum, delay normal gait restoration. The other area where the animals in the composite model differed from those in the isolated bone defect model is the self-mutilation of the foot on the operated limb.

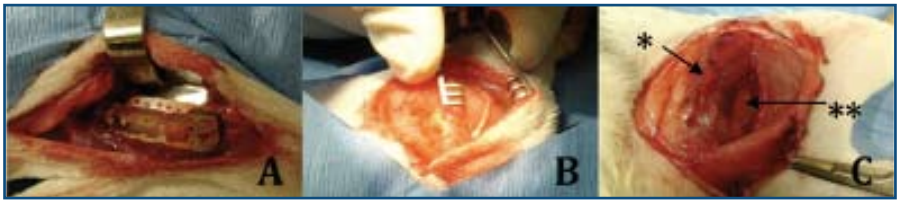


Figure II-31. Surgery pictures depicting (A) a perforated nanofiber mesh placed in the bone defect, (B) exposure of the sciatic nerve, and (C) the relative position of the muscle incision for creation of the bone defect (*) to the blunt dissection site for creation of a nerve defect (**).

Key Research Accomplishments

- Demonstrated that sustained (~7 day) release of BMP-2 from composite functionally modified alginate and PCL nanofiber mesh constructs restores function to massive bone defects in a rat segmental model by 12 weeks.
- Established a peripheral nerve defect model and demonstrated that oriented contact guidance nanofiber mesh scaffolds promote nerve regeneration and functional restoration.
- Completed a pilot surgery in January 2009 to create combined 8 mm bone and 15 mm nerve defects in a new composite injury rat model and treated animals as described earlier.
  - As hypothesized, return to normal gait function was slower following the composite injury compared to bone or nerve injury alone.
  - At 5 weeks, improved gait function and bridging of the defect with bone were observed.
  - Histological analysis revealed axonal regeneration bridging the 15 mm nerve defect.
- Completed vascular micro-CT imaging to help identify ligation points for studies on ischemic injury in Year 2.

Conclusions

A composite multitissue injury model is being 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 will include bone/nerve injuries and bone/vascular injuries. Once established, these models will be available for testing regenerative strategies developed by other AFIRM researchers. The researchers have established promising regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of a clinically approved inductive protein (BMP-2). A pilot study has been completed to establish the bone/nerve defect model and to evaluate composite injury repair.

Research Plans for the Next 4 Years

Year 2 milestones are to: (1) quantitatively evaluate the effects of sustained protein delivery (e.g., BMP-2 and BDNF) on recovery of limb function in the composite bone and neural defect model and (2) establish the composite bone and vascular defect model and characterize functional regeneration. Beyond Year 2, the researchers plan to quantitatively evaluate the relative efficacy of controlled spatiotemporal delivery of angioneurins (e.g., BDNF), angiogenic (vascular endothelial growth factor or HIF-1α), and osteoinductive (e.g., BMP-2) cues to restore function to limbs sustaining composite

injuries. They will specifically focus on factors that play a role in linking the formation of distinct tissues during development since they believe these will synergistically promote functional regeneration of damaged limbs. For example, HIF-1α activators have recently been shown to link angiogenesis and osteogenesis during skeletal development and promote fracture healing.

The second unique aspect of the researchers' approach will be to use deployment strategies developed at Georgia Tech (e.g., oriented or perforated nanofiber meshes and lipid microtubes) that provide spatiotemporal-controlled delivery. They believe controlling spatial distribution and temporal release kinetics is essential to maximizing the potency of delivered regenerative cues. They also plan to use the composite injury models to test promising limb regenerative strategies established by other researchers on the AFIRM team. The best of these approaches will then be transferred for evaluation in large animal models. For example, they plan to test the nanofiber mesh/alginate BMP-2 delivery system in a sheep segmental defect model in Year 2 in collaboration with Dr. Dietmar Hutmacher.

Planned Clinical Transitions

The researchers plan to pursue intellectual property protection to facilitate technology transfer to a biotechnology company interested in developing a clinical product for treating severe limb trauma.

Corrections/Changes Planned for Year 2

None.

Project 4.4.2, WFPC

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

Team Leader(s): *W.P. Andrew Lee, MD (University of Pittsburgh)*

Therapy: *Treat forearm or hand loss by transplantation with novel bone marrow/stem cell-based protocol*

Deliverable(s): *(Phase 1) Protocol that combines systemic stem cell-based therapy with local immunomodulation and (Phase 2) treatment for hand or forearm loss*

Key Accomplishment(s): *The researchers established a heterotopic hindlimb transplant model in miniature Yucatan swine using a novel immunomodulatory protocol and determined that it is a valid model to study the effects of induction therapy. They also performed hand transplantation on a former Marine who lost his hand in a training accident while on active duty.*

Introduction

Composite tissue allografts (e.g., hand transplants) are now a clinical reality and have been performed in multiple centers worldwide. To date, 45 hands have been transplanted globally. Apart from excellent and highly encouraging functional results, composite tissue allotransplantation (CTA) 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 consistent of recipient conditioning (induction therapy), donor bone marrow infusion, and monotherapy maintenance immunosuppression.

This program has two arms: a preclinical model of heterotopic hindlimb transplantation in Yucatan miniature swine and clinical trials of human hand transplantation. Research trials are parallel and complementary, and work in each arm will be detailed separately. The goal of Phase 1 in the preclinical swine model is to determine the optimal dose of bone marrow cells to be infused to establish stable mixed chimerism, which has not yet been determined in CTA. In the human clinical trial (Phase 2), the overall goal is to 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). Application of Phase 1 data to Phase 2 will allow targeted immunomodulation (using donor bone marrow stem cells and local treatment of the graft with peptide migratory inhibitors) enabling survival of limb CTA under minimal immunosuppression.

Research Progress – Year 1

Phase 1: *Establish a protocol that combines systemic stem cell-based therapy with local immunomodulation enabling graft survival without long-term systemic immunosuppressive treatment in a preclinical swine model for CTA.*

The researchers completed all animal experiments proposed for Year 1. They established a heterotopic hindlimb transplant model in miniature Yucatan swine as well as induction therapy using nontoxic cytodepletion by whole-body and thymic irradiation. In vitro analysis including chimerism testing, flow cytometry to assess the degree of cellular depletion following radiation, and cytokine studies by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are ongoing. Completed flow cytometry studies indicate that following cytodepletion, animals show approximately 80% T cell depletion, 100% B cell depletion, and 35% myeloid cell depletion, which is consistent with the depletion shown in human transplantation with alemtuzumab (Campath-1H). Bone marrow cell

retrieval and isolation from 6 donor animals produced between 1.6 and 6.6 billion cells, which is more than one order of magnitude greater than that required for infusion.

The researchers also tested the biological function of human CTLA4/Fc in an in vitro mixed lymphocyte reaction (MLR). In an alloantigen triggered baboon T cell proliferation assay, human CTLA4/Fc at 5 µg/mL concentration was able to inhibit proliferation by 80% (**Figure II-32**). In a pig antigen triggered baboon T cell proliferation assay, human CTLA4/Fc at 5 µg/mL concentration was able to inhibit proliferation by 90% (**Figure II-33**). Finally, the researchers established an ELISA to monitor serum fusion protein levels (data not shown).

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.*

The researchers established a protocol for the retrieval and isolation of bone marrow from cadaveric donor vertebral bodies. They also standardized bone marrow infusion techniques for use in hand transplant recipients and established screening protocols and accrual of candidates for hand transplantation. Furthermore, in March 2009 they successfully performed a hand transplantation on a former Marine who lost his hand in a training accident while on active duty. The patient is now at 4 months after the transplant on monotherapy immunosuppression without any adverse side effects. He is currently undergoing intensive hand therapy as an outpatient.

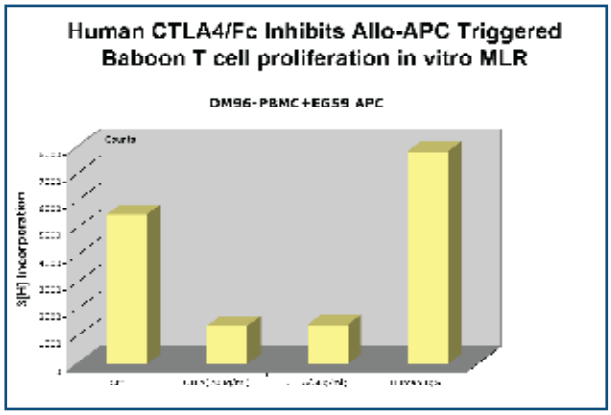


Figure II-32. Human CTLA4/Fc is potent in suppressing triggered T cell proliferation in a baboon.

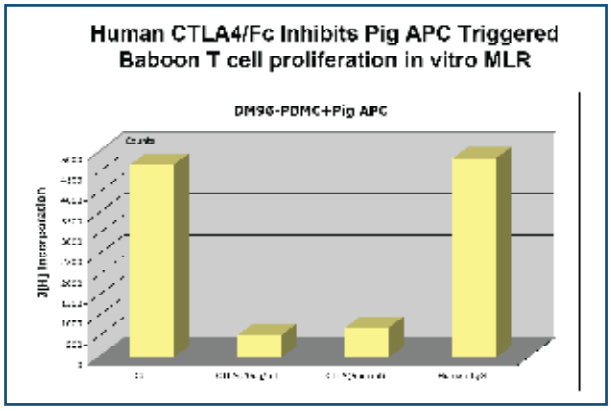


Figure II-33. Human CTLA4/Fc is potent in alloantigen suppressing pig xeno-antigen triggered in vitro MLR assay.

Key Research Accomplishments

- Established a heterotopic hindlimb transplant model in miniature Yucatan swine using a novel immunomodulatory protocol and determined that it is a valid model to study the effects of induction therapy.
- Produced and purified human CTLA4/Fc and anti-human CD154 mAb, established a bioassay, and tested the in vitro biological function of human CTLA4/Fc and anti-human CD154 mAb.
- Established a protocol for retrieval and isolation of bone marrow from cadaveric donor vertebral bodies.
- Performed hand transplantation on a former Marine who lost his hand in a training accident while on active duty.

Conclusions

The researchers have developed a preclinical heterotopic hindlimb transplant model for CTA using a novel immunomodulatory protocol. Preliminary data show that total

body and thymic irradiation results in a similar degree of cytodepletion as compared to Campath-1H in humans is well tolerated and is nontoxic to swine. Hence, this is a valid model to study the effects of induction therapy. Their modified protocol for bone marrow cell retrieval and isolation provides ample number of cells for infusion. Additional data will allow the researchers to study the basic molecular mechanisms of chimerism and its contribution to immunological tolerance in CTA. The researchers have applied their results from Phase 1 studies in performing hand transplantation using the Pittsburgh Protocol, a novel immunomodulatory strategy that aims to reduce maintenance immunosuppression necessary for successful CTA. Future application of this novel concept will hopefully allow for widespread adoption of CTA for reconstruction of upper extremity trauma.

Research Plans for the Next 4 Years

Based on the data obtained in Year 1, the researchers plan in Year 2 to supplement or replace the recipient conditioning with total body irradiation using fusion protein combinations active against IL15R and CTLA4Ig. In addition, they aim to prolong limb allograft survival in Year 3 by using targeted skin immunotherapy with leukocyte migration inhibitors in combination with optimal protocols from Year 2. Such targeted immunomodulatory protocols together with cell-based strategies could establish tolerance and ultimately eliminate the need for prolonged immunosuppression to maintain graft survival. Subsequently in Year 4, spaced dosing of tacrolimus monotherapy followed by weaning will be attempted under the cover of local immunotherapy.

Planned Clinical Transitions

For Phase 2 of this project, patients will be treated using the Pittsburgh Protocol to promote long-term hand transplant acceptance while minimizing the need for immunosuppressive drug therapy from Year 1 to Year 4. In Year 5, the researchers plan to translate findings from Phase 1 to Phase 2. They will design an optimized strategy combining targeted immunomodulation utilizing bone marrow stem cell/fusion protein induction and topical migratory inhibitors to further reduce maintenance immunosuppression and allow weaning of systemic drug therapy in hand transplantation. They note that this will overcome side effects related to high-dose immunosuppression, which will enable widespread clinical application of hand transplantation.

Corrections/Changes Planned for Year 2

None.

Project 4.4.1, WFPC

Blastemal Approach to Digit Reconstruction

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

Therapy: *Treat digit loss through epimorphic regeneration*

Deliverable(s): *Blastema-based strategy for inducing tissue and digit reconstruction*

Key Accomplishment(s): *Using the C57Bl/6 mouse digit amputation model, the researchers established a method for recruiting multipotential stem cells to the site of amputation by regional injection. They identified a partial genetic signature of the multipotential cell cluster (MCC) in vivo. They also isolated a more potent fraction of ECM degradation that shows in vitro chemoattractant results for perivascular stem cells.*

Introduction

The regeneration of lost limb and digit tissues as a result of trauma is being investigated through the concept of a "blastema-based approach." A blastema is the accumulation of preprogrammed stem cells that accumulate at the site of limb amputation in regenerative species such as the amphibian urodele (e.g., newt and salamander). In human fetal development, such structures exist and can replace lost tissues and organs, but this ability disappears at approximately gestational Weeks 16–18 and is replaced by the reparative processes of inflammation and scar tissue formation. The present work investigates mechanisms for recruiting large populations of endogenous stem cells to the site of limb and digit injury and then seeks to develop strategies to induce the formation of vascularized, innervated, functional limb and digit tissue to replace the damaged or missing structures.

Research Progress – Year 1

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.*

Using an established mouse model (C57Bl/6) of mid-second phalanx (P2) digit amputation, the researchers have definitively shown that endogenous multipotential stem cells can be recruited to the site of injury. They call this accumulation of cells a "multipotential cell cluster" or MCC. This induced migration of endogenous cells is caused by the local/regional administration of bioactive molecular derivatives of ECM (**Figure II-34**). The studies conducted in the first year of work show that the recruited cells express: Sca-1, RAR beta-1, Rex-1, Sox-2, and Pref-1. These markers are consistent with those found on multipotential stem and progenitor cells. Three of these markers, Sca-1, Pref-1, and Rex-1 were identified in studies before this past year. Sox-2 and RAR beta-1 have been identified in the past 10 months.

These cell markers are clearly associated with a multipotential phenotypic state; however, to further document the multipotentiality of these cells, the researchers have recently conducted a study in which these cells are microdissected from the amputation site 14 days after surgery and administration of the ECM compound. These dissected cells have been dissociated, placed in culture, and subjected to media conditions that promote differentiation along various cell lineages. The researchers have successfully shown the ability of these cells to form adipocytes, a mesodermal lineage cell type (**Figure II-35**), and neurons, an ectodermal lineage cell type (**Figure II-36**). They are currently expanding this list of cells that can be derived from the blastemal-like cell accumulation (referred to as an MCC).

The genetic signature is being further refined and confirmed by determining real-time PCR expression

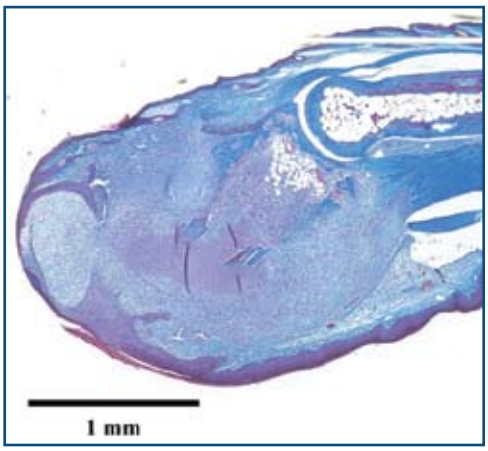


Figure II-34. The MCC present 14 days after amputation and treatment with ECM matricryptic peptides.

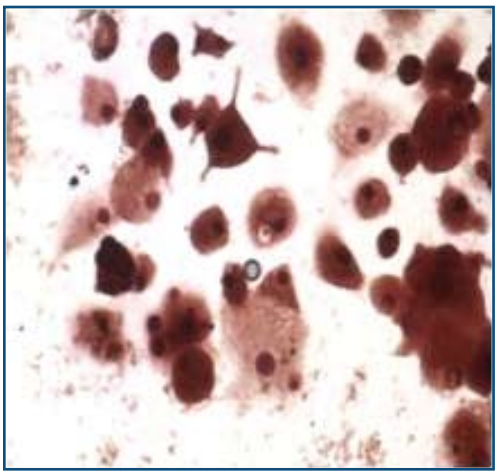


Figure II-35. Oil red O positive staining cells derived from the MCC when placed in lineage selective media. Most cells are large, round, and contain lipid vacuoles within their cytoplasm.

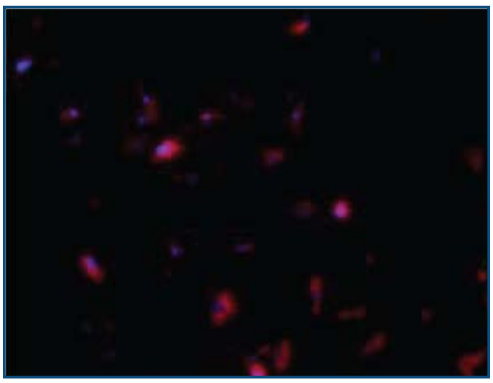


Figure II-36. Beta tubulin III positive staining cells derived from the MCC when placed in lineage selective (i.e., retinoic acid protocol) media. Most cells show dendritic processes in addition to the positive (fluorescent red) beta tubulin III stain.

profiles of the molecules that are represented by the above-mentioned markers. Other markers of multipotentiality are being investigated to further expand this genetic signature.

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

Enzymatically digested urinary bladder membrane has been shown to be chemotactic for stem cells. In an effort to identify the key chemotactic peptides from within the mix of peptides generated from enzymatic digestion, an approach was developed in which successive rounds of fractionation followed by analysis and selection was conducted. Digested urinary bladder membrane was fractionated initially with ammonium sulfate precipitation; the fractions containing simplified pools of peptides were then analyzed for their stem cell chemoattractive potential via Boydon chamber analysis. Fractions with the greatest chemoattractive potential were selected, concentrated, and refractionated via size exclusion chromatography. Peptide pool selection via chemoattractive potential was repeated, and the peptide fraction with the greatest potential was again selected. The process of fractionation, analysis, selection, and refractionation was repeated using ion exchange then reverse phase chromatography until a greatly simplified mix was generated and the reverse phase protein trace was running as single peaks. The peptide fractions with the greatest chemoattractive potential were again selected and analyzed via mass spectrometry, identifying short peptides that appear to represent fragments of some of the parent collagen molecules.

The researchers are in the process of repeating this experiment to document reproducibility. The individual molecules have been sequenced and if reproducibility is established, they will proceed to the step of synthesizing these molecules and investigating the ability to reproduce the bioactivity with a synthetic analog. Assuming success at being able to create molecules with the type of bioactivity that can induce a robust chemotactic response in vitro, the researchers will proceed to in vivo studies in which the synthesized bioactive molecules will be injected in various in vivo locations and the endogenous stem cell recruitment activity quantified (Specific Aim 3).

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

Through a reductionist approach in which intact porcine-derived ECM (urinary bladder origin) is solubilized and then separated by standard biochemical techniques such as size exclusion chromatography, ion exchange chromatography, reverse phase chromatography, and other methods as described for Specific Aim 2, the researchers

have been able to isolate fractions that contain bioactive degradation products of intact matrix molecules. Bioactivity is defined as the ability of these fractions to promote migration and/or proliferation of defined cell populations. The cell populations of interest include multipotential cells (a perivascular stem cell population recently described) and differentiated cells such as endothelial cells and fibroblasts.

Specific Aim 4: *Conduct a human digit reconstruction pilot study.*

Progress is dependent upon success in Specific Aims 1–3.

Key Research Accomplishments

- Using the C57Bl/6 mouse digit amputation model, established a method for recruiting multipotential stem cells to the site of amputation by regional injection.
  - Identified a partial genetic signature of the MCC in vivo including: Sca-1, Rar beta-1, Rex -1, Sox-2, and Pref-1.
- Recently isolated a more potent fraction of ECM degradation that shows in vitro chemoattractant results for perivascular stem cells.
  - This more potent fraction is currently being injected into the mouse model to determine in vivo efficacy.
- Sequenced two peptides that show chemotactic activity toward perivascular stem cells.
  - These peptides will now be re-evaluated for chemotactic property and then injected in vivo to determine efficacy.

Conclusions

The researchers have shown the ability to recruit endogenous multipotential cells to the site of injury in a nonregenerating mammalian system. The potential significance of this finding is important; however, the clinical utility will depend upon the ability to "instruct" these cells to form functional tissue. This ability will likely depend upon success at controlling the "microenvironmental niche." The niche defines the conditions and inductive stimuli that promote phenotypic differentiation, spatial organization, and functionality of the eventual tissue. To achieve this next step, this project will need additional resources, specifically, resources to develop a "biodome" (see "Planned Clinical Transitions" for more information).

Research Plans for the Next 4 Years

In Year 2, the researchers will identify in vitro bioactive molecules that can instruct, facilitate, or promote the formation of a blastema-like structure following injury. Year 3 work will evaluate the therapeutic efficacy of the molecules identified in Year 2 in the mouse model of digit amputation/regeneration. Year 4 and 5 work will develop a "biodome" to facilitate spatial and temporal organization of the recruited multipotential stem cells at the injury site for the purpose of functional tissue formation (see "Planned Clinical Transitions" for more information). The researchers will also identify potential industry partners. In Year 4, they will begin discussions with the FDA to evaluate the therapeutic molecules in humans.

Planned Clinical Transitions

The clinical utility of this approach will depend upon the ability to "instruct" the recruited cells to form functional tissue. This ability will likely depend upon the success at controlling the "microenvironmental niche." A "biodome" would represent a customized device to be placed over the site of injury and control microenvironmental conditions including hydration state, pH, oxygen tension, electrical potential, and other factors known to affect stem cell fate. In Year 4, the researchers plan to begin discussions with the FDA on evaluating the therapeutic molecules in humans. They hope to conduct a digit reconstruction pilot study on a human in 7–10 years based on successful completion of the specific aims.

Corrections/Changes Planned for Year 2

Additional resources and support will be needed to develop the "biodome" to facilitate spatial and temporal organization of recruited stem cells. This is not a trivial task and requires immediate support.

Project 4.4.7, WFPC

High-Throughput Technologies to Study Limb Regeneration

Team Leader(s): *Ron Stewart, PhD, and Jamie Thomson, PhD (University of Wisconsin)*

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

Therapy: *Method for activating and de-activating appropriate genes and gene networks to foster limb/digit regeneration*

Deliverable(s): *High-throughput method for identifying activated and deactivated genes and gene networks relevant to tissue regeneration*

Key Accomplishment(s): *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.*

Introduction

The researchers have been studying tissue regeneration using high-throughput technologies (microarrays and next-generation sequencing). In collaboration with Dr. Stephen F. Badylak's group, they have begun analysis of amputated mouse digit tips both from untreated mouse digits and those treated with ECM factors designed to enhance regenerative capabilities. The short-term goal of this collaboration is to identify genes and gene networks that are activated (or deactivated) during treatment. The longer term goal is to harness this knowledge in conjunction with methods from the researchers' prior work on the reprogramming of cells to activate or deactivate appropriate genes and gene networks to foster the regeneration of tissues.

Research Progress – Year 1

The researchers received 40 RNA samples from the Badylak group for microarray analysis. They completed a pilot experiment on 4 samples and a detailed time course experiment on 36 samples. They are using the NimbleGen whole genome mouse gene expression chip (NimbleGen 60mer chip, design MM8). Details on each experiment follow.

36-Sample Time Course Experiment

The researchers' 36-sample time course experiment included 3 replicates at 6 time points (ranging from day 0 to day 14) for both untreated mouse digits and those treated with ECM factors designed to promote regeneration. The researchers first performed a quality control study on all 36 samples, and 34 samples passed. Of these 34 samples, they chose 12 crucial samples for microarray analysis while the others served as potential replicates. These 12 samples had much higher quality RNA than the 4 RNA samples in the pilot experiment (discussion follows). They were labeled and hybridized for microarray analysis, and the researchers are now in the data analysis phase. Time course experimental data will provide crucial information on genes expressed early and late during the partial regeneration response seen in the ECM-treated samples. A time course experiment is crucial in that genes expressed very early in the time course are likely to be important for different processes than those expressed late (e.g., wound healing or matrix remodeling versus cell recruitment or de-differentiation).

4-Sample Pilot Experiment

The 4 samples in the researchers' pilot experiment included: mouse RNA to be used to evaluate protocols for RNA sample preparation and amplification ("RNA Test"), RNA from untreated, unamputated mouse digits ("UT"), 14-day post-amputation blastema (treated with ECM factors) ("ECM"), and 14-day post-amputation blastema (injected with saline) ("CTRL"). The amputation was performed as described in the P2 amputation model (**Figure II-37**).

Electrophoretic analysis indicates that the RNA is partially degraded

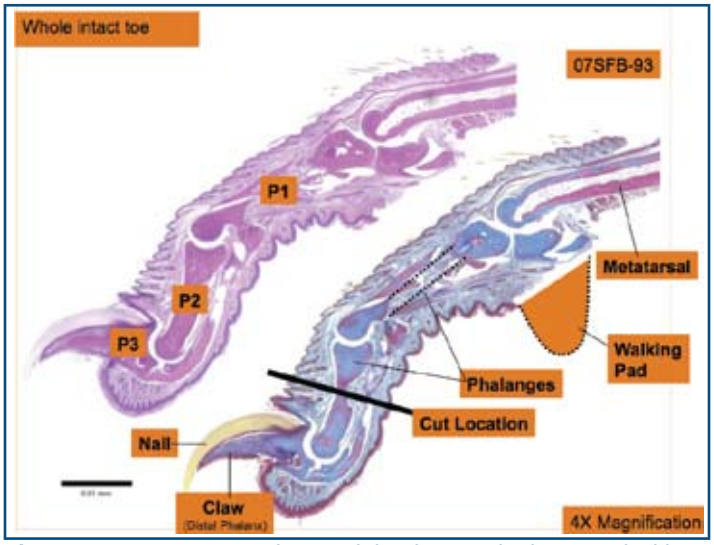


Figure II-37. P2 Amputation Model. The cut site is treated with various factors.

(**Figure II-38**). However, the mean fragment size (~110 bp) is still large enough to be detected on a microarray. In addition to the partial degradation issue, these tissue samples are very difficult to acquire. Thus, the researchers started with very small quantities (<100 ng) of total RNA, which are at least an order of magnitude less than the usual amount of starting material needed for microarray analysis. Because the RNA from UT, ECM, and CTRL is somewhat degraded, low in quantity, and very difficult to obtain, the researchers decided to first intentionally degrade the RNA test sample to a comparable size (~110 bp) and then run this sample on the mouse microarray (RNA test) before proceeding with the 3 other samples (UT, ECM, CTRL). While the RNA channel has an acceptable signal level, the RNA signal has an abnormally small dynamic range. Nevertheless, the researchers found approximately 1,000 genes to be expressed, indicating that the sample preparation procedures and amplification nominally worked even after partial degradation of the RNA.

Sample "ad" is a control RNA that is not degraded. Treated ("T" and "T10") and untreated RNA ("UT10") exhibit some degradation with a mean fragment size of approximately 110 bp.

While the results are mixed, because of the challenges in preparing the three other samples, the researchers proceeded with microarray gene expression analysis on these samples (UT, ECM, CTRL). The analysis shows some interesting preliminary results on the mouse digit tips. For instance, the mouse digit data show matrix metalloprotease-9 (MMP-9) to be one of the more highly upregulated genes in ECM-treated cases. MMP-9 (important for remodeling the ECM) is highly upregulated early in limb regeneration in the axolotl. One possible interpretation is that the mouse digit treatment is getting as far as some matrix remodeling steps (early steps in limb regeneration), but not getting further along, when MMP-9 would be less highly expressed in normal limb regeneration. The newer time course experiment will be able to further address this possibility as well as provide more detailed information on the treated and untreated mouse digit tips. The research-

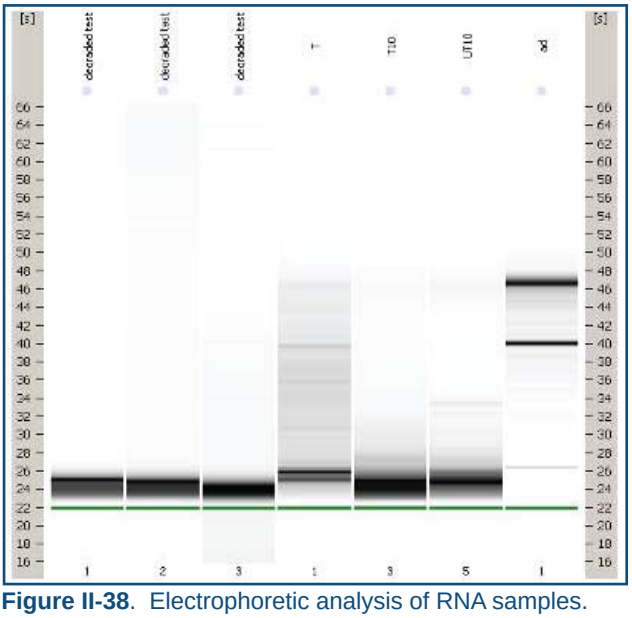


Figure II-38. Electrophoretic analysis of RNA samples.

ers will compare these data from the mouse with published axolotl blastema expression data to assess commonalities and differences.

In addition to the work on mouse digit regeneration, the researchers are utilizing the axolotl 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 regeneration. The researchers in the Thomson laboratory have begun an axolotl colony and will be performing RNA sequencing and microarray analyses of the limb blastema over the course of regeneration to identify activated or deactivated genes and gene networks. The blastema is likely to be composed of heterogenous cell types. The researchers are collaborating with another member of this consortium, Dr. Hyongsok Soh (University of California, Santa Barbara), in developing methods to manufacture affinity reagents for various rare precursor cell types present in the blastema. They will use these reagents in conjunction with Dr. Soh's dielectrophoretic cell sorter to distinguish cell types within the blastema. The sorted cells will then be tested for expression differences via RNA sequencing and epigenetic differences based on chromatin immunoprecipitation followed by sequencing (ChIP-seq). This information will be compared with nonregenerating blastemas and will allow the researchers to identify gene networks that are specifically activated in regenerating axolotl blastemas. They will also be able to identify the pattern of activation of these gene networks within the blastema.

Knowledge generated in the axolotl experiments will lay the foundation for activating blastemal-like activities in nonregenerating species such as the mouse. This work will also depend on reprogramming expertise in the Thomson laboratory being applied to the mouse digit regeneration model system developed by the Badylak group. The researchers plan on coordinating activities on the axolotl with functional testing strategies in the mouse to be performed in collaboration with Dr. Badylak.

Key Research Accomplishments

- Developed methods for sample preparation and RNA amplification from small-quantity RNA samples for microarray and next-generation sequencing analysis.
- Established data analysis pipelines for microarray and RNA sequencing analysis.
- Developed lentiviral-based methods for reprogramming cells based on altering transcriptional networks.
- Established methods for predicting gene networks based on co-regulation analysis.
- Completed pilot microarray study of mouse digit tips showing promising results with matrix remodeling genes and genes indicative of stem cell activity being expressed upon ECM treatment.
- Initiated a time-course microarray study that is currently in progress. High-quality RNA samples were produced and successfully processed for microarray analysis.

Conclusions

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. The ongoing collaboration with Dr. Soh on affinity reagent development and sorting technologies will allow this research team to dissect cell types within the axolotl blastema. Combined with the acquired gene network similarities and differences, this will give insight on how to manipulate the mouse model system to enhance digit regeneration by activating blastemal or MCC activities within the mouse digit.

A blastemal-based approach to limb regeneration, using the axolotl and mouse as model systems provides an excellent complement to the other work of the consortium. The blastemal-based approach has some longer term advantages. First, patterning is achieved as a byproduct of the process, taking advantage of the self-organizational capabilities of the blastema. Second, the regeneration is a scar-free process. Avoiding scar formation will be a crucial aspect of the success of this project. Third, a mature regeneration protocol would be minimally invasive. There are many significant challenges in activating blastemal-like activities in nonre-

generating organisms, and even a partial activation of these activities would represent a substantial advance in regenerative medicine.

Research Plans for the Next 4 Years

The research team will continue in Year 2 with microarray and/or deep sequencing RNA analysis to probe gene expression differences between ECM-treated mouse digits and controls. They will use this information in conjunction with existing literature on genes expressed in developing or regenerating limbs and digits to (1) characterize the ECM-treated phenotype and (2) identify genes and gene networks that likely need to be activated or repressed to augment mouse digit regeneration.

In Year 3 in collaboration with the Badylak group, the researchers will test genes and gene networks for the ability to augment mouse digit regeneration in the P2 amputation. Methods for transient expression or repression of particular genes will be developed. In Year 4, the researchers will continue with studies as defined in Year 3 and will define gene up- and downregulated in the axolotl. They will also determine active genes and gene networks in the regenerating axolotl limb. In Year 5, the cumulative data from Years 2–4 that are gathered from mouse and axolotl studies will be utilized along with the developed methods for gene activation and repression to guide the mouse digit work with the goal of improving digit regeneration.

Planned Clinical Transitions

Not specified.

Corrections/Changes Planned for Year 2

None.

Project 4.4.8, WFPC

Magnetophoretic Cell Sorting for Transplant Therapies

Team Leader(s): *Hyongsok Soh, PhD (University of California, Santa Barbara)*

Collaborator(s): *Badylak laboratory, UPMC, and Muschler laboratory, Cleveland Clinic*

Therapy: *Magnetophoretic cell-sorting device for improved transplant therapy outcomes*

Deliverable(s): *Systems for cell sorting that can isolate a small number of rare target cells*

Key Accomplishment(s): *The researchers developed a unique device that can achieve, for the first time, the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput. They also developed a device that allows purification of extremely rare cells from complex mixtures with unprecedented cell recovery.*

Introduction

Magnetic cell sorting allows high-throughput sorting of target cells based on surface markers. The technique is extensively used in biotechnology for a wide range of applications ranging from in vitro diagnostics to cell-based therapies. Existing methods (e.g., magnetic columns) suffer from two main disadvantages. First, separation is only based on a single parameter (i.e., the presence or absence of magnetization). Therefore, the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput is not possible. Second, current methods are unsuitable for the isolation of small numbers of rare cells from complex mixtures due to low rates of recovery. To address these critical problems, researchers in Dr. Hyongsok Soh's laboratory have developed two revolutionary methods of magnetic separation, and they are now utilizing these systems to isolate pluripotent, rare stem cells from tissues (in collaboration with the Badylak and Muschler laboratories).

First, the researchers have developed the Multi-Target Magnetic Activated Cell Sorter (MT-MACS), which makes use of microfluidics technology to achieve, for the first time, the simultaneous, spatially addressable sorting of multiple target cell types in a continuous-flow manner. Second, to handle extremely rare cells from small samples, they have developed the Continuous-Trapping Magnetic Activated Cell Sorter (CT-MACS). The researchers are currently working with the Badylak laboratory to adopt this separation technology to isolate blastema-like cells that express Sca-1 cell surface markers.

Research Progress – Year 1

Development of the MT-MACS. The MT-MACS makes use of microfluidics technology to achieve simultaneous, spatially addressable sorting of multiple target cell types in a continuous-flow manner. The researchers used the MT-MACS device to purify two types of target cells that had been labeled via target-specific affinity reagents with two different magnetic tags with distinct saturation magnetization and size. The device was engineered so that the combined effects of the hydrodynamic force produced from the laminar flow and the magnetophoretic force produced from patterned ferromagnetic structures within the microchannel result in the selective purification of the differentially labeled target cells into multiple, independent outlets. For the first time, the researchers demonstrated the capability to simultaneously sort multiple magnetic tags with >90% purity and >5,000-fold enrichment, and multiple bacterial cell types with >90% purity and >500-fold enrichment at a throughput of 10^9 cells per hour. They believe this innovative novel capability will have a significant impact for research as well as future clinical cell-sorting applications.

Development of CT-MACS Device for Rare Cell Isolation.
To isolate extremely rare cells from complex background matrices, the researchers have developed the CT-MACS device (**Figure II-39**). This device offers significant advantages over the conventional magnetic separation apparatus (e.g., magnetic columns) because it offers the capability to precisely control the hydrodynamic and magnetophoretic forces within the microchannel, enabling highly efficient manipulation of a small number of cells without any loss and imposing washing conditions that are stringent and reproducible. Thus far, the CT-MACS system has been used to successfully capture extremely rare cells (<200 cells/sample) from an excess of background cells (~10^9 cells/mL) with exceptional recovery (>90%).

Current progress on isolation of blastema-like progenitor cells from mice (in collaboration with the Badylak Laboratory, UPMC).
Currently, in collaboration with the Badylak laboratory at UPMC, the researchers are utilizing the CT-MACS chip to extract "blastema-like" progenitor (Sca-1 positive) cells from harvested mouse tissue samples and regenerated mouse toe tissues. Due to the low number of total cells (~10^4) that will be received from the tissues, high viability and cell recovery are critical. The CT-MACS device provides a key advantage over conventional magnetic columns because the sample size is extremely small (<100,000 cells), and the target cells occur at low frequencies (< 200 cells per sample). The schematic of the micromagnetic cell-sorting procedure is shown in **Figure II-40**.

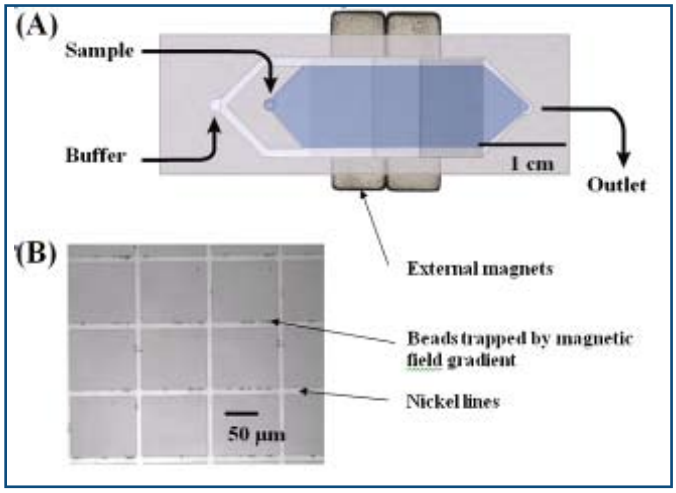


Figure II-39. CT-MACS device architecture. (A) Photograph of the microdevice with the external rare earth magnet. The device is 64 mm × 15.7 mm × 1 mm (L × W × H) and contains two inlets (sample and buffer) and one outlet. The microchannels inside the device are 30 μm in height and 12 mm in width. The microchannel contains microfabricated nickel strips with widths of 10 μm. During operation, the sample stream is flanked by two buffer streams. (B) Optical micrograph of the trapped magnetic beads. The beads are captured at the edge of nickel patterns where the magnetic field gradient is the strongest.

The first shipment of cells was used to assess their viability through the process. While refrigerated and suspended in Dulbecco's modified Eagle's medium through the course of overnight shipping and a full day of experimentation including incubation with magnetic beads, the bulk marrow sample yielded no detectable cell death compared to the time of harvest.

Key Research Accomplishments

- Developed the first MT-MACS chip, which can simultaneously sort multiple target cells with high purity, recovery, and throughput.
- Developed a custom CT-MACS device, which allows high-performance magnetic sorting of extremely rare target cells even in complex backgrounds containing a tremendous excess of background cells (e.g., whole blood and marrow samples).
- Collaborated with the Badylak and Muschler laboratories to use these microdevices to isolate extremely rare cells from mouse models.
- Preliminary tests with Sca-1-positive cells harvested from mice were successful but also revealed the need for refinements to the device design and labeling protocol.
- Built and tested a prototype second-generation device incorporating both MT-MACS and CT-MACS.

Conclusions

In this first year of AFIRM support, the researchers have developed two unique devices that solve severe

shortcomings inherent in current methods of magnetic cell sorting. In particular, they have developed the MT-MACS platform to achieve, for the first time, simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput. In addition, they have developed the CT-MACS device, which allows purification of extremely rare cells from complex mixtures with unprecedented cell recovery. A two-stage device incorporating a combination of both technologies promises even better performance with regard to purity.

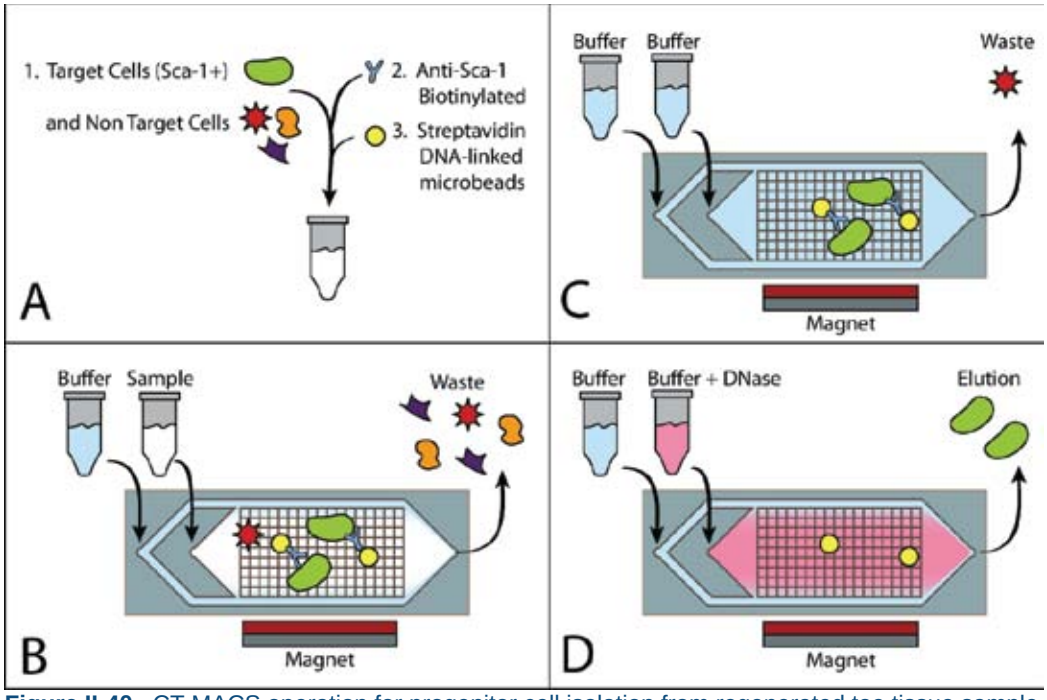


Figure II-40. CT-MACS operation for progenitor cell isolation from regenerated toe tissue sample. (A) Toe tissue is digested to release target and nontarget cells in solution. Anti-Sca-1-biotin antibody is added to bind to target cells. Streptavidin-DNA-linked microbeads are added to magnetically label target cells. (B) The sample is injected into the CT-MACS chip where all labeled target cells are captured and nontarget cells pass through uncaptured. (C) To wash out nonspecifically bound nontargets, more buffer is injected while maintaining the magnetic field. (D) Purified target cells are eluted by cleaving the DNA that links the antibody to bead with DNase enzyme. Collected cells may be analyzed or cultured externally.

To fully exploit the utility of the microfluidic devices, the researchers are collaborating closely with the Badylak and Muschler laboratories to isolate pluripotent progenitor cells from tissues of model animals. If successful, they believe their method will provide AFIRM researchers with the unprecedented capability to isolate rare stem cells with exceptional purity and recovery that cannot currently be matched by commercial instrumentation. They believe this unique cell-sorting capability will provide a critical technical solution to isolate the target stem cells to elucidate the mechanism of their function at the molecular level.

Research Plans for the Next 4 Years

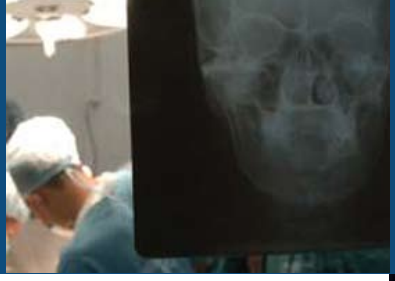
In the upcoming years (2–3), the researchers plan to develop a portable benchtop system so that collaborators within the AFIRM consortium can have access to this unique and powerful capability. In Years 3–5, they expect to scale up the system so that it can process larger volumes of samples for human applications. If successful, these new methods of magnetic-activated cell sorting will have a significant impact for research and clinical applications in which the sorting of rare target cells with high purity, recovery, and throughput are important.

Planned Clinical Transitions

Not specified.

Corrections/Changes Planned for Year 2

None.



III Craniofacial Reconstruction

Background

The AFIRM Craniofacial Reconstruction (CFR) Program aims to reduce the impact of devastating, disfiguring facial injuries on wounded warriors through the application of regenerative medicine. Current statistics show that 26% of all wounded warriors treated in U.S. military facilities suffer maxillofacial injuries, which include soft tissue face avulsions and facial burns in addition to craniofacial bone defects. Notably, craniofacial injuries rob victims of their physical identity. These injuries are therefore not just physically but also psychologically excruciating and impose tremendous costs on both the warrior and the military over the long term. Blast injuries and injuries from high-velocity projectiles, such as those encountered on the battlefield, present a range of therapeutic challenges and often require a staged repair. A significant need exists for the development of novel regenerative medicine approaches for the generation of both soft and hard tissues to overcome the current clinical barriers to craniofacial reconstruction.

Working along the continuum from bench research to translation and in collaboration with other AFIRM programs, the CFR Program aims to return warriors suffering these injuries to fully functional lives—independent, reintegrated, and with identities intact. To that end, the program is exploring a range of solutions from novel scaffolding and cell source materials to innovative approaches to the obstacles in transplantation of composite tissue allografts (CTAs) (e.g., hand transplants). The program comprises a multidisciplinary, multi-institutional collaborative research team to address the core issues associated with traumatic injuries to the craniofacial complex. Drawing on the strengths of each researcher on the team, an optimal set of complementary technologies is being identified to achieve hard and soft tissue regeneration. Over the next 4 years, with the resources and support of the AFIRM consortium, the CFR Program anticipates delivering advances that will dramatically improve the treatment of these devastating injuries.

Unmet Needs

Treatments in craniofacial reconstruction practices are currently inadequate to treat the unique and massive craniofacial deficits resulting from blast injuries. Massive craniofacial bone loss incurred in combat is presently reconstructed with nonresorbable synthetic bone materials, hard tissue replacement, or metallic devices that may transiently restore anatomical form and limited function. However, the currently available synthetic bone materials do not remodel and integrate with host tissue and can become infected and require extensive, multiple revision surgeries. Overall, contemporary surgical solutions are inadequate to regenerate massive bone loss in the craniofacial complex. Therefore, a biodynamic, biocompatible substitute for complex bone defects is a critical need in facial injury.

Soft tissue deficits may be treated with pedicled muscle and skin flaps and allogenic[1] skin substitutes. But these treatments do not restore functional muscle and nerve tissue; 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.

Current techniques and therapies neither mitigate movement-limiting scar contracture nor achieve complex soft tissue coverage esthetics. The gold standard treatment for reconstruction of the ear uses a patient's own rib cartilage as a graft material. However, autologous[2] rib cartilage grafts are limited in supply and provide inadequate dimensions of the ear cartilage tissue. An alternative approach utilizes a commercially available ear implant device (e.g., MedPor®), which is manufactured from linear, high-density polyethylene. This U.S. Food and Drug Administration (FDA)-approved implant is nontoxic, causes minimal foreign body reaction, and possesses adequate mechanical properties for use in nonload-bearing regions of the craniofacial skeleton. However, MedPor ear implants are often associated with complications, including inflammation, infection, erosion, dislodgement, and extrusion. A readily available, or readily generated, replacement tissue for complex soft tissue structures such as the ear is an unmet need in facial injury.

With severe facial injuries, loss of tissue can be massive. In such cases, there is no satisfactory surgical approach to facial replacement other than transplantation. However, 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 opportunist 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.

New patterns of injury have been observed in warfighters, including devastating craniofacial trauma. Repair and reconstruction of craniofacial injuries are exceedingly complex because of the issues involved in protecting vital senses; the unique challenges of repair, reconstruction, and regeneration in the presence of oral and periodontal bacteria; changes in repaired/replaced tissue over time; challenges in restoring/providing the capabilities for verbal and nonverbal communication and expression; and concerns related to managing not only the physical and cosmetic but also the psychosocial sequelae, frame of self-reference, and optimism of the patient. Therefore, planning the complex reconstruction and regeneration needs of wounded warriors with severe facial injuries is a critical need.

Areas of Emphasis

The Rutgers-Cleveland Clinic Consortium (RCCC) and Wake Forest-Pittsburgh Consortium (WFPC) are pursuing a complementary mix of research projects focused on various aspects of craniofacial reconstruction. Projects can be grouped into five "clinical challenge" topic areas: Bone Regeneration, Soft Tissue Regeneration, Skeletal Muscle and Nerve Replacement, Cartilage Regeneration (with a focus on the ear), and Virtual Modeling for Craniofacial Reconstruction. Addi-

[1] *Taken from different individuals of the same species.*
[2] *Originating from within the organism.*

Table III-1. AFIRM-funded projects per clinical challenge topic area.

Clinical Challenge Topic Area	Consortium	Project Number	Project Title
Bone Regeneration	RCCC	4.5.1	Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex
	WFPC	4.1.2	Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects
	WFPC	4.1.3	Novel Synthetic Bone
Soft Tissue Regeneration	RCCC	4.3.1	Composite Tissue Allograft Transplantation Without Life-Long Immunosuppression
	WFPC	4.1.4	Soft Tissue Regeneration
	WFPC	4.1.5	Injectable Implantable Engineered Soft Tissue for Trauma Reconstruction
Skeletal Muscle and Nerve Replacement	RCCC	4.1.2	Develop Innervated, Vascularized Skeletal Muscle
	WFPC	4.1.6	Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle
Cartilage Regeneration (Focus: Ear)	RCCC	4.5.4	Regeneration of Ear
	WFPC	4.1.1	Engineered Cartilage Covered Ear Implants for Auricular Cartilage Reconstruction
Virtual Modeling for Craniofacial Reconstruction	RCCC	4.5.5	Visualization of Patient-Specific Wounds and Injuries

tional details on projects in each of these topic areas can be found in **Table III-1** and subsequent sections of this chapter.

Bone Regeneration

Studies at RCCC

Overview: RCCC is developing biodegradable biomaterial platforms in Project 4.5.1, which researchers hope will provide compelling therapeutic solutions to regenerate craniofacial bone. The platforms incorporate bioactive bone-producing agents and will overcome the technical barrier to regenerate bone form and function in the craniofacial complex. In addition, the biomaterials will be sufficiently robust to bear load, an essential property for mid-face and jaw reconstruction. Furthermore, isolated stem cells and biomaterial surface modifications will support, enhance, and accelerate the regenerative soft and hard tissue healing cascades and mitigate scar formation and motion-limiting contracture.

Status at End of Year 1: The researchers have developed a bone/particle polymer composite bone void filler for the repair of craniofacial bone defects (**Figure III-1**). The product has passed ISO 10993 toxicity tests, which are internationally recognized standard tests for the biological evaluation of medical devices. Osteotech, Inc. has licensed the composite technology for development as a bone void filler. The researchers have submitted two provisional patent applications to the U.S. Patent and Trademark Office covering the composite bone void filler.

Research Plans for the Next 4 Years: During the upcoming year, the researchers will continue an FDA-inspired testing paradigm that emphasizes scaffold biocompatibility, biodegradability, cell interactions, and preclinical bone regeneration. They will perform a rabbit skull defect study at the U.S. Army Institute of Surgical Research (USAISR) during Year 2. They also plan to develop materials for reconstruction of the jaw in collaboration with USAISR. Lead candidate materials will be screened in a rabbit model before proceeding to a larger animal segmental defect model in Year 3.

Planned Clinical Transitions: The researchers will continue to engage actively with military surgeons, staff at USAISR, and clinical FDA consultants to ensure that they

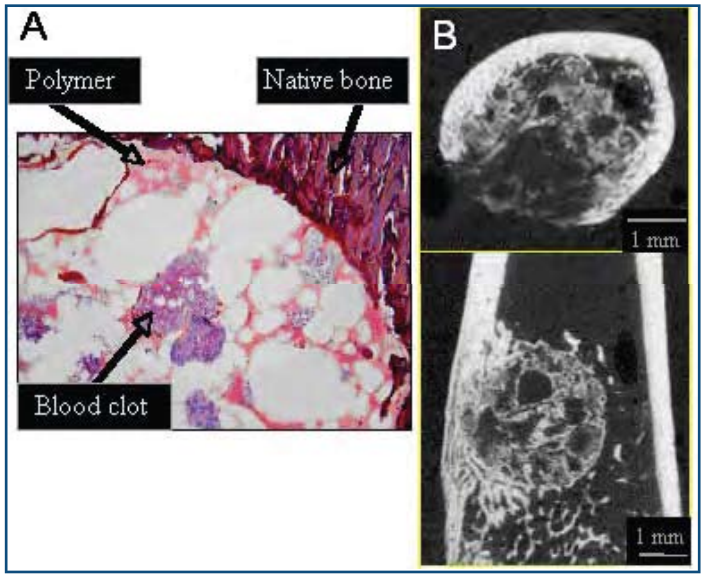


Figure III-1. (A) Histological section of the RCCC researchers' bone particle/polymer composite scaffold showing the scaffold-bone interface. (B) Computerized tomography images of the scaffold at 6 weeks showing new bone formation.

focus on the most expedient pathway to the clinic. Collaborators at Osteotech, Inc. will utilize data from the rabbit skull defect study in a 510(k) biomedical device application, which is currently targeted for the first quarter of 2010. Pending a successful regulatory filing, a clinical trial will be pursued.

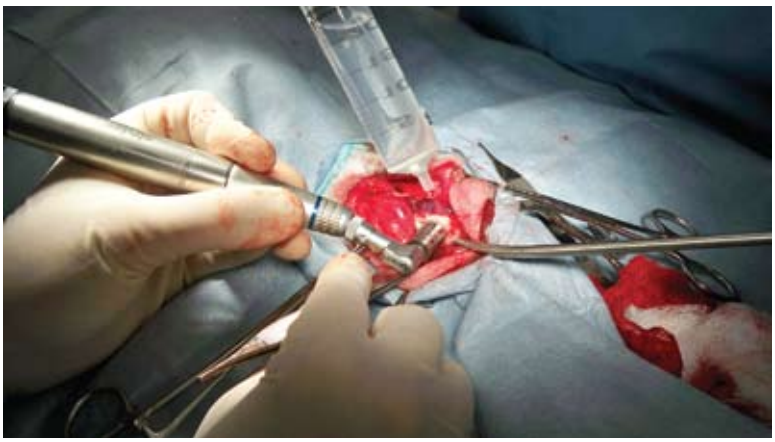
Studies at WFPC

Overview: Maintaining the proper anatomical relationship of the tissue adjacent to a bone defect is important, and Project 4.1.2 is focused on developing porous space maintainers that will serve this purpose. The researchers are also developing "in vivo bioreactor" technology to produce a bone flap with a vascular (blood) supply away from the site of injury that can be transplanted to the wound space once the wound has been optimized for reconstruction. In Project 4.1.3, WFPC researchers are developing nanostructured 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.

Status at End of Year 1: In Project 4.1.2, the researchers have fabricated and characterized a variety of porous space maintainer formulations presenting a range of porosities and mechanical properties. They have completed a pilot study focused on evaluating several space maintainer formulations for their ability to prevent soft tissue collapse in a nonhealing rabbit jaw defect model. The researchers have also characterized the release of antibiotics of interest from biodegradable microspheres following their incorporation within the space maintainers. In addition, they have identified a clinically viable antibiotic to treat a battlefield-relevant target bacterial species.

In Project 4.1.3, the researchers have successfully developed and characterized novel nanostructured bioactive bone cements. They have also initiated assessments of the cements in a rabbit skull defect model, and results show that the synthesized forms of cement materials are biocompatible. Finally, preliminary experiments in a rabbit ulna (forearm) defect model show the cements to have bone regenerating (bridging) properties.

Research Plans for the Next 4 Years: In Project 4.1.2, the researchers will continue to explore



WFPC researchers are creating a 10 mm nonhealing rabbit jaw defect model, which is used to explore various material formulations for bony space maintenance.

the release of antibiotics from the space maintainers in the rabbit jaw defect model. They will also continue to investigate the efficacy of an implantable "in vivo bioreactor" to prefabricate a vascularized bone flap. Finally, they will synthesize fumarate-based hydrogels and explore the controlled release of a variety of growth factors from these hydrogels. In Project 4.1.3, the researchers will continue to screen several versions of the synthesized bone cements in the rabbit ulna model to determine the best possible clinical candidate.

Planned Clinical Transitions: Working in collaboration with the Center for Clinical and Translational Sciences at the University of Texas Health Science Center at Houston, the researchers of Project 4.1.2 will prepare and submit forms and protocols for Institutional Review Board (IRB) approval of future clinical studies and FDA approval of their space maintaining devices. In Project 4.1.3, the researchers will continue to focus on further developing the bone cements to gather more data for FDA approval and eventual clinical trials.

Soft Tissue Regeneration

Studies at RCCC

Overview: RCCC is conducting two related studies under Project 4.3.1. Both of the studies are intended to make CTAs safer and more widely available to victims of disease and traumatic injury. In the first study, the researchers are testing the safety and efficacy of a therapeutic antibody, TOL101, as a conditioning agent in kidney transplant and CTA recipients.

In the second study, the researchers are developing a method for producing bioengineered donor-recipient chimeric cells that are capable of inducing lifelong immune tolerance in humans pre- or post-transplant.

Status at End of Year 1: The researchers have demonstrated the clinical feasibility of reconstructing massive post-traumatic segmental tissue loss in the face using allograft tissue by completing the first near-total face transplant in a civilian patient in the United States. This procedure further established the precedent for use of this therapeutic approach, even using conventional immunosuppression. The antibody TOL101 has undergone Good Manufacturing Practice (GMP) manufacturing, assurance testing, and pre-investigational new drug screening with the FDA. The therapeutic effect of the bioengineered chimeric cells was confirmed by prolonged survival of skin allografts.

Tyrosine-derived polycarbonate scaffolds developed at Dr. Kohn's laboratory (RCCC). The osteo-conductive potential of these scaffolds is being evaluated using a critically sized defect in the rabbit calvaria.

Research Plans for the Next 4 Years: The researchers will continue in the upcoming year to work actively with COL Robert Hale, MD, at USAISR and within other avenues, to define appropriate pathways, teams, and clinical protocols that will enable the methods they are developing to be deployed to treat injured warriors. They plan to assess the efficacy of their donor-recipient chimeric cells for adoptive transfer (i.e., passing on immunity from an immune individual to a non-immune one).

Planned Clinical Transitions: The researchers anticipate enrolling human patients in a TOL101 antibody clinical trial in late 2009, following extensive preclinical testing initiated at the request of the FDA. They anticipate completing initial human safety and efficacy testing (in approximately 30 human kidney transplant recipients) in the third quarter of 2010. The Cleveland Clinic granted IRB approval for a combined face and upper extremity transplant in April 2009. The team currently has an IRB submission for face and face plus bilateral upper extremity pending at the Brooke Army Medical Center (BAMC) IRB. The FDA will be engaged near the end of Year 2 using appropriate regulatory consultation to

design safety and efficacy trials that will be needed for the chimeric cells. The researchers project the first human therapeutic use of the chimeric cells in late 2011.

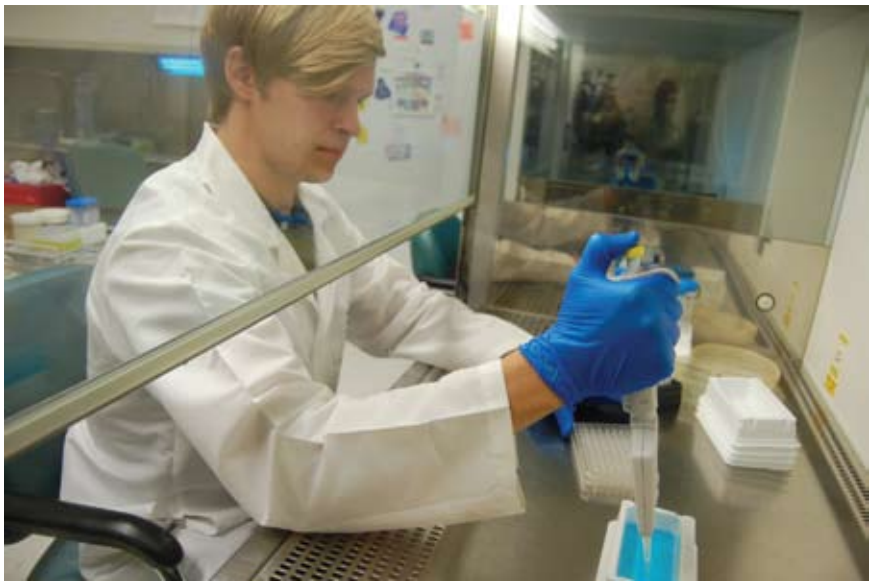
Studies at WFPC

Overview: A partnership between three leading institutions (Tufts University, University of Pittsburgh, and Wake Forest University) was formed to develop and deliver a clinically useful engineered soft tissue replacement that can either be used as a stand-alone therapy or integrated with composite tissue regenerative medicine therapy of burns, craniofacial injuries, and extremity injuries. While initiated as two separate AFIRM projects (Project 4.1.4, Soft Tissue Regeneration, and Project 4.1.5, Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction), the researchers quickly combined their unique talents into a strong and productive collaboration. The main scientific approach involves the use of autologous (i.e., a person's own) adipose stem cells and fibroblasts, combined with carrier biomaterials, to achieve vascularized soft tissues.

Status at End of Year 1: The researchers of Projects 4.1.4 and 4.1.5 developed four types of silk scaffolds (silk sponge, collagen type 1 coated with silk sponge, silk gel, and silk particles mixed with fibrin glue) and showed that each scaffold supported the growth of soft tissue. Preliminary results demonstrated that the silk gel and silk sponge scaffolds were the most promising configurations. The researchers developed a collagen hydrogel delivery system that releases a signaling protein that stimulates the growth of blood vessels. They implanted cell-hydrogel constructs subcutaneously (i.e., under the skin) in immunodeficient mice and have begun to evaluate the dimensional changes in the mice at various time points post-implantation. They also developed microspheres containing proteins known to promote blood vessel infiltration, which are expected to enhance tissue formation by the cell-hydrogel constructs.

Research Plans for the Next 4 Years: The researchers plan to continue to develop the silk scaffolds as well as maintain active interinstitutional collaborations over the next 4 years.

Planned Clinical Transitions: The silk-based biomaterial used in these studies has recently received FDA approval. Continued effort on the project will focus on the rapid advancement toward clinical trials with the technologies being developed.



Jedidiah McAtee isolates human adipose-derived stem cells, a process which involves enzymatic digestion (WFPC).

Skeletal Muscle and Nerve Replacement

Studies at RCCC

Overview: Injuries to the facial muscles significantly impact the health and self-esteem of the injured warfighter. In Project 4.1.2, RCCC researchers are engineering skeletal muscle with physiological connections to the host's neurovascular (i.e., nerve and blood vessel) network using biodegradable polymer scaffolds. They will implant these engineered muscle constructs in animal models, expose them to physiological stresses, and link them to the host neurovascular networks.

Status at End of Year 1: The researchers have successfully engineered immature muscle tissue through a self-assembly culture process. They are developing a biopolymer scaffold with a "muscle" layer and a "vascular" layer. They employed mesenchymal stem cells (MSCs) to support the formation of the prevascular network. The

researchers implanted the engineered immature muscles into adipose tissue of mice and determined that they express proteins involved with muscle contraction.

Research Plans for the Next 4 Years: The researchers will continue to optimize their scaffold design using mathematical and computational methodology. They will confirm the development and alignment of the muscle fibers, assess the cellular features of the developed muscle tissue, confirm blood vessel linkage with the host vasculature, and validate functionality of the engineered muscle through nerve excitability and muscle contractility measurements.

Planned Clinical Transitions: The research team is currently focused on the analytical and laboratory studies needed to validate the elements of the engineered skeletal muscle.

Studies at WFPC

Overview: Engineering skeletal muscle tissues with a patient's own cells would accelerate wound healing with cosmetic augmentation of the tissue defect and thus enhance restoration of tissue function. In Project 4.1.6, WFPC researchers will continue to develop the technology to further probe the feasibility and applicability of creating contractile skeletal muscle tissues through the use of a bioreactor system in conjunction with novel biomaterials/scaffolds and optimized bioreactor protocols.

Status at End of Year 1: The researchers have developed a detailed protocol for the production of bioengineered scaffolds seeded with well-characterized rat cell populations and conditioned in a bioreactor. They have recently published on their ability to generate an organized muscle tissue from human muscle precursor cells on a cell-free scaffold. They have more recently designed scaffolds that support the growth of skeletal muscle fibers. They have also created a custom-designed seeding chamber for their muscle scaffolds using an FDA-approved pharmaceutical-grade silicone rubber.

Research Plans for the Next 4 Years: In Years 2–5, the researchers will continue to evaluate the feasibility of implanting engineered rat skeletal muscle in a rat skeletal muscle defect model. They will also investigate the translation of engineered skeletal muscle in a craniofacial muscle defect in a larger animal model. Finally, they will determine the feasibility of using biopsies from humans in generating functional engineered skeletal muscle tissue.

Planned Clinical Transitions: The validation provided through the proof-of-concept studies will facilitate the clinical translation of the bioreactor technology for skeletal muscle development.

Laboratory engineered muscle tissue is preconditioned in a bioreactor system (WFPC).

Cartilage Regeneration (Focus: Ear)

Studies at RCCC

Overview: Reconstruction of the total external ear is one of the most difficult problems in the field of plastic and reconstructive surgery. In Project 4.5.4, RCCC researchers aim to expedite the development of a permanent, implantable, living external ear for the injured warfighter and to achieve cosmetic outcomes that meet patient expectations.

Status at End of Year 1: The researchers have successfully developed a sheep model for the subcutaneous implantation of ear-shaped constructs. They are designing these ear scaffolds to maintain the original three-dimensional shape of the construction upon implantation and to form functional cartilage from seeded cartilage and stem cells. They are refining the scaffold parameters using computer-assisted design techniques to achieve accurate architectural definition and to resist scaffold distortion when placed under the skin.

Research Plans for the Next 4 Years: In Years 2–3 of the project, the team will enhance cartilage formation from nasal-septal and auricular sources by optimizing cell-seeding density and bioreactor culture. If required, they will augment cartilage formation from primary cartilage cells by co-culture with MSCs. The researchers will also continue to evaluate candidate scaffold materials for their ability to support three-dimensional cartilage formation in both immunocompromised mice (using human cartilage) and immunocompetent sheep (using autologous cartilage).

Planned Clinical Transitions: The researchers should complete preclinical trials by the end of the second quarter in Year 4 and will analyze, audit, and report those studies to the IRB. The team will develop a clinical protocol for a pilot study during the first two quarters of Year 4 and will submit this to the IRB. They expect to begin a clinical trial by the end of Year 4.

Studies at WFPC

Overview: In Project 4.1.1, WFPC researchers seek to overcome the complications commonly associated with current implants for auricular reconstruction (e.g., MedPor) by coating nondegradable implants with engineered cartilage produced by the patient's own cells. Specifically, the researchers are engineering cartilage tissue that will entirely cover the abrasive, nonorganic ear implant, which would prevent implant exposure and extrusion, while maintaining appropriate mechanical properties. They aim to refine and optimize the processing system for a smooth translation to soldiers who require reconstruction of the ear.

Cartilage cells are seeded on an ear scaffold by WFPC researcher Jin San Choi, MS, with the goal of engineering an ear implant entirely covered by cartilage.

Status at End of Year 1: The researchers obtained cartilage cells from New Zealand white rabbits and human nose and rib cartilage tissues. They grew the cells in culture and characterized them using a variety of assays. They prepared hydrogels containing cartilage cells and implanted MedPor ear constructs into immunodeficient mice either alone or covered with the hydrogels. The researchers found that nontreated ear implants resulted in severe skin necrosis at 2 weeks post-implantation. They observed that the cartilage-covered ear implants were able to maintain device contour and placement without causing skin necrosis.

