FDA/NIST SPONSORED WORKSHOP

In Vitro Analyses of Cell/Scaffold Products

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National Transportation Safety Board
Conference Center
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I N D E X

P R O C E E D I N G S

MS. KAWALEY: Good morning, and welcome to the workshop on In Vitro Analyses of Cell/Scaffold Products. I am Bernadette Kawaley from the FDA Center for Biologics Evaluation and Research, Office of Communication, Training and Manufacturer's Assistance.

The Food and Drug Administration and the National Institute of Standards and Technology share an interest in facilitating this critical step in the development of bioengineered medical products and have collaborated to sponsor this event.

Today's workshop is scheduled to conclude at 5:30 p.m. The lunch period is from 12:15 p.m. to 1:30 p.m. and is on your own. There are several restaurants located throughout the promenade.

Please be mindful that there is no eating and drinking in the auditorium. Rest room facilities are located in the main lobby area.

Please refrain from using electronic devices such as BlackBerries and cell phones because the battery life will be drained due to this location. There is a phone available for use located in a

conference room near the rear of the auditorium.

This morning our first speaker will be Dr. Jesse Goodman, director of FDA Center for Biologics Evaluation and Research.

DR. GOODMAN: Well, welcome everybody, those from both the far and near. You know, having lived in Minnesota for a long time, I'm never very impressed by snow here. But we don't deal with it very well. And, of course, the national transportation system doesn't deal -- I shouldn't say that in the National Transportation Safety -- that's just a Freudian slip. But it doesn't deal with it very well either.

So I'm impressed that people have made it here. We have a lot more people registered. Hopefully, they too will make it. I would encourage people to think about sitting closer and being very engaged. At least the next time you get up, if there aren't a lot more people, come sit closer.

I'm actually very delighted to see what we hope will be a series of meetings or workshops that are really intended to bring together scientific and innovation expertise, both in the private and academic

sectors with the government sector in this area. I have thought from the day I got engaged in CBER's products, and still feel now, just a really incredible level of excitement about whatever you call the field, but these combination products, these tissue-engineered products, the application of engineering to biomedical sciences. And I sort of feel that this is a birth that's going on and that once we start seeing some of the possibilities come into being, it's going to be a revolution.

And I say this as somebody who doesn't have a particular personal stake in promoting it. So I'm very enthusiastic to see the number of people registered, to see the science moving forward and to see the excitement that's out there in the non-tissue engineering community about the possibilities of this.

And if a measure of that is -- I have a son, one son is an undergraduate in college who probably is the only one of my kids who'll go into medicine. But he just is constantly telling me how cool all the things that he reads about are and he's really interested in how you apply engineering to life

sciences and things like that.

So this is great. As I said, we hope it will be the first in a series. We're thinking about other areas, such as how you do clinical trials, some of the issues about manufacturing, et cetera. There's a list of things that people are considering.

I would really -- we would all welcome your feedback after this meeting. And I know Dr. Witten is going to talk next; Dr. Durfor. We welcome input about future meetings. This is very consistent with what we're trying to do at FDA and very much at CBER and CDRH, which is to help move fields forward so we can bring these kinds of promising innovations to patients and help build the science of product development, really.

And then I just want to thank those who were involved in organizing this, particularly NIST. And I'm not going to go through mentioning names, but it's great to partner with an expert scientific standard-setting group in the government.

And then also to just say that in this and many other areas, the Center for Biologics and the

Center for Devices are working very closely together and trying as much as we can to take a team approach to our interactions with you and not to be necessarily bound by what we've seen with other kinds of products, but to approach these with a fresh and open mind.

So with all that said, thank you all. And Dr. Witten is a good example of this, having come -- we were able to steal her away from the device center to the biologics center. So I think nobody could be better to help guide us in this field forward, working with CDRH.

So, again, welcome and thanks.

DR. WITTEN: Thank you, Dr. Goodman, for that introduction.

And what I'm going to talk about today briefly is what is the current FDA perspective and review of cell/scaffold products. And I'm going to provide an overview of some of the tools that we currently have and some of the areas in which I think that further characterization, tool development is needed. And that is really going to be the focus of this next day and a half workshop.

So this is just to set the stage to talk about why we're here, what are the goals of the workshop, and also talk about how that fits in or how this workshop fits into how FDA develops policy and guidance.

So I have our mission statement, responsible for protecting public health and regulate, as everyone knows, drugs, biological products and medical devices. But there is also a public health promotion aspect of this mission, and this is part of our effort to try to facilitate development of -- or enabling development and review of these cell/scaffold products at FDA.

So this is a promising area of scientific development, as Dr. Goodman mentioned. And the cell/scaffolds, there may be a lot of novel therapeutics that are coming down the road.

I think that one of the questions that we always ask when we see early clinical studies is what is the product that you're intending to study in the patients and characterize these products as a key challenge for sponsors and also for FDA. So developing and assessing the tools for product characterization is

a common interest of ours, FDA, NIST and the scientific community together.

I am going to just digress for a moment and talk a little bit about FDA regulations and policy development because I know a lot of the people in the audience are actually more involved in the basic science area. And so one question is how does this fit or how does FDA's perspective or efforts fit into scientific development and how would a workshop or what we learn from a workshop like this fit into our policy development.

So I'm just going to talk about our levels of regulation and policy development, constitution, statutes, which are laws, regulations, which is what FDA develops, and guidance documents, which give some more specific information about something like how to characterize your product or how to study it, or perhaps other areas of interest.

So I'm not talking about the constitution. I'll say the statutes is our law. So there's the Federal Food, Drug and Cosmetic Act, the Public Health Service Act, which is to prevent communicable disease

and has licensing provisions. And then there's other statutes, like the Administrative Procedure Act and Federal Advisory Committee Act, that speak to how we actually do our business. So how do we actually develop policy, how do we get public input, and how do we get advice? So those kinds of statutes would also apply to us.

The regulations are developed by FDA. So the laws are things that were given by Congress, and then FDA develops regulations to implement those laws because usually the laws are fairly general. They'll say the secretary of HHS will make sure products are safe and effective and don't transmit communicable disease, or have some other broad brush statements. In general, that's true, not always.

Then FDA will develop regulations which have the force of law. They're binding. It has a binding effect. And those will say how specifically we actually expect to implement the law that we're given. And there's an opportunity for public participation in regulation development by written comments, and FDA may hold workshops or other types of opportunity for public

participation.

Then there are guidance documents and those are documents -- they don't have the force of law. They describe FDA's interpretation of our policy on a regulatory issue and they can cover any area of medical product development. And I've listed a couple examples: design, production, promotion, manufacturing and testing of regulated products, inspection enforcement policies, et cetera.

And the guidance documents, which I'm going to give a number of examples of in this talk, give more specific information about how we actually expect you to -- or an option for how you can follow our regulations.

So these are some examples of guidance documents. We have a guidance on cell selection devices for point of care, production of minimally manipulated autologous peripheral blood stem cell. So this would be, for example, cell selection devices for use at the point of care to sort for cardiac cells for use in a cell therapy study for cardiac use. And the second one is an example of a cross-cutting guidance

I'm going to talk about a little bit later for products for knee cartilage repair. So that would give you some examples of the level of detail in the guidance.

So how do we develop guidances or any of this other policy? Well, there are formal rules about guidance development that I'm not going to go into. But one important way that we get input into guidance and policy development would include workshops like this one where there's a new area.

It's a challenge for FDA. It's a challenge for sponsors. And we certainly hear at meetings where does FDA stand on this; what does FDA want us to do. So this is really our opportunity to hear from the scientific community and the development community about what you think some of the key scientific questions are in the area of product characterization.

We have other ways to develop scientific review policy. Advisory committee input is important. We have research and research collaboration. We participate in standards committee. And there's actually a guidance that is out now that describes the ways in which FDA participates in standards development

across FDA, the Critical Path Initiative, which certainly speaks to developing scientific approach to product review in areas like this and then review experience.

We have the benefit and the privilege at FDA of seeing applications or having discussions with innovative product developers across a range of areas in a product type. So in some ways, although a lot of the information is information only available to us, we get to see both successes and failures -- early successes and failures across the development spectrum. And then we participate in meetings and through that scientific exchange also that informs our policy development.

So I'm not going to spend too long on the Critical Path Initiative. I have the website here. But this initiative recognizes that one of the challenges for evaluation of safety, efficacy and quality of medical products is that there are some cross-cutting tools that are needed that are -- that right now don't exist, that it might be of benefit rather than developing them product by product so that

each individual sponsor develops -- let's say in this case, there are characterization tools for their specific product, that some cross-cutting development tools would be of value to the field as a whole.

We have also critical path research. Our laboratory scientists conduct critical path research. Some of it is collaborative with the outside community and some of it is research just that is done in our own labs.

One example is our collaboration with NIST at CBER in the research on cell metrology. That's one of the focuses of our research program. Anne Plant, who's going to be moderating this session the next day, will be talking some about the cell metrology program.

There's the biomarkers consortium, is one specific example of the type of critical path effort. There's a public/private biomedical research partnership managed by the Foundation for NIH. We're a participant in this consortium. The goal of this consortium is to identify critical biomarkers as potential tools for cross-cutting policy development and to try to champion in some way development of these

cross-cutting tools, medical product development tools.

So I've listed here some of our outreach and collaborations at CBER. These are some of the things we do. We have liaison meetings with professional societies such as the ISCT, International Society for Cell Therapy, Regenerative Medicine Consortium, and the American Association of Tissue Banking.

We also have input into standards programs from a number of standards organizations. And we have other government collaborators, too, which I've listed here. We have very active collaborations with these groups including, for example, MATES, Multi-Agency Tissue Engineering group, which also will be discussed tomorrow. And, in fact, this workshop is part of our implementation of the MATES strategic plan.

We have a number of research collaborations and then workshops. This is on the list. But we've had a number of other workshops with the scientific community in the last year as well that I've listed here.

So I want to focus on this workshop and this workshop goal. The overall goal of the workshop is to

explore scientific advances to improve understanding of the cell/scaffold constructs within the framework of a product under consideration for clinical evaluation; so, in other words, characterizing a product when you're considering your clinical study.

There's two sessions. In vitro bench top characterization and that's -- which is today a systematic and high throughput analyses, which is the subject of tomorrow's discussion.

I'm not going to go into the organization of these. Dr. Durfor is going to discuss that in his presentation that follows mine.

So the speakers were all given questions, and the questions, you've all seen these. I'll just mention them.

What questions should be addressed when evaluating cell/scaffolds in preparation for the first human studies? And what test methods are available, and what analytical procedures need to be researched, developed or standardized for these products?

So I don't want to talk a lot about FDA. But since I'm going to talk about guidances, and since this

is a cross-center workshop, I just want to have this one slide up about FDA organization.

FDA, there's the Office of the Commissioner and then there's six centers. Three of them deal with medical products for evaluation: The Center for Biologics -- and I'm the director of the office in the Center for Biologics that regulates cell tissue and gene therapy -- the Center for Devices, which regulates medical devices for treatment, implants and diagnostic devices, and the Center for Drug Evaluation. So this workshop was cosponsored by CBER and CDRH in conjunction with NIST.

There's a number of regulatory pathways that these products might fall under. Some may be biologics devices. In general, the cell/scaffold products would be combination products. And since we're not here to talk about regulation, I'm not going to spend any time talking about specific regulatory pathways or jurisdictional issues since the focus of this meeting is characterization tools.

One of the things that we do tell sponsors when they come to us is that there's a number of

guidances, both in the Office of Cell and Gene Therapy and also in the Center for Devices, that may provide some insight to a product developer who is trying to figure out how to describe their product or test their product for their first in-man study.

So we always give people the advice to look and leverage existing guidances to support specific areas of tissue-engineered medical products. So the kinds of guidances could include CMC guidances for cellular products, general cell and gene therapy clinical guidance. There's some guidances for devices that may be applicable to scaffolds, and there's also clinical guidances that cross-cut product areas.

So although these guidances may not directly apply to the product that you're developing, they are a value to look at as a reference to give some insight into FDA thinking. So what I'm going to do is I'm going to just give some examples in the area of cell therapy and in the area of devices of the kinds of guidance and the kinds of information that you might be able to find.

So when we talk about cell therapy, of

course, a number of these may be standalone, just cell therapy products, not combination products. And here are some examples: There's pancreatic islet cells, stem and skeletal muscle progenitor cells for ischemic cardiac disease, stem cells for hematopoietic reconstitution and treatment of malignancies or metabolic storage diseases. We've seen stem cells for CNS applications and, of course, expanded autologous cartilage for joint repair. Those would be some of the examples of areas of interest.

So here are some of the cell therapy guidances that we point people to or cell therapy documents, perhaps I should have called it. There's a draft guidance on manufacturing, a guidance for human somatic cell and gene therapy. There's eligibility determination for donors of human cells, tissues and cellular tissue base products, which for development of allogeneic cell/scaffold product would be important for a developer to be aware of.

So these would all be resources for a product developer. And what do they cover? Topics like cell sourcing, how to characterize your starting cells or

tissues, information about cell banks and what we'd like to know about your cell bank.

It would be important to supply information about components in the reagents in your cell therapy product, and these would be some examples of some of the questions we'd ask about components in reagents and cell therapy development.

Manufacturing procedures are important, especially because the characterization tools for some of these may be somewhat lacking. So method of cell selection, culture components, irradiation, storage; in other words, exactly what you do to manufacture your cell therapy product, process residuals, what you do for prevention of product contamination.

Then there's some testing for cell therapies. And these are some of the things that we would ask a sponsor for a cell therapy to perform for us. But then, of course, the challenge is what happens if those cells are actually not the therapeutic in and of themselves. Perhaps the goal is to combine them with a scaffold, and these questions -- the cells may change and then you have the question about the combination

and not just the cells. So the questions and the answers may be somewhat different.

You have scaffold characterization and safety. And here would be some examples of scaffold materials that are in use. Resorbable and non-resorbable polymers, physiological materials. And there's certainly some more novel scaffold material we've seen under development for cell/scaffold products.

So here would be an example of a guidance document that might be useful for a cell/scaffold developer, although it's not specifically intended for the kinds of regenerative medicine products that we're talking about today. This is the guidance for the preparation of pre-market notification application for surgical mesh. So this slide and the ones that follow come from that guidance.

So in characterizing a surgical mesh, a sponsor would be asked about manufacture, including starting materials; the manufacturing process and where should cells -- what the sterilization methods and validation of the sterilization methods are, and how

it's tested for biocompatibility. Also, characterization of the scaffold in terms of its physical and strength characteristics and mechanical characteristics, and then how the properties change as a function of time if it's resorbable.

All these questions will most likely be relevant also for the scaffold that's used in a combination cell/scaffold product also.

Just like with the surgical mesh, the number of studies are going to be determined by how you're proposing to use it. So then you get to cell/scaffold. So I've provided some background about the approach if you have a cell therapy or a scaffold therapy, a scaffold product.

But what about these combinations? And what am I talking about when I talk about cell/scaffolds? Well, there's autologous or allogeneic cells on a matrix or wound repair, cell-seeded scaffolds for cardiovascular repair, encapsulated pancreatic islet cells, and collagen repair products. So these are all types of products that have been studied, reported in the literature, and some of them are on the market.

So what happens when you get to a cell/scaffold product? It becomes a challenge because we have the evaluation pathway for the left-hand part of this pipeline and we have an evaluation pathway for the right-hand part of this pipeline also with the scaffolds. But then when you put them together, there can be new questions that arise that may not be adequately addressed by just understanding the cells and the scaffold.

So these might be some examples: Impact of the device on the biologic; are there changes on the cells or the tissues? How can you evaluate if the scaffold is supposed to be a barrier to immune rejection; how can you evaluate its function there? The biologic may have an impact on the device in terms of degradation and other biocompatibility issues. And then, of course, the mechanical characterization can be a challenge, too.

So here in this area there's not as many for these combination products. There's not as many guidance documents that can be leveraged. I mean, we have the guidances in each area, but how do you look at

the construct as a whole?

There is this guidance, Preparation of IDE and IND. This is a cross-center guidance for products intended to repair or replace knee cartilage. So this is one place that people might look. It's a joint guidance. It reflects advisory committee input. It leverages the ASTM document that was written by the ASTM subcommittee.

The scope of this document is IND, IDE products to repair or replace articular cartilage of the knee, and it covers devices, biologics and combination products. It covers a number of topics in the area of manufacturing and CMC, non-clinical data and testing, and clinical study design.

So what to point to beyond this for some of these combination products is a challenge. And one of the things that we're hoping to get an understanding of from this workshop is some of the tools or some of the things that we might point people to once we get to the combination cell/scaffold product area.

I'd like to thank the workshop planning committee which, as Dr. Goodman already mentioned, has

representation from CDRH, CBER and NIST. And, of course, we at FDA are eager also to hear from the public. We don't make our policy in a vacuum, and we need input from across the community of research and users and, of course, patients, which is all of our focus.

So I'd like to thank you all and introduce the next speaker, which is Dr. Durfor. Charles Durfor is going to introduce the next session. And Charles Durfor is a senior reviewer in the Center for Devices and was also the lead reviewer for some of the first of the cell/scaffold products, some of the ones used for wound repair. So he's quite knowledgeable in this area, and we look forward to this session. Thank you.

I guess I should ask if there are any questions, although I'm really hoping that there will be a lot of questions for the speakers that follow. But if there are any questions about my presentation or what we're hoping to accomplish here today, I'd be happy to take them at this time.

I'm going to turn it over to you.

DR. DURFOR: But I want to first of all

welcome you all and thank you for coming. The weather was not the most kind to us today, but hopefully --

Thank you, and once again welcome. As we've just heard from Dr. Witten's talk, these are the objectives of what we're here for. And what I'd like to do as an introduction to this session is sort of give you a little bit of information as to how this workshop came about and why it's organized as it is organized.

So we are in this workshop focused on cell/scaffold products. Dr. Witten's talk has been, I think, very instructive, telling us that the FDA history with cellular therapies goes back a long way, back to 1989, and medical devices have looked at scaffolds as medical implants for much longer than that. So there's a great deal of experience in terms of cell products. Certainly, we don't know everything. No one knows everything, but there's some experience. And there's certainly experience as well with regards to medical device implantation.

It's when you put the products together that all of a sudden there's not as much information as

anyone would like. And so this workshop came out of -- and I was really -- it's really a pleasure to say there was a meeting that was held earlier this year by -- briefing both of our center directors, Dr. Goodman, who's kind enough to be here today, and Dan Schultz, to tell them about a strategic plan for MATES and about this area. They were the genesis of this workshop.

So to all of you, I thank you for the fact that the timing was short. To the presenters, the timing was very short. But this was something we felt really was important to do.

So a little bit about the workshop structure and as we've already heard once again -- or as I've just mentioned, there is a fairly strong amount of information about cells and implants, scaffolds. But the question becomes when you put the two of them together, things just change. So Dr. Witten has given us sort of a basic regulatory oversight of what the FDA considers, and then we're going to go on to a session that will now deal with science because that's really what this is all about.

Having been fortunate enough to be in the FDA long enough, I've seen a couple of other areas come forward and become very commercially exciting areas. But often as in any area, there are questions that are either real or hypothetical.

One that comes to mind is the issue many years ago with monoclonal antibodies. There was a great deal of concern about nucleotide DNA contamination, and that was a hypothetical concern that over time things became more clear and the approach to it became a little more relaxed. And that's partly what we're here to do today is to look at what are the real concerns, what are the hypothetical concerns, and to try and really focus things down on the key issues.

So the first part of doing that is to have two overview talks, one on biomarkers and then one on biomaterials. There are so many applications for this area. It's really hard to figure how to slice and dice. So we went very arbitrary.

We decided that we would divide the case studies, the seven case studies that follow, into a series of products that are either more structural in

nature and the others that are more biological or functional in nature.

We recognize that probably no one construct -- and I'll use that term to define cell/scaffold products. No one construct is solely structural or functional, but at least this is an approach for us to try and divide them up so that we can cover as many of the potential areas that may come forward as possible.

At the conclusion of the case studies this afternoon, Dr. Benton's going to give us an overview, a look at how FDA thinks about in vitro assays. And once again, why are we focusing in vitro assays? Well, this is once again a very broad area in need of a lot of conversation. And so we felt it was important to try and focus our talks and our approach for this workshop to one area, and that would be in vitro diagnostics or in vitro analyses of cells.

As Dr. Goodman has alluded to, we have great hope that there will be follow-up conferences and workshops that will tackle other important areas with these products. And so throughout the day today, as

you listen, as you learn, as you question, we hope that you will begin to formulate an opinion as to what should be an appropriate follow-up. And there are many different areas that we hope that you will deal with.

But today we're going to be having Dr. Benton talk about potency assays or in vitro assays and how their development parallels with the clinical development of these products so that it all comes together when you're ready to bring the product to commercial distribution.

At the end of the day, we will have a roundtable discussion, where both our speakers and we hope you will contribute your experience, your insight and help us focus down on those key questions that need to be thought about.

Why did we focus on products entering Phase 1 studies? Very simply, until that time or after that time, after you've begun Phase 1 studies, you'll be in a dialogue with the FDA and there will be some direct interaction. But often it's when you're beginning to prepare, where you don't have that interaction as much, is where you are searching for the right answers. So

we felt this was the best place, the biggest bang for the buck, if you will, to try and focus things down.

Now, the second day we move from our case studies in product areas to case studies in technology or in analytical procedures, and we open that morning with a presentation by Dr. Heineken.

Dr. Heineken is the chair of the Multi-Agency Tissue Engineering Working Group. His presence is a reflection that not only within the FDA are we both excited but serious about committing resources in an intelligent way to move these products forward, but that is an emphasis that is throughout the federal government.

Much to the Multi-Agency Tissue Engineering Working Group's credit, they have released a strategic plan in the last few months. There are copies that are out in the lobby. And if they're not there, right after break, they'll be more after lunch as we bring in box after box. We hope you take them. This is an attempt by federal agencies across the government, whether it be NASA, whether it be DOD, whether it be CMS, whether it be FDA -- all of us work together to

try and see how we can marshal our resources to really promote this area.

So I encourage you to grab a copy of the strategic plan. You'll note that MATES has its own website which is www.tissueenginnering.gov. Take advantage of that as well.

Then Dr. Plant of the National Institute for Science and Technology will chair the second day on developing techniques, and this is a perfect example of what we're trying to do within the government. Take advantage of our resources. And this is an area where NIST is extremely strong, and so we are extremely happy to be partnering with them, drawing upon their expertise as we move in this area.

At the end of the second day, or the partial second day, there will be a roundtable discussion on these technologies. I encourage you at that time to once again think about these technologies, think about what are their values, what are their limitations. Often, unfortunately, when I read the literature, I never find out what doesn't quite work. That makes it harder to publish.