Research Plans for the Next 4 Years: In Year 2, the researchers will continue to assess the biocompatibility and structural stability of the engineered cartilage ear implants. In addition, they will assess the host tissue response in the animal model. In Years 3–5, the researchers will begin identifying and selecting patients for enrollment in an initial clinical trial.

Planned Clinical Transitions: The researchers seek to continue to develop a system that requires a minimum tissue biopsy for cell isolation and expansion using different cell sources and to refine the cell delivery system to facilitate clinical translation of the cartilage-coated auricular implants.

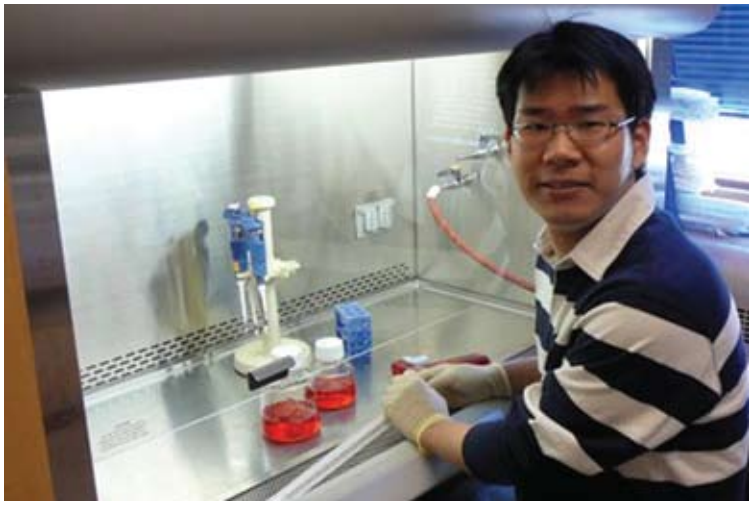
Virtual Modeling for Craniofacial Reconstruction

Studies at RCCC

Overview: In Project 4.5.5, RCCC researchers are developing a virtual reality visualization tool for patient-specific wounds and injuries. This tool should help integrate specific tissue regeneration strategies both within the CFR Program and throughout the AFIRM. The approach of the program will be staged, proceeding from the least biomechanically challenging to the most challenging zones in the cranio-maxillofacial complex.

Status at End of Year 1: During the first year of work, the SimQuest LLC team built the underlying infrastructure of a virtual reality system for visualizing patient-specific craniofacial wounds/injuries. The researchers assembled a beginning set of anatomically correct three-dimensional surface models of the face to serve as the foundation for its index into injury and wound data. They determined the level of tissue rendition necessary to capture iconic injuries for application in repair/regeneration integration and treatment. They also developed a database schema for information encoding.

Research Plans for the Next 4 Years: SimQuest's research plan is to develop the platform for translating AFIRM results from laboratory prototypes into clinical practice. Specifically, the technology to be developed comprises injury data analysis tools, patient-specific wound and injury models, and physics-based surgical simulation. The injury analysis tools identify successful or unsuccessful outcomes and serve to identify the common elements of treatment that may be the causative agents. The patient-specific modeling for the AFIRM will delve into the segmentation and modeling of targeted soft tissue structures such as specific muscles, nerves, vasculature, and scar tissue. New algorithms will be developed to segment these structures from computed tomography data. Once the patient-specific models are robust from a geometric standpoint, SimQuest will leverage its surgical simulation technology. Researchers will develop a simulation tool that can show how surgically moved or transplanted tissue will appear given the geometry and physics of the tissue.



Nathaniel Hwang, PhD, a postdoctoral associate in Dr. Langer's laboratory, is currently working on the Ear Tissue Regeneration Project in collaboration with Dr. Joseph Vacanti's group at Massachusetts General Hospital (RCCC).

Planned Clinical Transitions: The work that SimQuest is proposing builds upon several ongoing projects. The first of these is the craniofacial injury mapping project being performed in conjunction with the Walter Reed Army Medical Center. This project is developing the baseline capability for the injury analysis tools. As part of that project an IRB submission has been prepared and is currently being evaluated by the Walter Reed Army Medical Center IRB. As part of the AFIRM project, deployment of the injury tools will be extended to include the BAMC patient population and possibly others. IRB permission will be sought for this extension. The core focus of SimQuest is the development of surgical simulators targeted at training. The patient-specific modeling and simulation efforts of the AFIRM will feed into this product line and will be offered commercially through a variety of channels.

Project 4.5.1, RCCC

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial (CMF) Complex

Team Leader(s): *Jeffrey O. Hollinger, DDS, PhD (Carnegie Mellon University [CMU])*

Project Team: *Jinku Kim, PhD, Anuradha Karunanidhi, Joseph King, Sean McBride, Lyndsey Schutte, Aditi Sharma (CMU); Scott Guelcher, PhD, Shaun Tanner, Bing Li (Vanderbilt University); Joachim Kohn, PhD, Das Bolikal, PhD, Aniq Darr, PhD, Hanshella Magno (Rutgers – The State University of New Jersey, New Jersey Center for Biomaterials); Michael J. Yaszemski, MD, PhD, Mahrokh Dadsetan, PhD, Brett Runge, PhD (Mayo Clinic); Josh Wenke, PhD (USAISR); COL Robert Hale, MD (BAMC)*

Collaborator(s): *Subha Bhattacharyya, PhD, Todd Boyce, PhD, Osteotech, Inc. Eatontown, New Jersey*

Therapy: *Treatment of bone loss in the CMF complex*

Deliverable(s): *Injectable and implantable craniofacial bone scaffolds*

Key Accomplishment(s): *An injectable allograft bone/polymer composite bone void filler has been developed for the repair of craniofacial defects. The technology has been licensed to Osteotech, Inc., and the product has passed ISO 10993 toxicity tests. A 510(k) device application will be filed in the first quarter of 2010 pending the successful outcome of the rabbit calvarial defect study.*

Introduction

Massive bone loss to the CMF complex incurred in combat is reconstructed with either nonresorbable synthetic bone materials such as poly (methyl methacrylate [PMMA]), hard tissue replacement, or metallic devices (e.g., titanium) that may transiently restore anatomical form and limited function. When available, allogeneic and autogenous grafts are options. Soft tissue deficits may be treated with pedicled muscle and skin flaps and allogeneic skin substitutes. Contemporary surgical solutions are inadequate to regenerate massive osseous avulsion in the CMF complex. For these reasons, in the current research program, three potential biodegradable biomaterial platforms are being considered that may provide compelling therapeutic solutions to regenerate craniofacial bone: (1) mineralized bone particles/polyurethane (MBP/PUR), (2) tyrosine-derived copolymers, and (3) polyesters.

The platforms may incorporate bioactive angio-osteogenic agents and will overcome the technical barrier to regenerate osseous form and function in the CMF complex. In addition, the biomaterials will be sufficiently robust to bear load, which is an essential property for mid-face and mandibular reconstruction. Furthermore, isolated pluripotent cells and biomaterial surface modification will support, enhance, and accelerate the regenerative soft and hard tissue healing cascades and mitigate scar formation and motion-limiting contracture.

Research Progress – Year 1

Overall Goal: Design and develop therapeutics that will regenerate critically sized defects in rabbit parietal bones.

Task 1.A.1: Synthesis and characterization of injectable and implantable scaffolds.

Synthesis of PUR-based materials

The initial PUR formulation gelled too quickly (gel time less than 1 minute). To address this problem, a new catalyst was added to the formulation. The porosity of the composite increased with increasing catalyst concentration at a given water concentration, thereby giving more precise control over the porosity of the materials. The concentration of catalyst controlled the composite gel times to > 3 minutes and cure times to < 15 minutes, which were comparable to the values reported for calcium phosphate bone cements. High bone particle content was selected to yield the maximum achievable mechanical properties. To promote maximum bone ingrowth and remodeling rate, the porosity was adjusted in the range that also ensured stable mechanical properties. In this range, composites with wet compressive strengths of 7–10 MPa and wet compressive moduli of 175–300 MPa were prepared. These compressive strengths are in the low end of the range reported for calcium phosphate bone cements and greater than 0.4 MPa, which is the lower limit required for cranioplasty materials. No significant

difference in degradation rates for the composites was observed as a function of bone content. Degradation experiments with the lower porosity composites are in progress.

Subsequent experiments with alternative catalysts and bone particle processing methods showed that working times of approximately 3 minutes and porosities of 35%–40% could be achieved. Thus an improved, less expensive system was devised requiring mixing of copolymers, catalyst solution, and bone particles. The advantages of this approach included: (a) the allograft bone component will be prepared using existing, well-characterized Osteotech, Inc. technology; (b) the copolymer components will be packaged as manufactured without requiring further expensive processing; and (c) the catalyst solution will be easily blended and sterilized at a contractor's facility.

Synthesis of Tyr-PC-based materials

Three tyrosine-based copolymers were synthesized and fabricated as porous scaffolds for regeneration of parietal bones in a rabbit critically sized defect model. Scaffolds possessed a bimodal pore distribution with a highly interconnected, open pore architecture. A fast-track in vitro study was carried out to assess the relative rates of resorption. Due to deficiencies in these resorption rates, new tyrosine-based copolymer formulations were synthesized to maintain target mechanical and biodegradation properties. Gel permeation chromatography analysis of the scaffolds at different time points showed appreciable loss in molecular weight (about 35%). Due to deficiencies in the mechanical properties of these scaffolds, two additional tyrosine-based materials were synthesized and characterized. Scanning electron microscope (SEM) images show the pore architecture of the scaffolds (**Figure III-2**).

In vitro studies

In vitro biocompatibility of bone particle/PUR with pre-osteoblast cells dynamically seeded on the materials using spinner flasks was demonstrated. The cells appeared well attached and proliferated on the materials over 14 days of culture (**Figure III-3**). Also, the materials promoted osteogenic differentiation of the cells.

In vitro biocompatibility, differentiation, and mineralization of tyrosine-based copolymers with pre-osteoblasts were assessed using live/dead staining and scanning electron microscopy at days 1 and 4. A significant number of cells were viable and attached to the scaffolds. Using a standard cytotoxicity assay, it was demonstrated that the tyrosine-based copolymers did not reduce the metabolic activity of the cells compared to controls cultured on tissue culture plates. SEM images of cell attachment showed robust cell attachment throughout the scaffolds. Many of the individual pores were filled with the cell layers even after day 1 of culture and already started to produce extracellular matrix (ECM). Cell proliferation, enzyme activity, and calcium content produced by the cells indicated that three-dimensional porous tyrosine-based copolymer scaffolds significantly promoted osteogenic differentiation

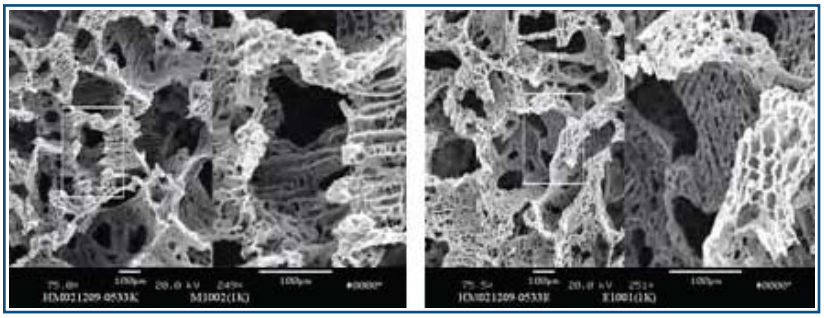


Figure III-2. SEM images of scaffolds made from M1002(1K), Lot HM021209-0533K, and E1001(1K), Lot 021209-0533E. These images were taken before ethylene oxide sterilization. Both images show the presence of highly interconnected pores.

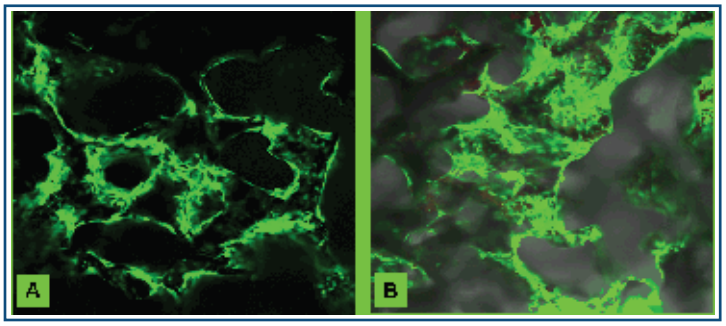


Figure III-3. Confocal microscopic images of MC3T3-E1 cells after 4 days of culture on tyrosine-derived polycarbonate scaffold (E1502) degassed for (A) 48 hours and (B) 14 days after sterilization.

and mineralization, as compared with the control (two-dimensional tissue culture plate).

Task 1.A.2: Pharmacokinetics

Release of osteogenic agent from high porosity (> 90%) implantable PUR scaffolds was funded by a separate Orthopaedic Trauma Research Program grant related to healing of infected segmental defects. It was shown that (a) a burst release of osteogenic agent followed by a sustained release for up to 21 days can be achieved using PUR scaffolds, (b) the burst release is tunable by varying the release strategy, and (c) released agent promotes new bone formation in a rat femoral plug model. Release of angio- and osteogenic agents from tyrosine-based copolymer scaffolds is in progress.

Task 1.A.3: Biological activity of released osteogenic agent

The osteogenic agent releasates from PUR and PUR composites were bioactive and showed significantly greater mineralization compared to the control (hMSC treated with exogenous bone morphogenic protein) after 14 days of culture in osteogenic media. The bioactivity determination of osteogenic agent releasates from tyrosine-derived copolymers is in progress.

Task 1.B: Regeneration of parietal bone: Rabbit critically sized calvarial defects (CSDs).

PUR composites were injected into femoral plug defects in rats. Histology revealed close contact between the polymer and the bone, and micro-CT images revealed new bone formation in the scaffold, suggesting the material was osteoconductive.

PUR composites were also injected into bilateral femoral plug defects in rabbits. Histology showed bone remodeling accompanied by degradation of the polymer phase. The materials' relatively high porosities facilitated migration of cells deep into the interior of the implant. Histological and x-ray studies suggested that after 6 weeks the scaffolds undergo substantial remodeling and integration with host tissue while supporting the ingrowth of cells and new tissue. Future studies will investigate their applicability for regenerating craniofacial bone in a CSD rabbit model.

The Institutional Animal Care and Use Committee protocol to study remodeling of composites in a rabbit calvarial model was approved by USAISR, and a preliminary study was conducted in which a CSD was made in the parietal bones of a rabbit.

The composite void filler was injected into the craniotomy. In the first injection, the material expanded substantially above the surface of the host bone. After approximately 20 minutes cure time, the material was removed from the craniotomy. The material did not adhere to the dura, and removal of the material revealed no discernible damage to the dura. A smaller amount of material was subsequently mixed and injected into the craniotomy. The working time was approximately 3 minutes, and after approximately10 minutes the material had fully expanded and was tack-free. A photograph of the injected bone void filler is shown in **Figure III-4**. Interestingly, when the material was first injected, the pulsation of the dura was readily observed. However, when the material had become tack-free after 10 minutes, the pulsation could no longer be observed. After the material was tack-free, soft tissues were closed in layers with resorbable 3-0 Dexon sutures. Based on these preliminary results, another pilot survival study was scheduled for June 10, 2009 at USAISR. Pending the successful outcome of these surgeries, the full study was scheduled for the first week in July 2009.

A rabbit calvaria protocol for use at Rutgers University was also approved by the Animal Care and Use Review Office. Scaffolds were fabricated from tyrosine-based copolymers and implanted in the calvaria model. The samples at week 12 were retrieved, and the histology and micro-CT analysis are in progress.

Task 2.A: Synthesize and characterize injectable bone particle/PUR composite delivery systems with tunable biological and mechanical properties.

Nonporous bone particle/copolymer composites with enhanced mechanical properties were prepared with higher bone content to obtain mechanical properties superior to those achieved for the cranioplasty biomaterial. As anticipated,

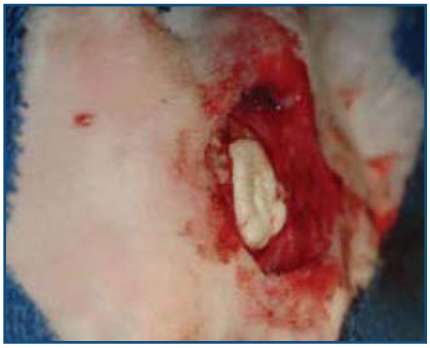


Figure III-4. MBP/PUR bone void filler injected in a rabbit calvarial defect.

strength and modulus decreased with increasing bone content due to the fact that at 64%/volume the system approached the close-packed limit. However, these high bone loadings were necessary to provide sufficient bone particle—particle contact such that the cells have a continuous path through the implant.

Task 2.B: Evaluate the potential of lead candidate biomaterials to promote bone regeneration in a goat alveolar cleft model.

Integration and remodeling were assessed in a rabbit distal femur model study separately funded by the Center for Military Biomaterials Research. PUR composite paste was compression-molded and implanted in bilateral rabbit distal femur plugs. After 6 weeks, histology showed regions of integration with host bone and remodeling. These data showed that nonporous allograft bone/polymer composites remodel in vivo by a creeping substitution mechanism, which was anticipated to be relevant to the injectable nonporous composites as well. The goat model study was planned for Year 2 but will be changed to a rabbit mandibular ridge augmentation study due to the greater clinical need for this.

Key Research Accomplishments

PUR Composites (CMU and Vanderbilt University Collaboration):

- Injectable porous bone particle/polymer composites with tunable porosities, mechanical properties, and working times have been fabricated. These materials have been shown to remodel in a rabbit distal femur model. Two provisional patent applications have been submitted to the U.S. Patent and Trademark Office.
- Osteotech, Inc. has licensed the composite technology for development as a bone void filler. The company is planning a 510(k) device application.
- A packaging strategy has been developed that is supported by preliminary accelerated stability tests. The composite has processing and mechanical properties comparable to those of calcium phosphate bone cements but is anticipated to promote improved adhesion to host tissue, higher strain at break, and faster remodeling. The clinical target is thus an improved cranioplasty biomaterial, which could potentially receive 510(k) regulatory approval.
- Sustained controlled release of osteogenic agent from the composite cranioplasty material has been achieved for up to 21 days.
- Low-porosity injectable bone/polymer composite cements have been fabricated with high bone content that produce wet compressive strengths of up to 60 MPa, more than 5 times stronger than the first-generation high-porosity material.
- Bone particle/PUR composites passed the ISO 10993 systemic toxicity test.
- In a nonsurvival test, the bone particle/PUR bone void filler was injected into a rabbit calvarial defect, and the working and tack-free times were comparable to those observed in vitro with no negative interactions with the dura observed.

Tyrosine-Based Copolymers (CMU and Rutgers University Collaboration):

- Tyrosine-based copolymer scaffolds were successfully prefabricated to fit into the CSD of rabbit skull.
- Tyrosine-based three-dimensional scaffolds induced significant osteogenic differentiation and mineralization of pre-osteoblasts, compared to two-dimensional tissue culture surfaces.
- In vitro cyto-compatibility and cell attachment data with tyrosine-based scaffolds showed no cytotoxicity and robust cell attachment.
- A study on the effect of ethylene oxide (EtO) sterilization on molecular weight loss is being carried out, and nuclear magnetic resonance analysis of EtO-sterilized scaffolds is ongoing. Gel permeation chromatography analyses of scaffolds that have been subjected to EtO sterilization and degassed show approximately 35% loss in molecular weight.

Polyester Copolymers (CMU and the Mayo Clinic Collaboration):

- Both injectable and implantable compositions based on two types of polyester copolymers have been identified for craniofacial applications.

Conclusions

Injectable polymer composites were shown to support new bone formation when injected into a rabbit femoral plug defect. Three-dimensional porous PUR composites supported cell attachment, proliferation, and osteogenic

differentiation of osteoprogenitor cells. By reducing the porosity, mechanical properties suitable for a cranioplasty were achieved. Osteogenic agent released from PUR composites was biologically active and induced new bone formation in a rat femoral plug model. The product design and development work on the cranioplasty biomaterial is being translated to a commercial product through collaboration with Osteotech, Inc.

Tyrosine-based copolymer scaffolds induced osteogenic differentiation and mineralization of pre-osteoblasts, and cell attachment was robust and showed no cytotoxicity. There was a significant loss in the molecular weight of these copolymers after EtO sterilization and degassing. A study will be performed on other scaffolds to determine whether this effect is composition dependent. Also, other methods for sterilization will be explored, specifically gamma radiation.

Research Plans for the Next 4 Years

During the upcoming year, the researchers will continue an FDA-inspired testing paradigm that emphasizes scaffold biocompatibility, biodegradability, cell interactions, and preclinical bone regeneration. The inclusion of imaging technology, solid freeform fabrication, and surface modification should enhance the utility of the CMF therapies. Regarding the injectable MBP/PUR composite void filler, the researchers will perform a rabbit calvarial defect study at USAISR during Year 2. The researchers also plan to develop materials for reconstruction of the mandible in collaboration with USAISR. Lead candidate materials will be screened in a rabbit model before proceeding to a larger animal segmental defect model in Year 3. Regarding the tyrosine-based copolymers and the polyesters, the researchers plan to complete the in vitro assays necessary for regulatory engagement. The best performing candidates will move into calvaria and mid-face surgeries in small and large animal models at USAISR and at Allegheny General Hospital in Pittsburgh.

Planned Clinical Transitions

The researchers will continue to engage actively with military surgeons, staff at USAISR, and clinical FDA consultants to ensure that they focus on the most expedient pathway to the clinic. Regarding the injectable MBP/PUR composites, collaborators at Osteotech, Inc. will utilize data from the rabbit calvarial defect study in a 510(k) biomedical device application, which is currently targeted for the first quarter of 2010. Pending a successful regulatory filing, a clinical trial will be pursued. Regarding the tyrosine-based copolymers and the polyesters, the researchers will identify and engage corporate entities that will aid in commercializing the products. Overall, the RCCC CFR Program will continue in Year 2 and beyond toward the commercialization of products that will help ameliorate severe craniofacial injury. This will include the regeneration of osseous craniofacial infrastructure, as well as an integrated approach to prevent infection, restore form and function to soft tissue adnexa (including neurogenic and myogenic elements), and mitigate scar contracture.

Corrections/Changes Planned for Year 2

CMU and Vanderbilt University will discontinue the collaborative development of a PUR-based composite for craniofacial bone regeneration. As a consequence of the disengagement, CMU will significantly increase collaborative interactions with Rutgers University and the Mayo Clinic. The Mayo Clinic collaboration will focus on polyesters and will be directed by Dr. Michael J. Yaszemski. In Year 2, the CFR Program will focus on therapeutic scaffolds that precisely mimic patient anatomies using ex vivo pre-casting, computer-aided design (CAD)/computer-aided manufacturing, and solid freeform fabrication. Moreover, the CMU collaboration with Rutgers University and the Mayo Clinic will exploit collaborative opportunities with the Cleveland Clinic, Therics, and SimQuest.

Vanderbilt University will move forward on the rabbit study with USAISR during Year 2. Osteotech, Inc. is planning a good laboratory practices study with the injectable bone void filler for the third quarter of 2009. Regulatory filings for a bone void filler for both orthopaedic and craniofacial applications are currently planned for the first quarter of 2010. In Year 2, the planned goat alveolar cleft model will change to a rabbit mandibular ridge augmentation model. The researchers anticipate that this model will accelerate the development of a mandibular ridge augmentation material, which is a compelling clinical need.

Project 4.1.2, WFPC

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

Team Leader(s): *Antonios G. Mikos, PhD (Rice University) and Mark E. Wong, MD (University of Texas Health Science Center)*

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. (3) Injectable system for delivery of growth factors necessary for bone regeneration and wound healing*

Key Accomplishment(s): *A variety of porous space maintainer formulations presenting a range of porosities and mechanical properties have been fabricated and characterized. Animal protocols have been prepared and submitted that focus on determining the efficacy of an implantable "in vivo bioreactor" to prefabricate a vascularized bone flap for subsequent transplantation into a large osseous defect in a large animal model.*

Introduction

Ballistic injuries resulting in significant soft and hard tissue loss are commonly encountered clinical scenarios in the current U.S. military combat theaters Operation Enduring Freedom and Operation Iraqi Freedom. This project seeks to develop a method to facilitate staged reconstruction of large osseous defects in the craniofacial region restoring function and esthetics in these injured personnel. The purpose of this research is to decrease the complications and infections associated with large bony reconstructions through three mechanisms: (1) by the initial implantation of a biocompatible, antibiotic-releasing space maintainer within the osseous defect during the early phases of treatment, (2) implantation of an "in vivo bioreactor" away from the site of injury that will allow for the generation of a vascularized bone construct, and (3) augmentation of the implanted vascularized bone flap within the recipient defect site with an injectable system for delivery of growth factors needed to promote bone regeneration and wound healing until sufficient integration of the bone flap has occurred.

Research Progress – Year 1

Craniofacial trauma is among the most debilitating forms of injury facing civilian and military populations due to the important esthetic and functional role of the craniofacial complex. Blast injuries and injuries from high-velocity projectiles such as those encountered on the battlefield often require a staged repair. Even when adopting a regenerative medicine approach, the definitive repair of bony defects will likely come only after control of local contamination and adequate soft tissue healing are achieved. During the initial stages of treatment and reconstruction, space maintenance at the site of bony defects is required to maintain the necessary space for delayed bone repair and also to give physicians an accurate scaffold over which to repair/regenerate soft tissue.

Currently, alloplasts such as PMMA are used as space maintainers due to their approved regulatory status and ability to be molded intraoperatively to fill complex defects. Wound dehiscence over these implants is a commonly encountered problem, typically occurring at later time points. This indicates insufficient wound strength rather than a deficiency in wound healing. The researchers hypothesized that a contributing factor in 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. Preliminary studies using a nonhealing rabbit mandibular defect with open communication to the oral cavity found greater wound dehiscence or a lack of wound healing around nonporous space maintainers when compared to porous space maintainers (Nguyen et al., *J Oral Maxillofac Surg*, submitted).

To further test the hypothesis that surface porosity of implants mitigates wound dehiscence in bony space maintenance applications, porous PMMA implants were fabricated with varying porosity and characterized with micro-CT and SEM. Implants were placed into unilateral 10 mm diameter nonhealing circular defects within rabbit mandibles possessing open communication with the oral cavity (**Figure III-5**). Wound closure and soft tissue integrity after 12 weeks of healing was assessed as part of a preclinical in vivo study. All surgeries have been completed (18 rabbits), and all implants have been retrieved. Gross observations of the integrity of the wound healing have been recorded for each sample upon harvesting (**Figure III-6**).

The results from the in vitro development studies demonstrate that porous PMMA implants can be fabricated in a controlled and reproducible manner using an aqueous phase containing carboxymethylcellulose (CMC) to impart porosity. Furthermore, varying the percentage of CMC present in the aqueous phase as well as the weight percentage of the aqueous phase in relation to the PMMA phase allows for control of implant porosity ranging from surface roughness without interconnectivity to interconnected pores throughout the implant. The ongoing in vivo study is elucidating further the role of pore and surface characteristics of the PMMA implants on wound healing and integrity and space maintenance within a bony defect using a nonhealing rabbit mandibular defect model.

Ongoing studies focus on the incorporation into and release from PMMA/CMC constructs of a highly clinically and battlefield-relevant antibiotic, colistin (effective against the *Acinetobacter baumannii-calcoaceticus* complex—the predominant organism recovered in trauma-related infections).

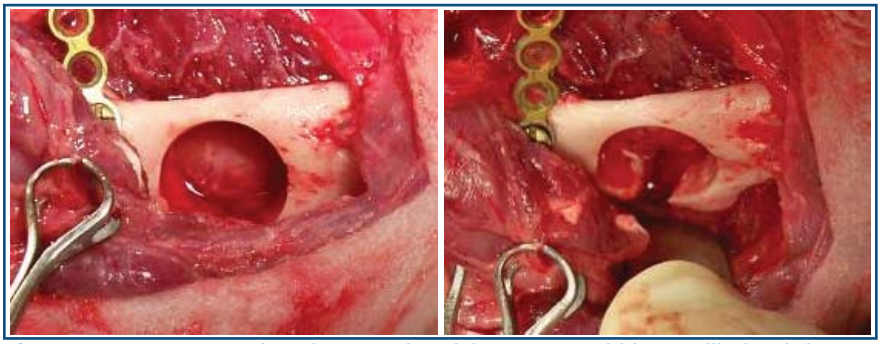


Figure III-5. Intraoperative photographs of the 10 mm rabbit mandibular defect with intraoral communication prior to the insertion of an experimental space maintaining implant. A metal bone plate (seen in photographs before final fixation) was used for mechanical reinforcement to mitigate fracture of the narrow margin of remaining mandible proximal to the defect at the request of the Institutional Animal Care and Welfare Committee.

Key Research Accomplishments

- Fabricated and characterized a variety of CMC/PMMA porous space maintainer formulations presenting a range of porosities and mechanical properties.
- Initiated identification of antibiotics to be released from the space maintainers as a prophylaxis against infection of the craniofacial wound site.
- Exploring and characterizing in vitro the release of the antibiotic(s) of interest from poly (L,D-lactic-co-glycolic acid) (PLGA) microspheres to be incorporated into the space maintainers. Identified a clinically viable antibiotic to treat a battlefield-relevant target bacterial species (colistin to treat *A. baumannii-calcoaceticus*).
- Initiated a pilot study to evaluate several CMC/PMMA space maintainer formulations for their ability to prevent soft tissue collapse in a mandibular CSD in a rabbit model.

Conclusions

The researchers have made considerable progress in the development of biocompatible antibody-releasing implants and an in vivo bioreactor, which can facilitate the staged reconstruction of large bone defects in the craniofacial region.

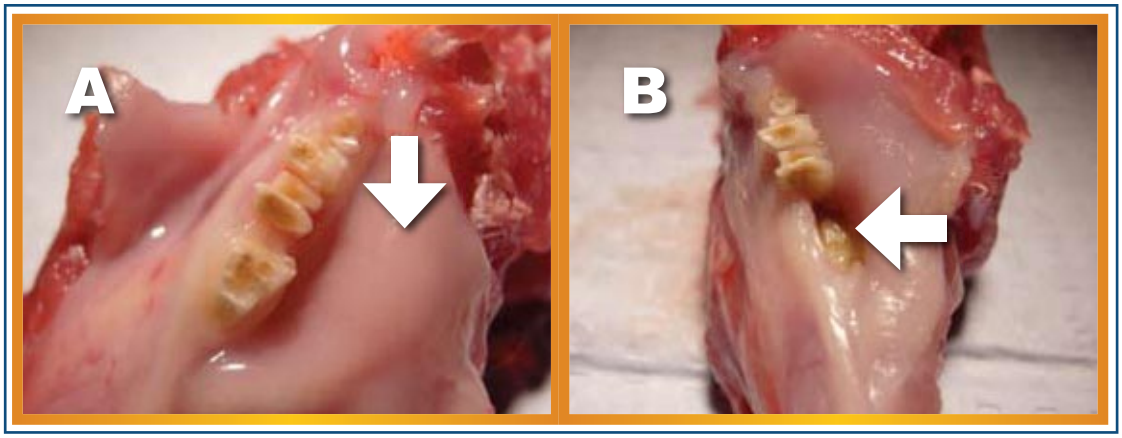


Figure III-6. Rabbit mandible sections harvested as part of an ongoing in vivo study. The section illustrated in (A) demonstrates healed soft tissue covering the implant (area indicated by arrow). The section illustrated in (B) demonstrates dehiscence of the soft tissue envelope surrounding the implant, which is observable at the point of the arrow.

Research Plans for the Next 4 Years

The researchers plan to explore the release of antibiotics from space maintainers in an infected rabbit mandibular defect model. They will also continue to investigate the efficacy of an implantable PMMA in vivo bioreactor to prefabricate a vascularized bone flap. Finally, they will continue to synthesize oligo (poly[ethylene glycol fumarate]) (OPF), fabricate OPF hydrogels, and explore the controlled release of multiple growth factors from fumarate-based polymeric materials.

Planned Clinical Transitions

The researchers are currently preparing institutional biosafety and animal protocols for submission to evaluate the release of antibiotics from the porous space maintainers in an infected rabbit mandibular defect model. They have prepared and submitted animal protocols to determine the efficacy of an implantable PMMA in vivo bioreactor to prefabricate a vascularized bone flap for subsequent transplantation into a large osseous defect in a large animal model. Working in collaboration with the Center for Clinical and Translational Sciences at the University of Texas Health Science Center at Houston, the researchers will prepare and submit forms and protocols for IRB approval of future clinical studies and FDA approval of space maintaining devices under development in Project 4.1.2.

Corrections/Changes Planned for Year 2

Apart from the selection of colistin, rather than tobramycin, as the battlefield-relevant antibiotic to treat *A. baumannii-calcoaceticus* to mitigate osteomyelitis, no deviations from the original proposal are projected for Year 2 of this project.

Project 4.1.3, WFPC

Novel Synthetic Bone

Team Leader(s): *Charles Sfeir, DMD, PhD (University of Pittsburgh)*

Therapy: *Structural and functional craniofacial bone replacement*

Deliverable(s): *Bone cements that incorporate bioactive NanoCaPs and ECM-derived materials*

Key Accomplishment(s): *Novel nanostructured apatitic bioactive bone cements and scaffolds based on the use of natural ECM-derived polymers that incorporate NanoCaPs were developed and characterized. The in vitro assessment of the cements and scaffolds was initiated, and the results show that the synthesized forms of the cement materials are biocompatible.*

Introduction

This project focuses on developing novel bone regeneration strategies for craniofacial reconstruction 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 also will be used for regenerating large osseous defects in the extremities where bone fracture is a major clinical problem contributing to nearly 50% of all injuries in armed forces personnel. The researchers are developing a synthetic bone-like environment that involves bioactive nanostructured amorphous and/or NanoCaPs, nanostructured CaP-based bioactive bone cements, and ECM-derived materials such as urinary bladder membrane (UBM). These nanostructured systems contain essential components to mimic the bone architecture, composition, and mechanical strength while providing the osteo-inductive and osteo-conductive characteristics required for bone tissue regeneration. The combined nanoscale hybrid system will incorporate bone morphogenetic proteins (BMPs) 2 or 7, which are known for their bone regeneration ability. The research strategy will create an organic/inorganic scaffold system that would simulate the unique composition and architecture of bone.

Research Progress – Year 1

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, critically sized calvarial defect rabbit model.

1. Cement synthesis

The first cement studied was based on only CaP powders having particles of 7–2 μm in size. Initial and final setting time of this cement was found to be 8 ± 1 minute and 17 ± 1 minute, respectively, at 298K. To incorporate macroporosity into this cement, a different amount of acidic calcium salt (ACS) was added to the cement. ACS is a relatively soluble calcium-rich phase and thus can be leached out from the cement to form pores of controlled sizes. The other major advantage of using ACS is that the dissolved ACS in vivo should react with the phosphate ions present in body fluids to form apatite of variable composition. This could lead to the replacement of ACS crystal by pores and to the growth of neighboring apatite crystals.

The setting characteristic of these cements (henceforth described as BCS cements) containing Nano-CaPs, ACS, BCS, and calcium phosphate was very similar to that of cements described earlier. The detailed phase evaluation of the BCS cements kept under phosphate buffered saline showed that the complete conversion of the cements into calcium-deficient hydroxyapatite (CDHA) occurred within approximately 15 days. The morphology of the formed CDHA from BCS cements was found to be different compared to that of ACS cements, and the size (length and diameter) of the CDHA whiskers formed in the case of BCS cements was in the nanometer range. This result suggests that the BCS cements may have a better in vivo resorption rate than ACS cements due to the nanoscale nature of the CDHA whiskers formed.

2. Synthesis of cement in combination with UBM

To improve the bioactivity of the BCS cements, UBM was added in the BCS cements. The presence of adhesion molecules, structural proteins, and growth factors inside the UBM may enhance the bone regeneration process. The addition of UBM into the BCS cements (henceforth described as UBM cements) leads to an increase in setting times of the cements. The phase analyses of these UBM cements using x-ray diffraction showed that the cements immersed in phosphate buffered saline completely convert into CDHA within 3 days, which is considerably shorter than ACS and BCS cements. However, the morphology of the CDHA formed in these UBM cements was found to be very similar to that of the BCS 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.

3. In vivo screening

A pilot in vivo experiment (no AFIRM funds were used) was carried out to screen several versions of the synthesized cements that will help to determine the best possible candidate for which resources will be dedicated in the immediate future to achieve clinical goals. The screening provided very encouraging in vivo data from the rabbit ulna defect model. The results utilizing cement alone and cement + BMP-2 scaffold systems showed bone regeneration (bridging) at 8 weeks post-surgery (**Figure III-7**).

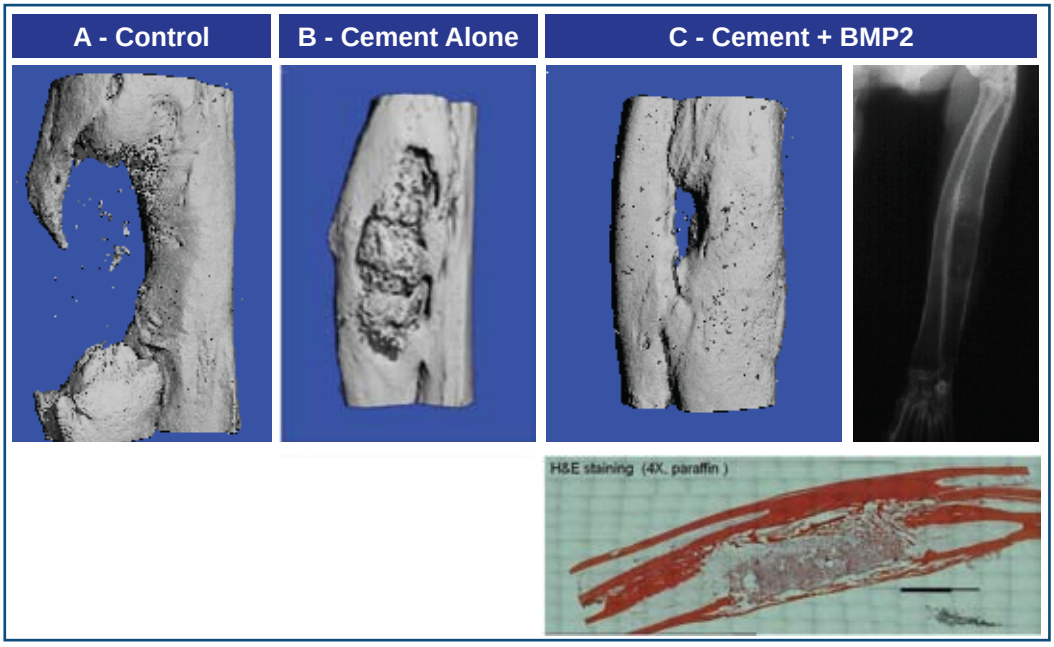


Figure III-7. Bone regeneration in the rabbit ulna defect model with synthesized bone cements alone (B) or with BMP-2 (C). Lower right: A section through the ulna stained with hematoxylin and eosin (H&E) shows the integrity of the bone cement.

Conclusions

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. They have initiated the in vitro assessment of these cements, and the results show that the synthesized forms of cement materials are biocompatible. The inorganic cements with or without BMP-2 appear to perform very well in vitro as well as in vivo (in the pilot study). Overall, these data show that the newly synthesized bioresorbable cements containing NanoCaPs with and without BMP-2 are very good candidates for further assessment as a therapy for bone regeneration.

Research Plans for the Next 4 Years

The researchers have initiated an in vivo screening experiment to rapidly determine if the NanoCaPs-containing materials will be optimal in the proposed bone regeneration strategy either with or without the incorporation of the osteo-inductive growth factor BMP-2.

Planned Clinical Transitions

The researchers will continue to focus on further developing the bone cements to gather more data for FDA approval and eventual clinical trials.

Corrections/Changes Planned for Year 2

No deviations from the original proposal are anticipated for this project.

Project 4.3.1, RCCC

Composite Tissue Allograft (CTA) Transplantation Without Life-Long Immunosuppression

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

Project Team: *Aleksandra Klimczak, PhD, Joanna Cwykiel, MSc, Arkadiusz Jundzill, MD (Cleveland Clinic)*

Collaborator(s): *Jim Herrman, PhD, CEO, Tolera Therapeutics, Kalamazoo, Michigan*

Therapy: *Transplantation without lifelong immunosuppression*

Deliverable(s): *Develop chimeric cells with tolerogenic potential as a supportive therapy for allograft transplants*

Key Accomplishment(s): *The first near-total face transplant in a civilian patient was accomplished in the United States in December 2008. The new, selectively blocking induction antibody (TOL101) for solid organ and CTA transplants has undergone GMP manufacturing, assurance testing, and pre-investigational new drug screening with the FDA. Donor-recipient cell fusion was accomplished both in vivo and ex vivo, and the therapeutic effect of donor-recipient chimeric cells was confirmed by prolonged survival of skin allografts.*

Introduction

In the current military conflicts in Afghanistan and Iraq, soldiers are experiencing unprecedented traumatic injuries from improvised explosive devices (IEDs). Such massive trauma often results in catastrophic injury or "polytrauma" to the victim, who may end up missing several limbs, being blind, and missing part or all of the face. While prosthetics and plastic surgery can help restore the injured warfighter to partial function, the potential ability to transplant large segments of vascularized tissue, allowing partial restoration of faces and limbs, will provide help and hope to victims of disease and traumatic injury. Having one's face restored to a more human appearance, especially, makes an amazing impact on an individual's morale, which can vastly aid their physical healing and rehabilitation and enhance their quality of life.

Successful CTAs, such as hand, larynx, abdominal wall, and very recently partial face transplants (International Registry on Hand and Composite Tissue Transplantation; www.handregistry.com), were performed in the clinic. CTAs that contain skin and lymphoid elements, such as lymph nodes and bone marrow, raise new challenges for transplant immunologists since the lymphoid elements may generate a high immunological response. To improve CTA outcomes, a supportive therapy with donor bone marrow transplantation was used in clinical practice; however, the clinical, technical, and biological criteria for using bone marrow transplantation as a part of cell therapy protocols are still being investigated. In such cellular cases, supportive therapy with donor-recipient chimeric cells may serve as an alternative method, one that will hopefully reduce complications of bone marrow transplantation such as long hematopoietic recovery and graft failure. Chimera refers to a person having two genetically distinct types of cells, for example, a nonidentical twin who shared a blood supply with a twin in utero and thus has cells/DNA from both twins in his or her body.

This project consists of two specific programs: (1) CTA with TOL101 antibody and (2) production of human chimeric cells for tolerance induction and rescue. Both programs aim to transform clinical immunomodulation methods to make allograft transplant of composite tissues safer and more widely available to victims of disease and traumatic injury. These methods will provide the injured warrior with the opportunity to receive large blocks of tissue and even whole limbs from a donor through the current tissue donor systems while reducing or eliminating the need for long-term immunosuppression as associated costs and risks. These same strategies are also relevant to advancement in solid organ transplantation.

Research Progress – Year 1

Project Goal: To transform methods for modulating immune responses to make allograft transplant of composite tissues safer and more widely available for both military and civilian patients who require restoration of the face and limbs.

Figure III-8. Schematic illustrating plan for transplantation of complex tissue allograft performed by Dr. M. Siemionow's team at the Cleveland Clinic in December 2008.

In Year 1, the research team began testing the safety and efficacy of a therapeutic antibody (TOL101; Tolera Therapeutics, Cleveland, Ohio) for use as a conditioning agent in kidney transplant recipients. Researchers also began optimizing a preclinical ex vivo technique for producing donor-recipient chimeric cells from host and recipient in the rat model. They plan to employ these cells as immunomodulators in the pretreatment setting prior to transplant and/or for post-transplant rescue. This supportive therapy with donor-recipient chimeric cells would represent a second and potentially transforming breakthrough in modulating immune reactions in patients with CTA and solid organ transplants.

Researchers tested preclinical models of bone marrow cell transplant and vascularized allograft transplants (e.g., limb and bone) combined with immunomodulating therapy. These tests resulted in stable multilineage chimerism for some myeloid cells and the majority of lymphoid cells with long-term (approximately 2 years) tolerance across major histocompatibility complex (MHC) barriers. Since MHC contains genes that control cell histocompatibility, which strongly impacts tissue type and transplant compatibility, the long-term tolerance should correlate with longer-term transplant success. In models that contained intact vascularized bone, the team's results suggest that the presence of certain tissue compartments may facilitate engraftment of hematopoietic cells. The Cleveland Clinic team demonstrated the clinical feasibility of reconstructing massive post-traumatic segmental tissue loss in the face using allograft tissue (**Figure III-8**). This procedure further establishes the precedent for use of this therapeutic approach even using conventional immunosuppression.

Key Research Accomplishments

- Researchers have confirmed the presence of donor-origin cells in the peripheral blood and bone marrow compartment of recipients at different time points. This confirmed the efficacy of the chosen immunodepletive protocol for chimerism induction.
- The research team has accomplished in vivo cell fusion in the first group of animals and has also created ex vivo donor-recipient cells by cell fusion.
- The first clinical observations confirmed the supportive role of donor-recipient chimeric cells created ex vivo by cell fusion for prolonged skin allograft survival.
- If successful, this novel approach will support tolerance-inducing protocols with cellular therapeutics in the transplantation field.

Conclusions

The researchers have successfully created ex vivo donor-recipient chimeric cells by cell fusion and have confirmed their beneficial effect for skin allograft survival in an animal model. To apply this technology to human chimerism production, the research team will produce chimeric human cells ex vivo with the ultimate goal of developing donor(s)-specific transferable tolerance. This supportive therapy with donor-recipient chimeric cells represents a new and potentially breakthrough modality in solid organ and CTA transplants. These advances should ultimately help

make face and limb transplants feasible for military and other trauma victims who have experienced massive tissue loss, as well as for cancer patients, who are currently excluded from being transplant candidates because of immunosuppression issues.

Research Plans for the Next 4 Years

During Year 1 of Project 4.3.1, the assessment of clinical safety of the researchers' immunomodulating approach was initiated in a Phase 1 study using TOL101 and bone marrow transplantation in the setting of renal transplantation. The researchers will continue in the upcoming year to work actively with COL Robert Hale, MD, at USAISR, and within other avenues, to define appropriate pathways, teams, and clinical protocols that will enable these methods to be deployed to treat injured warriors. Also in Year 2, work on this project will create ex vivo donor recipient chimeric cells by cell fusion as an application for human chimerism production. Planned experiments will create donor recipient chimeric cells and test their in vivo efficacy for adoptive transfer. Chimeric human cells also will be produced ex vivo with the ultimate goal of developing donor(s)-specific transferable tolerance. Work in Years 3–5 will be strongly dependent upon the result of ongoing work.

Planned Clinical Transitions

The researchers anticipate enrolling human patients in late 2009 in the TOL101 antibody trial, following extensive preclinical testing initiated at the request of the FDA. They anticipate completing initial human safety and efficacy testing (in approximately 30 human kidney transplant recipients) in the third quarter of 2010. The surgical techniques involved in CTAs are not regulated by the FDA. The Cleveland Clinic granted IRB approval for a combined face and upper extremity transplant in April 2009. The team currently has an IRB submission for face and face plus bilateral upper extremity pending at the BAMC IRB. The researchers project the first human therapeutic use of the ex vivo produced human cells in late 2011. The FDA will be engaged near the end of Year 2 using appropriate regulatory consultation to design safety and efficacy trials that will be needed for chimeric cells.

Corrections/Changes Planned for Year 2

Regarding the initial human testing of the TOL101 antibody in Project 4.3.1, the plan for testing in human kidney recipients has been delayed by approximately 6 months due to a request from the FDA to repeat some of the preclinical testing in nonhuman primates. This will be completed in cooperation with the University of Kentucky (John S. Thompson, MD).

Projects 4.1.4 and 4.1.5, WFPC

Soft Tissue Regeneration (4.1.4) Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction (4.1.5)

Team Leader(s): *David Kaplan, PhD (Wake Forest University; 4.1.4); J. Peter Rubin, MD (University of Pittsburgh; 4.1.5)*

Project Team: *David Kaplan, J. Peter Rubin, Kacey Marra, James J. Yoo, Sang Jin Lee*

Collaborator(s): *Tufts University*

Therapy: *Restoration of traumatic soft tissue defects*

Deliverable(s): *(1) Porous three-dimensional silk fibroin scaffold to provide sustained morphology, structure, and tissue function. (2) Vascularized fat and connective tissue pads. (3) Biodegradable hydrogel that releases angiogenic growth factors. (4) Biodegradable microspheres to deliver bioactive factors over long periods of time. (5) Novel hydrogel for cell delivery.*

Key Accomplishment(s): *Four types of silk fibroin scaffolds were developed, and each scaffold was shown to support the growth of soft tissue. A collagen gel delivery system was developed that releases vascular endothelial growth factor (VEGF) with the goal of enhancing vasculogenesis in vivo. Cell-hydrogel constructs were implanted in athymic mice, and the dimensional changes in the mice at various time points post-implantation are being examined. Polymeric microspheres containing proteins known to promote angiogenesis have been developed; they are expected to enhance tissue formation by the cell-hydrogel constructs.*

Introduction

Soft tissue defects represent a component of injury in a major percentage of military traumas, especially blast and burn injuries. The current standard of care for soft/fat tissue regeneration has relied on three approaches: (1) Surgical flaps that move adipose tissue from one site to another while maintaining an intact blood supply; (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 intact blood supply. However, the surgical flap approach is associated with medical risks, high costs, scarring, and functional loss. Additionally, free fat transplants often lose volume over time, attributed to traumatic rupture, avascular necrosis, and 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.

Therefore, restoration of traumatic soft tissue defects must begin with a strategy that will restore and maintain tissue size and shape to near normal dimensions. To address this challenge, a partnership between three leading institutions (Tufts University, University of Pittsburgh, and Wake Forest University) was formed 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.

While initiated as two separate projects (Project 4.1.4, Soft Tissue Regeneration, and Project 4.1.5, Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction), the researchers quickly combined their unique talents into a strong and productive collaboration. The main scientific approach will involve the use of autologous adipose stem cells and fibroblasts, combined with carrier biomaterials, to achieve vascularized soft tissues.

Specific Aims: (1) Engineering of vascularized connective tissue and fat pad, incorporating cellular elements and custom designed biomaterial scaffolds; (2) development of implantable and injectable composite vascularized soft tissue; (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.

Research Progress – Year 1

Project Goal: To engineer vascularized connective tissue and fat pads through the use of cell/scaffold combinations with an emphasis on the use of custom slowly degrading, biocompatible, porous silk

protein scaffold systems designed to restore and maintain cell-based soft tissue restoration for long time frames.

1. Design of custom, silk fibroin-based biomaterial scaffolds for soft tissue regeneration.
This first deliverable is a porous three-dimensional silk fibroin scaffold to provide sustained morphology, structure, and tissue function for at least 1 year while supporting cellular and vascular ingrowth to restore functional tissue.

Porous silk protein sponges were prepared and characterized, including control of a range of pore sizes and protein content during scaffold formation, related to mechanical properties and degradation properties of these material systems. Modifications to the silk sponges to enhance adipose tissue outcomes included coating with laminin, laminin-derived peptides, and vascular growth factors. Functionalization approaches included adsorption and carbodiimide coupling. Silk porous sponges, before and after functionalization, were characterized for structure and morphology to correlate processing conditions and chemistries to biomaterial features relevant to soft tissue reconstruction needs. Cell-based assays using adipose-derived stem cells (ASCs) were used to determine biological responses to the unmodified and modified scaffolds in vitro reflective of soft tissue outcome (**Figure III-9**). Thus a range of genotypic and phenotypic assays were employed.

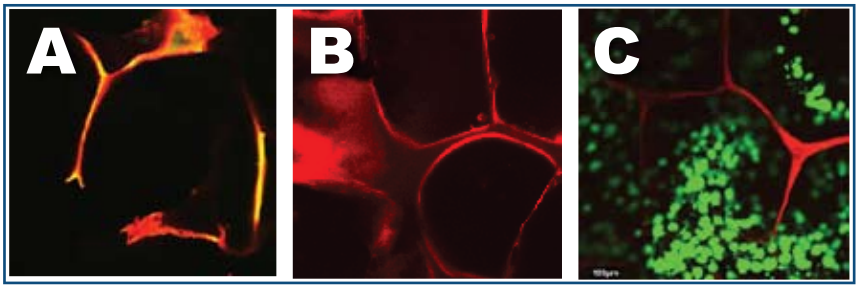


Figure III-9. Human ASCs (passage 3) were seeded onto silk sponges. Sponges were either coated with Type-1 collagen or left uncoated. Imaging: Confocal microscopy with live/dead stain (fluorescein diacetate and propidium iodide) was used to image the cells at varying time points. Confocal images using fluorescein diacetate and propidium iodide stain of seeded ASCs. (A) Uncoated sponge at 48 hours, (B) uncoated sponge at 5 days, and (C) collagen-coated sponge at 5 days. Notes: red = silk, green = live cells, pore sizes around 400 µm.

2. Use of ASCs and silk scaffolds for in vivo adipose tissue formation.
This deliverable is a vascularized fat tissue pad.

Four different types of silk scaffolds (silk sponge, collagen-type I coated silk sponge, silk gel, and silk particles mixed with fibrin glue) were seeded with primed ASCs (cultured for 7 days in adipogenic medium before seeding) labeled with CMFDA (CellTracker™ Green CMFDA [5-chloromethylfluorescein diacetate]) from Molecular Probes (Invitrogen). The seeded scaffolds were implanted subcutaneously into the dorsal region of a nude athymic mouse. Empty scaffolds were used as controls. The animals were followed for 17 days. At that time point, animals were sacrificed and scaffolds were explanted and assessed using three different stains: H&E, fluorescent, and Oil Red O. All four types of silk scaffolds used support adipose formation in vivo. Preliminary results show that silk gel and silk sponge are the two most promising configurations. (**Figure III-10**)

3. Design of angiogenic drug eluting collagen gel scaffold system.
This deliverable is a biodegradable hydrogel that releases angiogenic growth factors.
To formulate a collagen gel system that releases VEGF to enhance vasculogenesis in vivo, two concentrations of VEGF (100 ng or 25 ng) were incorporated in a collagen-based hydrogel. VEGF release was measured by enzyme-linked immu-

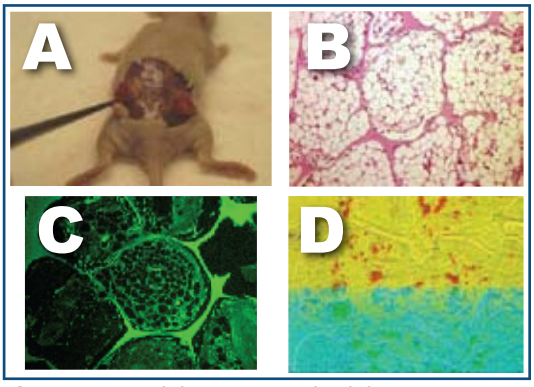


Figure III-10. (A) Macroscopic, (B) H&E, (C) fluorescent, and (D) Oil Red O microscopic images of the silk sponge seeded with 1 x 10^6 primed and labeled ASCs after 17 days are showing newly formed adipose tissue.

nosorbent assay at various time points up to 12 days. The researchers further examined VEGF release kinetics from muscle progenitor cells that were transiently expressing VEGF.

The release kinetics of the collagen gel with 100 ng VEGF were steady with approximately 20% of the total amount released on day 1. The release pattern was gradual over a period of 12 days until VEGF was completely depleted from the gel. When 25 ng VEGF was used, the release was rapid on the first day, which represented approximately 48% of the total amount of VEGF contained in the gel. The release was gradual thereafter, and VEGF was completely depleted from the collagen gel by day 9 (**Figure III-11**). VEGF production by the cultured cells steadily increased in the first 10 days, reaching 1 µg VEGF secreted from 5 x 10^6 cells. After 10 days, the amount of secreted VEGF decreased sharply until day 20 when only 0.1 µg VEGF was secreted from 5 x 10^6 cells. Lower VEGF amounts were detected until day 35.

4. Use of dermal fibroblasts and scaffolds for in vivo connective tissue formation. This deliverable is a vascularized connective tissue pad.

Table III-2. Experimental groups for in vivo study.

	Fibrin Hydrogel		Collagen (mg/mL)	HA (mg/mL)	Initial Cell Density (/mL)
	Fibrinogen (mg/mL)	Thrombin (U/mL)			
Fibrin	50	10	-	-	-
Fibrin	50	10	-	-	5 X 10^6
Fibrin/COL	50	10	5	-	5 X 10^6
Fibrin/HA	50	10	-	10	5 X 10^6

To evaluate the dimensional stability of cell-hydrogel constructs, the constructs were implanted subcutaneously in athymic mice (Charles River Laboratories, Inc.). Four experimental groups were implanted with different formulations of fibrin and collagen or hyaluronic acid (HA) (**Table III-2**). Dermal fibroblasts, suspended in the fibrinogen solution, were prepared by adding the same volume of thrombin solution using a double syringe applicator prior to implantation. The cell-hydrogel constructs were placed into the subcutaneous space, and the constructs were retrieved at 1, 2, and 4 weeks after implantation for analyses.

To evaluate the dimensional changes of cell-hydrogel constructs after implantation, dimensions of the retrieved samples were measured prior to implantation and at retrieval. The percentage of change in dimension at each time point was calculated by comparing the total dimension of the retrieved samples with the pre-implantation values.

5. Drug-eluting microspheres to enhance tissue formation.
This deliverable consists of biodegradable microspheres to deliver bioactive factors over long periods of time.
Based on previous results, dermal fibroblasts suspended in a fibrin/collagen solution showed the best dimensional stability when compared to the others. However, this formulation of cell-hydrogel constructs exhibited a reduction of approximately 50% of its structural dimensions. Enhancement of vascularization to implanted tissue may improve the dimensional stability of cell-hydrogel constructs. This could be achieved by the development of a dual-protein delivery system in which angiogenic proteins can be released to promote angiogenesis and enhance tissue formation in the cell-hydrogel constructs. To this end, microencapsulation techniques were employed to contain proteins, and release patterns of these proteins from polymeric microspheres were assessed. Biodegradable PLGA microspheres loaded with bovine serum albumin were prepared by a water-in-oil-in-water emulsion/solvent evaporation technique. Morphology of the microspheres was examined using a SEM; **Figure III-12**).

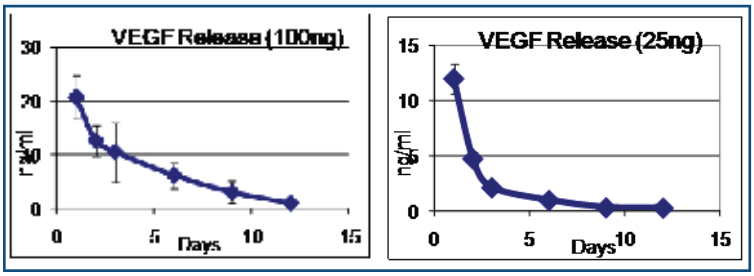


Figure III-11. Release patterns of different concentrations of VEGF incorporated in a collagen gel. VEGF concentration of 100 ng showed slower release kinetics as compared to the 25 ng formulation.

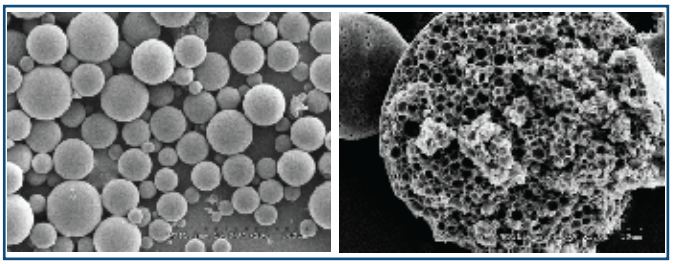


Figure III-12. SEM images of bovine serum albumin-loaded PLGA microspheres.

6. Development of double cross-linked injectable HA hydrogels for soft tissue engineering.
This deliverable is a novel hydrogel for cell delivery.

This aspect of the study aimed to develop novel biomaterials with improved ability to encapsulate and support adipose-derived stem cell adhesion and differentiation.

To modify HA, aldehyde-HA (HA–CHO) 1.0 g HA was dissolved in 100 mL nanopure H_2O at a concentration of 10 mg/mL. An aqueous solution of sodium periodate (0.5 M, 5 mL) was added dropwise, and the reaction was stirred for approximately 1.5–2 hours at room temperature in the dark. One milliliter of ethylene glycol was then added to inactivate any unreacted periodate. The reaction was stirred for 1 hour at ambient temperature, the solution was purified by exhaustive dialysis against H_2O for 3 days, and the dry product was obtained by freeze-drying.

Key Research Accomplishments

- Development and testing of custom silk fibroin scaffolds to support the growth of soft tissue.
- Generation of vascularized fat and connective tissue implants.
- Development of a primary human fibroblast culture system.
- Development of injectable hydrogel and acellular matrix particles as injection media.
- Development of injectable silk gel and silk particles as injection media.
- Development of a growth factor (VEGF) delivery system.
- Development of silk microspheres loaded with insulin and dexamethasone.
- Validation of ex situ injection model in mice.
- Development of a novel HA-based hydrogel that is able to encapsulate ASCs and demonstrates optimal gelation and degradation properties.
- Demonstration of a degradable and biocompatible protein scaffold for soft tissue reconstruction, based on silk, which will take more than 1 year to degrade in vivo. Further, these scaffolds are mechanically robust to hold size and shape during this time frame, they can be easily sterilized, and the silk-based biomaterial recently has received FDA approval (Serica, Inc.).

Conclusions

The researchers of Projects 4.1.4 and 4.1.5 developed four types of silk fibroin scaffolds (silk sponge, collagen-type 1 coated with silk sponge, silk gel, and silk particles mixed with fibrin glue) and showed that each scaffold supported the growth of soft tissue. Preliminary results demonstrated that the silk gel and silk sponge scaffolds were the most promising configurations. The researchers developed a collagen gel delivery system that releases VEGF, which is aimed at enhancing vasculogenesis in vivo. The researchers implanted cell-hydrogel constructs (containing fibroblasts with either fibrin, collagen, or HA) in athymic mice and have begun to evaluate the dimensional changes in the mice at various time points post-implantation. They also developed polymeric microspheres containing proteins known to promote angiogenesis, which are expected to enhance tissue formation by the cell-hydrogel constructs.

Research Plans for the Next 4 Years

The combined efforts in Projects 4.1.4 and 4.1.5 have led to the development of a silk-based, degradable, and biocompatible protein scaffold for soft tissue reconstruction. The researchers have made considerable progress during the first year of the study and plan to continue the work described in the proposal as well as maintain active inter-institutional collaborations over the next 4 years.

Planned Clinical Transitions

The silk-based biomaterial used in these studies has recently received FDA approval. Continued effort on the project will focus on the rapid advancement toward clinical trials with the technologies being developed.

Corrections/Changes Planned for Year 2

None.

Project 4.1.2, RCCC

Develop Innervated, Vascularized Skeletal Muscle

Team Leader(s): *Cathryn Sundback, ScD, Joseph Vacanti, MD (Massachusetts General Hospital [MGH])*

Project Team: *Craig Neville, PhD, Mei Li, MS, Eric Finkelstein, PhD, Caitlyn Dickinson (MGH); Mindy Ezra, Joachim Kohn, PhD (Rutgers – The State University of New Jersey, New Jersey Center for Biomaterials)*

Collaborator(s): *Scott Goldman, Kensey Nash Corporation*

Therapy: *Repair and regenerate muscle damaged in facial and limb injuries*

Deliverable(s): *Innervated, vascularized skeletal muscle*

Key Accomplishment(s): *Biodegradable polymer scaffolds were tested that improved the ability to handle engineered myooids for skeletal muscle regeneration. Vascular-like networks and immature muscle were produced in vitro using biopolymer gels. MSCs were employed to support the formation of the prevascular network. The engineered immature muscles were implanted into adipose tissue of mice and found to express muscle contractile proteins. Work is ongoing to characterize the invasion of the host vasculature into the engineered myooid.*

Note: This project is AFIRM research fully supported in Year 1 by leveraged funding (DoD Award No. W81XWH-04-2-0031, Center for Military Biomaterials Research).

Introduction

Traumatic injuries to the head, neck, and particularly the face are the major contributors to the mortality and morbidity of military personnel in the current conflicts in Iraq and Afghanistan. Although the head, face, and neck comprise only 12% of the total surface area exposed during combat, these body areas have sustained proportionally more injuries than any other body region. Injuries to the facial muscles significantly impact the health and self-esteem of the injured warfighter. These muscles are small relative to limb muscles, and few autologous muscles are available to restore function. Transplanted facial muscles can restore function but require lifelong immunosuppression; the risks associated with immunosuppression are difficult to justify for non-life-threatening injuries. Consequently, the smaller facial muscles, like the orbicularis oculi muscle, are excellent candidates to be engineered as these muscles will significantly improve the quality of life of the maimed warfighter without inducing additional risk. The technological challenges in engineering these muscles are achievable in the short term because of their small size, required contractile force, and readily available, effective, autologous cell sources.

The ultimate goal of the AFIRM is to move therapies for the treatment of severe tissue trauma from the use of artificial prosthetics to the capability to repair and regenerate the most severe tissue damage in soldiers with facial injuries and limb amputation. As a critical step toward this goal, the team at MGH is engineering innervated, vascularized skeletal muscle tissue that, in the near term, could be employed to reconstruct muscle injuries. Currently, no products are commercially available to reconstruct skeletal muscle tissue or to restore muscle function.

Research Progress – Year 1

The objective of this project is to engineer innervated, vascularized skeletal muscle, with physiological connections to the host's neurovascular network, using tyrosine-derived polymer scaffolds. These scaffolds will be elastomeric with overall mechanical properties matched to native skeletal muscle tissue. When exposed to in vivo dynamic forces, the elastomeric construct will transmit these forces to the developing muscle tissue, enhancing differentiation and alignment of the skeletal muscle myotubes. Additional skeletal muscle tissue maturation will occur when effective neuromuscular junctions are established with the host neural network. The near-term objectives are to optimize the scaffold parameters in vitro to develop prevascularized skeletal muscle tissue. In parallel, muscle innervation will be

simulated by electrical stimulation to determine its impact on muscle maturation and function. Finally, these engineered muscle constructs will be implanted in animal models and exposed to physiological stresses and anastomosed to the host neurovascular networks. These mechanical and biological signals will be harnessed to drive neural ingrowth and optimize neuromuscular junction formation and skeletal muscle maturation.

In Year 1, this research team has tested biodegradable polymer scaffolds that improved the ability to handle engineered myooids for skeletal muscle regeneration. The researchers successfully engineered immature muscle tissue through a self-assembly culture process. To engineer vascularized muscle tissue using the MGH self-assembly protocol, a biopolymer scaffold is being developed with a muscle layer and a vascular layer. MSCs were employed to support the formation of the prevascular network (**Figure III-13**). The engineered immature muscles were implanted into adipose tissue of mice and found to express muscle contractile proteins (**Figure III-14**). Work is ongoing to characterize the invasion of the host vasculature into the engineered myooid.

Key Research Accomplishments

- Several scaffold concepts continue to be tested that will improve the ability to handle engineered myooids and temporarily support the myooids until sufficient mechanical integrity develops.

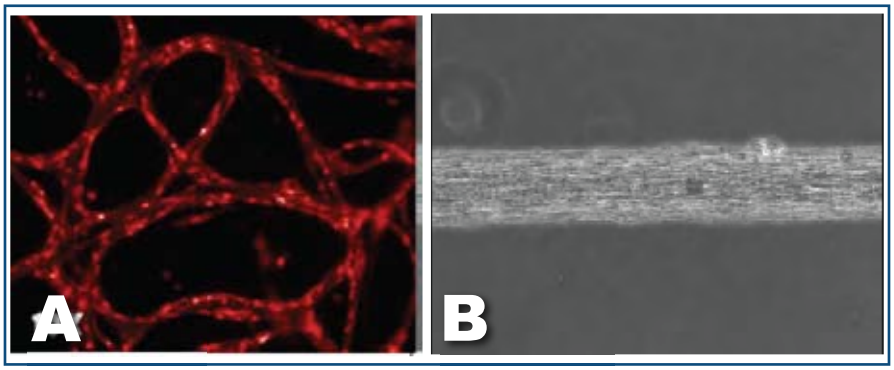


Figure III-13. Engineered endothelial networks and vascularized immature muscle tissue in double fibrin layer. (A) Endothelial cells and MSCs co-cultured in top "vascular" fibrin layer formed stable endothelial networks (day 3 post-differentiation). (B) Myoblasts and fibroblasts co-cultured on top of double fibrin layer self-assembled into three-dimensional immature muscle tissue that spontaneously contracted (day 14).

- Both vascular-like networks and immature muscle have been formed in vitro in double layer fibrin gels; the bottom gel is designed to support muscle self-assembly, and the top gel layer is designed to support prevascular network formation. The MSCs, employed to support the formation of the prevascular network, were determined to prevent fibrin degradation. Investigation is ongoing.
- Engineered immature muscles were implanted into highly vascularized adipose tissue. These muscle constructs were explanted and continued to express muscle contractile proteins. Work is ongoing to characterize the invasion of the host vasculature into the engineered myooid.

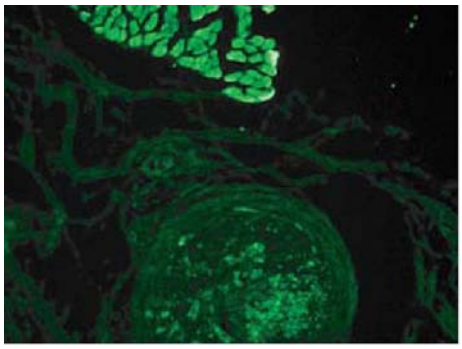


Figure III-14. Explanted engineered skeletal muscle. The bright green tissue in the upper portion is native muscle while the circular-shaped green tissue in the lower center is the engineered myooid. The engineered tissue continues to express appropriate muscle markers.

Conclusions

The team at MGH is investigating concepts to support in vitro engineered muscles to improve ability to handle, allow in vivo implantation, and support the muscle tissue until sufficient mechanical integrity develops. Initial results indicate that three-dimensional muscle tissue can self-assemble and a prevascular network can form using this biopolymer scaffold. However, the MSCs employed to support vascular network formation also prevented scaffold degradation and disrupted myotube formation. Finally, the first implanted engineered muscles have been explanted from immunocompromised rodents and continue to express appropriate contractile muscle markers.

Research Plans for the Next 4 Years

Several scaffold concepts will be investigated using a combinatorial-computational method to design and synthesize the scaffold using a series of tyrosine-based copolymers. The scaffold design and resorption rate will continue to be optimized to achieve the required elastomeric properties, degradation rates, and architecture. To confirm differentiation and alignment of myotubes, expression of muscle-specific proteins associated with the contractile apparatus MyHC will be assessed by RNA and protein analysis, as well as visualized by immunofluorescence. Future work is planned to assess cell morphology of the developed tissue by histological examination. Maturation of the vascular network and muscle tissue, as well as host axonal ingrowth, will be demonstrated using a variety of immunohistochemical staining assays. Functional innervation will be validated and anastomosis with host vasculature will be confirmed. Functionality of the engineered muscle will be determined through excitability and contractility measurements.

Planned Clinical Transitions

The research team is currently focused on the analytical and laboratory studies needed to validate the elements of the innervated skeletal muscle. It is expected that the developed tissue will be further assessed for cell morphology and functionality in Year 3.

Corrections/Changes Planned for Year 2

None.

Project 4.1.6, WFPC

Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle

Team Leader(s): *George J. Christ, PhD*

Therapy: *Skeletal muscle reconstructive procedures required to repair complex facial injuries*

Deliverable(s): *A skeletal muscle tissue implant capable of generating clinically relevant force/tension*

Key Accomplishment(s): *A standard operating procedure (SOP) for use of rat muscle precursor cells (MPCs) was developed. An organized muscle tissue from human MPCs has been generated in vitro on a decellularized scaffold, as well as an aligned polycaprolactone (PCL)/collagen scaffold. Myotube formation has been demonstrated both in culture and on the surface of the aligned PCL/collagen scaffold. A custom-designed seeding chamber for muscle scaffolds has been created using an FDA-approved pharmaceutical-grade silicone rubber.*

Introduction

Current management 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 researchers' long-term goal is creation of 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 through use of 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 soft tissue function. The initial clinical application will be repair and restoration of craniofacial battlefield wounds.

Research Progress – Year 1

The researchers recently have published on their ability to generate an organized muscle tissue from human MPCs in vitro on a decellularized scaffold (porcine bladder acellular matrix; BAM) and more recently have extended those findings to the aligned PCL/collagen scaffolds that are the subject of this project (**Figure III-15**). Some representative examples of myotube formation derived from rat MPCs are illustrated in Figure III-15. The researchers have evidence for myotube formation both in culture and on the surface of the aligned PLC/collagen scaffold.

To improve the efficiency of their cellular seeding protocol prior to bioreactor preconditioning, the researchers have created a custom-designed seeding chamber for the muscle scaffolds (**Figure III-16**). Specifically, they have changed the material from the colored silicone rubber originally used to an FDA-approved pharmaceutical-grade silicone rubber. The new material is easier to work with, clear, and more malleable, all of which confers advantages when manipulating the seeded scaffolds within the seeding chamber. However, while they have optimized to a large extent the cellular seeding and bioreactor protocols, they acknowledge the need to make further modifications to the PCL/collagen scaffold. More specifically, remodeling and integration of the PCL/collagen scaffold with host tissue in vivo are occurring at a slow rate, and this is impacting the formation of functional (i.e., contractile) muscle. However, they expect to resolve this issue quickly based on their familiarity with this material.

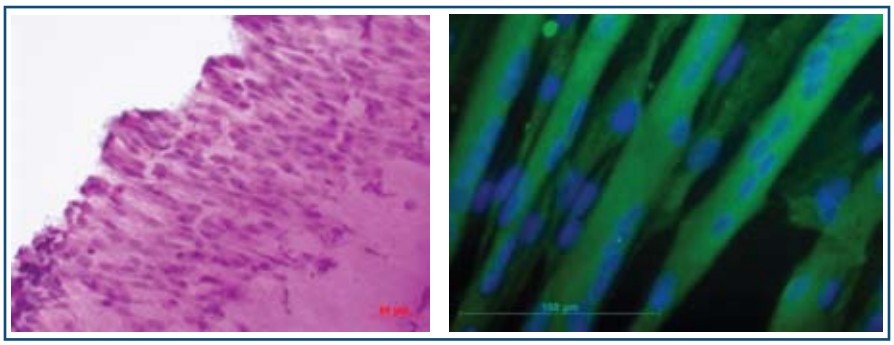


Figure III-15. Tissue organization in vitro. The left panel shows an H&E section taken parallel to the surface of a PCL/collagen-aligned scaffold seeded with MPCs. Clear evidence for myotube formation on the surface of the scaffold prior to bioreactor preconditioning. The image on the right shows a representative immunofluorescence stain for MyoD and DAPI showing myotube formation of rat MPCs in culture. Positively stained myotubes have been obtained when probing rat MPCs with actin, desmin, and MHC (data not shown). The rat MPC SOP is complete.

Key Research Accomplishments

- Completed initial observations/characterization of engineered tissue function in vivo, which indicates the necessity to further modify/optimize the PCL/collagen-based scaffold system.
- Introduced the latissimus dorsi defect model and completed retrieval and physiological analysis of the first control defect.
- Performed cell seeding, bioreactor preconditioning, and implantation of multiple bioengineered skeletal muscle constructs.
- Established an assay for the neuromuscular junction.

Conclusions

The researchers have made considerable progress toward the development of an organized muscle tissue from human MPCs that will be capable of generating clinically relevant force/tension.

Research Plans for the Next 4 Years

In Years 2–5, the researchers plan to (1) evaluate the feasibility of implanting engineered rat skeletal muscle in a rat skeletal muscle defect model, (2) investigate the translation of engineered skeletal muscle to a craniofacial muscle defect in a larger animal model, and (3) determine the feasibility of using biopsies from humans in generating functional engineered skeletal muscle tissue.

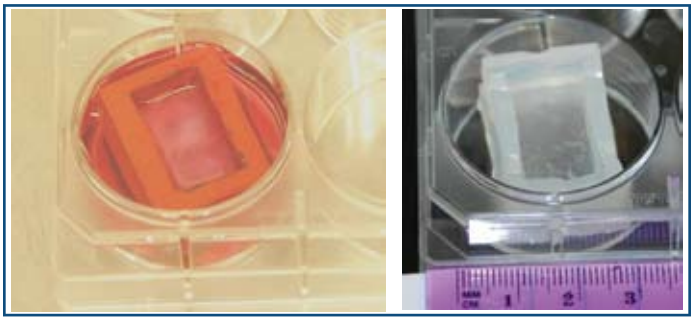


Figure III-16. Picture of the custom-made seeding chamber containing a scaffold in the center of the chamber in a 6-well plate. In this scenario, the seeding chamber is draped with the BAM.

Planned Clinical Transitions

The researchers have developed a detailed protocol for the production of bioengineered scaffolds seeded with well-characterized rat cell populations and conditioned in a bioreactor. They gathered preliminary data documenting the respective facility with all of the assays and methods required to evaluate the proof-of-concept studies required to document the utility of the proposed approach. The validation provided through the proof-of-concept studies will facilitate the clinical translation of the bioreactor technology for skeletal muscle development.

Corrections/Changes Planned for Year 2

The researchers encountered one significant "negative" finding in Year 1. Specifically, they observed that the originally proposed PCL/collagen scaffold apparently remodels too slowly for the proposed use for bioengineered skeletal muscle (4 or more months required for significant yet still incomplete remodeling with other characteristics unsuitable for the proposed application); therefore, they are now working on modifications to that scaffold system to increase the utility of this biomaterial. They expect to solve this issue quickly and have other scaffolds that they can use in the interim (e.g., BAM and other collagen-based scaffolds) to continue to progress with the critical proof-of-concept studies. Thus, the researchers anticipate no delays in milestones or deliverables as they proceed with the proposed work.

Project 4.5.4, RCCC

Regeneration of Ear

Team Leader(s): *Joseph Vacanti, MD, PhD, Cathryn Sundback, ScD (MGH)*

Project Team: *Mack Cheney, MD, Tessa Hadlock, MD, Robin Lindsay (Massachusetts Eye and Ear Infirmary)*

Irina Pomerantseva, MD, PhD, Ken Rask, Erik Bassett, Katherine Kuligi, Mark Randolph, David A. Bichara, MD, Xing Zhao, Matt Johnson (MGH); Fan Yang, PhD, Nathaniel Hwang, PhD, Daniel Anderson, PhD, Robert Langer, PhD (Massachusetts Institute of Technology [MIT])

Collaborator(s): *Scott Goldman, Kensey Nash Corporation*

Therapy: *Reconstruction of the external ear*

Deliverable(s): *Permanent implantable living external ear*

Key Accomplishment(s): *The MGH-led team has demonstrated tissue engineering, scaffold concepts, and animal models that will ultimately be combined in a successful ear replacement clinical trial: Ovine and human cartilage has been engineered; ear-shaped scaffolds have been prototyped, which will maintain complex three-dimensional ear architecture when exposed to physiologic forces; and an ovine model has been developed for heterotopic implantation of ear-shaped autologous constructs.*

Introduction

Conventional artillery and IEDs often inflict serious disfiguring injuries to the head and extremities. Surgeons have limited options for facial reconstruction in people who have suffered blast injuries; the challenge is compounded by the complex geometry and diversity of craniofacial tissue. Reconstruction of the total external ear is one of the most difficult problems in the field of plastic and reconstructive surgery. Standard surgical treatment of microtia in adolescents involves several surgeries to harvest rib cartilage, sculpt an ear shape, and place it under a skin/fascia envelope. Results can be a crude replication of the external ear. One alternative is to implant a porous polyethylene strut under the skin/fascia envelope that simulates the contours of the ear. But the plastic material is inflexible; it is only available in predetermined shapes and sizes, and the implants are predisposed to extrusion and infection.

Cartilage can be successfully engineered in vitro and in vivo by seeding isolated cartilage cells (chondrocytes) onto porous/fibrous scaffolds. This research group has successfully engineered ear-shaped cartilage using various biodegradable scaffolds and cartilage cells in animals with compromised immune systems. Also, in a swine model, the team tested the use of connective tissue surrounding the cartilage (perichondrium) to improve the flexibility of engineered ear-shaped cartilage. The engineered ear design must maintain its complex three-dimensional structure and shape even when exposed to mechanical forces imposed by the overlying tissue envelope. The goal of this project is to expedite the development of a permanent, implantable, living external ear for the injured warfighter and to achieve cosmetic outcomes that meet patient expectations.

Research Progress – Year 1

Specific Aim 1: Optimize in vitro culture of tissue-engineered cartilage. The researchers determined the cell concentration required for the creation of a tissue-engineered ear. The average adult human male ear has a cartilage volume of approximately 3–4 cm^3, and 60 x 10^6 cells/mL was the minimum concentration needed to form high-quality cartilage. The team is using cartilage cells or a combination of cartilage cells and MSCs to form cartilage. Both cell types are easily obtained, can be cultured, and have been demonstrated to form cartilage.

Specific Aim 2: Evaluate the ability of FDA-approved, degradable, biocompatible biomaterials with properties appropriate for ear tissue engineering to support chondrocyte growth and matrix production. The team is testing scaffold materials made from various polymers and scaffolds built from degradable, biocompatible fibrillar collagen. Both porous polyester and collagen scaffolds showed uniform cartilage-cell distributions irrespective of scaffold type and seeding methodology.

Specific Aim 3: Evaluate cartilage formation and scaffold distortion, and demonstrate a

clinically functional engineered ear. Researchers evaluated cartilage formation on the scaffold materials both in vitro and in vivo in mice, using both cartilage cells and stem cells. The team employed a permanent metallic structure to define and maintain a scaffold shape during healing; this framework was coated with porous polymer or collagen to create a cell-friendly environment (**Figure III-17**). The group tested its biologically active, engineered ear implants versus traditional polymer ear implants in a sheep model with mixed results in both groups. Nevertheless, none of the experimental tissue-engineered ears have extruded through the skin, and the contours of the engineered ear construct are more naturally defined (**Figure III-18**).

Technology Readiness Level (TRL) 3: Researchers have integrated the basic concepts of cell isolation, expansion, scaffold fabrication, and tissue engineering; they are currently testing these in a simulated environment. They have carried out proof of concept via implantation studies in immunocompromised mice, which demonstrated minimal construct deformation over a 12-week period. They have observed native-like contiguous cartilage formation using human naso-septal cartilage cells. They have implanted various cell-hydrogel-polymer hybrids in the ovine model and are currently awaiting results. They plan to develop methods to utilize a limited number of harvested cells to advance to TRL 4 and begin clinical trials.

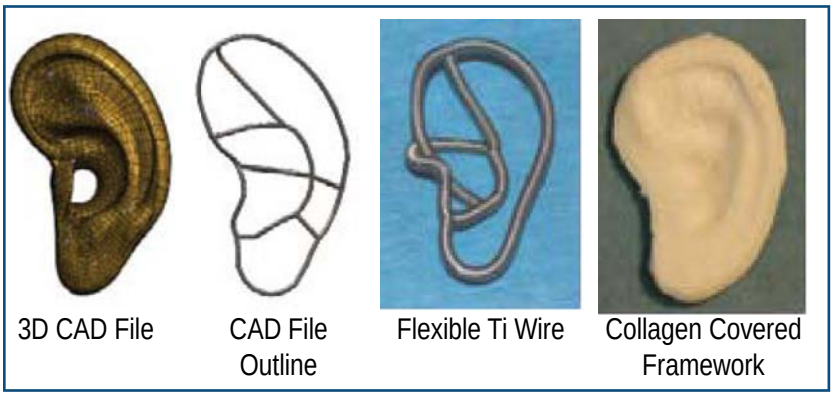


Figure III-17. Ear-shaped scaffolds have been designed using CAD principles and have been tested with numerous materials.

Key Research Accomplishments

- Developed an ovine model for subcutaneous implantation of ear-shaped constructs.
- Engineered cartilage from both ovine and human cartilage cells and MSCs in immunocompromised mice.
- Designed ear-shaped scaffolds using CAD principles and tested the scaffolds with numerous materials.

Conclusions

This research group has reached several milestones in this program to engineer replacement external ears. Researchers successfully developed a large animal model to help tailor the architecture and to select the materials for ear-shaped scaffold prototypes. They are designing these ear scaffolds to maintain the original three-dimensional shape of the construct upon implantation and to form functional cartilage from seeded cartilage and

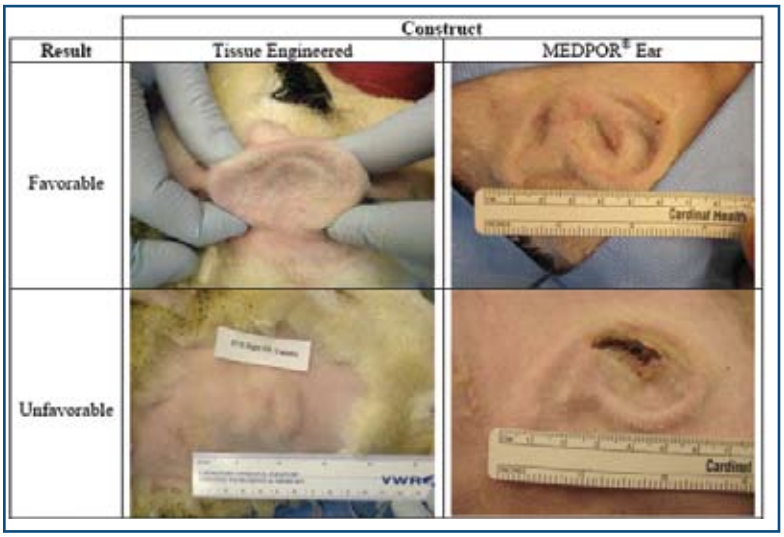


Figure III-18. Both favorable and unfavorable results have been achieved for both groups. However, none of the experimental tissue engineered ears have extruded through the skin. Furthermore, better aesthetic results have been achieved in the tissue engineered ear group (top left) relative to the MedPor group (top right).

stem cells. They have identified many experimental parameters to successfully form cartilage from chondrocytes and stem cells in vitro. They have estimated the amount of cartilage tissue that is needed to generate a contiguous cartilage matrix of a given volume. They are refining the scaffold design, using CAD techniques, to achieve accurate architectural definition and to resist scaffold distortion when placed under the skin/fascia envelope.

In summary, this team of researchers and surgeons is on schedule to initiate a clinical trial, beginning in July 2013, which is intended to demonstrate the safety and feasibility of the final external ear construct. In preparation, scaffolds have been developed from biocompatible FDA-approved materials with an embedded permanent framework to define the ear shape and prevent distortion. These scaffolds have been used to engineer human and animal cartilage in animal models.

Research Plans for the Next 4 Years

The researchers made significant progress in Year 1 on the MGH–MIT program toward ear regeneration. As a result, they identified two challenges: (1) how to select an FDA-approved (or -approvable) scaffold material that supports three-dimensional toleranced cartilage formation in immunocompetent animals and (2) how to identify appropriate autologous cell sources. In Years 2–3, the team will enhance cartilage formation from nasal-septal and auricular sources by optimizing cell-seeding density and bioreactor culture. If required, in Years 2–4, they will augment cartilage formation from primary cartilage cells by co-culture with MSCs. In Years 2–4, the researchers will continue to evaluate candidate scaffold materials for their ability to support cartilage formation in two animal models: immunocompromised mice, using human cartilage, and immunocompetent sheep, using autologous cartilage. In Years 3–4, the researchers will maintain the three-dimensional implanted ear shape by optimal choice of scaffold material and reinforcing geometry, which should allow the implant to withstand physiologic contractile forces and distortion during scaffold resorption. The team will obtain FDA-approved or minimally modified synthetic materials from the MIT Langer laboratory and processed collagens and decellularized ECM materials from Kensey Nash. Ultimately, Kensey Nash will supply the program with GMP-compliant scaffold materials. Based on the described work, the researchers expect the TRL to progress from current level 3 to level 4 by the end of Year 4.

Planned Clinical Transitions

Researchers expect to perform a pilot clinical trial immediately upon completion of Year 4. They should complete preclinical trials by the end of the second quarter in Year 4 and will analyze, audit, and report those studies to the IRB. The team will develop a clinical protocol for a pilot study during the first and second quarters of Year 4 and will submit this to the IRB. They expect to obtain an approval for a pilot clinical study from the IRB upon completion of Year 4. They will discuss the regulatory requirements with the FDA prior to initiating the exploratory clinical trial.

Corrections/Changes Planned for Year 2

None.

Project 4.1.1, WFPC

Engineered Cartilage-Covered Ear Implants for Auricular Cartilage Reconstruction

Team Leader(s): *James J. Yoo*

Therapy: *Reconstruction of the external ear.*

Deliverable(s): *Engineered cartilage tissue covering the MedPor ear implant*

Key Accomplishment(s): *The researchers prepared fibrin hydrogels with various concentrations of fibrinogen and thrombin, mixed cultured chondrocytes with the hydrogels, and implanted constructs subcutaneously into athymic mice. They harvested the mice at 4, 8, 12, and 24 weeks post-implantation and performed compression testing, histological and immunohistochemical analyses, and biochemical evaluations. While nontreated ear implants resulted in severe skin necrosis at 2 weeks post-implantation, the cartilage-covered ear implants were able to maintain device contour and placement without causing skin necrosis.*

Introduction

Traumatic injuries are 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 life-threatening danger, it is functionally and cosmetically debilitating and hinders injured soldiers to return to society. The gold standard treatment method for auricular reconstruction uses a patient's own rib cartilage as a graft material. However, autologous rib cartilage graft is limited in supply and provides inadequate dimensions of the ear cartilage tissue. An alternative approach utilizes a commercially available ear implant device, such as MedPor, which is manufactured from linear high-density polyethylene. This FDA-approved implant is nontoxic, causes minimal foreign body reaction, and possesses adequate mechanical properties for use in non-load-bearing regions of the craniofacial skeleton.

Although the synthetic ear implant advantageously eliminates the morbidity associated with the rib cartilage, the use of the MedPor ear implant often is associated with complications that include inflammation, infection, erosion, and dislodgement. As a result, MedPor ear 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 temporo-parietal tissue flap from the side of the head to cover the implant, which provides vascularized tissue cushion against the abrasive implant. In this project, the researchers have developed an engineered cartilage that entirely covers the abrasive MedPor implant, which would prevent implant exposure and extrusion while maintaining appropriate mechanical properties. Creation of cartilage tissue using a soldier's own cells would bring benefits and minimize the morbidity associated with implant dislodgement (**Figure III-19**).

The strategy for developing an engineered cartilage tissue covering the MedPor ear implant consists of two steps: (1) surface modification of the device's innate hydrophobic characteristics to achieve hydrophilic environment for cells and (2) coverage of chondrocyte-hydrogel conjugation to MedPor implant to achieve cartilage tissue cushion against the device. In this project, the researchers plan to further refine and optimize the processing system for a smooth translation to soldiers who require auricular reconstruction.

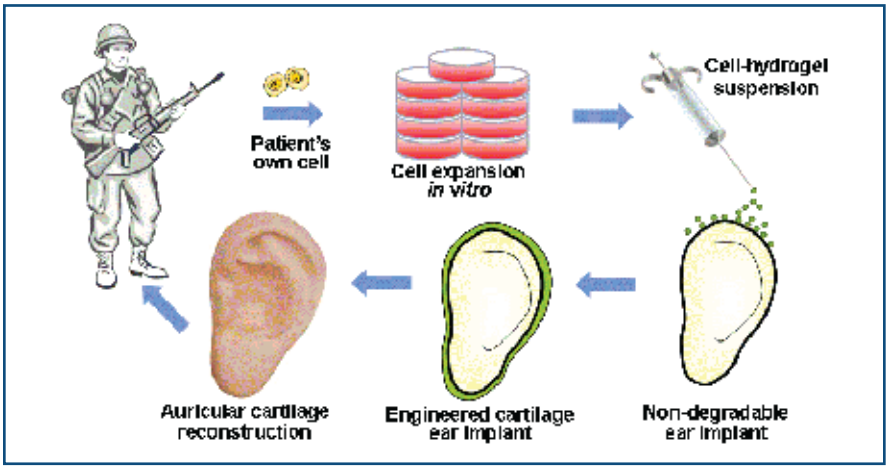


Figure III-19. Strategy for engineering cartilage-covered ear implants for auricular tissue reconstruction.

Research Progress – Year 1

1.1. Isolation and expansion of chondrocytes from auricular, nasal, and costal cartilages. Auricular chondrocytes were obtained from fresh ear cartilage tissue biopsies from New Zealand white rabbits (Charles River Laboratories Inc., Wilmington, Massachusetts). Human nasal and costal chondrocytes were obtained from discarded human nose and rib cartilage tissues. Cartilage was dissected after carefully removing the perichondrium. Cartilage pieces were minced into 1 mm^3 pieces. These were suspended in Ham's F12 medium containing 0.2% type II collagenase (Worthington Biochemical, Freehold, New Jersey) and incubated at 37°C for 4 hours. After enzymatic digestion, the resulting cell suspension was passed through a 100 m filter to collect cells. The chondrocytes were grown and expanded in low glucose Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum, 1% L-glutamine, and 1% streptomycin penicillin at 37°C, 5% CO_2 until a sufficient number of cells were obtained. All reagents for cell culture were purchased from Invitrogen (Gibco® Cell Culture, Carlsbad, California). After isolation, chondrocytes were characterized using phenotypic expression and proliferation assays (**Figure III-20**).

1.2. Fabrication and characterization of fibrin hydrogels. The concentrations of fibrinogen and thrombin, cell density, Ca2+ concentration, and pH can be modified for optimizing fabrication conditions for hydrogel systems used in tissue engineering. To evaluate the initial properties of a hydrogel system for use in auricular cartilage reconstruction, fibrin hydrogels with various concentrations of fibrinogen and thrombin were prepared. In addition, when the concentration of fibrinogen in the gels was held constant at 80 mg/mL and the thrombin concentration was varied, the stiffness of a hydrogel containing 10 U/mL thrombin was significantly different than that of a gel containing 100 U/mL of thrombin.

1.3. In vivo evaluation of chondrocyte-fibrin constructs. Primary auricular chondrocytes isolated from a biopsy of rabbit ear cartilage were expanded until sufficient numbers of cells were obtained. The phenotype of these cells was confirmed by immunocytochemistry for type II collagen (data not shown). The cells were then mixed into hydrogels containing varying amounts of fibrinogen and thrombin at different cell densities. Constructs from these six experimental groups were implanted subcutaneously into athymic mice and harvested 4, 8, 12, and 24 weeks after implantation (n=3). By 24 weeks, the chondrocyte-fibrin constructs from group EC-5 were the most similar to native auricular cartilage in color (opaque white), and they maintained their initial shape and size.

1.4. Compression testing. Using compression testing, the stiffness of the retrieved constructs was measured at each time point. Stiffness of the constructs was

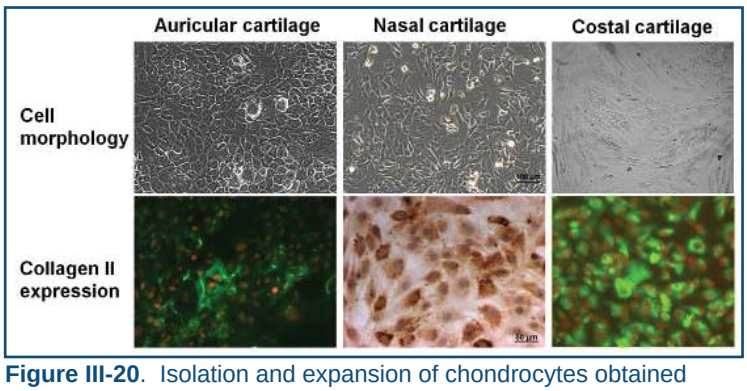


Figure III-20. Isolation and expansion of chondrocytes obtained from auricular, nasal, and costal cartilage tissues.

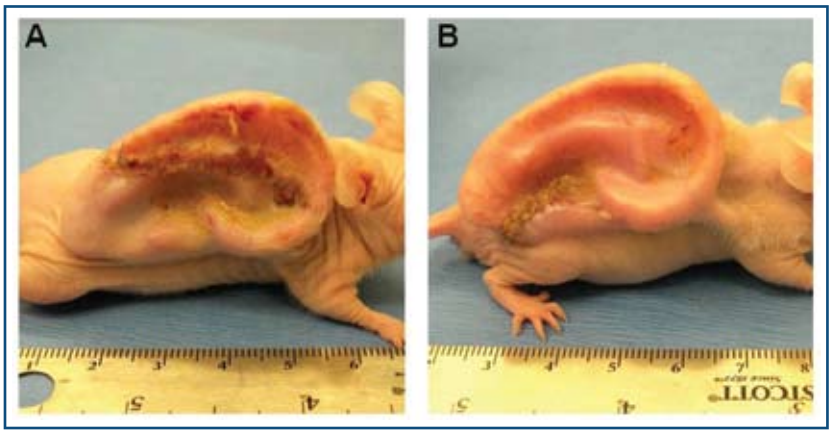


Figure III-21. Post-implantation at 2 weeks; (A) ear implant only and (B) engineered cartilage-covered implant.

increased by decreasing fibrinogen concentration. These results show that fibrinogen concentration and initial cell density significantly affected the mechanical strength of the engineered cartilage tissue. In addition, stiffness of the constructs in all experimental groups gradually increased with time.

1.5. Histological and immunohistochemical analyses.
Histological staining of the retrieved samples revealed that a new cartilaginous matrix had been formed in the chondrocyte-fibrin constructs in all experimental groups. The chondrocytes in the newly formed tissue demonstrated the same morphological characteristics as those in native cartilage, with cells located within typical chondrocyte lacunae, surrounded by cartilaginous matrix.

1.6. Biochemical evaluations.
The water content of the constructs was determined by comparing wet and dry weights. All experimental groups exhibited a slight decrease in water content with time, most likely due to gel contraction or matrix production.

1.7 Engineered cartilage-covered ear implants.
Grossly, the engineered cartilage-covered ear implants placed in athymic mice showed no evidence of skin necrosis, implant exposure, or extrusion. However, nontreated ear implants (control) resulted in severe skin necrosis at 2 weeks after implantation (**Figure III-21**). This study to date indicates that cartilage-covered ear implants are able to maintain device contour and placement without causing skin necrosis. Further studies are being performed to demonstrate consistency.

Key Research Accomplishments

- Completed a proof-of-concept study in a mouse model.
- Completed a pilot study to optimize a fibrin hydrogel system.
- Completed the optimization of a cell delivery system.

Conclusions

The researchers have made considerable progress toward the development of cartilage-coated auricular implants that are able to maintain device contour and placement without causing skin necrosis.

Research Plans for the Next 4 Years

In Year 2, the researchers will continue with assessment of the biocompatibility and structural stability of the engineered cartilage ear implant. In addition, continued testing of the engineered ear implant will be performed to assess the host tissue response in the animal model. In Years 3–5, the researchers will begin identifying and selecting patients for enrollment in the initial clinical trial.

Planned Clinical Transitions

The researchers seek to continue to develop a system that requires a minimum tissue biopsy for cell isolation and expansion using different cell sources, to continue to develop SOPs for autologous cell sourcing and an associated expansion system, and to refine the cell delivery system to facilitate clinical translation of the cartilage-coated auricular implants.

Corrections/Changes Planned for Year 2

In this project, the researchers are implanting ear cartilage in a rodent ex situ model using human cells. However, they acknowledge that this model does not entirely reflect the clinical scenario in the craniofacial region. They are considering adding a large animal study that would involve implantation of engineered ear cartilage in the auricular region using autologous cells. The objective would be to further confirm the clinical applicability and to develop SOPs for surgical methods.

Project 4.5.5, RCCC

Virtual Reality Application for Modeling and Visualization of Patient-Specific Craniofacial Wounds and Injuries

Team Leader(s): *Timothy Kelliher, Howard Champion, MD (SimQuest, LLC)*

Deliverable(s): *Visualization of patient-specific wounds and injuries*

Key Accomplishment(s): *Built the underlying infrastructure of a virtual reality system for visualizing patient-specific craniofacial wounds, injuries, and treatment strategies that will permit high-quality comparisons of expected outcomes based on existing medical imaging data. By creating a database schema that supports the most important data, identified in partnership with the National Capital area Craniofacial Reconstruction Team at both the microscopic (10-3 m) and cellular (10-6 m) levels, and creating an initial visualization pipeline from existing software tools, there is a solid foundation for the desired virtual reality system.*

Introduction

Widespread use of IEDs against the United States and other forces in Operation Iraqi Freedom, combined with the general effectiveness of body armor in minimizing lethal torsal injuries, has resulted in new patterns of injury in survivors that include devastating craniofacial trauma. Repair and reconstruction of craniofacial injuries are exceedingly complex because of the issues involved in protecting vital senses; the unique challenges of repair, reconstruction, and regeneration in the presence of oral and periodontal bacteria; changes in repaired/replaced tissue over time; challenges in restoring/providing the capabilities for verbal and nonverbal communication and expression; and concerns related to managing not only the physical and cosmetic but also the psychosocial sequelae, frame of self-reference, and optimism of the patient.

Research Progress – Year 1

During this first year of work, the SimQuest team has worked with other members of the AFIRM team to:

- Develop the requirements for a virtual reality application that enables visualization of patient-specific wounds and injuries and the integration of specific treatment strategies, including wound healing, repair, flaps, hard tissue implants, and specific tissue regeneration strategies to include skin, soft tissue, muscle, nerve, and vessel with or without absorbable platforms.
- Ascertain the granularity of tissue rendition necessary to capture iconic injuries in these body areas and to fully understand the repair/regeneration integration and treatment.
- Develop a database schema that is sufficient to encode this information.
- Acquire the necessary software to begin working with selected team members in the coming years.

SimQuest team members reviewed current literature on craniofacial imaging and reconstruction and met with experts to determine the range and granularity of needs for tissue rendition in the virtual reality simulation. This work focused on the needs of facial reconstruction and transplant procedures. The team has assembled a beginning set of anatomically correct three-dimensional surface models of the face to serve as the foundation for its index into injury and wound data. To achieve the granularity of tissue required, these models needed to be expanded to encompass not just surface definitions but also volumetric models such as those found in voxel phantoms. There are a multitude of voxel phantoms of the head and body developed by various groups. Although none of these is ideally suited to this application, they do provide a springboard from which a suitable phantom can be

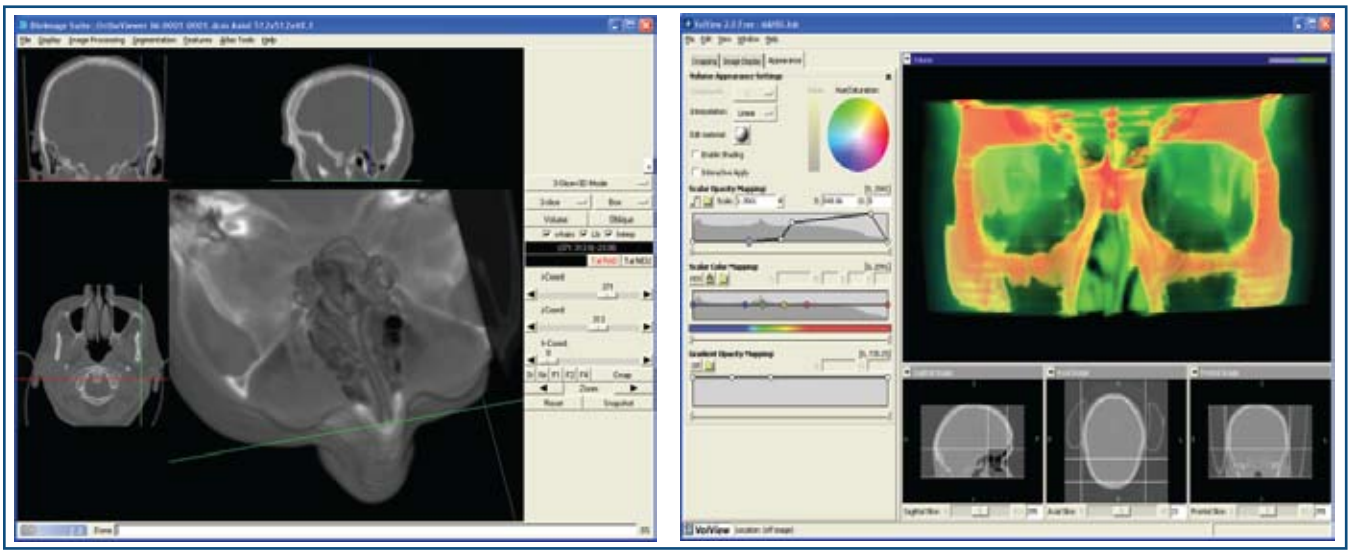


Figure III-22. Baseline imaging and modeling tools. Pictured left, Image analysis and manipulation tools, BioImage Suite (Yale), VolView (Kitware), DicomWorks (Puech), Slicer3 (SPL). Pictured right, Modeling tools, Paraview (Kitware); Open development, ITK, VTK.

built. Voxel phantoms come from the materials and communication modeling communities. In the medical imaging community, these are referred to as image or volume atlases.

The three-dimensional modeling tools that SimQuest has been investigating are aimed at completion of a suitable image-guided atlas. The SimQuest team evaluated three-dimensional modeling tools and development platforms to determine which are appropriate for use in working with the AFIRM team (**Figure III-22**). VtkEdge (a library of advanced visualization and data-processing techniques that complement the VTK [The Visualization Toolkit] and ITK) (Insight Segmentation and Registration Toolkit) offers a clear distinction in flexibility of use and range of support. Although VTK has strong presence in the solution, its rendering engine may need to be replaced with a package that is more suited to real-time applications such as OpenSceneGraph.

Key Research Accomplishments

- Development of a virtual reality application that enables visualization of patient-specific wounds and injuries and integrates specific treatment.
- Determination of the granularity of tissue rendition necessary to capture iconic injuries for application in repair/regeneration integration and treatment.
- Development of a database schema for information encoding.

Conclusions

The work summarized has significant translational implications and paves the way for further development. SimQuest has been coordinating with the Cleveland Clinic and USAISR to determine the best near-term use of its technology and to identify which gaps need to be addressed. The area where there is likely to be the largest immediate impact is investigating healing effects on tissue volumes for facial reconstructive and transplant procedures.

Research Plans for the Next 4 Years

SimQuest's research plan is to develop the platform for translating AFIRM results from laboratory prototypes into clinical practice. Specifically, the technology to be developed comprises injury data analysis tools, preparation of patient-specific wound and injury models, and physics-based surgical simulation. The injury analysis tools create a statistical basis for determining high-impact areas of focus and for tracking effectiveness of surgical and regenerative techniques. They identify successful or unsuccessful outcomes and serve to identify the common elements of treatment that may be the causative agents. CTA and regenerative tissue therapies require precise knowledge of the state of hard and soft tissues. Algorithms for creating the hard tissue model of the patient are

being developed under an externally funded program. The patient-specific modeling for the AFIRM will delve into the segmentation and modeling of targeted soft tissue structures such as specific muscles, nerves, vasculature, and scar tissue. New algorithms will be developed to segment these structures from computed tomography data.

Once the patient-specific models are robust from a geometric standpoint, the next phase is to employ SimQuest's surgical simulation technology. Leveraging this technology requires enhancing the three-dimensional geometric model with information about the mechanical properties of the constituent tissues and tissue replacements. An application will be developed that bundles the patient-specific models and simulation technology into a clinically deployable package. The simulation tool will be able to show how surgically moved or transplanted tissue will appear given the geometry and physics of the tissue. This will provide clinicians with details of the way tissues will interact and measures of the volume of transplant tissue necessary to achieve satisfactory coverage.

Planned Clinical Transitions

The work that SimQuest is proposing builds upon several ongoing projects. The first of these is the craniofacial injury mapping project being performed in conjunction with the Walter Reed Army Medical Center (WRAMC). This project is developing the baseline capability for the injury analysis tools. As part of that project an IRB submission has been prepared and currently is being evaluated by the WRAMC IRB. Once accepted, this will establish the ability to deploy the injury tools to WRAMC and begin collecting patient data. As part of the AFIRM project, deployment of the injury tools will be extended to include the BAMC patient population and possibly others. IRB permission will be sought for this extension. While it is beyond the scope of the currently proposed project, this research provides a foundation for creation of a national CTA repository that would track these procedures, candidate transplant recipients, and a potential donor pool. SimQuest is an industrial research and development company whose core focus is the development of surgical simulators targeted at training. The patient-specific modeling and simulation efforts of the AFIRM will feed into this product line and will be offered commercially through a variety of channels.

Corrections/Changes Planned for Year 2

None.

IV Scarless Wound Healing

Background

Scar formation following injury is a major clinical problem for U.S. soldiers and civilians alike, leading to disabilities in form and function. The annual economic burden of this problem in the United States has been conservatively estimated at more than $4 billion. Attempts to address this problem have traditionally focused on the molecular components of wound healing in the hopes of identifying a single gene or transcription factor responsible for scar formation. To date these strategies have not been successful in significantly altering the fibroproliferative process (scar formation) in humans. A better understanding of the pathophysiology of scar formation points toward the need to control the wound environment and the responding cells that contribute to the synthesis and organization of the healed tissue.

Wound healing begins at the onset of injury and can continue for months, even years in some tissues. Many researchers have investigated scarless wound healing, and countless resources have been expended toward that end. For any approach to be successful, however, it must be truly integrated in its scope so as to encompass all phases of wound healing, beginning with initial injury and spanning well beyond putative remodeling phases. For scarless wound healing to become a reality, a comprehensive, multifaceted program has been developed. The AFIRM approach encompasses a continuum of technologies aimed at the various stages of wound healing. Collectively, they form a powerful tool designed to mitigate the normal tissue repair response and promote the underlying cellular machinery toward regenerative processes. These projects represent a coordinated effort to block every stage of scarring and address every phase of wound healing in a single research program, with the overarching aim of developing a new wound management paradigm.

Unmet Needs

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. The devastating consequences of scarring are perhaps the single biggest problem facing our injured warriors. However, the burden of scarring is also enormous for U.S. civilians as more than 50 million operative procedures and more than 3 million traumatic lacerations occur each year and all heal with scarring. The annual economic burden of this problem in the United States has been conservatively estimated at more than $4 billion. Thus, there is an enormous unmet clinical need to reduce scarring in soldiers and civilians.

Researchers are developing a variety of topical treatments for wound healing and scar reduction. However, it is difficult to maintain bioactivity of locally applied therapeutic agents because of problems with lack of retention of the agent in the wound, poor tissue penetration, and instability of protein therapeutics in the protein-degrading environment of the wound. Moreover, deep injuries and multiple sites of injury further limit the usefulness of local treatment. Hence, a systemic approach to tissue repair is an unmet need.

Current therapies for hypertrophic scarring are ineffective because of limited understanding into the pathogenesis of the underlying fibroproliferative process. Prior research has contributed fragments of insight into the disease process but has been largely descriptive because of the lack of an appropriate animal model. Animal models currently in use, such as the rabbit ear or red Duroc pig, are in species for which many molecular biology techniques are not available. While some insights into the pathogenesis of fibroproliferative disease have been gained, these models do not reproduce the factors thought to initiate the human disease. Therefore, development of an appropriate and effective animal model of hypertrophic scarring is another unmet need.

Areas of Emphasis

AFIRM researchers are pursuing a complementary mix of research projects focused on various aspects of scarless wound healing. Projects can be grouped into four "clinical challenge" topic areas: Control of Wound Environment Mechanics, Therapeutic Cell/Molecular/Gene 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.

Control of Wound Environment Mechanics

Studies at Wake Forest-Pittsburgh Consortium (WFPC)

Overview: Recently, the Gurtner laboratory developed the first rat model of hypertrophic scarring[1] based on increasing the skin stress of healing wounds. The researchers found that the skin's properties correlated with the amount of scarring following wounding. In Project 4.5.1, the researchers are examining the role of mechanical stress in scar formation. They are developing a novel device that can actively control wound environment mechanics to mitigate scarring and fibrosis (i.e., the formation or development of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process). The research team ultimately aims 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.

Status at End of Year 1: The researchers determined that the red Duroc pig is an ideal choice for biomechanical skin studies. They created computer-modeled wound simulations that allow for precise characterization of region-specific skin mechanics. They also developed the first generation of safe, durable, and modifiable pressure-sensitive adhesive (PSA) devices that can modify mechanical forces and subsequently alter the amount of scarring and fibrosis after an injury.

Research Plans for the Next 4 Years: The researchers of Project 4.5.1 plan to utilize their swine and human biomechanical skin data to further refine their first-generation PSA devices. They will continue to test the PSAs on incisional swine wounds and expand the range of wounds to excisional wounds of various sizes. They will also localize proteins in cells in swine skin samples through the technique

[1] *Hypertrophic scars are typically elevated, thick and fibrous, a shade of red or purple, stiffer than the surrounding skin, and limited to the original boundaries of the wound.*

Table IV-1. AFIRM-funded projects per clinical challenge topic area.

Clinical Challenge Topic Area	Consortium	Project Number	Project Title
Control of Wound Environment Mechanics	WFPC	4.5.1	A Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation
Therapeutic Cell/ Molecular/Gene Delivery to Wounds	WFPC	4.5.2	Regenerative Bandage for Battlefield Wounds
		4.5.5	Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies
	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
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 Regenerative Healing
Scar Mitigation	WFPC	4.5.6	Delivery of Therapeutic Compounds into Injured Tissues
		4.5.7	Scar Mitigation via Matrix Metalloproteinase-1 Therapy

known as immunohistochemistry to assess the recapitulation of a regenerative wound environment. They will tailor second-generation PSA devices to specific body regions.

Planned Clinical Transitions: In collaboration with Neodyne Biosciences, Inc., the researchers of Project 4.5.1 have begun a pilot clinical trial on 10 human volunteers undergoing elective surgical procedures. The researchers will continue to recruit patients for this trial and will maintain close follow-up with the initial group of volunteers.

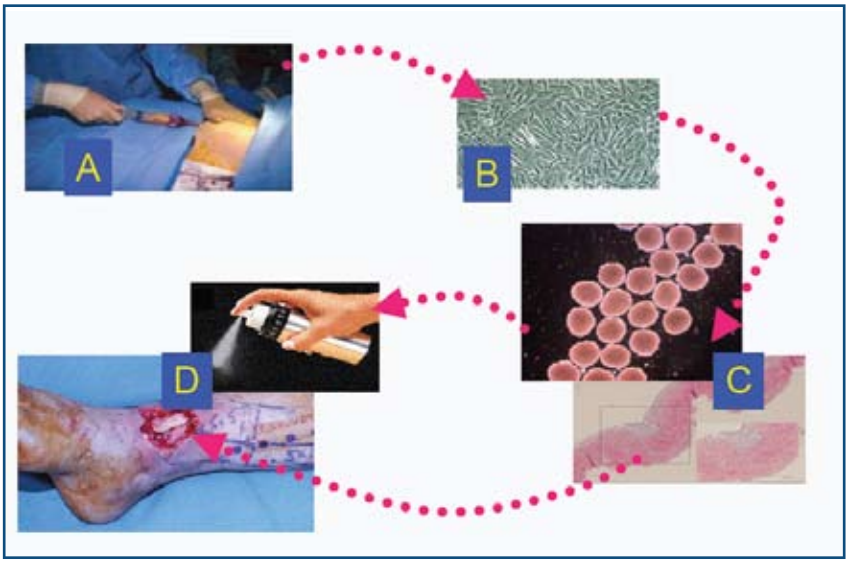
Therapeutic Cell/Molecular/ Gene Delivery to Wounds

Studies at WFPC

Overview: A novel approach is needed to minimize the development of inflammation and fibrosis in a wound in the initial days following injury while promoting the regeneration of tissue. The researchers of Project 4.5.2 are 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 human amniotic fluid-derived stem cells (AFSCs). The goal of this bandage is to maintain an acute wound in a pro-regenerative state and prevent the onset of scarring, fibrosis, and infection. The researchers of Project 4.5.5 have previously used a variety of techniques to identify candidate genes that are differentially expressed in healing fetal wounds. They ultimately aim to use these gene products to modulate the adult wound environment so as to abolish or mitigate scar formation in healing adult wounds. They are specifically evaluating the use of nanoparticles as a nonviral means of delivering molecules in animal wound models.

Status at End of Year 1: In Project 4.5.2, the researchers have developed modifiable hydrogel scaffolds that can be used to deliver wound-healing drugs. They utilized microprinting technology to create patterns of proteins important in wound healing on the hydrogel scaffolds. They have also seeded the bioscaffolds with AFSCs and fibroblasts. The researchers have shown that the bioscaffolds can be used to deliver wound-healing drugs. Initial experiments in mice reveal that the bioscaffolds integrate well with the host tissue (i.e., they maintain their architecture, do not incite a robust inflammatory response, and allow the incorporation of cells). In Project 4.5.5, the researchers have found a gene, CCT-eta, that is normally decreased in healing fetal wounds to be increased in healing adult wounds. They developed a nonviral nanoparticle-mediated delivery system that can be used to selectively decrease the expression of CCT-eta in a complex wound milieu.

Research Plans for the Next 4 Years: In Project 4.5.2, the researchers plan to further refine their hydrogel scaffold processing techniques and experiment



Adipose tissue-derived therapies for wound healing, tissue repair, and scar management (RCCC). Adipose (fat) tissue can be harvested using minimally invasive suction methods (A). Stem cells are then isolated and expanded in a culture dish (B). The fat-derived stem cells can be formed into discrete modular units (C) and/or seeded onto biocompatible scaffolds (C). The formulated therapies can be delivered by a variety of methods (e.g., spray, injection, or implantation) to open wounds (D) with the intent to assist and enhance wound healing.

with additional hydrogel materials. They will continue to develop methods to incorporate stem cells into their hydrogel scaffolds. They will develop more complex patterning on the scaffolds and test them with additional components of the extracellular matrix (ECM) (e.g., laminin). They plan to develop an intradermal (i.e., within the skin) scaffold implantation model, which may permit a better assessment of the scaffolds than the current subcutaneous (i.e., beneath the skin) implantation model. In Project 4.5.5, the researchers will continue to optimize their methodologies and will extend their analyses to a burn model. In later years, they will examine the effects on scar formation of additional gene products known to be differentially expressed in healing fetal wounds.

Planned Clinical Transitions: In Project 4.5.2, the research team anticipates the start of a clinical trial (with or without stem cells) by Year 4. The researchers of Project 4.5.5 aim to begin a clinical trial by the end of Year 5.

Studies at Rutgers-Cleveland Clinic Consortium (RCCC)

Overview: Burn injuries carry risks to survival, independence, and function, both in the short term and over time, as the injuries mature with scarring and contractures. In Project 4.6.3, RCCC researchers are establishing an animal model in which intravenous and cell therapies for burn healing will be tested, specifically using Peptide 12 (P12), curcumin, and mesenchymal stem cell (MSC) intravenous treatment to prevent cutaneous scarring. Project 4.7.1 aims to develop novel autologous therapies (i.e., donor and recipient are the same person) and products derived from adipose tissue for the promotion of wound healing and tissue repair and for the prevention and treatment of severe scarring. Adipose tissue in the human body is unmatched in abundance, ease of harvest, and expandability. The researchers are developing a therapeutic platform called Modular Niche Therapy™ (MNT™), which involves the formulation of self-assembled spheroid aggregates or "modules" that can function as "regenerative hubs" for tissue repair. MNT provides potential advantages related to delivery, manufacturing, shipping, inventory, and therapeutic efficacy.

Status at End of Year 1: In Project 4.6.3, the researchers completed the testing of crude curcumin in the rabbit ear hypertrophic scar model. They found that crude curcumin produced a modest, but significant, reduction of scarring and a significant improvement in wound healing in the model. They also determined that pure curcumin had similar effects to crude curcumin on wound healing at low and medium doses but had a pronounced effect on wound healing at a high dose. Research is under way to evaluate the effects of both human bone marrow-derived MSCs from the Caplan laboratory and rabbit adipocyte stem cells on rabbit ear wound healing and scar prevention. The research team of Project 4.7.1 formulated and cultured adipose-derived stem cells (ASCs) in three-dimensional multicellular aggregates and demonstrated the superiority of this approach over ASCs grown in flat cultures. They found that the ASCs generate a variety of factors pertinent to wound healing. In addition, they determined that ASCs can support the viability, morphology,

and growth of human skin cells and other wound-related cells and accelerate healing in preclinical animal models. They plan to begin a clinical trial entitled "Autologous Fat Transfer for Scar Prevention and Remodeling (AFT-SPAR)" early in Year 2.

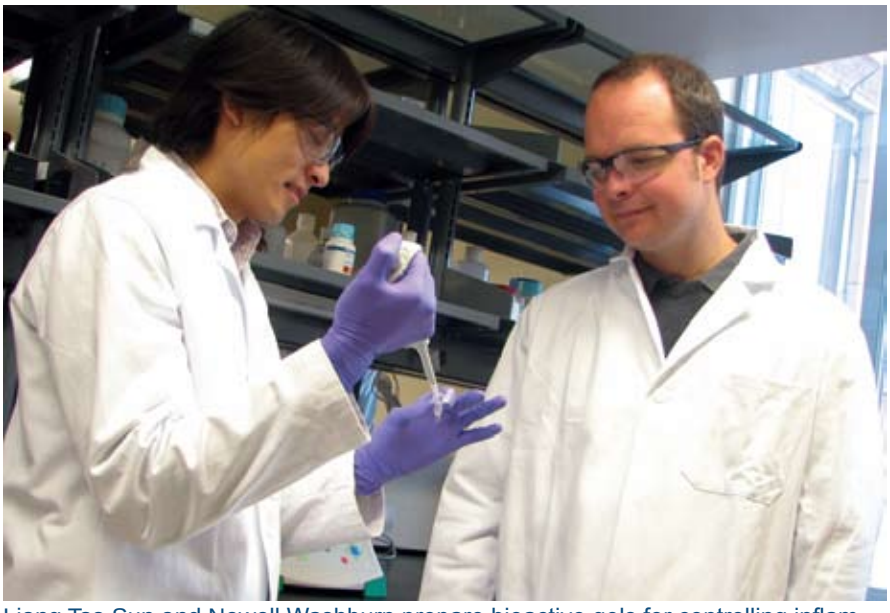
Research Plans for the Next 4 Years: The researchers of Project 4.6.3 plan to evaluate P12 as well as to further evaluate pure curcumin in their rabbit ear model. They will also examine the effects of human bone marrow-derived MSCs and rabbit ASCs on wound healing and scar prevention in the rabbit ear model. In addition, they plan to extend studies to the rabbit ear ischemic model and the rabbit ear ischemia/reperfusion model. The overall approach and early results of Project 4.7.1 are aligned with the development of several therapeutic products over the next 4 years. For example, the researchers plan to develop a method for injecting ASCs into wounds to alleviate soft tissue defects. They will also develop scaffolds seeded with ASCs and determine whether these scaffolds lead to an increased development of blood vessels and thereby result in expedited "graft take." They are also planning to develop an aerosolized delivery of MNT, which they hope will ultimately allow for the spray delivery of ASCs in multicellular aggregates to wounds.

Planned Clinical Transitions: The researchers of Project 4.7.1 have filed a patent portfolio related to ASCs and the MNT platform through the University of Virginia. The technology is being licensed to a start-up company (GID Group) founded by Dr. Katz and colleagues. Dr. Katz' team intends to pursue regulatory approval through the United States in conjunction with strategic partners and/or joint ventures. To this end, GID Group has completed a pre-Investigational New Drug (IND) meeting with the U.S. Food and Drug Administration (FDA) with regard to the use of autologous ASCs for cutaneous wound healing. Tumorigenic and toxicology studies are needed prior to the preparation of a formal IND application and initiation of clinical trials.

Attenuation of Wound Inflammatory Response

Studies at WFPC

Overview: Following injury, intense inflammatory responses initiate an integrated process of wound healing that terminates in the formation of scar tissue. Redirecting this process toward a regenerative outcome requires controlling the inflammatory response, and therapies that function locally would provide the basis for new approaches to improving outcomes following burn, trauma, or other injury where native repair responses result in the formation of scar tissue. In Project 4.5.3, the research team is developing gels that contain monoclonal antibodies or peptides (short proteins) with specific affinities for cytokines and other mediators of inflammation. By decreasing levels of cytokines that lead to inflammation and increasing levels of cytokines that prevent inflammation, the researchers hope to reduce pathological inflammatory processes at the wound site, thereby increasing the likelihood of scarless wound healing. In Project 4.5.4, the researchers are studying two related processes highly relevant to scar formation: inflammation and fibroblast activity. The researchers believe that control of these two processes will serve to establish an optimal foundation for therapies and interventions leading to regenerative healing.

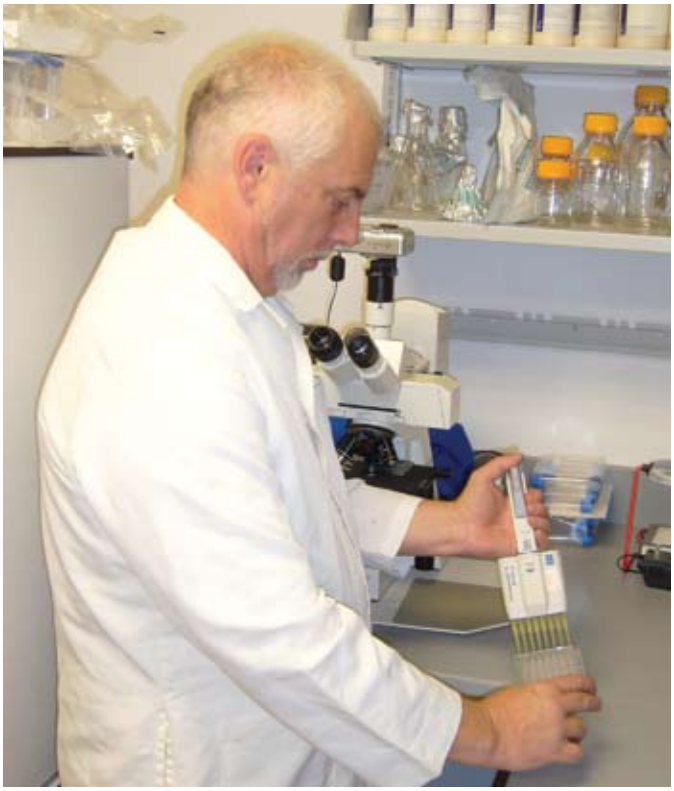


Liang Tso Sun and Newell Washburn prepare bioactive gels for controlling inflammatory responses (WFPC).

Status at End of Year 1: In Project 4.3.5, the researchers created gels coupled to antibodies against the pro-inflammatory cytokines TNF-α (tumor necrosis factor) and IL-1β (interleukin). They found that gels coupled to TNF-α alone could reduce inflammation at the wound site. However, the maximum reduction of an inflammatory response occurred when both TNF-α and IL-1β were coupled to a gel. In Project 4.3.4, the researchers have identified topical treatments that promote healing while reducing the amount of scarring in skin incisional wounds in the rat. They have shown that the treated wounds have greater tensile strength and lesser amounts of collagen, which is a principal component of scar tissue.

Research Plans for the Next 4 Years: The researchers of Project 4.5.3 plan to test their inflammation-attenuating gels in a rat burn model of scar formation during Years 2–3. They aim to design formulations that reduce inflammation, tissue death, and scar tissue formation. In Year 3, they will examine the consequences of their inflammation-attenuating gels on bacterial colonization at the burn site. In Year 4, they will transition to a large animal model. In Project 4.5.4, the researchers will complete analyses of the topical treatments and their effects on healing using biochemical assays for inflammatory markers and collagen. They will select the most optimal treatment regimen. By Years 4 and 5 they will design interventions geared toward the development of a wound treatment regimen for a future clinical trial.

Planned Clinical Transitions: In Project 4.5.3, the researchers note that meeting their milestones for Years 2–4 would position their technology for potential clinical trials. They have applied for patents related to their technology, and a spin-off company has licensed the technology and is preparing to manufacture the bioscaffolds. The researchers of Project 4.5.4 have selected therapeutic agents that have the advantage of already being approved for use in humans. This should facilitate the pathway toward a Phase 1 clinical trial. They will first test their treatments in an animal model. They hope to identify a treatment that can be advanced to a clinical trial. The researchers anticipate the clinical trial commencing after Year 5 of the project.



Dr. Koepsel prepares an enzyme assay for MMP-1 0223 (WFPC).

Scar Mitigation

Studies at WFPC

Overview: Transforming growth factor-β1 (TGF-β1) is a major factor responsible for wound repair, but its activity also results in scar formation and fibrosis. The researchers of Project 4.5.6 are developing the methodology to alter the protein decorin by adding a peptide sequence that causes the resultant decorin fusion protein to specifically home to injured tissue, inhibit excessive TGF-β1 activity, and aid in wound healing and scar reduction. Matrix metalloproteinases (MMP-1) have been implicated to play a role in the healing of muscle scars. It has been shown that injection of MMP-1 directly into a muscle scar can improve muscle regeneration by breaking down the collagen fibrils within the scar tissue. The researchers of Project 4.5.7 are examining the interaction between MMP-1 and collagen in an in vitro model of scarring, followed by in vivo studies in rats and dogs.

Status at End of Year 1: The researchers of Project 4.5.6 produced and purified recombinant

decorin fusion protein and achieved targeted delivery of the fusion protein into regenerating tissue following intravenous injection in mice. They also demonstrated that the decorin fusion protein reduced TGF-β1 activity and inhibited scar formation in mice during wound healing. The researchers of Project 4.5.7 developed the methodology to produce highly purified MMP-1 for use in preclinical trials. They also developed a method to watch the activity of MMP-1 on a collagen surface in real time.

Research Plans for the Next 4 Years: The researchers of Project 4.5.6 plan to identify and develop peptides (chains of amino acids, the building blocks of proteins) that home to regenerating tissues, penetrate into tissue, and make blood vessels permeable and able to receive therapeutic agents. They have dubbed the scheme the "CendR pathway," and they intend to describe its benefits for regenerative medicine. In Project 4.5.7, the researchers are planning numerous preclinical studies (dose range, efficacy, etc.) on MMP-1 in rats over the next 4 years. Based on the rat studies, a dog trial will assess the effectiveness of MMP-1 therapy in a large animal model and will further test recovery of function.

Planned Clinical Transitions: In Project 4.5.6, the researchers plan to move their decorin fusion protein into clinical trials within the next 4 years. They will seek venture capital funding or sell the rights to the fusion protein to a commercial party to move it into clinical trials as soon as possible. In Project 4.5.7, the researchers aim to transition their project into a clinical trial by the end of Year 5. The timing of when the clinical trial will actually begin will depend on the data accumulated over the next 2–3 years.

Project 4.5.1, WFPC

A Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation

Team Leader(s): *Geoffrey C. Gurtner, MD, Michael T. Longaker, MD, PhD, Reinhold Dauskardt, PhD (Stanford University)*

Collaborator(s): *Neodyne Biosciences, Inc.*

Therapy: *Control of wound environment to minimize scarring*

Deliverable(s): *Battlefield-ready, region-specific devices capable of stress-shielding mechanical forces to minimize scar formation*

Key Accomplishment(s): *The researchers determined that the red Duroc pig is an ideal choice for biomechanical skin studies. They developed finite element methods (FEM) to model wound stresses, which allow for precise characterization of region-specific skin mechanics. They also developed the first generation of safe, durable, and modifiable PSA devices that could modify mechanical forces and subsequently alter scarring and fibrosis after injury.*

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. The researchers found that intrinsic skin mechanics correlated with the scarring phenotype following wounding, as low mechanical stress fetal wounds exhibited minimal fibrosis and stiffer human skin displayed robust scarring. These findings prompted them 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. Ultimately, they 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.

Research Progress – Year 1

This first year of research has yielded promising and exciting new findings highly relevant to developing stress-shielding devices.

Aim 1: *To understand the fundamental mechanical properties of unwounded and wounded swine skin in various mechanical stress environments.*

The researchers subjected swine skin to biomechanical studies and found: (1) swine resting strain was between 2.4% and 6%, (2) Young's modulus (ratio of stress over strain) ranged from 5 to 10 MPa, and (3) swine resting stress is between 0.12 and 0.6 MPa. These data correlate with adult human resting stress levels, which are known to be approximately 0.16 MPa. The red Duroc pig is known to be an excellent animal model for the study of hypertrophic scarring based on histologic characteristics. The researchers now show that the Duroc pig is also an ideal choice for biomechanical skin studies. They demonstrated that there is a direct correlation between strain and stress at physiological strain levels in swine skin (**Figure IV-1**).

As shown in **Figure IV-2**, the researchers also developed FEM to create computer-modeled wound simulation. The FEM calculations allow for precise characterization of region-specific skin mechanics.

Aim 2: *To minimize scarring by creating a regenerative stress state in healing swine wounds.*

The researchers developed a polymeric PSA device capable of biaxial stress-shielding of wounds, the properties of which can be

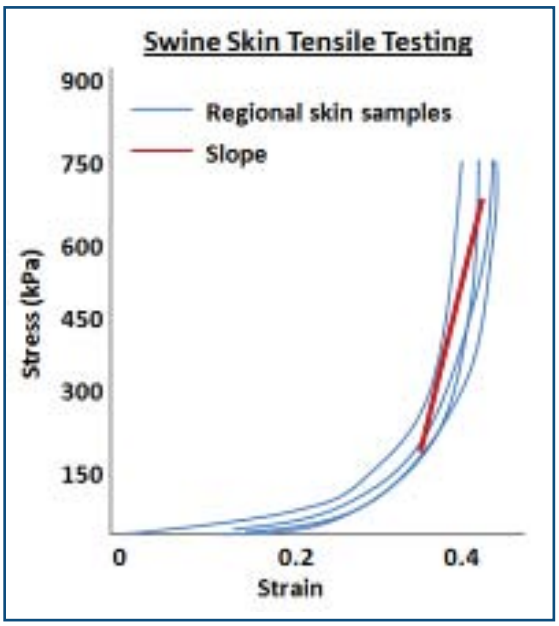


Figure IV-1. Tensile testing of samples demonstrates linear elastic properties with a direct correlation of strain and stress at physiological strain levels.

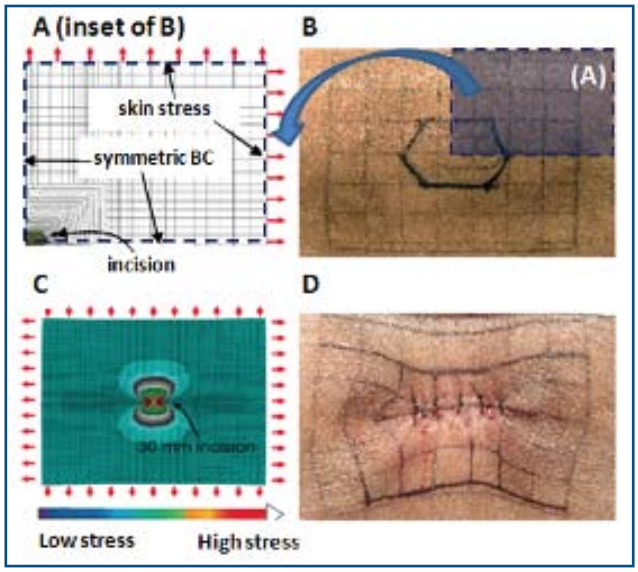


Figure IV-2. FEM calculations used to estimate the stresses surrounding incisions (A) designed to elevate skin stresses. Incision shape (B) and the elevated principal stresses surrounding the wound (C). Gross image of wound immediately after excision and closure (D). BC = boundary conditions.

maintained for over a week without adverse complications to adjacent skin. Polymer properties, such as thickness, size, and elasticity, can be modified based on wound characteristics. The researchers have shown a protective "shielding" effect with the first generation of PSAs on incisional wounds. They observed that incisions under compression (i.e., stress-shielded) displayed minimal scarring on gross and histologic exam compared to incisional wounds under either natural or induced tension (**Figure IV-3**). The shielded wounds also showed minimal dermal fibrosis on collagen fiber examination under polarizing light (Figure IV-3A3), which is in contrast with incisional wounds under induced tension (Figure IV-3C3). Natural tension wounds were intermediate in scarring phenotype (Figure IV3-B1-B4). This gradient of intrinsic skin stresses correlated positively with resultant wound fibrosis and provides strong evidence that modulation of mechanical wound environment can affect fibrosis.