So question things. See how these might be applicable. Find out where there are limitations. Find out where there is value.

At the end of the workshop, we are very fortunate to have Professor Nerem here, and he will serve as rapporteur for us. And I've asked him to consider three topics that he will help summarize for us.

First will be a scientific summary of what we've all discussed. So it's important for all of us to discuss it, not only to sit and listen, but to get up and share information. The second thing I've asked Professor Nerem to comment on is his opinion as to the scope of what maybe the next workshop, the follow-up workshop would be. So once again, he's here to listen. He's here to give us his insight.

The third thing I've asked Dr. Nerem to consider -- and once again we would appreciate your insight as well -- is when all is said and done and this workshop is over, and you're back on your plane or your train or your car, what would be the written output that you would like to see from NIST and FDA

regarding this workshop. How can we somehow take the information we've discussed and put it in a written form that will be of value? And we will look to Professor Nerem to give us his insight.

Well, that brings us back once again to why we're here. We're here to learn what are the key questions to ask when you're bringing a cell/scaffold product into its first study; what sort of analytical procedures, test methods are available; which need further work, standardization, development to make them very valuable and looking at the safety, the purity, the potency and the consistency of cell/scaffold products. The way this works is we all share information. So we encourage you to participate, to share your knowledge, and then we will have a very hopefully dynamic discussion.

That said, it's my pleasure to introduce the first speaker today, Dr. Rocky Tuan. Dr. Tuan is the branch chief for the Cartilage Biology and Orthopedics Branch within the National Institutes of Arthritis and Musculoskeletal and Skin Diseases.

Dr. Tuan received his bachelor degree in '72,

his PhD in 1977 from Rockefeller University. He performed postdoctoral research at Harvard Medical School, first in the Department of Orthopedic Surgery and then from 1978 to 1980 in the Department of Developmental Biology at Mass General.

In 1980, Dr. Tuan was appointed as assistant professor in the Department of Biology at the University of Pennsylvania and in '86 he was promoted to associate professor.

In 1988, Dr. Tuan joined the Thomas Jefferson University as the director of orthopedic research and as a professor. Subsequently, during his tenure at Thomas Jefferson, he served as the vice chairman in the Department of Orthopedic Surgery with a joint appointment in the Department of Biochemistry and Molecular Biology.

In 1997, Dr. Tuan established the nation's first cell and tissue engineering PhD program at Thomas Jefferson with the mission of training the next generation of cross-cultural biomedical scientists.

In the fall of 2001, Dr. Tuan joined the Intramural Research Program at NIH as the chief of

Cartilage Biology and Orthopedics Branch. His interests now include both basic and clinical research directed towards understanding the mechanisms of regulating cartilage function and the basis of cartilage and orthopedic diseases in the development of functional cartilage tissue substitutes.

Today Dr. Tuan will provide us with an overview of cellular biomarkers with a focus on how this information can be used to characterize cells found on cell/scaffold products.

Dr. Tuan, thank you very much for your time.

DR. TUAN: Sorry about the PC to Mac transition. It usually takes a little while.

So I want to just thank again the FDA and NIST for organizing this workshop. This is an incredibly timely topic. And I was sitting there listening to Celia's talk and then I -- she really put everything into great perspective as to why this is important and why we should be here turning up our brain power and try to really come up with some perspectives for the future.

So what I'm going to -- I think Buddy and I

have you can say enviable or also an extremely challenging task of trying to give you an overview of some of the things that are critical issues in this area. So what I'm going to do today is to give you an idea of sort of the overview of cellular biomarkers.

Now, it is obviously not possible to summarize these things in 30 minutes or even less than that. So what I'd like you to come away with at the end of my presentation is to really think about -- I'll give you an example -- some of the examples of what are the needs, why are we doing this in the first place, and how to address these needs, how to evaluate whatever we're trying to do, and what are the challenges and also what next.

Now, I will select some things from areas that I know a little bit more about in terms of musculoskeletal as an example. But by and large, I'll try to be as conceptual as possible.

Now, for musculoskeletal needs, you go with official documents. For example, this one, which is the 2004 Surgeon General's report on bone health, you can see that, first of all, it's a pretty big book and

there's a lot of data in there. And also this is the bone and joint decade, so it really underscores the importance of musculoskeletal health.

If you look at the statistics -- again, this is excerpted from the surgeon general's book. The surgeon general's book, you can see that the numbers are pretty staggering. These are millions and millions and the symbols are immediately telltales. I don't have to go into specifics. So this is important. Bone health is important.

This next slide tells you that cartilage health is also very important. Again, musculoskeletal diseases represent the number one reason any of us goes to see a doctor. And in terms of the musculoskeletal diseases, degenerative joint diseases rank as number one in terms of prevalence. And among those, osteoarthritis is the big hitter. It affects more than 10 percent of the population of the U.S., for example. And if you're over 65, chances of having at least one joint affected is more than 50 percent and is an equal opportunity disease, affects both genders equally.

Now, the other thing is that on the

left-hand side, you can see, say, in osteoarthritis you can have the narrowing of joint space, fibrillation of the surface cartilage and then eventually leading to deformation of the joints. So this is why this is an important disease.

Now, what can we do about this currently? I think all of you know that the ultimate solution, the final solution, of course, is total joint replacement, plastic and metal. Total joint arthroplasties have worked very well, and there's nothing wrong with it. The only thing is that it is a replacement, So at the end, it will need to be replaced again.

So what can we do about this? What other options do we have? Well, you can be somewhat palliative and you can inject lubricants of sorts or pain relievers, and those are used. Or you can think about cells, and there are procedures that are currently done and also FDA-approved in many forms that can be used to fix this.

Now, one is autologous cartilage transplantation. That is basically similar to hair plug transplant. You are not really making any new

cartilage. You're just moving cartilage from a less weight-bearing area, which is sort of in the back of the joint, to the front where you rub a little bit more. So as you can see, this is not a permanent solution; it's just like hair plug transplant.

So there's a cellular approach, and this is equivalent to the hair plug transplant. You take that plug and you disassociate the plugs into cells. These cells are then grown up and then they are then implanted, rather, in a collagen carrier underneath a periosteal flap that you now sew onto this defect to make like a trampoline. Underneath the trampoline, you inject these cells. And the cells will take, and eventually you fill up that space.

So those are the biological alternatives. So you can see here that -- another view of what the challenge is. You have a joint that is pretty badly deformed over here on the right where all this matrix is gone. And if you were to look at an early phase of this particular disease, what happens is that you have a degeneration of the surface cartilage. It's a structural damage. It's a structural tissue that has

gone bad.

So for now let's talk about how we can deal with this. So the one approach is, of course, to see if we can come up with a functional substitute, not just a replacement like plastic and metal. So what we have is a defect, and if we could find something that would look like a defect, and then we could shape it and put it in such a way that it can go right back in there and integrate.

So that's the sort of example of what we want to do here, cells with scaffolds and try to get this thing to work. Now, in other words, what we're trying to do is to take medicine from preventative, palliative, or restorative reconstructive to a regenerative phase, a regenerative stage. This is what we're aiming for, tissue regeneration. And one of most promising approach is tissue engineering.

So now, tissue engineering, let's just briefly talk about this. And I'm sure the later speakers will talk about this in even more detail.

In order for tissue engineering to work, there are essentially three components, three

requirements. You have the productive component. You have the conductive component, and then you have the inductive component. These are not necessarily exclusive in terms of their description.

The productive component consists of cells. And those are the cells that you can either get differentiated cells, say, exactly like what you find in that tissue that you want to make or something that is a progenitor cell, that will become the tissue that you want to make.

Then, of course, you need to put all these things into some type of scaffold. Most of the tissues that we've talked about, even the functional tissues, have a structure. They're not just floating around in isolation. They have a shape, form and structure that needs to be reproduced. So in order to do that, what we try to do is to produce a scaffold that has the rudiments of that structure in place already.

Then, of course, the final one is inductive component. I don't have time and this is not the topic for this particular workshop, either. So it's really the biologically active components or influences that

you need to make this whole go to its maximal stage.

So, what cells? Well, a cell type that has been talked about a lot and, of course, has political, ethical, moral, all kinds of implications, of course, are the embryonic stem cells. And again, this is not the workshop to talk about it, but this is just a *Time* magazine version of how you get an embryonic stem cell; whether and how these cells are going to pan out and in terms of -- particularly in terms of funding and so forth, it's to be seen.

Now, embryonic stem cells, of course, are cells that have pluripotency, meaning they can become any tissue type you want. The challenge here is how to direct them so that they can become only the cell types that you want and stop there and not go any further. So that's the key. This is in addition to all the other complications that I mentioned earlier.

Now, of course, some of you may have seen these -- this is actually the copy from last year's publication -- the so-called iPS cells, induced pluripotent stem cells that made the news, in fact, in a very big way November 20th, just not too many days

ago. And these are the cells -- this is the procedure originally published by Shinya Yamanaka in Kyoto where he took four genes. He found four genes, that when put into an adult cell, dumb fibroblast, will make these cells pluripotent. First, he did it in mouse, which was last fall. And then on November 20th, together with Jamie Thompson at University of Wisconsin, they succeeded in doing in human cells.

So what you have done is in principle produce a human stem cell equivalent. And what these cells can do in the context of what we are interested in, cell/scaffold reproduction or tissue regeneration, awaits further investigation. But it's very exciting.

So another cell type -- and again, this was alluded to earlier -- that's quite a favorite and certainly an attractive kind of cell type are adult stem cells. And among these cells, in addition to hematopoietic stem cells, are the mesenchymal stem cells. These are cells that look like fiberglass. Sort of like everything tastes like chicken, they look like fiberglass. If you take a mesenchymal stem cell and show it to somebody, they can't tell by looking at

it they look like fiberglass, but they're able to differentiate into multiple cell types, particularly along the connective tissue lineages, the so-called mesenchymal lineages.

They're found in a lot of tissues. They're different than hematopoietic stem cells. What characterizes the most is the cell renewal ability. I should say extended cell renewal ability. They're not infinite compared to the embryonic stem cells, but it's pretty okay.

So here's a cartoon that tells us what the potential may be for these cells. And so this is the cell that a lot of people want to use as the beginning cell type for the cell/scaffold construct.

So where do they come from? Here's just a very quick diagram, a table of some of the tissue sources. The original mesenchymal stem cells were discovered in bone marrow by Alexander Friedenstein in 1960s. He called them colony-forming, unit fiberglass, CFUFs. And so that remained a favorite among a lot of people.

Other new favorites include those from

muscle, adipose, blood and peripheral blood, and other types of tissues. I put some arrows there for the cells that we use in our laboratory and that we consider potentially candidate cell types.

Now, I want to tell you what these cells are good for. For us, they're good for two reasons. There are other reasons that other people have, of course. The first is that they can become cartilage or chondrocytes.

Here's sort of a standard type of experiment. You take these cells, and you spin them in the bottom of a tube so they aggregate and form a little tiny ball. And you wait a couple weeks. You give them all kinds of goodies. At the end, if you were to look at the cells, it becomes a little tiny nodule of cartilage. It's very nice. It's beautiful looking cartilage.

The other thing you could do is you put them in a dish and you give them all kinds of goodies again. And after about two to three weeks, they become bone cells. They express markers such as alkaline phosphatase, and they produce mineral, and they turn on

the genes. Beautiful. Beautiful bone cells.

The challenge here is not so much on what the cells can do; it's what you can make their activities become or contribute to it being a piece of product. So that is where a scaffold comes in. You have to put these cells in a context such that you can actually do something with it.

So biomaterials, native, synthetic, natural, whatever you want or a combination, hybrid molecules and so forth, you need to put the cells into this particular biomaterial so you form a three-dimensional construct. There are different types of forms of biomaterials. The chemistry is itself very complicated and comprehensive. I don't have time to go into it.

But, basically, there are the following types of shape and form they come in. It could be a foam. It could be fibers. It could be hydrogels. It could be beads. It could be a combination of these things in whatever way you want. So those are the kind of things that we work with.

Now, with this the approach, then, is as follows: You got a scaffold. You got some cells.

Let's say you have progenitor cells. The advantage of a progenitor cell or stem cell is that you can make more of them. If you use differentiated cells, whatever you got is what you have.

So obviously, usually we don't have spare parts in our body except for fat. So therefore, you're kind of limited in that sense. So stem cells are attractive in that sense. So you can have stem cells. And, of course, you choose your goodies, and that's where the biology comes in, the bioactive factors or environment or what have you. And then you want to probably do some type of physical stimulation. It could be mechanical. It could be oxygen tension or whatever you have, your electricity or whatever you want, some type of physical stimulation. You need to somehow -- if it's a vascularized tissue, you need to stimulate vascularization. And then you should have at the end an engineered tissue.

Now, what I put down here is that what I think is going on here. What is tissue engineering? Tissue engineering is assisted development, just like assisted living, not quite the same as the lifestyle

before but not bad. Somebody's helping you. You're getting somewhere. You're getting the job done, whatever. The basic pleasures of life are there.

So, assisted development, now, it's a little bit different. It's quite different, in fact, from regular development. First of all, time, of course is much shorter. Secondly, the cells that you ask to help out in this have never done this before, most of the time; they're just sort of naive. But you are building a home, and you say get in here and do the stuff. Here's my scaffold, what I think you will like and I want you do the job. So it's assisted development.

So, we want to make a tissue. What is a tissue? Now, I just picked this out from a histology textbook. Your standard sort of garden variety tissue is made of cells. In this particular case, it's a very synthetically active cell because you can see all the ribosomes and all the rough endoplasmic reticulum. It usually doesn't live in a void. It lives in a matrix. That matrix is usually made of structures that are fibers or things that hang on to fibers and so on. It is a dynamic context.

A dynamic context is a sense that the cell, of course, is the one who is responsible for synthesizing everything outside and inside the cell, whereas as tissue engineers, we're doing it slightly differently. We already made the stuff outside the cell. We can't make the stuff inside the cell unless you do gene therapy. But you make the stuff outside or you stick the cell in there, and you say, hey, love it; do something good.

But we are trying to reproduce this structure as I've shown on the slide here. In other words, if you go into cartoon form, you will have a cell that's, of course, regulating itself. So as you can see, the arrow inside the cell is making all kinds of transcription factors and signaling molecules and ribosomal proteins and what have you, 10,000 things inside the cell.

It is also making various bioactive molecules outside the cell that tells itself what to do as well as other cells what to do and, of course, is making the extracellular matrix, which supports it, but also can be informational at the same time because extracellular

matrix has informatic ligands that actually the cells can read.

Now, there are many cells in the tissue. In fact, there are many, many cells. And the key thing is that these cells also interact with each other. And when they interact with each other, if you want to make a good tissue, there should be a positive interaction that enables the function of the tissue to develop its function and to integrate with the rest and so on and get on with life with the host.

So this is what we want. We want to make something like this that will actually make everybody happy doing what we want them to do. So in order to define what we are trying to, whether we are getting there or not, we need biomarkers. Biomarkers have already been defined earlier, and I'm just stating it again.

This is just a -- I think it's a Wikipedia definition actually. So it's got to be correct. So it's a characteristic that's objectively measured and evaluated as an indicator of a normal biological process, a pathological process, or pharmacological

response to a therapy or intervention. That's actually a pretty good definition from Wikipedia.

I think the traditional biomarkers we're all familiar with, blood pressure, heart rate, those are the things that if you don't -- if those things are not looking too good, you are in trouble. So those are -- I think we can all understand the importance of that. The more recent ones, like PSA for prostate cancer and various markers for it, to exposure to environmental toxins and so on and so forth.

Now, in our context, what should we look for? Now I'm going to go through this part a little slower because this -- the conceptual part of my talk.

We are looking for cellular markers that will tell us a story. And the first story we want, of course, is the positive story. Good news, we always want good news first. Are we getting there? How are we doing? Can we use it now? That type of thing.

So I've listed some of these here, and we can do some discussion later. The first group that I think will be very important are markers of the desired tissue, the end result. Now, we actually know a lot

about these things. If you are a musculoskeletal biologist, you know about bone. You know about cartilage. You know cardiovascular. You know about blood vessels and so on.

So you actually -- we already know the properties of that final tissue. So the first one you want to look at, of course, is whatever corresponds with the mature tissue function of the phenotype. In the case of cartilage, it would be collage type II. In the case of muscle, it would be myosin heavy chain and so forth and so on. So that's number one.

The second one is something that corresponds with the progression towards the desired phenotype. You're not there yet, but you're on your way. So in our case, in the case of cartilage, one of the earliest signs that something is happening along the chondrogenic pathway would be a transcription factor called Sox9. If we see that, usually we're pretty happy. Hey, very soon we're going to have cartilage.

The third type, the third example would be those that correspond to a phenotype that's resistant to pathology. Now, all tissues have a certain ability

to stay healthy. We suppress bad influences, bad environmental things, and bad circulating, nasty molecules.

So in the case of cartilage, for example, there are these things called tissue inhibitor,called metalloproteinase. What they do is they inhibit proteases that chew up your matrix. So usually, a good tissue, a good for us cartilage tissue, would have a reasonable amount of TIMP. So that would be something you also want to look for.

The next one would be a phenotype responsive to anabolic signals. It's almost like monitoring appetite. Instead of taking heart rate, you just watch how much food the kid is eating. If the kid is eating a lot of food, things are pretty okay. Anabolic response element, if we can it, that'd be nice.

Now, mechanical properties, of course, that'd most likely relate to structural tissues as opposed to functional tissues, except those also have a structure. And so I think one of the things we want to do is that if you want to say whether I'm getting there or not in the case of cartilage is that you do some compressive

material property testing. If it's got the right viscoelasticity, well, then you're probably getting there.

Then, of course, functional imaging is an area that is extremely powerful, lots of cool toys out there. So if you can use some way of using a functional imaging type of thing that use very specific contrast agents or parameters, if there are telltale, they'll be wonderful.

Now, other examples or other types of positive cellular biomarker, in addition to the final desired tissue type, I think we also sometimes want to make something new and improved; actually a tissue plus what we want, something that's even better than what you had in the old days.

So a novel tissue type will be kind of cool. So a novel tissue type will come with enhanced performance. So now, this is where creativity comes in and innovation. You say, well, I want to make a piece of cartilage. But I actually want it to have a little bit more of this type of extracellular matrix versus the other type because I think it will last longer.

Well, if that's the case, you should look for those things.

The third type are called the markers of therapeutically manageable tissue type. Sometimes what you want is you want to make a tissue type or a construct that has the ability to respond to therapeutic agents in a more enhanced manner. Therapeutic agents work, but it depends on receptors. It depends on enzyme pathways and so on and so forth.

Maybe you can build that in. Now, you can build it in either by non-gene dependent method or you can use gene based approaches, what have you. But at the end of the day, you would want to look for that.

And then the final positive marker that I want to talk about is markers of the response state of a host. Now, this is not totally in vitro, although you can certainly come up with composites in the laboratory that simulate some type of host/graft interaction.

So you can find -- remember, the host is not necessarily just sitting there doing nothing just receiving your graft. Ideally, you would want the host

to help out. So you would want a construct using cell and scaffold that actually has certain activity that would stimulate the host to respond. So if you can find out what that is, that will be a very nice marker as well.

All right. So those are the positive ones. Those are the good news. Now come the bad news. You also want to know the bad news as quickly as possible. I listed some of these, and again, no specifics here but I think just the general guideline.

Cell death is something you want to monitor for sure, lack of functional integration with the host tissue, either mechanical or signaling aspects, or the coordination of biological activities. Clearly, whatever you put in at the end of the day has to function in a coordinated manner together with the surrounding tissue and maybe even systemically with the host. And then a very, very important type of negative cellular biomarker is the harmful effect markers. And I listed just a few of them here. I'm sure there are others.

Inflammatory reaction, we worry about that

all the time. We can have the best construct, whatever we want. But if once we put it in, we have inflammatory reactions, proinflammatory cytokines come spewing out, chemokines and so forth, we are in deep trouble. So we want to find a way to measure those responses as quickly as possible.

Immune reaction. Now, there are two types of immune reaction. There are the quiet immune reaction, which are dependent on T cells, B cells and the recognition of epitopes and the production of antibodies and all that stuff. That's really easy to monitor. There are very sophisticated methodologies available.

In addition to that particular type of acquired immunity, which we're more familiar with, there's also innate immune response. And that's dependent on cellular activity, which is endocytosis of foreign particles by macrophages and monocytes and so forth.

That you also have to monitor, the moment you put that in. Are macrophages going to swarm and grab onto your construct and just chew it up in no time?

You've got to find out quickly, very quickly. You can do some type of chemotactic assay or what have you, but you need to know that.

Degradation of extracellular matrix. Sometimes the bad influences could be a very slow seeping into your tissue type of bad reaction. It could be that your construct for whatever reason is slowly but surely being stimulated after you put it into a native environment to produce enzymes that would degrade the surrounding tissue. That would be really bad. So you fix that hole, but you now make a bigger crater. So that's not what you want.

Then finally, metabolic reactions, will this construct for some reason stimulate undesirable metabolic reactions from the surrounding tissue? So those are the negative things.

So on top of all these things -- and, again, this was alluded to earlier -- there's also the scaffold performance issue. I've just listed three of these. You can go into more detail later.

Degradation profile, critical. If it's a biodegradable material, you want to time it so that its

degradation profile is in sync with the building up process that's inherent in that construct.

Toxicity as well as local acidosis. For example, those are critical issues as well related to the scaffold biomaterial.

All right. So I'm going to give some examples here. And,again, by no means, as I said, they're not comprehensive at all. Some of these may be relevant and some of them may not be as relevant to the topic you have in mind.

First of all, you can measure chemical markers, ions, oxygen, et cetera. Those are pretty easy to measure. If they can give you an indication of what you want, information you want to get out, go ahead and use those.

Metabolic markers, small metabolites, cellular energy stays with the cell. Imaging markers, MRI, Micro-CT, secreted bioactive molecules, such as hormones, peptide or steroid hormones, growth factors, signaling molecules, et cetera. Extracellular matrix, it could be the macromolecules themselves or subpeptide epitope of those molecules. You can have cellular

products. It could be proteins, nucleic acids, mRNA, what have you. And then physical biomarkers such as mechanical properties, I mentioned that earlier.

So let's say you want to measure these things. How are you going to do this? And again, other people will give you more concrete examples. I just thought of four different types of assay systems that certainly can be used as a platform to develop enabling technologies.

The first is -- will be the most desirable, is basically in situ analysis. You stick something in. On the other end, there's a number. It comes out and says good, bad, whatever it is. Yeah, you got a number. You got a readout. That would be really nice. If you have that, it would be short and sweet.

So ions and oxygen, obviously, you can do that because there are specific electrodes. There are also probes that have ligands already coded onto the tip of the probe so you can stick it in there and some type of indirect reaction. But nevertheless, you get a readout.

The second type of analysis is a little bit

more indirect. You basically have this in vitro construct sitting in a dish and you can sample the soup or sample a little piece of the material, whatever. And you can go assay for some type of activity or some type of chemical level of material.

You can also have -- another way of indirect assay is to have a sentinel of some sort just sitting in the dish. It could be a solid phase conjugated material just sitting on the side and it glows every time some particular molecule is produced because it recognizes certain things and so on. So it could be a cell, another cell, which is just a reporter cell. It will sit there. If the tissue type, the construct you're making, is going to produce something, it will again give you a readout.

Well, then you have other things, the sort of destructive analysis. This will involve completely rendering your construct into smithereens. So you can take that, extract that. And you can, of course, measure gene expression, profiles using RT-PCR, or micro array, or what have you, or you can measure proteins by a certain activity or proteomic approaches,

high throughput type of thing.

Then finally, you have a more physical type of analysis where, for example, integration of a particular construct to the host tissue is of interest to you. You can certainly do this in vitro. You can make a ring, make a hole in the middle, stick something in there, and then wait a certain period of time and use a piston to try to push that out. That will give you the mechanical characteristic of that interface, which in itself is also a biomarker.

So the requirements, therefore, for all these types of things -- I just came up with three of these. First of all, the assay must have specificity, reproducibility and accuracy. So sometimes you can have all of them in just -- by doing one assay. Sometimes you're going to have to combine a couple of them. So together you do five assays, the overlap is the stuff in the middle on that Venn diagram will be very specific. That's okay, too.

But at the end of the day these assays -- if you do, it must be specific -- have to be reproducible, and namely the technology is such that the variations

are not so big that you're always looking at noise. And it has to be very accurate. It goes without saying.

The second thing I think is important is that biomarkers must have interpretive value on the developmental and functional state of the engineered construct. Now, it will be very desirable to have that, meaning that the biomarker itself is not an itinerant standby type of associate but rather a functional player in that tissue developmental process. Ideally, that's what you want.

You actually want this marker at the end to actually tell you, yes, I have the tissue that I want. You must know that. So I think it's critical that preferably if you have a bunch of biomarkers that you're working with, work on the ones that can do that.

The third thing is that the biomarkers should preferably also provide information of the nature of host response to the engineered construct. Again, it goes a little bit beyond the in vitro setting of today's part of the workshop. But we must know what the host is doing when this material is introduced into

the organism.

So how am I doing on time?

DR. DURFOR: One minute, two minutes.

DR. TUAN: One or two minutes, okay.

So I am just going to go skip all of these things because I actually wanted to tell this story because I think it's important to have time to discuss things. Sorry about that. There we go.

Okay. So to conclude in the last minute, I have the following thoughts. And first of all I want to say it is really great to be doing work in this field because I think it's really one of the most exciting areas of biomedical research that encompasses both basic science and engineering technologies. So it's very cool, point number one.

Point number two, cell/scaffold constructs maintain under enabling conditions that are currently being used as a building blocks for tissue engineering and regeneration. So I think how to optimize these constructs really should be just one of the most desirable things to do.

Now, evaluation of the efficacies of these

cell/scaffold constructs must be based on the use of critical biomarkers that assess both the developmental and maturation characteristics of the target tissue. And then analysis of these biomarkers should provide information on, number one, improving development of the construct. The markers that we have should have sufficient information that will allow us the next time to do a better job.

Secondly, enhancing the functional integration of host tissues. And then finally, reducing potential unfavorable effects. And then finally, understanding the nature and regulation of cellular responses in cell/scaffold constructs should lead to their development as functional tissue analogues in vitro to first of all study the mechanism of pathogenesis; because it's very important that if we make something that really behaves like a piece of tissue, now what you have done is you have created a model, an analogue to investigate why you get the disease in the first place, pathogenesis analysis.

Secondly, the high throughput screening for potential therapeutic agents because now you have an

isolated tissue that can respond based on the parameters that you have set for biomarkers.

All right. So all of that said, I just want to challenge you to be a salamander. I think Bob Nermem talked about this before in one of his talks. And so here's regeneration in salamander. This guy is not funded by any federal agency, and he doesn't have a fiscal year budget to work with, but he can do everything. And I listed some of the time scale that a salamander uses. That's pretty amazing. This is -- and we're talking about complete regeneration.

So that's what we should aspire to, to be a salamander. And so I just want to say that it's a pleasure to be here and to share with you some of my thoughts on biomarkers. Thank you very much for your attention.

DR. DURFOR: Questions? Please step to the microphone.

UNKNOWN QUESTIONER: I guess the problem we have with regenerative medicine, what is our goal?

The analogy is you have a pothole. There's an absence of something and you put something back in.

So do you now compare it to a normal road that never had the defect? Do you compare it to a road that has a hole that now you've filled it with something?

And what's the important aspects? All of the biomarkers you talked about were biological activity related to structural. So is our goal structural repair or is it a clinical outcome that gives you a comparator? And if that comparator -- if that's what you're saying, what is that comparator to, and how does that relate to biomarkers?

DR. TUAN: Yeah, excellent point. I wish I had answers to all the questions that you raised.

So first thing first. So you mentioned what should we compare these parameters once we have them -- what is our control essentially? So in the case of a cartilage repair, for example, it will be extremely challenging, for example, for us to restore something to its pristine state. I think that is a goal that we all would like to achieve, but I don't think it's that easily doable.

Now, having said that, what are the scores? What are the sort of the clinical performance scores

that we use? Well, they are pretty well established: pain scores, performance scores, mobility scores and so on and so forth. And then some of them are based on radiological and imaging type of scores.

So we can say all, right, at the end of the day we end up with ICRS score from 4 or 5 to 1 to 2. That's not bad. And then pain subsides and the patient is able to perform certain functions. So I think at the end of the day it is clinical outcome that has to be the ultimate decider, so to speak, for whether we have achieved our goal.

The biomarkers, what do they do? Again, I tried to make this point. We probably should not use one single biomarker for anything. It's going to be a combination cocktail, a repertoire of biomarkers that one major function is to tell us whether we are moving in the right direction because we just don't know. We try different things. It's very important whether we've moving in the right direction.

Secondly, that they are correlated with some type of functional outcome at the end, in this case, mostly structural because it's what I do in our work.

And so the biomarkers are not themselves completely -- how should I say? They don't tell the whole story, but they are giving you the indication.

Now, I also pointed out that it would be very nice to make a tissue that is therapeutically responsive. There's nothing wrong with pharmacological or pharmaceutical type of intervention. There's nothing wrong with that.

The problem is sometimes our tissue is degenerated or damaged to such a state, diseased to such a state, it doesn't respond anymore. So if we can make sort of the next step, which is to make a tissue that is now pharmacologically responsive, that would be very nice. So you take one particular medication a day and you feel better, before you couldn't even get there. So I think there's different types of expectations, and we should be open to all of these.

Now, your second part is whether it is structural or functional. I don't know. I mean there are needs in both camps, obviously, and some of the tissues actually do both.

So I think for the functional tissues that do

not necessarily have, say, a mechanical requirement, a physical, mechanical requirement as, say, in cartilage, then maybe the challenge is -- I shouldn't say that they're less. It's a different type of challenge because then they have to be responsive to, say, metabolic signals and all those things. How do you tune in these other quote, unquote receptors? And that's the other thing.

So at any rate, I wish I had answers for your question, but those are critical points, clearly.

MS. SEAVER: Sally Seaver, Seaver Associates.

Wonderful talk. I really enjoyed the overview, especially at the end saying that we need to understand why the organism degenerated in the first place, or the organ.

DR. TUAN: Yes.

MS. SEAVER: I'm going back to a baby step here. One of the issues that you have when you transition from the research lab into the clinical lab is then going into the new language of the pharmaceutical industry. And you used three terms, specificity, reproducibility and accuracy.

And I would really -- and I can tell you that in ICH land where you -- which is harmonization of term land, those have slightly different meanings. But I really would like to know what your definition of accuracy is because it's not clear to me.

DR. TUAN: Yeah, I guess, you're 100 percent right. I'm looking at it from putting on glasses from the laboratory, obviously. Accuracy to me -- I mean so reproducibility and accuracy are not synonymous because you can reproducibly be getting wrong data.

MS. SEAVER: Right.

DR. TUAN: So I remember in my old chemistry labs, you would do elemental analysis. And if you would really screw up, you just -- every time you get the same wrong percentage of sodium in your unknown that you got.

So reproducible is important because, experimentally, there's got to be protocol that can be established. It's a standard operating protocol that if you do this, you will get this. So that's what I mean by reproducibility. Accuracy means that whatever you get actually is exactly what it is. So that's

my --

MS. SEAVER: So more like truth?

DR. TUAN: What's that?

MS. SEAVER: More like truth. It's a real --

DR. TUAN: Yeah, more like truth.

MS. SEAVER: Okay.

DR. TUAN: Yeah.

MS. SEAVER: All right. Just a quick comment. What you're calling reproducibility in the land of ICH or pharmaceutical development is called precision.

DR. TUAN: Precision, okay, yeah. No, I agree with you.

MS. SEAVER: And accuracy has a different thing, which I thought. And I agree, it's a reliable result.

DR. TUAN: Yeah, reproducibility is precision, is what I meant.

MS. SEAVER: Thank you.

DR. TUAN: Yeah.

MS. HUNSECKER: Thanks, Rocky. That was a really comprehensive overview and --

DR. TUAN: I tried.

MS. HUNSECKER: You may recognize that some of us will probably try to plagiarize liberally in times to come.

But I'd like to challenge you to go a little beyond what you've -- this very comprehensive approach, especially in light of the end -- where you ended this in terms of looking at a regenerative model.

I think although it's implicit in some of your explanations, I think it might be helpful to be even more explicit, to go beyond when we look at the host environment, to go beyond the factors that prevent us from integrating the construct or incorporating a construct, but also to look more at the regenerative, if you will, aspects, the remodeling aspects, because, especially in a lot of the constructs we're working on now, remodeling is one of those things we look for as an evidence of a healthy state of affairs.

So I was wondering if you'd like to comment on whether you see that as too far in the future to be talking about practically now or if we could incorporate that into your schema.

DR. TUAN: I'm really glad you pointed that -- I was looking at my slide last -- slides actually this morning and I was wondering whether I should include that because -- particularly after I stuck the salamander slide on there.

You're a hundred percent right. Ideally, what we want is not just assisted development. It is really assisted, then real development. And that has to come from the host in a regenerative fashion as opposed to a repair fashion.

However, the in-between, which is once you put a repaired tissue in there that fully integrates, there's no reason why they can't basically invite the host tissue to come in and participate. So at the end, that completely repaired or reconstructed tissue is a hybrid. Some of it is we put in, some of it because of what we put in, and the host responds in a favorable way.

So, yeah, it would be ideal. So that's what I meant when I said one of the markers -- it would be nice to have a cellular biomarker that actually measures the response of the host. It could be a

constructive response or integrative response.

So, yeah, it is somewhat in the future because right now we're just fixing potholes, at least we're focused on that. But it would be nice if the road actually grows back and just pushes into that hole, and then fills it up.

MS. HUNSECKER: Especially in terms of a conference like this, I think it would be helpful to think about those -- start to think about those aspects now because at some point we're going to have to say if we continue to monitor what happens when we put constructs in, where is the end point? And if we look at remodeling as one of the aspects, then that could be considered part of the monitoring in terms of timelines, and where we say, okay, we're done now. We don't have to have to be as rigorous in our monitoring.

DR. TUAN: Right. So that's why -- kind of what I was trying to include in this novel tissue type, that I'm saying that what we put in is not just a recreation of what we would like to replace, but it's actually a tissue type that has stimulatory activity, bioactivity.

And then you need a different type of cellular marker now, which probably would mean that the cells have to be genetically engineered somehow or altered in such a way that that effect will -- because normally that tissue doesn't repair. That's why we need to do the job. So I think we would end up with a novel tissue type.

Nadya.

MS. LUMELSKY: Thank you, Rocky. It was excellent.

I very much want to -- before I say what I want to say, I very much want to second what you and Rosemary were saying. It's the contribution of endogenous remodeling should be the -- we should kind of push into that direction. So we work from both ends.

But what I wanted to say is that -- it's kind of a common question. It seems to me that what -- it very much depends on the tissue you're working with, how good it should be -- referring to the previous question. In the case of cartilage, we have total joint replacement. So that's how we want to be better

with the tissues we produce because, as you said, they work well but they will need to be replaced. So the tissue constructs we're making, we need to know that long term they're going to be better than the joint replacement.

So now, how do we ask this question? How do we analyze our tissue construct so that we know that those things are going to be better?

In this context, I want to come back to your biomarkers. As you said, very rightly pointed out, it's not going to be one biomarker; it's going to be combination of biomarkers. And in the real life, probably what we're going to deal with is 20 biomarkers, and some of them are going to be excellent, and some of them are not going to be so good, and some of them are going to be mediocre.

So we're going to be dealing with a space of biomarkers with a very complicated arrangement of values in terms of what we are looking for. So it seems to me that we need to start devising strategies, and they're going to be some very specific strategies depending on the tissues; but to assign weights somehow

to make sense out of this space we're going to get from the analysis of the biomarkers.

So I wanted to hear if you can comment on that and how -- what kind of strategies we can use.

DR. TUAN: Yeah. So one way to deal with it is to borrow a page, obviously, from the developmental biologists. So what they usually do is they say I want to make, say, cartilage. So they cut cartilage development into five stages. Each stage they do a massive profiling of everything, and then they pick their most favorite one. They say, well, let's knock this one out and see if the animal makes cartilage. If you do this, it doesn't make cartilage, okay. Good, you need that and so on and so forth.

So we don't have to do this. I mean they've already done it. So we can borrow what they have, their score sheet, and say, well, the top three in this category, this category, this category would be this.

Now, the trouble there is that they're dealing with an animal who's already on the path, doing cartilage. We are recreating this. So the players, even assuming they are the same players, they may not

show up at the same time in the same dosage.

So I think we have to be very careful. Yes, we borrow a page from developmental biology, but because what we're doing is kind of weird, it's not developmental biology. So we have to be very careful. So we cannot -- like you said, a combination approach has got to be the way to do it. But as a starter, I think that's what we ought to do.

Now, the other thing that we also work backwards is that we look at a diseased tissue and we say what it's missing. So they don't have that in developmental biology because they're just going one way.

But on the other hand, if we take a disease model -- particularly when you look at early disease stages, which is the difficult part, obviously, because usually you don't hear about it until the person is ill. If you have that model and that's actually where tissue engineering or cell/scaffold constructs would be very helpful, we can make in vitro models and we induce the disease.

Then we can actually find out what are the

things that are changing. So then we can say, well, we want these not to be there because negative markers are just as good as positive markers anyways. So I think that's what I would try to do.

And then all of this is dependent on -- the question was asked earlier about precision, accuracy and so on. It's really dependent -- a lot of it is in the technology. If we are wishy-washy in our data because it's fuzzy, then we'll never find out. So I think technology -- enabling technologies must be there to make this happen. So that's how I would go about it.

MS. PLESHKO: Nancy Pleshko, Exponent.

Rocky, that was a great talk, and I was really happy to see biomarkers put in a much broader context than just cellular, so that's really important.

I had a comment, actually, regarding the first question about the comparators and whether or not the clinical outcome is ultimately what we should be looking at. And I think it's important to remember that there are a lot of different pathways to possibly get to the same clinical outcome.

So even though the patient might end up with the same pain score or whatever, and it looks great with their ACI or knee replacement, that going back and kind of looking along the way at the characteristics and everything that we're talking about today is always still going to be important, sort of following the trail of how we got there.

And so not just okay, they look great clinically, but kind of what are the characteristics that got them there and how, as we're talking about, reproducible is it, et cetera. So it's just sort of an important thing --

DR. TUAN: Yeah, you're right. I mean, the clinical outcome, to some extent at the end, is the final arbitrator of whether something is going to go into a patient. But we need to find out what are the components that contribute to the clinical score, and that's science. That's biology. That's engineering, et cetera, et cetera.

So I think all those pieces have to be there. If we know which one contributes to that clinical outcome, I think we have a better way of controlling

what the clinical outcome is.

MS. PLESHKO: I agree.

DR. BERTRAM: Dr. Durfor, one other question, if I might.

DR. DURFOR: Yes.

DR. BERTRAM: Let me add my accolades to all the others, a fantastic and enjoyable presentation.

DR. TUAN: Thank you.

DR. BERTRAM: One question I've got, I liked your breakout of conductive, inductive and productive. It's a nice way of thinking about it.

I'd like to ask this question within the context of the inductive aspect of your thinking; specifically, since most of us who are involved in developing products want that final clinical outcome to be adequate to show that the patient has actually received something of benefit.

One of the questions, though, all of us depend on that inductive environment to actually give that clinical outcome. Clearly, we know there are differences in the patient's ability to heal.

Do you or have you considered any potential

biomarkers where we could actually evaluate before the patient is considered for this type of therapeutic approach? Biomarkers that could be done in vitro, easy sample, that would give us signals that this patient is a good patient to pick and this patient may not be as good.

DR. TUAN: Yeah, outstanding question. I just came back from a periprosthetic osteolysis workshop that the American Academy of Orthopedic Surgeons put together. Again, it was mentioned that this is a very successful procedure, obviously. But there are people who, for unknown reason -- their implants will fail prematurely. So there's been all kinds of speculation as to why, so the compatibility, the sort of personalized medicine type of thing.

One of the things that people talk about, of course, is metal allergy; is it possible that that can be a contributing factor because ions do come out and so on and so forth.

So let's just say that's a contributing factor. We don't know how important it is, but let's say it is. Then the genetics should be a criterion.

So there are multi-center studies going on right now that, again, that are sort of based on outcome because you won't find out until the implant actually fails to see if metal allergy, for example, can be a contributing factor to premature osteolysis and implant failure.

So along that same direction, just based on what you just said, let's say we made something that we think is going to work really nicely, but there may be an incompatibility. The incompatibility may be based on some genetic polymorphism, just as to pharmaceuticals, the same thing. We may be able to find that out.

So I think, yeah, that's an excellent point. It would be a biomarker that's not of the scaffold but more so on the person, the host, the potential host, and what the interaction will be. So I take that. That was a great suggestion.

DR. DURFOR: Thank you all. We are scheduled for a break now from 10 after 10, which is in a minute, until 10:25. At this point, we will reconvene. So please be here at 10:25. Thank you.

(Whereupon, a recess was taken.)

DR. DURFOR: Thank you. It's my great pleasure to introduce Dr. Ratner. Dr. Buddy Ratner is the Michael and Myrna Darland endowed chair of bioengineering and a professor of chemical engineering at the University of Washington. He's also the director of the University of Washington Engineering Biomaterials Research Center.

Dr. Ratner received his PhD in 1972 in polymer chemistry from Polytechnic Institute of Brooklyn. From 1985 to 1996, he directed the National ESCA and Surface Analysis Center for Biomedical Problems. In 1996, he assumed the directorship of the University of Washington Engineering Biomaterials Center, which is an NSF engineering research center.

Dr. Ratner is also the editor of the *Journal of Undergraduate Research in Bioengineering*, a past president of the Society of Biomaterials, and an author of well over 400 publications and numerous texts, one of which is "Biomaterials Science: An Introduction to Materials in Medicine."

Dr. Ratner is a fellow of the American

Institute of Biomedical and Biological Engineering, the American Vacuum Society, and a fellow of the Biomaterials Science and Engineering.

He served from 2002 to 2003 as the president of the American Institute for Medical and Biological Engineering and vice president of the Tissue Engineering Society International from 2003 to 2005.

His current research includes biomaterials, tissue engineering, polymers, biocompatibility, surface analysis of organic materials, self assembly, nanobiotechnology, and RF plasma thin film deposition.

I can think of no better person that to ask and to hear from today about characterization of biomaterials than Dr. Ratner. Thank you.

DR. RATNER: Good morning, and thank you, Charles, for the opportunity to I guess give one of the lead-off talks anyway in this symposium.

Rocky and I were both contemplating exactly how one would structure such a talk. The topic is gigantically broad. And I think his approach was great, and it actually I think meshes pretty well with what I'm going to do.

So let's see. It's a workshop, in vitro analyses of cell/scaffold products. And I was asked to overview characterization of biomaterials, and it seems scaffolds are made of biomaterials. We have porous materials, gels, and decellularized tissue, which is used as a scaffold.

I tried to think from a biomaterials sense how we characterize materials and how we would bring that to scaffolds, and it came out to be a pretty awesome size list of mechanical and thermal properties, morphological characteristics, chemical characteristics, stability, biological characteristics. And all these are in vitro, so there are many possibilities for the scaffold characterization.

I think two general points that this talk has to build on -- one thing is that multi-parameter characterization will be essential. No one method is going to allow you to understand your scaffold, and not all methods will be relevant in every case. There's a lot of individuality depending on the nature of the tissue engineering and the type of scaffold. So we'll see those things come along.

Here's some more general things. I have the title, Originally Core Issues in Scaffold Characterization. I decided again fitting in the theme to mesh with Rocky, and I got stuck on the word "physical," physical scaffold characterization.

So a couple of thinkings here. For one thing, in mechanics, I think in a scaffold we want to match the mechanical properties to the tissue. Do we have a flexing environment? Do we have a relatively static environment? And think about the different mechanics of bone, cartilage, skin, heart, liver or brain as an example, with an essentially decreasing modulus as you go down that list.

We also have to worry about things like pore size, pore geometry, pore distribution. And closely related to that is interconnectivity, of course, and the percent of void space.

And then we also want to worry about cell interactions, but that's maybe more biological. But that really does go down to surface properties. Somehow the cell or the biological milieu is putting down proteins on the surface, or we're putting down

proteins in advance on a surface that will stimulate or trigger cell attachment, proliferation, differentiation, these sort of processes. So surface properties could be key there.

We might want to do controlled release of active molecules, very commonly done actually and probably one of the important themes.

Then there are the other physical areas concerned with the biodegradation, the ultimate departure of our scaffolds. And here we have issues of rate, of degradation, of mechanics versus time. So, yes, we're interested in mechanics of scaffolds, but as we degrade, the mechanics also degrade in property. And, of course, in degradation we worry about the cytocompatibility of what comes out of the degradation process.

Then contamination issues are kind of a, let's say, central concern probably to FDA regulatory thinking and also to the ultimate success and reproducibility, or maybe I'll say precision of scaffolds.