Aim 3: *To bring the device to clinical trials and field use.*

Aim 3 utilizes the skin mechanics data obtained in Aims 1 and 2, which showed that human and swine skin biomechanical properties correlate well. First-generation polymeric PSA devices have been refined on swine and translated to human clinical devices. These devices are durable, resilient, and transportable, and studies in Aim 2 show that they are readily modifiable to adjust to different wound regions of the body. The researchers' Institutional Review Board has been fully approved, and initial (pilot) clinical trials have begun on human volunteers (N=10) undergoing elective surgical procedures. This study is being performed by Neodyne Biosciences, Inc., which is a start-up company spun out of Stanford University that has licensed the technology. Preliminary results on the first 10 patients are promising.

Key Research Accomplishments

- Measured the mechanical properties of swine skin in various regions and correlated these data with unwounded human skin.
  - Determined that intrinsic stress levels were similar between swine and humans, thus validating the choice of animal model.
- Developed FEM to model wound stresses, which allows for precise characterization of region-specific skin mechanics.

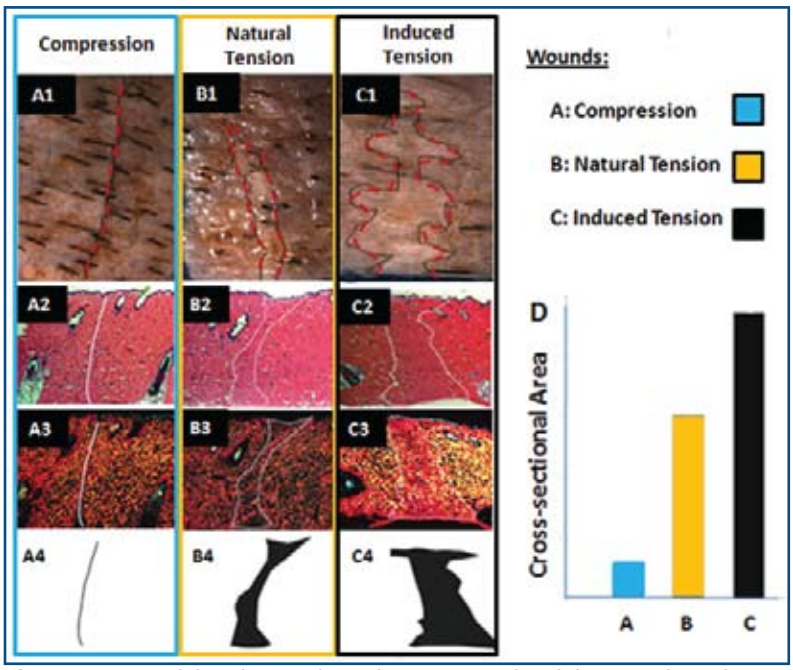


Figure IV-3. Incisional wounds under compression (A), natural tension (B, no PSA), and induced tension (C). Gross images show minimal scarring with wound compression (A1), moderate scarring with natural tension (B1), and excessive scarring with induced tension (C1). This is also seen on cross-sectional imaging with hematoxylin and eosin (A2, B2, C2) and polarized light (A3, B3, C3). Scar areas demonstrate nearly 10-fold differences between wounds under compression and tension (D).

- Created first-generation PSA devices that are safe and maintain continuous surface contact in swine and humans.
  - Determined that PSA devices could be used on incisional swine wounds to effectively and reliably regulate wound stress and fibrosis following injury.
- Demonstrated that stress shielding wounds reduce scar formation while the induction of tension increases scar formation (observed on both gross exam and histology).

Conclusions

During the first year of this study, the researchers accomplished a variety of tasks that will allow the project to advance. To their knowledge, they are the first to demonstrate that regional variations in stress and strain in red Duroc swine and human skin are similar. They have developed a robust computer model to predict wound behavior based on their unwounded swine and human skin data and have shown that it accurately predicts scarring phenotype. Additionally, they have developed novel polymeric PSA devices that can effectively modify wound mechanical stress in swine incisions by modulating the level of fibrosis. Future experiments will utilize wounds of different types (incisional versus excisional), sizes, and locations. Furthermore, the researchers have translated these promising results into initial pilot studies in human volunteers undergoing reconstructive surgical procedures. Overall, this innovative approach to wound fibrosis has yielded both promising insight into wound repair and novel therapeutics to address this significant biomedical burden.

Research Plans for the Next 4 Years

The researchers plan to utilize the biomechanical skin data obtained from swine and human to further refine their first-generation PSAs. They will continue to test devices on incisional swine wounds and expand the range of wounds to excisional wounds of various sizes. They will also perform immunohistochemical (IHC) testing of swine skin samples to assess the recapitulation of a regenerative wound environment. Second-generation PSA devices will be tailored to specific body regions.

Planned Clinical Transitions

The researchers' preliminary data demonstrate that this novel mechanical approach to wound repair is effective in swine and has significant potential for modulating wound repair and scar formation in humans. Initial (pilot) clinical trials have begun on 10 human volunteers undergoing elective surgical procedures. This study is being performed by Neodyne Biosciences, Inc. The researchers will continue to recruit patients in the upcoming year and will maintain close follow-up with the initial group of volunteers.

Corrections/Changes Planned for Year 2

None.

Project 4.5.2, WFPC

Regenerative Bandage for Battlefield Wounds

Team Leader(s): *Geoffrey C. Gurtner, MD, Michael T. Longaker, MD, Anthony Oro, PhD (Stanford University)*

Therapy: *Improved wound healing and reduced scarring*

Deliverable(s): *Regenerative bandage that promotes fetal-like wound healing instead of scarring*

Key Accomplishment(s): *The researchers have (1) developed modifiable, drug-delivering hydrogel scaffolds, (2) patterned fibronectin on hydrogel scaffolds using microprinting, (3) seeded stem cells into the bioscaffolds and demonstrated biocompatibility, and (4) initiated experiments in mice, which reveal that the hydrogel scaffolds are biocompatible, maintain their architecture, do not incite a robust inflammatory response, and allow cellular incorporation.*

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 AFSCs 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 back in the United States.

The researchers aim to develop absorptive dressings with matrix composition resembling fetal microarchitecture (Aim 1), test the regenerative potential of AFSCs and matrix separately (Aim 2), incorporate AFSCs into a biomimetic hydrogel to create a regenerative dressing for field use (Aim 3), and maximize wound healing and skin regeneration.

Research Progress – Year 1

Aim 1: *To design hygroscopic dressings mimicking fetal micropatterning.*

Differences between scarring and scarless wound healing may be partially explained by ECM differences in collagen architecture between fetal and adult dermis. Fetal dermal microarchitecture provides essential structural and chemical signals required for regeneration and thus plays a pivotal role in directing cells toward a regenerative pathway. The researchers established pullulan as a biocompatible and biodegradable hydrogel replacement of hyaluronic acid (HA). They then fabricated and micropatterned hydrogel matrices, using microfiber substrates pullulan and collagen, to mimic the dermal architecture of embryonic skin (**Figure IV-4**). They optimized pullulan-collagen hydrogel scaffold pore size and cross-linking chemistry (**Figure IV-5**). This architecture is incorporated in their regenerative bandage and will promote the initiation of a regenerative healing response.

The researchers also developed microprinting and polydimethylsiloxane stamping for linking fibronectin domains on the hydrogel scaffold to create a structural matrix replicating native fetal-like dermal architecture (**Figure IV-6**). In addition, they covalently linked small molecules (e.g., deferoxamine) on pullulan for controlled release in vivo and site-specific

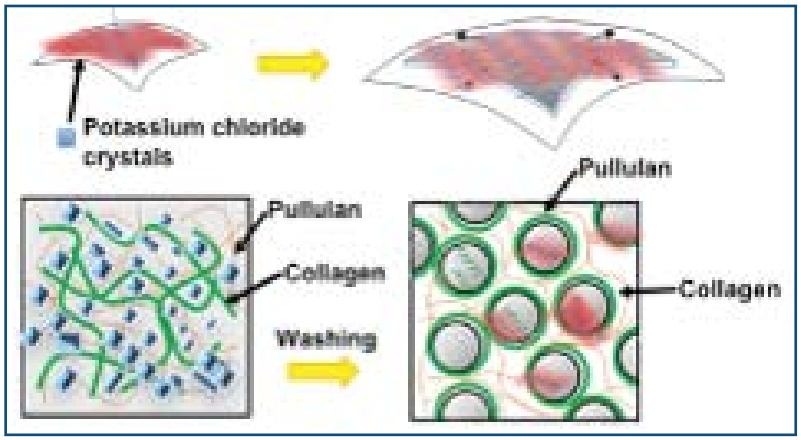


Figure IV-4. Induction of scaffold pores and porosity by in-gel crystallization with potassium chloride (KCl) salt followed by dissolution.

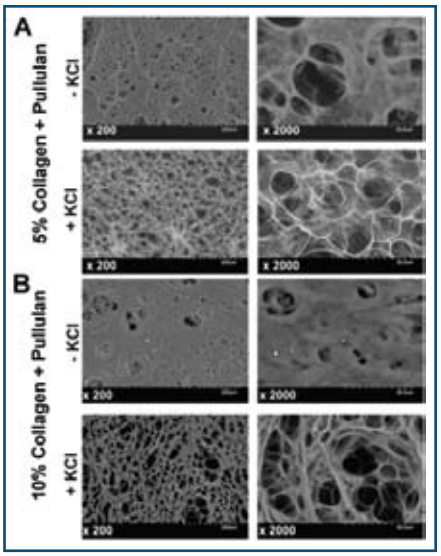


Figure IV-5. Pullulan-collagen hydrogel scaffold pore formation: scanning electron microscopy of (A) 5% collagen and pullulan with the addition of KCl salt demonstrated an ideal pore size (50–100 microns) compared to (B) 10% collagen and pullulan with the addition of KCl salt.

increase of the pro-neovascularization transcription factor hypoxia inducible-factor-1α.

Aim 2: *To determine the ability of AFSCs to maintain wounds in suspended animation.*

There is a critical window during initial phases of wound healing (inflammatory and proliferative) when the foundations for fibrosis and contraction are laid so that the body can rapidly repair itself. Since wound repair elements like stem cells cannot rapidly access the entirety of a large wound, the body instead quickly contracts and lays down collagen to seal the wound. On swelling of the hydrogel in an aqueous medium, the research team found that dispersed collagen forms micro-reticular domains similar to fetal matrix. The researchers believe the placement of a hydrogel bridge with a fetal-like matrix will allow stem cells to directly deploy to the wound environment, obviating the need for delayed cellular migration. Thus, the body will not need to lay down scar tissue but instead can utilize the dermal bridge and applied AFSCs to maintain a regenerative phase.

As an initial approach, the researchers have placed murine bone marrow-derived MSCs directly on hydrogel scaffolds in vitro to determine their ability to incorporate within them. They will transition to AFSCs after these preliminary studies since they determined that the pullulan-collagen hydrogel scaffolds induced tubule formation when co-cultured with endothelial cells and ASCs. They will determine the ability of AFSCs to limit scarring and maintain wounds in suspended animation. Initial experiments in mice show that the pullulan-collagen hydrogels are biocompatible, maintain their architecture, do not incite a robust inflammatory response, and allow cellular incorporation.

Aim 3: *To utilize federally funded AFSCs to determine their regenerative capacity in wounds delivered via micropatterned dressings.*

Federally funded, approved AFSCs will be utilized to promote tissue regeneration in combination with various matrices. The combination of AFSCs and numerous agents (e.g., protein engineered epidermal growth factor) and antimicrobi-

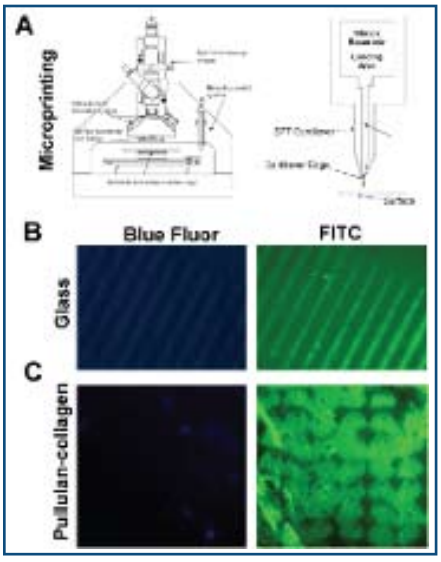


Figure IV-6. Directional microprinting. (A) Microprinting device schematic. (B) Linear arrays of microprinted FITC (fluorescein isothiocyanate)-labeled fibronectin on glass. (C) Microprinting array of FITC-labeled fibronectin domains directly onto collagen-pullulan hydrogel scaffold.

als will make these dressings an advanced version of current FDA-approved biological dressings (e.g., Integra, Alloderm, and Apligraf).

Based on data from Aims 1 and 2, the researchers will place matrices seeded with AFSCs on wounds of various depths to determine their regenerative capacity. Currently, they are in the process of placing green fluorescent protein (GFP)-labeled MSCs into matrices and incorporating them into their previously published excisional wound-healing model and their developing incisional murine model. The researchers will determine the dressing's ability to aid in the regeneration of wounds and will translate these results into AFSC-based therapies for wound regeneration.

Key Research Accomplishments

- Developed porous, modifiable pullulan-collagen hydrogel scaffolds.
  - Determined that micro-domain pore sizes can be modulated to mimic those found in fetal collagen patterning.
- Utilized microprinting technology to pattern fibronectin on the pullulan-collagen hydrogel scaffolds.
- Modified pullulan hydrogels to deliver small molecules (deferoxamine) into murine wounds.
  - Observed that the molecules maintain their efficacy and are effectively released by the dressing biomaterial.
- Seeded MSCs and fibroblasts into the bioscaffolds and demonstrated excellent cellular biocompatibility.
- Determined through preliminary in vivo studies in mice that the pullulan-collagen hydrogels are biocompatible, maintain their architecture, do not incite a robust inflammatory response, and allow cellular incorporation.

Conclusions

By developing a biocompatible hydrogel matrix capable of supporting stem cell activity and micropatterning molecules known to regulate cellular proliferation, researchers in the Gurtner laboratory have completed the initial steps to recapitulation of the fetal microenvironment. Utilizing their matrix, they aim to refine micropatterning techniques and test ligands involved in the regenerative process. The addition of stem cells to their scaffold will be important in initiating and maintaining a regenerative wound environment. The ultimate aim is to utilize this stem cell-impregnated hydrogel to create a regenerative bandage that promotes fetal-like wound healing instead of scarring. By treating soldiers immediately post-injury with the acute regenerative bandage (consisting of a fetal biomimetic matrix and human AFSCs), they hope to hold an acute wound in a pro-regenerative state of "suspended animation" until definitive therapy is provided in the United States.

Research Plans for the Next 4 Years

In Years 2–5, the researchers plan to further refine their hydrogel processing techniques and experiment with other hydrogel materials to assess cellular incorporation. They will test more complex patterning with matrix components such as laminin and RGD (arginine-glycine-aspartic acid) peptides. They will characterize matrix components in murine fetal skin and attempt to recapitulate this environment in hydrogels.

The researchers will continue to develop methods to incorporate stem cells into their hydrogels. They will also develop an intradermal scaffold implantation model that may permit better assessment of scaffolds compared to the subcutaneous implantation model. These aims will allow the fabrication of a regenerative bandage based on fetal matrix components and patterning to deliver stem cells for optimal wound repair.

Planned Clinical Transitions

The research team could be ready (with or without stem cells) to initiate a clinical trial by Year 4.

Corrections/Changes Planned for Year 2

None.

Project 4.5.5, WFPC

Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies

Team Leader(s): *Sandeep Kathju, MD (Allegheny Singer Research Institution)*

Therapy: *Reduction of scar formation after injury*

Deliverable(s): *Nanoparticle-mediated gene delivery to wounds*

Key Accomplishment(s): *The researchers determined that CCT-eta (eta subunit of the chaperonin-containing T-complex polypeptide) is increased in healing adult wounds, which is in contrast to levels of CCT-eta in healing fetal wounds. They demonstrated the use of a nonviral nanoparticle-mediated delivery system to selectively decrease gene expression in a complex in vivo wound milieu. They designed and validated a siRNA-expressing plasmid vector that will serve as a potent tool for further experimental manipulation of CCT-eta in wounds in conjunction with their delivery systems.*

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, and entrapment of nerves. In addition, psychosocial damage is associated with severe facial disfigurement. Burn injuries are particularly prone to extensive and crippling hypertrophic scarring.

Researchers in Dr. Kathju's laboratory have investigated mammalian fetal wound healing as a model of scarless healing after integumentary injury. Until the beginning of the third trimester, mammalian fetuses heal their injuries regeneratively, without attendant scar deposition. This property, intrinsic to fetal tissues and not simply the result of the protected uterine environment, must necessarily derive from differential gene utilization in the healing fetal wound milieu versus the scirrhous adult wound milieu. The researchers have previously used differential display, polymerase chain reaction suppression subtraction hybridization, and microarray analysis to identify multiple candidate genes that are differentially expressed in healing fetal wounds. They ultimately aim to use these gene products to modulate the adult wound environment so as to abolish or mitigate scar formation in healing adult wounds.

The researchers have chosen for initial testing candidate gene CCT-eta, found to be specifically reduced in healing fetal wounds. They are evaluating technologies that would allow for efficient transfection of their candidate molecular agents into healing skin wounds. In particular, they are evaluating nanoparticle-complexed formulations as a nonviral means of molecular delivery in animal wound models.

Research Progress – Year 1

The researchers have verified that, in contrast to healing fetal wounds, CCT-eta is actually increased in healing adult integumentary wounds, highlighting its attractiveness as a candidate gene responsible for the distinct phenotypes of these two processes. They also established an in situ hybridization protocol in the laboratory that has identified multiple cell types (including fibroblasts) that upregulate CCT-eta locoregionally in response to wounding in an adult organism (**Figure IV-7**). Additionally, they established an IHC protocol that confirms at a protein level the findings of these in situ hybridization studies with CCT-eta (**Figure IV-8**). Both the in situ hybridization and IHC protocols will also become more applicable for the examination of the effects of other candidate genes downstream in this investigation.

The researchers have made initial evaluations of several systems for the molecular/gene delivery to a healing wound milieu. They determined that nanoparticulate

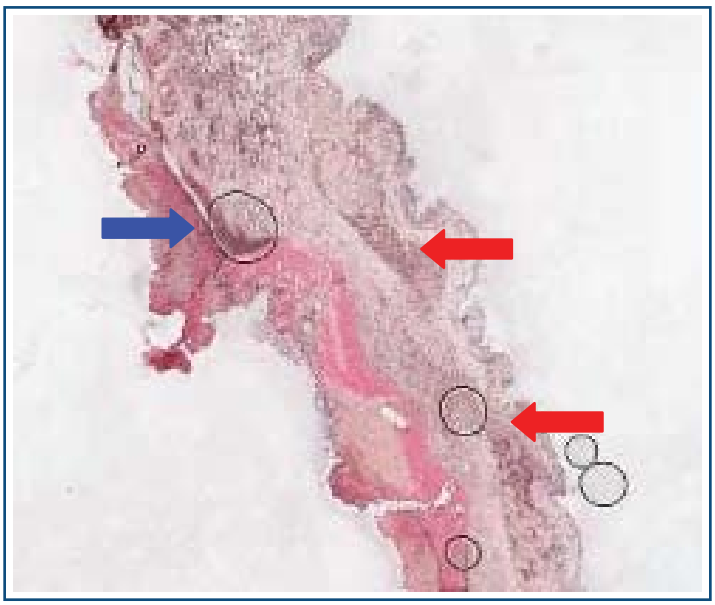


Figure IV-7. In situ hybridization for CCT-eta in an adult wound. The dark brown staining represents accumulation of CCT-eta message. Note the intense staining in the panniculus carnosus immediately adjacent to the zone of wounding (red arrows) and in the leading edge of the tongue of migrating epithelium beneath the eschar/coagulum.

administration of molecular agents using atelocollagen and agarose matrix are feasible and nontoxic to the surrounding tissues. In at least one system (complexes in agarose matrix), they demonstrably reduced the amount of CCT-eta expressed in a complex wound milieu. The researchers also designed and constructed a plasmid vector capable of expressing a siRNA against CCT-eta, enabling for the first time the ability to downregulate CCT-eta using DNA rather than RNA reagents, which entails multiple advantages. Last, they have demonstrated in a fibroblast cell line that their plasmid effectively reduces the expression of CCT-eta at the protein level, reinforcing its potential utility as an interventional agent in vivo.

Key Research Accomplishments

- Established in situ hybridization and IHC protocols for determining the levels of CCT-eta genes and proteins, respectively, in adult wounds.
- Determined that CCT-eta is increased in healing adult integumentary wounds, which is in contrast to levels of CCT-eta in healing fetal wounds.
- Identified multiple cell types that upregulate CCT-eta in response to wounding in an adult organism.
- Determined that multiple nanoparticulate carriers of molecular constructs, including atelocollagen and agarose, can be used to modulate gene expression in a wound milieu.
- Designed and validated a siRNA-expressing plasmid vector that can be used to manipulate CCT-eta in wounds.

Conclusions

This work represents significant progress in applying the lessons learned from scarless healing fetal wound biology to scirrhous adult wound healing. Using a single differentially expressed gene product, CCT-eta, as a model molecule, the researchers have for the first time been able to use a nonviral nanoparticle-mediated delivery system

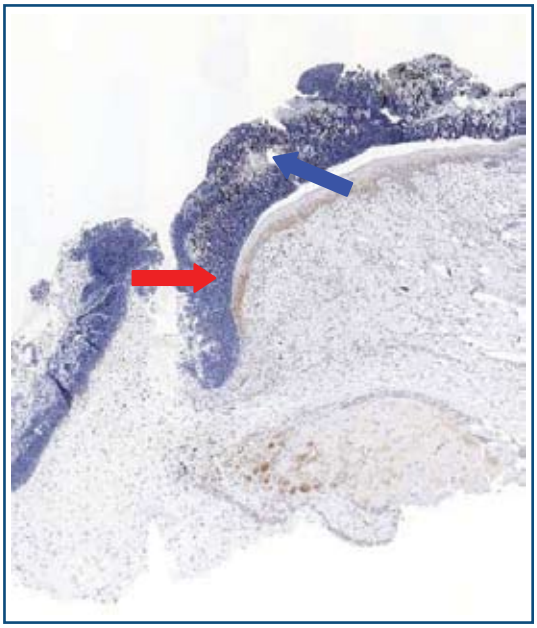


Figure IV-8. IHC demonstration of CCT-eta expression in a healing, full-thickness, integumentary wound. As with in situ hybridization, note the dense staining in the migrating tongue of epithelium (blue arrow), in the cells of the panniculus carnosus bordering the injury zone (red arrow), as well as staining of individual fibroblasts and inflammatory cells.

to selectively decrease gene expression in a complex in vivo wound milieu. Further studies will better define the experimental parameters and dosages needed to optimize these effects and will allow for the evaluation of other fetal wound-specific gene products in vivo.

In parallel work, the researchers have designed and validated an siRNA-expressing plasmid vector that will serve as a potent tool for further experimental manipulation of CCT-eta in wounds in conjunction with their delivery systems. This DNA vehicle will also allow the examination of even more promising nonviral delivery techniques including the nanostructured calcium phosphate particles that are being evaluated in another AFIRM project. In addition, although the researchers' initial studies have focused on integumentary wounds, their methodologies can be applied to animal models for tendon and burn injury.

Research Plans for the Next 4 Years

In Year 2, the researchers will continue to evaluate different parameters for siRNA application into healing wounds using both agarose and atelocollagen to try to determine the optimal and most efficacious experimental scheme. They will also extend this analysis to a burn model in addition to the incisional/excisional wound model they have thus far investigated. In later years, in addition to looking at the effects of agents against CCT-eta, they will examine the effects on scar of other gene products known to be differentially expressed in healing fetal wounds.

Planned Clinical Transitions

The researchers aim to transition this project into a clinical trial by the end of Year 5.

Corrections/Changes Planned for Year 2

The Kathju laboratory plans to introduce two significant new components into the project in Year 2. First, the Allegheny Singer Research Institution has recently acquired equipment that allows the delivery of ultrasonic energy to small animals. An institute collaborator on other projects has used this equipment in an animal model with some preliminary success. The researchers hope to apply this modality to wound healing as it is noninvasive and holds the promise of significantly increasing their ability to manipulate the healing wound microenvironment. Second, there is a growing body of evidence that, in the case of burn wounds, chronic infection may play a major role in the scar burden that forms (as well as contribute to more acute and serious complications, including sepsis and death). The bacteria in these chronic infections likely persist in biofilm configuration. The researchers have expertise in the study of bacterial biofilms, and they will evaluate a novel antibiofilm therapy in a burn model to determine its effect on wound healing and scar formation.

Project 4.6.3, RCCC

TLI and Promote Non-Scar Healing After Burns and Severe Battle Trauma: P12, Curcumin, and MSC Intravenous Treatment to Prevent Cutaneous Scarring

Team Leader(s): *Thomas Mustoe, MD (Northwestern University)*

Project Team: *Seok Jong Hong, MD, Sheng-Xian Jia, MD, PhD, Jennifer Nunez, MD, PhD, Yanan Zhao, MD (Northwestern University)*

Collaborator(s): *Richard Clark, MD (Stony Brook University)*

Therapy: *Enhance healing and attenuate scarring following burn injury*

Deliverable(s): *IV treatment with curcumin, P12, and MSCs*

Key Accomplishment(s): *During Year 1, initial experiments with crude curcumin demonstrated a modest, but significant, reduction of scarring in the rabbit ear model at a calculated tissue dose. In addition, the project team has started to evaluate the effects of both human bone marrow-derived MSCs and rabbit ASCs on rabbit ear wound healing and scar prevention.*

Introduction

The laboratory is investigating the different wound healing capabilities and the potential for scar-free healing of three agents with proven anti-inflammatory, anti-apoptotic, and/or granulation tissue proliferative properties: MSCs, curcumin, and P12. These agents are being tested within the rabbit ear hypertrophic scar model with the ultimate goal of identifying novel treatment modalities that can be efficiently translated into clinical practice to promote non-scar healing and wound regeneration.

Research Progress – Year 1

Aim 1: *Promotion of wound healing and prevention of hypertrophic scar using topical and systemic administration of chemical agents within the rabbit ear hypertrophic scar model, curcumin, and P12.*

In Year 1, the researchers completed testing on curcumin. Initial experiments with crude curcumin demonstrated a modest, but significant, reduction of scarring in the rabbit ear model, developed in the Mustoe laboratory, at a calculated tissue dose of 0.2 M. Higher doses (1 and 2 M) had no effect. In contrast, wound healing was significantly improved by both 0.2 and 1 M doses. Using pure curcumin, wound healing was improved similarly at the low and medium doses, but the higher curcumin dose (2 M) demonstrated an even more striking effect. The results of experiments to determine the effect of pure curcumin on rabbit ear scarring are pending and will be continued into Year 2. Pending the availability of P12, tests on this agent will commence presumably by the fall of 2009.

Aim 2: *Promotion of wound healing and prevention of hypertrophic scarring using topically and systemically administered MSCs.*

Research is under way to evaluate the effects of both human bone marrow-derived MSCs from the Caplan laboratory and rabbit ASCs on rabbit ear wound healing and scar prevention (**Figure IV-9**).

Key Research Accomplishments

- Determined that low doses of crude curcumin produced a modest, but significant, reduction of scarring in the rabbit ear model.
- Demonstrated that low and medium doses of crude curcumin led to a significant improvement in wound healing in the rabbit ear model.
- Determined that pure curcumin produced wound healing in the rabbit ear model in a dose-dependent manner.

Conclusions

Curcumin has proven effective in reducing scarring and improving wound healing within the rabbit ear hypertrophic scar model. Curcumin formulation and delivery are still being optimized within animal

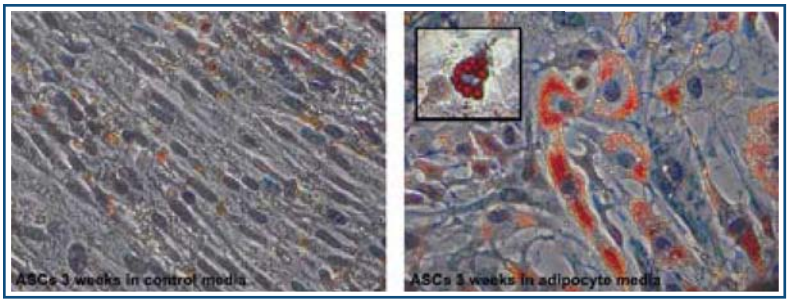


Figure IV-9. ASCs incubated with adipocyte differentiation media versus control media. Lipids are stained with Oil Red O. ASCs show considerable lipid development and organization compared to controls suggesting that ASCs isolated from rabbits are multipotent.

models. The experimental work with human bone marrow-derived MSCs has just begun; the research team hopes to determine whether human bone marrow-derived MSCs promote wound healing within the rabbit ear hypertrophic scar model, as has been found in other animal models.

Research Plans for the Next 4 Years

The researchers of Project 4.6.3 plan to evaluate P12 as well as to further evaluate pure curcumin and MSCs in their rabbit ear model. In addition, they plan to extend studies to the rabbit ear ischemic model and the rabbit ear ischemia/reperfusion model.

Planned Clinical Transitions

Not noted in the progress report.

Corrections/Changes Planned for Year 2

None.

Project 4.7.1, RCCC

Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management

Team Leader(s): *Adam J. Katz, MD (University of Virginia)*

Project Team: *Ning Yang, PhD (University of Virginia)*

Collaborator(s): *Bud Brame, LifeNet Health, Virginia Beach, Virginia*

Therapy: *Scar prevention and management*

Deliverable(s): *AFT-SPAR Autologous Adipose Cell-Based Therapies*

Key Accomplishment(s): *Studies to date have shown that human ASCs generate soluble and matrix factors pertinent to wound healing. They can support the growth of keratinocytes and other wound-related cells and accelerate healing in vivo in preclinical models. A clinical trial for scar prevention and scar remodeling with AFT-SPAR will commence early in Year 2.*

Introduction

Severe blast and burn injuries result in extremely challenging functional and deformational deficits related to soft tissue scarring. The need to regenerate and/or replace subcutaneous (adipose) tissue and/or cutaneous (dermal) tissue in soft tissue wounds is often minimized or completely overlooked. However, the reconstructive surgeon realizes the importance of this complex clinical objective and has made soft tissue reconstruction an integral aspect of surgical approaches to limb amputation, burn injury, and trauma. Tissue engineering approaches to cutaneous and subcutaneous tissue replacement have become common in recent years, with a goal to provide predictable and reproducible platforms based on scientific principles. The current general model for these engineered approaches involves the use of progenitor and/or stem cells in conjunction with matrix carriers or bioengineered scaffolds. ASCs (often interchangeably referred to as stromal, progenitor, or preadipocyte cells) are a logical, practical, and potentially ideal cell source for these objectives.

The purpose of this research program is to develop novel autologous therapies and products derived from adipose tissue for the promotion of wound healing and tissue repair and for the prevention and treatment of severe scarring. The clinical objectives for reconstruction span from the subcutaneous layer to the dermal-epidermal layers. Adipose tissue enables an autologous paradigm because it is unmatched in abundance, ease of harvest, and expandability. The use of ASCs as the cellular foundation of the dermal component of skin constructs may provide expedited manufacturing time frames for autologous tissues and/or reduce the donor site deformity associated with the initial tissue/cell harvest.

Research Progress – Year 1

Dr. Adam Katz has developed a therapeutic platform called MNT that involves the formulation of self-assembled spheroid aggregates or "modules" that can function as "regenerative hubs" for tissue repair. MNT provides potential advantages related to delivery, manufacturing, shipping, inventory, and therapeutic efficacy.

The research and development scope of the program ranges from the tissue to the cellular level. On the tissue level, the University of Virginia team has planned and received funding for a small clinical trial entitled "Autologous Fat Transfer for Scar Prevention and Remodeling (AFT-SPAR)." The goal of this clinical trial is to explore and define evidence-based methods, techniques, and treatment guidelines for AFT-SPAR therapies. The objective of this initial safety and feasibility study will be to see whether (1) AFT performs in the subacute phase of injury (2–4 weeks) to prevent scar formation as the healing process occurs and (2) AFT can impact the remodeling of longstanding scars (at least

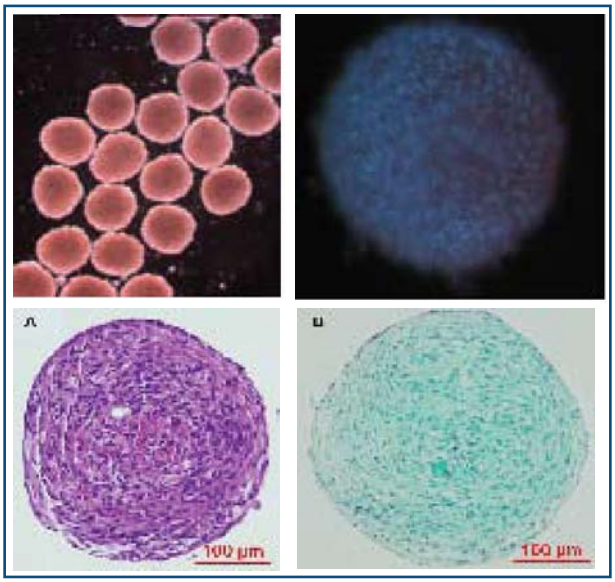


Figure IV-10. ASC multicellular aggregates (MAs) are reproducibly formed using a hanging droplet technique. Top Left: multiple, uniform-sized ASC MAs prepared using cells pre-labeled with DiI fluorescent dye, maintained in suspension culture. Top Right: Cell nuclei have been stained blue, providing additional perspective on the cellular topography of these aggregates. Stained sections demonstrate the presence of abundant cellularity (ASCs) embedded within as self-generated ECM, composed of abundant collagen. Bottom Left: Hematoxylin and eosin staining, Bottom Right: Trichrome staining (100x).

6 months old) from the initial injury. Strong collaboration with the U.S. Army Institute of Surgical Research (USAISR) has and will continue to play a role in implementing this clinical trial.

On the cellular level, the University of Virginia team has completed its first year of laboratory studies focused on the development of novel autologous adipose cell-based therapies and constructs for the repair, replacement, and/or regeneration of hypodermal, dermal, and/or epidermal defects. Results to date confirm that the combination of the intrinsic characteristics of stem cells and their microenvironment shapes their properties and defines their potential.

A key strategy has been to formulate and culture ASCs in three-dimensional MAs (**Figure IV-10**). The team has shown that this approach compared to ASCs grown in flat cultures confers enhanced biological potency and a more reproducible phenotype/system. Together, these findings have implications for regulatory objectives and reproducible and predictable quality control associated with scale-up manufacturing for translational objectives. In addition, the MA platform provides a flexible and practical system for manufacturing, shipping, and inventory logistics that may provide commercial advantages compared to prefabricated layered constructs currently used.

ASCs are able to support the viability, morphology, and growth of human keratinocytes (the principal cell type found in the epidermal layer of human skin), and it appears that these interactions are primarily mediated by direct interactions between the two cell types. As such, ASCs may prove useful for wound healing and skin repair challenges via direct interactions with keratinocytes, but further in vitro and in vivo studies are required. ASCs may be a viable substitute (partial or total) source of dermal fibroblasts (the principal cell type found in the dermal layer of human skin) for cutaneous challenges and provide advantages of availability so that donor site morbidity is lessened and/or product manufacturing time frames are significantly reduced.

Key Research Accomplishments

- Formulated and cultured ASCs in three-dimensional MAs and demonstrated that this approach confers enhanced biological potency and a more reproducible phenotype/system compared to ASCs grown in flat cultures.
- Determined that ASCs generate soluble and matrix factors pertinent to wound healing.
- Determined that ASCs can support the viability, morphology, and growth of human keratinocytes and other wound-related cells and accelerate healing in vivo in preclinical models.

Conclusions

Plans for translation of these therapeutic products is based around a patent portfolio related to ASCs and the MNT platform, which has been filed through the University of Virginia and licensed to a start-up company founded by the principal investigator Dr. Katz and colleagues. GID Group has completed a pre-IND meeting with the FDA in regard to the use of autologous ASCs for cutaneous wound healing. Tumorigenic and toxicology studies are needed prior to the preparation of a formal IND application and initiation of clinical trials. Dr. Katz and his team intend to pursue these

objectives within the context of the AFIRM program with additional extramural funding (e.g., National Institutes of Health [NIH]), (eventual) corporate funding from the private sector, and the development of strategic partnerships.

Research Plans for the Next 4 Years

This project's overall approach and early results are aligned with development of three specific therapeutic products over the next 4 years. *Modular (Injectable) Adipogenesis for Soft Tissue Defects:* Funding for this subproject has recently been augmented by the award of an NIH R21 grant. Continued work will include in vivo implantation studies and tumor toxicology studies. *Dermal Replacement/Repair:* Two distinct but related strategies for this project include ASC-seeded scaffolds and aerosolized MNT. Continued in vitro studies will aim to optimize the logistics of efficient scaffold seeding—testing strategies that involve freshly isolated cells seeded at the "point of harvest" versus strategies that involve the use of culture-expanded cells. In vivo studies will be initiated to determine if scaffolds seeded with ASCs undergo enhanced neovascularization, resulting in expedited "graft take." For aerosolized delivery of MNT, vehicles will be explored and an attempt made to define basic parameters for successful, viable spray delivery of ASC MAs to in vitro targets and ultimately to in vivo wounds. *Sprayable Bilayered MNT for Composite Dermis/Epidermis:* Continued studies will focus on understanding the in vivo efficacy and morphogenesis of composite aggregates (composed of ASCs and keratinocytes) as well as tumor toxicology studies.

Planned Clinical Transitions

A patent portfolio related to ASCs and the MNT platform has been filed through the University of Virginia and licensed to a start-up company (GID Group) founded by Dr. Katz and colleagues. GID Group is in the process of fundraising and related efforts aimed to consolidate additional ASC intellectual property and translational devices related to ASC isolation. It is also in discussions with several potential manufacturing/clinical trial-"competent" partners, including one of the largest tissue procurement companies in the country. Finally, GID Group has also founded and launched a prototype STEM (Science and Technology Enhancing Medicine) Center™ in Spain, through which the researchers anticipate the early and expedited testing of various cell-based therapies in the clinical realm. Dr. Katz' team intends to pursue regulatory approval through the United States in conjunction with strategic partners and/or joint ventures. To this end, GID Group has completed a pre-IND meeting with the FDA with regard to the use of autologous ASCs for cutaneous wound healing. Tumorigenic and toxicology studies are needed prior to the preparation of a formal IND application and initiation of clinical trials. In addition, there is a continued need to refine chemistry, manufacturing, and control requirements and manufacturing standard operating protocols for the MNT platform in general. The Katz team intends to pursue these objectives within the context of the AFIRM program with additional extramural funding (e.g., NIH), (eventual) corporate funding from the private sector, and the development of strategic partnerships.

Corrections/Changes Planned for Year 2

None.

Project 4.5.3, WFPC

Multi-Functional Bioscaffolds for Promoting Scarless Wound Healing

Team Leader(s): *Newell Washburn, PhD*

Collaborator(s): *USAISR*

Therapy: *Modulate local inflammatory responses to reduce scarring and promote healing*

Deliverable(s): *Bioscaffolds (gels) that contain antibodies or phage-derived peptides with affinities for mediators of inflammation*

Key Accomplishment(s): *The researchers created uncrosslinked HA gels by coupling monoclonal antibodies to HA and created crosslinked HA gels by covalently attaching the RGD peptide to HA-mAb gels. They determined that HA gels could modulate macrophage phenotype without coupled antibodies. They determined that the maximum reduction of an inflammatory response was achieved when antibodies against both TNF-α and IL-1β were coupled to the HA gels.*

Introduction

Following injury, intense inflammatory responses initiate an integrated process of wound healing that terminates in the formation of scar tissue. Redirecting this process toward a regenerative outcome requires controlling the inflammatory response, and therapies that function locally would provide the basis for new approaches to improving outcomes following burn, trauma, or other injury where native repair responses result in the formation of scar tissue. Researchers in the Washburn laboratory are developing bioscaffolds that contain monoclonal antibodies or phage-derived peptides with specific affinities for cytokines and other mediators of inflammation. They hypothesize that cytokines that are bound to the bioscaffold in an inactive conformation (binding at the cytokine domain that interacts with the cell receptor) will be neutralized while those that are bound to the bioscaffold in an active conformation (binding away from the cytokine domain that interacts with the cell receptor) will have an increased biological activity. By downregulating pro-inflammatory cytokines and upregulating anti-inflammatory cytokines, the researchers hope to modulate the inflammatory microenvironment, preventing pathological inflammatory processes or increasing the efficacy of therapeutic agents.

Research Progress – Year 1

Aim 1: *Animal testing of bioscaffolds functionalized with antibodies against IL-1β and TNF-α.*

Monoclonal antibodies were coupled to HA using carbodiimide chemistry to form a highly viscous solution referred to as uncrosslinked HA-mAb gels. The mAb concentrations in the gels were measured using PAGE (polyacrylamide gel electrophoresis) and a modified enzyme-linked immunosorbent assay (ELISA).

The researchers covalently attached the RGD peptide to the HA-mAb gels via a poly(ethylene glycol)-acryoyl chloride linker. They tested the biological activity of uncrosslinked and crosslinked HA-RGD-mAb conjugates subcutaneously in healthy rats. They delivered the gels to the site and sutured them closed. Rats were sacrificed after 4 days, and samples were collected that included the injury site and underlying muscle.

The characteristic healing responses 4 days following treatment with saline (**Figure IV-11**, left panel) and HA-RGD without mAb (Figure IV-11, center panel) formed the baseline for this study. There is evidence for closure of the incisional wound site in both treatments although it appeared to have progressed further in the site treated only with saline. Comparable numbers of invading macrophages were observed in sections stained for CD68 (cell surface marker used to identify macrophages). However, a lower fraction of HA-RGD-treated animals stained positively for CCR7 (associated with the cytotoxic M1 phenotype) compared to

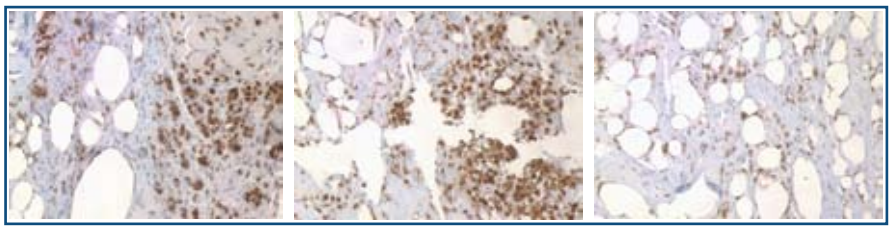


Figure IV-11. Tissue section from site treated with saline (left), HA-RGD (center), HA-RGD-anti-TNF-α stained for CD68. Extensive infiltration of mononuclear cells is evident in the saline-treated section as well as in the site treated with HA-RGD. Significant reductions in macrophage invasion were observed in the site treated with HA-RGD-anti-TNF-α.

saline-treated animals, which suggests that the HA-RGD gel itself is modulating the macrophage phenotype. The results of testing gels that only contained mAb against IL-1β were not found to significantly inhibit macrophage invasion or phenotype. However, sites treated with HA functionalized with anti-TNF-α demonstrated relatively high numbers of invading macrophages of the M2 (noncytotoxic) phenotype but delayed healing. This indicates that the HA matrix itself may influence macrophage phenotype but that targeting a single cytokine may not effectively alter the inflammatory response.

Quantification of the IHC data shows the following trends. Reductions in macrophage invasion were observed across multiple sites, as well as reductions in the number of cells expressing the M1 phenotype. These results suggest that only neutralizing TNF-α may have similar effects on macrophage responses at sites of acute inflammation.

It is important to note that in most cases, the uncrosslinked HA-RGD-mAb gel was no longer observed at the injury site. Presumably it diffused to surrounding tissues over time since it behaved like a viscous solution. This would make it difficult to apply topically since it would tend to run off the wound site. A crosslinked formulation of the HA-RGD-mAb gel with the same biological activities would make an attractive candidate for modulating the inflammation at a chronic wound site.

Analyzing macrophage phenotype using IHC, researchers in Washburn's laboratory did not observe any significant change in the fraction that stained positively for markers of the M2 phenotype compared to saline controls. This suggests that these crosslinked gels are not capable of neutralizing the activities of pro-inflammatory cytokines on a biologically relevant timescale, further suggesting that this is due to the slow rate of diffusion of small proteins like IL-1β, whereas diffusion of a cytokine to a nearby cell receptor is favored.

The researchers' understanding of how uncrosslinked and crosslinked gels function in vivo is summarized in **Figure IV-12**. Cytokines are released by cells at the wound site and diffuse into the extracellular environment. Interactions with the matrix compete with receptor binding. In the case of the uncrosslinked gels, cells are embedded within a medium containing a high concentration of high-affinity antibodies that specifically bind the cytokine and prevent it from reaching the cell receptor. In the case of crosslinked gels, cells adhere to the surface of the antibody functionalized gel but do not penetrate into it. Cytokines produced by cells have the shortest diffusion distance to cell receptors. Any cytokine that diffuses into the matrix is presumably sequestered with high affinity, but it appears that the majority of cytokines reach their receptors.

Key Research Accomplishments

- Demonstrated that neutralization of TNF-α alone may provide significant reductions in inflammatory signaling.
- Determined that maximum reduction of inflammatory responses occurs when both

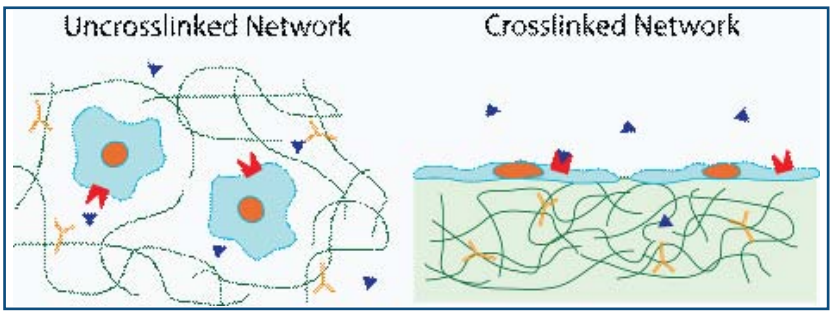


Figure IV-12. Schematic representation of interactions between inflammatory cells and uncrosslinked (left) and crosslinked (right) HA-RGD-mAb gels. Two cells are depicted in each picture, each with one receptor that can bind the cytokine (triangle). Antibodies in the green HA matrix are shown as Y.

TNF-α and IL-1β have been neutralized.

- Demonstrated that HA gels can modulate macrophage phenotype without antibodies.
- Identified material design parameters that optimize the activities of covalently attached monoclonal antibodies.

Conclusions

The trends in the researchers' preliminary animal data are summarized in **Table IV-2**. As expected, acute inflammation was observed at the wound site treated with saline, characterized by extensive invasion of mononuclear cells that express markers for the cytotoxic M1 macrophage phenotype. After 4 days, significant tissue ingrowth was observed at the site. Following treatment with the HA-RGD control gels (no monoclonal antibodies), significant mononuclear cells were observed, but many more stained positively for the noncytotoxic M2 macrophage phenotype marker, suggesting the HA matrix itself influences the macrophage phenotype. Tissue in-growth was still observed but with a few more remaining voids than the saline-treated site.

Treatment with HA-RGD functionalized with anti-IL-1β yielded identical results to treatment with the HA-RGD control, indicating that neutralization of IL-1β did not significantly inhibit inflammation. The sites treated with HA-RGD conjugated with anti-TNF-α have similar levels of macrophage invasion as the other HA-RGD sites, but the sites displayed a noticeable lack of healing, suggesting that neutralization of TNF-α somehow resulted in an inhibition of processes associated with inflammation.

Sites treated with HA-RGD functionalized with anti-IL-1β and anti-TNF-α displayed significant reductions in macrophage invasion and very few signs of healing. This suggests that simultaneous neutralization of these cytokines arrested the inflammatory response. Interestingly, a crosslinked version of the identical formulation of HA-RGD-(anti-IL-1β/anti-TNF-α) had nearly identical inflammatory responses to the saline-treated control, which suggests that material geometry is a critical parameter to consider in developing gels that will regulate pro-inflammatory cytokines.

Research Plans for the Next 4 Years

In Years 2–3, the researchers plan to test inflammation-attenuating gels in a rat burn model of scar formation in collaboration with USAISR. Formulations that reduce hyperinflammation, tissue necrosis, and scar tissue formation will be screened in thermal injury models. In Year 3, the researchers will have identified lead formulations and will perform tests that involve bacterial challenges to the injury site to determine the extent to which attenuation of pro-inflammatory cytokines results in increased bacterial colonization at the burn site. In Year 4, tests on larger body surface area models will be performed to test the effects on immunosuppression as well as tests in large animal models, such as pigs, that have tissue responses more similar to humans.

Planned Clinical Transitions

Meeting the milestones outlined earlier in Years 2–4 would position this technology for potential clinical trials. The researchers have applied for patents related to this technology, and a Carnegie Mellon University spin-off company has licensed the technology and is preparing to manufacture the bioscaffolds.

Corrections/Changes Planned for Year 2

Research direction was refocused to target pathways to clinical use for treating burns. Scar-forming animal models will be tested. Collaborations with Project 4.5.4 will be initiated to investigate the effects of attenuating different mediators of inflammation with these bioscaffolds.

Table IV-2. Treatment condition results from preliminary animal experiments.

Treatment Condition	Result
Saline	Macrophage invasion (mostly M1 phenotype)
HA-RGD	Macrophage invasion (more M2 phenotype)
HA-RGD-(anti-IL-1β)	Identical to HA-RGD treatment
HA-RGD-(anti-TNF-α)	Macrophage invasion (more M2 phenotype)
HA-RGD-(anti-IL-1β/anti-TNF-α)	Decreased macrophage invasion (more M2)
Crosslinked HA-RGD-(anti-IL-1β/anti-TNF-α)	Same as saline treatment

Project 4.5.4, WFPC

Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration

Team Leader(s): *Patricia Hebda, PhD*

Project Team: *Joseph E. Dohar, MD and Tianbing Yang, PhD*

Therapy: *Attenuate local inflammatory responses to reduce scarring and promote healing*

Deliverable(s): *Combinatorial anti-inflammatory topical therapy to reduce scar formation*

Key Accomplishment(s): *The research team demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and prostaglandin E2 (PGE2) can attenuate the wound inflammatory response following skin incisional wounds in the rat, which leads to a reduced amount of scarring and the promotion of healing.*

Introduction

The Hebda laboratory 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.

The researchers in Hebda's laboratory propose to use a combinatorial, yet specific anti-inflammatory therapy to significantly reduce or eliminate scarring associated with dermal wound healing. The first two targets selected for testing were cyclo-oxygenase-2 (COX-2) and TGF-β1, both proven players in inflammation and scar formation. They hypothesize that a combined treatment of inflammation will result in a synergistic effect not achievable with single-agent therapies. The proposed mechanism of action is a reduction in the chemoattractant gradients normally used by invading fibroblasts along with a diminished pro-fibrotic cue.

The second goal of this study is to precisely characterize the role of fibroblasts in the development of fibrosis. While this issue has been addressed extensively by other researchers, it remains unclear how much of the fibroblast response to injury is an intrinsic property or a response to soluble wound factors. The researchers propose 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 them to determine if the fibroblast phenotype is a dynamic one, largely influenced by the wound environment. Should this be the case, then prevention of fibrosis/scarring could be primarily a matter of reducing pro-fibrotic signals in the wound bed.

Research Progress – Year 1

The researchers have previously conducted studies that support the current proposal (i.e., local delivery of anti-inflammatory or antifibrotic agents can influence the wound healing response). In vitro work has revealed that PGE2 has significant fibroblast modulatory potential. The researchers' data indicate that PGE2 can decrease both migration and collagen synthesis by adult dermal fibroblasts, but fetal fibroblasts are resistant. Interestingly, keloid fibroblasts, a well-established fibrotic fibroblast phenotype, are also inhibited by PGE2 to the same extent as adult cells. The researchers also found that PGE2 in vitro can block the upregula-

tion of collagen I and III synthesis following TGF-β1 administration. These in vitro data are strongly suggestive that a COX-2 blocking approach (i.e., downregulating all inflammatory mediators produced by this enzyme), combined with the selective addition of PGE2 (found to have anti-inflammatory effects in addition to its inhibition of fibroplasias), may indeed reduce scarring in an animal wound model while promoting more regenerative healing.

Aim 1: *To determine the potential of combinatorial anti-inflammatory therapy in decreasing subsequent fibrotic fibroblast activity in the wound bed.*

The researchers have established a rat model to determine the combined effects of the COX-2 inhibitor nimesulide and PGE2 on healing of full thickness skin wounds by clinical assessment, histologic evaluation, and biochemical analysis. Nimesulide is an established anti-inflammatory molecule. PGE2 decreases fibrosis and promotes healing in several tissues including skin. The combination of these activities may work together to improve healing outcomes.

Either excisional wounds or incisional full thickness wounds were made in the dorsal mid-region of rats. Treatments were applied in a biocompatible hydrogel on the day of injury. After 14 days the animals were euthanized and the incisional full thickness wounds surgically removed. Wound tensile strength is a way to measure healing as determined by collagen synthesis and integration into the surrounding unwounded tissue. Tensiometry measurements were made, and the results demonstrate that both nimesulide and PGE2 significantly increased the regain of tensile strength. However, the combination of the two had variable results, which are currently under further study. Nimesulide-treated excisional wounds were larger on post-wound days (PWDs) 14 and 21 but did not reach statistical significance.

Histological observation indicated that both treatments produced more advanced healing compared with either vehicle alone. However, nimesulide-treated wounds showed greater vasodilation while PGE2 treatment reduced inflammation and increased fibroplasia. Compared to vehicle-treated excisional full thickness wounds, nimesulide and PGE2 showed reduced TGF-β1 levels on PWD 7, a point when wound collagen production is upregulated, indicating that these agents are effective in modulating this process (**Figure IV-13**). This downregulation of TGF-β1 correlates with the decreased abundance of collagen observed by picrosirius red, polarized light microscopy. Interestingly, the combination treatment showed reduced TGF-β1 levels on PWD 21, which may have implications for the later stage of wound remodeling.

Key Research Accomplishments

- 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, which leads to reduced scarring and increased healing. This determination was based on:
  - Clinical assessment of healing
  - Wound histology
  - Tissue levels of ECM components
  - Tissue biomechanics—tensiometry
  - Collagen organization—tissue morphometrics

Conclusions

These results demonstrate that early, short-term treatment with anti-inflammatory agents can attenuate the wound inflammatory response with downstream effects on healing. Nimesulide and PGE2 each have specific and potentially useful effects on the healing process. The finding that nimesulide- and PGE2-treated wounds have less collagen deposition yet exhibit higher regain of tensile strength supports the premise that controlled modulation of inflammation can decrease fibrosis without impeding the healing process. These results are very encouraging that the experimental plan is feasible and the milestones achievable. The work will continue with additional studies to verify and optimize results to date and then progress to the second aim, which will introduce donor cells into the wounds.

In compartment syndrome and in burn injuries, the host response to the inciting trauma must be minimized while healing and tissue regeneration are encouraged. Blood vessels and nerves are susceptible to damage and may require restoration through regen-

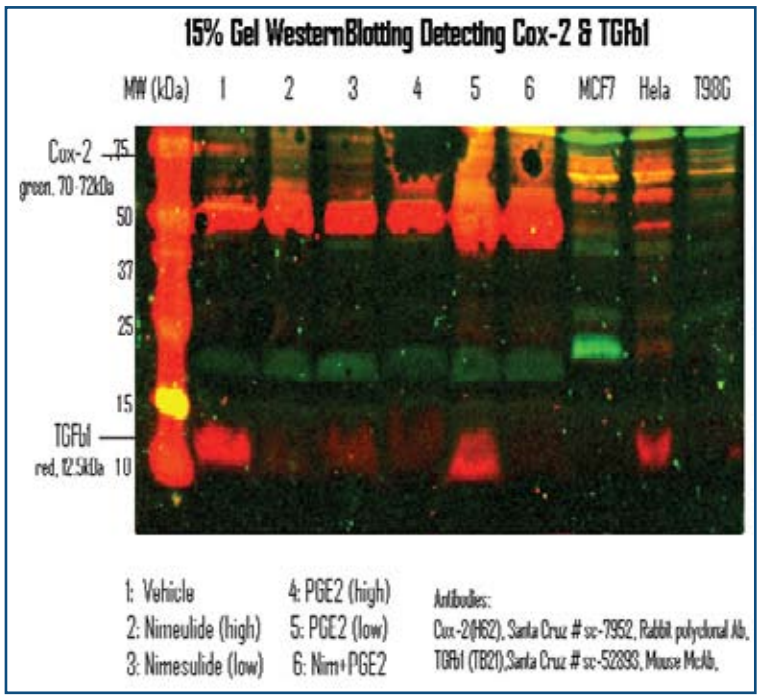


Figure IV-13. TGF-β1 and COX-2 Levels in Rat Skin Wounds. Following 4 days of treatments, the levels of TGF-β1 were markedly lower as confirmed by western blotting in all of the treatment groups on PWD 7 comparable in all groups. Changes in COX-2 were not detected in any group on PWD 7. Other time points may need to be considered for COX-2.

erative modalities. Inflammation, which activates fibroblasts in the soft tissue, must be downregulated and measures need to be taken to reduce or reverse the risk of fibrosis, which could result in permanent impairment and scarring. Once the initial responses to injury have been addressed and the patient has been stabilized, there is the second concern for optimizing subsequent wound healing to reduce scarring and fibrosis and to promote regenerative healing and restore function. The objectives and deliverables of this project build upon these clinical goals, and the researchers anticipate opportunities to collaborate on various projects in these other AFIRM program areas.

Research Plans for the Next 4 Years

Another rat wounding experiment is under way to optimize the treatments and expand upon the initial experimental design. In addition, the researchers will prepare cultures of donor fibroblasts and characterize, expand, and store these cell strains for planned experiments under Aim 2.

In Year 2, the researchers plan to complete analyses of the topical treatments and their effects on healing using biochemical assays for inflammatory markers and collagen and also selecting the most optimal treatment regimen. Some of the original goals for Year 2 have already been accomplished. Hence, the research team will be able to move ahead to the next objective, which involves precisely characterizing the contribution of the fibroblast phenotype to the overall degree of tissue fibrosis. For this work, they have been preparing rat fibroblasts of several distinct phenotypes (with respect to their healing properties), as well as adult rat stem cells derived from bone marrow; each cell strain is labeled with a stable and benign fluorescent tag—the GFP. Each cell phenotype will be tested as donor cells in animal wound healing models and can be identified in the healing wounds because of the GFP marker. The researchers will be able to tell how well each type of cell strain survives and distributes itself in the wound bed and how it contributes to the healing process. They will use markers for inflammation and collagen production, as well as wound histology and tensiometry, to determine the qualitative and quantitative effects on healing. The researchers feel that this part of the project should carry over into Year 3, during which time they plan to complete the analysis of fibroblast contribution to healing outcome.

In Years 4 and 5, the researchers will address Aim 3 (the design of interventions) based on the results of the first two aims, which provide a wound environment for rapid, regenerative healing. They will combine the anti-inflammatory treatments with donor fibroblast delivery to see whether the combination enables donor fibroblasts (of favorable phenotypes) to have a greater impact on healing outcomes. The focus of these studies

will be geared toward the development of 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. 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 2

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. They are also planning to work toward the integration of this approach with other projects in the Scarless Wound Healing Program. For example, they anticipate using Dr. Washburn's HA-based matrix as a sustained release delivery vehicle for their agents.

Project 4.5.6, WFPC

Delivery of Therapeutic Compounds into Injured Tissue

Team Leader(s): *Erkki Ruoslahti, MD, PhD*

Therapy: *Systemic treatment to regenerate tissue and reduce scarring at wound sites*

Deliverable(s): *Systemically delivered wound-targeted recombinant fusion protein*

Key Accomplishment(s): *The researchers produced and purified a target-seeking antifibrotic agent (recombinant decorin fusion protein) in mammalian expression vectors and baculovirus. They achieved targeted delivery of the agent into regenerating tissue following intravenous injection in mice. They also demonstrated that the agent reduced excessive TGF-β1 activity and could inhibit scar formation in mice during wound healing.*

Introduction

It is difficult to maintain bioactivity of locally applied therapeutic agents because of problems with lack of retention of the agent in the wound, poor tissue penetration, and instability of protein therapeutics in the protease-rich environment of the wound. Moreover, deep injuries and multiple sites of injury further limit the usefulness of local treatment. Clearly, systemic approaches to tissue repair would be valuable. Another problem with injury recovery is scar formation. The response to tissue injury in adult mammals seems to be focused on the quick sealing of an injury, which results in scar formation in the majority of the tissues. The two main elements of scar formation are the proliferation of fibrous astrocytes and smooth muscle cells and enhanced ECM production by these cells, both of which limit regeneration in adult tissues. In contrast, fetal tissues heal by a process that restores the original tissue architecture and results in no scarring. TGF-β1 is a major factor responsible for wound repair, but its activity also results in scar formation and fibrosis. Researchers in Ruoslahti's laboratory have designed a wound-targeted recombinant fusion protein that specifically homes to injured tissue and inhibits excessive TGF-β1 activity and has other beneficial effects on tissue regeneration.

Research Progress – Year 1

Production of decorin fusion-proteins in mammalian expression system.

The researchers designed wound-targeted recombinant decorin fusion proteins by adding the wound homing peptide CAR sequence (CARSKNKDC) to the C-terminus of human decorin (**Figure IV-14**). They also cloned the following control proteins into pcDNA3.1 mammalian expression vector: native decorin (DCN), bovine serum albumin targeted with CAR peptide (BSA-CAR), and decorin targeted with mutated CAR homing peptide (DCN-CAR-M). They have previously shown that the mutation of two basic amino acids in the CAR sequence to neutral ones (CARSKNKDC mutated to CAQSNNKDC) results in impaired wound-homing properties: CAR-M sequence is essentially inactive in wound homing. The resulting proteins (DCN, DCN-CAR, BSA-CAR, and DCN-CAR-M) were produced as secreted proteins in a mammalian cell expression system. The yields of purified fusion proteins were 10–20 mg per liter of cell culture media, providing ample material for characterization and in vivo treatment studies.

In SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), decorin and the decorin-homing peptide fusions migrated as sharp bands at 45 kDa with a smear above it (Figure IV-14). The sharp bands correspond to the core proteins, and the smear is caused by heterogeneity in the chondroitin sulfate chain attached to most of the molecules. Decorin produced in a baculovirus expression system

runs on SDS gels as a sharp band since baculovirus is not capable of incorporating a GAG side chain into decorin (Figure IV-14). Mass spectrometry confirmed protein identity as decorin, and differential scanning calorimetry produced a sharp peak with a melting temperature of Tm=49.3°C, indicating native protein folding.

The researchers established the biological activity of their wound-targeted recombinant decorin fusion protein and control fusion proteins in vitro. They subsequently validated the decorin fusion protein by therapeutically delivering it and control proteins to wounds. They achieved targeted delivery of the decorin fusion protein into regenerating tissue following intravenous injection in mice (**Figure IV-15**). The researchers conducted an experimental treatment trial that showed the decorin fusion protein to reduce scar formation while control proteins did not (**Figure IV-16**). They demonstrated that the decorin fusion protein could inhibit TGF-α-dependent scar-associated processes. They note that their fusion protein can serve as a selective TGF-α therapeutic; it can reduce scar formation by inhibiting TGF-α but does not compromise the immune defense at the site of the injury.

Key Research Accomplishments

- Produced and purified a target-seeking antifibrotic agent (recombinant decorin fusion protein) in mammalian expression vectors and baculovirus.
- Established the in vitro biological activity of the decorin fusion protein.
- Achieved targeted delivery of the decorin fusion protein into regenerating tissue following intravenous injection in mice.
- Demonstrated that the decorin fusion protein could inhibit TGF-α-dependent scar-associated processes.
- Determined that the decorin fusion protein could inhibit scar formation in mice during wound healing.

Conclusions

The researchers have demonstrated the validity of their homing peptide-based targeting technology in a wound healing model. Combining the inherent activities of decorin with a targeting moiety yields a compound with enhanced specificity and potency. This approach may help make systemic enhancement of tissue repair and regeneration a feasible option in the care of the wounded soldier. Going forward, the researchers will test the wound-homing peptides and other candidate peptides from their homing peptide collection for their ability to target

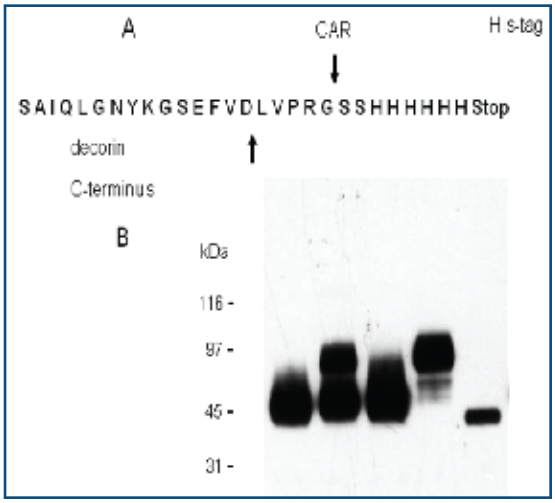


Figure IV-14. Cloning and production of decorin fusion proteins. (A) A schematic showing the fusion of the CAR peptide sequence and a his-tag to C-terminus of full-length human decorin cDNA. (B) Gel electrophoretic analysis of recombinant proteins. The recombinant proteins were expressed in mammalian cells, purified on a Ni-column, separated on gradient SDS-PAGE gels, and detected with a monoclonal anti-6-histidine tag antibody. Recombinant proteins from left to right: DCN, DCN-CAR, DCN-CAR-M, BSA-CAR, and DCN produced in a baculovirus expression system.

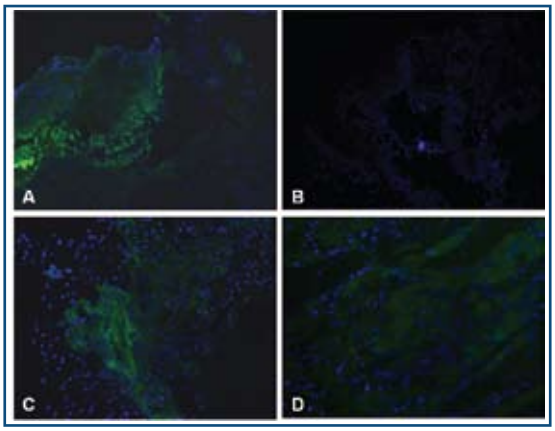


Figure IV-15. CAR peptide homing to acute injury. (A-D), fluorescein-conjugated CAR-peptide (A, C) or fluorescein-conjugated control-peptides (B, D) (500 µg) were intravenously injected into mice 48 hours after induction of skin incision wounds (A, B) and skeletal muscle crush-injuries (C, D), and the injured tissue was collected 4 hours later and examined for the presence of the peptides (green, i.e., FITC-fluorescence). The nuclei were stained with DAPI (blue). Magnification 200x.

injuries in internal organs. They will also make the homing peptides available for collaborators to target their compounds to tissue injuries. A high priority is to publish the wound healing data, which will hopefully generate interest in the pharmaceutical/biotechnology community, allowing the project to move into clinical trials.

Research Plans for the Next 4 Years

The researchers plan to identify and develop peptides that home to regenerating tissues, penetrate into tissue, and make blood vessels permeable for the extravasation of therapeutic agents and their penetration through extravascular tissue. They have dubbed the scheme as CendR pathway, and their intention is to describe its benefits for regenerative medicine.

Planned Clinical Transitions

The research team plans to move targeted decorin into clinical trials within the next 4 years. They will either seek venture capital funding or sell the rights to targeted decorin to a commercial party to move it into clinical trials as soon as possible.

Corrections/Changes Planned for Year 2

The researchers intend to test the possibility of providing unique tissue penetration for therapeutic agents by using CendR pathway, which was recently identified in their laboratory. The CendR pathway is based on peptides that activate a transport pathway through vascular walls and tissues. The CendR system could enhance the penetration of systemically administered agents into wound tissue. Local permeabilization of scar tissue may also become possible.