So let me go down that kind of list here.

Again, I have these different things in blue here. I'm just going to take each of these chunks and kind of blow them up and talk about them a little.

So we start with mechanical properties, and there's a bunch of standard things. And, in fact, I noticed many of the talks that will follow me will do a much better job on this. But let me make just a few comments because I think it's an important part of the overall characterization of any biomaterial and particularly scaffolds.

So we can talk about a stress-strain behavior from which we get a parameter often called a modulus. We can also talk about a strain to failure. How much can we stretch the thing before it breaks? Flex fatigue testing over time, again, anything in a flexing environment you have to know how many times you can flex it before it's going to fail.

Then there's a whole range of viscoelastic or dynamic mechanical properties. And again, the issues kind of in purple on this slide are modulus matching, the decay of properties during degradation, ultimate strength and toughness.

And then for tubular and hollow structures, adverse pressure becomes important. So I have a picture of a testing apparatus. I have no stock or financial interest in any testing company, but a few companies are making test instruments that have particular focus for biomaterials and soft tissue. And they've been actually very helpful because rather than have to fabricate everything from scratch, one can get a commercial instrument these days that is in the correct parameter space to analyze many of the types of tissues we have.

So one of these companies, for example, showed a very nice plot that talks to -- again, so just a very basic type of data we might get, the stress-strain curve. So one puts various stresses on it and sees how far it stretches. And in this particular curve, the data set was evaluating acellular dermal matrices that potentially are useful in tissue engineering after four weeks implantation.

Of course, what you'd look -- it's a little hard to see it here, but these points here represent the natural or the undegraded natural tissue, native of

the abdominal wall. And what you try and do is emulate that in these types of tissues for this type of replacement. So it gives an idea of how one can get some data that takes you into the right mechanical zone of these tissues.

What I'm going to do throughout the talk -- that was again a data set from somebody else. But I can talk, I think, more reliably about data that comes out of our own group. And I'm going to really focus now on two types of data, one coming from decellularized natural tissues and another from a type of scaffold called sphere-templated scaffold that we're making a lot of. And not so much then present these two approaches, the tissue engineering to you, but I'm going to give a number of examples on how we've taken characterization methods and brought it to these types of systems.

So let me tell you about this sphere-templated material. We've been having some very good luck with this. And the way these things work, sort of described in this little slide, is we start with some microspheres and we sieve them to get a

uniform size fraction or size cut. And then we shake them so they compact down into almost a 2D or three-dimensional close-packed crystal, sort of close-packed billiard balls, if you will.

And then we gently heat these microspheres so the edges of the beads fuse or center together. It gives us a sintered cake. And then we surround them with a liquid, a monomer in this case. And then we polymerize the monomer, solidify around the beads. And finally, we use a solvent to solubilize out the beads, which leaves us with an interconnected system where every pore space is exactly the same size.

The reason why we're so excited about this is one biological data set that I'm not going to elaborate on. But I want to tell you why we're focusing on this class of materials. What we've found, if we implant these in many different tissue spaces -- I'll tell you the list of them in a little while -- is if the spherical pore size is correct -- and we found to be 35 microns to be about right -- one gets hugely enhanced angiogenesis in the implant site.

Many tissue engineering scaffolds, for

example, have pore sizes up around 160 microns, pretty common area, which has almost no spontaneous induction of angiogenesis. And if you look at the zero pore size, the solid slab of material, the control -- I don't have it on here, but it looks almost exactly like the 160 micron.

So what we've seen -- and again, I'm not going to elaborate on this. I'm just trying to show you why we're so interested in these scaffolds -- is if you get the right pore size, you get sort of a vascularized integrated healing, or if the pore size is lower or higher, one gets much more fibrotic avascular-type healing. So we're sort of focusing, obviously, on that key pore size.

So, again, one of the types of characterizations -- I'll go a little bit more into this a little bit later -- involves just a simple visualization, which could be very nice. We do a type of visualization called a digital volumetric imaging, which literally involves a section or cutting material and reconstructing the slices taken off the microtome into a 3D image.

So you see up there an image of this scaffold and also scanning electron microscopy. Now, the particular scaffold that we're showing here, I talk about the microspheres with a polymer and polymerizing.

Well, the polymer we used in this case was a fibrin glue and we added thrombin and polymerized it. And you can see the very uniform pore structure we get in this. And, in fact, if we take the microscopy and look even higher, you can see fibrin strands that line each of these little pore spaces.

So, again, using basic mechanical tests and tools to start characterizing these materials. For example, as we make these, we look at the Young's modulus, the slope of stress, the initial portion of the stress-strain curve as a function of the amount of fibrinogen concentration that we're putting in. Well, we find if we increase the fibrinogen concentration, we can start to get better mechanics to our system.

But we've also been using an interesting crosslinking agent called Genipin. Genipin is derived from an Asian gardenia plant. In fact, it's sold in Asian medicinal shops. But it has an interesting

crosslinking reaction on tissues, and it's very nontoxic.

So, again, we take these types of constructs made of, for example, natural materials, fibrin or -- in fact, we do this also with decellularized natural tissue and crosslink with Genipin. And, again, we can measure this increase in Young's modulus as we increase the concentration of Genipin and get some idea of the strength of our materials.

So that's just a little bit. We'll hear a lot more about mechanics, particularly the dynamic mechanical methods; give you a lot more information about materials.

Let's go on to morphological characteristics, though. Again, these are pretty straightforward. Almost everybody does some light microscopy of SEM. And actually, one can gain a tremendous amount of information by just looking at materials. So there's a whole range of different types of scaffolds that are shown in this illustration. And again, one gets a very visual feeling for what the scaffolds look like thanks to the SEM methodology.

But you can do a lot more quantitative things with it, too. Back in the 19th century, Henry Darcy, he was a French hydrologist, and he was wondering how one understands the percolation of water through soils, and developed a law we call Darcy's law, a very actually pretty simple equation which helps us understand this sort of percolation.

For example, we have taken the morphometric information, the pictures, if you will, of these scaffolds, analyzed or did an image analysis of the morphology. In fact, there's a paper that came out in *Physical Review B* in 1986 that talks about how to take these images and convert them into an understanding of the structural spaces. Combine that with Darcy's law and about five solid pages of mathematics later; we're able to come up -- in the references there, if anybody wants it, to plow through that.

But we're able to come up with a bunch of parameters that allowed us to calculate, based on permeability and image analysis data, critical throat size, interconnects between these cells, hydrologic permeability, the tortuosity, where if liquid is moving

straight through, it would be 1, 1.2, shows some tortuosity and the total porosity.

So we're able to do a pretty complete characterization of the interconnectivity in a quantitative way of our materials. So taking the pretty picture, if you will, the picture that gives this intuitive feeling for the material and then going to a quantitative description.

So that's some of the things you can do with morphological characteristics. I've spent most of my career working on chemical characteristics and in particular, understanding surface and bulk characteristics. And, again, we'll elaborate on some of these.

The surface methods provide information relevant to biological interactions and to contamination issues. And the bulk methods are critical for complete characterization.

I don't want to put down anybody that's focusing on bulk materials, but there's been such a large history in the chemistry community, the chemistry world, on the chemical characterization of materials,

that largely it's been reduced to a pretty good practice.

And one could use infrared, NMR, size exclusion chromatography, thermal analysis to get a very good bulk characterization of materials. So I'm not going to elaborate on those. I'm going to talk more about surface characterization.

So I often say we have a basic repertoire of surface analysis tools we can use: the technique called ESCA or XPS, SIMS and RMS spectrometry; the atomic force microscopy that gives both surface information but also gives sort of a morphological-type information; contact angle method and infrared. This is our tool chest that we can use.

The reason why it's nice to have these tools is they each look a different depth into the material. So some of the methods, simple contact angles -- for example, the AFM, static SIMS look on the very most, outermost 5 to 10 angstroms. Other techniques, such as ESCA or Auger spectroscopy, are going to go down 100 angstroms into a material. And then techniques like attenuated total reflected, it's infrared. Pretty easy

to do on just a laboratory infrared spectrometer, but that technique penetrates probably well into the bedrock of the city of Washington by the time you get finished on this diagram. But still surface analysis, still a few microns deep.

So let's see. Let me just talk about the ESCA technique and show you some things we can do with scaffolds. There are two acronyms that are used for this technique. They're both exactly the same. It's created no end of confusion. But one is called ESCA. The other is X-ray photoelectron spectroscopy.

The gear that does it, it often runs about $1 million a machine, although there are some lower-cost machines. And the NIH through -- actually, these days, through -- the NIBIB Center has been funding a national resource, the NSEG BIO, that allows investigators to come in from all over the country -- in fact, all over the world -- and use this type of equipment, which is optimized for biological work.

So what do you get from this ESCA method? Well, it analyzes, as I said, the outermost, maybe 50 to 100 angstroms. And, interestingly, the first thing

you get is all the elements present, except hydrogen and helium. And that's pretty good right away. There's not a lot of analytical techniques that will tell you every element that's in the surface of the material. The amounts of each element, it is quantitatively, plus or minus 10 percent under average conditions, and with a little care, you can get easily plus or minus 1 percent.

But as well as telling the elements, we also get something called the molecular environment. So as well as telling if you have carbon present, we can tell if we have carbon as a hydrocarbon or as a hydroxyl, or carbon bound in a carbonyl, or carbon bound to fluorine. These are pretty distinctive sort of things.

We can do non-destructive depth profiles through that outermost 100 angstroms, what's on the outermost outer surface and work our way down. There's some other kinds of information there I won't be going down to. But to go down to No. 7, elemental imaging. One can do a spatial map of the surface with maybe 10 micron resolution.

The other technique I'm going to show for

scaffolds is this technique called secondary ion mass spectrometry, and anybody that's done mass spectrometry understands the data that comes off. It's a mass spectrum. But we're taking the molecules in the spectrum just right off the surface.

Again, the machines, the hardware -- getting up a million dollars a machine. And, again, the NSEG BIO center, for example, NIH resource makes this type of instrumentation available to biomedical researchers, although many universities have these, too.

What do we get from the SIMS technique? Well, we get a very high mass resolution, get mass fragments. And since we get an exact mass to many, many decimal points, we like can tell very precisely what those mass fragments are. It has an exquisitely high analytical sensitivity. You will learn things about your material you probably never wanted to know when you do this analysis.

It has a very high spatial resolution. We can do maps at least a tenth of a micron, X, Y resolution. People are talking about doing 40 nanometer maps, but a tenth of a micron is routine for

mapping spatial distributions. It has a very shallow sampling depth. We're looking right at the outermost surface, which might be what cells or proteins see. And we can do a -- using some recent techniques, we can start doing some depth profiling through the materials.

Then finally, the contact angle technique. I call it the five-dollar surface analysis method. With those million-dollar machines, you need basically a protractor and a magnifying glass to do contact angle work. And it can be performed in any lab, and it's very surface sensitive. But there are, in fact, many artifacts that can come up in getting the data. The data's hard to interpret. And with scaffolds, I think it's minimally useful because of the porous nature of the scaffolds.

So let's talk about the application of these methods to scaffolds. Again, let's go back to the sphere-templated materials. Here's another image of one. This one is made out of the polyHEMA hydrogel, the material of soft contact lenses. And these particular hydrogel scaffolds have shown excellent healing in subcutaneous sites. They heal

percutaneously with the restoration of the epidermis and the dermis. They heal in heart muscle, which is very subject to scarring. We've implanted them in the vaginal wall. We've had wonderful integration, but the material is not biodegradable. And so for a tissue engineering scaffold, it has that limitation.

One of our major interests is the tissue engineering of cardiac muscle. This is funded through a bioengineering research partnership through NHLBI. And we're using these porous materials -- in fact, the HEMA porous materials. And we make them into rods of materials that are cultured with cardiomyocyte cells that are derived from the George W. Bush H7 human embryonic stem cell line. So I don't get arrested here talking about this.

But anyway, we -- my colleague Chuck Murray has developed a proliferating line of human cardiomyocytes that were derived from these embryonic stem cells, and we culture them in rods of these sphere-templated materials.

Then these are -- the intent, in fact, what we're doing on an animal scale now is injecting these

directly into the cell wall. The gray area here is supposed to represent a myocardial infarction. We're trying to inject rods of cardiac muscle, regenerating cardiac muscle directly into these healing zones with a heart.

So what we do need, though -- the reason why I showed that, what we need is a biodegradable form of our material. And one of my PhD students, Sarah Atzet, has made some pretty complicated chemistry here. But she's made a polycaprolactone, which she calls a macro initiator. And here's the polycaprolactone section. These bromo groups, isobutyryl bromide, at the ends that allow us to grow the polyHEMA in very controlled molecular weight segments. This is catalyzed by copper. It's a reaction called the ATRP reaction. So what Sarah's making are these materials that have polycaprolactone crosslinks and also have polycaprolactone segments.

The interesting thing about these is the only HEMA blocks break down to 5 kilodalton, and they're totally water soluble, and they're very easily cleared by the body. And so we can get this precise control of

this material.

Well, we have this new material and we started characterizing it. So, for example, we can take the ESCA method and do an analysis of this polycaprolactone, or materials that are crosslinked with the polycaprolactone, and see only peaks for carbon and oxygen. That's nice. That's what we were hoping to see. We didn't see any bromine, no copper, which would be the residual products of the ATRP chemistry.

If we adsorb gelatin into this material as an attachment factor to help cells attach, you can see the nitrogen peak that appeared. So we can get very good quantification of the amount of a cell attachment factor on those materials.

We also made a very high crosslinked material. And lo and behold, we got something that kind of surprised us; we saw silicon peaks. And this is silicon contamination. It's tremendously ubiquitous.

And the good thing about ESCA is it's told us it's there. We can go back, clean up our synthesis,

and ensure we have no silicon. And there's no reason why we'd ever want any silicon in this particular construct. So, again, the ESCA's helped us out quite a bit already.

Another thing we're trying to do with this construct is covalently immobilize molecules to it. So we're adding the methacrylic acid. We're adding carboxylic acids to this material. And let's see, the carboxylic acids as shown here, and then using a chemistry, which is a carbodiimide chemistry and N-hydroxysuccinimide, to put on a very common leaving group that allows ready mobilization of molecules.

Again, these surface analysis methods have been very good for characterizing these sort of things. We can go from the classic spectrum shown in Curve A of the polyHEMA material, a very clear shape, and as we start reacting -- then you're reacting with the carbodiimide -- we can see the disappearance of the carbon EDC. We can see the amide bond forming from that reaction and characterize just the whole extent of the reaction.

In fact, here's just the nitrogen signal.

And you can watch just the nitrogen signal just increase as we activate with this carbodiimide activation. So it becomes a very good monitoring tool for, again, getting the control and reproducibility that we'd like to see in these systems.

Then we went to the SIMS systems. Now, this is a series of SIMS spectra done on a variety of different -- the sphere-templated materials, actually, some are solid slabs. Some are non-biodegradable. Some are biodegradable. The reason why I show it, of course, they're mass spectrum. We have a mass scale on the bottom. But they're awesomely a complicated spectrum, almost overwhelming in the amount of information that's contained in a spectra that's like this.

So we've turned to a analytic mode to help us digest that tremendous amount of data. And, in fact, the SIMS spectra of data we put into Excel spreadsheet, it's really boring tables of numbers. But what we really don't want, we don't really want data. We want information.

So we're using processes to extract

information from data. And there's a whole set of methods called multivariate analysis, sometimes called chemometrics. And they allow us to identify trends that might be hidden in huge datasets. They make use of large amounts of data. Sometimes we say let's plot X against Y because we can't handle all the data.

Imagine having a graduate student plotting every one of those peaks against another variable. The graduate student would take five years and then go on the next set of plots. It's a hard way to get a PhD.

So this just allows us to throw all the data into the computer. We use all the data, not just that which we think is important. It helps us generate hypotheses and trends.

What's going on there in the simplest way is that we look at X, Y plot with a whole bunch of points. You look at that. You see no trend in that set of points. But if we can take that dataset with some more information and look at in three dimensions, had an X, Y and Z, and flip it into the third dimension, you'd see there's a huge correlation in another dimension.

But what the computer does is do this

correlation through multidimensions. The computer really doesn't care about 60-dimensional space or anything. It can clearly do a 60-dimensional plot. We get the three dimensions.

But the computer does that easily, and it gives us trends through space of hugely complicated datasets. That's what's happening. And, again, there's a whole bunch of methods. We don't have time to go into the -- it's linear algebra methodology that's used frequently to analyze these. There's a whole bunch of acronyms.

We use the principal component analysis to look at groupings or clusterings. And we use partial least squares to look at trends. But there's a whole bunch of these methods that are available.

So using the principal component analysis, for example, we can easily distinguish on our matrices we have by these clusters of points. This has taken all the SIMS data and asking us what's different between these spectra.

It tells us that the HEMA plus protein is here. If we add the N-hydroxysuccinimide to the

surface, it's here, and if we add the protein, it's here. So we can get very distinct groupings of materials using this sort of method.

We've taken this even further and done a very thorough analysis using many of the different peaks, polycaprolactone, HEMA peaks, copper bromine peaks. In fact, all those SIMS spectra, here's this sort of list of different samples studies.

We're putting all this data into the computer, and what we're finding is we can separate out and get very unique clusterings. Now, these clusterings are called scores. So we see this cluster of peaks, and it says there's a trend. There's a similarity in these.

We can take any one of these clusters and do what's called a loading plot and ask which SIMS peaks are contributing to it. This is not a hidden miracle. It's simple mathematics. And we can get these loadings, which ones contribute to making it. So we can backtrack and find out why they're similar and why they're different. It's a tremendously rich source of information.

So there are some conclusions that are coming out, the polycaprolactone-containing scaffolds, we're just starting this analysis work now. But interestingly, we see residual copper only in the non-biodegradable samples. In our biodegradable ones, we can't find any copper, even with the huge sensitivity of the SIMS method. We do see some very low levels of bromine. They're below the detection of ESCA, but the SIMS is very sensitive. We can optimize to try and get rid of that.

And also, it turns out the surface of our gels, the surface of these spherical structures, are rather rich in polycaprolactone, where the HEMA component or the hydrogel component is more under the surface.

Another thing we can do with the SIMS method is we can do a spatial imaging of the surface. At the bottom here, we have a secondary ion image. This is the sort of image you get from a scanning electron microscope.

But what we've done is taken this image and focused on different mass fragments. On sodium, we see

no sodium here. If we look at C_3, a propyl type group, you can see distribution of a lot of hydrocarbon type features.

We can focus even finer on these features. But we can get an image of how these things are distributed along the surface and what's there on the surface with actually very high spatial resolution. It's a very good characterization method.

Well, finally, one of the other methods in the physical characterization of materials is we're working on degradable scaffolds here and do they really degrade.

So, again, my PhD student Sarah Atzet is working on this; measured degradation in three ways. She looked at a swelling ratio, a tensile modulus and a mass loss. And for these polycaprolactone systems, the nondegradable material, here's a swelling ratio as a function of time under various conditions, including in the presence of a lipase, an esterase. And you can see the nondegradable one; the swelling ratio doesn't change over 100 days. But if we add various concentrations of lipase, particularly the highest

concentration, we start to break it down. So esterase that's in the body probably are having some effect on the polycaprolactone.

You can look at the tensile modulus. And, again, the tensile modulus degrades with the presence of lipase. The materials become weaker where the nondegradable one does not degrade in the presence of lipase.

We can also look at mass loss and find that the highest mass loss corresponds to the highest lipase concentration. So, again, we can characterize it, understand in a very quantitative, thorough way the degradation properties.

One last question is what's coming off. Is it toxic? And here we're using a colorimetric assay for cell proliferation. So the more color, the more lively the cells are, if you will. And the latex control pretty well wipes out the cells.

But the degradation products after degrading -- actually, we can rapidly degrade these in KOH. So we degrade in KOH, neutralize and look at the toxicity of the total degradation products for these

things and find that they're basically nontoxic. So we can get a pretty thorough characterization there.

Let me just finish up with two or three comments on decellularized tissues. Again, these make very interesting scaffolds because they have a very great potential to stimulate cell, particularly differentiation processes.

And Professor Steve Batalac has noted that the different types of decellularized scaffolds seem to interact with unique types of cells. For example, this is a decellularized esophageal scaffold, so no cells but the extracellular matrix of the scaffold. And this one will simulate esophageal cells. And he also did this with bladder and small intestine and looked at this.

But using the ToF SIMS scores, we can see these clusters; the ToF SIMS easily distinguishes the difference between the three different types of scaffolds I showed in the SEM images of the scaffolds.

So what we can do now is go back and look at what is at the surface. We can take these loading spots, look at what it is giving the spectromatic

difference and attempt to relate that to how they're interacting with the cells. And here's one of these loading plots. Again, you can see the distinctive peaks that correlate positively and negatively with our cell parameters.

So let me finish up. We have, I think, an impressive tool chest to bring to bear on scaffold characterization. We can distinguish different scaffold types, observe degradation, measure contamination.

And I think ultimately we're going to have to have the harder question of what do we really need for optimal tissue engineering once we find good scaffolds that really do reconstruct tissue in much the way that Rocky told us about. I think then we can go back and use these surface analytical tools to understand them better, to ensure we're getting reproducibility that we need for real tissue engineered products.

With that, let me again acknowledge my funders and the investigators, researchers and students who did a lot of the work. And thank you very much for your attention.

DR. DURFOR: Questions?

MR. McNAMEE: Thanks very much.

As you were talking about the different methodologies for identifying products, what sort of characteristics of the monomers that are used to make the polymers could be -- would there need to be to use them as biomarkers during the degradation process so that you could follow in vivo as these matrices degrade?

What would you have to engineer into the polymer science in order to make it easier for that biomarker identification?

DR. RATNER: Well, it's an interesting point to think about engineering handles right into the scaffold to watch the degradation events. So one would want to see what effect the engineered handle has on the ultimate biology, which is really our goal. But one could think about doing that.

In the meantime, we do have very good analytical techniques. Again, I didn't even talk about the techniques for measuring the molecular weights and things like that as we watch these things degrade. We

have just such a great set of analytical tools that allow us to precisely identify the products that came out of these scaffolds that the handles could make it a sort of pseudo-biomarker, if you will. It could make it easier to do.

But I think we have other methods in place. For example, the polycaprolactone would break down into caproic acid. And if I wanted to directly measure caproic acid formation, I have lots of methods to do that.

DR. DURFOR: If I could, before you ask your question, would each questioner please take the time to introduce themselves? Thank you.

MS. CATALANO: Hi, I'm Jennifer Catalano from the Center for Biologics.

I was very impressed by your principal component analysis data. It looks wonderful, even in the first two principal components that you're getting such nice clusters. And I use microarray data, and so we have the same type of analysis that we do.

And I think in terms of regulatory -- in terms of the regulation and I think of in terms of

specifications, setting ranges where you would set passes or failures for your product that's going to go through.

And so I'm wondering what your thoughts are on using principal component analysis to set specifications and whether that would be relatively difficult or easy, mathematically speaking.

DR. RATNER: Yeah, very good questions. One of the joys of publishing refereed journals is dealing with the referees, and they've asked us about the statistical significance also of these sort of works.

So we've actually developed a protocol for putting sort of the pseudo-area error bar surrounding each of these clusters, how reliable, how meaningful are they in a statistical sense. And so now we can tell. With a 95 percent confidence, this is a unique identification of a unique species, and I'm sure we published that. We can get you the reference to that.

But the other thing about -- I'm glad you noted how nice our clusters are. I don't want to say we cheat. But one of the optimization procedures we do is first we put all the data into it and we get some

clusters that don't look as nice.

But you can sort of see what clustering trends are happening. It tells us which peaks are more important, and then we focus our energies on just those peaks. And then we can get these very, very fine clusters. So it becomes a good method to optimize the PCA.

MS. PLESHKO: Nancy Pleshko, Exponent. I have one comment and one question.

The comment is regarding the infrared spectroscopy for chemical analysis. We do quite a bit of infrared spectroscopic imaging. And so with that technique you get very high resolution characterization of the surface or of the full-depth histological section, as I know that you're aware of.

And I think it's used not infrequently, and it could be a really good technique for looking at things like biomaterial degradation and interaction of a biomaterial with the host tissue. So that's just the comment.

DR. RATHER: Yeah, it was on my list. I just couldn't address everything. But in my own lab, we

also do a tremendous amount of infrared spectroscopy. So I am totally committed to doing it, and it's a very useful technique. But since it was developed about 1900, there's also a very strong base for interpretation. So it's not so new to people.

MS. PLESHKO: And the question is, with either the ESCA or the ToF SIMS, can you get actual maps of biological crosslinks? That would be very, very useful. And it's a question that we also get asked a lot, and it's of great interest in biomaterials and studying aging tissues like cartilage.

DR. RATNER: Well, one of the things we've been doing is applying the principal component analysis. And this is work being done by Professor Bonnie Tyler, who's applying the principal component analysis to images; not just to spectra but to images.

And what we've been able to do, for example, with the ToF SIMS, is look at distributions of proteins on surfaces, not just that we have this protein but how they're distributed on a surface. And so if there are crosslinks that have distinctive features, you might be

able to see them if you have the right resolution.

DR. DURFOR: I have a question, if I might.

DR. RATNER: Sure.

DR. DURFOR: In everyone's highly desirable world, you'd be able to take these scaffold analyses methods and apply them to scaffolds containing cells, and in many cases you can. But often the limitation is sample preparation and what is and what isn't possible.

So as from your perspective in terms of what's possible now and as new methods of sample preparation come forward, how do you see these analyses methods being applied to cell scaffold constructs?

DR. RATNER: Yeah, a number of different comments there. For one thing, there's a whole set of methodologies that I think we've pioneered at the University of Washington, which involve taking samples, rapidly freezing them at extremely low temperatures, and putting them at the ultra-high vacuum environment, and presumably having some degree of preservation through the freezing, which allows us to look at live cells.

There are a few groups. There's a group at

Penn State under Nick Winograd that's actually done great work looking at receptors on cell surfaces by SIMS on frozen hydrated cells. So I think there's potential to bring this over to cell/scaffold.

The other part of it, though, is that the cells do spew out this nice extracellular matrix that would make things work well. So you really lose the ability to see your scaffold anymore once the cells do that.

Yeah, Rocky?

DR. TUAN: So, Buddy, along the same direction there, what kind of chemical analytical tools do we have to look at in a vital way that interface between the cell and the scaffold? Because, obviously, that interaction is what guides the success or the failure of that construct.

So freezing is one way, but then the cells are gone. But is there some way we can do it in a vital manner based on other types of imaging methodologies?

DR. RATNER: We've published on a interference contrast microscopy. It's related to

confocal and related to -- it's based upon the effect if you -- an internal reflection. If you put your finger on a glass of water, you can see your fingerprint magnified usually.

And we've looked at the contact points of cells with different materials and gotten real-time data on interaction of cells with materials using this sort of methodology. So that might be taken on to look more in real time about how the cells are attaching and interfacing with materials.

DR. TUAN: I guess one way to do it is actually enable the cell to be a readout as well. FRET or other types of fluorescence resonance where -- you have to change the cell, obviously. The cell becomes a little reading machine that tells you what's on the surface. I mean those are -- so now you're interfacing with the cell on the material.

DR. RATNER: I fully agree. But the information that cell's trying to tell you, as you pointed out, is very complicated.

DR. TUAN: Very complicated, yeah.

DR. RATNER: We got to learn to read what the

cell is telling us.

Do we have time for some more questions? Please, yeah.

MR. KRISCO: Peter Krisco from National Institutes of Health. I really enjoyed your talk and the variety of techniques you presented.

I agree with your point that mechanical properties of the scaffold should be matched to tissues. But depending on the tissues, some of them exhibit really high heterogeneity at a microscopic level. Could you comment on that and what kind of techniques could be used for analysis at that level?

DR. RATHER: You mean the -- I'm just trying to understand the question. You mean the final tissue engineering construct or do you mean the starting scaffold?

MR. KRISCO: Either one.

DR. RATHER: Well, again, these imaging techniques are terrific, especially when combined with the mathematics of principal component analysis.

But looking at heterogeneities, both in principal and in the finished construct, are pretty

easily on the scaffold. If there are non-uniform areas, you can get them to show up in enhanced colors that look great in slides but actually have a real mathematical meaning. So I think the methods are there to characterize, down to a tenth of a micron at least, heterogeneities as one goes across both tissues and scaffolds.

I think -- Tim, yeah.

DR. BERTRAM: Thank you, Buddy. Very good insights. Oh, Tim Bertram, Tengion.

Quick question for you. It's an extension really of what you've showed here.

Have you found any of the in vitro assays that you've presented here to match any of the clinical measurements? In particular what I'm thinking about is this MRI, a clinical tool that is giving us some molecular insight in trying to understand degradation of that scaffold in vivo.

Have you found anything that matches and bridges those two?

DR. RATNER: Well, fortunately, I was asked to talk to the in vitro situation. So I kind of

bypassed that question.

The answer is that at this time, no. But we do have all that lipase data, for example, I showed you, and hydrolysis data on this. And these materials are now in long-term implants in animals, so we are hoping to get at least a -- to see how meaningful our in vivo data is compared to long-term in vivo implantation. The kinetics we're seeing, the disappearance match is what see in vivo. So we're trying to do that, but it's challenging.

DR. DURFOR: Once, again, let's thank Dr. Ratner.

MR. AZEKE: Excuse me. Hello. I have one more question, if you have time for that. I'm in the side room here. Thank you very much for your talk. John Azeke, University of Florida.

We are currently doing some studies with fiber constructs, and we would like to look into the actual fibers, the bulk property of the fibers themselves within this larger scaffold. And I liked some of the information you had on the depth profiling of the different methods, but which of those would you

recommend if we were trying to look at individual fibers in larger 3D structure and see the bulk properties of those fibers?

DR. RATNER: Well, you know the SIMS methodology, for example, easily works to a tenth of a micron. What is that, a 100 nanometers? So you'd in principle be able to look at some pretty fine fibers individually, look at each individual fiber. Also, many people are doing nanotextile testing now., so you can start to get mechanical properties of an individual nano fiber.

So I think the tools are in place. I don't think it's too challenging. And then you can use things like finite and element analysis to put together the properties of the individual fibers to get the composite or total fabric or scaffold, and how it behaves from the individual fibers.

MR. AZEKE: Thank you.

DR. DURFOR: Okay. I'm going to go ahead and introduce our next speaker while we get the AV part of it up.

It is my great pleasure to introduce

Dr. David Kaplan. Dr. Kaplan is the endowed Stern Family Professor of bioengineering, professor and chair of the Department of Biomedical Engineering at Tufts University. Professor Kaplan holds faculty appointments in the Tufts University School of Medicine and also the University School of Dental Medicine and the Department of Chemistry.

His research focus is on biopolymer engineering with the goal of trying to understand structure-function relationships, with an emphasis on studies related to biomaterials and functional tissue engineering.

He has published well over 400 papers, edited eight books, and continues to pursue topics related to bioengineering polymers, stem cell biology, and the context of biomaterial signaling and functional tissue engineering. He also directs the NIH Tissue Engineering Resource Center that involves the coordination of Tufts, MIT and Columbia University, and the bioengineering and biotechnology program at Tufts.

It is my great pleasure to introduce him and have him give you a talk today on in vitro

characterization of hard tissue constructs with a structural role. Professor Kaplan.

DR. KAPLAN: So good morning, everyone, and thanks very much to the organizers for the opportunity to talk to you this morning.

So this is the task they gave to me, to tell you about in vitro characterization of bone, ligaments, tendons and cartilage, and they ascribed 20 minutes for me to do all this. So need I say more?

So I'm going to really just focus a little bit on bone and a little bit on ligament initially and just give you a quick view of how we normally grow an analyze those tissues in vitro. And I will spill over a little then into in vivo issues related to those tissues because I don't think you can segregate completely the in vitro from the in vivo if you really want to direct the in vitro studies appropriately.

Then I'm going to really try and leave time at the end to talk about what I would say are three areas we feel pretty passionate about in terms of directions we need to go for both in vitro and in vivo to build on characterization tools and opportunities.

So that's a lot to cover in 20-plus minutes.

So this is very familiar to all of you. Let's introduce bone tissue engineering as one of the hard tissues I was asked to cover, bone generation in vitro. And then this shows a little bit integrated in vivo as well. This is a one-stop slide to cover it.

At the top you see all of the efforts to generate scaffolds depends on what you're trying to grow. Buddy beautifully introduced the role of pores and so on. I'll come back to that.

You eventually make some kind of three-dimensional porous scaffold onto which, in our hands, we generally seed with different stem cell sources. These then go into suitable bioreactors, either static culture or some kind of dynamic culture in order to optimize conditions. I'll again come back to that in a minute as well. And out of that, after introducing the appropriate biochemical and chemical stimulants to the cells, we can generate suitable bone-like plugs.

Ultimately, by Micro-CTs, you see some beautiful mineralized deposits. And these can then be

taken into in vivo studies to see if the in vitro characterization have meaning in vivo when we're done. And that's what you see on the left.

In the in vivo side, we can look at mechanically-unloaded specimens, such as in cranial defects at the top portion there or in mechanically-loaded critical size femur defects.

So, in general, what do we do? Anytime we're growing this kind of bone-related tissue, we will look at many genetic markers, nut we'll also look at biochemical markers. You see calcium. We'll also look at a variety of biochemical markers on the left such as calcium deposition. Again, this is all in vitro as well as alkaline phosphatase activity.

On the right side, you see all the traditional histological characterizations as markers for what you'd like to see, and Rocky did a fantastic job of reviewing all the challenges and issues there are as well.

And to us, if we're doing something in vitro, ultimately what we want to look at is what's at the bottom, which is evidence for the distribution and

content of mineralized tissue. Otherwise, all the histology and other markers really don't have a lot of meaning.

And you can see one simple outcome in some of our older work at the bottom. If we use a protein scaffold on the bottom right that degrades very, very slowly, we get robust mineralized deposits. On the left, a scaffold that degrades very readily such as collagen, you lose integrity. You lose transport. You get the so-called doughnut effect, and you only get mineralized tissue on the perimeter.

So this is an easy way to start, but then you have to take that to the next level. And, again, this builds off very directly from what Buddy just mentioned. If you're trying to grow bone -- you all realize bone is different depending on where you are and what you want to implant in the human body. So here we can simply control bone-related mineralized morphology simply by regulating the scaffold design at the top based again on controls of pore size distributions.

So you see very small pore sizes to the left,

150 microns up to almost 500 on the right. And you see at the bottom by a more magnified Micro-CT analysis, a cortical-like bone on the left and trabecular-like bone on the right.

So this again is a marker or a nice measure by using CT as a really good way with just in vitro analysis to not only direct the kind of bone you're going to make but look at it during the process to see where you're going.

I should remind you again. These are again some of our protein-based scaffold designs that are very slow degrading systems. And they afford the opportunity then to template the mineralized deposits from the stem cells in vitro as they're differentiating into osteoblasts.

Then we can go into the mechanically-unloaded system here. These are the cranial defects. And you see all the traditional histological analysis shown with variance being empty scaffolds all the way over to fully tissue-engineered bone on the right side before implantation into the animal model, in this case a mouse.

You see at the bottom again the real concrete evidence for mineralized restoration of function by implanting the tissue-engineered version. But, again, marker-wise, we can follow all these markers. And I'll be mentioning this again in a few minutes.

We're going to show you some nice mechanically-loaded defects. In the transition, they apparently degraded away. But what you would see here again is the use of Micro-CT as a very powerful tool to track the healing in vivo from what we just showed you in vitro as early evidence of growing highly mineralized bone tissue.

The point of this slide was to say if you simply focus by analysis of the distribution and content of mineralized tissue, you may get fooled. And you also need to look at the biomechanical integration of that tissue, which was on the bottom right to show you you get improved interfacial stabilization and mechanical properties by pre-growing these tissues towards bone before implantation, compared to controls where they were not pre-grown or scaffolds alone.

Looks like all the figures are going to be

tortured. So this will be interesting. So here -- that's supposed to be a nice cable structure at the top middle, but it got turned around.

So let me shift from bone for a minute to ligaments. And so ligaments will be the other tissue I'll talk a little bit about due to the time. So now we have a whole different set of constraints, a whole different set of guidance principles that we must consider. And for any ligament and most other tissues like this, mechanics becomes absolutely central to what you're trying to do. So here we construct textile engineered ligament scaffolds based on some of our fiber protein systems. These are seeded again with our stem cell sources. Depending on the study, we use different stem cell sources, all human stem cell lines, though.

Here we have to be more creative about the environment that we use. So this is a complex bioreactor system, top right and bottom left, where we apply three-dimensional forces, mechanical forces, to the tissues, both in rotation and in tension, to induce the stem cells towards ligament-like outcomes. Then we

have all the markers we need to follow to assess what kinds of tissues are coming out of this process. The issue here is that there aren't very defined biochemical stimulants that we can provide to the stem cells to get them to specifically differentiate into ligament-like tissue.

So underlying this would therefore be in vitro assessments of the mechanical profiles of the scaffolding that you're going to use. And if the goal is a human ACL, then as you see in the gray zone there, we try and recapitulate, at least, if not more, the mechanical properties that we need. And you can see the ultimate tensile strength, stiffness, yield point, elongation, all critical components to understand for these kind of mechanically demanding tissues.

Inserted you see fatigue testing. Obviously, this has to last a long time if it's going to be used clinically. So we need to make sure the materials chosen survive the rigors of that tissue environment.

Then as with bone, we can go to traditional markers to see how we're doing. Obviously, microscopy and so on, which Buddy talked about. And then look at,

in this case, transcript levels of various markers as indications of ligament-like tissue. And these can be superimposed with immunocytochemistry and so on.

So those are easy. Those are bone and ligament as two examples. I just want to highlight the complexities ahead and the challenges ahead.

So let's say we're going to grow an osteochondral graft. So we're not just going to grow bone, and we're not going to grow cartilage or ligament, we're going to grow two tissues at the same time. So now the issues of characterization in vitro, regardless of what we do in vitro, becomes compounded.i

So here's one way we do this. This is where we prepare first at the bottom left a series of protein-based microspheres, where we load in our growth factors. In this case, we were using IGF at BMP-2. The goal is to grow cartilage or bone, thus the choices. And then we use a poragen, a salt poragen, sodium chloride. And we use a gradient maker to deposit, then reverse gradient. So the two growth factors combined with forming the poragenic morphological structure of the scaffold.

And when we're done, as you see in the middle, a very porous, reticulated structure which has embedded in the walls -- a little hard to see at the bottom by SEM -- these little microspheres, which will slowly release then the growth factors.

Then the goal here is to have growth factors at one end of this three-dimensional solid, spongy scaffold optimized towards inducing the stem cells towards cartilage, at the other end towards bone, and something should happen in the middle.

We come back to traditional assessments then to do this. And you can see we can follow by genetic transcript levels or by calcium quantitation for markers in vitro what we're getting in terms of bone outcomes or in the way of cartilage outcomes depending on the other markers.

You get some interesting synergies we don't have time to talk about, but, nonetheless, these are not interfacial tissue outcomes that can be tracked with the same kinds of assessments that we've already talked about for bone and cartilage. It's just a little more difficult to interpret, as well as the

histology that goes with this, and more traditional staining that both Buddy and Rocky talked about earlier.

So from traditional approaches like I just showed you, the outcomes in vitro are what I would call fairly standard today. We have to look at biochemistry and structure. We have tools to do that. We have genetic markers, real-time. RTPCR is the standard today. Cell biology, we tend to look at some of this, not all of this. And obviously, mechanical properties are critical.

I put at the bottom, though, as I was putting this talk together, the fact that what we do is great. But there's so many things we could be doing, or still don't do, to further improve outcomes and measures of outcomes. And I picked after this a couple of examples just to highlight the challenges that still exist but also makes the science quite exciting, I think.

So first of all, we worry about source material for scaffolds. It's critical. We tend to not spend a lot of time on it. I'll show you one example where it's important. We can spend more time on

scaffold features.

We can worry about matching degradation rates, as was mentioned before, to tissue remodeling. I'll show you at the end one of the coming tools which is modeling to do that and have better predictive tools. Most of us have not yet integrated immune cells into the process in vitro. I think that's something that many labs are starting to do. We need to do more of. Many of us are just beginning to integrate the issue of vasculature, which Rocky mentioned briefly. I'll show you one of those tools coming as well, where we try and integrate the endothelial cells with other cells.

Markers, they're great. I've showed you some. But when do you measure them, how often, and where are their meanings, cultivation conditions, the complexity of mechanics? And I'll come back to vascularization at the end because tissue size and integration become critical, and they're severely limiting the field today in terms of what we can do with clinical benefit down the road.

So that's a lot in the last 10, 15 minutes.

So briefly, here are the challenges. So we talked about markers. Well, if you look just at the bone field today, you can come up with a laundry list of markers. And, again, Rocky talked about this.

Some are early, some are mid, some are late stage. Which ones are critical, how do we prioritize these, and which ones should we follow remains an open question. We tend to believe when we see calcium deposition as a mature marker, that's the way to go. And these others fill in the gap, particularly collagen Type 1, but depends on which lab is doing the work.

If we look at scaffold sources, here's using collagen as a scaffold source. And I just highlighted in a box there are three commercial sources of collagen, one from Sigma, one from Roche. Again, like Buddy, I have no vested interest in these companies. But you can see they're drastically different when you buy them and use them in terms of what the composition is and what the molecular weight distributions are of the collagen chains, which are on the right as the controls.

The Roche and Sigma look pretty good. The

Calbiochem looks like it's a mess. Why do we care what that source is? For many studies we've done and other labs, it's absolutely critical in terms of how the stem cell signaling is going to be affected towards what those cells will do to that matrix, how fast they'll remodel the matrix, and, in fact, what the sort of senescence-related markers will be for the stem cells.

So if the collagen is already digested before you used it in your scaffold, you'll start to up-regulate different integrins for those cells, and that will take those cells down a pathway you may not want to go.

We show you one mature marker here of using stem cells grown on different collagen types, and the outcomes are completely different, depending on what the structure of that collagen is when you initiate the experiments, all due to changes in these cryptic epitopes that again induce different downstream pathways.

We can also go to more traditional materials approaches, make phase diagrams, as I show you here, for some of our protein scaffolds, spongy scaffolds.

And at the top, I show you some derived and made in an entirely aqueous-based process. And at the middle there, you see HFIP. That's derived in an all-organic solvent-based process.

The process isn't so important, other than the outcomes. The one on the top tends to have a fairly low content of crystalline content in the protein when we're done. At the bottom, it has a high content. If we implant those, the one at the top will degrade away in weeks to months and the one at the bottom will take years. So you really have to know what you're doing with the material that you use at the biomaterial side so that the cells will get off on the right foot and do what you want.

Here's an example from soft tissues, so not really relevant to the discussion. But it highlights what I'm trying to stress. At the top, the four quadrants there are using four different degradable scaffold systems today, all the ones you know about: PLGAs, collagens, and then some of our silk systems.

In vitro, by all the markers, they all look

fine. We get Oil-O-red. We get all the adipose-related markers you would want, no problem. We can see it with mesenchymal stem cells from bone marrow. We can use adipose-derived stem cells. All work fantastic.

You take those and implant them in vivo in the bottom. The scaffolds from collagen and PLGA are gone before you can retrieve the samples after a couple of months. And so what we may dial in -- in vitro has no meaning in vivo if we haven't considered that crossover at the longevity and these other markers that become important.

We can look at how cultivation conditions in vitro affect outcomes as well. So this is just a simple way to show you on the top; on the left side, static culture and on the right side, a more dynamic culture, just spinner flasks. These are the same porous protein scaffolds where we're seeing with our human bone marrow-derived mesenchymal stem cells, and just using microscopy to look for bone nodules and outcomes over time. They all do fine.

But if you look closely, you see markers

measured different ways -- I'm not going to spend a lot of time on this -- where you see significant differences in some of those intermediary markers that I mentioned before. So you have to consider this. In general, the spinner flasks do a lot better, as you would expect, because of transport.

We can track mechanics over time on the top left. Those markers don't always track to improve mechanics. And so you have to be very careful about matching which markers to which outcomes that are meaningful for the bone outcomes you're after in this case.

So I have two slides just to tell you it gets worse because here we're growing something like a temporomandibular joint. So think about the complexity here of all the markers we've talked about. I'm not going to have time to go into this.

Secondly, those of you who study the spine, we have the bone and the cartilage, everything else, in there, and the complexity just gets surmounted again with the biomechanical complexity. So the marker issue becomes critical, depending on the tissue type you're

after and going to focus on.

So just very quickly, I want to go over something that comes out of a workshop that a few of us attended last year. This was headed by David Butler and colleagues. And out of that is coming a report that'll probably be published this coming year in *Tissue Engineering*. It's in review now. David said it was okay to talk about some of what's in the report because there were about 500 authors on it at this point.

But the goal there was to look at what we had to do to transition from in vitro to clinical relevance in essence. And so I highlighted a few things here. First of all, for general goals, obviously, faster recovery, shorter -- short-term, long-term functional benefits, be it pain as was mentioned before or mechanical support, we want to be able to improve the sort of the loss of disease progression and, obviously, morbidity issues and so on.