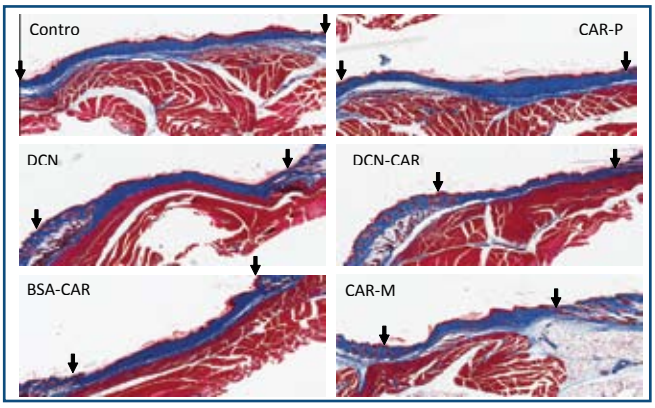


Figure IV-16. Reduction in scar formation and scar width during wound healing in mice treated with homing peptide-targeted decorin. Panel shows representative microscopic fields from the wounds of animals on day 21.

Project 4.5.7, WFPC

Scar Mitigation via Matrix Metalloproteinase-1 Therapy

Team Leader(s): *Alan Russell, PhD, Richard Koepsel, PhD*

Therapy: *Remediation of muscle scarring*

Deliverable(s): *MMP-1 treatment to heal muscle scars*

Key Accomplishment(s): *The researchers developed a method to produce pure homogeneous MMP-1 enzyme for use in preclinical trials, modified MMP-1 with a collagen binding peptide, developed the Dual Polarization Interferometer (DPI) as a platform for determining real-time MMP activity, and demonstrated differences in activity of MMP-1, PEG-MMP-1, and peptide-MMP-1.*

Introduction

This project builds on previous research that demonstrated a possible connection between MMP-1 and the healing of muscle scars. Scarring often occurs during healing of skeletal muscle injuries. This scarring inhibits complete healing of the muscle and can result in significant loss of function. MMP-1 is an enzyme normally involved in remodeling of ECM and works by hydrolyzing type I collagen. It has been shown that injection of this enzyme directly into a muscle scar can improve muscle regeneration by breaking down the collagen fibrils within the scar tissue. In this project, Russell and Koespel's research team will first look at the interaction between MMP-1 and collagen in vitro with the aim of gaining a better understanding of relevant parameters. In doing so they can make some realistic predictions of the enzyme's behavior in vivo and improve their preclinical study design. Once they have the relevant in vitro data, they will be able to reduce the number of animals in the initial studies, accelerating the progress toward clinical trials.

Research Progress – Year 1

Aim 1: *In vitro analysis of MMP-1 activity against model scar tissue.*

In this aim, the researchers will: (1) determine the mass of each form of the enzyme (the purified zymogen pro-MMP-1, an active recombinant MMP-1, and the active enzyme after modification with polyethylene glycol) required to degrade a given mass of scar, (2) determine the catalytic stability of the enzyme in simulated in vivo conditions, (3) determine the rate of diffusion of the enzyme from simulated scar, and (4) assess the use of multiple doses of the enzyme for scar degradation. The knowledge gained from these tasks will be used to determine the optimal treatment modalities for subsequent animal studies.

Data Obtained Pertaining to Aim 1

New method for the production of rhMMP-1

Previous production and purification of rhMMP-1 resulted in an enzyme preparation that was extensively contaminated with proteolytic fragments. A new method was tested wherein the protein was produced in cells that were zinc-limited and purified in zinc-free buffer. When MMP-1 is purified in the presence of zinc, there are numerous degradation bands due to the nonspecific protease activity of the enzyme. The researchers found that a more homogenous product results when no zinc is present. The ability to produce pure homogeneous enzyme is critical for any possible future clinical trial.

Interaction of MMP-1 with collagen

Previous work on this aim identified Michael Raghunath's laboratory in Singapore as having the capability to test MMP-1 in an in vitro model of scarring. This experiment shows that both MMP-1 and PEG-MMP1 can degrade collagen and that the choice of using

MMP-1, PEG-MMP-1, or MMP-1 modified with a collagen-binding peptide will depend on which of the three variants has the least tendency to migrate from the scar site. These researchers are addressing this issue by determining which of the enzymes can bind most avidly to fibrillar collagen.

To better predict the amount of enzyme that will need to be injected into a scarred wound, the researchers have undertaken studies of the binding affinity of the enzyme to a collagen-coated surface. Results from these studies indicate that an ELISA can measure small amounts of rhMMP-1 but may not be able to distinguish between small differences in binding affinity. Several repeats of this experiment have given similar results, but they are not sufficient to accurately measure binding constants.

In addition to MMP-1and PEG-MMP-1, a newly developed, modified MMP-1 has been produced. This new MMP-1 is modified with a synthetic collagen-binding peptide derived from von Willebrand factor. The peptide, GWREPSF-CALSGGGG-NHS, was bound to MMP-1 in a manner analogous to PEG modification. Initial enzyme activity measurements show that the peptide modification does not affect enzyme activity against a small-molecule substrate. Further characterization is under way. Because of the likely addition of six or more peptide chains on the MMP-1 molecule, the researchers expect an increase in binding to collagen. This will allow a reduction in the amount of protein and frequency of treatments at the scar site. The ELISA binding experiments were expanded to include the peptide-modified MMP-1 but with similar results. To get past this hurdle, new methods for determining the interaction between MMP-1 and collagen have been developed.

MMP-1 interactions with collagen at the molecular level

The researchers have devised a new method to precisely determine the binding of MMP-1 to collagen. It is likely that this method will also be able to determine the activity of MMP-1 on collagen on a surface. The method uses the Farfield Analight Bio200, a DPI. In their first set of experiments, the researchers used the DPI to attempt to measure the binding and release of MMP-1 to a layer of collagen covalently attached to the surface of the instrument's flow chip (**Figure IV-17**). The silica chip was amine modified, and a bifunctional NHS cross-linker was attached to the surface (Figure IV-17A-C). Collagen was injected into the chip's flow chamber and allowed to crosslink to the surface. Free, unreacted, cross-linker was blocked by injection of ethanolamine (Figure IV-17C-D) and MMP-1 was injected (Figure IV-17E-F). At the end of the MMP-1 injection, the resumption of buffer flow washed off all loosely bound enzymes with tightly bound enzymes remaining on the surface (Figure IV-17, first arrow). As flow continued over the next 37 minutes (Figure IV-17, second arrow), the mass decreased by 1.23%, with density and thickness also changing by similar magnitudes (data not shown). The relative role of MMP-1 release from the surface and cleavage of the collagen in this experiment has yet to be resolved. Trials using varying amounts of enzyme concentrations close to the binding constant should resolve such issues.

The second set of experiments with the DPI followed a similar protocol to the first except that HEPES (free acid) was used as the buffer. In addition, the chip was unmodified and collagen was deposited by physioadsorption; the blocking compound was a casein hydrolysate, and there was no ethanolamine injection. While the results consistently showed huge effects, they will need to be tweaked to gain highly reliable quantitative accuracy. There were variations in the collagen layers, the enzyme-loading parameters were not optimized, and there was some obscuring of the binding reaction due to the presence of trace amounts of glycerol in the

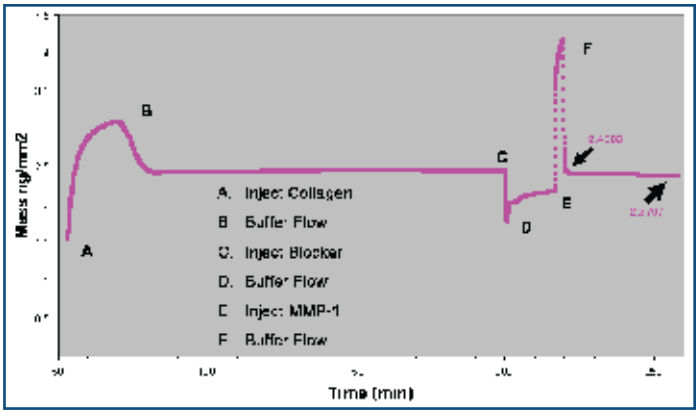


Figure IV-17. Surface mass plot of a DPI analysis of MMP-1 binding to collagen.

enzyme preparations that were not completely removed in the buffer exchange procedures used to prepare the enzymes for the DPI.

Binding constants for MMP-1 can be obtained from the DPI data by measuring the on-and-off rates of a range of MMP-1 concentrations injected across collagen layers. **Figure IV-18** shows the graphs of the thickness changes that occur during injection of 500 µg/mL MMP-1 across the collagen and gelatin layers.

The DPI demonstrates that for the first time the researchers can watch the interaction between collagen and MMP in real time and follow the reaction kinetics on a molecular scale. With the experimental modifications, they should be able to determine binding efficiency and get a good measure of how much enzyme will be required to hydrolyze a given mass of scar.

Aim 2: *Determine the efficacy of MMP-1 against muscle scars in rats.*

The tasks in this aim will provide the parameters of a treatment protocol that will be translated to a large animal (canine) model. The rat studies described in Aim 2 are scheduled to start in September 2009.

Aim 3: *Translate the findings of the rat studies to a canine model.*

This task will be a pilot study to determine whether the technology can be scaled up and used for treatment of severe injuries similar to those encountered in the field. Successful completion of these tasks will allow the researchers to proceed to advanced preclinical trials and then to clinical trials within 5 years of the completion of this project. The canine studies are scheduled to start in April 2012.

Key Research Accomplishments

- Developed a method to produce pure homogeneous MMP-1 enzyme for use in preclinical trials.
- Modified MMP-1 with a collagen-binding peptide.
- Developed the DPI as a platform for determining the activity of MMPs at a molecular level in real time.
- Demonstrated the differences in activity of MMP-1, PEG-MMP-1, and peptide-MMP-1.

Conclusions

During this reporting period, the researchers have refined the method for production of MMP-1, resulting in the ability to produce pure protein with a minimum of degradation artifacts. This will allow the production of enzyme suitable as a therapeutic product. MMP-1 or indeed any of the other MMPs may be useful for therapy of conditions other than muscle scars (for example, burn wounds and surgical scars), and this method will allow production of high-purity enzyme. The researchers have also developed a method to watch the activity of MMP-1 on a collagen surface in real time. This breakthrough science will add to the understanding of the molecular mechanisms of MMPs as well as other enzymes. This work could result in small-molecule interventions that can regulate MMP activity or better direct tissue remodeling. Finally, the use of the DPI to measure MMP-1 activity will likely lead to a platform for estimating dosage regimens for clinical and preclinical trials.

Research Plans for the Next 4 Years

The MMP-1 enzyme studies are

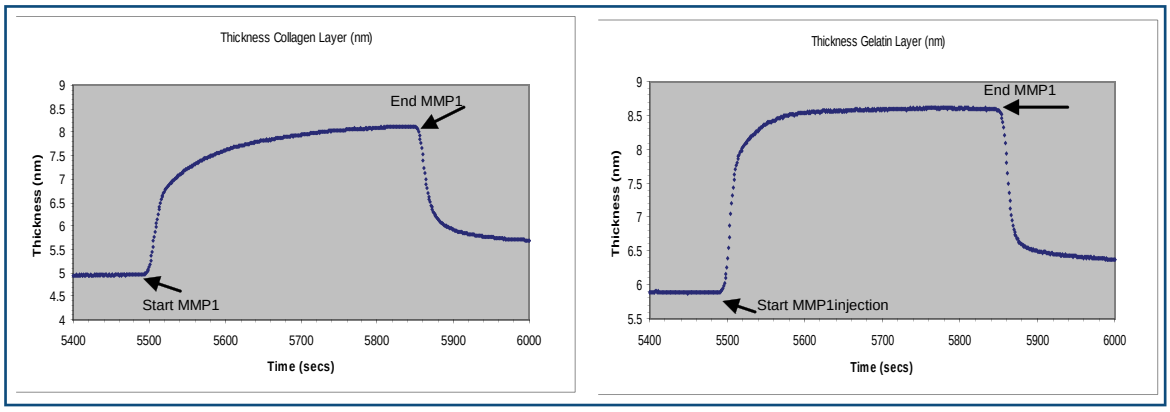


Figure IV-18. MMP-1 binding to collagen and gelatin layers.

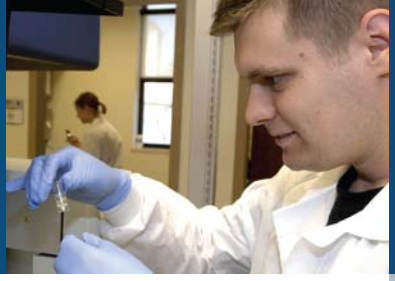
scheduled to continue into the first half of Year 2, and the results will assist dosing formulation for the preclinical studies. Studies in rats are currently being planned and protocols are being developed. The rat studies will begin in the second half of Year 2 and extend through Year 4. They will start with a small dose-ranging trial based on the binding data gathered during Year 1–1.5. A second trial will determine the effectiveness of the peptide-modified MMP-1 compared to native enzyme. A third trial will assess the optimal time after injury for administration of the enzyme therapy, and a final rat trial will investigate the use of a multiple dosing regimen. Based on the rat studies, a dog trial will assess the effectiveness of the enzyme therapy in a large animal model and will further test recovery of function.

Planned Clinical Transitions

The researchers aim to transition their project into a clinical trial by the end of Year 5. The timing of when the clinical trial will actually begin will depend on the data accumulated over the next 2–3 years.

Corrections/Changes Planned for Year 2

The original protocol called for a rat model based on preliminary work with mice, which used a laceration injury. The researchers are working on a revised model, which will be an excision injury in the rat gastrocnemius muscle. They believe that this model better represents the type of injury they hope to treat with MMP-1 therapy.



V Burn Repair

Background

Changes in weaponry have resulted in a higher frequency of burns, often in combination with other severe injuries. These injuries are costly in both human and economic terms and carry risks to survival, independence, and function, both in the short term and over time as the injuries mature with scarring and movement-limiting contractures. The current standard of care for burn injuries includes early excision of the damaged tissue and autografting (i.e., transplanting tissue from one part of the body to another), and these procedures have not fundamentally changed in over 30 years. Finding novel solutions to the challenge of burn injuries will improve outcomes for wounded warfighters suffering not only from burns but from other debilitating and disfiguring injuries where vasculature, wound coverage, or scarring are problematic. It is anticipated that the therapies developed by the AFIRM will serve the military population as well as later translate to the civilian population, where there are more than 1 million burns in the United States each year, resulting in 900,000 hospital days, 4,500 deaths, and more than $1 billion annually in treatment costs and lost productivity.

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, secondary to barrier disruption, which leads to 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 and can have a devastating effect both acutely, with deep second-degree burns often extending to become full-thickness third-degree burns leading to increased tissue loss, longer healing times, and excess morbidity and mortality, and chronically, with increased scarring, wound contractures, and poor quality of life. Therapies, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and antioxidants have not yet shown substantial benefit in preventing injury extension. Hence, a critical unmet need is the 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 water-soluble cream, is capable of inhibiting the growth of both gram-positive and gram-negative bacteria and penetrates the eschar (i.e., 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 a disruption in the body's acid-base balance if applied over an extensive surface. Silvadene, a suspension of silver sulfadiazine in a water-soluble base, does not induce pain or disturb the acid-base balance but fails to penetrate the eschar well and often does not protect against the bacteria *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 bacteria) or even pancytopenia (a medical condition characterized by reduced levels of white and red blood cells and platelets). Silver nitrate is active against a broad spectrum of bacteria but cannot penetrate the eschar and is caustic, damaging to otherwise viable tissue. Hence, new topical treatments are needed to prevent infection in burn patients.

After burn wound excision, 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. Hence, a critical need exists to speed granulation tissue formation in excised wound beds to improve graft survival and to speed re-epithelialization of 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 skin allografts (i.e., tissue grafted from one individual to a genetically nonidentical member of the same species) and porcine skin xenografts (i.e., tissue grafted from one species to an unlike species) are the two most readily available skin substitutes, but they are (a) less adherent to the wound bed than fresh autografts, (b) less able to control the bacterial population of the underlying wound, and (c) usually do not become well vascularized (i.e., 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 with limited success. An effective synthetic skin substitute should be compatible with the patient's own tissue, not elicit an immune response, be nontoxic, 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 skin-like neodermis or granulation tissue (i.e., the fibrous connective tissue that replaces a clot in healing wounds).

Burn injury progression, wound infection, delays in grafting, and grafting with inadequate material, such as highly meshed autograft, all can lead to increased scarring with the possibility of severe wound contractures (see **Figure V-1**). The

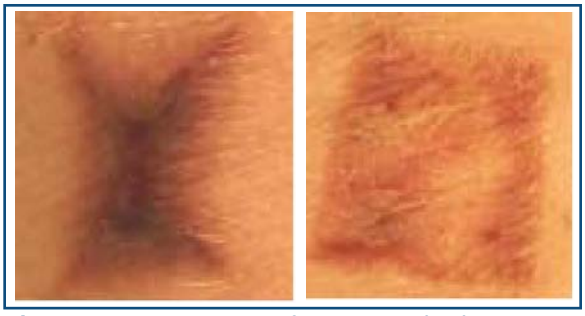


Figure V-1. Appearance of representative burns at 28 days after injury (RCCC). Contracted control wound (left) and noncontracted TGF-β (transforming growth factor-beta) peptide antagonist treated wound (right).

Table V-1. AFIRM-funded projects per clinical challenge topic area.

Clinical Challenge Topic Area	Consortium	Project Number	Project Title
Intravenous Treatment of Burn Injury	RCCC	4.6.1	Prevent Burn Injury Progression, Reduce Inflammation, and Induce Healing in a Rat Hot Comb Model
		4.6.2	Bone Marrow-Derived Mesenchymal Stem Cell (MSC) IV Treatment to Prevent Burn Injury Progression
Topical Treatment of Burn Injury	RCCC	4.6.4	Topical Iodine Treatment of Burns to Prevent Infection
		4.6.5	Topical P12 Treatment of Burns Using Fibro-Porous Mat
		4.6.6	Curcumin-Loaded Nanospheres as a Topical Therapy to Limit Burn Injury Progression and to Promote Non-Scar Healing
	WFPC	4.2.3	Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues
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 Bio-Printing of Skin for Battlefield Burn Injuries
Skin Products/ Substitutes	RCCC	4.7.2	Burn Repair with Autologous Engineered Skin Substitutes
	WFPC	4.2.1a	Tissue Engineered Skin Products
		4.2.1b	Tissue Engineered Skin Products/Comparative Skin Study
		4.2.6	Amniotic Fluid Stem Cells for Burn

therapies and innovative technologies 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

AFIRM 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.

Intravenous Treatment of Burn Injury

Studies at Rutgers-Cleveland Clinic Consortium (RCCC)

Overview: Burn injuries carry risks to survival, independence, and function, both in the short term and over time, as the injuries mature with scarring and contractures. Finding novel solutions to the challenge of burn injuries will improve outcomes for wounded warfighters. RCCC is conducting three projects that share the idea of treating burns through intravenous (IV) therapies. In Project 4.6.1, RCCC researchers are testing biologic agents that may prevent burn injury progression, reduce inflammation, induce healing, and inhibit scarring using established animal models. In Project 4.6.2, RCCC researchers are studying the regenerative capacity of mesenchymal stem cells (MSCs) with a focus on the potential of MSCs to regenerate tissue in massive skin wounds.

Status at End of Year 1: Project 4.6.1 began as a focused screening investigation for agents that may inhibit burn injury progression when administered intravenously. Screening experiments were performed in a rat hot comb burn model. The researchers identified two agents that significantly inhibited burn injury progression: fibronectin peptide P12, which is known to inhibit cell death, and the

A polymer synthesis robotic accelerator at Rutgers – The State University of New Jersey.

neutraceutical curcumin, which is found in the spice turmeric and is known to have anti-inflammatory, anticancer, antioxidant, wound healing, and antimicrobial properties. In Project 4.6.2, the researchers focused during the past year on establishing suitable animal models and identifying the most practical modes of delivery of MSCs in these models. They are pilot testing MSCs in the rat hot comb burn model of Project 4.6.1, the autologous engineered skin substitutes (ESSs) of Project 4.7.2, the rabbit ear scarring model of Project 4.6.3 (see the Scarless Wound Healing chapter), and a mouse excisional skin defect model that was established in their laboratory. Animal protocols and documentation have been approved, all laboratories have MSCs, and the test systems are now in place with sufficient staff and resources to proceed.

Research Plans for the Next 4 Years: The researchers of Project 4.6.1 intend to establish proof of principle in the most relevant animal model to the human condition to move P12 and/or curcumin forward to clinical trials. Since porcine skin closely resembles human skin, it is ideal to be used as the burn progression model. The researchers have begun testing optimal doses (according to the rat burn model) of P12 and curcumin in the porcine hot comb burn model and expect the first results to be available by August 2010. In Project 4.6.2, the researchers will test the efficacy of MSCs in animal models of skin wounds and burns, which will establish the preclinical basis for the testing of MSCs in clinical trials focusing on battlefield wounds. They will track the presented MSCs so that their site of action can be established. They will use immunocytochemical and histological analyses to establish the MSC dose-dependent kinetics of wound regeneration in each experimental situation.

Planned Clinical Transitions: The researchers of Project 4.6.1 have scaled up the Good Manufacturing Practice (GMP) production of both P12 and curcumin. Once proof of principle is obtained, they plan to file for orphan drug status with the U.S. Food and Drug Administration (FDA). They note that Phase 1 clinical trials could commence by Year 3. The program leader of Project 4.6.2, Dr. Arnold Caplan, is a founder of Osiris Therapeutics, Inc., which is a leading company in the preparation of human bone marrow-derived MSCs for a variety of inflammatory disorders. Several Osiris studies have advanced to Phase 2 clinical trials. The researchers note that a pathway to commercialization will be fairly clear once proof of principle is obtained in a relevant animal model. They are therefore considering moving immediately to the porcine model of Project 4.6.1 to determine whether their therapeutic approach to prevent burn injury progression is effective.

Topical Treatment of Burn Injury

Studies at RCCC

Overview: Prevention or reduction of battlefield infections would optimize wound healing and improve

Electrospun polymer fiber mat.

Rutgers University graduate student Charles Florek separates an electrospun polymer fiber mat from a stack of mats that he has prepared for use in animal studies.

outcomes following acute trauma and thermal injuries experienced by the warfighter. In three related projects, researchers at RCCC are testing the topical application of three therapeutic agents for the treatment of burns: iodine, peptide P12, and curcumin. In Project 4.6.4, researchers are testing the delivery of pure molecular iodine to a burn injury in an absorptive dressing that is optimized to achieve wound healing. In Project 4.6.5, researchers are engineering a drug delivery scaffold for the topical therapy of large, acute burn injuries that are unable to close. They are exploring the effects of sustained release of the fibronectin-derived peptide P12 in this scaffold model. In Project 4.6.6, researchers are testing the topical delivery of curcumin using nanospheres in a gel. In concurrent AFIRM projects (described in the preceding section), RCCC researchers are testing two of these agents, P12 and curcumin, for IV delivery in the treatment of burns.

Status at End of Year 1: In Project 4.6.4, the research team identified a polymer (repeating structural unit) 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 and moist, formalin-treated polyvinyl alcohol (PVA) sponge that releases molecular iodine into wounds in a controlled manner as fluids released from the wounds are absorbed. The researchers have begun to analyze the I-Plex device, including PVA-iodine complex preparation and the loading and release rates of iodine from both starch-foamed and air-foamed sponges. They determined that starch-foamed PVA sponges are best suited for in vivo studies. In Project 4.6.5, the researchers used a process known as electrospinning to create fibroporous scaffolds that can bind and deliver fibronectin-derived peptide P12 to wounds. They fully characterized the physical and chemical properties of the electrospun scaffolds and initiated release studies of P12 from the scaffolds. In Project 4.6.6, the research team demonstrated that curcumin, which is highly lipophilic (i.e., easily dissolves in fats, oils, and lipids but not in water), could be readily entrapped within a core of nanospheres derived from the amino acid tyrosine. They have formulated curcumin-loaded nanospheres to contain high and stable concentrations of curcumin. They determined that nanosphere-released curcumin was able to permeate to deep layers of human cadaver skin.

Research Plans for the Next 4 Years: In Project 4.6.4, the researchers plan to achieve proof of principle of the I-Plex wound dressing in a porcine-infected burn model, which has already been established at Stony Brook University (SBU). They also plan to develop better methods of estimating iodine pickup/loading rates from the PVA sponges. In Project 4.6.5, the researchers plan in Year 2 to conduct mechanistic studies of the electrospun scaffolds that were formulated during Year 1. Once the researchers identify a technique that loads P12 effectively onto their scaffold and releases it over a 1- to 7-day time interval, they will initiate proof-of-concept and safety studies of P12 in porcine wound and burn models. In Project 4.6.6, the research team plans during Year 2 to conduct proof-of-concept and safety studies of the nanosphere-curcumin formulation using a porcine burn model.

Planned Clinical Transitions: By the end of Year 2 or early in Year 3, the researchers of Project 4.6.4 plan to file a pre-market Notification Application (510(k)) with the FDA for their I-Plex wound dressing. They expect the I-Plex device to be in clinical trials following FDA clearance by Year 3 or 4. In Project 4.6.5, the researchers expect to initiate Investigational New Drug-related talks with the FDA by Year 3. They expect to make plans for clinical trials in Year 4. They also expect the necessary P12 stability and safety data to be obtained simultaneously in collaboration with researchers of the parallel AFIRM Project 4.6.1. In

Larisa Sheihet, PhD, Rutgers research professor testing the wettability of electrospun fiber mats.

Project 4.6.6, the researchers plan to initiate discussions with the FDA and the Center for Devices and Radiological Health for pre-investigational device exemption (IDE) review by Years 3–4 of the project. They will simultaneously conduct Good Laboratory Practice (GLP) safety and efficacy studies. In Year 4, they plan to initiate human clinical trials.

Studies at Wake Forest-Pittsburgh Consortium (WFPC)

Overview: Keratins are tough, fibrous structural proteins found in structures that grow from the skin (e.g., hair and nails). WFPC researchers of Project 4.2.3 are exploiting the heat-protective properties of topically administered keratins to ameliorate the progression of injury immediately following a burn. The researchers seek to deliver a topical therapy that would limit the progression of the "zone of coagulation" that develops following a thermal injury thus limiting the overall size and severity of the burn. Specifically, they plan to develop an in vitro model to study the mechanism of keratin's heat-protection that had been observed in earlier animal studies, determine the underlying mechanism, formulate an optimized keratin-based burn treatment that preserves damaged skin and reduces total burned surface area (TBSA), validate this treatment in pigs, and perform a human clinical trial on a prototype keratin burn dressing.

Status at End of Year 1: The researchers of Project 4.2.3 successfully developed a mouse skin cell isolation and culture system, which they are using to test the effects of several subtypes of keratin on recovery from thermal treatment as measured by cell proliferation assays. They performed preliminary burn injury studies in mice and pigs. In mice, they found that wounds treated with a keratin gel healed significantly faster than wounds treated with a control gel. In pigs, they observed that the regrowth of skin over thermal wounds was more rapid in keratin-treated animals versus controls.

A prototype system has been developed that uses ink-jet technology to print skin cells directly on burns (WFPC).

Research Plans for the Next 4 Years: The researchers hypothesize that certain keratin subtypes may be more potent than others. They will study the effects of keratin subtypes on thermal recovery using cell proliferation assays. They will begin to investigate changes in gene expression using microarrays. They will have a contract laboratory perform a comprehensive series of safety tests (required for a future clinical trial) on the keratin samples.

Planned Clinical Transitions: FDA-required safety studies are being conducted to enable a first-in-man study and eventually a human clinical burn trial. The researchers plan to request a pre-IDE meeting with the FDA in the third quarter of 2009. They are condensing the time allocated for animal model testing so that a clinical trial is now expected to commence during Year 5.

Wound Healing and Scar Prevention

Studies at WFPC

Overview: Replacement of the barrier function of skin is critically important to the survival of armed forces personnel while reducing scarring, and improving regenerated skin function would certainly increase the likelihood of returning to active duty. Regenerative medicine holds the potential to provide a functional replacement for skin. WFPC's projects related to wound healing and scar prevention support a variety of innovative technologies, including spray device delivery of stem cell replacements for skin cells (Project 4.2.2), artificial extracellular matrix (aECM) proteins for tissue repair following burn injury (Project 4.2.4), and the application of ink-jet technology to "print" skin onto excised burn wounds (Project 4.2.5). The research being conducted in these studies and importantly, clinical translation of the resulting technologies, may create a paradigm shift in the treatment of injured armed forces personnel.

Status at End of Year 1: In Project 4.2.2, the researchers have established a cell spray model that can "seed" the burn site with a desired cell type (e.g., fetal skin stem cells). They have also developed a Petri dish-based capillary membrane model of an active wound dressing that supports the growth of stem cells. In addition, they have established laboratory methods for the isolation and cell culture of fetal skin stem cells. In Project 4.2.4, the researchers have completed the design and construction of two new aECM proteins with properties that will be advantageous for use in regenerative therapy. They also prepared thin film matrix constructs that will be used to evaluate the wound-healing ability of the aECM proteins. In Project 4.2.5, the researchers completed the design and development of the portable skin printing device. They achieved delivery of skin cells directly onto skin defects in a mouse model using the device. They also determined that printed constructs healed faster than controls and were able to self-organize into skin that appeared almost identical to normal mouse skin.

Research Plans for the Next 4 Years: In Project 4.2.2, the researchers plan to test the suitability of human fetal skin stem cells for cryopreservation (i.e., storage at ultralow temperatures) by applying and comparing different regimes. They will evaluate logistical considerations for the establishment of a cell bank for human fetal skin stem cells. They will also test the effects of proteins called growth factors on these cells. In Project 4.2.4, the researchers will identify key design parameters of their aECM proteins that facilitate wound healing in cell culture studies on their thin film matrix constructs. They plan to begin animal studies with their aECM constructs by the beginning of Year 3 and complete physical characterization of the matrix constructs by the end of Year 3. The results of animal studies and physical characterization will guide the redesign and optimization of second-generation aECM constructs in Year 4. In Project 4.2.5, the researchers will continue to develop the portable skin printing device and will complete the rodent studies in Year 2. They will also complete the initial printer prototype for a pig model. In Years 3–5, the researchers will demonstrate the applicability of the skin bio-printing delivery system in a pig burn wound model. They will develop cell banks for a human clinical trial and refine and optimize the skin printing system for clinical use.

Planned Clinical Transitions: In Project 4.2.2, the researchers will prepare the spray device for FDA approval during Year 3. They will prepare for a human clinical trial and test the spray device for FDA approval during Year 4. They anticipate the commencement of a clinical trial for the spray device in Year 5. In Project 4.2.4, animal studies on second-generation constructs in Year 5 will provide the basis for clinical evaluation of the use of the aECM proteins in burn repair. In Project 4.2.5, progression to porcine burn injury models is anticipated within the next 2–3 years. The researchers plan to begin a clinical trial on the portable skin printing device in Year 5.

Skin Products/Substitutes

Studies at RCCC

Overview: 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 researchers of Project 4.7.2 aim to design and test new prototypes of ESSs that restore skin color and develop vascular networks thereby resulting in improved outcomes in recovery from life-threatening burns. They also seek to initiate commercial availability of the original model of ESSs.

Status at End of Year 1: The researchers have established advanced models of ESSs with pigmentation and vascularization. They demonstrated feasibility for

WFPC researcher Dr. Joerg Gerlach with the skin cell spray device.

advancement to animal studies in Year 2. Clinical studies with ESSs at the U.S. Army Institute of Surgical Research (USAISR) have been proposed and will continue to be pursued for the prospective benefits of wounded warfighters.

Research Plans for the Next 4 Years: In Years 2–3, the researchers plan to study the regulation of various types of skin cells in ESSs, including melanocytes, keratinocytes, endothelial cells, and fibroblasts. They also plan to measure the efficiency of pigment expression after transplanting ESSs in immune-deficient mice, test tumorigenicity in immune-deficient mice, and fabricate a matrix with interconnected channels that mimic a vascular network. In Years 4–5, the researchers plan to study the regulation of pigment expression in transplanted ESSs. They also plan to transplant endothelialized ESSs to mice and perform a quantitative evaluation of the perfusion.

Planned Clinical Transitions: The researchers aim to translate ESSs to clinical trials. If plans for this clinical study proceed without delays, the researchers feel that the first burn patients will be treated at USAISR by July 2010.

Stem cells derived from amniotic fluid are expanded in a WFPC laboratory.

Studies at WFPC

Overview: The need for an "off-the-shelf" skin replacement that is instantly available and alleviates the need to take a split or full thickness skin graft has long been sought. Project 4.2.1 aims to deliver an off-the-shelf, composite skin substitute for use in full-thickness thermal injuries. Two industry partners, Organogenesis, Inc. (Project 4.2.1a) and Intercytex (Project 4.2.1b), are rapidly developing separate and distinct products that will be tested in clinical trials. The goal of Project 4.2.6 is to utilize stem cells from amniotic fluid-derived stem cells (AFSCs) or comparable cells from other perinatal sources (e.g., placenta), to develop an improved off-the-shelf bioengineered skin product for the treatment of extensive burns.

Status at End of Year 1: In Project 4.2.1a, the researchers established a project management structure with the initiation of Design Control. They also established cell banks for porcine skin cells. They refined the porcine self-assembly skin matrix. Finally, they began development of a porcine wound model and, in parallel, a human skin matrix. In Project 4.2.1b, the researchers have made substantial progress in the development of a "biological dermis" composed of human fibroblast cells. They developed a casting dish for preclinical production of the biological dermis and identified a suitable freeze-drying process. They developed a burn pig protocol. They characterized maturation of the skin constructs by high-powered microscopy and tissue-staining techniques. Finally, they identified ultrasound stimulation protocols that greatly increase the mechanical and biological handling characteristics of the constructs. In Project 4.2.6, the researchers demonstrated that human AFSCs (hAFSCs) express CD146, a cell surface marker protein recognized as a marker of perivascular cells (i.e., cells flanking blood vessels). They also isolated and expanded CD146-positive perivascular cells from placenta (canine and human). They demonstrated enhanced skin wound healing by hAFSCs in immune-deficient mice.

Research Plans for the Next 4 Years: In Project 4.2.1a, the researchers during Year 2 plan to complete final development and validation of the porcine model. Multiple iterations of both porcine and human tissue constructs will be developed to determine the spectrum of feasible constructs and the optimum conditions for culture and processing. The researchers will complete the final preclinical implementation in Year 3. They will also execute the porcine burn model. Clinical trial preparations will begin in Year 4.

In Project 4.2.1b, the researchers plan to produce material for the burn pig study, perform the first

pig implantations in Pittsburgh, analyze the results, and determine which iteration(s) is suitable for taking forward. They also plan to determine options and timings for epithelialization (i.e., growth of skin over a denuded surface). They will conduct follow-up pig studies to fully determine the remodeling needed to achieve skin replacement. They also plan to conduct the first human study to compare human versus pig remodeling.

In Project 4.2.6, experiments in Year 2 will focus on testing conditions previously reported to induce skin-like cells from human MSCs (hMSCs), which share many markers with hAFSCs. The researchers will begin in Year 2 to try to combine immature hAFSCs with candidate skin stem cells to attempt to drive the differentiation of hAFSCs into epidermal skin cells (e.g., keratinocytes and melanocytes). In Year 3, the researchers will begin to assess the interaction of cells of a dermal lineage with hAFSC-derived epidermal lineage cells both in cell culture and in animal models. Bioprinting of skin, being developed in a parallel AFIRM burn project, will offer another, potentially more rapid and accessible, approach to assess the interaction of hAFSC-derived dermal and epidermal cell types in animal models. In Years 4–5, the main focus will be on further optimization of differentiation to generate the skin equivalent and testing in rodent models.

Planned Clinical Transitions: The research team of Project 4.2.1a anticipates the start of a clinical trial by Year 5 of the project. The researchers of Project 4.2.1b aim to begin a clinical trial focused on their ICX-SKN constructs by the first quarter of 2012. Transition of Project 4.2.6 to clinical studies is anticipated to occur 2 years after the 5-year project ends. Success in bio-printing with undifferentiated stem cells and hAFSC-derived epidermal lineage cells could potentially accelerate clinical translation of the hAFSCs.

Clinical Trials

Two clinical trials have been added to the Burn Repair Program at WFPC.

Clinical Trial 1
A Comparative Study of the ReCell® Device and Autologous Split Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries
Principal Investigator – James H. Holmes IV, MD
This is an FDA-approved trial for ReCell. ReCell is a technique whereby a small (~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 dermal-epidermal junction are harvested and immediately applied to an excised burn wound via a syringe at an expansion ratio of 80:1 (~320 cm^2). From the approximately 4 cm^2 of skin, approximately 320 cm^2 of burn can be "grafted" using the patient's own cells without the need for any culture techniques. This technology has the potential to radically alter modern burn surgery. Patient enrollment will begin in the fall of 2009 with the trial completion estimated to be 18–24 months later.

Clinical Trial 2
In Vitro Expanded Living Skin for Reparative Procedures
Principal Investigators – Sang Jin Lee, PhD; James J. Yoo, MD, PhD; James H. Holmes IV, MD
This initial Phase 1 trial will analyze the safety of harvesting, expanding, and then grafting a piece of skin from a burn patient. An approximately 40 cm^2 split-thickness piece of skin will be harvested at the initial operation from a burn patient who is anticipated to require multiple acute operations. The skin will be expanded in a bioreactor to approximately 100 cm^2 over 2 weeks. The skin will then be "grafted" back onto the patient in the standard manner, with or without meshing, at the next operation. This technology also has the potential to significantly alter modern burn surgery as it will produce an alternative for generating "more" skin for grafting when faced with limited donor sites.

Cells are applied to an excised burn wound via a syringe at an expansion rate of 80 to 1 (WFPC clinical trial).

Project 4.6.1, RCCC

Prevent Burn Injury Progression, Reduce Inflammation, and Induce Healing in a Rat Hot Comb Model by Determining the Optimal Doses and Treatment Times for Systemically Administered MSCs, Curcumin, Pentoxifylline, and P12

Team Leader(s): *Richard Clark, MD (SBU)*

Project Team: *Adam Singer, MD, Fubao Lin, PhD, Monica McTigue, PhD (SBU)*

Collaborator(s): *Corporate Partner: NeoMatrix Formulations, Inc.*

Therapy: *Prevent burn inflammation and injury extension with IV therapy*

Deliverable(s): *IV curcumin and IV fibronectin P12*

Key Accomplishment(s): *Four agents were screened for their ability to inhibit burn injury progression when administered intravenously: curcumin, a purported antioxidant; deferoxamine, a potent iron chelator; pentoxifylline, a tumor necrosis factor-alpha inhibitor; and P12, an anti-apoptotic fibronectin peptide. Both curcumin and P12 demonstrated remarkable ability to inhibit burn injury progression in a rat hot comb model.*

Introduction

Battlefield polytrauma secondary to blasts and explosions is increasingly common, affects multiple sites, and is complex. Progressive extension of burns and other battlefield injuries can have devastating effects, both acute and chronic. In the short term, deep second-degree burns often become full-thickness third-degree burns, which lead to increased tissue loss, longer healing time, excess morbidity, and mortality. Longer-term, chronic effects include increased scarring, wound contractures, and poor quality of life. Therapies such as NSAIDs and anticoagulants (heparin) have not shown substantial benefit in preventing burn injury progression to date. This project will focus on therapies that researchers hope will ultimately prevent burn injury progression, reduce inflammation, induce healing, and inhibit scarring in burn victims.

Reperfusion injury is the medical term for tissue damage that results when blood supply returns to damaged tissue (such as a burn) after a period of ischemia; this can include inflammation. Reperfusion injury has three major consequences: cytokine release, generation of reactive oxygen species, and markedly increased programmed cell death (apoptosis). The researchers identified five therapeutic agents with low-risk profiles that target those consequences: (1) MSCs derived from human bone marrow MSCs; (2) pentoxifylline, which inhibits tumor necrosis factor-alpha production; (3) curcumin, a potent antioxidant; (4) deferoxamine, a potent iron chelater; and (5) P12, a peptide made from fibronectin, which has a remarkable ability to decrease programmed cell death. Curcumin, deferoxamine, pentoxifylline, and P12 are being studied in Project 4.6.1 while MSCs are being examined in Project 4.6.2.

Using a rat hot comb burn model, the researchers seek to determine the optimal doses and treatment times for systemically administered P12, curcumin, pentoxifylline, and deferoxamine. They also plan to determine the effects of P12 and curcumin on oxidative stress-induced cell death in in vitro models.

Research Progress – Year 1

Project 4.6.1 began as a focused screening investigation for agents that may inhibit burn injury progression. Screening experiments performed in a rat hot comb burn model demonstrated that P12 maximally inhibited burn injury progression (90% inhibition when administered at 10 mg/kg in lactated ringers 1 and 24 hours after burns) while curcumin was more potent but had a less maximal effect (65% inhibition at 3 µg/kg). Pentoxifylline had minimal activity. The ability of IV P12 to inhibit burn progression at 7 days post-injury is shown in **Figure V-2**. Since it is doubtful that curcumin works as a direct antioxidant (this would

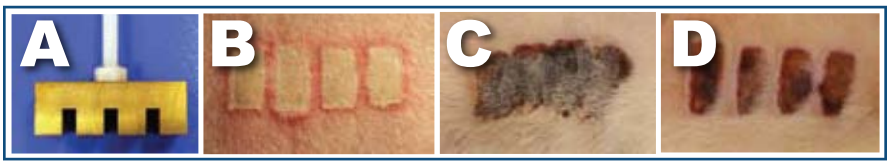


Figure V-2. IV infusion of P12 1 and 24 hours after burn inhibits injury progression in a rat hot comb model. (A) Brass comb. (B) Burn immediately after injury. (C) Burn at 7 days after injury without treatment. (D) Burn at 7 days after injury that had been treated with 10 mg/kg P12.

require millimolar intracellular levels), the researchers hypothesized that it worked indirectly by binding iron, a known attribute. Although curcumin-bound iron is not completely inactive, this binding has been touted to prevent the Fenton reaction (an iron-dependent pathway that generates hydroxyl radicals from hydrogen peroxide). Therefore, the researchers tested deferoxamine, a potent FDA-approved iron chelator that holds iron in an inactive state, in the rat burn model. Surprisingly, it had minimal activity. When P12 and curcumin were tested in vitro for their ability to inhibit oxidative and cytokine stress in adult human dermal fibroblasts and human dermal microvascular endothelial cells (HDMECs), P12 had a striking ability to do so at 30 µM (the calculated in vivo effective concentration) while curcumin had no effect at 10–50 nM (the calculated in vivo effective concentration). At micromolar concentrations (levels often used in cell culture studies), curcumin was cytotoxic.

Key Research Accomplishments

- Established a rat hot comb model for burn injury progression. Screened several agents but found that only P12 and curcumin significantly inhibited burn injury progression.
- Established in vitro models using normal adult human dermal fibroblasts for oxidative and cytokine stress-induced cell death. Found that P12 inhibited oxidative and cytokine stress-induced death of adult human dermal fibroblasts.

Conclusions

The researchers demonstrated that P12 and curcumin significantly inhibit burn progression in a rat hot comb model. They established two in vitro models of oxidative stress-mediated cell death. P12 was found to protect cells from both hydrogen peroxide and hypoxanthine/xanthine oxidase-mediated killing, suggesting that P12-induced cell resistance to oxidative stress may play an important role in preventing burn injury progression. Curcumin cannot protect cells from oxidative stress-mediated cytotoxicity when added together with oxidative stress, suggesting that other mechanisms are involved in its effects on preventing burning injury progression. These results indicate that P12 or curcumin could be developed into therapies to prevent burn progression and reduce morbidity and mortality in patients with extensive and severe burns.

Research Plans for the Next 4 Years

The researchers intend to establish proof of principle in the most relevant animal model to the human condition to move P12 and/or curcumin forward to clinical trials. Since porcine skin closely resembles human skin, it is ideal to be used as the burn progression model. This large animal model choice has been confirmed by Jules Mitchel, President, Target Health, a virtual pharmaceutical company in New York, New York. The researchers have begun testing optimal doses (according to the rat burn model) of P12 and curcumin in the porcine hot comb burn model and expect the first results to be available by August 2010.

Planned Clinical Transitions

The researchers have scaled up the GMP production of both P12 and curcumin. As soon as they obtain proof of principle from experiments with either of these agents, they plan to file for orphan drug designation with the FDA (both agents meet the criteria for orphan drug designation, that is, an unmet need in a disease or disorder affecting less than 200,000 individuals in the United States per year). The researchers have already initiated plans for this filing through their contacts at Target Health. In addition, they have acquired estimates for protein stability tests for P12 and toxicokinetic studies for either agent through MicroConstants, Inc., San Diego, California and WIL Research Laboratories, LLC, Ashland, Ohio, respectively. It is possible, if all goes well, that Phase 1 clinical trials could commence by Year 3.

Corrections/Changes Planned for Year 2

None.

Project 4.6.2, RCCC

Bone Marrow-Derived Mesenchymal Stem Cell Intravenous Treatment to Prevent Burn Injury Progression

Team Leader(s): *Arnold Caplan, PhD, Case Western Reserve University*

Collaborator(s): *Dr. Glenn Prestwich (University of Utah); Dr. Richard Clark (SBU); Dr. Steven Boyce (University of Cincinnati); Dr. Thomas Mustoe (Northwestern University)*

Therapy: *Treatment of burn injury progression*

Deliverable(s): *IV bone marrow-derived MSCs*

Key Accomplishment(s): *A mouse excisional skin defect wound model was developed that allowed quantitative fluorescent imaging of the localization of infused adult MSCs.*

Introduction

Adult bone marrow-derived MSCs can differentiate into many types of cells and can thus be active components in tissue engineering, helping reform injured tissues. MSCs also have a newly discovered, profound therapeutic use, which is their ability to secrete bioactive molecules that calm or neutralize massive, chronic inflammatory processes while establishing the microenvironment to enhance tissue-intrinsic regeneration events. The key to their therapeutic efficacy is the manner in which they are applied or directed to the wounded or injured tissue. The researchers are testing hMSCs in cases of huge excisional wounds and burns. They hope the regenerative capacity of MSCs, and their newly discovered ability to secrete anti-inflammatory molecule, will help heal large burns in both military and civilian wounded.

Research Progress – Year 1

The researchers have begun to track human bone marrow-derived MSCs in a mouse excisional wound model. The research is being conducted at the Caplan laboratory at Case Western Reserve University in consultation with Dr. Richard Clark of SBU, Dr. Steven Boyce of the University of Cincinnati, and Dr. Thomas Mustoe of Northwestern University. The team's activity for this past year was directed at establishing suitable animal models, identifying the most practical modes of delivery, and testing the therapeutic use of hMSCs in different yet relevant circumstances. Notably, the use of hMSCs in rodent models has been established in the Caplan laboratory using other disease models.

Ongoing work includes (1) Dr. Clark's comb/burn model; (2) Dr. Boyce's skin substitute; (3) the mouse excisional skin defect, which the team established and conducted initial experiments with to optimize the surgery and to pick reasonable, measurable, outcome parameters (**Figure V-3**); and (4) Dr. Mustoe's rabbit ear scarring model, for which Drs. Caplan and Mustoe designed rabbit experiments to use hMSCs to determine if infusion versus onlay delivery tech-

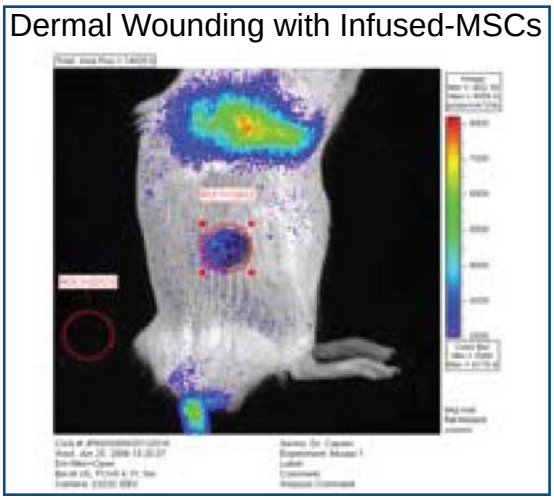


Figure V-3. Systemically introduced luciferase-marked MSCs can be visualized noninvasively in a live subject following infusion of MSCs.

niques could be used to inhibit the scar formation routinely observed in this model.

Initial experiments in the rat hot comb model performed at SBU found that MSCs provided by the Caplan laboratory, but cultured in the Clark laboratory, demonstrated minimal activity in the rat hot comb model. Several issues, however, arose about the protocol; thus, repeat experiments are pending. MSCs have also been provided by the Caplan laboratory to the Mustoe laboratory (Project 4.6.3) and Boyce laboratory.

Key Research Accomplishments

- Established the test systems with sufficient staff and resources to proceed.
- Received approval on all animal protocols and documentation.
- Completed pilot experiments in June 2009.

Conclusions

The research team is now well situated to provide a multipronged test and optimization of the delivery of therapeutically active hMSCs to different skin wound sites as a proof of concept of their efficacious activity in clinically relevant animal models.

Research Plans for the Next 4 Years

In Year 2, the research team will test the efficacy of MSCs in animal models of skin wounds and burns to establish the preclinical basis for the testing of allogeneic MSCs in clinical trials focusing on battlefield wounds. Using the four experimental animal situations outlined previously, they plan to complete statistically relevant trials that can test the optimal delivery technology and optimal dose of MSCs for wounds and burns. They will track the presented MSCs so that their site of action can be established. They will use immunocytochemical and histological analyses to establish the MSC dose-dependent kinetics of wound regeneration in each experimental situation. By using selected animal situations, they will attempt to identify the key regulatory step(s) or molecule(s) that controls the efficacious activity of MSCs. Where possible, the researchers will compare MSCs from different tissues (e.g., fat, muscle, and marrow) in models following the completion of experiments using marrow-derived MSCs.

Planned Clinical Transitions

The program leader of this project, Dr. Arnold Caplan, is a founder of Osiris Therapeutics, Inc., which is a leading company in the preparation of human bone marrow-derived MSCs for a variety of inflammatory disorders. Several Osiris studies have advanced to Phase 2 clinical trials. The researchers note that a pathway to commercialization will be fairly clear once proof of principle is obtained in a relevant animal model. They are therefore considering moving immediately to the porcine model to determine whether their therapeutic approach to prevent burn injury progression is effective.

Corrections/Changes Planned for Year 2

None.

Project 4.6.4, RCCC

Topical Iodine Treatment of Burns to Prevent Infection

Team Leader(s): *Richard Clark, MD (SBU); Mason Diamond, DDS, PhD (Rutgers – The State University of New Jersey, New Jersey Center for Biomaterials [NJCBM])*

Project Team: *Niraj Ramachandran, PhD (Rutgers – The State University of New Jersey, NJCBM)*

Collaborator(s): *Sol Rosenblatt, Plas Tech Company*

Therapy: *Prevention of wound infection following burn injury*

Deliverable(s): *Molecular iodine-releasing polymer wound dressing*

Key Accomplishment(s): *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.*

Introduction

Unlike most injuries encountered in hospitals, battlefield wounds are polytraumatic in nature, involving multiple mechanisms of injury to multiple anatomical sites. In burn-related injuries that affect more than 40% of the total body surface area, 75% of deaths stem from infection. Antibiotics and antimicrobial agents may help after the fact, but their side effects include microbial resistance, allergic potential, and tissue toxicity. If clinicians can prevent or reduce battlefield infections, wound healing will be optimized and outcomes improved following acute trauma and thermal injuries experienced by the warfighter. Serious morbidity and mortality from wound infections are not limited to the battlefield; antibiotic-resistant bacterial infections now increasingly occur in the hospital, which poses a major health risk to the general population. Infection control is a key determinant in successful management of both acute and chronic wounds in civilian and military populations.

The ideal product for preventing infection would target a broad spectrum of microbes, be biocompatible, not produce resistant bacteria, and be unlikely to cause sensitivity or allergy. Topical iodine has been an effective antimicrobial for several centuries. It is effective against all deleterious microbes, does not produce resistance in bacteria, and does not produce allergies. All of the aforementioned factors make iodine ideal for traumatic wounds requiring immediate and effective treatment. The researchers of this project seek to deliver pure molecular iodine to a burn injury in a matrix that is optimized to achieve wound healing.

Research Progress – Year 1

Since some infected wounds produce exudates from tissue necrosis, the researchers chose to employ a dressing that manages microbial wound contamination and infection and also absorbs exudates. The I-Plex absorbent antimicrobial wound dressing is a nonsterile, nonadherent and moist, formalized PVA absorptive dressing that contains complexed and small amounts of free molecular iodine (**Figure V-4**), which is released in a controlled manner into the wound as exudates are absorbed.

Initial proof-of-concept and biocompatibility testing was conducted

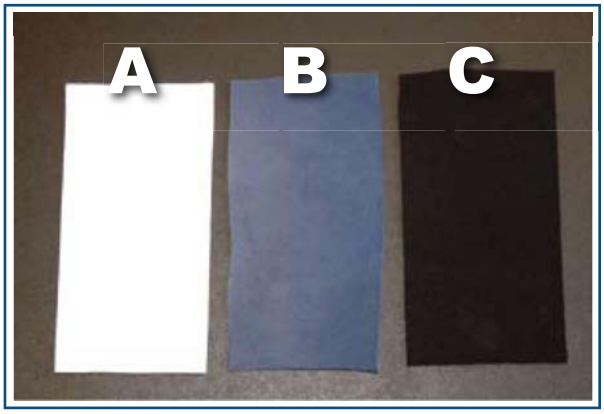


Figure V-4. PVA sponge samples: (A) before iodine complexation, (B) partially (light blue) saturated with molecular iodine, and (C) fully (black) saturated with molecular iodine.

more than 15 years ago. Under the RCCC AFIRM program, the NJCBM at Rutgers, in collaboration with Dr. Richard Clark of SBU and the original inventor (retired chemist Sol Rosenblatt), is spearheading the effort to establish product specifications, test methods, and determine biocompatibility. The researchers plan to compile and file a pre-market Notification Application (510(k)) with the FDA. They expect that the I-Plex dressing will be included in clinical trials (as a nonsignificant risk device) following FDA clearance.

In Year 1, the researchers conducted a preliminary analysis on the I-Plex device, including PVA-iodine complex preparation. The researchers used two kinds of PVA sponges for complex formation: starch-foamed and air-foamed. Currently, only the starch-foamed sponge will be used for the preliminary rat study to be carried out at SBU. The team established a preliminary specification of maximum iodine loaded in the PVA sponge as approximately 4%–6% by weight of the total device. They estimated the percentage of iodine released from the sponge. Using the media method, researchers established that all of the iodine is released within 5 to 6 hours. The rate of release of iodine from the air-foamed and open-celled sponge is much faster than the starch-foamed sponge. An important observation is that even though at the end of 5 hours only about 30%–35% of elemental iodine is detected, the sponge has been rendered completely white, indicating that all of the bound iodine has been released.

Key Research Accomplishments

- Completed feasibility studies for the preliminary evaluation of the I-Plex device, including fabrication of the PVA sponge and complexing iodine with the sponge.
- Estimated the iodine loading and release rates from the PVA sponge for both starch- and air-foamed materials.
- Scaled up and used the complexing procedure for developing samples for the pilot rat study to be carried out at SBU during the summer of 2009.
- Established a quality control procedure in which samples made from each batch were tested for the weight of iodine loaded.
- Performed ultraviolet-visible absorption spectra of the iodine complexing bath after complexing to match the absorption spectra for different batches after iodine was loaded.

Conclusions

Iodine-PVA sponges provide a very simple, convenient, nonadherent bandage, which can be conveniently used in military applications where a premium is placed on keeping out dirt, maintaining an antimicrobial environment around the wound, and absorbing exudates. Bound elemental iodine is released from the I-Plex sponge only in the presence of the organic material that is found in the wound. The PVA sponge turns white when placed on a wound site, indicating that the iodine has been released. This dressing will be used in ongoing research on wound-healing studies in rats and pigs. The researchers' ultimate goal is to employ this sponge to prevent infection. They note that the sponge can serve as an alternative to antibiotics, which treat infection after the fact, when more damage has been done and more side effects may occur. This should improve morbidity and mortality and reduce complications and healing time for patients suffering from both acute and chronic burn wounds for both the military and civilians.

Research Plans for the Next 4 Years

The research team plans to achieve proof of principle in a porcine-infected burn model, which has already been established at SBU. They also plan to develop product specifications and evaluate biocompatibility. However, the researchers note that several important issues need to be addressed before proceeding to animal studies. First, the formalized PVA sponge used in this application is a crosslinked matrix, and determining what forms of iodine are incorporated into the sponge is difficult. The iodine pickup and loading rates were estimated using two methods (bath depletion and direct weight gain of the sponge), which are both rough estimates. The team aims to develop better methods during Year 2. Second, the distribution and density of iodine species loaded onto the PVA sponge device need to be better characterized. Third, since this material is obtained from an external source, the research

project team needs specifications and quality control data that are not yet available. Fourth, after the team establishes what kind of iodine species is loaded (polyiodides or polyiodines), it will establish methods for testing the release of each individual species. The current release testing method, used in Year 1, detects only molecular iodine.

Planned Clinical Transitions

By the end of Year 2 or early in Year 3, the researchers of this project plan to file a pre-market Notification Application (510(k)) with the FDA. They expect the I-Plex Absorbent Antimicrobial Wound Dressing to be in clinical trials (as a nonsignificant risk device) following FDA clearance by Year 3 or 4.

Corrections/Changes Planned for Year 2

The researchers initially planned to investigate the possibility of integrating Symbollon's molecular iodine generating system with a two-phase polymer system from the Kohn laboratory. By the summer of 2008, after carefully reviewing the required components of such a system, Dr. Kohn concluded that the feasibility of integrating these systems was very low. He noted that available tyrosine-derived polymers are only soluble in organic solvents; hence, creating delicate balanced mixtures of the water-soluble Symbollon redox system within these polymers would be difficult. However, Dr. Kohn was able to identify another polymer system that would be feasible for the release of molecular iodine. The system, called the I-Plex Absorbent Antimicrobial Wound Dressing, is a nonadherent, moist, formalin-treated PVA absorptive dressing. The PVA sponge contains complexed (and small amounts of free) elemental iodine, which is released into the wound as exudates are absorbed by the polymer.

Project 4.6.5, RCCC

Topical P12 Treatment of Burns Using Fibroporous Mat

Team Leader(s): *Richard Clark, MD (SBU)*

Project Team: *Lauren Macri,(SBU); Stefan Salomon, PhD, Joachim Kohn, PhD (Rutgers – The State University of New Jersey, NJCBM)*

Therapy: *Speed generation of a viable wound bed and reduce reharvest time of autograft donor sites*

Deliverable(s): *P12-polymer composite*

Key Accomplishment(s): *Electrospun tyrosine-derived polymer mats were fabricated that bind and deliver fibronectin-derived peptide P12 in vitro. The polymer compositions were tuned for controlled biodegradation rates and drug (P12) release.*

Introduction

Although burn care has advanced over the years, there is still a critical need to develop treatments that promote robust burn wound healing to reduce any complications and impairments associated with autografting. The overall aim of this project is to engineer a drug-delivery scaffold for the topical therapy of large, acute burn injuries that cannot be closed. The researchers seek to determine whether the sustained release of the fibronectin-derived peptide P12 can prevent burn injury progression, accelerate tissue formation in third-degree burns with excised wound beds, facilitate skin graft survival, and speed growth of new tissue in donor sites.

Research Progress – Year 1

The researchers accomplished the tasks of fabricating and characterizing the engineered drug delivery scaffold during Year 1. First, the electrospinning process, which uses electrical charges to synthesize long, continuous fibers (**Figure V-5**), was used to fabricate a fibroporous mat made of tyrosine-derived copolymers for the delivery of P12. Second, the stability of P12 in the organic solvent, hexafluoroisopropanol (HFIP), which was used during the electrospinning process, was assessed. The researchers found that P12 remains biologically active after exposure to HFIP. However, HFIP has recently been shown to interact with P12 thereby causing conformational changes and subsequent prevention of efficient binding and/or release of P12. Third, the physical and chemical properties of the electrospun fibroporous mats were fully characterized. The researchers observed no significant change in fiber diameter and morphology of mats containing P12 compared to control mats (**Figure V-6**). The loading of P12 in fibroporous mats was shown to produce a fibrillar distribution of P12 within the sample. Fourth, the effect of polymer composition and the presence of P12 on the degradation of fibroporous mat were investigated. The researchers confirmed that an increased percentage of hydrophilic and charge-bearing copolymer components accelerates the degradation of the mat, which is in agreement with previous reports for tyrosine-derived materials. In addition, they found that the lifetime of

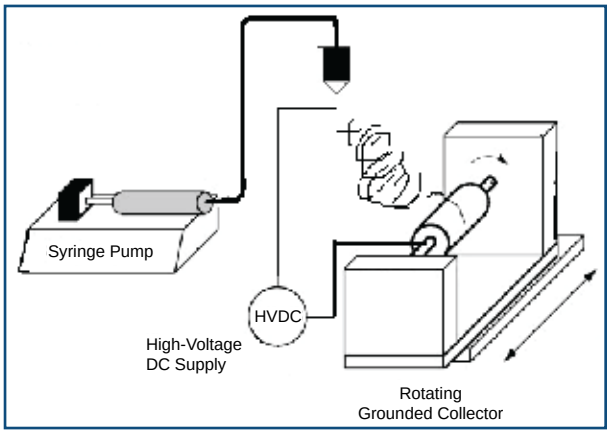


Figure V-5. The electrospinning process (adapted from T. J. Sill and H. A. von Recum, *Biomaterials* 29, 1989 [2008]).

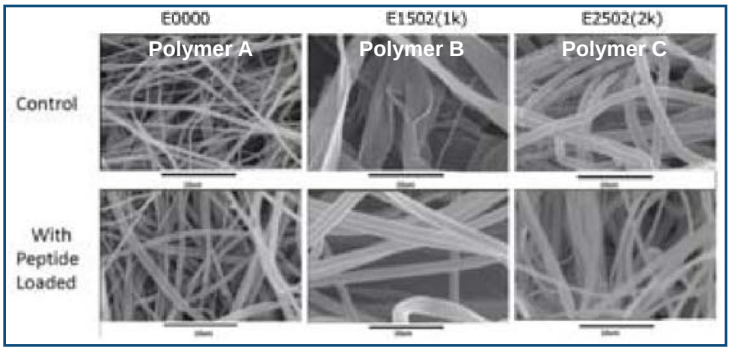


Figure V-6. Scanning electron microscope images of electrospun fibroporous mats (magnification = 5000x, scale bar = 10 µm) made with three different tyrosine-based copolymers.

the P12-loaded fibroporous mats was dramatically lengthened when compared to unloaded fibroporous mats. Finally, the research team has initiated the release studies of P12 from a fibroporous mat.

Key Research Accomplishments

- 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.
- Achieved fabrication and characterization of the engineered P12 delivery scaffold: P12-containing fibroporous mats were successfully produced using the electrospinning technique.
- Evaluation of P12 release kinetics from electrospun fibroporous mat is ongoing.

Conclusions

The research team's novel engineered construct is composed of an electrospun fibroporous mat fabricated from a tyrosine-derived biomaterial. Researchers believe that this mat may simultaneously degrade and deliver a novel, bioactive P12 over a 1-week time period, a time relevant to the most crucial stages of cutaneous wound repair after third-degree burns. The electrospun fibroporous mat is derived from the tyrosine-derived copolymers, which are biocompatible and possess tailorable degradation kinetics, depending on the specific chemical composition including the presence of P12. The researchers believe that this novel technology may (1) prevent burn injury extension and (2) promote the formation of granulation tissue in excised third-degree burns and at the interface where implanted autografts, allografts, or engineered skin replacements connect with the underlying host tissue. Future work will include in vitro biological studies and in vivo pig excisional wound-healing studies.

Research Plans for the Next 4 Years

The researchers plan during the upcoming year to conduct mechanistic studies aimed at testing the biocompatibility and conductive/inductive capabilities of the electrospun fibroporous mats that were formulated during Year 1. In vitro tests with adult human dermal fibroblasts and human epidermal keratinocytes will be used in these studies. Once they identify a fabrication technique that loads P12 effectively and releases it over a 1- to 7-day time interval, they will initiate proof-of-concept and safety studies of P12 in porcine models of excisional wounds and excised burns.

Planned Clinical Transitions

The researchers expect to initiate Investigational New Drug-related talks with the FDA by Year 3. They expect to make plans for clinical trials in Year 4. They expect the necessary P12 stability and safety data to be obtained simultaneously in collaboration with researchers in the parallel AFIRM Project 4.6.1.

Corrections/Changes Planned for Year 2

The research team found that only 3%–4% of P12 was binding to the tested polymers, which they feel may be secondary to the fabrication technique. The researchers plan to focus on further optimizing the electrospinning process, simultaneously with investigating an alternative fabrication technique (e.g., compression molding).

Project 4.6.6, RCCC

Curcumin-Loaded Nanospheres as a Topical Therapy to Limit Burn Injury Progression and to Promote Non-Scar Healing

Team Leader(s): *Larisa Sheihet, PhD (Rutgers – The State University of New Jersey, NJCBM); Richard Clark, MD (SBU)*

Project Team: *Joachim Kohn, PhD, Stefan Salomon, PhD (Rutgers – The State University of New Jersey, NJCBM); Adam Singer, MD (SBU)*

Therapy: *Prevent burn inflammation and injury extension with topical therapy*

Deliverable(s): *Curcumin-loaded nanosphere gel*

Key Accomplishment(s): *The feasibility of formulating curcumin-loaded tyrosine-derived nanospheres for topical delivery was demonstrated. The nanospheres significantly enhanced penetration of curcumin to human cadaver skin deep epidermal strata in vitro, and fluorescent model compounds in porcine deep epidermis in vivo, compared to skin penetrations achieved with propylene glycol (PG) (control) formulations.*

Introduction

Although respiratory distress is often the critical issue immediately after burn injury, skin loss becomes the major problem within the next 24 hours, secondary to barrier disruption, which leads to fluid and heat loss and possible infection. Burn injury progression, wound infection, delays in grafting, and grafting with inadequate material such as meshed autograft can all lead to increased scarring with the possibility of severe wound contractures. The therapy proposed in Project 4.6.6, topical application of curcumin in a nanosphere form, could potentially address one or more of the primary issues stated above and thus may reduce more severe wound scarring and contractures in the long term.

Research Progress – Year 1

Aim 1: *Design, prepare, and characterize the curcumin-loaded nanospheres and their gel formulations.*

The researchers demonstrated that curcumin, which is highly lipophilic (i.e., easily dissolves in fats, oils, and lipids but not in aqueous solutions), could be readily entrapped within a core of tyrosine-derived nanospheres. This formulation provided high and stable concentrations of curcumin, which may potentially improve its bioavailability. Curcumin entrapped in nanospheres is seven times more stable than a similar concentration dispersed in phosphate buffered saline (PBS). Additionally, the researchers demonstrated that curcumin's stability within nanospheres was similar to the stability measured in curcumin-PBS containing 4% (w/v) bovine serum albumin (BSA). Although 4% w/v BSA-PBS solution could be considered a much cheaper vehicle for curcumin delivery, the researchers suspect that curcumin release will be more controlled when administered via nanospheres. The nanospheres release curcumin in a rate-controlled manner, which could help maintain curcumin levels within the therapeutic window, preventing both toxic and nontherapeutic doses. These results confirm that the use of a properly designed delivery vehicle can significantly improve the efficacy of difficult-to-uptake drugs.