You can see additional criteria here that become very important. We want to do better than the best available technologies. It has to be safe. We

want to maintain cell viability where possible and we want to get integration. I bring this up because if we don't keep these issues in mind, all the in vitro markers again become less meaningful as we move ahead.

In this report, you'll see six or seven tissues. For the bone, I've already talked about. The markers are up here. We care about mechanical function, integrationl, and physiological functions as the primary goals.

Due to time, I'm going to skip to the ACL since I mentioned the ligaments. The needs are obviously for traumatic rupture and replacement. The current options are there. In terms of patellar tendons and hamstring replacements, outcomes, mechanical again becomes the primary marker we worry about and all the biological effects have to come in with that. So common themes from this become function, structure and biology. We have plenty of markers to do pretty well with most of those.

So I want to talk in these last few minutes just about three examples of where I think we need to go with new markers and new opportunities for this for

any of the hard and even soft tissue. One is better imaging modalities.

So all the imaging we tend to do now in our newer work is focused on the endogenous signals from the cells and the scaffolds and the new matrix that's generated. So we want to avoid dyes. We want to avoid labeling cells, as was mentioned before, to study remodeling and so on.

So, for example, in collaboration with Irene Georgakoudi in our department, we look at a combination of things like second harmonic generation combined with two photon excited fluorescence emission. And by doing so, I just show you how you can track scaffold processing and structures very systematically, going from fibers to films, stretch films, hydrogels, porous scaffolds. It doesn't matter. You can use and exploit these tools, not having to add anything into the system, just borrowing from what those structures are inherently.

Then you can superimpose upon that what the cells are doing, again, through their endogenous metabolic signatures. So here we're tracking over time

the differentiation of our stem cells, our human stem cells on a surface towards bone. And you can watch and see the morphology of the cells. You can track the nucleus, and you can see the change in metabolic profiles as those cells are undergoing differentiation.

Then finally, you can plot metabolic ratios, which is a good indication of what those cells are doing as they differentiate. And you can see those profiles change depending on the scaffold type you use, and that becomes very important in relating the cells to the matrix.

Then finally, you can begin to superimpose on top of that the new matrix deposition that happens as the cells degrade the original scaffold and as the cells are differentiating. And for bone, obviously, we want to see that nice green fibrous structure that's the new collagen, again, from second harmonic. And so this, again, is just all the endogenous signaling.

So you could track the original matrix as the cells are going through differentiation and new matrix enumeration by the cells all in a dynamic way, which is very important for what we do.

Second main point for the future is vascularization. I mentioned this before. If we're going to grow suitable -- most of the tissues we're talking about with suitable size, we have to even in vitro figure out how to deal with pre-vascularization.

Buddy mentioned one really nice approach based on his spherical system. We use a number of approaches. I'll just show you one very briefly. But we can use either design, sort of directed engineering, which is a single-channel approach. I'll show you that one. We can use cell-directed engineering approaches, which is also a very viable one, or you can use traditional microfab approaches, which becomes very important. We have to do this with all degradable materials. That's the trick. So that's not always easy. But there are nice systems now that we use routinely to accomplish all three of these in the lab at this point.

So the one I'll mention is to use a single tube. This is a protein tube. You can see two examples on the side there. You can make these virtually impenetrable to even small molecules, or you

can make them very porous, as you see on the right, so you can proteins through the walls, so they begin to look and feel like a vascular tube.

Mechanically, they match in terms of all the other things you'd like to see. And so you can start to grow tissue in a slab gel where you have pre-vascularized conduits, where those conduits behave as part of a tissue system. And we take this in lots of different directions that I apologize we don't have a lot of time to talk about. But you can quantify. That's the important thing here as well. You can track oxygen levels and figure out what's the minimum size unit you need per vessel to maximize whatever cell and tissue function you want, and then you can start to combine those to make larger tissues.

Then the last very brief point I wanted to make, and then I'll stop, is the third direction I think that's very important to the field -- and it was mentioned a bit as well by Buddy for some of the analytical work -- is that of quantifying what happens between cells and matrices.

So we do this by flux modeling. In this

case, we feed these cells different structural states of collagen, and we look at quantitatively how those cells remodel that collagen and lay down on the right new extracellular matrix. And the differences are significant in terms of what you feed the cells and what they're placed on versus what the cells are going to do and how rapidly they will do it.

So we see this as a very valuable tool in vitro to prescreen and understand how changes in matrix design affect new matrix generation, both in vitro and ultimately in vivo.

So that's really quick. Sorry. Here's the group, the fantastic group of students and post-docs I'm fortunate to work with in our lab. And then also let me thank the great collaborators within Tufts, outside of Tufts, strong funding support, and also to the NIH for our resource center in tissue engineering.

I'll be happy to answer any questions. Thanks.

DR. DURFOR: We do have time for one or two questions. Oh, actually, why don't I yield the floor? Please, go ahead.

UNKNOWN QUESTIONER: (Inaudible).

DR. KAPLAN: Danny, I completely agree. I also think it spills over to staying with in vitro models that you want to develop for the disease states so you can study intervention techniques. So I agree.

MR. DALEY: Mike Daley, Tigenics, Inc. I guess I was also struck by the problem with all these in vitro models, et cetera, is we can establish what is good, what is bad. We can then establish our goal is to be good. But the problem is there's that gray area in between. And it not only relates again to biomarkers, to engineering and constructs, and what is required when you put three-dimensional things together, et cetera, it's recapitulating probably -- the goal is to recapitulate the normal tissue. I think we're fooling ourselves if we're going to in two decades overcome what has taken millenniums to occur. So if that's our goal, we might as well all go home.

The question I have is I still don't know what is the goal. Is the goal to just get the regenerative part? Is it the goal to get the clinical

part? If the clinical part is the problem, you have your suggestions of pain function, et cetera.

Patients have the same amount of pain before and after, but they are functionally better off. Is that a good outcome? Patients have less pain but have decreased function. Is that an okay outcome? Patients go back to full sports activity, but have the same pain, less function. Is that okay?

I don't know. And the problem is is when we transition and take this and do the translational medicine from what we're trying to do from biomarkers, from tissue engineering, and bring it to the clinic -- and hopefully our goal is to bring value to the patient that needs these types of products.

I don't know what our objective is. I don't know from our standpoint, from basic biology to the clinic, what we're trying to achieve, and we have to know when we do clinical trials. Pick one, that's called a primary outcome measure. That's what you hang your hat on. That's what you win or lose. And I don't see that coming from that transition and where we're moving towards that common goal.

So I guess I'd like to push it back. The question is what do you see is the -- what is the goal, coming to from all of these markers and all these models, et cetera, for achieving an appropriate tissue engineering product?

DR. KAPLAN: I'm not really sure even how to answer that because it depends on -- you keep -- since I have -- as you think, there's one goal, and I would say there's many goals for what we're doing and talking about here. Some are purely in vitro goals and some are obviously in vivo goals for translational medicine. So it depends on what you're making and why you're making it.

I'll give you just one example from what Nadya asked about, the prior question. In many cases, many of us are growing these tissues strictly in the laboratory, simply to develop disease models to then study therapeutic options that will go to treat patients.

So those goals are going to be very different than if we're going to implant a piece of bone for someone who's lost a segment from cancer. There, you

clearly want to improve function for that human being. It may be mechanical function. It may be less pain. It could be a combination. So it depends on what your goal is.

On the ligament example, there are some options to repair torn ACLs today that mainly involve, as I mentioned, taking part of another tendon or ligament and replacing the damaged ligament. Do they work? Sure. Are they optimal? No, because now you have two sites of pain and suffering. So if we can find something that alleviates that second surgical site, that's an improvement.

What's the measure? That's going to be obviously restoration of mechanical function. Can the patient go play soccer if that's their goal? It may simply be someone who wants to be able to get around the house. So it depends on the patient.

MR. DALEY: (Inaudible). And that's where you should be. It's like a pyramid. You move towards that point, and I guess both from the basic biology, cell markers, the constructs of tissue engineering, we all have to move towards that point.

I guess what it is is -- you're right. You just said well, it depends. It doesn't depend when you do a phase 3 clinical study. There's only one. It's that Jack Palance type of thing. We're all after that. What is it that's important to you or important to the (inaudible)?

DR. DURFOR: I appreciate that comment, and it's very important. But it also underlines why we focused this workshop on bringing products into the clinic.

I think your question is an important one. I think it's something that you learn as you use a product. But at this point in time, what we're focusing on today is understanding in vitro assays as you begin to move into the clinic.

Thank you very much, Dr. Kaplan.

So we will move from hard to soft tissue. And it's really a treat for me to introduce Dr. Parenteau.

Dr. Parenteau was trained as an anatomist and cell and developmental biologist. From there, she went on to look at the behavior, the manipulation, and the

understanding of human cells and tissues and how they're used to make tissue engineering products.

Her past experience has involved many years as the senior vice president and chief scientific officer of Organogenesis. She also went to form -- to be a cofounder and CEO of a biotech company that was looking at adult stem cell technology designed to try and address diabetic and metabolic disorders.

Her awards are many, including being recognized as one of the R&D 100 Award in 1990 and the Best of Biotech Award in 2000.

Dr. Parenteau has also served as the U.S. authority on the cell biology and cell therapy information for the U.S. government multi-MATES funded assessment, the W-Tech study in 2000 that tried to assess worldwide where was tissue engineering going.

With all of that said, it is truly a pleasure to introduce Dr. Parenteau to come and talk to us today about in vitro characterization of skin constructs. Thank you very much.

DR. PARENTEAU: Well, thank you. It's a pleasure to be here to talk about skin constructs with

a structural role. And I'm probably the first speaker that's actually going to give you an industrial perspective, an applied science perspective.

The one thing that you can do today that we couldn't do 15-plus years ago is learn from experience. And much of what I'm going to talk about has been in the public domain for quite some time now. But what I'll focus on is why we did certain things the way we did and what we gained from it.

And once I came out with this odd phrase, "hindsight is bliss," everybody thought about it and then started laughing because they realized what I had done was combine ignorance is bliss with hindsight is 20/20, and I came up with this odd phrase. And it served as a running joke amongst us for many years. But rather, the message behind it was quite serious. And that was rather that the insight that we were gaining from our preclinical work was the bliss part. And ignorance never is bliss. Ignorance is just risk.

So what you want is a clear vision of what's ahead, and you get that through an awareness of what has gone before, and I hope this talk helps in some of

that; and then awareness of what's there around you that you can bring into your problem from other disciplines and other fields and other disease states; and then, of course, the insight that you gain from in vitro analysis.

Breakthrough is defined as productive insight in the dictionary, "productive" being the operative word. In vitro analysis will give you a sense of what your product should be. And from the questions we've heard this morning, you can tell that people are grappling with that. It's going to give you insight and start to understand -- you're going to start to understand mechanism, the hows and whys, long before you reach the clinic.

It's going to all -- all this combined is going to contribute to your foundation. You're going to lay your safety arguments and the scientific validation for your technology, your approach to the problem, and even the clinical indication you're shooting for, all that combined to a reduction of risk.

Structure function products, to me, are like chicken and egg. Structure isn't just manmade because

the biological processes are going to modify and give rise to structures on their own just like Buddy mentioned. And these structures then in turn, impart function and influence other functions, and then you change the structure.

So how do you deal with such a dynamic and complex thing? Well, you separate it into the different elements, and you make sure you understand each one.

The first is the biological character, and that's, of course, contributed by the cellular component of what you're doing. Certainly, identification is key. And depending on what your cell source is, this will be more or less challenging.

For us, we were taking skin tissue, deriving keratinocytes and fibroblast skin cells, and then we were making skin. And we were going to use it in a skin application. So that was a highly homologous application. The more non-homologous you get, the more challenging some of this is going to be. So the identification wasn't really the issue. But for us, we were doing an allogeneic therapy. So for us, cell

purity was key because we wanted to be sure that our processes were not co-propagating things like endothelial cells, which are professional antigen-presenting cells. And so this became important.

But to say I have 50 zillion keratinocytes is meaningless without understanding what they are because the keratinocytes population is a differentiating heterogeneous population as most parenchymal cell types will be. And so you have to understand what the character has to be and then how it relates to how it's going to behave in your process.

So how do you understand the character of your population? Well, the best -- one of the great ways for differentiating cell populations is to look at a lot of growth-related parameters. And I don't have time to really go into some of that, but if you're interested in probing more and understanding that, I can point you in the right direction. So come see me. That's a talk amongst itself. But in things like fibroblasts, which are hard to get a handle on sometimes, they are what they make. And so for that,

you look at biosynthesis, and you understand that.

That will be particularly important for, say, cell-produced scaffolds. For instance, your question will be, am I making a fibrous scar in this dish or am I making a mesenchymal tissue, for example. And the way you'll find that out is by looking at the collagen and the proportion of glycosaminoglycans and all that over time, and then relating that to what the developmental biologists are telling you characterize these tissues or the matrix biochemists.

So then it becomes an issue of behavior in the process. And in that, you're looking for certain cell response and interactions because the cells in your process are going to be different than what you wanted the cells to do, which is more of a regenerative phenotype to get your cell bank or to get enough cells to begin with. Now you probably want them to be functional.

So you're looking at proliferation versus differentiation, and you want to find a range that defines that component and also what it should be for your product, what your goal is. And then the

character of them is they become apoptotic, proliferative, differentiating, biosynthetic? All those are very fundamental things that can be easily assayed for and analyzed, and those are very insightful things that you will want to know.

The next is biochemical character. So you've had your biological component. You get your arms around what the cells should be doing, but you need more. And you're going to see there's a layering of biological information and biochemical information, even physical information that then builds your story. And it's not just about a composition at any one time because you have biosynthesis. You'll have changes to the matrix over time, and you'll also have -- it's not just structural.

They're going to be production of factors. And production of cellular factors does two things for you. It starts to lay a foundation for you to be able to understand what you might be able to contribute to the recipient, but it also gives you the insight of what those cells think they are in that tissue.

For example, are they of a injury

inflammation phenotype? Are they of a regenerative phenotype? Are they of a repair fibrotic phenotype, or are they of a differentiated stable phenotype? And the way you find that out is by understanding what cytokines and growth factors characterize or those states, and then you look for those in your tissues.

Even though I am a cell and development biologist, I will put in a plug for physical character because it adds mechanical testing. Even if you're doing something like skin, say, versus a vascular graft, where you think, well, the mechanics aren't as important -- but mechanical testing is another layer of information and insight that can be very valuable.

And don't pooh-pooh the simple. Even when we were doing cell contracted collagen lattices, just the fact of gel contraction told you something about the collagen process, febrile formation, cell interaction, all those things. We found out fibroblasts have a certain maximum density. They regulate that. They make decorin. They make tenascin. You want to characterize each thing, and you want to build your knowledge as you go along.

So mechanics, for example, if you're doing a, say, cell produced matrix, as two skin companies are now and two vascular graft companies are, looking at tension and collagen over time -- and you'll hear me say "over time" a lot because you have to have time as an element in your testing in a biological process -- will give you so much more information than either one alone. You can look at configuration comparisons, growth factor influences, and cellular contribution.

Of course, then it's all about how it all comes together. And you will want to characterize what you have in the end very well. But it's all been building with time. And you've been making decisions during that process, trying to understand the biology, what you can and cannot do, tweaking your process, going around to finally get to something where you say okay, now I know what I will achieve.

In skin, one of the best ways to understand what you've done if you have an epidermis is to look at it. So it can be rather simple. You see character as a population. It's an indicator of normalcy, which is

very nice. It's verification your process ran as expected.

Because you're looking at it over time, you're looking for representation of differentiated strata, presence of stratum corneum, whether there are nuclei, whether the envelope formed prematurely. And you're drawing in all that the pathologists can teach you about it, you're drawing in all that the dermatologists can teach you on it, and you're using all this knowledge to put to your thing by just interpreting your histology slide.

Of course, this is an in vitro test, so pretty pictures are not enough information because pictures can fool you. And so you validate what you see by immunohistochemistry and biochemistry, essentially adding that biochemical character to it.

For example, for immunohistochemistry, we looked at integrin expression over time, keratin expression over time, differentiated keratinocyte markers, and where they formed in the strata. All those things were biologically relevant to a specific issue in keratinocyte biology. We looked at keratin

subspecies by biochemistry to confirm, and then we looked at lipid biosynthesis because we know that would be important for barrier function.

So barrier function is the most stringent test you could possibly ever do for normal keratinocyte differentiation. It is the goal in life of the epidermis to make barrier, and a lot of things have to come together. Just like things -- mechanics help you understand how the extracellular matrix molecular have come together, this is the epidermis' structural component.

And it can be measured simply by percutaneous absorption with tritiated water or transepidermal water loss going through. And it's a functional measure of differentiation with time. And so it supports your morphological findings and your biochemical findings. And so now you have a picture. And that picture told us that what we were making in this skin construct was a phenotype of a freshly healed wound. And that ended up making perfect sense.

In the literature at the time, I'll just mention that there was an article written during the

development -- this was late eighties, I hate to say -- saying, well, epidermis generated in vitro, is it normal? Oh, we're not seeing this and we're not seeing that. This is abnormal. It wasn't abnormal at all.

If you really understand where you are in the continuum of biology, then you say, okay, this is a freshly healed wound, so it's still expressing a little keratin 6 and 1, but it's normal, it's normal. And when you're going for a regulated product, anytime you can say it's normal and relate it back to in vivo, that's like gold to you.

Cornification. This is the chicken and egg here. Cornification adds a structural and functional component. It protects the underlying living layer. So it adds a robustness to the clinical product, and it allows clinical meshing, which is a practical feature that's important for clinical use.

However, it also provides physiological feedback, and that translates to its impact even to survive on animals. And if we take a skin construct with no barrier and engraft it, it fails. If we take

one with barrier, it takes as a skin graft on a mouse and lasts for the life of the mouse.

And so that tells you -- now, these things tell you two things. One is, if you choose to do a skin construct with a stratum corneum and one without, those are two different products. If you choose to do one that has a stratum corneum that you achieve barrier function in, then that also is two different products.

So you want to use your preclinical research, the bulk of it in vitro because that's where you get that mechanism to make those critical decisions. To say that just adding keratinocytes and fibroblasts to a wound, it doesn't matter what configuration, is hogwash, absolute hogwash. It's a disrespect of biology for us to think so. So be careful. You want to make very -- you're going to be making very important decisions no matter what you're doing. I'm just showing you how it was done in skin.

The other thing I want to mention is bioequivalence, which can send shivers up people's spines, this complex product. But what are we really looking for with bioequivalence? We're looking for

consistency.

So if you've done your characterization, you understand the components going in. You know your process. You've looked at it, and you know the character of your construct, then you -- in fact, Sally, who asked a question earlier, and the team put together the characterization for the PDR -- you actually can get a handle on some of these things.

So how did we do it in skin? Well, we could now look at ability to meet morphological criteria. We could look at time to maturation, so essentially, cell behavior within the process. We could look at barrier function, a cytokine profile, and I'll talk a little bit more about that. And then, of course, tie it together with in vivo by making that link, by engrafting it on athalamic mice.

So the practical benefits of a really strong preclinical plan are, to my mind, immeasurable because they set your process parameters. They help you achieve reproducibility. And if you have trouble with reproducibility, it usually means you don't quite have your arm around maybe one or two parameters. And it

helps you look at mechanism of action, function and safety even before you set foot in the clinic. Remember, it's all about reduction of risk and being sure you've made the right thing for your technology.

So the one thing I'll show you -- well, two examples of that. And one is response to wounding because we were doing wound healing. And this was an assay that was developed actually by Jonathan Garlick when he was a graduate student, and he was sort of learning skin equivalents from us. He was at SUNY Stony Brook at the time with Lauren Teichman. And then Jan Hardin Young, my partner, and Joe Lanning and Carrie Isaacs modified it for our commercial use.

And this assay allowed us to be able to say that the skin construct we made was living. And it's an in vitro assay. And what happened is you take the skin construct, mesh it because we could. We were then able to put it on a fresh dermal matrix, expand it some to create a series of small wounds. And we then analyzed what happened.

And sure enough, you can see that morphologically, the epidermis migrated and healed the

wound and went through a regenerative process as well as rose repair and reformed stratified epidermis over those gaps. However, a picture is okay. But was it really mimicking what was known about wound healing in humans?

And indeed, we looked at protein expression over time, looking at pro-inflammatory cytokines and growth factors and also gene expression over time, using semi-quantitative PCR, confirming and strengthening then what we were looking at. And then we made the in vivo link by engrafting then onto an athalamic mouse and looking at gene expression and saying, yes, indeed, this could happen. So then you know okay, the skin construct is inherently capable of going through the injury and healing response, and that is what we are supplying to the patient.

The immunology was an issue for us from a safety perspective because we were doing allogeneic cells. And this is another area people have a bit of a phobia, I think, about. And the first thing is the product has to work. So you want to be honest with yourself and make sure that you're going to make the

best product. And if it means doing allogeneic cells, then that's what it has to be.

So keratinocytes and fibroblasts are nonprofessional antigen presenting cells. We felt, based on our work and the literature, that it was the co-stimulatory pathway that was to blame. And by using antibodies to link to the co-stimulatory pathway of the T cell in the presence of the keratinocytes and fibroblasts, we were able to say, yes, indeed, that's where we think is the mechanism of why we're not seeing the response. Of course, the patients were monitored, both humoral and cell-mediated immunity.

I just want to bring up the cytokines just for a second because a lot of people are interested in the modulatory properties of mesenchymal stem cells. I just want to let you know that over ten years ago, Joe Lanning did describe that keratinocytes, certainly, because that was our focus, produced TGF-beta and PGE2 in response to gamma, which is a perfectly normal part of the injury inflammation response. And so that could quell a T cell proliferation assay. But we figured that was a modulatory thing, and we wanted to get at

mechanism. So we let that play out, and then we ran the assays.

We also looked at the process of sensitization here, this time modifying the T cell assay by using HLA-matched dendritic cells. And just to say, the allogeneic cells didn't sensitize nor was the response different if the recipient -- if the patient's T cells, say, would have been prior sensitized.

So the take-home message is first you can't -- you can be Bill Belichick, and you still won't win the Super Bowl without a great team. So make sure you have the expertise. Your science has to be sophisticated. It can be simple but sophisticated. And make a logical plan based on questions. The best way to get answers is by asking questions. It's going to help you make informed decisions and build a firm scientific rationale.

And as you'll see, all the things that I showed you weren't terribly fancy. And if I were doing it today, 10 years later, 15 years later, would I do it all that much differently? Probably not. It's the

relevance that counts.