Aim 2: *Investigate the percutaneous penetration of curcumin-loaded nanospheres in two models of non-burned skin: in vitro cadaver skin and in vivo porcine skin.*

Ex vivo skin permeation tests on human cadaver skin confirmed that nanospheres significantly enhanced permeation of curcumin and Nile red to deeper layers of human cadaver skin, compared to a non-particles-based vehicle, PG (**Figure V-7**). Measured curcumin penetration was about 30% higher when applied in nanosphere formulation. The in vivo porcine skin model also showed that nanospheres, when formulated in a gel or water-based suspension, delivered significantly higher amounts (2.3-fold and 1.4-fold, respectively) of Nile red (lipophilic model compound)

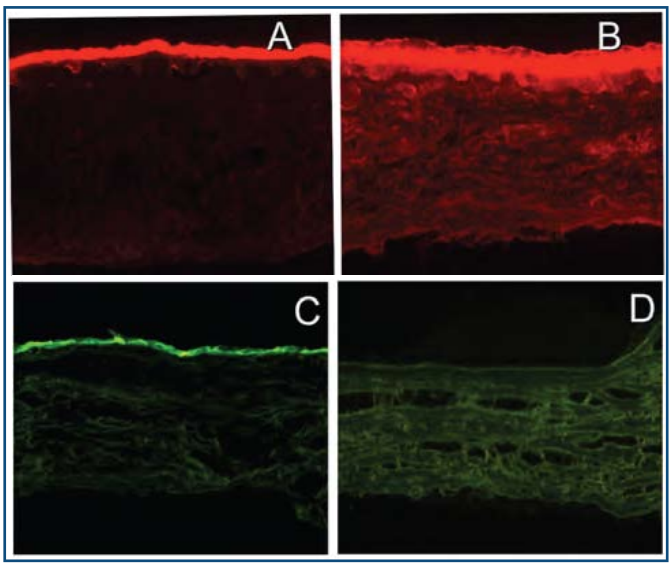


Figure V-7. Cross-sectional images of skin sections obtained following 24 hours of passive permeation.
(A) PG-Nile red, (B) Nile red-loaded nanospheres, (C) PG-curcumin, (D) curcumin-loaded nanospheres.

compared to the PG formulation. Formulation of the nanospheres in gel significantly increased their efficacy, compared to both water-based solutions and PG, due to viscosity that provided close skin contact and superior hydration.

Key Research Accomplishments

- Formulated curcumin-loaded nanospheres to contain stable high amounts of curcumin compared to curcumin dispersion in PBS.
- Confirmed non-concentration-dependent and sustained-release profile of curcumin from curcumin-loaded nanospheres.
- Determined that nanospheres significantly enhanced permeation of lipophilic Nile red and curcumin to deeper human cadaver skin layers, compared to a nonparticulate vehicle, PG.
- Demonstrated that nanospheres formulated as gel or aqueous suspension delivered significantly higher amounts of Nile red to the porcine skin compared to the PG formulation. The gel formulation delivered greater amounts of Nile red than the aqueous solution.

Conclusions

The nanosphere approach offers a way to increase the skin concentration of agents while mitigating the damage to skin barrier function that occurs when other approaches are used, such as chemical enhancers or iontophoresis (the process of introducing a drug in ionized form through intact skin by applying a direct electric current). This research team's results highlight the potential benefit of curcumin-loaded nanospheres for the topical treatment of burns, especially given the increased curcumin stability and improved skin penetration that is a prerequisite to reduce or stop burn progression.

Research Plans for the Next 4 Years

The research team plans during the upcoming year to conduct proof-of-concept and safety studies of the nanosphere-curcumin formulation using an in vivo porcine burn model.

Planned Clinical Transitions

The researchers plan to initiate discussions with the FDA and the Center for Devices and Radiological Health for pre-IDE review by Years 3–4 of the project. They will simultaneously conduct GLP safety and efficacy studies. In Year 4, they plan to initiate human clinical trials.

Corrections/Changes Planned for Year 2

None.

Project 4.2.3, WFPC

Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues

Team Leader(s): *Mark Van Dyke, PhD (Wake Forest University)*

Collaborator(s): *KeraNetics LLC*

Therapy: *Immediate treatment of burn injuries with keratin-based biomaterials*

Deliverable(s): *Keratin hydrogel*

Key Accomplishment(s): *The researchers developed a porcine heat shock model and a standard method for the production of keratin biomaterials. They established a primary mouse skin cell isolation and culture system. They initiated ISO 10993 safety testing on keratin biomaterial. They determined that re-epithelialization occurred more rapidly when keratin hydrogel treatment was applied in the pig thermal burn model.*

Introduction

Keratins are a family of structural proteins with unique physical, chemical, and biological characteristics. They can be derived from numerous tissues, but the keratins from hair fibers are especially suited to biomaterials development for human clinical application. Using oxidative and reductive chemical treatment, keratins can be uncrosslinked and functional keratin subtypes purified from fiber extracts. These purified keratins can be modified in a variety of ways to create numerous structures such as films, foams, coatings, gels, and fibers, each with a unique and in many cases, tailored set of physical, chemical, and biological properties. A number of different cell types can interact strongly with keratins through integrin and other membrane receptors and influence cell behavior.

The potential clinical benefit of a keratin biomaterial for treatment immediately following burn injury is a reduction in TBSA. TBSA is directly linked to outcomes, including length of hospital stay, scarring, treatment cost, and mortality. Anything that reduces TBSA will have a positive influence on these parameters. Moreover, keratin gels may also help reduce inflammation and scarring and promote more rapid epithelialization of burns and skin graft donor sites. All of these aspects will be studied during the conduct of this project using in vitro and in vivo models.

The specific aims of this project are to (1) investigate the thermoprotective characteristics of keratin biomaterials in vitro and (2) test the thermoprotective characteristics of keratin biomaterials in rodent and pig burn injury models.

Research Progress – Year 1

As evidence of keratin biomaterials' capability to control cell behavior, initial pilot studies were conducted in which keratin biomaterials showed an ability to increase cell adhesion, proliferation, and migration. Upregulation of genes that are important to the regenerative function of several cell types has also been demonstrated. Burn studies in mice and pigs have also shown favorable outcomes. In one chemical burn study, wounds treated with keratin hydrogel healed significantly faster than wounds treated with a conventional biomaterial hydrogel. In a thermal injury model in pigs, re-epithelialization was more rapid in keratin-treated wounds compared to conventional treatment (**Figure V-8A** and **V-8B**).

These data suggest a role for keratins in the salvage of cells and tissue after burn injury. Any technology that serves to protect cells from thermal injury or promotes cell survival would be of tremendous value as an immediate post-burn treatment. Tissue preservation in the zone of stasis will prevent a burn wound from growing larger during the nascent stages of recovery when cell death normally contributes to an overall increase in TBSA. Keratin biomaterials may be able to provide stability to the wound while promoting normal healing and be deployable directly

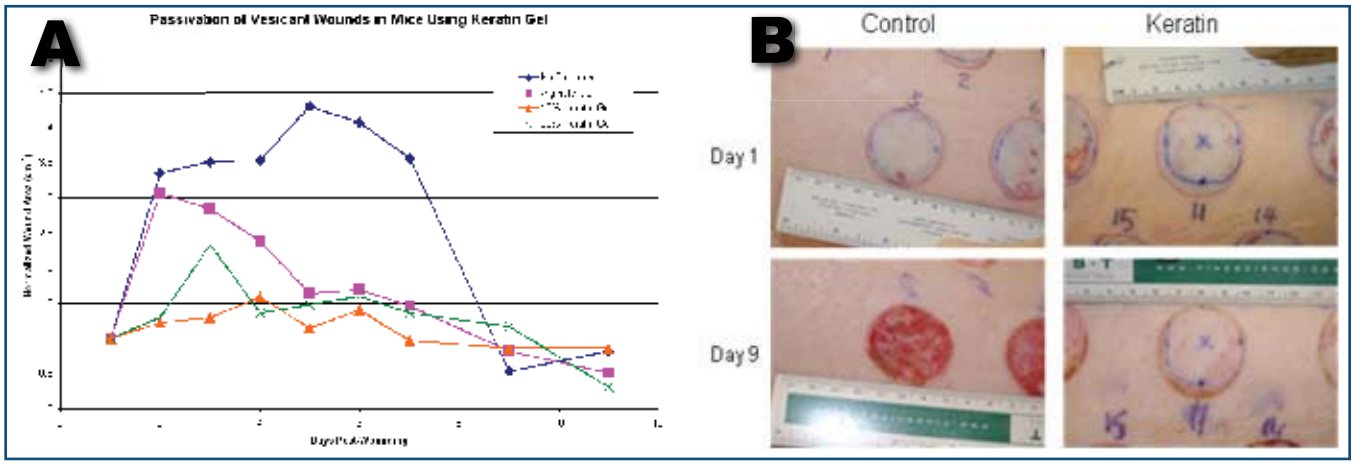


Figure V-8. Results of (A) a chemical burn model in mice and (B) thermal burn model in pigs. Wound sizes in the untreated and conventional hydrogel treatment groups increased dramatically by day 2 due to tissue loss in the area surrounding the burn in mice. Wound size was appreciably more stable in the keratin treatment groups. In the pig model, re-epithelialization occurred more rapidly when keratin hydrogel treatment was applied. These data suggest a role for keratin in the preservation of cells and tissue in the zone of stasis surrounding a burn. Moreover, rapid re-epithelialization may also result from keratin treatment.

to the field so that they can be used immediately after burn injury. However, the mechanism of cell and tissue salvage is not known, and an optimal formulation and treatment protocol have yet to be devised. The researchers hypothesize that keratin biomaterials act on the cell surface receptors of thermally injured cells in the zone of stasis, including vascular cells, fibroblasts, and keratinocytes. Further, certain keratin subtypes may have greater efficacy than others. What remains to be demonstrated are the specific structure-activity relationships between keratin and skin component cells and the signaling mechanisms by which these keratins promote cell survival.

The researchers have identified a new source of human hair feedstock and qualified it for use in the production of keratin biomaterials. An initial set of quality control tests were developed, validated, and used to evaluate several commercial sources of human hair. These include molecular weight, viscosity, amino acid content, purity, trace metal content, and biological activity. Based on these assays, a primary supplier has been chosen, and a long-term supply contract will be negotiated. In a related project using non-AFIRM funding, a GLP-compliant laboratory for pilot-scale production is being established. Initial equipment has been specified and is currently being ordered. A lease agreement has been signed to establish the laboratory in the same building as the AFIRM. This laboratory will be capable of producing keratins on the kilogram scale.

The researchers have sectioned and stained pig tissues from a pilot thermal injury study. The tissues are currently undergoing histomorphometric analysis for initial burn depth, inflammation, granulation, vascularization, re-epithelialization, and scarring. An addition pilot wound-healing study has been initiated, also using non-AFIRM funding. Recently, Dr. Lillian Nanney at the Vanderbilt University School of Medicine has begun an excisional wound-healing study in pigs. The goal of this study is to assess re-epithelialization in skin graft donor sites. An initial group of two pigs was completed in March 2009 and a second set of two pigs completed in May 2009. The tissues are being evaluated histologically.

The researchers are continuing work with the in vitro heat shock model. A primary mouse skin cell isolation and culture system has been established and is currently being used to test the effect of several keratin subtypes on recovery from thermal treatment as measured by proliferation assays. The research team will also begin investigating changes in gene expression using microarrays. Keratin samples have been prepared and characterized using the laboratory's standard battery of quality control assays and have been sent to a contract laboratory for the following series of ISO 10993 tests:

- Cytotoxicity
- Irritation
- Sensitization
- Acute systemic toxicity
- Mutagenicity
- Pyrogenicity
- Bioburden

Key Research Accomplishments

- Developed a porcine heat shock model.
- Developed a standard method for the production of keratin biomaterials.
- Initiated ISO 10993 safety testing on keratin biomaterial.
- Established a primary mouse skin cell isolation and culture system (in vitro heat shock model).
- Began two porcine wound-healing studies that will support the submission of an IDE application.
- Began testing keratin materials in the pig model to determine the basis of their thermoprotective mechanism and ability to accelerate wound healing. Demonstrated that re-epithelialization occurred more rapidly when keratin hydrogel treatment was applied.

Conclusions

A standard method for the production of keratin biomaterials, including a commercial source of human hair and quality control assays, has been developed. This technology is slated for transfer to a partner company, KeraNetics LLC, beginning in August 2009. Two porcine wound-healing studies are under way that will support the submission of an IDE application package to the FDA later this year. ISO 10993 safety testing is also under way. Testing of keratin materials to determine the basis of keratin's thermoprotective mechanism and accelerated wound healing has been initiated and will be used as the basis to optimize the technology. An optimal formulation will then be tested in animal models of burn injury and assessed against current standards of care.

Research Plans for the Next 4 Years

Since their heat shock model was developed faster than expected, the researchers are accelerating the time line for the project. They plan to complete the mechanistic investigation during the upcoming year. Based on feedback to their annual review, the researchers have decided to drop the rodent wound studies that were originally proposed and will switch to a pig model that they have previously used. They anticipate conducting the preclinical pig studies in Year 3 with an optimized burn dressing prototype. The researchers plan to add a part-time postdoctoral fellow to the project to accelerate the time line for mechanistic and animal experiments.

Planned Clinical Transitions

The researchers are condensing the time line for animal model testing; a clinical trial is now expected to commence during Year 5. Due to the funding being provided by a partner company, KeraNetics, the researchers have been able to perform some preliminary animal studies in pigs. In addition, KeraNetics is establishing a GLP-compliant facility to produce keratins that can be used in human clinical trials. The researchers have also initiated FDA-required safety studies, which they expect to complete in the next few months. They are confident that compressing the time line for the animal studies, in addition to the safety testing, will allow them to approach the FDA about a clinical trial. Through KeraNetics, they plan to have their first FDA meeting in the fall of 2009. If the safety studies are promising and the initial meeting with the FDA is successful, they may be poised to enter into a small first-in-man study in 2010 with an IDE and Institutional Review Board (IRB) approval. They note that funding will need to be identified for this study.

Corrections/Changes Planned for Year 2

Since their heat shock model was developed faster than expected, the researchers are accelerating the time line for the project. They plan to complete the mechanistic investigation during the upcoming year. Based on feedback to their annual review, the researchers have decided to drop the rodent wound studies that were originally proposed and will switch to a pig model that they have previously used. They anticipate conducting the preclinical pig studies in Year 3 with an optimized burn dressing prototype. The researchers plan to add a part-time postdoctoral fellow to the project to accelerate the time line for mechanistic and animal experiments.

Project 4.2.2, WFPC

Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device

Team Leader(s): *Joerg Gerlach, MD, PhD (University of Pittsburgh)*

Therapy: *Stem cell delivery for the treatment of burn wounds*

Deliverable(s): *(1) Optimized cell isolation and spraying methodologies and (2) FDA-approved spray device that can deposit fetal skin stem cells into wounds*

Key Accomplishment(s): *Established an in vitro cell spray model and an in vitro wound capillary membrane model of an active wound dressing for the support of progenitor cells, which work with fetal skin fibroblasts and keratinocytes. Established laboratory methods for the isolation and cell culture of fetal skin stem cells.*

Introduction

The treatments available to burn patients have had a dramatic effect on survivability, yet the functional outcome of a severely burned patient is still poor. Our bodies have an innate tendency to respond to injury by producing scarring with all the resulting consequences. A host of graft materials are available to treat full-thickness burns. Although these therapies have improved patient outcomes, they all share severe scarring as an unattractive feature. They are simply poor means by which to induce fetal-like wound-healing responses. Healing without scarring is a natural process for the fetus, but once we are born we rapidly lose this ability.

Regenerative medicine research seeks to accelerate regenerative processes that essentially recapitulate fetal-like wound healing in the adult. The living skin equivalents that are available to patients today are early attempts to deliver regenerative medicine to burned patients. In a landmark study by Hohlfeld (*Lancet* 366:840-842), fetal-derived skin cells were banked and then used as a source to culture living skin equivalents that were grafted onto pediatric patients. Although these fetal-derived constructs produced a more fetal-like healing response (i.e., rapid wound closure and no hypertrophic granulation tissue), the approach still delivered the cells in a traditional matrix. Hohlfeld demonstrated that the fetal cells were not detectable after several weeks; therefore, the cells established an appropriate environment for healing in the wound bed.

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 dermal fibroblasts and epidermal keratinocyte progenitor cells. The researchers have developed a clinically implemented spray device (**Figure V-9**) that can "seed" the burn site with any given cell type. They have found that by separating cells spatially from each other during delivery, they can minimize many of the problems associated with covering the area with a graft (**Figure V-10**). The first clinical tests in Berlin, Germany, used expanded keratinocyte cultures that were cultured ex vivo and then delivered to the wound site of burned patients. The researchers propose to combine efforts of their clinical trial in Berlin and the work of Hohlfeld by exploring the use of the skin spray device with banked fetal-derived skin stem cells.

Research Progress – Year 1

This project focuses on in vitro studies. The long-term clinical goal of this project is to use a cell spray deposition system to deposit fetal skin stem cells into a wound (Figure V-10). The fetal skin cells would have the potential to differentiate into basal keratinocytes and other terminally differentiated skin cells. The researchers feel

Figure V-9. Prototype of the spray head and the processor-controlled pneumatic.

that the sprayed delivery of highly proliferative skin cells that establish a fetal-like healing response prior to their elimination from the body will benefit patient outcomes. Moreover, expanding skin progenitor cells in vitro may form the basis of an off-the-shelf skin-cell-based therapy product that can be made readily available for immediate therapy and that does not require complex individual patient in vitro cell culture.

The researchers isolated fetal skin cells under IRB exemption. They developed methods for the cell culturing of the fetal skin cells. Methods to isolate fetal skin stem cells included a mechanical "dry swab off" method using the surface of collagen-coated dry polystyrol cell culture dishes followed by immediate covering with cell culture media, collagenase tissue digestion, mechanical scraping with a scalpel, trypsin tissue digestion, and capturing outgrowth from tissue on a collagen-coated dish surface (after the tissue settles on the culture media-covered culture dish). The researchers established tissue donation and sourcing logistics and collaborations. After extensive discussions and literature searching, they decided to focus on tissues younger than gestation week 9. The rationale is that fetal skin at this age consists only of one layer. In addition, hair structures with subsequent progenitor specialization have not yet developed before week 9. The markers for a functional assay were determined in the literature review. The antibodies, fluorescence-activated cell sorting, and staining methods are in preparation. The researchers have begun using immunofluorescence microscopy to identify surface markers of fetal epidermal skin progenitor cells and dermal fibroblasts. Initial clonal growth has been enabled and compared to adult skin cells. Cell stability was tested up to seven passages.

Overall, the researchers have established an in vitro cell spray model and an in vitro wound capillary membrane model of an active wound dressing for the support of progenitor cells, which work with fetal skin fibroblasts and keratinocytes.

Key Research Accomplishments

- Established an in vitro cell spray model and an in vitro wound capillary membrane model of an active wound dressing for the support of progenitor cells, which work with fetal skin fibroblasts and keratinocytes.
- Established laboratory methods for the isolation and cell culture of fetal skin stem cells.

Conclusions

This project was designed to expand successful work previously performed in Germany, in which fetal skin cells were sprayed onto excised burn wounds using a precision device. Optimization of isolation and banking techniques for cells are well under way with completion expected during Year 3 of the project. Preparation of documentation needed to obtain FDA

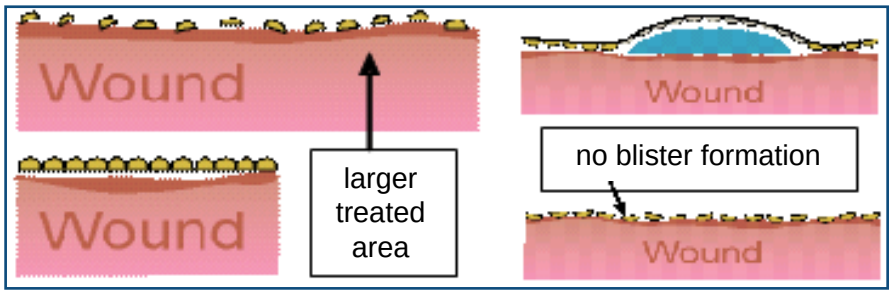


Figure V-10. Using sprayed cells results in reducing the required cell number, a larger treatment surface for therapy, and avoiding blister formation. Reducing the cell number speeds up application time, reduces in vitro differentiation, and better preserves basal keratinocyte progenitor cells.

approval of the spray device is also under way. Ultimately, a clinical trial is anticipated to begin by Year 5 of the project.

Research Plans for the Next 4 Years

During the second year of the project, the researchers will continue to optimize cell isolation and banking techniques. They will evaluate logistical considerations for the establishment of a cell bank and will establish and characterize cell clones. They will also optimize the spray device for stem cell application. They will complete cell isolation and banking techniques and prepare the spray device for FDA approval during the third year of the project. Characterization of cell isolation and banking techniques will be completed during the fourth year of the project. During Year 4, the researchers will also prepare for a human clinical trial and test the spray device for FDA approval. During the upcoming years, the researchers plan to test the suitability of human fetal skin progenitor cells for cryopreservation by comparing several methods.

Planned Clinical Transitions

The researchers will prepare the spray device for FDA approval during Year 3. They will prepare for a human clinical trial and test the spray device for FDA approval during Year 4. They anticipate the commencement of a clinical trial for the spray device in Year 5.

Corrections/Changes Planned for Year 2

None.

Project 4.2.4, WFPC

Artificial Extracellular Matrix Proteins for Regenerative Medicine

Team Leader(s): *David A. Tirrell, PhD*

Therapy: *Regenerative therapy for burn repair*

Deliverable(s): *Optimized aECM proteins for clinical burn repair*

Key Accomplishment(s): *The researchers have completed the design and construction of two new artificial ECM proteins with high-affinity fibronectin-derived cell-binding elements combined with elastin-like domains. They also prepared the first generation of thin film matrix constructs to be used for aECM protein evaluations.*

Introduction

The researchers of this project are developing aECM proteins for use in regenerative therapies for burn repair. Artificial ECM proteins are designed by combining elements drawn from natural ECM 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. During the 5-year span of the project, the researchers plan to complete the design and expression of optimized ECM proteins, prepare acellular and cell-seeded matrix constructs, complete cell culture studies of wound healing in ECMs, and conduct animal-model studies of burn repair. The researchers note that studies completed in this project will allow for a follow-up evaluation of aECM proteins for use in clinical burn repair.

Research Progress – Year 1

This project was significantly delayed due to unforeseen administrative and contractual issues. The researchers began work in February 2009 and made significant progress in the initial 3-4 months of work. They have completed the design and construction of two new aECM proteins with high-affinity fibronectin-derived cell-binding elements combined with elastin-like domains, which provide the needed mechanical properties:

((VPGIG)2VPGKG(VPGIG)2)6-FN10 or FN910-((VPGIG)2VPGKG(VPGIG)2)6

FN10 and FN910 represent full-length domain 10 and domains 9 and 10, respectively, from fibronectin, and the VPGXG elements represent the elastin-like domains.

Previous generations of aECM proteins prepared in the researchers' laboratory differed from these new proteins in two important ways. First, the cell-binding domains in previous generations were composed of only short RGD (arginine-glycine-aspartic acid) sequences derived from FN10.1. The researchers' preliminary studies suggest that inclusion of full-length FN10 and FN910 enhances the rates of cell spreading and wound healing in in vitro assays. Second, earlier proteins included epitope tags to aid in identification and purification. In the design of the FN10 and FN910 aECM proteins, the epitope tags have been removed to enhance biocompatibility. Both tagless versions have now been expressed, and the researchers are optimizing expression levels. They will examine variation in the length and sequence of both ECM domains, as well as variation in the design of the N-terminal region of the protein, to enhance expression yields. They note that cell

culture studies of wound healing will be initiated as soon as adequate quantities of protein are in hand. The researchers also completed the first generation of thin film matrix constructs, which will be used for the evaluations of aECM proteins.

Key Research Accomplishments

- Completed the design and construction of two new aECM proteins with high-affinity fibronectin-derived cell-binding elements combined with elastin-like domains.
- Completed the preparation of first-generation thin film matrix constructs.

Conclusions

Successful expression of first-generation aECM proteins has been achieved and provides a basis for subsequent evaluation in vitro and in vivo. From a burn perspective, numerous opportunities exist to couple this technology with existing burn therapies or with evolving technologies in the AFIRM Burn Repair Program. In addition, it is possible that this technology could be the platform/scaffold for a future skin substitute.

Research Plans for the Next 4 Years

With the first generation of ECM proteins in hand, the researchers have begun cell culture studies of wound healing on thin film matrix constructs. These studies will constitute a major focus in Year 2 and will identify key design parameters (i.e., protein chain length, identity, and density of cell-binding domains) that facilitate rapid healing. Animal studies (pig burn model) on first-generation constructs will begin late in Year 2 or early in Year 3. The researchers will complete physical characterization (determination of elastic modulus and tensile strength) of matrix constructs by the end of Year 3. The results of animal studies and physical characterization will guide redesign and optimization of second-generation ECM constructs in Year 4.

Planned Clinical Transitions

Animal studies on second-generation constructs in Year 5 will provide the basis for clinical evaluation of the use of the ECM proteins in burn repair.

Corrections/Changes Planned for Year 2

None.

Project 4.2.5, WFPC

In Situ Bio-Printing of Skin for Battlefield Burn Injuries

Team Leader(s): *James J. Yoo, MD, PhD*

Therapy: *Immediate burn wound stabilization with functional recovery*

Deliverable(s): *In situ portable skin printing system*

Key Accomplishment(s): *The researchers designed and developed a portable skin printing device. They achieved delivery of skin cells directly onto skin defects in a mouse model using the device. They also determined that printed constructs healed faster than controls and were able to self-organize into skin that appeared almost identical to normal murine skin.*

Introduction

Burn injury is a common source of morbidity and mortality on the battlefield, comprising 10% of all casualties. Most battlefield burns are massive injuries and require grafts for coverage and repair since any loss of full-thickness skin of more than 4 cm in diameter will not heal by itself. Current treatment options such as autografts and 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 soldiers. Therefore, a new approach that permits immediate burn wound stabilization with functional recovery is necessary.

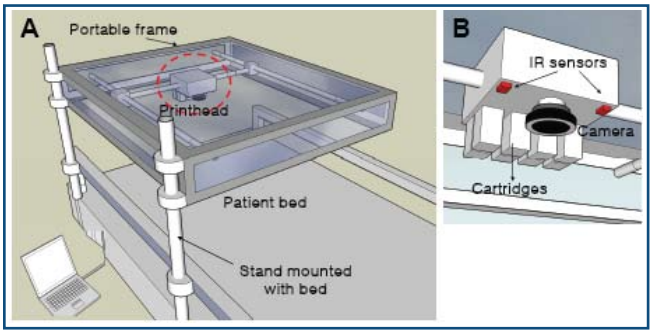


Figure V-11. Schematic of a portable skin printer.

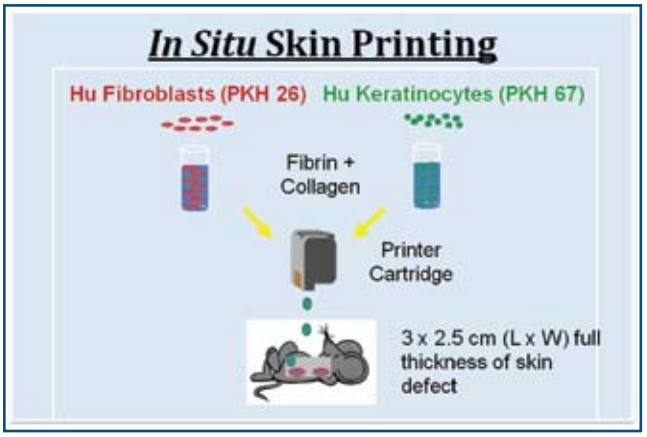


Figure V-12. Study overview of in situ skin printing.

To address the present limitations, the researchers aimed to develop a novel delivery system that would allow for on-site in situ repair of battlefield burn injuries using tissue-engineered skin grafts produced with a portable skin printing system (**Figure V-11**). The unique advantages of the proposed in situ skin bio-printing system include the ability to (1) treat massive burns immediately after stabilization of the wound on the battlefield as skin cells and matrices can be accurately delivered onto the injured sites and (2) deliver several dermal cell types and matrices simultaneously onto target sites to generate anatomically and functionally adequate dermal tissues. The delivery of major skin tissue elements onto the injured site would allow for a rapid restoration of the skin and may minimize scarring and enhance cosmetic recovery.

Research Progress – Year 1

To demonstrate that skin cells can be printed directly onto skin wounds, human keratinocytes and fibroblasts were loaded into the skin printer (**Figure V-12**). Cultured human fibroblasts and keratinocytes were labeled with fluorescent membrane bound markers (keratinocytes–green; fibroblasts–red). A full thickness skin defect (L x W, 3 cm x 2.5 cm) was surgically made on the dorsal surface of athymic mice to compare printed constructs against untreated controls. The mice were examined every week for 3 weeks and sacrificed at week 3 for histological analysis.

Printed human keratinocytes and fibroblasts, prelabeled with membrane bound dye, were identified under fluorescent microscopy. Hence, the printed cells were able to survive in vivo after in situ printing onto the wound. Printed constructs healed faster than controls and were able to self-organize into skin that appeared almost identical to normal murine

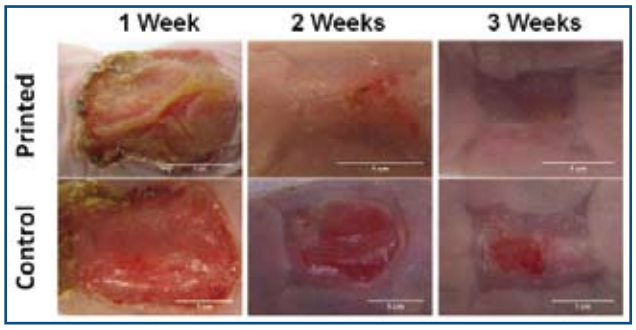


Figure V-13. Comparison of wound healing over 3 weeks demonstrating closure of the wound with printed cells while the wound remains open in the untreated group.

skin (**Figure V-13**). Histological examination following staining with hematoxylin and eosin (H&E) revealed that the epithelial thickness and dermal structure were of printed skin and were comparable to normal skin (**Figure V-14**). An AE1/AE3 immunohistochemical stain for cytokeratin revealed the presence of keratinocytes in the epidermal layer and showed that the printed constructs had a normally layered epidermis (Figure V-14).

Key Research Accomplishments

- Designed and developed a portable skin printing system and associated software.
- Achieved delivery of skin cells directly onto skin defects in a mouse model using the skin printing device.
- Determined that printed constructs healed faster than controls and were able to self-organize into skin that appeared almost identical to normal murine skin.
- Designed a noncontact valve delivery system.
- Completed scale-up of the bio-printing hardware with a laser sensor capturing system.

Conclusions

The researchers have developed a portable skin printing system that will allow for on-site in situ repair of battlefield burn injuries. Using the device, they were able to print labeled human keratinocytes and fibroblasts directly onto skin defects in a mouse model. They subsequently determined that printed constructs healed faster than controls and were able to self-organize into skin that appeared almost identical to normal murine skin. The delivery of major skin tissue elements onto an injured site via the portable skin printing device will allow for a rapid restoration of the skin and may minimize scarring and enhance cosmetic recovery.

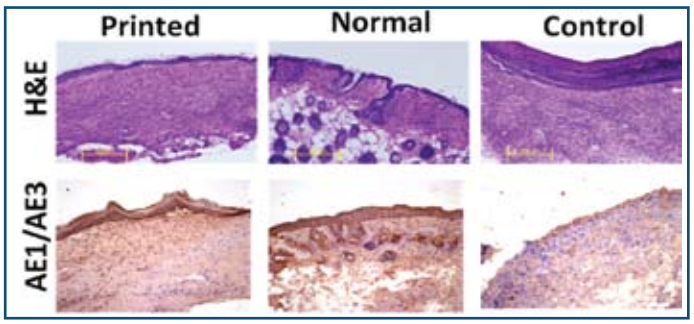


Figure V-14. Morphological analysis of printed skin. (Top) H&E staining of printed, normal, and control sections obtained at 3 weeks post-surgery. Printed skin is virtually indistinguishable from normal skin. (Bottom) AE1/AE3 immunostain for cytokeratins demonstrates viable epithelium in the printed skin.

Research Plans for the Next 4 Years

In Year 2, the researchers will continue to develop the portable skin printing device and will complete the rodent studies. They will also complete the initial printer prototype for a pig model. In Years 3–5, the researchers will demonstrate the applicability of the skin bio-printing delivery system in a pig burn wound model. They will develop cell banks for a human clinical trial and refine and optimize the skin printing system for clinical use. They anticipate starting a clinical trial in Year 5.

Planned Clinical Transitions

Initial small animal model testing has been successful, and progression to porcine burn injury models is anticipated within the next 2–3 years. The researchers plan to begin a clinical trial in Year 5 of this project.

Corrections/Changes Planned for Year 2

None.

Project 4.7.2, RCCC

Burn Repair with Autologous Engineered Skin Substitutes

Team Leader(s)/Project Team: *Steven Boyce, PhD, Dorothy Supp, PhD (University of Cincinnati)*

Collaborator(s): *Lonza Walkersville, Inc., Walkersville, Maryland*

Therapy: *Treatment of deep burn injuries*

Deliverable(s): *Autologous ESSs*

Key Accomplishment(s): *Advanced models of ESSs with pigmentation and vascular analogs were established during Year 1, and feasibility was demonstrated for advancement to animal studies in Year 2.*

Introduction

Soldiers who experience combat-related burn injuries often have extensive, deep wounds that need prompt and effective closure. Conventional skin grafting cannot harvest enough skin from one donor to cover large burn wounds. Autologous ESSs have been developed and tested clinically as an adjunctive treatment for burn repair. ESSs are formed from cells (cultured keratinocytes and fibroblasts) attached to a collagen-based matrix. This composite of cells and biopolymers is designated as a medical device by the FDA. ESSs have been shown to be efficacious in the treatment of excised, full-thickness burns of greater than 50% of the total body surface area (**Figure V-15**). Completed clinical studies with ESSs show a reduction in the requirement for harvesting of autologous skin to complete wound closure. Successful completion of product development may allow reductions in morbidity and mortality for soldiers who are casualties of combat-related burns covering large portions of their bodies. Although ESSs reduce the requirements for harvesting skin autografts, there are two major deficiencies: (1) incomplete pigmentation, which does not resolve with time, and (2) the absence of a vascular plexus, which limits the thickness and rate of engraftment of ESSs.

The overall goal of this project is to design and test new prototypes of ESSs for improved outcomes in recovery from life-threatening burns and to initiate commercial availability of the original model of ESSs. The specific research aims are to overcome the two major limitations of current ESSs, that is, to (1) restore skin color in ESSs by adding human melanocytes and regulating pigment expression after transplantation and (2) develop a vascular network in ESSs by adding HDMECs, which undergo morphogenesis to promote earlier perfusion and increase engraftment. Because clinical studies have demonstrated successfully that an initial model of autologous ESSs is effective for treatment of life-threatening burn injuries, the second, commercial objective of this project is to translate ESSs to clinical trials to serve burned soldiers.

Research Progress – Year 1

Restoration of Skin Color: The first step of this research was to test and verify that the transplantation efficiency of skin pigment cells, melanocytes (HMs), could be increased to make restoration of color a practical step in ESS fabrication. Selected conditions resulted in normal dendritic morphology of HMs. In addition, keratinocytes (HKs) exhibited normal proliferation and cellular migration. The second step was to inoculate HM-HK cultures in two stages to form a stratified epithelium. ESSs inoculated with selected HM:HK ratios developed an epidermal substitute that was normally stratified and keratinized. The researchers grafted ESSs with increasing HM densities to full-thickness wounds on athymic mice to assess pigmentation in vivo. They have initiated tumorigenicity testing as required by the FDA to confirm the safety of

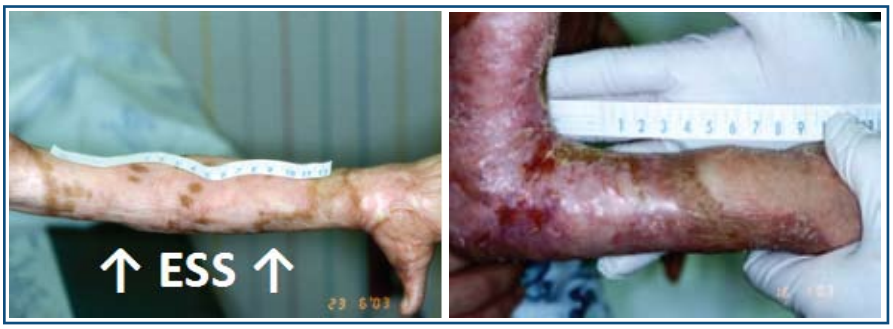


Figure V-15. ESS success (left) and EpiCel™ failure (auto keratinocyte sheets) (right).

cultured melanocytes. Histological examination of injected HMs confirmed the survival of normal melanocytes without tumor formation.

Hence, a model for ESSs with regulated pigmentation was established and qualitative results were demonstrated. Each of the project's subaims is proceeding toward quantification to provide predictable technologies to restore human skin color in engineered skin. Together with regulation of cell density and efficient transplantation of melanocytes, completion of tumorigenicity studies will enable consideration of clinical studies with pigmented engineered skin to match skin color in burn patients. Researchers have calculated the proper melanocyte density needed to completely restore pigmentation for any individual's body surface. These factors for cellular requirements are fully consistent with current capabilities for propagation of human melanocytes within time intervals (4–8 weeks) for treatment of extensive burn injuries with engineered human skin.

Development of a Vascular Network Within the ESSs: The first step of this aim employed an engineered biopolymer matrix with transverse channels as vascular conduits. To facilitate anastomosis of vascular conduits in ESSs to blood vessels in the wound, biopolymer matrices were fabricated with perforations that extended from the wound bed to the epithelial surface. Research is ongoing to optimize the diameter of the channels/perforations when the matrices are hydrated. In the second step, the researchers studied by microscopy the morphogenesis of fibroblasts and HDMECs on collagen-glycosaminoglycan matrices. To reduce draining of cell suspension through the perforations, a monolayer of human fibroblasts or a layer of fibrin was prepared as an attachment surface for the biopolymer matrices. These results demonstrate that a model for ESSs with vascular conduits was established with qualitative results. Although several paracrine pathways among skin cells have been found in human skin, the regulation of vasculogenesis in vitro is not yet fully understood, and the proposed experiments should elucidate some of these cell-cell interactions. After generating ESSs that are consistently and accurately populated with channels that are lined with HDMECs, the researchers will perform animal studies.

Translation of ESSs to Prospective Clinical Trials: Research for this aim is being conducted in collaboration with Lonza Walkersville, Inc., which currently holds the licenses to the ESS technology. During the first year of the project, initial testing of ESSs was conducted in burn patients in paired-site, randomized, prospective studies. Pediatric patients with life-threatening burn injuries were treated with ESSs. All patients treated with ESSs survived and have returned to the daily activities of living.

An IDE protocol has been active since 1998 for clinical testing of autologous ESSs (formerly called cultured skin substitutes). During 2007–2008, two subjects with burns involving 90% or more of the total body surface area were treated with ESSs as an adjunctive therapy to split-thickness skin autograft. In a patient with 94% burns, ESSs were applied surgically and compared to autograft for qualitative outcome and reduction of donor skin harvesting. Approximately 50% of this patient's total body surface area was closed permanently starting with donor skin of less than 1% total body surface area.

To plan for initial clinical trials in military patients with burns, the research team requested advice from the Multicenter Clinical Trials Group (MCTG) of the American Burn Association. Dr. Steven Wolf, who directs clinical research at Brooke Army Medical Center participates in the MCTG. The MCTG will provide advice to assemble a Data Safety Monitoring Board for this prospective study. Advice from the MCTG will be coordinated

with the Clinical Trials Office of RCCC-AFIRM to develop a clinical protocol for use at USAISR.

An application to the AFIRM for clinical studies with ESSs was submitted in October 2008 by the University of Cincinnati but was rejected. The director of the RCCC-AFIRM consortium petitioned successfully for revision of the application, which was resubmitted in March 2009 by Lonza Walkersville, Inc. and remains under review at the time of this report. The Principal Investigator of this project remains committed to bringing ESS technology to clinical trials for catastrophic burns in military personnel.

Key Research Accomplishments

- Established advanced models of ESSs with pigmentation and vascularization.

Conclusions

Autologous ESSs have been demonstrated to provide medical benefits to patients with life-threatening burns. The researchers of this project have established advanced models of ESSs with pigmentation and vasularization. They demonstrated feasibility for advancement to animal studies in Year 2. Clinical studies with ESSs at USAISR have been proposed and will continue to be pursued for the prospective benefits of wounded warfighters.

Research Plans for the Next 4 Years

In Years 2–3, the researchers plan to study the regulation of melanocyte survival in ESSs in vitro by co-culture with epidermal keratinocytes and the regulation of endothelial cell survival by co-culture with fibroblasts. They also plan to measure the efficiency of pigment expression after transplantation to athymic mice, test tumorigenicity in athymic mice using clinical melanoma cells as positive controls, fabricate a biopolymer matrix with interconnected channels as vascular conduits, and localize HDMECs in perforated matrices (and verify their localization by immunochemistry). In Years 4–5, the researchers plan to study the regulation of pigment expression in transplanted ESSs. They also plan to transplant endothelialized ESSs to mice and perform a quantitative evaluation of the perfusion. Finally, they will plan for clinical transplantation.

Planned Clinical Transitions

The researchers aim to translate ESSs to clinical trials. Because an initial prototype of ESSs has been studied clinically for several years, the regulatory and logistical requirements for clinical trials are well understood. In addition, a commercial developer, Lonza Walkersville, Inc., currently holds a license to the ESS technology. During Year 1, supplemental funding for clinical studies through the AFIRM became available, and Dr. Steven Boyce, the Principal Investigator, collaborated in two applications for support of initial clinical studies through the AFIRM. The second application was approved for partial funding ($500,000) and is currently being activated. It is anticipated that initial funding will be followed by additional funding ($1 million), which together will support:

1. Completion of protocols for current GMP-compliant manufacturing and characterization of the ESS device.
2. Preparation of regulatory protocols for the FDA and study protocols for the IRB at USAISR and the U.S. Army Human Subjects Research Review Board. These protocols will be designed for electronic data collection by the Clinical Trials Office of the AFIRM.
3. Recruitment of a Clinical Research Organization to staff and operate the clinical trial.
4. Enrollment and treatment of 10 burn patients at USAISR as a Phase 1 clinical study.

If plans for this clinical study proceed without delays, the researchers feel that the first burn patients will be treated with autologous ESSs at USAISR by July 2010.

Corrections/Changes Planned for Year 2

None.

Project 4.2.1a, WFPC

Tissue Engineered Skin Products

Team Leader(s): *Vincent Ronfard, PhD (Organogenesis, Inc.)*

Therapy: *Human cell-based therapy for treating burn injury*

Deliverable(s): *Porcine and human self-assembly dermal matrices*

Key Accomplishment(s): *The research team 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.*

Introduction

The overall objective of this project is the development of an advanced human cell-based therapy for the treatment of deep, extensive burns. The project strategy is to develop and rapidly test porcine versions of Organogenesis, Inc.'s proprietary human cell-based, self-assembled wound therapy. The most successful embodiment will then be subsequently tested in a pilot/pivotal clinical trial. This strategy allows for changing the construct rapidly based on experimental feedback and offers the ability to test multiple constructs simultaneously in an immunologically competent animal model.

Research Progress – Year 1

The researchers have made progress on a number of fronts over the past year. They set up the laboratory for porcine cell culture and establishment of the porcine self-assembled matrix. They established a literature database, held an on-site observation of burn care by project team members, consulted with burn care experts to refine the Target Product Profile, and identified key opinion leaders in the area of burns for consultation and product testing. The researchers established legal agreements with Yann Barrandon for the development and conduct of porcine wound model experiments and the establishment of a porcine fibroblast cell bank. They did not need a legal agreement for porcine keratinocytes since they were already banked at their facility.

The researchers established porcine fibroblast and porcine keratinocyte Master Cell Banks. They optimized culture medium for porcine fibroblasts, characterized dermal matrix using histological methods, and performed initial proof of concept for use of biomaterial scaffolding for fibroblast growth. Porcine keratinocytes and porcine fibroblasts can be observed in **Figure V-16a** and **V-16b**, respectively. The researchers completed the initial phases of manufacturing optimization, including increasing surface area to meet the developing Target Product Profile. They also explored methods to increase matrix thickness and achieved an increase in product thickness by culture reseeding. They began the development of a large square culture device.

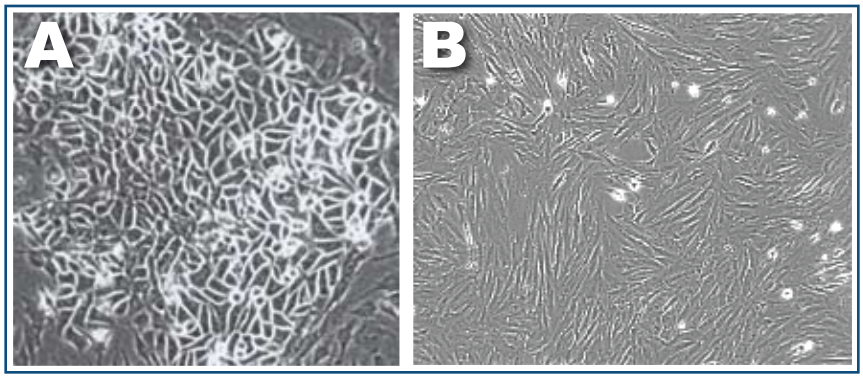


Figure V-16. (A) Porcine keratinocytes after tissue dissociation (magnification 10x). (B) Porcine fibroblasts after tissue dissociation (magnification 20x).

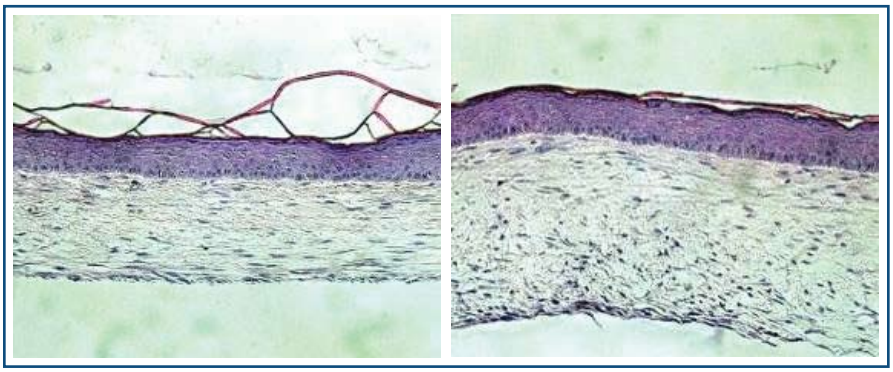


Figure V-17. Human skin equivalents were grown in serum-free medium for 25 days (with or without additional growth factor). The optimization of media will allow the development of a thicker matrix (twice the amount).

They also began the initial stages of the production of a human dermal matrix by optimizing the medium, performing a histological characterization, and improving matrix thickness (**Figure V-17**).

Key Research Accomplishments

- Established a project management structure including the initiation of Design Control.
- Established porcine fibroblast and keratinocyte cell banks.
- Refined the porcine self-assembly dermal matrix.
- Began development of a porcine wound model and, in parallel, a human dermal matrix.

Conclusions

The researchers have made progress in the development of a porcine version of their target product. Initial grafting of the porcine construct will be performed within the next 6 months after Institutional Animal Care and Use Committee approval. Because Organogenesis, Inc. has previously developed a successful cell-based wound therapy product, the researchers have been able to enlist not only research and development but also manufacturing, regulatory, legal, clinical and medical, commercial, and other functions in the product development. This allows the assessment and development of the target product from all perspectives necessary for successful full-scale manufacture and marketing of a human burn wound treatment.

Research Plans for the Next 4 Years

The researchers will continue the next phase of Design Control during Year 2. Completion of this phase will result in a final product description, business case analysis, high-level intellectual property assessment, regulatory pathway definition, product team definition, and a final development time line. Simultaneously, and in parallel, final development and validation of the porcine model will be completed, and multiple iterations of both porcine and human tissue constructs will be developed to determine the spectrum of feasible constructs and the optimum conditions for culture and processing. They expect approximately 30 iterations of porcine tissue construct and 20 iterations of human tissue construct. The researchers will complete the final preclinical implementation in Year 3. This is consistent with Design Control phases 3 and 4. They will also execute the porcine burn model. They will prepare for a clinical trial in Year 4. They will complete large-scale run(s) manufacturing (process, product development), which is in alignment with Design Control phases 5 and 6. They anticipate the initiation of a clinical trial in Year 5.

Planned Clinical Transitions

The researchers aim to begin a clinical trial by Year 5 of the project.

Corrections/Changes Planned for Year 2

None.

Project 4.2.1b, WFPC

Tissue Engineered Skin Products/ Comparative Skin Study

Team Leader(s): *Paul Kemp, PhD (Intercytex)*

Collaborator(s): *Biopharma Technology Ltd, University of Pittsburgh*

Therapy: *Human cell-based therapy for treating burn injury*

Deliverable(s): *Biological dermis produced by human fibroblast cells*

Key Accomplishment(s): *The researchers developed 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.*

Introduction

The need for an "off-the-shelf" skin replacement that is instantly available and alleviates the need to take a split or full-thickness skin graft has long been sought. Several "living skin equivalents" and "living dermal equivalents" have been developed and approved by the FDA. Although these materials were initially designed to work as skin grafts, the dermal component of these products rapidly degraded in the wound environment, releasing the cells, which then secondarily contributed to wound healing. This failure to provide a constant structural element was due to the quantitative and qualitative nature of the ECM into which the cellular elements were deposited and is illustrated in **Figure V-18**. The intended aim in these first-generational constructs was of gradual implant remodeling while maintaining a structure sufficiently robust enough to resist degradation in the wound as shown in Figure V-18a. With these first-generation materials, the actual wound response resembled that shown in Figure V-18b, where the implant rapidly degraded and produced a failed element within a wound that then slowly healed.

The aim of this project is to produce a biological dermis (ICX-SKN) that is produced by human fibroblast cells, is instantly available, can be applied in one surgical procedure, and can withstand the harsh environment of a deep dermal burn. Once grafted, it is then incorporated rapidly by the host, vascularized, epithelialized, and subsequently remodeled into new host tissue, thus alleviating the need for skin grafting and reducing the amount of burn scar. A reiterative approach is being developed to optimize the biological characteristics of such a dermis.

Research Progress – Year 1

1. Development of a 10 x 10 cm casting tray

A 10 x 10 cm casting system was developed to scale up the ICX-SKN constructs, requiring exploration of manufacture routes/options, identification of suppliers, and costing issues. A tray insert to be used in combination with a commercially available dish was selected and prototyped from the six options identified.

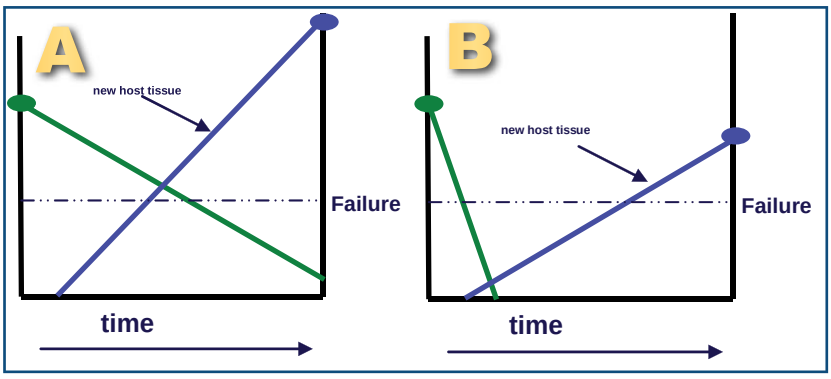


Figure V-18. Successful wound healing depends on the ability of an implant to resist degradation.

2. **Development of the freeze-drying process**

Freeze-drying of the matured pSKN matrix is a step in the manufacture of ICX-SKN that allows preservation of the matrix. This allows stockpiling material for further processing and disassociation of the long pSKN maturation process from downstream processing of the final ICX-SKN product. Freeze-drying had to be developed for the 10 x 10 cm pSKNs and due to technical complexity and equipment, the process was developed in collaboration with experts at Biopharma Technology Ltd. in the United Kingdom. Several freeze-drying cycles were investigated. The best results were obtained when 10 x 10 cm matrix was prepared for freeze-drying by washing out total media with triple WFI (water for injection) wash, followed by a cycle of: freeze-drying, freezing with annealing to -50°C, one cycle of defrosting to -10°C, then re-freezing to -50°C, and finally drying at -50°C.

A regime of thermal treatment and primary drying steps with temperatures and hold times was created by Biopharma Technology Ltd., which could ultimately be transferred to a contract manufacturer with GMP accreditation to successfully freeze-dry batches of 10 x 10 cm pSKN intermediates, standardizing the process and improving matrix structure, cell-friendliness, and mechanical strength.

3. **Product and process characterization of SKN**

Scanning electron microscopy was used to determine changes in the pSKN matrix during maturation. Results indicated that during maturation the overall thickness initially decreased but remained constant after 14 days. In addition, matrix density increased and pore size decreased, which suggests increased collagen deposition. Immunostaining using collagen 1 antibodies further supported increase in collagen deposition during maturation, which in turn provides greater mechanical strength and a more structured matrix.

4. **Effects of ultrasound stimulation on SKN**

Initial studies indicate that the novel use of ultrasound stimulation during maturation increases the quantity of collagen and improves pSKN matrix structure. Rheological analysis shows an increase in strength stimulated pSKN constructs, 56%–74% and 36%–48% increase at day 21 and day 49 of maturation, respectively, compared to controls.

Key Research Accomplishments

- Developed a casting dish for preclinical production of the ICX-SKN biological dermis.
- Identified a suitable freeze-drying process so that the ICX-SKN constructs can be stored and inventoried before recellularization and use.
- Developed a burn pig protocol (in collaboration with the University of Pittsburgh).
- Characterized maturation of the ICX-SKN constructs by scanning electron microscopy and immunostaining.
- Identified ultrasound stimulation protocols that greatly increase the mechanical and biological handling characteristics of the constructs.

Conclusions

The researchers are making significant progress in the development of a biological dermis composed of human fibroblast cells. The experiments completed to date have laid the foundation for the planned burn pig study, which will reveal the in vivo characteristics of the skin constructs.

Research Plans for the Next 4 Years

The researchers plan to produce material for the burn pig study, perform the first pig implantations in Pittsburgh, analyze the results, and determine which iteration(s) is suitable for taking forward. They also plan to determine epithelialization options and timings. They will conduct follow-up pig studies to fully determine the remodeling needed to achieve skin replacement. They also plan to conduct the first human study to compare human versus pig remodeling.

Planned Clinical Transitions

The researchers aim to begin a clinical trial focused on their ICX-SKN constructs by the first quarter of 2012.

Corrections/Changes Planned for Year 2

The requirement to develop porcine fibroblast and keratinocyte cell banks has been superseded since Organogenesis, Inc. already has these and the possibility exists to use their cell banks in the future.

Project 4.2.6, WFPC

Amniotic Fluid Stem Cells for Burn

Team Leader(s): *Mark E. Furth, PhD (Wake Forest University)*

Therapy: *Bioengineered skin product for the treatment of extensive burns*

Deliverable(s): *Bioengineered multipotent stem cells from hAFSCs as a skin equivalent/substitute*

Key Accomplishment(s): *The researchers demonstrated that hAFSCs express CD146, a cell surface marker protein recognized as a marker of perivascular cells. They also demonstrated enhanced skin wound healing by hAFSCs in immune-deficient mice. They also found initial evidence for the expression of stratified epithelial lineage markers p63 and cytokeratin 14 (CK14) by some hAFSC lines in response to in vitro differentiation conditions.*

Introduction

Several bioengineered skin products utilizing allogeneic cells have proven safe and effective in human testing for wound healing, and some have shown value in treating burns. These products do not cause acute rejection, and dermal cells may be able to persist over relatively long periods. However, there is evidence that allogeneic epidermal cells do not engraft permanently, but rather that they are eventually rejected and must be replaced by the recipient's own cells. In the case of extensive burns, the limited amount of remaining epidermis makes this problematic. Although only autologous cells provide a perfect genetic match, a careful analysis of donor pools indicated that as few as 10 carefully selected self-renewing stem cell lines potentially could offer "the maximum practical benefit" for matching of human leukocyte antigen (major histocompatibility) antigens. This number appears small enough to enable manufacturing and maintenance of inventory for rapid delivery in treatment settings.

The goal of this project is to utilize broadly multipotent stem cells from human amniotic fluid (hAFSCs), or comparable cells from other perinatal sources (e.g., placenta), to help develop an improved off-the-shelf bioengineered skin product for the treatment of extensive burns. The researchers anticipate that the differentiation of hAFSCs to the keratinocyte lineage will be essential for their use in the production of the epidermal component of a bilayered living skin equivalent. Longer term, they will attempt to differentiate the cells toward melanocytes to enable production of pigmented skin equivalents.

Research Progress – Year 1

1. Stem cell sourcing: Relationship of hAFSCs to perivascular/MSCs and isolation of perivascular cells from placenta.

A recent report by members of the AFIRM consortium showed that MSCs can be prospectively identified in multiple human organs as perivascular cells that express CD146 (together with the related marker NG2 and PDGFR-beta) and do not express hematopoietic, endothelial, and myogenic markers. The perivascular cells described by Crisan et al. resemble hAFSCs in their ability to differentiate into various mesenchymal lineages (endodermal and ectodermal lineages were not reported). In addition, some of their clones have a greater capacity for self-renewal than has typically been described for MSCs.

The observed similarity in surface marker expression (especially CD146) of hAFSCs to a subset of perivascular cells with properties of MSCs that can be isolated from placenta (canine or human), together with previous data on the isolation of hAFSC-like cells from this source, suggests two action points. First, it supports the decision to focus initially on differentiation protocols that promote the generation of epidermal-like cells from MSCs. Second, it supports the exploration of placenta as a source

of stem cells that may be equivalent to AFSCs but somewhat easier to obtain.

2. Dermal function: Promotion of wound healing by hAFSCs.

At the time of formulating the proposal for this AFIRM project, it was not clear whether it would be essential to drive in vitro differentiation of hAFSCs to dermal fibroblasts or whether the undifferentiated cells could be used for this purpose. Based on a report of a skin equivalent utilizing undifferentiated MSCs, together with the strong degree of overlap of expression of many markers (although not CD146) that were noted between MSCs, hAFSCs, and human fibroblasts, the researchers considered it likely that hAFSCs could be utilized for dermal constructs without prior differentiation in culture. A relevant test of this hypothesis is to assess the ability of hAFSCs to participate in skin wound healing in vivo. The researchers found that undifferentiated hAFSCs could contribute to healing skin puncture wounds in severe combined immunodeficient (SCID) mice (**Figure V-19**). Using a human nuclear antigen (HNA) as a marker, human cells were observed in healing wounds at day 7 (**Figure V-20**) but were not detectable after day 14 (not shown). This suggests that AFSCs will be able to substitute for foreskin-derived dermal fibroblasts in skin equivalents without the need for prior differentiation in culture.

3. Expression of keratinocyte lineage markers by hAFSCs.

A key task for the initial period of the project is the discovery of methods to promote the differentiation of hAFSCs, or similar stem cells, toward the keratinocyte lineage to be able ultimately to generate the epidermal component of a skin equivalent. While continuing to optimize the development of new, low-passage hAFSC lines, and comparing hAFSCs with related stem cells that may be even easier to source, the researchers have initiated differentiation experiments toward this goal. Based in part on personal communication from Dr. Robert Christy, USAISR (San Antonio, Texas), their initial efforts have focused on conditions claimed to promote the production of epithelial-like cells from human bone marrow-derived MSCs, as well as human adipose-derived stromal cells, another source of MSCs (R. Christy, personal communication).

The researchers found initial evidence for the expression of stratified epithelial lineage markers p63 and CK14 by some hAFSC lines in response to in vitro differentia-

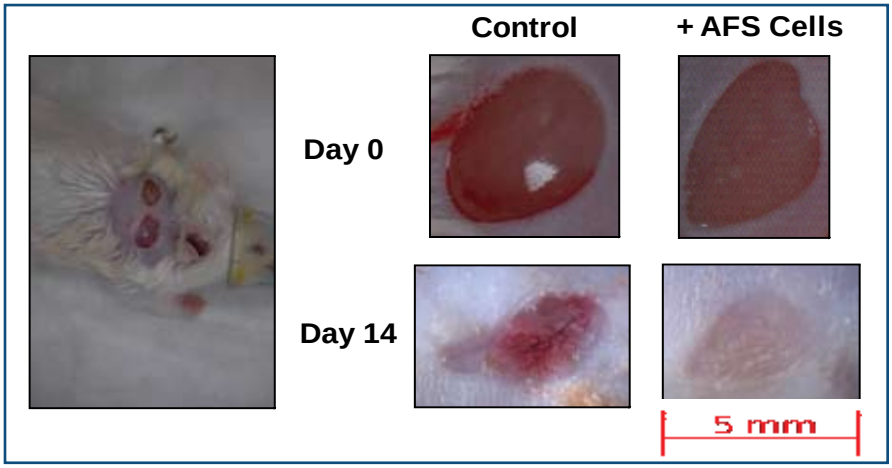


Figure V-19. Skin wound healing enhanced by hAFSCs. Paired punch biopsy wounds in SCID mice (left). At day 14 wounds treated with hAFSCs show ~90% closure compared to ~60% closure in controls ($p < 0.05$) and show increased granulation (dermis).

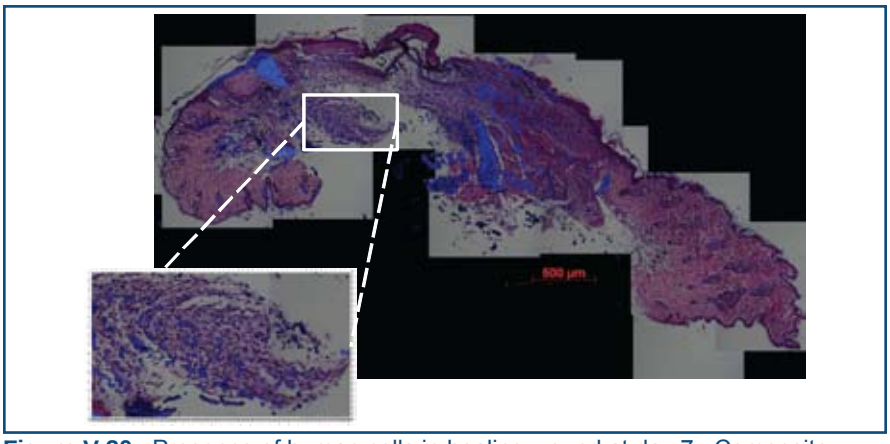


Figure V-20. Presence of human cells in healing wound at day 7. Composite image of wound treated with hAFSCs. Photograph shows H&E staining overlaid with immunofluorescence for HNA (blue), identifying human cells, from adjacent sections. Human cells (HNA+) were no longer detected after day 14.

tion conditions. Albeit preliminary, the observations of expression of CK14 and p63, especially the Np63 isoforms, demonstrate that certain essential functions of stem/progenitor cells in the basal layer of stratified epithelia such as skin can be turned on in hAFSCs. In carrying out the work plan, the highest immediate priority will be to optimize the commitment of hAFSCs (or closely related stem cells) to epidermal stem/progenitors able to yield functional keratinocyte-like cells. In addition to following up the positive preliminary data obtained using one published set of conditions—in particular, to determine whether all of the components of the rather complex serum plus growth factor cocktail, are essential—the researchers intend to test several additional sets of conditions reported for both MSCs and embryonic stem cell differentiation toward the keratinocyte lineage.

Key Research Accomplishments

- Demonstrated that hAFSCs express CD146, a cell surface marker protein recognized as a marker of perivascular cells.
- Isolated and expanded CD146-positive perivascular cells from placenta (canine and human).
- Demonstrated enhanced skin wound healing by hAFSCs in immune-deficient mice.
- Found initial evidence for the expression of stratified epithelial lineage markers p63 and CK14 by some hAFSC lines in response to in vitro differentiation conditions.

Conclusions

The data obtained to date provide support for some of the key assumptions underlying the project plan and point toward additional proof-of-concept studies. The results support the researchers' general concept that a class of broadly multipotent, strongly self-renewing stem cells that are free of some of the potential drawbacks of embryonic stem cells (e.g., teratoma formation) will have utility as a novel allogeneic cell source for skin equivalents, specifically for burn patients. As discussed in the initial project plan, ultimately, the application of the hAFSCs for this purpose will be conditioned strongly by advances in the development for burn treatment of skin equivalents based on the "traditional" allogeneic cell source for such products (foreskin).

Research Plans for the Next 4 Years

The principal rate-limiting target remains induction of differentiation of hAFSCs to cells of the epidermal lineage. Experiments in Year 2 will focus on testing conditions previously reported to induce keratinocyte-like cells from hMSCs, which share many markers with hAFSCs. Four distinct published protocols are being compared with the goal of defining an optimized protocol that may incorporate elements of each protocol. The key initial target is efficient expression of epidermal basal cell markers p63 and CK14; additional skin stem/progenitor cell markers will be tested as well. The researchers will begin in Year 2 to try to combine undifferentiated hAFSCs with candidate epidermal stem/progenitors to enhance differentiation. Although the main approach will be through in vitro constructs, progress with in vivo systems such as bio-printing in other AFIRM projects may also allow parallel experiments in vivo. Once progress toward epidermal keratinocyte lineage is achieved, the researchers will initiate significant efforts to differentiate hAFSCs toward the melanocyte lineage (anticipated in Year 3).

In Year 3, the main goal will be to begin to assess the interaction of dermal lineage cells (or undifferentiated hAFSCs capable of functioning in this lineage) with hAFSC-derived epidermal lineage cells both in vitro and in vivo. The principal approach will be to utilize a skin equivalent paradigm from an AFIRM Burn Repair Program corporate partner and determine whether the hAFSC-derived cells can substitute for the existing cell sources. Bio-printing of skin, being developed in a parallel AFIRM burn project, will offer another, potentially more rapid and accessible, approach to assess the interaction of hAFSC-derived dermal and epidermal cell types in vivo.

In Years 4–5, the main focus will be on further optimization of differentiation to generate the skin equivalent and testing in rodent models.

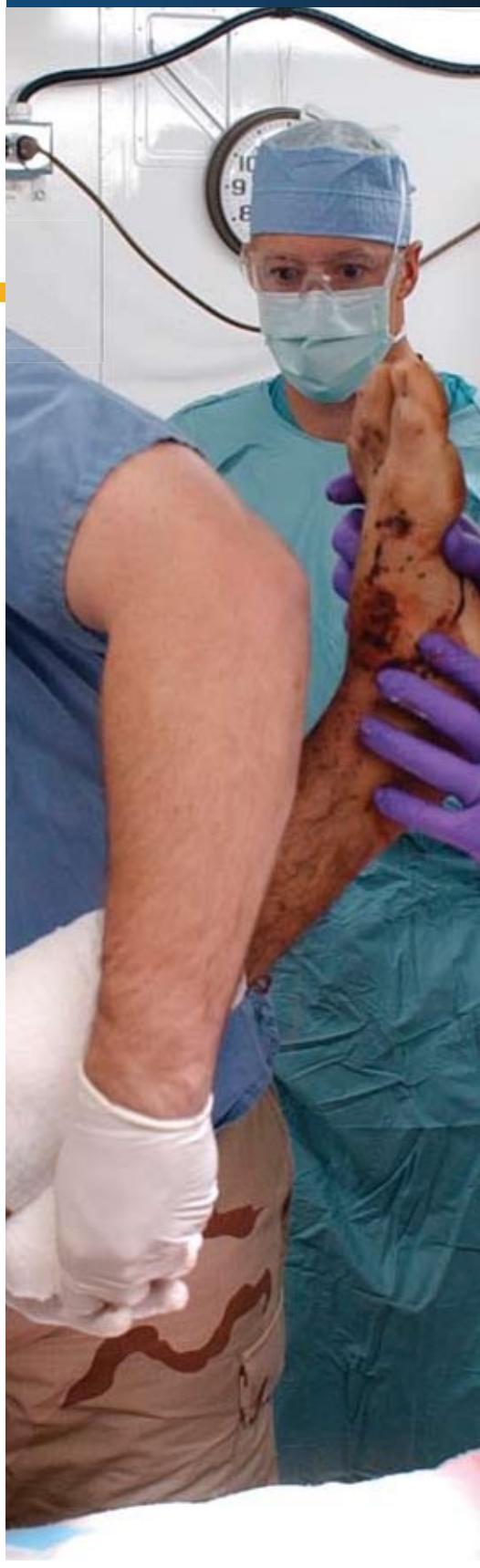
Planned Clinical Transitions

Transition of this 5-year project to clinical studies is anticipated to occur 2 years after the project ends. Success in bio-printing with undifferentiated stem cells and hAFSC-derived epidermal lineage cells could potentially accelerate the clinical translation.

Corrections/Changes Planned for Year 2

Because of evidence that undifferentiated hAFSCs can function in vivo in skin wound healing, the researchers hypothesize that the stem cells may substitute for dermal fibroblasts in skin equivalent constructs without prior in vitro differentiation. They plan to perform tests in vitro using co-culture technologies, such as cell sheets (recently reported with MSCs) and, potentially, in vivo using bio-printing. If the bio-printing technology continues to advance, it may permit accelerated epidermal lineage differentiation of hAFSCs in vivo. The incorporation of this technology in Year 2 would be a significant change relative to the initial plan. Since it is now known that hAFSCs and perivascular cells isolated from placenta share critical surface markers (e.g., CD146), the researchers have greater flexibility in the potential sourcing of stem cells from placenta in addition to amniotic fluid. As appropriate additional hAFSCs and hAFSC-like placental cell clones become available, they will be tested in the paradigms developed with currently available hAFSC lines.

VI Compartment Syndrome



Background

In the arms and legs, thick layers of connective tissue called fascia surround groups of muscles, holding them in place and protecting them. Within these layers of tissue that do not readily expand are confined spaces, called compartments, which contain 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 a muscle after surgical intervention. CS can also result from 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.

The overall goal of the AFIRM CS Program is to reduce the impact of CS on the wounded warrior through the application of regenerative medicine. The projects seek to increase the salvage of injured limbs that have been affected by CS with an interdisciplinary approach based upon a combination of stem cells and inductive biodegradable scaffolds for the reconstruction of functional compartment tissues.

Unmet Needs

AFIRM researchers have identified unmet needs for wounded warriors. The first set of needs can be characterized as prevention and early treatment, specifically, the ability to decrease the probability that an injured extremity will develop CS and to limit the risk of complications (infection and further tissue loss) following fasciotomy. The second set of needs is related to late treatment and repair, specifically, the ability to regenerate tissue lost as a consequence of CS. Untreated CS can lead to Volkmann's contracture, which is a permanent shortening of musculature of the hand at the wrist and results in a claw-like deformity

Table VI-1. Projects funded by AFIRM per clinical challenge topic area.

Clinical Challenge Topic Area	Consortium	Project Number	Project Title
Prevention/Early Treatment of Compartment Syndrome	RCCC	4.1.1	Compartment Syndrome Early Stage Biomaterials-Based Therapies
Cellular Therapy of Compartment Syndrome	WFPC	4.3.1	Cellular Therapy for the Treatment of the Consequences of Compartment Syndrome
		4.3.2	Use of Bone Marrow-Derived Stem Cells for Treatment of Compartment Syndrome
Biological Scaffold-Based Treatment of Compartment Syndrome	RCCC	4.1.2*	Engineering Skeletal Muscle Replacements
	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

**The first task of this project was to demonstrate that a bioresorbable scaffold can be used to engineer functional muscle fibers that contract upon stimulation. As the researchers gained more experience with their experimental system, they realized that the most promising application for this technology is in the engineering of small muscles such as the one that moves an eyelid. Since many warriors with facial injuries lose their ability to move the eyelid, there is a significant need for a functional restoration of eyelid movement. Accordingly, Project 4.1.2 was redirected to an eyelid muscle model and moved to the Craniofacial Reconstruction Program.*

of the hand and fingers. Advanced CS can lead to permanent paralysis due to the failure of muscles and nerves in an affected compartment to recover. 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 can regenerate muscle, nerve, and blood vessels lost to CS and other battlefield wounds and that will provide an improved functional recovery for the injured soldier.

Areas of Emphasis

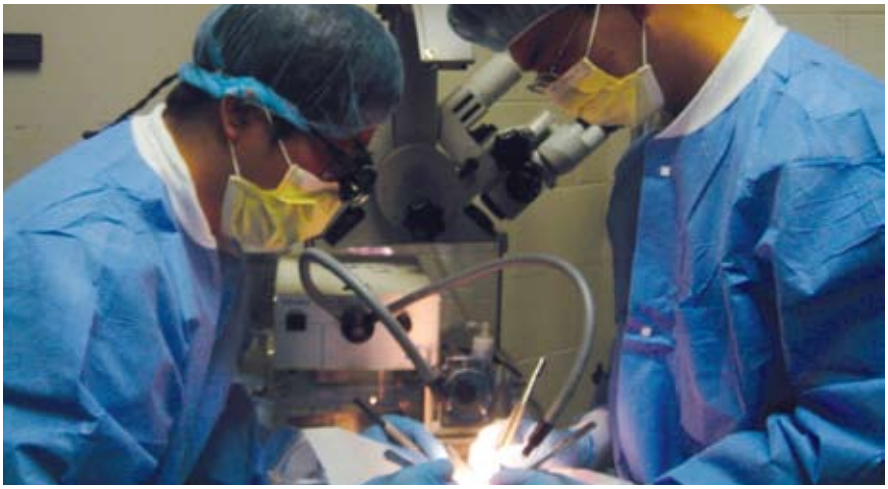
AFIRM researchers are pursuing a complementary mix of research projects focused on various aspects of treatment of CS. Projects can be grouped into three "clinical challenge" topic areas: Prevention/Early Treatment of CS, Cellular Therapy of CS, and Biological Scaffold-Based Treatment of CS. Additional details on projects in each of these topic areas can be found in **Table VI-1** and subsequent sections of this chapter.

Prevention/Early Treatment of Compartment Syndrome

Studies at Rutgers-Cleveland Clinic Consortium (RCCC)

Overview: There is a period of time after injury and before a CS becomes established when it may be possible to modify the equilibrium of fluid flow into the tissues and reduce the risk and incidence of CS. In Project 4.1.1, RCCC researchers addressed this potential opportunity using a heretofore-unproven membrane sealant technology. They designed aqueous solutions of copolymers that would seal and repair damaged cell membranes and thereby repair damaged tissues. They also designed topically applied biomaterials (e.g., sol-gels) that provide a hydrated, mechanically stable environment and have the capacity to deliver therapeutic agents into the tissue site for tissue healing and infection control.

Status at End of Year 1: At the start of Year 1, the researchers had identified a polymer system as a possible membrane sealant. As work progressed, the researchers obtained results on surface tension and membrane interactions using a Langmuir microbalance. However, they determined that the copolymers did not work as well as a commercially available surfactant, poloxamer P188. They acknowledged that any new family of polymeric surfactants that may be synthesized would require more



Drs. Fujimoto and Hashimure operating in Dr. William R. Wagner's laboratory (WFPC).

than 5 years to advance to clinical trials. It was therefore decided early on that this part of the project would not be continued, and the work on this concept would be terminated.

Regarding the second aim, the researchers assumed that a topical sol-gel-based delivery system for pro-survival cytokines could be used to treat early CS. They chose a cytokine called platelet-derived growth factor (PDGF) for the delivery system. However, even after making improvements in the sol-gel nanostructure, they could not demonstrate the delivery of a therapeutically effective dose of PDGF, and suitable 7-day release profiles of PDGF were only obtained when the total dose of PDGF in the delivery system was significantly below the therapeutically effective range. This aim was discontinued at the end of Year 1 as the project was not viewed as being sufficiently advanced compared to competitive commercial technology.

Research Plans for the Next 4 Years: A review of the performance of Project 4.1.1 by the RCCC Executive Committee revealed a number of weaknesses in the research program. The most significant finding was that a company outside of the AFIRM had advanced new technologies useful for the treatment of CS. The competing technology appears to have advantages over the technology proposed in Project 4.1.1 and is so advanced that it is expected to reach clinical trials before Project 4.1.1 could deliver a clinical therapy. Accordingly, the Executive Committee reached the unanimous decision that the work performed under Project 4.1.1 within RCCC is not competitive and that further funding of this line of research would not be in the best interest of wounded service members.

Cellular Therapy of Compartment Syndrome

Studies at Wake Forest-Pittsburgh Consortium (WFPC)

Overview: WFPC researchers are using human muscle-derived and bone marrow-derived stem cells to reconstruct functional compartment tissues following the development of CS. In Project 4.3.1, the research team is using clinically proven human muscle-derived stem cells (hMDSCs) delivered by local injection to promote the reconstruction of functional tissue composed of muscle and tendon. In Project 4.3.2, the researchers are harvesting bone marrow-derived stem cells and injecting them locally within extremity wounds that have been complicated by CS in an effort to amplify a regenerative response

WFPC researcher Dr. Johnny Huard is examining muscle sections stained with hematoxylin and eosin (H&E).

and modulate adverse inflammatory responses.

Status at End of Year 1: In Project 4.3.1, the research team created a model of CS in a rat leg muscle using a combination of a tourniquet and an external compression device and performed a microscopic evaluation of the model using a variety of stains. They isolated, characterized, and banked human and rodent muscle-derived cell populations that will be used in stem cell repair strategies. They also examined muscle biopsies obtained from human CS patients using a variety of stains and a procedure that reveals differences in genes and found that the marker profile of several genes in human CS biopsies was appreciably different from that of human control biopsies. In Project 4.3.2, the researchers developed a large animal (pig) CS model. They also developed an automated bone marrow stem cell harvesting and isolation system with associated protocols. They completed a comparative analysis of bone marrow stem cells in uninjured and CS-injured pigs.

Research Plans for the Next 4 Years: In Project 4.3.1, the researchers will focus on reproducing the data that have been reported for Aims 1 and 2 thus far in the rat model. They will increase the number of animals in each of the experiments and optimize conditions to ensure proper outcome and allow for the publication of a detailed study of their murine model of CS. They will also begin to inject hMDSCs into their CS model. The researchers will also examine more human muscle biopsies to allow them to better understand the variability among human CS patients. In Project 4.3.2, the researchers will primarily focus on determining clinically relevant stem cell administration paradigms, most importantly, the number and timing of stem cell treatments required. Years 3 and 4 will entail finalizing standard operating procedures for the preparation of automated bone marrow stem cells. They will perform U.S. Food and Drug Administration (FDA) cytotoxicity and safety studies with non-AFIRM support.

Planned Clinical Transitions: In Project 4.3.1, a transition to clinical trials is planned in Years 3 and 4 for the local injection of hMDSCs. The researchers' initial results using a common antihypertensive and congestive heart failure drug (losartan) are very promising, which may accelerate a transition to human clinical trials in Year 2 or 3 of the project. In Project 4.3.2, FDA cytotoxicity and safety studies will be performed in Years 3 and 4 with non-AFIRM support. Visits with the FDA through the regulatory consultant will culminate in preparation of the paperwork needed for a human clinical trial for 20 patients. The clinical trial for extremity injury treatment will begin in Year 5.

WFPC researcher Dr. Sheila Ingham is working on the development of a CS rat model.

Biological Scaffold-Based Treatment of Compartment Syndrome

Studies at WFPC

Overview: WFPC researchers are developing animal models of CS and implantable scaffolds that can be used to treat this potentially devastating condition. In Project 4.3.3, the researchers are focused on abdominal CS. They are investigating approaches where biodegradable scaffolds with elastic properties are implanted that will encourage cell migration and remodeling of the abdominal wall tissue. In Project 4.3.4, the researchers are creating models of peripheral CS in the rabbit and dog. They are also utilizing the

inductive properties of the extracellular matrix (ECM) as a scaffold for the reconstruction of functional compartmental tissue in these animal models. In Project 4.3.5, the researchers are inducing a patient's own stem cells to develop into muscle tissue through the use of a target-specific scaffolding system.

Status at End of Year 1: In Project 4.3.3, the researchers have created biodegradable, elastic scaffolds for reconstruction of the abdominal wall after the development of CS. They have integrated their scaffolds with ECM from the dermal layer of the skin as well as MDSCs to aid in abdominal wall regeneration. They have also created an abdominal wall defect model in the rat for the assessment of their biodegradable scaffolds. In Project 4.3.4, the researchers have established reproducible rabbit and dog models for the development of peripheral CS. They also determined that ECM derived from another species could support the growth of human microvascular endothelial cells, 3T3 fibroblasts, and perivascular stem cells in the rabbit and dog models. In Project 4.3.5, the researchers have implanted their scaffold system into damaged muscle and determined that it can mobilize stem cells from the host to move into the muscle tissue and develop into a variety of cell types, including bone, muscle, fat, and blood vessel cells.

WFPC researcher Dr. Kerry Daly is working on a regenerative medicine approach to CS.

Research Plans for the Next 4 Years: In Project 4.3.3, the researchers will continue to evaluate their biodegradable, elastic scaffolds in the small animal (rat) model. If successful, the scaffolds will be assessed in a large animal (porcine) model. In Project 4.3.4, the researchers will continue to refine their rabbit and dog models of peripheral CS through the addition of associated trauma to simulate the natural causation of the condition. They will also evaluate the effects of adding ECM derived from another species to the host ECM in the two models of CS. They will assess the recruitment of the host's cells into the compartment over time. In Project 4.3.5, the research team will continue studies focused on incorporating muscle tissue-inducing agents combined with the biomaterials scaffolding system. They seek to demonstrate functional improvement of injured muscle tissue in an animal model.

Planned Clinical Transitions: The researchers of Project 4.3.3 expect to be exploring partnering opportunities with industry for the use of the developed materials in the coming years. Upon successful completion of Aim 2, they will meet with the FDA to determine the preclinical data that would be required to justify filing for an investigational device exemption. The researchers anticipate the start of clinical trials in Year 5. The researchers of Project 4.3.4 anticipate transitioning to clinical trials following Year 3 or 4. Project 4.3.5 is a basic research project and is not slated for clinical trials during the lifetime of the award.

Project 4.1.1, RCCC

Compartment Syndrome Early-Stage Biomaterials-Based Therapies

Team Leader(s): *Paul Ducheyne, PhD (University of Pennsylvania); David Devore, PhD (Rutgers – The State University of New Jersey, New Jersey Center for Biomaterials [NJCBM])*

Project Team: *Jaehyun Kim, PhD, Christine Knabe, PhD, Shula Radin, PhD, Jonathan Garino, MD (University of Pennsylvania); Niraj Ramachandran, PhD, Joachim Kohn, PhD (Rutgers, NJCBM)*

Collaborator(s): *Thomas Walters, PhD (U.S. Army Institute of Surgical Research [USAISR])*

Therapy: *Treat early-stage CS*

Deliverable(s): *A toolkit for early-stage CS therapies based upon: (1) biodegradable tyrosine-based block copolymer membrane sealants, (2) bioresorbable silicon-based sol-gels for delivery of bioactives, and (3) composites made from the tyrosine-based copolymers and sol-gels as absorbent wound dressings*

Key Accomplishment(s): *The researchers identified a tyrosine-based triblock polymer system as a possible membrane sealant and obtained results on surface tension and membrane interactions using a Langmuir microbalance. They also developed silica-based sol-gel microparticles with controlled nanoporosity that provided highly controlled in vitro delivery of PDGF. However, all of the proposed CS studies were terminated in RCCC due to analysis of competitive commercial technology.*

Introduction

The high-energy shock wave caused by improvised explosive devices produces severe crush injuries and avulsive tissue wounds that can lead to CS, which has become a devastating problem in the battlefield. CS occurs when elevated intramuscular pressure decreases vascular perfusion of a muscle compartment to a point no longer sufficient to maintain viability of the muscle and neural tissue contained within the compartment. If left untreated or diagnosed late, CS leads to amputation or death.

The intent of Project 4.1.1 was to develop a toolkit for early-stage CS therapies based upon: (1) biodegradable, biocompatible tyrosine-based block copolymer formulations, (2) bioresorbable silicon-based ceramic sol-gels, and (3) composites made from the tyrosine-based block copolymers and sol-gels as absorbents and wound dressings. Aqueous solutions of triblock copolymers were designed to seal and repair damaged cell membranes and thereby repair damaged tissues (Aim 1). Sol-gel microparticles were designed to encapsulate and deliver therapeutic agents to the damaged tissue for tissue healing and infection control (Aim 2). The combination of the sol-gel and copolymers in composite dressings was designed to absorb extracellular fluid from an injured muscle compartment so as to reduce increase of hydrostatic pressure and also to serve as active wound dressings to deliver therapeutic agents post-fasciotomy (Aim 3). Scale-up of the technologies was planned to commence within 24 months.

The project involving polymeric surfactants was led by Dr. David Devore at Rutgers, the sol-gel technology was led by Dr. Paul Ducheyne at the University of Pennsylvania, and the composite wound dressing was a close collaboration between their groups. Work on sol-gel/copolymer wound dressings for delivery of anesthetics was an ongoing collaboration between the Rutgers and Pennsylvania groups, funded by a separate Congressionally Directed Medical Research Programs grant, which was anticipated to enable acceleration of the wound dressing envisioned for CS therapy.

Research Progress – Year 1

Aim 1: *Reduce the risk and incidence of CS by delivering a prophylactic triblock copolymer surfactant solution that will seal damaged membranes.*

A potential prophylactic approach to treat blast injuries that received preliminary evaluation at USAISR was based on resealing and repairing damaged cell membranes with polymeric surfactants. A library of biodegradable, biocompatible copolymer surfactants was therefore to be evaluated as potential "vascular bed sealants." These were intended for systemic intravenous or regional intra-arterial delivery, which could be implemented in the battlefield or in any later clinical setting. Much of the underlying polymeric surfactant technology required for these "membrane sealants" had already been developed.

The preliminary Langmuir film balance studies using available copolymers were not promising, and it was clear that alternative polymer compositions would need to be designed and synthesized to achieve the high levels of membrane surface activity required for therapeutic efficacy beyond what had been attained with a commercially available surfactant poloxamer P188. It was therefore decided early on that this part of the project would not be continued and the work on this concept would be terminated.

Aim 2: *Delivery of pro-survival cytokines into the tissue site during early care using bioresorbable silica-based ceramic sol-gel drug delivery systems.*

Silica-based ceramic sol-gel technology had been developed by the Ducheyne group with tunable porosities capable of controlled delivery of a broad range of hydrophilic and hydrophobic therapeutic agents such as growth factors, antioxidants, and antibiotics. Drug molecules were incorporated in the nanosized pore channels and were released by diffusion through the aqueous phase that penetrates into these pores. Sol-gel powders in a size of about 10–20 μm could be easily injected using standard 22 French needles. Sol-gels had been synthesized with various growth factors or molecules mimicking the size of growth factors or other large biological molecules, and the Ducheyne group had achieved controlled release with molecules up to a size of 70 kDa. All growth factors used expressed their biological function.

In this project, conditions were determined for synthesizing sol-gel powders with controlled release profiles for PDGF. Controlled release of PDGF from the sol-gel powders was successfully achieved in vitro. However, because the overall project was not viewed as being sufficiently advanced compared to technology developed by Twin Star Medical of St. Paul, Minnesota, the project was terminated at the end of Year 1, and no further studies of other bioactive agents in the sol-gels were pursued.

Aim 3: *Improve the rate of resolution of swelling following fasciotomy through the use of biomaterial wound dressings designed to absorb extracellular fluids from exposed tissues and deliver therapeutic agents to promote healing.*

Biocompatible hydrogels had been used to prepare flexible, mechanically strong composite wound dressings containing the silica sol-gels described in Aim 2. This was part of an ongoing, separate collaborative project between Drs. Devore and Ducheyne on delivery of local anesthetics funded by the Congressionally Directed Medical Research Programs. For this AFIRM project on CS, the tyrosine-based copolymer hydrogels were to be designed to absorb more than 100% of their weight in body fluid while maintaining their flexibility, adhesion, and mechanical integrity. The composite dressings would also enable delivery of selected therapeutic agents to the wound site, specifically PDGF and one or more antibiotics.

One recent approach for the treatment of CS was the application of tissue ultrafiltration using a system designed and developed by Twin Star. In this process, interstitial fluid was removed through semi-permeable hollow fibers of 1.3 mm outside diameter using a vacuum manifold with a negative pressure of 500 mm Hg. It was demonstrated with this device in an in vivo porcine model that removal of only about 1 mL of interstitial fluid results in a reduction of intramuscular pressure so that compartment pressure is restored to a normal range. It was therefore postulated that absorption of interstitial fluid by means of a biodegradable tyrosine-based hydrogel may achieve comparable intramuscular pressure reduction and prophylaxis without the need for a complex vacuum manifold device. However, a subsequent analysis of competitive technologies (ACT) made it evident that the hydrogels were not likely to provide fluid uptake levels comparable to what was achieved by either commercial hydrogel polymers or by the Twin Star ultrafiltration system. Therefore, Aim 3 of the project was not pursued.

Key Research Accomplishments

- Identified a tyrosine-based triblock polymer system as a possible membrane sealant and obtained results on surface tension and membrane interactions using a Langmuir microbalance.
- Determined that time-dependent, sustained in vitro release kinetics for PDGF from sol-gels could be controlled by modifying the nanostructural properties of the sol-gel.

Conclusions

Regarding the polymeric surfactant membrane sealants, the insertion of the tyrosine-based copolymer into lipid monolayer films was

deemed to be very low compared to the commercially available poloxamer P188. The relatively poor performance of the tyrosine-based copolymer is ascribed to its strong self-association into nanospheres even at extremely dilute concentrations. Substantial synthetic modifications in the structure of the tyrosine-based copolymers would be required to create a family of new polymeric surfactants that may be competitive with the poloxamer. It is not consistent with the objectives of the AFIRM to pursue such basic research and hence this work was discontinued.

Regarding the sol-gel delivery of PDGF, the researchers demonstrated a correlation of release kinetics to the nanostructure of the sol-gel, which enabled the design of optimal nanostructure properties for the release of PDGF. They achieved a time-dependent, sustained release by modifying the sol-gel processing and drying methods. However, even after making improvements in the sol-gel nanostructure, the researchers could not demonstrate the delivery of a therapeutically effective dose of PDGF. This aim was also discontinued as the project was not viewed as being sufficiently advanced compared to competitive commercial technology.

Research Plans for the Next 4 Years

A review of the performance of this project by the RCCC Executive Committee revealed a number of weaknesses in the research program. The most significant finding was that a company outside of the AFIRM had advanced new technologies useful for the treatment of CS. The competing technology appears to have advantages over the proposed technology and is so advanced that it is expected to reach clinical trials before this project could deliver a clinical therapy. Accordingly, the Executive Committee reached the unanimous decision that the work performed under Project 4.1.1 within RCCC is not competitive and that further funding of this line of research would not be in the best interest of wounded service members.

Planned Clinical Transitions

Not applicable.

Corrections/Changes Planned for Year 2

All programs and projects in RCCC are subjected to periodic reassessments. In each case, the projects are evaluated for their impact on the wounded warrior and for their ability to address unmet clinical needs. The performance of the project team is also evaluated for how successfully the team meets its milestones and complies with RCCC's reporting requirements. Further project evaluation criteria include an assessment of the likelihood that a therapy or product would reach clinical trials within the AFIRM's 5-year contract period. Finally, projects are compared to competitive projects from outside RCCC through an ACT.

The RCCC Executive Committee reviewed the performance of the CS Program (Projects 4.1.1 and 4.1.2). In its evaluation, the committee carefully considered the above-mentioned criteria based on several inputs, including detailed presentations by the project leaders (who were fully engaged in the review process), progress reports, and technical information submitted by the project leaders throughout the year. An ACT was an integrated part of the review.

The review process revealed a number of weaknesses in the CS Program. The most significant finding was that a company outside of the AFIRM had advanced new technologies useful for the treatment of CS. The ACT revealed that the competing technology can be used pre- or post-fasciotomy, either to decrease pressure in the compartment or to deliver agents through multiple catheters. This competing technology is so advanced that it will surely reach clinical trials before Project 4.1.1 can deliver a clinical therapy.

Accordingly, the Executive Committee reached the unanimous decision that the work performed under Project 4.1.1 within RCCC is not competitive and that further funding of this line of research would not be in the best interest of wounded service members. In addition, Project 4.1.2 (Muscle Replacement) was redirected to the regeneration of the muscle moving the eyelid and reassigned to the Craniofacial Reconstruction Program. By these actions, RCCC eliminated its own CS Program. It is important to note that work to alleviate the consequences of CS continues in other parts of the AFIRM. RCCC regards the termination of its CS Program as the best course of action since it will result in (1) the elimination of duplicate efforts between the two AFIRM consortia and (2) an opportunity for better strategic realignment of RCCC's funding with its most promising projects.

Project 4.3.1, WFPC

Cellular Therapy for the Treatment of the Consequences of Compartment Syndrome

Team Leader(s): *Johnny Huard, PhD, Shay Soker, PhD*

Collaborator(s): *USAISR, University of Pittsburgh Medical College (UPMC)*

Therapy: *Cell-based treatment for CS*

Deliverable(s): *(1) Murine model of CS, (2) hMDSC approach for treating CS, (3) angiogenic agent(s) for treatment of CS, and (4) biological approach to preventing/eliminating scar tissue and improving muscle healing after CS injury*

Key Accomplishment(s): *The research team created a model of CS in the rat tibialis anterior (TA) muscle using a combination of a tourniquet and an external compression device and performed a histological analysis of this model. They also isolated, characterized, and banked human and rodent muscle-derived cell populations that will be used in stem cell repair strategies. In addition, they examined muscle biopsies obtained from human CS patients using a variety of stains and reverse transcriptase-polymerase chain reaction (RT-PCR) and found that the marker profile of several genes in human CS biopsies was appreciably different from that of human control biopsies.*

Introduction

In general, musculoskeletal injuries strongly impact the Army in terms of human suffering, direct and indirect monetary costs, loss of time for work or training and, perhaps most importantly, military readiness. These injuries alone account for a large number of disabled soldiers. According to the Medical Surveillance Monthly Report issued by the Department of Defense, musculoskeletal injuries were the leading cause of hospitalization of active duty members in 1993. In 1997 and 2001, these injuries again were highlighted on the list of frequent causes of service member hospitalization, ranking second and fifth overall in these years, respectively.

Among the musculoskeletal injuries that result from battlefield trauma that need the most clinical implementation is CS. CS occurs when the circulation and function of tissues within a closed space are compromised and, ultimately, cause tissue necrosis (leading to contractures, muscle weakness, and sensory deficits), rhabdomyolysis, renal failure, and even death. In fact, acute CS is a potentially devastating condition for soldiers as the pressure within an osseofascial compartment rises to a level that decreases the perfusion gradient across tissue capillary beds, usually leading to cellular anoxia, muscle ischemia, muscle degeneration, and fibrosis. 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 including severe pain and lack of pulse in the limb and is supplemented by compartment pressure measurements.

The specific aims of this project are to (1) determine and compare the regenerative capacities of hMDSCs with that of human myoblasts after implantation in injured skeletal muscle after CS, (2) investigate the effect of angiogenesis on the regenerative capacity of hMDSCs injected into injured skeletal muscle after CS, and (3) develop biological approaches to prevent and eliminate scar tissue and improve muscle healing after CS injury.

Research Progress – Year 1

The researchers conducted research to establish the best animal model in rodents. They tried several models during the first year of this project. They obtained three approved Institutional Animal Care and Use Committee protocols from their universities for the creation of CS in rats. The animal models created were based on the knowledge that to increase pressure, CS can be established by diminishing the compartment size and/or by increasing the compartment content. Attempts to create CS in rats by increasing the compartment pressure by saline and plasma injection were inconsistent due to the properties of rodent fascia. Better reproducibility was obtained when a tourniquet was used, and the best results were attained when a tour-

niquet and an external compression device were used on the hindlimb.

The results of histological examination of the affected TA muscles (injured via a tourniquet and external compression device) are shown in **Figure VI-1**. The tourniquet and external device induced ischemia and subsequently enucleation of the central core of the TA, which was confirmed using a number of nuclear stains and dyes, as well as by probing for nuclear-specific antigens such as histone H3. The muscle fiber disintegration and lack of nuclei within the TA's central core represents a necrotic compartment. H&E staining at 7 days post-injury revealed intact muscle fiber architecture at the periphery that was lost more centrally in the TA muscle. Muscle fiber integrity and endothelial cell presence was then examined in damaged and control TA muscles. Damaged muscle peripheries showed some preserved muscle fiber architecture and an increased presence of microvasculature when compared to the enucleated TA central core. This enucleated, ischemic central core represents a necrotic muscle compartment. This decellularized compartment devoid of capillaries will serve as a target for Aim 2, which proposes to combine stem cell and angiogenic therapies.

Figure VI-1 also reveals a buildup of mononuclear cells along the periphery of the injured muscle. The researchers are currently in the process of characterizing these infiltrating cell types. Interestingly, Masson's trichrome staining reveals immature collagen deposition and fibrosis along the front of these infiltrating mononuclear cells. This fibrotic front is most easily seen in the injured muscle. A closer examination of injured muscle fibers in the TA central core revealed the loss of muscle fiber–fiber integrity. The fibers also seemed to be lacking nuclei. In parallel, the histology of muscle biopsies obtained from human CS patients (sources: USAISR in Fort Sam Houston, Texas, and the UPMC) was examined to emulate the conditions in the animal model and better understand the histology and molecular profile of this syndrome in humans. The time at which muscle is collected is important, with later stages showing severe muscle damage. To compare human biopsies with rat CS tissue, the researchers performed RT-PCR studies of certain genes from the human biopsies and rat sections. They found that the marker profile of several genes was appreciably different from that of control human biopsy.

The researchers have immunostained CS-injured muscle with a blood vessel marker (CD31) to establish a baseline for future work on angiogenic factors. They have also immunostained muscle sections with anti-dystrophin to establish a baseline of damage in CS-injured muscle and controls to facilitate future stem cell therapy of CS injuries. They also isolated, characterized, and banked human and rodent muscle-derived cell populations that will be used in stem cell repair strategies.

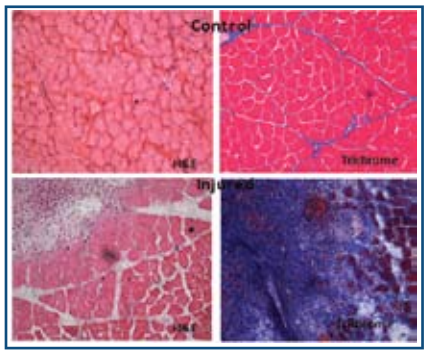


Figure VI-1. Comparison of control and injured TA after 7 days.

To achieve Aim 3, the researchers conducted research capitalizing on previous studies in murine models to inhibit scar formation to facilitate better muscle tissue regeneration. The effect of an orally active, nonpeptide angiotensin receptor antagonist (losartan) on one CS patient was examined in the clinic, and dramatic improvement was seen in his muscle injury. More patients and data obtained from randomized and double-blinded experiments may provide additional insight into the mechanism of repair by losartan and its effects on CS patients.

Recently, the researchers achieved live cell labeling of human umbilical vein endothelical cells, pericytes, and myoblasts, which allows for the imaging of individual cells when grown together in vitro (**Figures VI-2** and **VI-3**).

Key Research Accomplishments

- Created a model of CS in the rat TA muscle using a combination of a tourniquet and an external compression device that allows the pressure to drop as it supports the fascia and performed a histological analysis of this model.
- Immunostained CS-injured muscle with a blood vessel marker (CD31) to establish a baseline for future work on angiogenic factors.

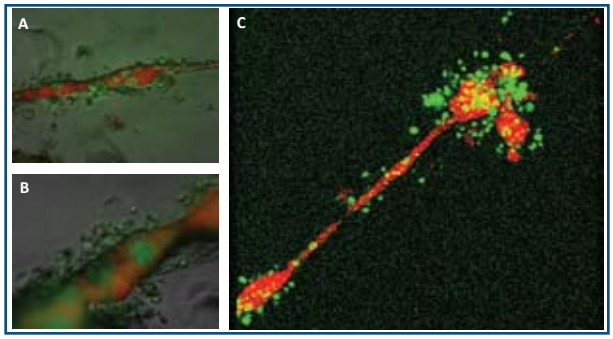


Figure VI-2. Pericytes (green) grow along side and surround human umbilical vein endothelial cells (HUVECs - red) when grown together in matrigel. Cells were grown in matrigel for 5 days before being imaged with a Zeiss Axiophot inverted fluorescent microscope at either 10x magnification (A) or 20x magnification (B). In (C), a Z-stack (10x) was obtained with a Zeiss LSM 510 confocal microscope, and a three-dimensional image was constructed using the LSM Image Browser software.

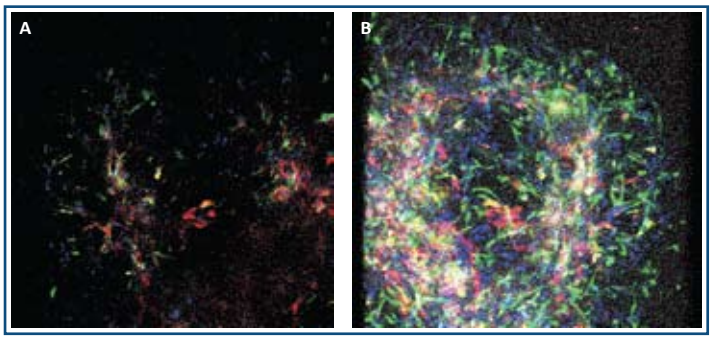


Figure VI-3. Pericytes (blue), human umbilical vein endothelial cells (HUVECs - red), and GFP+ mouse myoblasts (green) are able to be visualized by confocal microscopy when grown together in matrigel. Cells were grown in matrigel for 8 days before being imaged with a Zeiss LSM 510 confocal microscope at 10x magnification (A). In (B), a Z-stack was obtained, and a three-dimensional image was constructed using the LSM Image Browser software.

- Immunostained muscle sections with anti-dystrophin to establish a baseline of damage in CS-injured muscle and controls to facilitate future stem cell therapy of CS injuries.
- Isolated, characterized, and banked human and rodent muscle-derived cell populations that will be used in stem cell repair strategies.
- Examined muscle biopsies obtained from human CS patients using a variety of stains and RT-PCR to better understand the histology and molecular profile of this syndrome in humans.
- Studied the effect of an orally active, non-peptide angiotensin receptor antagonist (losartan) on one CS patient in the clinic and observed dramatic improvement in his muscle injury.

Conclusions

The researchers have made progress toward each of the three goals of this project. Their creation and characterization of a murine model of CS will lay the foundation for future studies. The researchers believe that with more patients and additional data obtained from randomized and double-blind experiments, they will be able to gain considerable insight into the mechanism of repair by losartan and its effects on CS patients.

Research Plans for the Next 4 Years

The researchers will focus on reproducing the data that have been reported for Aims 1 and 2 thus far in the rat model. They will increase the N size for each of the experiments and optimize conditions to ensure proper outcome and allow for the publication of a detailed study of their murine model of CS. At the same time, they will be conducting other experiments (described in Aims 2 and 3) in which injections of hMDSCs will be made into the CS model. Some of the in vivo studies can be evaluated for maximum success of the experiments and survival of the animals. Institutional Animal Care and Use Committee approval of any modifications will be sought. The researchers also plan to examine more muscle biopsies from the USAISR and UPMC to allow them to better understand the variability among human CS patients.

Planned Clinical Transitions

A transition to clinical trials is planned to occur in Years 3 and 4 for local injection of hMDSCs. The initial results using a common anti-hypertensive and congestive heart failure drug (the angiotensin converting enzyme inhibitor, losartan) are very promising, which may accelerate a transition to human clinical trials in Year 2 or 3 of the project.

Corrections/Changes Planned for Year 2

None.

Project 4.3.2, WFPC

Autologous Bone Marrow-Derived Stem Cells to Treat Extremity Injury Complicated by Compartment Syndrome

Team Leader(s): *Kenton Gregory, MD*

Collaborator(s): *Oregon Laser Institute*

Therapy: *Cell-based treatment for CS*

Deliverable(s): *Large animal model of CS and treatment for extremity injury complicated by CS using bone marrow-derived stem cells*

Key Accomplishment(s): *The researchers developed a large animal (porcine) injury and CS model, and an automated bone marrow stem cell harvesting and isolation system with associated protocols. They completed a comparative flow cytometry analysis of bone marrow stem cells in uninjured and CS-injured pigs.*

Introduction

Massive soft tissue wounds to extremities are among the most common battlefield injuries sustained by soldiers in current military conflicts. Secondary swelling or compression creates ischemia and infarction of muscle, nerve, and vascular tissues (CS), which dramatically magnifies the injury. Emergent fasciotomy can abrogate the ischemic process but creates additional injury. In many cases, the ECM remains intact, but the cells have died to an extent that endogenous regenerative capacity is exceeded, leaving a dysfunctional, atrophic limb. Soldiers developing CS have prolonged recovery times and rarely recover complete function. The research team for this project seeks to improve cellular regeneration by amplifying the endogenous host response to severe injury through autologous bone marrow stem cell therapy. Shifting the balance from atrophy and scar formation to replacement with functional cells by amplifying the body's natural response to injury is the key to this new treatment.

Animal and clinical trials of autologous bone marrow-derived stem cell therapy to treat extremity injury, as well as acute myocardial infarction, have shown early safety and efficacy. Whether the regenerative effect is due to stem cell-mediated paracrine effects or cell engraftment has not been proven, but improved functional outcomes are significantly positive. The researchers have developed a large animal model of CS and are determining parameters critical to a successful regenerative strategy, including time of cell harvest and administration, numbers of needed treatments, and practical stem cell separation technologies. Research in earlier phases of their program has shown a significant bone marrow amplification of stem cell niches and a local increase in stem cell homing signals within large animal models 1 week after extremity injury and CS. Based on this work, the researchers hypothesize that bone marrow harvest of stem cells after 1 week of injury will result in an efficacious therapeutic aliquot of stem and progenitor cells.

Research Progress – Year 1

Specific Aim 1

CS Animal Model

The research team established a large animal model of CS in a skeletal muscle. They noted that the domestic juvenile swine has proven to be an adequate model to perform initial studies. However, they acknowledged that there are limitations to this particular animal model. One drawback to domestic swine is the animals' rapid growth rate. Swine may gain up to 0.75–1.0 lb per day. This rapid growth rate has made it difficult to plan studies beyond 3 months. There is also the issue that juvenile animals are not at the sexual maturity of an adult human, which is the targeted population group for clinical studies. The researchers also noted that the healing rates among juvenile animals may be much faster than their adult counterparts.

Now that the researchers have developed an injury and CS model with the inexpensive domestic juvenile swine, they will perform therapeutic long-term studies with Sinclair mini-swine. This breed of mini-swine has a leg structure that is similar to a domestic swine. In addition, the Sinclair swine do not develop a potbelly to the degree that the Yucatan swine do, thus avoiding the abdomen dragging on the ground. An alternative would be to use adult sheep, which have a thicker muscle compartment fascia compared to a swine and may allow the use of saline instead of plasma infusions. Sheep also have some housing advantages over swine.

While most clinical trials of stem cells for clinical regenerative strategies involve the use of a single injection of cells, it is generally believed that optimal results will be attained with a series of treatments. Accordingly, a therapeutic trial is being performed in swine comparing a series of injections over the month following injury. The researchers are comparing: (1) a single injection at 1 week, (2) injections at 1 and 2 weeks, and (3) injections at 1, 2, and 4 weeks. They hope to determine whether there is any benefit of added sequential injections into the injured muscle.

Flow Cytometry

The researchers developed polychromatic flow cytometry protocols for the study of adult porcine bone marrow stem cells. They completed a comparative analysis of bone marrow stem cells in uninjured and CS-injured pigs, examining changes in stem cell surface markers. They demonstrated that bone marrow cells from pigs express known markers of mesenchymal stem cells (CD29, CD90, and CD44), endothelial cells (CD144, CD31, and VEGFR2), perivascular cells (CD146 and CD105), myogenic cells (CD56 and CXCR4), and stem cells (c-kit and Sca-1). Preliminary results suggest that the porcine CS injury animal model upregulates expression levels of certain identifiable cell phenotypes. Data demonstrated that CD90, CD56, CD144, CD105, CD144, and VEGFR2 showed statistically significant increases in response to trauma at different time points.

The researchers plan to perform additional flow cytometry analyses of cell surface markers on freshly isolated bone marrow-derived cells isolated from control and injured pigs at earlier time points. If the specific bone marrow stem cell populations with skeletal muscle remodeling ability can be identified, isolated, and enriched, this will greatly enhance the ability to use these cells for a clinically relevant therapy for the repair of injured damaged muscle.

Cell Labeling

The researchers showed that different quantum dot cell trackers do not load equally into pig bone marrow cells. The red fluorescent quantum dots (> 625) were found to be retained longer in the pig bone marrow stem cells than the other fluorescent colors (e.g., green, yellow-green, yellow, and orange).

The researchers showed that the uptake of all quantum dots was highly dependent upon the use of the coating peptide. A time-course study revealed that 1 hour was best for optimal loading of the quantum dots into the bone marrow stem cells. The organic CM-DiI fluorescent dye was found to load all the bone marrow stem cells within 10 minutes. Proliferation studies revealed that the CM-DiI-loaded bone marrow stem cells had similar proliferation rates compared to unloaded bone marrow cells.

Cell Invasion and Colony Forming Unit Assays

The researchers noted that studies from many other tissue types have shown that several homing factors are upregulated after injury, which appears to be important in attracting bone marrow to the sites of injury. They commented that the role of homing factors has not been studied in CS injury. They developed a cell invasion assay protocol for adult porcine bone marrow in response to the homing factor SDF-1 (**Figure VI-4**). 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.

The researchers noted that there is evidence from other studies that preconditioning stem cells in low-oxygen environments confers to these cells a greater ability to migrate to injured sites compared to untreated cells. They stated that the use of hypoxic-treated bone marrow cells may result in a better treatment of the damaged tissues after CS injury.

Specific Aim 2

The researchers developed clinically relevant bone marrow har-

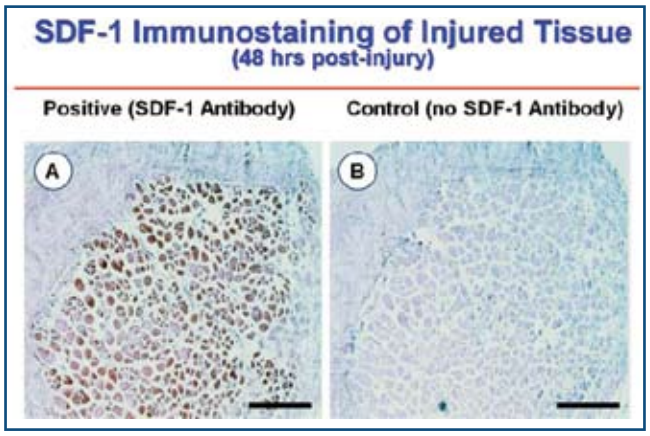


Figure VI-4. Immunostaining for the homing factor SDF-1 in the injured porcine TA muscle. Note that just 48 hours after injury there is substantial positive SDF-1α staining within the tissue.

vest and cell isolation techniques, protocols, flow path and software development, and validation for the automated SEPAX Cell Separation platform. They evaluated all systems in acute and chronic swine studies. The research team began FDA regulatory pathway development with Darin Weber with the Biologics Consulting Group (Seattle, Washington) and the Bio-Safe Regulatory Group.

Key Research Accomplishments

- Developed a large animal (porcine) injury and CS model.
- Developed an automated bone marrow stem cell harvesting and isolation system with associated protocols.
- Completed a comparative flow cytometry analysis of bone marrow stem cells in uninjured and CS-injured pigs.
  - Preliminary results suggest that CD90, CD56, CD144, CD105, and VEGFR2 are upregulated in response to trauma at different time points.

Conclusions

The researchers have successfully developed a large animal model of CS and an automated, closed/sterile flow path, small-footprint bone marrow stem cell isolation and preparation system, which can be deployed with minimal facility requirements. This approach to accelerating the healing and regeneration of tissue lost to battlefield blast and other extremity trauma offers a unique, safe, and practical opportunity to improve functional recovery for the injured soldier.

Research Plans for the Next 4 Years

Year 2 and part of Year 3 of this project will primarily focus on determining clinically relevant stem cell administration paradigms, most importantly, the number and timing of stem cell treatments required. If the large animal trials prove safety and efficacy of this concept, Years 3 and 4 will entail finalizing standard operating procedures for bone marrow harvest and disposable flow paths and software for the preparation of automated bone marrow stem cells. FDA cytotoxicity and safety studies will be performed with non-AFIRM support. Visits with the FDA through the regulatory consultant will culminate in the preparation of an Investigational New Drug and submission to the Institutional Review Board and FDA for human clinical trials for 20 patients. Year 5 will be the human clinical trial for extremity injury treatment.

Planned Clinical Transitions

In Years 3–4, FDA cytotoxicity and safety studies will be performed with non-AFIRM support. Visits with the FDA through the regulatory consultant will culminate in the preparation of an Investigational New Drug and submission to the Institutional Review Board and FDA for human clinical trials for 20 patients. The human clinical trial for extremity injury treatment will begin in Year 5.

Corrections/Changes Planned for Year 2

Aside from completion of the stem cell administration study that was somewhat delayed due to animal model development and contracting delays, no significant changes are proposed.

Project 4.3.3, WFPC

Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy

Team Leader(s): *William R. Wagner*

Collaborator(s): *Johnny Huard, PhD*

Therapy: *Treatment for abdominal CS*

Deliverable(s): *Biodegradable elastomeric scaffolds for fascial reconstruction*

Key Accomplishment(s): *The research team has produced abdominal wall patch materials, a series of novel dermal ECM digests and electrospun poly(ester urethane) urea (ePEUU) blends, tissue constructs combining MDSCs and ePEUU, and a small animal model for in vivo assessment of abdominal muscle patches.*

Introduction

Abdominal CS represents the pathophysiologic consequence of a raised intra-abdominal pressure, which causes progressive hypoperfusion and ischemia of the intestines and other peritoneal and retroperitoneal structures. Damage control laparotomy is performed for repair of the primary injury and hemorrhage control. In many cases, massive edema of the bowel, caused by primary injury and/or operative procedures, precludes the primary closure of the abdominal wall fascia, which may lead to secondary abdominal CS. Open abdomen management has been adopted in the initial stage of treatment. After successful avoidance of abdominal CS, however, the large abdominal wall defect needs to be reconstructed. In that circumstance, when autogenous tissue reconstruction is planned, mobilization of the components of the abdominal wall is difficult, leading to repairs under tension and an increased incidence of hernia formation over time.

Many techniques are advocated for repair of these defects. The most commonly applied approaches are with the use of prosthetic materials. The most obvious advantages to the use of prosthetic materials are their ready availability and the fairly simple techniques of implantation. The disadvantages to prosthetic materials are the risks of intestinal fistula, prosthetic infection, adhesions, and recurrent hernias. Excellent clinical and animal results with biodegradable material derived from animal ECM have been obtained with these materials in a variety of placements. The disadvantage of these materials is that the mechanical properties, particularly elasticity, are limited.

Given these observations, the researchers hypothesized that application of an engineered tissue based upon an elastic biodegradable synthetic material, ePEUU, would result in improved outcomes in the reconstruction of the abdominal wall and other sites of fascia reconstruction. The elastic and biodegradable properties of this material may facilitate the generation of a mechanically appropriate tissue both in the early and late stages of healing for an extensive abdominal wall defect. The researchers are investigating approaches where scaffolds are implanted that will encourage cell migration and remodeling of the wall tissue, as well as approaches where cell-seeded scaffolds are implanted for wall regeneration.

Research Progress – Year 1

The research team successfully produced abdominal wall patch materials by electrospinning the biodegradable elastomer, ePEUU, under dry conditions and conditions of media wetting. These materials have been characterized with biaxial mechanical testing to show similarities to the native abdominal wall tissue and differences from the

stiffer control material, expanded polytetrafluoroethylene (ePTFE). The researchers noted that they were particularly interested in their findings with wetted electrospinning since this technique led to markedly different properties from traditional dry electrospinning and better mimicked the nonlinear tensile properties of native abdominal wall. They created a series of novel blends of dermal ECM (dECM) digests and ePEUU using electrospinning (**Figure VI-5**). The resulting composite materials markedly increase the mechanical properties of dECM and provide a new material for abdominal wall repair (and potentially for a variety of other applications). The research team has initiated biocompatibility testing with these materials.

The researchers collaborated with Dr. Johnny Huard to create a tissue construct by combining MDSCs and ePEUU. PEUU was electrospun while the cells were electrosprayed from culture medium in a microintegration process. The researchers have shown that they can obtain tissue constructs with very high cell densities (**Figure VI-6**) and that cell viability is consistently high (in excess of 95%). They have begun examination of the biaxial mechanical properties of these constructs. They are working to minimize the formation of lamellae in these structures. Once the constructs have been characterized, the researchers will evaluate them in abdominal wall implantation.

The researchers created, ahead of schedule, the small animal (rat) model for the in vivo assessment of abdominal patches (including peritoneum, rectus muscle, and fascia). They initiated studies with defect reconstruction by dry and wet ePEUU sheets as well as ePTFE, which is currently used clinically as a control. They created abdominal wall defects (1 x 2.5 cm size, including peritoneum, rectus muscle, and fascia) in the Lewis rat model. In the initial proposal for in vivo experiments, they considered the creation of 2 cm wide x 3 cm long abdominal wall defects. However, recent studies use smaller abdominal defect models for rats. Therefore, they changed the defect size from 2 x 3 cm to 1 x 2.5 cm. They successfully established this abdominal wall defect model without morbidity and mortality up to 8 weeks after surgery (**Figure VI-7**).

Key Research Accomplishments

- Produced abdominal wall patch materials.
- Produced a series of novel dECM digests and ePEUU blends.
- Created tissue constructs combining MDSCs and ePEUU.
- Created a small animal model for the in vivo assessment of abdominal muscle patches.

Conclusions

The researchers have made substantial progress toward the development of an elastic MDSC-seeded cellularized fascia tissue construct for the repair of lifesaving fasciotomy and the encasement of the new compartment tissue. They have developed innovative biocompatible matrices and characterized them as integrated tissue constructs for fascial reconstruction.

Research Plans for the Next 4 Years

As mentioned before, the researchers are ahead of schedule and have a small animal model in use. They are currently collecting data from explanted samples and characterizing constructs mechanically and histologically over the implant period. The implants that are currently being evaluated are acellular (i.e., they do not utilize microintegrated stem cells). In the coming year, the researchers will broaden these in vivo studies in terms of the implant

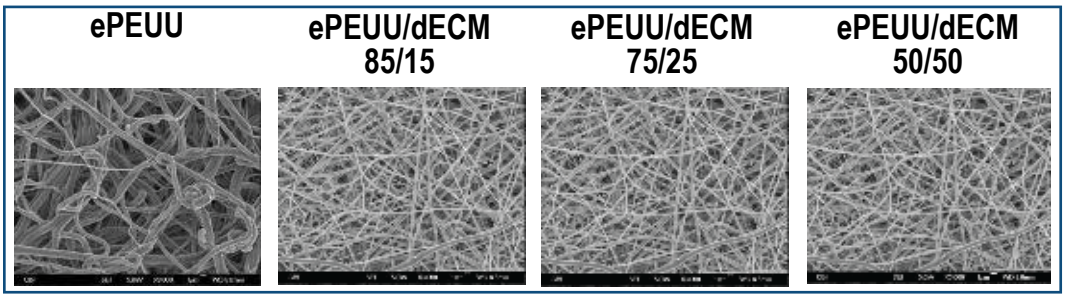


Figure VI-5. Scanning electron microscope images demonstrating the fibrous morphologies of ePEUU blended with dECM at (A) 0, (B) 15, (c) 25, and (D) 50 wt% dECM.

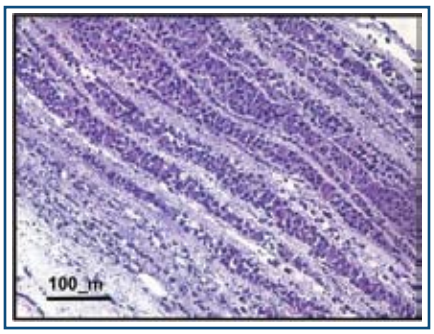


Figure VI-6. Established cellular microintegration technique. H&E staining of microintegrated MDSE with ePEUU fibers after 1-day static culture.

periods (4, 8, and 12 weeks) and assessments, including the histological biocompatibility, adverse events, intestinal fistula, prosthetic infection, and recurrent hernias for each of the implanted constructs or biomaterials. They anticipate the submission of a full manuscript on the first phase of these results by the end of 2009. The researchers will also further evaluate the cell microintegrated and ECM-polymer blend scaffolds both in terms of in vitro characterization and in the small animal model. They plan on utilizing GFP (green fluorescent protein) transgenic MDSCs to allow cell tracking in the in vivo studies with the microintegrated scaffolds but to date have had some difficulty in isolating the GFP cells. This effort will continue and, if necessary, other cell tracking methods will be used (e.g., acute cell membrane dyes and lacZ transfection).

With optimization of scaffold design ongoing in Years 2 and 3, the researchers plan to initiate large animal trials with the porcine model later in Year 3 using acellular constructs. They will build complexity 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 they reach the large animal model, the researchers anticipate being able to evaluate their fascial reconstruction technique in concert with their approaches of other AFIRM CS projects.

Planned Clinical Transitions

The researchers expect to be exploring partnering opportunities with industry for the use of the developed materials in the coming years. Upon successful completion of Aim 2, they will meet with the FDA to determine the preclinical data that would be required to justify filing for an investigational device exemption. Once this milestone is met, clinical trials can commence. The researchers anticipate the start of clinical trials in Year 5.

Corrections/Changes Planned for Year 2

There have not been any substantial changes for Year 2. The researchers have already developed the small animal model. This work originally was to be done in the first part of Year 2. The small animal model tasks, however, are extensive and the researchers will be moving through material evaluation, starting with the simplest constructs. Year 2 will be focused on the in vivo evaluation of constructs, but they will also be doing in vitro characterization of the more complex tissue constructs that will enter small animal trials late in Years 2 and 3.

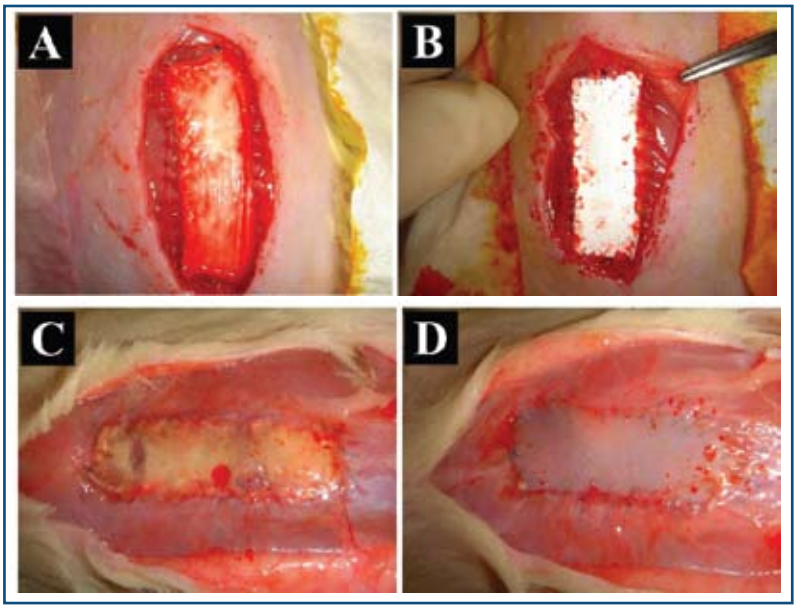


Figure VI-7. Macroscopic appearance of implanted ePEUU with media wetting during processing (A) and ePTFE (B) at surgery, appearance of implanted ePEUU with media (C) and ePTFE (D) 8 weeks after surgery.

Project 4.3.4, WFPC

Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome

Team Leader(s): *Stephen F. Badylak, DVM, PhD, MD*

Collaborator(s): *University of Pittsburgh*

Therapy: *Treatment for peripheral CS*

Deliverable(s): *Methods for (1) in situ decellularization of a tissue compartment and (2) reconstructing functional compartmental tissue in animal models utilizing the inductive properties of ECM as a scaffold.*

Key Accomplishment(s): *The researchers established reproducible rabbit and dog models for the development of peripheral CS. They also established the biocompatibility of exogenous ECM for supporting the growth of human microvascular endothelial cells, 3T3 fibroblasts, and perivascular 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 researchers of this project are 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, and pericardium) 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 in situ decellularization of the necrotic tissue while retaining the native ECM. Stated differently, the ECM within the compartment would be isolated from its original cell population (which has now become necrotic), and this matrix would then be used as a template for tissue reconstruction.

Research Progress – Year 1

The research team is pursuing two specific aims: (1) development of a method for in situ decellularization of a tissue compartment and (2) determination of the efficacy of autologous intracompartmental ECM alone or autologous intracompartmental ECM combined with either stem cells or exogenous xenogeneic ECM for the reconstruction of functional compartmental tissue in animal models.

Specific Aim 1

The researchers have made considerable progress toward the creation of peripheral CS in the rabbit and dog preclinical animal models. They established a method of complete decellularization of the anterior tibial compartment in the models. They noted that this method can be accomplished in 1 hour and 40 minutes with the perfusion of a mixture of enzymes and detergents (**Figure VI-8**).

Specific Aim 2

Although the researchers have not yet attempted intracompartmental reconstruction of functional skeletal

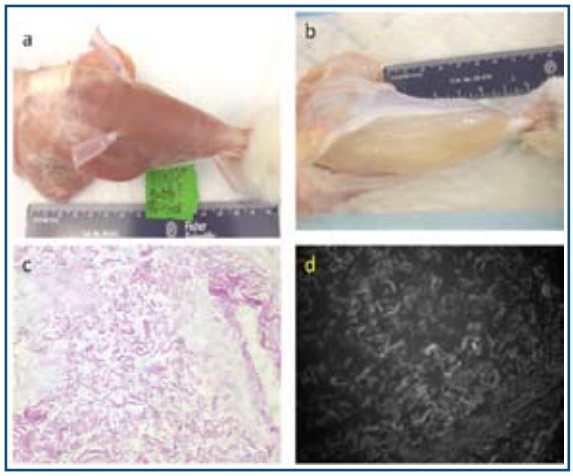


Figure VI-8. Ex vivo decellularization of the anterior tibial compartment in a rabbit limb (a and b) is accomplished in 1 hour, 40 minutes of perfusion with a mixture of detergents and enzymes. The normal histological architecture of the compartment is absent and no nuclei are visible (c and d). c – Canine compartment, H&E staining, 200x. d – Canine compartment ECM, DAPI, 200x.

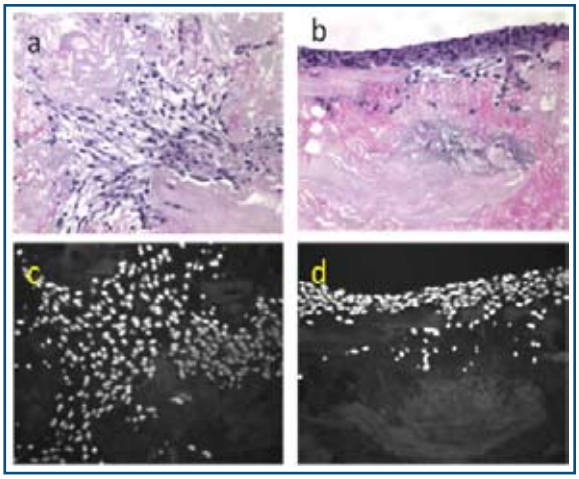


Figure VI-9. 3T3 fibroblasts on compartment ECM after 28 days in culture. Fibroblasts are infiltrating into the deeper layers of the ECM (a and b – H&E, c and d – DAPI).

muscle tissue, they have conducted a parallel study in which exogenous xenogeneic ECM was used. They established biocompatibility of the ECM for supporting the growth of human microvascular endothelial cells, 3T3 fibroblasts (**Figure VI-9**), and perivascular stem cells.

The researchers have also begun a comparative study of muscle-derived ECM with urinary bladder matrix and liver-derived matrix for their relative abilities to support in vitro cell growth and to produce degradation products with chemotactic effects upon multipotential progenitor cells.

Key Research Accomplishments

- Established a method for the development of peripheral CS through the complete decellularization of the anterior tibial compartment in the rabbit and dog.
- Established the biocompatibility of exogenous ECM for supporting the growth of human microvascular endothelial cells, 3T3 fibroblasts, and perivascular stem cells.

Conclusions

The researchers have developed two animal models of peripheral CS that are suitable for the evolution of potential therapeutic interventions.

Research Plans for the Next 4 Years

During Year 2, the researchers will continue to refine their animal models by the addition of associated trauma to simulate the natural causation of this condition and will evaluate the addition of exogenous ECM to the autologous ECM in the two models. They will evaluate the recruitment of autologous cells into the compartment over time.

Planned Clinical Transitions

The researchers anticipate transitioning to clinical trials following Year 3 or 4 of this project.

Corrections/Changes Planned for Year 2

None.

Project 4.3.5, WFPC

Material-Induced Host Cell Recruitment for Muscle Regeneration

Team Leader(s): *Sang Jin Lee*

Therapy: *Treatment of CS through muscle tissue regeneration*

Deliverable(s): *Demonstration of muscle tissue regeneration using a target-specific scaffolding system*

Key Accomplishment(s): *The researchers demonstrated (1) host stem/progenitor cell mobilization into implanted biomaterials, (2) the differentiation of recruited cells into multiple cell types, and (3) muscle stem/progenitor cell mobilization into implanted biomaterials in vivo. They also characterized the infiltrating host cells within the implanted biomaterials.*

Introduction

CS is a common traumatic injury that results in muscle, nerve, and vessel damage due to increased pressured within a confined space in the body. Although CS can affect any limb or muscle compartment, including the abdomen, it mainly occurs after trauma to the lower leg. 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 a continued problem long term. Various management therapies have been introduced, including physical therapy, muscle transplantation, and myoblast cell therapy, using MDSCs or progenitor cells. However, none has entirely addressed the problems associated with the long-term consequences of CS in wounded soldiers.

The researchers aim to utilize 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 regenerated 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 muscle tissue regeneration using a target-specific scaffolding system (**Figure VI-10**).

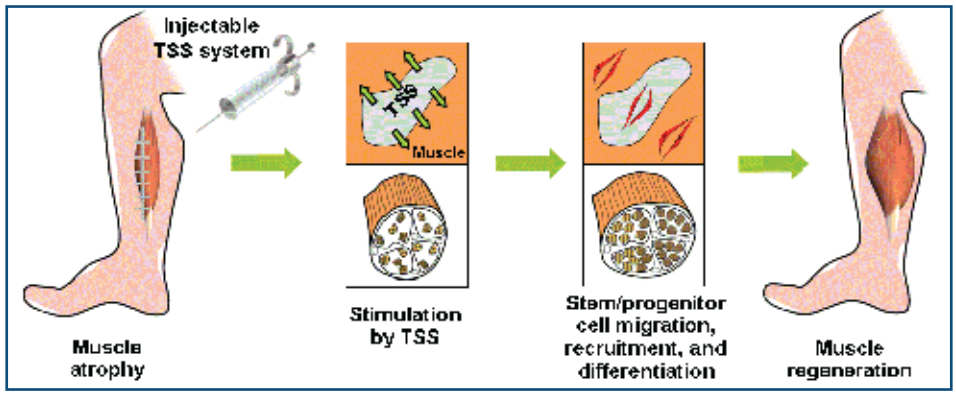


Figure VI-10. Schematic diagram of the muscle tissue regeneration paradigm of Project 4.3.5.

Research Progress – Year 1

During the past year, the researchers successfully completed the tasks proposed for Year 1 and met all of the milestones. They demonstrated that host stem/progenitor cells can be mobilized and recruited into the implanted biomaterials. They also demonstrated that these stem/progenitor cells are capable of differentiating into multi-lineage cells (e.g., osteogenic, adipogenic, myogenic, and endothelial cells). The researchers found that implantation of biomaterials in the muscle region led host muscle stem/progenitor cells that express PAX3, PAX7, and myoD to migrate within the scaffold (**Figure VI-11**). Their ongoing work includes the incorporation of myogenic-inducing factors into the biomaterials system, which would permit in situ muscle differentiation.

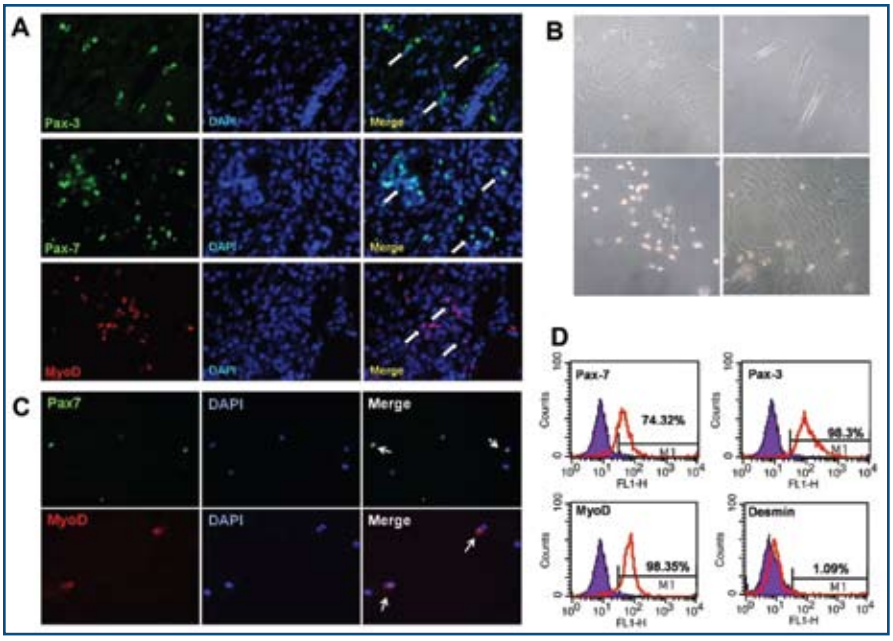


Figure VI-11. Immunofluorescent staining for Pax3, Pax7, and myoD of (A) retrieved biomaterials, (B), (C) isolated infiltrating host cells. (D) FACS analysis for expression of Pax3, Pax7, myoD, and desmin of infiltrating cells.

Key Research Accomplishments

- Demonstrated host stem/progenitor cell mobilization into implanted biomaterials.
- Demonstrated the differentiation of recruited cells into multiple cell types, including osteogenic, adipogenic, myogenic, and endothelial cells.
- Determined that muscle stem/progenitor cells could be mobilized into implanted biomaterials in vivo.
- Characterized the infiltrating host cells within the implanted biomaterials.

Conclusions

The researchers have made substantial progress toward the development of a reliable biomaterial system that can initiate host cell mobilization, recruitment, and differentiation into muscle tissue in vivo for the treatment of CS.

Research Plans for the Next 4 Years

In Year 2, the researchers will continue studies focused on incorporating myogenic-inducing agents combined with the biomaterials system. In Year 3, the researchers plan to demonstrate myogenic differentiation of the recruited stem/progenitor cells in a rodent model. In Year 4, they plan to show functional improvement of injured muscle tissue in vivo. In Year 5, they will evaluate the therapeutic potential and long-term stability of the myogenic-inducing biomaterials.

Planned Clinical Transitions

This basic research project is not slated for clinical trials during the lifetime of the award.

Corrections/Changes Planned for Year 2

None.

VII AFIRM Program Statistics

Introduction

As indicated throughout this annual report, the AFIRM program is composed of a host of researchers across many research institutions. While the previous chapters demonstrate the depth of their research projects, the breadth of the program also can be seen from a global perspective, viewing the research consortium as a whole, rather than as many individual components. This chapter demonstrates the extent and quality of scientific and technical expertise being applied to the problems of regenerative medicine. This chapter also demonstrates tangible, scientific outcomes attributable to AFIRM-supported research: inventions disclosed, patent applications filed, research or review articles published or accepted, and conference and meeting presentations and posters presented or accepted. The AFIRM program data shown in this chapter are based on the first program year (PY1). For the purpose of this annual report, PY1 is defined as the period from the initiation of research projects in May 2008 through the end of May 2009.