So the ideal preclinical plan for any of you doing this in earnest, as they used to say, you want to generate insight every step of the way. And you want to help it give you information to determine the components, define your products and set your process parameters.

There should be a reason for everything that you put into your process, and it helps you determine how and why your thing works. It creates that important foundation for safety, and it supports probable efficacy. And that reduces your strategic risk immensely, and it continues -- it's like money in the bank for a small company. It continues to contribute.

Just to mention, I had to put in a few mouse experiments there because the in vivo and in vitro work best in partnership.

So plan for success. And when I mean success, I mean to the marketplace, not to Celia or Chuck's desk, but to the marketplace because that helps you prioritize things and not ignore certain issues.

And you want to develop a sense of what you need early because it's less costly. It's less risky. And any weakness that you have, any stone you did not unturn will come back to bite you. And even the hottest technologies can reach roadblocks. So your preclinical plan is invaluable because your second chances are rare.

The other thing that's different about today that even wasn't true a couple years ago is that Jan Hardin Young now are involved in helping others succeed in this area. And in the spirit of the workshop, we've put together two white papers on a high-value preclinical plan and closing the information-to-knowledge gap, both of which we consider very important for success.

We also have a couple educational resources. I know there's at least one person in the audience that has taken advantage of this, and we have two podcasts in applied science for some training for some staff, if people are interested. And we also have a free ezine on applied science as well.

You can reach us on the web and e-mail me at

any time. And I'm here at the meeting. We have a few reprints if someone -- they are not too yellow. But if people want nitty-gritty of some of the assays we did, we have that. And if you'd like us to send you a white paper, please let me know. Thank you.

DR. DURFOR: We have time for one or two questions if you have any.

MS. HUNSECKER: My question is a little bit philosophical. I think one of the messages that you're bringing home to us is that you have to constantly pay attention to what you're learning and you sort of have to evolve your process as you're learning and incorporate new information.

But also, if you're thinking of terms of commercial endpoint, there comes a time when you have to really nail down a lot of the parameters that you're using because your data has to be, as you say, consistent. And once you come up with certain -- there has to be a point in your preclinical process where you fix some parameters even if the data continue to evolve in the course of your development.

So from a -- I know you can't give specifics

because every case is individual. But do you have any general guidance for people in terms of how do you keep your eye on the science but at the same time paying attention to the fact that the road to the clinic has to be done with something very tangible, very reproducible and very predictable?

DR. PARENTEAU: Well, I'll give you one example. When we developed our method of keratinocyte propagation, we then were at a crossroad, and now I'm talking 1989.

But anyway, we had to make a decision because we now had technology where we could do an epithelial sheet graft. We could also engraft just a dermal matrix with cells, fibroblasts. And we already knew enough to know that we could do full a bi-layered skin construct because we were driving at the time in doing an in vitro product, which was what won the R&D 100 Award in 1990. So we had a decision to make on what the clinical product should be. And it was based on the fact that skin grafts worked. Okay? And so we felt that the closest we could get to a skin graft, the better off that we would be.

So you have to focus on what the patient needs first. So no matter what you're doing, what is the clinical problem? And that's where your focus should be.

And then you go back and say do I yet have technology that can meet that, and you ask yourself those fundamental questions. And then you'll know whether you're ready or not because too many people -- even when people still go to doing wound healing assays using fibroblasts, I would say to them -- if they work for me, I'd say show me where fibroblasts increase the rate of wound closure and show me how that is. Why? Because I'm not going to spend 5, 10 $20 million doing a product like that with the endpoint to that gentleman's -- clinical endpoint of wound closure without knowing that.

So it's back to, again, what stimulates, what powers wound closure in vivo, and then am I contributing to that. So it takes a lot of integrated scientific information.

Like I said, you have to be sophisticated science. You have to get it beyond phenomenology.

Tissue engineering has been criticized by maybe the biologists because -- in being phenomenology. So you can't run on phenomenology if you're doing a product. You have to know.

So what do I need in the clinic? What can I do? Where do the two meet? What's my timeline? How much money do I have? And will I be able to do it? And what expertise do I need to achieve what I need if I find there's a gap in between?

DR. BERTRAM: Nancy, Tim Bertram, Tengion. Thank you. Very nice presentation.

Quick question for you. I did like your bioequivalency in vitro test. That was an interesting challenge you took on. I'm curious, though. How did you establish the appropriate predictive endpoints that allowed you to judge bioequivalence in vitro?

DR. PARENTEAU: Because we -- well, you start by characterizing what your product is. So once you have what defines your product -- and in our case, we had that quite well defined. We then essentially -- bioequivalence is a matter of achieving that product, those characteristics.

DR. BERTRAM: If I might follow up --

DR. PARENTEAU: So essentially a freshly healed wound.

DR. BERTRAM: No, I understand that. The question isn't that.

DR. PARENTEAU: Sorry.

DR. BERTRAM: It's more about -- no, I wasn't clear -- the clinical relevance. So to say that your product is bioequivalent to another product, you have to have a defined bioequivalence.

DR. PARENTEAU: It's not bio -- sorry, sorry. I think you misunderstood. It's not bioequivalent to another product. It's bioequivalence within -- when you're changing cell strains.

DR. BERTRAM: Oh, so you used it as an established way to make the change control.

DR. PARENTEAU: Yes, yes.

DR. BERTRAM: Got it. Thank you.

DR. PARENTEAU: So my question is the idea of going for stem cells and then putting a lot of problems upfront, it depends. Depending on what your cell source is, it's going to shift your burden of -- your

difficulties in achieving certain things. But we were using normal cells. But we had to change them once in a while. So we needed to show that we could do that.

DR. RATNER: Buddy Ratner, University of Washington.

Your comment about biologists having criticized tissue engineers for our poor understanding of the total biology of the systems is -- maybe has some validity. But on the other hand, I've taken to criticizing biologists for the reductionist approach, which has sown the gates, the complexity of the in vivo environment. I think what we're learning to do, we're setting new ground here as a field by developing a conducive, inductive environment. And then saying that built within the biology -- if you don't turn it down the wrong path, built within the biology, you can get things going down the right way.

DR. PARENTEAU: Well, you know I've been -- my mantra for a while now, for several years, has been that the biologists and the engineers need to get together and have more.

When we did the skin construct, I was a

biologist. I was sort of thrown into this field. I happen to be using a cell contracted collagen lattice as a matrix because that was the company's technology. But I was a -- I'm a cell and developmental biologist, and I came in with -- so the issue there is that product is a biological product more than anything else. But we need to get both together. Sometimes I just feel so bad that all that great work that you're doing and Dave is doing and everybody -- I know NIH is getting together multidisciplinary teams to address that, but it's so needed to bring things forward. You guys need to be enabled as much as they need to be enabled by you. Thanks.

DR. DURFOR: Let's thank Dr. Parenteau again.

Okay. We're running a little late, and I apologize for that. We're going to have a lunch break from 12:30 to quarter to 2. We're going to give you some advice as to where to eat.

(Whereupon, a lunch recess was taken.)

A F T E R N O O N S E S S I O N

DR. HURSCH: Okay. Welcome back. I hope everybody found something for lunch.

I'm Deb Hursch, and I'm from the Office of Cellular Tissue and Gene Therapies, and I'll be moderating this afternoon's session.

Our first speaker this afternoon is Keith Gooch. Dr. Gooch is an associate professor of biomedical engineering at Ohio State University, where his lab focuses on regulation of differentiation, growth and remodeling of cells and tissues. He received his PhD in chemical engineering at Penn State with John Frangos and did postdoctoral work in the lab of Robert Langer at MIT.

His talk today will be on the in vitro characterization of cardiovascular constructs, vascular grafts as a model system.

Welcome, Dr. Gooch.

DR. GOOCH: I'd like to start by thanking the organizers for asking me to speak and to present some of my views.

Just as the organizers were challenged with

trying to cover the most sampling of the many different tissues that we might want to do and chose cardiovascular, I can't cover all the cardiovascular. So within the cardiovascular system, it's very logical to talk about the heart. There's great work going on with heart valves. And when I was preparing my talk, I was hoping that Michael Sachs, who's speaking tomorrow, would cover that. And he's confirmed that, so it'll be some work on heart valves presented tomorrow.

There's also ongoing work, not quite as developed, on the idea of heart patches. A promising area, but one I won't speak on just for the sake of time. And within the vasculature, my personal interests are both in the microvascular and the conduit vessels. But, again, for the sake of time, I choose only the conduit vessels.

So if we think about the conduit vessels, the larger vessels that can be used for a number of clinical applications, coronary artery bypass grafts, in the pulmonary circulation, in the peripheral circulation as well as AV fistulas for individuals who are undergoing hemodialysis. So there's a number of

approaches to tissue engineering, and I don't have the time to summarize them. But I thought they'd be worthwhile, at least mentioning.

So people are taking cells and synthetic scaffolds -- and I think that's been highlighted in a number of the previous talks -- as well as some examples of cells and maybe what we call processed ECMs, like highly purified collagen or fibrin gels, which have also been mentioned.

There's also ideas of taking cells and instead of giving them a matrix, allowing them to make their own matrix, having the cells in their cell-derived ECM.

An idea that I work on in my lab, and a few other labs around the country work on, is the idea of taking ex vivo vessels and directing the remodeling. So maybe taking a small vessel and growing it as an attach unit to a larger vessel or taking a vessel that doesn't perform too well and remodel it as an attach unit.

Another approach -- the first were all done in vitro or ex vivo. Another approach is to do a completely in vivo approach capitalizing on the ideas

of Sparks manual, which is basically an idea of implanting something subcutaneously or in the intraperitoneal cavity, allowing a foreign body process to occur.

People have also generated tubes that could be useful for tissue engineering. But the net effect of all these different processes, you end up with a tube. And I choose one that I thought was a pretty picture. But the question is besides maybe a pretty picture, what makes a good tube or what would make it a good vessel because all of them ultimately end up with something that looks crudely like a blood vessel.

So the question I'm going to try to address today, or several, is a given tube, a good tissue engineered blood vessel. And I'm going to try to ask that on two levels. The first one, is that tube good enough to publish and merit animal studies? And the other one, is it good enough to merit clinical studies?

I think the answers are somewhat related. And, actually, I think the first question is actually pretty easy because we have 30 years of background of people publishing on tissue-engineered blood vessels

and a number of years, tens of years, of people doing animal studies. So we have a lot of data on that.

What we don't know too much about is what's the metrics that one would use for their first clinical studies. So what question -- as a speaker, I was asked to specifically address what questions should be asked when evaluating cell/scaffold products in preparation for the first human studies. So I'm going to look back to the animal studies and the previous publications to try to address the second.

So thinking very crudely in a very reductionist point of view, if we think about a blood vessel, what is its function? I think you could break it down into two -- and again, I acknowledge this is very crude and simplified -- two absolutely essential functions.

It has to hold the blood. If it leaks, it hemorrhages, it's not working. It also has to carry the blood. If it becomes occluded or thrombosed, it's no longer conducting the blood. It's useless. So I'd say those are absolutely essential functions in vessels.

There are essential functions that are of vasculature on a global scale that a single vessel might not need to do. For example, we have to have vaso-regulation, the ability for our vessels to dilate and contract; otherwise, we perish. But a single vascular graft may or may not have to do that.

In the same way, our vessels need to remodel as we grow and age or undergo physical stimulation. It's absolutely essential that occurs. But maybe a grafted vessel wouldn't have to do that.

So I think what I'm going to try to do is focus on the first two as essential functions and think about the second two as examples of nonessential ones. And, of course, when we think about vascular grafts, in some ways they're like any other engineered tissue. They're going to have some standard functions. It doesn't matter if it's a vascular graft or cartilage or another tissue. There are some very basic things. It needs to be immunocompatible. It needs to be biocompatible, non-prone to infection. Since those are common to other areas, I'm not going to speak on those.

So essential functions, it needs to be able

to contain the blood. To do that, it has to have an adequate mechanical strength. And so what has been traditionally done in the past is to assess that in what I've called acute evaluation. Basically, one takes their engineered blood vessel or their tube and cannulate it, put tubes in, put fluid in, increase the pressure until it bursts. It's a pretty dramatic effect. It's a very easy endpoint, and it's characterized.

So data taken from one recent study from Cytograft's work, where they were looking at their tissue engineered blood vessels and comparing it to human arteries and human veins. And you can see the middle column. They're comparable. So acutely, it has a very similar burst strength than the native tissue. So it's able to contain the blood for at least short periods of time.

Well, you need to engraft not necessarily a vessel but something you want to graft in, you need to sew in. So how well does it integrate with the surrounding tissue? It has a lot to do with how well it can be sutured, and how well the sutures are

retained can also be quantified.

So it's something that -- some very essential functions of it, the ability to maintain, contain the blood, either through the wall or through the ends, has to do with this burst strength or suture retention.

So many other groups have presented this type of data. And though initially 30 years ago, when Weinberg and Bell were doing their first tissue engineered blood vessels, burst strength was a key problem, I think that's a solved problem. There's many approaches to make a strong enough vessel that has good suture strength. So it's essential. A number of groups have solved it.

What else does it need to be able to do? It needs to carry blood. Typically, or in the past with people's publications, when they try to evaluate their engineered vessels, some people -- again, I'm trying to limit myself to in vitro characterization Forming a blood clot is obviously an in vivo phenomenon, but we can look at it ex vivo or in vitro.

So people have perfused it. For example, here's data showing perfused, heparinized human blood

through a tissue-engineered construct that was either endothelialized on the right or unendothelialized on the left. And you can see platelet deposition.

So what the researchers took from this was saying that the endothelialization of the graft prevented the platelet accumulation, which is one of first steps in thrombosis. So from this in vitro study, they inferred that it would perhaps in vivo not be non-thrombogenic.

Alternatively, you can look at secreted anti-thrombogenic factors. For example, Joyce Bishop in her "Nature of Medicine" paper in 2001 looked at nitric oxide production, which is a soluble agent which inhibits platelet activation. So, again, just thinking about two of the essential ones, ability to contain blood and to conduct the blood.

We can also think about the nonessential ones, saying that it would be, I would say, very nice if the blood vessel has it, maybe not absolutely essential. One might be vasoactivity, as I already mentioned. One way to assess this is using a myograph. Basically, a vessel is cut into a small ring and then

the ring is put in between two protrusions. And basically, the force exerted by the vessel on those protrusions are felt.

And you can add vasoactive compounds that either cause it to constrict or relax and the forces can be -- and here is some data from Laura Nicholson's paper in *Science* from 1999, where she looks at the vasoactivity of some her tissue engineered blood vessels. And she sees the vasoactivity's about 10 percent of what a native vessel might be expected to be. But the key point is the vessel is responsive to the vasoactive agents.

Well, so far I've spoken about how people in the past have looked at the function of the vessels. Of course, function and structure are highly related. So another way to assess the potential for the function is look more directly at the structure.

Almost all the papers in the past that have looked at engineering vessels have given a representative histology. For example, biochemical assays, such as the collagen and elastin, which is the two major ECM components of the vessels. DNA perhaps

is the surrogate of the cell number. We can do more sophisticated instead of doing simple histology. We can have immunohistochemistries or look at a phenotype of the cells, for example. Are the cells in the media, are they really smooth muscle cells? Are they making the appropriate proteins that a smooth muscle cell? Are they in the contractile phenotype? Are they in the secretory phenotype? So these types of things can be assessed by immunohistochemistry. Are the endothelial active or activated? So these types of things have typically been accessed.

So what I think I've tried to do kind of quickly is just go through 30 years of literature where people have been engineering and publishing on tissue engineered blood vessels and to say sort of what's good enough to publish and what's maybe reasonably enough to merit an animal study.

But, again, the purpose of the workshop, or as it was explained to me, is to think about, as we get to the first clinical trial, what are the types of things we want to look at and how might in vitro characterization aid in that?

So the real question I tried to address, is it good enough to merit clinical studies? And in one way, I think it's been addressed. It's been addressed twice at least because there's been two clinical studies with human tissue engineered grafts.

A Japanese group published in 2001, with a follow-up in 2005, making cell construct implants for different cardiovascular applications, primarily in the pulmonary circulation in humans. And after five years with 42 patients, they have relatively impressive results. And the results are ongoing.

So at least once people have thought very carefully about is the technology ready to go into humans. And the second time where I think people have looked critically is what other technology is ready to go in humans or not is the look by Nicholas L'Heureux at Cytograft and his colleagues there.

They have recently published -- I think it was in October -- in *New England Journal of Medicine* of this year the results of 10 or 12 AV fistulas that were done. Both of these were done outside the States, so they weren't assessed here. But, again, so we have

information that they have.

So I spoke to L'Heureux about when he was trying to present his work, was there additional information that he was presenting that's not in his papers; was there particular work to go through the agencies? And he described his work as being characterized well in the published literature.

So the type of work that I referred to in the past as characterization of published evaluation of engineered vessels was what he used to go forward, not in the States but overseas. Not to say that's an appropriate evaluation, but it was -- that's how it was done.

So the question is, is it going to be good enough to merit clinical studies and what can the in vitro studies do. So I think that we can think about it in respect to the safety, the efficacy, and the consistency and what can the in vitro studies do.

But before I speak about the in vitro studies, I think I'd be remiss -- because I really do believe that animal studies really are the key to evaluating engineered tissues. And I can speak about

the merits of the in vitro system, and I think there are many. But I think they need to be coupled with thinking about the merits of the animal studies.

With the animal studies, it becomes an interesting challenge because now we're trying to do human tissue and it raises the question do you want to do human tissue in an animal, in which case now you put it in a xenographic environment and its behavior might be very different because it's in a xenograph environment; or do you want to make the vessel, say, porcine tissue and then put it in a pig? Now you can have a allograft. You can have autologous transplantation if you want but now also take pig vessel. And I think there are merits to both approaches, and they need to be carefully considered.

One consideration, though, I think about is thinking about designing human vessels and testing them, whether they're tested in the lab or putting those human vessels into animals. And I think there's two reasons for that. I think there's already been a lot of species variability shown in engineering of vessels. Approaches that work very well in one tissue

type, say rodents, doesn't translate to pigs, or what works with human cells doesn't translate the other direction. For example, approaches that worked very well with human cells, when they tried to do animal models didn't work so well. They couldn't create their vessels. So there's a lot of species specificity.

But having spoken about the in vivo, thinking more about the in vitro, so how can we characterize the safety of the vessel? I think we can actually do some useful work with in vitro characterization of the vessel.

I think as I get started on the bottom of the list, there's always going to be those standard things for any engineered tissue, and I'm not going to dwell on those. Obviously, the system can't be prone to infection once it's implanted. And it itself cannot be non-sterile or at least free from external contamination.

But I also think that with our in vitro characterization we can talk about this ability to contain blood. For example, the burst strength that we've already talked about, that's a well established

assay. Clearly should be done on in vitro tissue before we would be thinking about going clinical as well as suture strength, again, something definitely to do.

But as I mentioned earlier, when I was talking about these two things, the burst strength and the suture strength, both of those are acute assays. Well, what about the chronic ability of the material to withstand mechanical stimuli? I think this is quite important because one of the characteristics of most of the engineered vessels is they're rich in collagen and they're deficient in elastin. I think that's a fair characterization of almost all the vessels.

Well, why is that important? Well, one of the standard animal models of studying aneurysms is to have a vessel in vivo, inject elastase into to digest the elastin in the wall and gradually let the vessel form an aneurysm.

So what it suggests is in vivo if you have a very strong material because a lot of the strength comes from the collagen, not so much of the acute strength comes from the elastin, you can have a very

strong tissue on the short term, but it can begin to aneurysm.

So the point being, acute evaluations of the mechanical properties might not be predictive of the chronic. And this becomes a question of well, how can we evaluate it? If it's a hip joint or something, purely mechanical, we can put it on a system and give it 1 million, 10 million load cycles in a very short period of time and simulate a lifetime's worth of stress.

I don't think that's something that's appropriate to think about doing with a soft biomaterial like a vessel because its ability to respond to the stresses isn't just its response to the passive mechanical loading but how it grows and remodels in response to the stresses.

How might we use -- perhaps, the best way to address that would be with in vivo studies. But how might we be able to use in vitro or ex vivo studies to address that question through the chronic ability of the vessels to resist mechanical loads?

Well, we have ex vivo perfusion systems which

we can culture and grow vessels in. As I mentioned, this is an area that my lab works in. There's a number of others. We can take intact vessels from humans or from animals, culture them ex vivo, outside the body for a period of up to a month -- the longest we've gone for -- and they maintain their viability and their activity.

So what we can conceivably do is do the same types of things with engineered vessels. We can take engineered vessels ex vivo and put them in some type of perfusion system and see how they behave long term. The system's relatively simple. The diagram's probably more complicated than it needs to be.

It's basically a perfusion loop which fluid is pumped around. By changing aspects of the system, you can control flow, pressure, paucity. And it's well instrumented, so you can assess the mechanical environment and also maintain the viability of the vessel.

For example, when you put a porcine carotid artery in one of these systems, you can maintain it at normal intensive pressure. There's no tendency to

dilate for a period of nine days, ten days. You can put it under a hypertensive environment, and you can see it begins to dilate. And this raises a question and maybe a challenge of interpreting data like this.

Is that an active biological response? Is that desirable? The material is responding to the biological environment by increasing its diameter or is this just passive creep and the vessel's going to go on to form an aneurysm?

From this type of data, I don't think you can discern. We do know that under hypertension, vessels do dilate acutely. They remodel in that direction. So this might be a typical or reflective of in vivo response, saying that this ex vivo assay is capturing that behavior, or it may be a creep. I don't think it's creep from other studies we do. You can inhibit this by a number of pharmacological inhibitors like MMPs. So to us, it suggests it's more of an active.

But the point being is that we can take these ex vivo systems and take the vessels and expose them to in vivo-like environment for long periods of time and see how they change their ability to contain the blood.

Are they forming an aneurysm, for example, or do they rupture and leak?

Well, what about in vitro characterization of efficacy? Well, we talked about the important role of carrying blood. And I've already alluded to people's in vitro studies of thrombosis, platelet adhesion or secretion of molecules that might regulate platelet activation.

I also think that there's at least the potential of using an ex vivo or an in vitro system to study stenosis, basically the occlusion of the vessels. I'll get to that.

But what I want to say before I get to that is that I think though we can study efficacy or we can study things that might be related to efficacy in our in vitro system. I think we can pick out the really bad vessels using an in vitro system. I don't think we're going to be able to say this is a good vessel and this is a better vessel using an in vitro system. Let me be controversial, but let me explain why I have that view.