Personnel

A substantial workforce has been funded through the AFIRM program to conduct research on regenerative biology and medicine, from faculty members to undergraduate students (**Figure VII-1**). Notably, more than 80 research faculty were funded through the AFIRM in PY1. Another 68 postdoctorate associates and fellows and 80 scientific and technical staff were funded through the AFIRM.[1] Finally, with more than 40 graduate students and more than 20 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.

[1] The numbers may be slight overestimates due to some individuals working on multiple subprojects. Where available, the names of scientists and students who worked on more than one subproject have been identified, and they are included only once in the results. Not all individuals who worked on the program were named.

In addition to the many scientists directly supported by the AFIRM program, many others conducting research for the program were not directly supported with AFIRM funds (shown as the brown bar extensions in **Figure VII-2**). Technical expertise is being leveraged through the contributions of these scientists to achieve the goals of the program. For example, 17 faculty contributed to the Wake Forest-Pittsburgh Consortium (WFPC) research projects without being funded by the AFIRM. Additionally, 12 postdoctorate fellows, 12 graduate students, 14 undergraduate students, and 28 staff scientists and technicians at WFPC and 13 undergraduate students at the Rutgers-Cleveland Clinic Consortium (RCCC) contributed to AFIRM research in the first program year without being funded by the program (Figure VII-2).

The AFIRM program is composed of five overarching research program areas: Limb and Digit Salvage, Craniofacial Reconstruction, Scarless Wound Healing, Burn

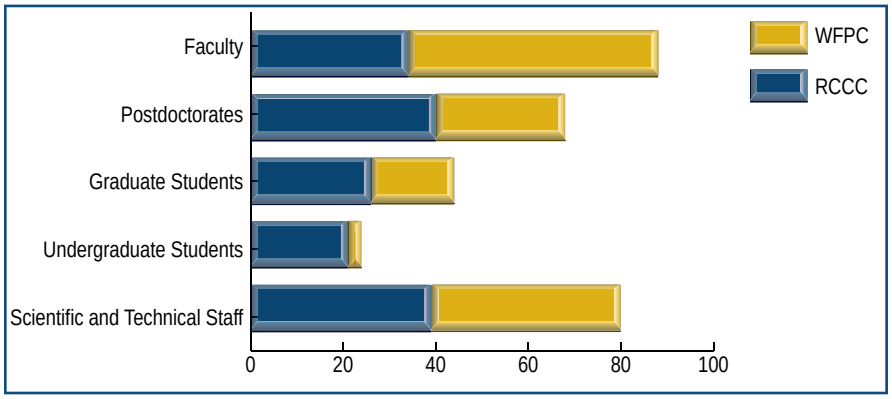


Figure VII-1. Numbers of scientists and students supported through the AFIRM program during PY1[2].

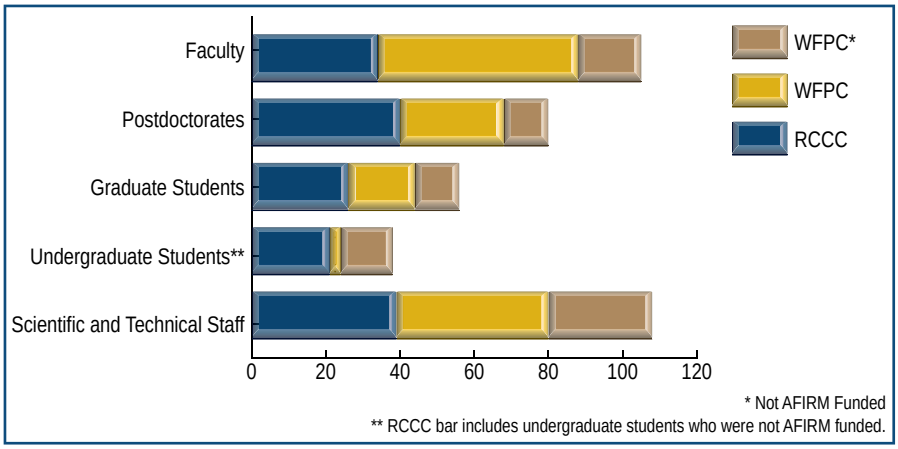


Figure VII-2. Numbers of scientists and students who conducted research on AFIRM projects during PY1.

[2] This is defined as the number of unique individuals supported by the AFIRM program during any part of the first program year. See footnote 1 for caveat regarding these data.

[3] A small number of researchers (13 from RCCC and at least 6 from WFPC) worked on projects in two or more different program areas. Figure VII-3 only counts each person once, and their distribution across the program areas is approximately proportional to the program areas on which they all work.

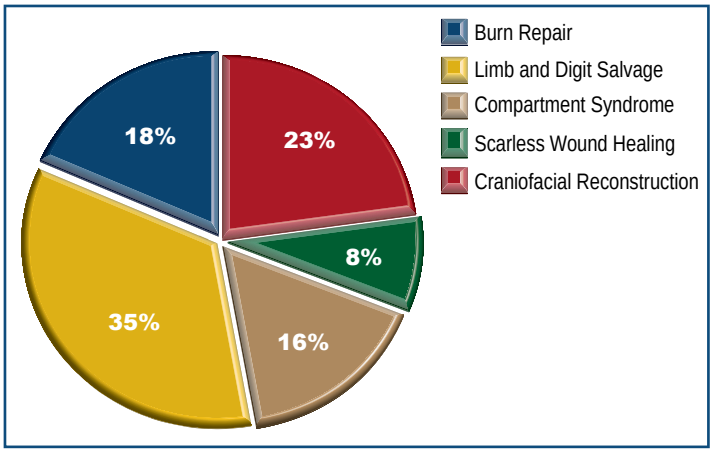


Figure VII-3. Percentage of personnel conducting research in the AFIRM program across the five program areas.

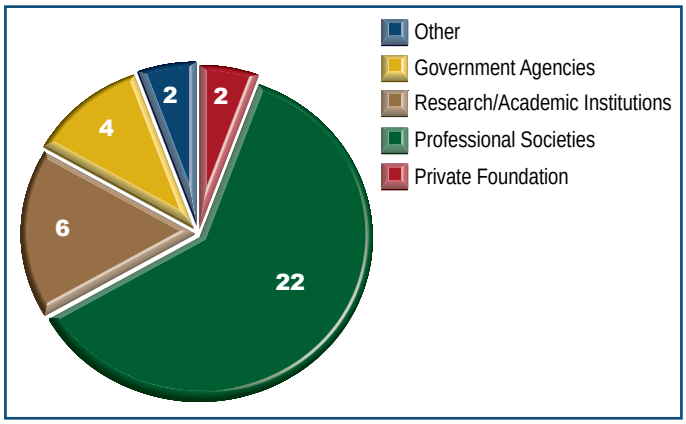


Figure VII-4. Distribution of honors and awards to AFIRM faculty by type of conferring organization.

Repair, and Compartment Syndrome. **Figure VII-3** depicts the approximate proportion of all personnel, funded and unfunded, who worked on the different program areas in PY1.[3]

Honors and Achievements

The AFIRM program's faculty are highly accomplished in their respective scientific fields. Between May 2008 and May 2009, 36 honors and awards were conferred upon AFIRM faculty, as self-reported by the researchers. These honors include awards from private foundations, selection to membership or leadership positions in professional societies, honorary degrees from academic institutions, and awards for distinguished careers from government agencies. The distribution of the honors received is displayed according to the type of conferring organization in **Figure VII-4**.

The complete list of honors and awards received by AFIRM faculty during PY1 is presented in **Appendix A**.

Another highlight of the AFIRM program is the substantial recruitment of young talent into the field of regenerative medicine. For example, more than 90 students (56 graduate students and 38 undergraduate students) received practical scientific training through AFIRM-sponsored research projects in PY1 (shown in Figure VII-2). The first AFIRM-supported graduate students to complete their degree requirements include two PhD recipients and one master's degree recipient in PY1.

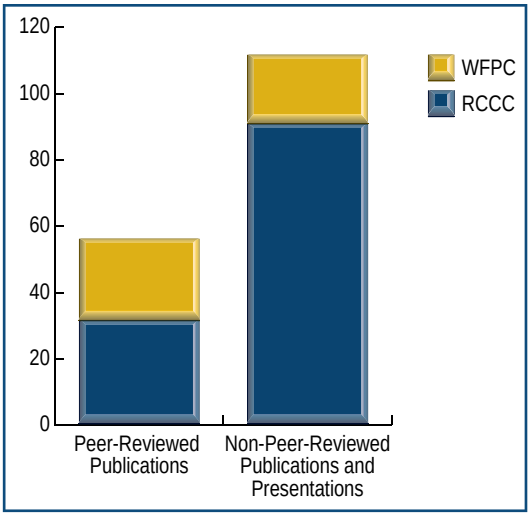


Figure VII-5. Dissemination of AFIRM-sponsored research findings to the scientific community in PY1.

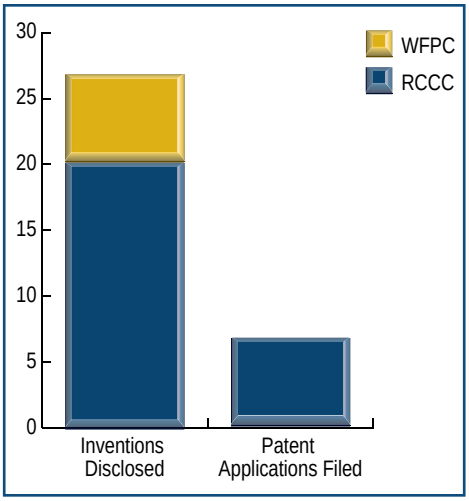


Figure VII-6. AFIRM-attributable invention disclosures and government patent applications filed in PY1.

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:

Peer-Reviewed Publications
Research or review articles accepted to, in press, or published in peer-reviewed journals or peer-review edited books between May 2008 and May 2009. Research or review manuscripts submitted to a journal or in preparation are not included in this annual report.

Non-Peer-Reviewed Publications and Presentations
Meeting symposia, invited talks, oral presentations, and posters delivered or accepted between May 2008 and May 2009 are included 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.

In the first program year, 59 peer-reviewed publications resulted from AFIRM-sponsored research, and 118 presentations and non-peer-reviewed publications resulted from the research (**Figure VII-5**).[4] The complete list of AFIRM researchers' publication and presentation citations is presented 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 government patent office for the intellectual property.

Many of the AFIRM program's principal researchers were already developing regenerative medicine-related research products at the time the program was initiated. Products developed entirely before the AFIRM program existed are not recognized as AFIRM program outcome accomplishments.[5] However, products initially developed prior to AFIRM support but refined during the AFIRM program period are considered AFIRM program outcome accomplishments as are all

[4] Articles published or accepted and presentations and posters delivered or accepted in 2008 or 2009 were included if the self-reported citations did not list the month of publication.

[5] Definitions of AFIRM-attributable inventions, patent applications, and patents were developed to standardize the self-reported program data (see Appendix C).

newly disclosed, self-reported intellectual property.

For the first year of the program, there was an expectation that the initial AFIRM-sponsored research would lead to the disclosure of inventions to institutional technology licensing offices and, to a much lesser extent, the filing of patent applications. This pattern was realized in PY1 with the filing of 27 invention disclosures and 6 government patent applications (**Figure VII-6**). Since government patent applications would have to have been reviewed by institutional technology license offices this same year, it is not surprising that only six government patent applications were filed in PY1 that are attributed to AFIRM support.[6] No patents attributable to AFIRM support were awarded to AFIRM researchers during this first year of funding, which is expected considering the length of time for a patent to be issued.[7]

The complete list of inventions disclosed and patent applications filed in PY1 that are attributable to AFIRM-sponsored research is shown in **Appendix C**.

[6] The 6 filed patent applications are also counted among the 27 invention disclosures in PY1. A patent application filed during the first program year must have been disclosed as an invention in the same program year; otherwise, the intellectual property could not be attributed to AFIRM-supported research and development.

[7] 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; however, accelerated examination status for patent application reviews can reduce the time an application is in pendency at the USPTO to 12 months or less.

Appendix A: Honors and Awards to AFIRM Faculty

During the reporting period from May 2008 to May 2009, 36 honors or awards were received by AFIRM faculty, as self-reported.

Rutgers-Cleveland Clinic Consortium

Bannazadeh, M (Cleveland Clinic): Charles C. Guthrie Award for Basic Science Research, Midwestern Vascular Society, 2009.

Clark, R (Stony Brook University): President, Society for Investigative Dermatology, 2009.

Ducheyne, P (University of Pennsylvania): William Hall Award, Society for Biomaterials, 2008.

Hollinger, J (Carnegie Mellon University): Clemson Award for Applied Research, Society for Biomaterials, 2008.

Langer, RS (Massachusetts Institute of Technology): Distinguished Chemist Award, New England Institute of Chemists, 2009.

Langer, RS (Massachusetts Institute of Technology): Honorary Degree, Harvard University, 2009.

Langer, RS (Massachusetts Institute of Technology): Honorary Degree, Mount Sinai School of Medicine, 2009.

Langer, RS (Massachusetts Institute of Technology): University of California, San Francisco Medal, University of California, 2009.

Rosen, J (Dartmouth University): Humanitarian Award, Lifetime Contributions for People with Disabilities, U.S. Health and Human Services, 2009.

Siemionow, M (Cleveland Clinic): Honorary Academic Appointment, Professor of Surgery, Karol Marcinkowski University of Medical Sciences, Poznan, Poland, 2008.

Siemionow, M (Cleveland Clinic): Medal Gloria Medicnae, Polish Medical Association, 2009.

Siemionow, M (Cleveland Clinic): Polish Order of Merit, the Commander's Cross Polonia Restituta Award, The President of Poland, 2009.

Vacanti, J (Massachusetts General Hospital): The Flance-Karl Award, American Surgical Association, 2009.

Yaszemski, M (Mayo Clinic): Brigadier General, U.S. Air Force Reserve, 2009.

Yaszemski, M (Mayo Clinic): Kappa Delta Award (Elizabeth Winston Lanier Award), American Academy of Orthopaedic Surgeons, 2009.

Wake Forest-Pittsburgh Consortium

Guldberg, RE (Georgia Institute of Technology): Chair-Elect, TERMIS-NA, 2009.

Gurtner, G (Stanford University): James Barrett Brown Award (best plastic surgery paper), American Association of Plastic Surgeons, 2008.

Lee, WPA (McGowan Institute): Best Paper in CTA, AFIRM All-Hands Meeting, 2009.

Mikos, AG (Rice University/University of Texas Health Science Center): Chemstations Lectureship Award, American Society for Engineering Education, 2009.

Mikos, AG (Rice University/University of Texas Health Science Center): Distinguished Scientist Award, Houston Society for Engineering in Medicine and Biology, 2008.

Soh, HT (University of California, Santa Barbara): ALA Innovator Award, Association for Laboratory Automation, 2009.

Thomson, J (University of Wisconsin): Meira and Shaul G. Massry Prize, Meira and Shaul G. Massry Foundation, 2008.

Thomson, J (University of Wisconsin): Member, National Academy of Sciences, 2008.

Tirrell, M (University of California, Santa Barbara): Fellow, Indian National Academy of Engineering.

Tirrell, M (University of California, Santa Barbara): Member, American Academy of Arts and Sciences.

Wagner, WR (McGowan Institute): Carnegie Science Award in the Life Sciences, Carnegie Science Center, 2008.

Wagner, WR (McGowan Institute): College of Fellows for Biomaterials Science and Engineering, International Union of Societies for Biomaterials Science and Engineering, 2008.

Wagner, WR (McGowan Institute): Deputy Director of the National Science Foundation Engineering Research Center: “Revolutionizing Metallic Biomaterials,” National Science Foundation, 2008.

Wagner, WR (McGowan Institute): Pittsburgh Innovator Award, The University of Pittsburgh, 2008.

Wagner, WR (McGowan Institute): President-Elect, American Society for Artificial Internal Organs, 2009.

Wagner, WR (McGowan Institute): Professor to Watch, Pittsburgh Business Times, 2008.

Wagner, WR (McGowan Institute): Program Chairman, American Society for Artificial Internal Organs, 2009.

Wagner, WR (McGowan Institute): Representative from the Society for Biomaterials to the International Union of Societies for Biomaterials Science and Engineering, International Union of Societies for Biomaterials Science and Engineering, 2009.

Wagner, WR (McGowan Institute): Vice President at Large, American Institute for Medical and Biological Engineering, 2008.

Wong, ME (Rice University/University of Texas Health Science Center): Distinguished Service Award, Houston Society of Oral and Maxillofacial Surgeons, 2009.

Appendix B: Peer-Reviewed Publications and Other 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 between May 2008 and May 2009. Additionally, book chapters are included as peer-reviewed publications. The articles shown in **Tables 1a and 1b** were self-reported by AFIRM researchers.

Table 1a. Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium

Clark RAF, Pavlis M. Dysregulation of the mTOR pathway secondary to mutations or a hostile microenvironment contribute to cancer and poor wound healing. *J Invest Dermatol* 129:529-531, 2009.
Clark RAF. Oxidative stress and "senescent" wound fibroblasts as potential therapeutic targets. *J Invest Dermatol* 128:2361-2364, 2008.
Clark RAF. Synergistic signaling from extracellular matrix-growth factor complexes. *J Invest Dermatol* 128:1354-1355, 2008.
Dadsetan M, Hefferan TE, Szatkowski JP, Mishra PK, Macura SI, Lu L, **Yaszemski MJ**. Effect of hydrogel porosity on marrow stromal cell phenotypic expression. *Biomaterials*, 2008 (14):2193-202.
Dadsetan M, Szatkowski JP, Shogren KL, **Yaszemski MJ**, Maran A. Hydrogel-mediated DNA delivery confers estrogenic response in nonresponsive osteoblast cells. *Journal of Biomedical Materials Res A*, 2009.
Dadsetan M, Knight A, Lu L, **Windebank AJ, Yaszemski MJ**. Stimulation of neurite outgrowth using positively charged hydrogels. *Biomaterials* 30, 3874-3883, 2009.
de Ruiter GC, Onyeneho IA, Liang ET, Moore MJ, Knight AM, Malessy MJ, Spinner RJ, Lu L, Currier BL, Yaszemski MJ, Windebank AJ. Methods for in vitro characterization of multichannel nerve tubes. *J Biomed Mater Res A*. 2008; 84(3): 643-51.
de Ruiter GC, Spinner RJ, Alaid AO, Koch AJ, Wang H, Malessy MJ, Currier BL, **Yaszemski MJ**, Kaufman KR, **Windebank AJ**. Two-dimensional digital video ankle motion analysis for assessment of function in the rat sciatic nerve model. *J Peripher Nerv Syst*. 2008; 12(3): 216-22.
de Ruiter GC, Spinner RJ, Malessy MJ, Moore MJ, Sorenson EJ, Currier BL, **Yaszemski MJ, Windebank AJ**. Accuracy of motor axon regeneration across autograft, single-lumen, and multichannel poly(lactic-co-glycolic acid) nerve tubes. *Neurosurgery*. 2008; 63(1): 144-53.
Gordon CR, **Siemionow M**, Zins J. Composite tissue allotransplantation: A proposed classification system based on relative complexity. *Transplant Proceedings*, 41(2): 481-4, March 2009.
Kempen DH, Kruyt MC, Lu L, Wilson CE, Florschutz AV, Creemers LB, **Yaszemski MJ**, Dhert WJ. Effect of autologous bone marrow stromal cell seeding and bone morphogenetic protein-2 delivery on ectopic bone formation in a microsphere/poly(propylene fumarate) composite. *Tissue Engineering Part A*, 2009 (3):587-94.
Kempen DH, Lu L, Classic KL, Hefferan TE, Creemers LB, Maran A, Dhert WJ, **Yaszemski MJ**. Non-invasive screening method for simultaneous evaluation of in vivo growth factor release profiles from multiple ectopic bone tissue engineering implants. *Journal of Controlled Release*, 2008 (1):15-21.
Kempen DH, Lu L, Hefferan TE, Creemers LB, Maran A, Classic KL, Dhert WJ, **Yaszemski MJ**. Retention of in vitro and in vivo BMP-2 bioactivities in sustained delivery vehicles for bone tissue engineering. *Biomaterials*, 2008 (22):3245-52.
Kempen DH, Lu L, Heijink A, Hefferan TE, Creemers LB, Maran A, **Yaszemski MJ**, Dhert WJ. Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration. *Biomaterials* 2009, (14):2816-25.
Kim J, Hefferan TE, **Yaszemski MJ**, Lu L. Potential of hydrogels based on poly-ethylene glycol) and sebacic acid as orthopedic tissue engineering scaffolds. *Tissue Engineering Part A*. 2009.
Johnson PA, Demtchouk A, Patel H, Sung H-J, Treiser MD, Gordonov S, Sheihet L, Bolikal D, **Kohn J**, Moghe PV. Interplay of anionic charge, poly(ethylene glycol), and iodinated tyrosine incorporation within tyrosine-derived polycarbonates: Effects on vascular smooth muscle cell adhesion, proliferation and motility. Accepted to *J Biomed Mater Res A*, 2009.
Lee KW, Wang S, **Yaszemski MJ**, Lu L. Physical properties and cellular responses to crosslinkable poly(propylene fumarate)/hydroxyapatite nanocomposites. *Biomaterials*, 2008 (19):2839-48.
Macri L, **Clark RAF**. Tissue engineering for cutaneous wounds: Selecting the proper time and space for growth factors, cells and the extracellular matrix. *Skin Pharmacol Physiol* 22:83-93, 2009.
Nasir S, Bozkurt M, Klimczak A, **Siemionow M**. Large antigenic skin load in total abdominal wall transplants permits chimerism induction. *Ann Plast Surg*. 61(5): 572-9, November 2008.

Table 1a. Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Nasir S, Bozkurt M, Krokowicz L, Klimczak A, **Siemionow M**. Correlation of chimerism with graft size and revascularization in vascularized and nonvascularized skin allografts. *Ann Plast Surg* 62(4): 430-8, April 2009.
Sheihet L, Chandra P, Batheja P, **Devore D, Kohn J**, Michniak B. Tyrosine-derived nanospheres for enhanced topical skin penetration. *Internat J Pharmaceutics*, 2008, 350, 312-319.
Siemionow M, Klimczak A, Unal S, Agaoglu G, Carnevale K. Hematopoietic stem cell engraftment and seeding permits multilymphoid chimerism in vascularized bone marrow transplants. *American Journal of Transplantation*. 8(6):1163-76, June 2008.
Siemionow M, Klimczak A. Tolerance and future directions for composite tissue allograft transplants – Part II. *Plast Reconstr Surg* CME article, 123(1): 7e-17e, January 2009.
Siemionow M, Nasir S. Immunologic responses in vascularized and nonvascularized skin allograft. *J Reconstr Microsurg*, 24(7): 497-505, Oct. 2008. Epub 2008 September 16.
Siemionow M, Sonmez E. Face as an organ. *Ann Plast Surg*, 61(3):345-52, September 2008.
Siemionow M, Klimczak A. Immunodepletive anti-a/b-TCR antibody in transplantation of composite tissue allografts: Cleveland clinic research experience. *Immunotherapy*. (in Press).
Thakur RA, Michniak BB, Florek CA, and **Kohn J**. Electrospun nanofibrous polymeric scaffold with targeted drug release profiles for potential application as wound dressing. *Internat J Pharmaceutics*, 2008, 364, 87-93.
Wang H, Sorenson EJ, Spinner RJ, **Windebank AJ**. Electrophysiologic findings and grip strength after nerve injuries in the rat forelimb. *Muscle Nerve*. 2008; 38(4): 1254-65.
Wang H, Spinner RJ, Sorenson EJ, **Windebank AJ**. Measurement of forelimb function by digital video motion analysis in rat nerve transection models. *J Peripher Nerv Syst*. 2008; 13(1): 92-102.
Wang H, Spinner RJ, **Windebank AJ**. Quantitative evaluation of movement and strength of the upper limb after transection of the C-7 nerve: Is it possible in an animal model? *J Neurosurg Spine*. 2009; 10(2): 102-10.
Wang S, Kempen DH, Simha NK, Lewis JL, **Windebank AJ, Yaszemski MJ**, Lu L. Photo-cross-linked hybrid polymer networks consisting of poly(propylene fumarate) and poly(caprolactone fumarate): Controlled physical properties and regulated bone and nerve cell response. *Biomacromolecules*, 2008 (4):1229-41.
Wang S, Kempen DH, **Yaszemski MJ**, Lu L. The roles of matrix polymer crystallinity and hydroxyapatite nanoparticles in modulating material properties of photo-crosslinked composites and bone marrow stromal cell responses. *Biomaterials*, 2009; (20):3359-70.
Yazici I, Cavusoglu T, Comert A, Vargel I, Cavusoglu M, Tekdemir I, **Siemionow M**. Maxilla allograft for transplantation: An anatomical study. *Ann Plast Surg*. 61(1):105-13, July 2008.

Table 1b. Peer-Reviewed Publications: Wake Forest-Pittsburgh Consortium

Adams JD, Kim U, Soh HT. Multi-target magnetic activated cell sorter (MT-MACS). *Proceedings of the National Academy of Sciences, USA,* (105) 18165-18170, 2008.
Braun G, Pallaoro A, Wu G, Missirlis D, Zasadzinski J, Tirrell M, Reich N. Laser-activated gene silencing via gold nanoshell-siRNA conjugates. *ACS Nano*, DOI: 10.1021/nn900469q, 2009.
Choi JS, Lee SJ, Christ GJ, Atala A, Yoo JJ. The influence of electrospun aligned poly(varepsilon-caprolactone)/collagen nanofiber meshes on the formation of self-aligned skeletal muscle myotubes. Biomaterials. 2008 Jul;29(19):2899-906. Epub 2008 April 9.
Ebert AD, Yu J, Rose FF, Mattis VB, Lorson CL, Thomson JA, Svendsen CN. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature*. 2009 January 15;457(7227):277-80.
Ishikubo A, Mays J, and Tirrell M. Behavior of cationic surfactants in poly(styrene sulfonate) brushes. Ind Eng Chem Res, 47, 6426-6433, 2008.
Johnen C, Steffen I, Beichelt D, Bräutigam K, Witascheck T, Toman N, Moser V, Ottomann C, Hartmann B, Gerlach JC. Culture of subconfluent human fibroblasts and keratinocytes using biodegradable transfer membranes. *Burns* 2008;34:655-663.

Table 1b. Peer-Reviewed Publications: Wake Forest-Pittsburgh Consortium (cont.)

Karmali P, Kotamraju V, Kastantin M, Black M, Missirlis D, Tirrell M, and Ruoslahti E. Targeting of albumin-embedded paclitaxel nanoparticles to tumors. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 5, 73-82, 2009.
Kastantin M, Ananthanarayanan B, Karmali P, Ruoslahti E, and Tirrell M. Effect of the lipid chain melting transition on the stability of DSPE-PEG (2000) micelles. *Langmuir*, 25 (13), 7279-7286, 2009.
Kim U, Qian J, Kenrick SA, Daugherty PS, Soh HT. Multi-target dielectrophoresis activated cell sorter (MT-DACS). *Analytical Chemistry*, (80) 8656-8661, 2008.
Kim YJ, Teletia N, Ruotti V, Maher CA, Chinnaiyan AM, Stewart R, Thomson JA, Patel JM. ProbeMatch: Rapid alignment of oligonucleotides to a genome allowing both gaps and mismatches. *Bioinformatics*. 2009 June 1;25(11):1424-5.
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Smitthipong W, Neumann T, Gajria S, Li Y, Chworos A, Jaeger L, Tirrell M. Noncovalent self-assembling nucleic acid-lipid based materials. *Biomacromolecules*, 10, 221-228, 2008.
Smitthipong W, Neumann T, Chworos A, Jaeger L, Tirrell M. Supramolecular materials comprising nucleic acid biopolymers. *Macromol Symp* 264, 13-17, 2008.
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Appendix B: Peer-Reviewed Publications and Other Publications and Presentations

Tables 2a and 2b display non-peer-reviewed publications and all presentations. These publications and presentations were self-reported by AFIRM researchers. 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 between May 2008 and May 2009. 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" between May 2008 and May 2009 are included in the following tables. 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 2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium

Aurora A and **Derwin KA**. Engineering Allograft Fascia Lata as an Augmentation Device for Musculoskeletal Repair, 9th NJ Symposium on Biomaterials Science and Regenerative Medicine, New Brunswick, New Jersey, October 29-31, 2008 (presentation).
Aurora A and **Derwin KA**. Engineering Allograft Fascia Lata as an Augmentation Device for Musculoskeletal Repair, Army Science Conference, Orlando, Florida, December 1-4, 2008 (poster).
Aurora A and **Derwin KA**. Reinforced Fascia Lata as an Augmentation Device for Musculoskeletal Repair, AFIRM All Hands Conference, St. Petersburg, Florida, January 14-15, 2009 (abstract, poster and presentation).
Balint EB, **Gatt CJ, Dunn MG**. Development of a Tissue Engineered Scaffold for Meniscus Replacement, **26th Army Science Conference**, Orlando, Florida, December 1-4, 2008 (manuscript).
Batheja P, Chandra P, Rai V, Michniak-Kohn B, **Devore D, Kohn J**. An In Vitro Skin Equivalent for Evaluation of Skin Absorption of Compounds, presented at the Armed Forces Institute of Regenerative Medicine Army Science Conference, Orlando, Florida, December 1-4, 2008.
Boyce ST, Simpson PS, Kagan RJ. 2008. Survival of burn involving 90% of the total body surface area after treatment with autologous engineered skin substitutes. Proc 26th Army Science Conference; Report LO-2.
Boyce ST, Simpson PS, Kagan RJ. Survival of Burn Involving 90% of the Total Body Surface Area After Treatment with Autologous Engineered Skin Substitutes. Presented at the Armed Forces Institute for Regenerative Medicine All Hands Meeting, St. Pete Beach, Florida, 2009.
Boyce ST, Swope VB, Zimmerman RL, Supp DM. Regulation of Pigmentation by Epidermal Melanocytes in Engineered Skin Substitutes. Presented at the Armed Forces Institute for Regenerative Medicine All Hands Meeting, St. Pete Beach, Florida, 2009.
Brzezicki G, Siemionow K, Klimczak A, **Siemionow M**, McLain R. Ischemic Conditions Result in Lack of GFAP Expression in Satellite Cells of the Dorsal Root Ganglion (oral presentation), 51st Annual Meeting, Ohio Valley Society of Plastic Surgeons, Cleveland, Ohio, May 16-18, 2008.
Brzezicki G, Siemionow K, Klimczak A, **Siemionow M**. Anti-Inflammatory and Neuroprotective Properties of the Epineural Sheath Promote Regeneration after Lumbar Root Injury in the Rat Model (poster presentation), 88th Annual Meeting of American Association of Plastic Surgeons, Rancho Mirage, California, March 21-24, 2009.
Brzezicki G, Siemionow K, Klimczak A, **Siemionow M**. Epineural Sheath Patch and Bone Marrow Stromal Cells Promote Regeneration in Dorsal Root Ganglion Injury in Rat Model (oral presentation), Composite Tissue Transplantation Session, Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Chen BK, de Boer R, Knight AM, Wang H, Spinner RJ, Malessy MJA, **Yaszemski MJ, Windebank AJ**. Growth Factor Releasing Microspheres and the Effect on Peripheral Nerve Regeneration. AFIRM All-Hands Meeting, St. Petersburg, Florida, January 13-15, 2009.
Clark RAF, Lin F, Macri L, Tonnesen MG, Mosher DA. Fibronectin Growth Factor-Binding Domains Are Required for Fibroblast Survival and Growth in Response to PDGF-BB: Implications in Wound Healing. Presented at AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Clark RAF, Lin F, Tonnesen MG. Fibronectin contains four novel, evolutionarily conserved growth factor-binding peptides required for fibroblast survival. J Invest Dermatol 129:S6, 2009.

Table 2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Costache AD, Sheihet L, Knight DD, **Kohn J**. Modeling of Polymer-Drug Interactions in Biodegradable Tyrosine-Based Nanospheres Using Molecular Dynamics Simulations and Docking in 6th International Nanomedicine and Drug Delivery Symposium (NanoDDS'08), Toronto, Canada, 2008.
Dadsetan M, Hefferan TE, Heine-Gelder A, Spelsberg TC, Lu L, Maran A, **Yaszemski MJ**. Proliferation and Differentiation of Osteoblasts Encapsulated in Hydrogels. Armed Forces Institute of Regenerative Medicine All- Hands Meeting, Saint Petersburg, Florida, January 13-16, 2009.
Dadsetan M, Yaszemski MJ. Doxorubicin release from microspheres encapsulated within oligo (polyethylene glycol) fumarate hydrogel. *PMSE Preprints*, 2009, accepted.
Dadsetan M, Yaszemski MJ. Incorporation of electrical charge into oligo (polyethylene glycol) fumarate hydrogel for cartilage regeneration. *PMSE Preprints*, 2009, accepted.
Darr A, Lovas B, Saini S, Muschler G, Kohn J. The Optimization of Tyrosine Derived Polycarbonate Scaffolds for Repair of Bone Defects. Presented at the Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, 2009.
de Boer R, Knight AM, Malessy MJA, Spinner RJ, **Windebank AJ**. Role of Microsphere Delivered Nerve Growth Factor (NGF) and Glial Cell Line Derived Neurotrophic Factor (GDNF) in Peripheral Nerve Regeneration. Annual Meeting of American Society for Peripheral Nerve, Maui, Hawaii, January 9-11, 2009.
de Boer R, Knight AM, Malessy MJA, Spinner RJ, **Yaszemski MJ, Windebank AJ**. AFIRM Nerve Regeneration Project 1: Growth Factor Releasing Microspheres and the Effect on Peripheral Nerve Regeneration. Number KP-13. Army Science Conference, Orlando, Florida, December 1-4, 2008.
de Boer R, Knight AM, Wang H, Malessy MJA, Spinner RJ, **Windebank AJ**. Microsphere Delivery of Nerve Growth Factor (NGF) and Glial Cell Line Derived Neurotrophic Factor (GDNF) in Supporting Peripheral Nerve Regeneration in Polymer Scaffolds. Abstract Number: 950266. American Neurological Association 133rd Annual Meeting, Salt Lake City, Utah, September 21-24, 2008.
Devore D, Kohn J. Polycarbonates as an Engine of Innovation. Presented at the Armed Forces Institute of Regenerative Medicine Army Science Conference, Orlando, Florida, December 1-4, 2008.
Duggan W, Grykien C, Klimczak A, Nair D, Gatherwright J, **Siemionow M**. 85. Augmentation of the Regeneration of Peripheral Nerve Defects with the Transplantation of Donor Derived Bone Marrow Stromal Cells. Plastic Surgery 2008 Abstract Supplement, 122(4S): 73, October 2008.
Duggan W, Grykien C, Klimczak A, Nair D, **Siemionow M**. Neural Regeneration of Peripheral Nerve Defects Promoted by Donor Derived Bone Marrow Stromal Cells. Supplement to Plastic and Reconstructive Surgery, 121(6S), Session 2C, Abstract 48C, June 2008 Supplement.
Gatt CJ, Dunn MG. Load-Bearing Scaffolds for Soft Tissue Regeneration, 9th NJ Symposium on Biomaterials Science and Regenerative Medicine, New Brunswick, New Jersey, October 29-31, 2008 (presentation).
Gordon CR, Alam D, Bernard S, Djohan R, Papay F, Zins JE, Coffman K, **Siemionow M**. Using a FACES score to create a face transplant candidate registry. Proceedings of the American Transplant Congress, *Amer J Transplant*, S2(9): 433, 2009.
Gordon CR, **Siemionow M**, Zins J. Composite tissue allotransplantation: A proposed classification system based on relative complexity. Proceedings of the American Transplant Congress, *Amer J Transplant*, S2(9): 1309, 2009.
Hafeman AE, **Hollinger JO, Guelcher SA**. Release of BMP-2 and Tobramycin from Injectable, Biodegradable Polyurethane Scaffolds for Enhanced Bone Fracture Healing. Army Science Conference, December 1–4, 2008, Orlando, Florida.
Hafeman AE, Zienkiewicz K, Li B, Davidson JM, **Guelcher SA**. Injectable Biodegradable Polyurethane Scaffolds for Tissue Regeneration. TERMIS-NA Annual Conference and Exposition, December 7–10, San Diego, California (oral presentation).
Hivelin M, Nasir S, Klimczak A, Krokowicz L, **Siemionow M**. 20. Immunotolerance Induction for Composite Tissue Transplantation by Donor Presensitization with Recipient Bone Marrow. Plastic Surgery 2008 Abstract Supplement, 122(4S): 17, October 2008.
Jia S, Nuñez JM, Roy N, Zhao Y, **Mustoe TA**. Northwestern University, Chicago, Illinois. Therapy to Limit Injury and Promote Non-Scar Healing Within the Rabbit Hypertrophic Scar Model. Presented at the Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, 2009.

Table 2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Jundzill A, Brzezicki G, Klimczak A, Gatherwright J, **Siemionow M**. Improving Nerve Regeneration of Allogenic Epineurium with Donor Derived Bone Marrow Stromal Cells Bridging Peripheral Nerve 20 mm Defect (oral presentation), Composite Tissue Transplantation Session, Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Jundzill A, Brzezicki G, Klimczak A, Gatherwright J, **Siemionow M**. Improving Nerve Regeneration of Allogenic Epineurium with Donor Derived Bone Marrow Stromal Cells Bridging Peripheral Nerve 20 mm Defect (oral presentation), 54th Annual Meeting of the Plastic Surgery Research Council, Pittsburgh, Pennsylvania, May 27-30, 2009.
Jundzill A, Brzezicki G, Klimczak A, Gatherwright J, **Siemionow M**. Improving Nerve Regeneration of Allogenic Epineurium with Donor Derived Bone Marrow Stromal Cells Bridging Peripheral Nerve 20 mm Defect (poster presentation), American Transplant Congress, Combined Annual Meeting, American Society of Transplantation/American Society of Transplant Surgeons, Boston, Massachusetts, May 30-June 3, 2009.
Jundzill A, Klimczak A, Brzezicki G, Gatherwright J, Sonmez E, **Siemionow M**. Effect of Chimerism Induction After Bone Marrow Transplantation into Alternative Anatomical Compartments (oral presentation), 54th Annual Meeting of Plastic Surgery Research Council, Pittsburgh, Pennsylvania, May 27-30, 2009.
Jundzill A, Klimczak A, Brzezicki G, Gatherwright J, Sonmez E, **Siemionow M**. Effect of Chimerism Induction After Bone Marrow Transplantation into Different Anatomical Compartments (poster presentation), American Transplant Congress, Combined Annual Meeting, American Society of Transplantation/American Society of Transplant Surgeons, Boston, Massachusetts, May 30-June 3, 2009.
Jundzill A, Krokowicz L, Klimczak A, Mielniczuk M, Grykien C, **Siemionow M**. Pulsed Acoustic Cellular Therapy Induces Expression of Pro-Angiogenic Factors in Muscle Microcirculation (oral presentation), 51st Annual Meeting, Ohio Valley Society of Plastic Surgeons, Cleveland, Ohio, May 16-18, 2008.
Jundzill A, Sonmez E, Klimczak A, Gatherwright J, **Siemionow M**. Prolongation of Composite Tissue Allograft Survival Using Alternative Routes of Bone Marrow Transplantation (poster presentation), American Transplant Congress, combined annual meeting, American Society of Transplantation/American Society of Transplant Surgeons, Boston, Massachusetts, May 30-June 3, 2009.
Kanunanidhi A, Kim J, Li B, Schutte L, **Guelcher S, Hollinger J**. Osteogenic Differentiation of Human Mesenchymal Stem Cell in Response to Exogenous and Released Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) from Polyurethane (PUR) Composites to Treat Craniofacial Bone Defects, AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Kempen D, Lu L, Hefferan T, Creemers L, Heijink A, Maran A, Dhert W, **Yaszemski MJ**. Intermittent PTH (1-34) Administration Enhances BMP-2 Induced Ectopic and Orthotopic Bone Formation (oral presentation). 55th Annual Meeting of the Orthopaedic Research Society, Las Vegas, Nevada, February 22-25, 2009 .
Klimczak A, Agaoglu G, Yazici I, Unal S, Kulahci Y, **Siemionow M**. B-Cell Chimerism Contribution to Face Allografts Survival in Face Allograft Models Containing Vascularized Bone Marrow Component (oral presentation), 54th Annual Meeting of Plastic Surgery Research Council, Pittsburgh, Pennsylvania, May 27-30, 2009.
Klimczak A, Agaoglu G, Yazici I, Unal S, Kulahci Y, **Siemionow M**. B-Cell Chimerism Contribution to Face Allografts Survival in Face Allograft Models Containing Vascularized Bone Marrow Component (poster presentation), American Transplant Congress, Combined Annual Meeting, American Society of Transplantation/American Society of Transplant Surgeons, Boston, Massachusetts, May 30-June 3, 2009.
Klimczak A, Agaoglu G, Yazici I, Unal S, Kulahci Y, **Siemionow M**. The Impact of Various Components of Facial Allograft on Chimerism Induction in Different Face Transplant Models (oral presentation), 25th Annual Meeting of American Society for Reconstructive Microsurgery, Maui, Hawaii, January 10-13, 2009.
Klimczak A, Hivelin M, Nasir S, Krokowicz L, **Siemionow M**. Effect of Donor Sensitization on the Immunotolerance in Composite Tissue Transplantation (oral presentation), 51st Annual Meeting, Ohio Valley Society of Plastic Surgeons, Cleveland, Ohio, May 16-18, 2008.
Klimczak A, Sonmez E, **Siemionow M**. Effect of Chimerism Induction after Donor Bone Marrow Transplantation into Different Recipient Compartments (oral presentation), 51st Annual Meeting, Ohio Valley Society of Plastic Surgeons, Cleveland, Ohio, May 16-18, 2008.
Klimczak A, Sonmez E, **Siemionow M**. Efficacy of In Vivo Created Donor/Recipient Chimeric Cells Depends on Time of Maturation (oral presentation), Composite Tissue Transplantation Session, Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.

Table 2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Klimczak A, Unal M, Demir Y, **Siemionow M**. 63. Supportive Therapy with Donor bone Marrow Cells and Presence of Regulatory CD4+/CD25+ T-Cell Promotes Face Transplant Survival. Plastic Surgery 2008 Abstract Supplement, 122(4S): 53, October 2008.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Effect of Immunotherapy with Donor Bone Marrow Transplantation Under Selective Immunodepletive Protocol of Anti-alpha/beta-TCRmAb and Cyclosporine A Supports Facial Allograft Survival (poster presentation), 9th New Jersey Symposium on Biomaterials Science and Regenerative Medicine, New Brunswick, New Jersey, October 29-31, 2008.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Face Transplant Survival Is Promoted by Donor Bone Marrow Cells and Presence of Regulatory CD4+/CD25+ T-Cell (electronic poster presentation), XXII International Congress of the Transplantation Society, Sydney, Australia, August 10-14, 2008.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Facial Allograft Survival Supported by Immunotherapy with Donor Bone Marrow and Development of Regulatory CD4+/CD25+ T-Cell (poster presentation), 2008 American Transplant Congress, Toronto, Ontario Canada, May 31-June 4, 2008.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Regulatory CD4+ / CD25+ T-Cell Supported Facial Allograft Survival After Immunotherapy with Donor Bone Marrow (oral presentation), 53rd Annual Meeting, The Plastic Surgery Research Council, Springfield, IL, May 28-31, 2008.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Regulatory CD4+/CD25+ T-Cell Supported Facial Allograft Survival After Immunotherapy with Donor Bone Marrow. Supplement to Plastic and Reconstructive Surgery, 121(6S), Session 2C, Abstract 59C, June 2008 Supplement.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Selective Protocol and Immunotherapy with Donor Bone Marrow Supports Facial Allograft Survival by Presence of Regulatory CD4+/CD25+ T-Cell (oral presentation), 1st American Conference on Reconstructive Transplant Surgery (ACRTS): Prospects and Future of a New Era in Reconstructive Surgery, Philadelphia, Pennsylvania, July 18-19, 2008.
Klimczak A, Unal M, Demir Y, **Siemionow M**. Supportive Therapy with Donor Bone Marrow Cells and Presence of Regulatory CD4+/CD25+ T-Cell Promotes Face Transplant Survival (oral presentation), Plastic Surgery 2008, Annual Meeting of the American Society of Plastic Surgeons, Chicago, Illinois, October 31–November 4, 2008.
Knight AM, Dadsetan M, de Boer R, Giusti G, Walker-Santiago R, Wang H, Shin AY, **Yaszemski MJ, Windebank AJ**. Biodegradable Polymers as Scaffolds in the Repair of Peripheral Nerves. AFIRM All-Hands Meeting, St. Petersburg, Florida, January 13-15, 2009.
Knight AM, de Boer R, Dadsetan M, Giusti G, Wang H, Borntrager A, Shanfeng S, Spinner RJ, **Yaszemski M, Windebank AJ**. Regeneration Supported by Biomaterials PCLF, OPF and PLGA, and the Effect of NGF and GDNF Across a 10mm Gap in the Rat Sciatic Nerve. Program 703.1. Society for Neuroscience, Washington, DC, November 15-19, 2008.
Lewitus D, Vogelstein J, Xia X, **Kohn J**, Harshbarger S. Novel Biodegradable Regeneration-Type Neural Interfaces for High Resolution Stimulation and Recording from Peripheral Nerves, Presented at the Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, 2009.
Li B, Kim J, Karunanidhi A, Schutte L, **Hollinger J, Guelcher S**. Sustained Release of rhBMP-2 from Polyurethane Scaffolds Promotes New Bone Formation in Rat Femoral Defect. Presented at the AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Li B, **Guelcher SA**. Controlled Release of BMP-2 from Injectable Polyurethane Scaffolds. TERMIS-NA Annual Conference and Exposition, December 7–10, San Diego, California (poster).
Luangphakdy V (Cleveland Clinic). Rapid Systematic Assessment and Advancement of Effective Grafting Bone Materials. Poster and podium presentation presented at the AFIRM All-Hands Meeting, St. Petersburg, Florida (January 14-16, 2009.
Macri L, Tonnesen MG, Lin F, **Clark RAF**. Fibronectin growth factor-binding peptides protect adult human dermal fibroblasts from oxidative and cytokine-induced cell death. J Invest Dermatol 129:S6, 2009.
Macri LK, Salomon S, Ezra M, **Kohn J, Clark RAF**. Characterization of the Delivery of a Fibronectin-Derived Peptide from an Electrospun Fibroporous Mat for the Treatment of 3rd Degree Burns. Presented at the AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Maran A, Dadsetan M, Brophy CM, **Yaszemski MJ**. Polymer-Mediated Controlled Delivery Prolongs the Anti-Tumor Effects of 2-Methoxyestradiol in Bone Cancer Cells (oral presentation). The 9th New Jersey Symposium on Biomaterials Science and Regenerative Medicine, New Brunswick, New Jersey, October 29, 2008.

Table 2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Muschler G. Bone Defects – Cell Therapies. Podium Presentation, Extremity War Injuries IV: Collaborative Efforts in Research, Host Nation Care, and Disaster Preparedness, Washington, DC, January 21-23, 2009.
Muschler G. Orthopaedic Challenges and Stem Cell Therapies for Bone Regeneration. Podium Presentation, TERMIS-NA. San Diego, California, December 7, 2008.
Muschler G. Project 4.2 highlighted and described in presentation to the 9th New Jersey Biomaterials Symposium, October 29, 2008.
Nasir S, Hivelin M, Klimczak A, **Siemionow M**. Different Cell-Based Therapeutic Approaches in Face Allografts. Supplement to Plastic and Reconstructive Surgery, 121(6S), Session 1A, Abstract 3A, June 2008 Supplement.
Pumberger M, Dadsetan M, **Yaszemski MJ**. Effect of Electrical Charge on Chondrocyte Attachment, 35th Annual Northeast Bioengineering Conference (oral presentation), April 3-5, 2009.
Ramachandran N, Mason D, Rosenblatt S, **Clark RAF, Kohn J**. Iodine-Complexed Polyvinyl Alcohol Sponges for Absorbent Antimicrobial Wound Dressing. Presented at the AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Rosen J. Project Update on Compartment Syndrome Program, Armed Forces Institute of Regenerative Medicine All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Runge MB, Dadsetan M, Baltrusaitis J. Ruesink T, Yaszemski MJ. Biocompatibility of polycaprolactone fumarate-polypyrrole composite materials: Effect of anionic dopant on cell viability. *PMSE Preprints*, 2009, accepted.
Runge MB, Dadsetan M, Baltrusaitis J. Ruesink T, Yaszemski MJ. Evaluation of electrically conductive and non-conductive porous three-dimensional scaffolds. *PMSE Preprints*, 2009, accepted.
Runge BM, Dadsetan M, Baltrusaitis J, Ruesink T, Yaszemski, MJ. Evaluation of electrically conductive and non-conductive porous three-dimensional scaffolds. *PMSE Preprints*, 2009, accepted.
Runge BM, Dadsetan M, Maran A, **Yaszemski MJ**. Electrically conducting 3-dimensional porous scaffolds for bone regeneration. *PMSE Preprints*, 2009, 100, 655-656.
Sheihet L, Salomon S, Zhaveri K, Kilfoyle B, Singer A, **Clark RAF, Kohn J**. Curcumin-Loaded Nanospheres as a Therapy to Limit Burn Injury Progression and to Promote Non-Scar Healing. Presented at the AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Sheihet L, **Devore D**, Gounder MK, Rubin EH, **Kohn J**. Nanospheric Chemotherapeutic and Chemoprotective Agents in 2008 Era of Hope, Breast Cancer Research Program, Baltimore, Maryland.
Sheihet L, **Devore D**, Gounder MK, Rubin EH, **Kohn J**. Tyrosine-Derived Nanospheres for Anti-Tumor Drug Delivery in 8th World Biomaterials Congress, Amsterdam, The Netherlands, 2008.
Sheihet L, Gounder MK, Chandra P, Batheja P, **Devore D**, Rubin EH, Michniak B, **Kohn J**. Tyrosine-Derived Nanospheres for the Delivery of Lipophilic Therapeutics in 9th New Jersey Symposium on Biomaterials Science, New Jersey, 2008.
Siemionow M, Bozkurt M, Grykien C, Krokowicz L, Klimczak A, Froimson J, Nair D. Contribution of Donor Derived Bone Marrow Stromal Cells and Neurotrophic Factor for Regeneration of Peripheral Nerve Defects (oral presentation), Eurohand 2008, XIIIth Congress of the Federation of European Societies for Surgery of the Hand (FESSH), Lausanne, Switzerland, June 19-21, 2008.
Siemionow M, Duggan W, Klimczak A, Gatherwright J, Nair D. Peripheral Nerve Defect Regeneration Augmented by Transplantation of Epineural Tube Supported with Bone Marrow Stromal Cells: A Preliminary Report (oral presentation), 9th New Jersey Symposium on Biomaterials Science and Regenerative Medicine, New Brunswick, New Jersey, October 29-31, 2008.
Siemionow M, Hivelin M. Face transplantation: Clinical application of the concept. Polish Journal of Surgery, 80(10): 1041-1053, October 2008.
Siemionow M, Klimczak A, Unal S, Agaoglu G. Maintenance of Donor-specific Chimerism Despite Osteopontin-dependent Bone Fibrosis in Vascularized Bone Marrow Transplantation Model (oral presentation), Eurohand 2008, XIIIth Congress of the Federation of European Societies for Surgery of the Hand (FESSH), Lausanne, Switzerland, June 19-21, 2008.
Siemionow M, Sonmez E, Klimczak A. Technical and immunological aspects of face transplantation. *Polish Journal of Surgery*, 81(1): 68-74, 2009.
Singer AJ, Taira BR, McClain SA, Rosenberg L, **Clark RAF**. Mechanism of burn wound progression in a porcine comb model. *J Burn Care Res* 30:S130, 2009.

Table 2a. Presentations and Non-Peer-Reviewed Publications: Rutgers-Cleveland Clinic Consortium (cont.)

Sonmez E, Klimczak A, **Siemionow M**. Efficacy of Chimeric Cell Transplantations to Induce Donor Specific Tolerance Induction, Harvested at Different Time Periods Post-Transplant After Bone Marrow Transplantation. Supplement to Plastic and Reconstructive Surgery, 121(6S), Session 2C, Abstract 63C, June 2008 Supplement.
Sonmez E, Klimczak A, **Siemionow M**. P14. Comparison of Chimerism Induction After Donor Bone Marrow Transplantation into Different Recipient Compartments. Plastic Surgery 2008 Abstract Supplement, 122(4S): 103, October 2008.
Taira BR, Singer AJ, Lin F, McClain SA, Lim T, Andersen R, **Clark RAF**. Administration of Crude IV Curcumin After Burn Injury Halts Burn Injury Progression. European Burns Association Congress Annual Meeting, Lausanne, Switzerland, September 2-5, 2009.
Taira BR, Singer AJ, McClain SA, Lin F, **Clark RAF**. Comparison of two partial thickness burn models. J Burn Care Res 30:S55, 2009.
Tanner S, Kim J, Karunanidhi A, Schutte L, Jacobs M, Bhattacharyya S, **Hollinger J, Guelcher S**. Development of Injectable Allograft Bone/Polymer Composite Scaffolds to Treat Craniofacial Bone Defects, abstract submitted to AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Tanner S, Kim J, Karunanidhi A, Schutte L, Jacobs M, Bhattacharyya S, **Hollinger J, Guelcher S**. Development of Injectable Allograft Bone/Polymer Composite Scaffolds to Treat Craniofacial Bone Defects AFIRM All-Hands Meeting, St. Pete Beach, Florida, January 14-16, 2009.
Tanner SA, Zienkiewicz KL, **Guelcher SA**. Allograft Bone/Polymer Composites Support Bone Remodeling in a Rabbit Distal Femoral Plug Model. Abstract submitted to the Society for Biomaterials Annual Meeting, 2009.
Vaughn A, **Gatt CJ, Dunn MG, Kohn J**. Development of Resorbable Polymer Fibers for Tissue Engineered Scaffolds for Anterior Cruciate Ligament (ACL) and Meniscus Repair and Replacement. Presented at the AFIRM All-Hands Meeting, St. Pete Beach, Florida, 2009.
Wang H, de Ruiter G, de Boer R, Spinner RJ, **Yaszemski MJ, Windebank AJ**. Evaluation of Rat Limb Function Following Nerve Injury and Repair. AFIRM All-Hands Meeting, St. Petersburg, Florida, January 13-15, 2009.
Wang H, **Windebank AJ**, Spinner RJ. Impact of C7 Transection on the Upper Limb: Quantification of Motor Function in a Rat Model. Annual Meeting of American Society for Peripheral Nerve, Maui, Hawaii, January 9-11, 2009.
Yoshii T, Hafeman AE, Nyman JS, Zienkiewicz KL, Gutierrez GE, Mundy GR, **Guelcher SA**. Injectable, Biodegradable Polyurethane Scaffolds with Local Lovastatin Delivery for Enhanced Bone Regeneration. Orthopaedic Research Society Annual Meeting, February 23–25, 2009.

Table 2b. Non-Peer-Reviewed Publications and Presentations: Wake Forest-Pittsburgh Consortium

Adams JD, Kim U, Soh HT. High Throughput, Multi-Target Magnetophoretic Separation, Proceedings of MicroTAS Conference, 2008.
Bhatt KA, Vial IN, Wu K, Kelantan M, Dauskardt RH, Longaker MT, Gurtner GC. Stimulating the Regenerative Potential of Adult Wounds Through Mechano-Modulation of Skin Stresses. Abstract/presentation, Stanford University 2nd Annual Resident Research Day, May 12, 2009.
Brandacher G, Schneeberger S, Gorantla VS, Moore LR, Pulikkotil B, Wachtman GS, Donnenberg AD, Lee WPA. Vertebral Bone Marrow Cell Retrieval and Isolation for Composite Tissue Allotransplantation. AFIRM All-Hands Meeting 2009, St. Petersburg, Florida, January 2009.
Galvez MG, Chang, EI, Glotzbach JP, Major M, Vial IN, Thangarajah H, Rajadas J, Gurtner GC. Pullulan Delivery Film for Targeted Ischemic Preconditioning. Abstract/presentation, Plastic Surgery Research Council 54th Annual Meeting, May 30, 2009.
Gerdon AE , Qian J, Zhang Y, Adams J, Oh S, Soh HT. High Sensitivity Protein Detection Using Micro-Magnetic Aptamer PCR (MAP), Proceedings of MicroTAS Conference, 2008.
Hashizume R, Fujimoto KL, Hong Y, Amoroso N, Tobita K, Keller BB, Sacks MS, Wagner WR. Abdominal wall reconstruction with an elastic, biodegradable implant leads to cellular ingrowth and better mimicry of native mechanical properties. ASAIO J 55-163, 2009.

Table 2b. Non-Peer-Reviewed Publications and Presentations: Wake Forest-Pittsburgh Consortium (cont.)

Kretlow JD, Shi M, Young S, Kasper FK, Wong M, Mikos AG. Porous Poly(Methyl Methacrylate) Implants for Osseous Space Maintenance and Promotion of Soft Tissue Healing Around Craniofacial Bone Defects. 2009 Tissue Engineering and Regenerative Medicine International Society World Congress, Seoul, South Korea (accepted for oral presentation).
Kretlow JD, Shi M, Young S, Kasper FK, Wong ME, Mikos AG. Porous Poly(Methyl Methacrylate) Implants for Osseous Space Maintenance Following Craniofacial Injury. Southwest Society of Oral and Maxillofacial Surgeons Annual Meeting. St. Augustine, Florida: Southwest Society of Oral and Maxillofacial Surgeons 2009 (oral presentation).
Kretlow JD. Osseous Space Maintenance in Regenerative Medicine. U.S. Army Institute of Surgical Research Open House. Fort Sam Houston Texas: United States Army Institute of Surgical Research 2009 (oral presentation).
Lavasani M, Usas A, Lu A, Clark K, Pollett JB, Huard J. Human Muscle-Derived Progenitor Cells Express Neuronal and Glial Cell Markers In Vitro and Promote Peripheral Nerve Repair. Muscle-Derived Stem Cells Spontaneously Express Neuronal Markers In Vitro and Promote Peripheral Nerve Repair. Armed Forced Institute of Regenerative Medicine, St. Pete, Florida, 2009.
Machingal MA, Vishwajit V, Bishwokarma B, Zhao W, Yoo JJ, Christ GJ. Mouse latissimus dorsi as a model system for evaluating tissue engineered skeletal muscle. FASEB J. 2009 23:468.42009.
Qian J, Liu X, Zhang Y, Xiao Y, Gerdon A, Soh HT. Enhanced Microfluidic Systematic Evolution of Ligands by EXponential Enrichment (EM-SELEX), Proceedings of MicroTAS Conference, 2008.
Shi M, Kretlow JD, Young S, Kasper FK, Wong M, Mikos AG. Antibiotic-Releasing Porous Poly(Methyl Methacrylate) Scaffolds for Osseous Space Maintenance and Infection Control. 2009 Tissue Engineering and Regenerative Medicine International Society World Congress, Seoul, South Korea (accepted for oral presentation).
Shi M, Kretlow JD, Young S, Kasper FK, Wong ME, Mikos AG. Characterization of Porous Polymethylmethacrylate Scaffolds for Osseous Space Maintenance. Houston Society for Engineering in Medicine and Biology Conference. Houston, Texas: Houston Society for Engineering in Medicine and Biology 2009 (oral presentation).
Shi M, Kretlow JD, Young S, Kasper FK, Wong ME, Mikos AG. Porous Poly(Methyl Methacrylate) Implants for Osseous Space Maintenance Following Craniofacial Injury. 2009 Armed Forces Institute of Regenerative Medicine Conference. St. Pete Beach, Florida: Armed Forces Institute of Regenerative Medicine 2009 (oral and poster presentations).
Stem and Progenitor Cells for Engineering of Functional Soft Tissues for Musculoskeletal Injuries, Gordon Conference on Musculoskeletal Biology and Bioengineering, Andover, New Hampshire, July 23, 2008.
Wachtman GS, Unadkat JV, Gorantla VS, Brandacher G, Schneeberger S, Lee WPA. Immunosuppression Minimization in a Preclinical Model of Composite Tissue Allotransplantation. AFIRM All-Hands Meeting 2009, St. Petersburg, Florida, January 2009.
Wachtman GS, Unadkat JV, Gorantla VS, Brandacher G, Schneeberger S, Lee WPA. Immunosuppression Minimization in a Preclinical Model of Composite Tissue Allotransplantation. Robert H. Ivy Society of Plastic and Reconstructive Surgeons, Pittsburgh, Pennsylvania, March 2009.
Wachtman GS, Unadkat JV, Gorantla VS, Brandacher G, Schneeberger S, Lee WPA. Titration of Bone Marrow Cell Infusion in a Preclinical Model of Composite Tissue Allotransplantation. Ohio Valley Society of Plastic Surgeons, Indianapolis, Indiana, May 2009.
Wang L, et al. Bone Marrow Response to Extremity Injury with Compartment Syndrome in a Large Animal Model. AFIRM Meeting.
Wong VW, Galvez MG, Rajadas J, Gurtner GC. Collagen Hydrogel Scaffold Fabrication Through Salt-Induced Phase Inversion (abstract/presentation. Stanford University 2nd Annual Resident Research Day, May 12, 2009.
Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J. Blocking Myostatin Improves the Success of Muscle Cell Transplantation. Armed Forced Institute of Regenerative Medicine, St. Pete, Florida, 2009.

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 using this rigid definition for the analysis, the following nonvalidated definitions were applied to self-reported intellectual property milestones:

- A self-reported invention disclosure filed with the inventor's institutional technology licensing office between May 2008 and May 2009 is attributed to the AFIRM program in PY1.
- A self-reported patent application filed with a government patent office between October 2008 and May 2009 is attributed to the AFIRM program in PY1.
- No self-reported patent issued in 2008 or 2009 is attributable to the AFIRM program. (According to the USPTO Performance and Accountability Report Fiscal Year 2008, the average patent pendency period for Biotechnology and Organic Chemistry is approximately 35 months. Even applications filed under accelerated examination status can take up to 12 months before final disposition.) In subsequent years, a patent award will be attributable to the AFIRM based on the original patent application filing date meeting the minimal criteria for patent applications above.

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 able to (1) identify patent applications filed for self-reported inventions and (2) identify and validate filing dates for patent applications.

Fifteen government-filed patent applications were self-reported by AFIRM researchers. Eight of these applications were filed prior to October 2008, and another one was filed in June 2009. These nine patent applications are not included in the count of first year patent applications attributed to the AFIRM program. The remaining six applications shown in the following table were all reported by researchers in the Rutgers-Cleveland Clinic Consortium. Of the six patent applications included, one did not have a patent filing number or serial number. This one patent was included because the self-reported filing date of May 30 may not have resulted in the issuance of the application filing number by the time the researchers submitted their annual report.

Patent Applications – Rutgers-Cleveland Clinic Consortium

Reinforced Tissue Graft (PCT/US2009/38570). Filed on March 27, 2009. Derwin K (Cleveland Clinic).

Transport of Biologically Active Molecules into a Cell, Mitochondrion, or Nucleus (#12/354,142). Filed on January 15, 2009. Sarkar G, Bolander ME, Mandal D, Mahlum EW, and Yaszemski MJ (Mayo Clinic).

Biocompatible Polymer Ceramic Matrices. Filed on May 30, 2009. Ducheyne P and Devore D (Rutgers).

Injectable Bone/Polymer Composite Void Fillers Prepared from Lysine-Derived Prepolymers (#61/109,892). Filed on October 30, 2008. Guelcher SA, Hafeman AE, and Davidson JM (Vanderbilt University).

Injectable Composite Bone Void Fillers and Tissue Regeneration Scaffolds (#61/120,836). Filed on December 8, 2008. Guelcher SA, Hafeman AE, Davidson JM, and Nanney LB (Vanderbilt University).

Katz-Spray: Spraying Device and Related Method for Cell Aggregates and Cell Aggregate Suspension Thereof (PCT/US2009/033220). Filed on February 5, 2009. Katz, AJ (University of Virginia).

Appendix C: Patent Applications and Invention Disclosures

Invention disclosures are not publicly reposed in standard databases; therefore, the AFIRM consortium reports are the only information source for these records. The provided information did not always indicate a date when filed with the institutional technology licensing office, and some 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 between May 2008 and May 2009. 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.

In total, 21 invention disclosures were made by AFIRM faculty during this period (see Chapter VII, Figure VII-6). In addition, the six patent applications listed previously that were filed in PY1 are also included in the sum of invention disclosures based on the assumption that the invention must have been disclosed to the institutional technology licensing office this program year to be attributed to AFIRM-sponsored research.

Invention Disclosures: Rutgers-Cleveland Clinic Consortium

Design for Tissue-Lined Bioabsorbable Stent. Filed in February 2009 (Cleveland Clinic).

Photo Self-Crosslinkable Hydrogels: Novel Synthesis of Poly (Ethylene Glycol) Muconate (PEGM), Poly (Ethylene Glycol) Muconyl itaconate (PEGM, and Poly (Ethylene Glycol) Itaconate (PEGI) (Mayo Case #2008-165, pending). Gruetzmacher JA and Yaszemski MJ (Mayo Clinic).

Conducting polymeric composites of polycaprolactone fumarate and polypyrrole for nerve regeneration (Mayo Case #2008-317, pending). Runge BH, Dadsetan M, and Yaszemski MJ (Mayo Clinic).

Conducting Polypyrrole-Polyproplene Fumarate Scaffolds for Bone Regeneration (Mayo Case #2008-380, pending). Yaszemski MJ, Runge MB, and Dadsetan M (Mayo Clinic).

Novel Biogradable Crosslinkable Polymer for Stereolithography (Mayo Case # 2008-063, pending). Gruetzmacher J and Yaszemski MJ (Mayo Clinic).

Neoteric Crosslinkable Biodegradable Copolymer for Use in Stereolithography (Mayo Case # 2008-064, pending). Gruetzmacher J and Yaszemski MJ (Mayo Clinic).

Electrical Conductive Hydrogel Composites of Oligo(Poly Ethylene Glycol) Fumarate and Polypyrrole (pending). Runge MB, Dadsetan M, and Yaszemski MJ (Mayo Clinic).

Injectable Composite Bone Void Fillers Prepared from Lysine-Derived Prepolymers (Invention Disclosure VU0914). Filed on September 7, 2008. Guelcher SA, Tanner SA, and Zienkiewicz KL (Vanderbilt University).

IV Curcumin to Prevent Burn Injury Progression. Filed on December 28, 2008. (Stony Brook University).

Tissue Engineered Fibrocartilage Replacement. This invention is a device, and method to implant the device, for a scaffold for regeneration of the meniscus of the knee and other fibrocartilagenous structures. (University of Medicine and Dentistry of New Jersey).

"Katz-ECM": Generation of ECM and Clinical Applications of Such from Human SCs Cultured in 3-D Suspension. Katz, AJ (University of Virginia).

Invention Disclosures: Rutgers-Cleveland Clinic Consortium (cont.)

"Katz-Free": Serum-Free Adipogenesis of Human ASCs Formulated as 3-D Aggregates. Katz, AJ (University of Virginia).

Katz-Wound: Methods and Compositions Useful for Wound Healing. Katz, AJ (University of Virginia).

Injectable Resorbable Allograft Bone/Polyurethane Bone Cement (Invention Disclosure VU9101). Guelcher SA, Tanner S, and Zienkiewicz K (Vanderbilt University).

Invention Disclosures: Wake Forest-Pittsburgh Consortium

Ultrasound Stimulation. Kemp, P (Intercytex).

Novel Nanostructured Smart Injectable Bone Cements for Bone Regeneration. Sfeir C (McGowan Institute).

Intelligent Biodegradable Pullulan Regenerative Matrix for Tissue Engineering. Gurtner G (Stanford).

Matricryptic ECM Peptides for Tissue Reconstruction (Pitt Ref No.: 01877). Filed on December 12, 2008. Badylak SF and Tottey S (McGowan Institute).

System for Bone Regeneration. Kolambkar Y and Guldberg RE (Georgia Institute of Technology).

Micromagnetic Aptamer-Based Protein Sensor. (University of California, Santa Barbara).

Multi-Target Magnetic Activated Cell Sorter (UC Docket #: UCSB-521PRV; 2008-657-1). (UCSB).

Appendix D: Index of Team Leaders, Project Team Members, and Collaborators

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Donald Lindorfer of Rutgers – The State University of New Jersey is acknowledged for his work with the people-based RCCC images.

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