So if we think about clinical data of

different vascular grafts, saphenous veins from the legs are widely used and internal mammary artery from the chest wall is widely used. The five-year patency rate, just basically meaning is it still carrying blood, after a period of time is about 50 to 70 percent for saphenous vein. Pretty good. The patency rate for the IMA or the internal mammary artery is 93 percent. So basically, you have a failure of 1 out of 2 times or 1 out of 16 times or 1 out of 10 times, depends on what's number.

So one vessel is much, much, much better than the other. Why? We don't know. There's a lot of people who claim I think it's the intactness of the IEL of the IMA. Or other people say, well, if you look at the endogenous nitric oxide production of the IMA, it's much higher or the ability to handle oxidated stress is much better.

So a lot of these studies -- almost all these studies have been based on in vitro characterization. My point being is we have two pretty good vessels, the saphenous vein and the IMA. When we study them very carefully ex vivo, we can't come to a consensus of why

one's doing better than the other.

So I think I could argue through the reverse. If we had two vessels and we didn't know how they perform in vivo, purely looking at their in vitro characterization, it would be hard to determine a good vessel from a very good one. But, again, I still think there's good merit at looking at in vitro characterization. Why? Because I think we can pick out vessels that are likely to perform poorly.

One way when we can think about looking at their in vitro characterization is that we can actually study stenosis of vessels ex vivo. So when we have intact vessels, not an engineered vessel but an intact vessel and we culture it in vivo, we can manipulate the environment -- and I can talk about the details of that later if anybody's particularly interested -- so that we can take a saphenous vein, for example, the graft from the leg that doesn't do particularly well, and by exposing it to a certain stimuli -- actually, the same type of stimuli it would typically see during a grafting procedure -- you get two types of undesirable remodeling.

You get hypertrophy in the lumina; basically intimal hyperplasia with lumen being the internal area where you're supposed to be carrying blood is becoming occluded. Why? Because you have a proliferation of new cells.

You can see in both of these vessels there's a dark ring in the internal regions. That's the intima, the IEL, and on the panel on the middle, you can see there's a large deposition of new cells inside the IEL growing and basically occluding the area where the blood should be flowing.

Another thing which I think is more subtle, but is actually more occurring pretty dramatically here, is that not only are you getting hyperplasia going in that the vessel's growing in to close off its response, but the vessel is remodeling in.

So you might say well, how are they different? In one you have proliferation of new cells and hypertrophy or new addition of material. In the second, you have the same material, but it's changing its non-loaded configuration. So basically, instead of being a vessel that looks like this, it's grown like

this so the inside's thinner. The walls get thicker to compensate.

So you can have two types of remodeling in these in vitro systems. And so I think that we can use these systems potentially to look at types of stenosis that might occur in tissue-engineered vessels as well.

In contrast, though, if you look at a cultured carotid artery, an example of an artery that would potentially do fairly well in a grafting procedure under the same chemical and stimulation, it doesn't develop this intimal hyperplasia or inward remodeling. So the point is I think these in vitro model systems can have a potential to look at stenosis.

If we think about in vitro characterization and consistency, I think this is the one thing that in vitro can do incredibly well. We can look at biochemical composition. We can look at anything we want. You can do gene arrays. You can do sRNA arrays. We can characterize it as much as we wish. We look at cellular viability, proliferation phenotype. You can look at the dimensions of the vessel, the organization, the structure, the components in the walls. You can

study the mechanical properties.

I think this level of characterization might be useful because I think we could do better than has typically been done. For example, people look at burst strength. Another important aspect is the compliance of the vessel. This has been studied in some engineered vessels, but the analysis is very incomplete.

By using the same type of perfusion system I already mentioned, we can do a proper biomechanical analysis of the vessels. I won't go into the details because I see the moderator's already standing up. But basically, what we get out of it is very specific stress-strain behavior, not something with the compliance or some heuristic level response.

You can get extremely specific stress-strain behavior out of these types of testing. You can do parameter fitting, and you can do statistical analysis to say this vein has a very different mechanical property than another one as opposed to doing something as simple as a burst stress, which is very often done and only occasionally done, something to look at

compliance. So I think there's abilities to do this better.

The same way we can look at elastin or things like that, or structural components that most people look at. I don't think they look at it too carefully because they don't have a lot, and they often don't acknowledge that or highlight that issue as strongly as they might.

But we can look at histologies, and we say well, there's elastins all in each. We can do a biochemical assay, which you can see in the bottom left, there's about the same amount of elastins each. If you think about this more carefully, you can do quantitative morphometrics and look at the elastin fibers and look for breaks in the strand. That's something we can do with quantitative image analysis. And disruption of elastin pre-moves the development of intima hyperplasia, and we can see in the panel on the lower right, vessels under different conditions have extremely different continuity of the elastin. Even though there's the same amount, whether you look at histology or biochemical, by doing quantitative

morphometrics, you can get more information.

So I think consistency is really easy to access. It has a lot of potential to spot, I think, problems and to look at the desirability of the product. I think the problem is the consistent product is not necessarily a functional one. So we can have a highly consistent vessel, but it may not do well in vivo. I think that's obvious, but I think it's a point we need to think about.

We can also think about the existing vascular grafts. So I do a lot of work where I get excised human saphenous veins that's discarded after surgery. And I can see there's a huge variation in these tissues. If I look at the amount of intima hyperplasia, it might go from .5 millimeters up to 3, so sevenfold. Any characteristic, I can look at these vessels; they vary a lot. But they're routinely used, and they routinely work fairly well.

And I think a reasonable question is. is the engineered vessels -- the one we want to eventually make and go into clinical trials -- how consistent does it have to be relative to something we use every day in

the clinic, 300,000 times a year for bypass grafts? Should it be as consistent? Does it need to be more consistent since it's engineered and maybe it doesn't have to meet the standards?

I think that's a reasonable question. I'm not trying to offer an answer, but I think it's something that we need to think about. We can measure consistency extremely well. How useful of a metric it is for predicting function is not clear unless one is extremely careful about what one is measuring.

And the question is on what level of standard do we want to have consistency relative to, say, a native tissue? I will skip this because I acknowledge that I think I am out of time and I want to save time for discussion.

So hopefully what I've convinced you, or at least presented some information on, is that we can think about the vessels from the essential functions or nonessential functions. There's very good ways to assess these in vitro.

And though not all these assays would necessarily be useful, I think a lot of them can give

good insights into predicting the potential performance of engineered vessels. And I think a very good use of these in vitro studies is to spot potential problems, and that working in conjunction with the animal studies are quite strong together.

Thank you for your attention.

DR. HURSH: We have time for a couple of questions if there are questions from the audience.

So I was curious, how do you achieve your stenosis in vitro?

DR. GOOCH: Okay. So to get a stenosis in vitro of a saphenous vein, you expose it to arterial levels of oxygen. That's, I think, incredibly interesting. It's not what we're talking about today, but I think it's really interesting because when one takes a saphenous vein from a leg and puts it in the coronary circulation, it's exposed to arterial levels of oxygen. If you have an antioxidant, it doesn't happen.

UNKNOWN QUESTIONER: (Inaudible). You're absolutely correct in terms of the differences between the various species. And doing trans-species, it's

really very, very difficult to -- even if you're using a (inaudible) cells, the proteins are different, particularly (inaudible).

So do you think, in your mind, as you're thinking about heart valves and blood vessels being issues, where potentially being put into yourself down the road or to one of your child, would you like to have a primate (inaudible)?

DR. GOOCH: Yeah, and then it becomes a question of do you want human within -- nonhuman/primate or do you want human/nonhuman primate back into nonhuman/primate?

So, for example, the *New England Journal of Medicine* paper that I referred to, that was their standard. They did mice, and they did primates. So I think it would be a very useful step and very doable. It's not unrealistic a request.

DR. NYBERG: Scott Nyberg from Mayo Clinic. You showed the data that there is clinical application. What's the biggest barrier from additional clinical studies? Say the work you're doing, what is the biggest barrier in this field?

DR. GOOCH: Well, you're asking specifically the work I do? I potentially think the ex vivo remodeling of vessels is an interesting idea, but about three years ago, or maybe more, I began to think about it as more of a useful scientific tool.

As we've talked about the potential tissue engineering and the study of pathology, for example, intimal hyperplasia -- to answer your question, I think what you're trying to ask, though, was more general. Was it within the overall field?

DR. NYBERG: Right, if it works, if the vascular conduits work in a low pressure pulmonary venous system, why haven't they been scaled up to arterial systems? Is it a strength of the construct or --

DR. GOOCH: So the work from the Japanese group, I think it was a strength issue. However, the data that I showed is currently -- that published just two months ago in *New England Journal of Medicine* -- is done with humans with a AV fistula. So at least the upstream version of it, side of it, is exposed to arterial levels of pressure.

The reason they went with the AV fistula wasn't to do with the pressure, but if you had failure. So obviously, the bleeding there is going to be an issue and potentially life-threatening. But it wasn't the same as putting it inside a chest cavity or other locations. So they chose that location not due to pressure but sort of safety in case of failure.

DR. BERTRAM: Yeah, Tim Bertram. Thank you. Insightful presentation. I have a question.

You were just about, I thought, to answer it when you started talking about the pathology. But that actually is the question.

And that is is, I was impressed that you've been able to use the in vitro systems to get at deeper insights into what I think many would consider to be kind of an in vivo question, restenosis being an example.

The vessel has many other challenges. The burst strength, which is common, that's an easy one to imagine. However, there are other pathologies, intimal hyperplasia, for example, post-implant or post to the insertion, inter-positional, for example.

Have you considered using in vitro methods to begin to assess pathologies that may develop as a way to predict or screen through various vascular prototypes?

DR. GOOCH: Again, to answer your specific question, no, I haven't for my own research group. Is it a reasonable concept to pursue? Yes, I've thought of it. In the general field, it'd be very reasonable.

So, yes, one can look at intimal hyperplasia and one can look at other aspects. You could look at calcification potentially. Those types of pathologies could be explored.

DR. BERTRAM: So then to make it specific -- sorry for one follow-up. So I know we can. Have you done that?

DR. GOOCH: No. I have not.

DR. BERTRAM: Oh, okay.

DR. GOOCH: And to my knowledge, others have not either.

DR. HURSH: Okay. I think we need to move on, so --

Our next speaker is Tim Bertram. Dr. Bertram

is a senior vice president for science and technology at Tengion. He received a doctor of veterinary medicine and a PhD from Iowa State University. He has been a senior scientific executive at Pfizer, SmithKline Beecham and Procter & Gamble and has active academic appointments, including one to the Industrial Advisory committee at the UCLA's bioengineering department.

His talk today will be on an in vitro analysis of a scaffold cell product, Tengion's autologous neobladder construct. Welcome, Dr. Bertram.

DR. BERTRAM: Thank you. A couple of things. One, I'd like to thank the organizers for the invitation. I've been very impressed with the speakers and so now to the audience, hopefully, I can continue that. If not, then there's a drop. I know it's a postprandial depression. So be patient with me, I'll try to wake you back up. And Melissa's to follow. So I'm sure it'll get more exciting as we go along.

What I was asked to talk to you about was to give a background on Tengion, what we do, our development pathway and then some specific applications

of how we're using in vitro methods to characterize the product that we've got, its purity, its potency, and the use of in vitro assays as a mechanism, if you will, to move into the clinics.

Then lastly, we'll talk about some challenges and opportunities and suggestions of what we might consider for new assays to develop.

What I'd like to do as far as the background is basically provide a very high-level overview of how Tengion got to where it is. Many of the works and discussions that were talked about by Nancy, some talked about by Rocky, and a number of them mentioned by other speakers have been done and were done using in vitro methods as a means to select what particular product and product components that might be developable.

That's actually the key point and where I'm going to focus this presentation today. So in about 10 to 15 years of research, using a number of these different in vitro assays to select cell, understand cell, biomaterial compatibility, to look at different biomaterials that might be appropriate to engineer a

complex organ, such as the bladder, these studies were done and a series of decisions were taken.

These decisions ultimately culminated in a -- actually a series of clinical studies. Now, the clinical studies were reported recently in The Lancet. But what that provided in the foundation for Tengion, which licensed the technology and utilized that information that had been done over the years, was it allowed us to understand the in vitro methods, extrapolate them importantly to how they might work, or act, or predict the animal work, the preclinical development work as Nancy talked about. But even greater, how, in fact, these assays might in fact lead forward and actually be functional, and be able to be applied into a clinical situation.

That, in fact, is what I would like to share with you. I've always been asked to give a brief overview of how these assays are being used in our current FDA-regulated activities.

We currently have three Phase 2 studies ongoing, focusing on patients that have bladder failure. The evaluation of other populations is being

considered as we go forward. But, importantly, what we're doing is we're working very closely with FDA as we go through and try to explore how we can use the information that's been gathered to make these products.

Again, what I'll do for the purposes of this presentation today is show how we've actually deployed in vitro methods to get to this place.

So at a high level, our development program used preclinical studies. These were both done in vitro and in vivo. And as Nancy indicated, many of the extrapolations between the in vitro and the in vivo were done during these investigative studies.

As someone mentioned, the preclinical program is really set up to translate forward. If you will, it's the in vitro aspect of the clinical trial. Carrying that forward is quite important, but being able to have in vitro methods that can actually begin to predict that are quite significant.

In particular, those in vitro methods were relevant into the biomaterials characterization. Buddy characterized a number of and demonstrated a number of

different assays that were used. We've used various assays of that nature to characterize the various biomechanical properties of the scaffold that composes our product.

In addition to that, though, we use in vitro methods to look at bioprocess. That, in fact, is probably the most significant place. And was discussed earlier, Nancy again talked about bioequivalency and the use of in vitro methods to make change control and manufacturing.

That's actually going to be where I focus down later parts of the talk. But I want you to just benchmark that. That is a place where we have used in vitro methods, I think, to quite good effect.

The second stage of our development package is actually in the process control. And, again, this is a place where in vitro methods play a significant role in the development package, not only in biomaterial testing, assessment, what the outcome is after it's been placed either into the animal or should it have been placed into another situation, looking at its degradation such as in humidified environments or

dry environments.

Also, then in the product characterization, in the purity and potency, this is where the in vitro assays are particularly useful and can be deployed to substantial effect to ensure that the product has its consistency and be able to communicate to the regulatory agencies the information that they know your product actually is consistent and it's going out in a way that you're going to get that outcome in the clinics, which is really, for our company -- not every company but for our company, the clinical outcome and the clinical utility is what we focus on. And anything else is really just part of either supporting that activity or is not relevant for the development of this product.

Then lastly, in the clinical program where we do our toleration and efficacy studies, these three aspects of the way we look at the clinical program would, in fact, deploy in vitro methods largely from the product production and possibly to test any outcomes following, for example, some of the standard methods that one might do; for example, urinalysis in

our particular case.

So the point here is is that in vitro methods actually are used and deployed throughout the entire development process, but they're selectively cut and selectively deployed to achieve the development objective in order to get the product into a commercial setting.

So as part of the product production, our scale-up, in vitro methods, again, played a very, very significant part. However, they more represent the pinnacle, if you will, the peak of a mountain of data that rests below them. The mountain of data includes the extensive amount of in vivo testing that's come, particularly as a result of the preclinical studies.

That, coupled with the clinical testing and looking to see that the outcome of the product matches the in vitro measurement point, allows us then to begin to make those extrapolations between what we've done and chosen to use as a measure of the product and what that -- whether or not that's actually predictive of what actually goes into the patient.

Ultimately, these are combined in a way that

we would carry those methods, the in vitro test method or the appropriate methodology, forward into a manufacturing and commercial environment.

But the in vitro method forms a foundational element and eventually may form a surrogate mark through the development process to monitor and step into the development procedures.

So what, in fact, are some of the specifics and how are we using it? We have a product that is exactly as was described here. It is a cell and a scaffold. Those two elements form the raw materials. So in vitro methods can be used to assess those raw materials.

We actually, though, think about the deployment of in vitro methods not only on the raw material characterization in the process but also in the characterization of the final construct that goes out.

And, in fact, this is going to be one of the things that I'm going to challenge folks, I hope, such as Buddy and Rocky and others, who are thinking, and possibly NIST -- who are thinking about new development

tools. As one tries to non-destructively, non-invasively sample that product, which is a living product as Nancy points out, which is a product that's now sealed to comply with GDPs and GMPs, and it's a product that's about to be placed into a human, how can we peer into that product and get some of the key and critical assessments using methods that are purely in vitro?

I'll talk about that. But in general, we look at these two as slightly separate. Even though the in vitro methods may be similar, they're used for different purposes.

Basically, what we do, then, is we take the biopsy from the patient. We have an autologous product. Those cells are isolated that we want to use, the urothelial cells. The smooth muscle cells are isolated and expanded. Again, in vitro methods then form a significant component of our whole production process.

The scaffold itself, as I already talked about, is characterized through the different methods Buddy chatted about, and then we form the construct.

And it is the construct where the unique challenges, I believe, exist for us to look at new in vitro methods, particularly as it's being packaged and shipped to the clinical site and the physician for ultimately surgical implantation, and then the regeneration of the bladder as it exists for that patient who has bladder failure.

So now to take a step deep into where exactly these in vitro assays are used and how we deploy them to generate a product.

In the case of the raw materials, looking at that aspect of our process, clearly, what's critical is the phenotypic analysis of those cells. This was clearly articulated by Rocky. It was talked about by others, that what you've got to do is make sure that the cells that you're putting on are the cells that you believe are appropriate. In vitro methods are paramount.

One of the things that's a little bit different here -- and I would like to have the audience recognize -- many of the things that were talked about of what we can do with characterizing the phenotypic expression of the cell, at this point in development

when you're actually beginning to manufacture or produce that product, you don't do all of the possible assays. You've got to check and decide which one or two, or whatever's appropriate, that actually tells you you've got the right cell.

In our case, with smooth muscle, we chose alpha-actin. If there are any smooth muscle cell biologists in here, I know there are a hundred other different phenotypic markers that could be used, but that was the one that was chosen because of its consistency and its ability to be most predictive for what that outcome would be.

Again, the methods that are used, any number of different analytical methods can be deployed when assessing a particular phenotypic expression. We happen to use flow cytometric, or as on occasion, we would use manual methods, such as cytospin. Either way, the use of these in vitro methods allows us to capture and characterize that cell type and to know what we're dealing with.

The second cell type is urothelial cell. Again, in vitro methods are critical. And, again, like

the smooth muscle cell, if anybody's a urothelial cell biologist in here, there are many different endpoints one can choose.

However, in this case, the cytokeratins are very specific. They mark epithelial cells. Because we have an autologous product, anything that was isolated would have been an urothelial cell because the sample was taken from the bladder. So it's autologous and homologous. So the expression of cytokeratin would be done in the urothelial cells. Same basic analytical tools are used, again, deployed with different purposes.

One of the things that is quite significant, and it is an area where there are opportunities for growth, is, in fact, evaluating the propagation characteristics of the cells. Because in a production environment you've got these cells growing in closed systems, the ability to monitor those cells using some form of imaging technology, which has been alluded to by some of our speakers, actually is a very powerful tool.

To date, there are some types of methods

available, but they are not ideal, and they don't allow you to look at things such as cell factories which have multiple layers. So there's some challenges to some of that technology, but those methods allow you then to do your phenotypic analysis.

I won't go into detail on the scaffold. Buddy did a fantastic job on that. I'm going to jump forward.

The other thing that has received no mention today or so far, Melissa may, but in vitro methods go beyond just the scaffold and the cell when one's looking at the application of these in vitro methods for the production of a tissue-engineered or regenerative product.m And specifically here, I'm referring to the bioburden or the ability to assess whether or not there is some form of contamination. This is critical. A whole set of guidelines have been developed, the good tissue practices, which will actually focus on this in a concern for cross-contamination.

I would introduce here, just for the purposes of broadening our discussion and make sure we've

covered the waterfront, that we consider some in vitro methods that allow us to get rapid assessments of things such as mycoplasma, endotoxin, bacteria.

I think here is another area where there are certain assays that are available. I'll talk to those in a minute. But there are opportunities to improve, particularly taking those opportunities and giving them at the time of product release.

The next area where we use in vitro methods is actually in the area of the construct. This is an area that, as I said, presents a relatively unique challenge. Again, the components of what we've got have to be characterized. We have cells on a scaffold. So those two same elements have to be evaluated in some way.

However, in this case, we can't reach in. We can't take a sampling out of the cells that we're expanding. We've got now a contained and specific construct, and to do any destructive testing on it means that the patient does not get what you've promised them to give them, which is a regenerative template.

As such, developing mechanisms and in vitro methods that allow you to go in and begin to look at the cell function without actually taking that cell out.

The approach we've taken is to use metabolic assays, and we've used actually two different assays, including glucose uptake and lactate production. That allows us in a mixed population cells, smooth muscle cells, and urothelial cells, to look at cell viability, cell growth characteristics. And it also allows us then to understand if the cells are still metabolically active.

It's very much analogous to what Nancy pointed out when they did their assay for skin growth. Is that a living product? Is there something alive in that? And you have to show that that exists. And so this would be an analogous in vitro assay to that kind of thing.

One of the physical dimensions -- again, because we cannot touch it, the size and shape are evaluated. It has to go out looking like a bladder. This isn't a kidney. This isn't another hollow

internal organ. So those are assessed.

As far as the final product release, one of the things -- and I alluded to this in the previous slide I wanted to talk a bit more about -- is the bioburden.

If anybody here represents these companies, your products are clear, they're qualified, validated, they're useful. But I do think that we can begin to think about some assays that are actually more specific, that actually may allow us to get answers back more quickly. Particularly for us, a challenge because we have an autologous product, the bladder, where the sample it is taken from is not a sterile environment. So bioburden is something that we deal with every single day. And to get an assessment of that and to be able to evaluate the potential risks to the product are quite significant.

What I'd like to do now is turn this 90 degrees, so basically showing you how these in vitro assays have been used in this part of the process and then how, in fact, these are used to make decisions in the production process.

So there are parts where in vitro assays become go/no-go assays. Basically, if the product as it's being prepared for clinical use doesn't pass one of these tests, the product is not provided for implantation.

We look at this -- we have three of those. We consider bioburden, fitness for use and purity to be the major key points of the end process characterization; the test center, as those are outlined on the left-hand side of the slide.

What I wanted to show this group, and really the only purpose of putting this slide up is if you look in the middle column in the manufacturing steps. If you look to see how the in vitro method is deployed, it's deployed in multiple places at multiple times.

And so the data that's accumulated over time on a given lot actually can be used to follow that particular production process. And by doing multiple samplings, you not only increase your confidence that the in vitro assay has given you the data that you want, but it's also telling you that your product that's going to come out at the end as actually working

properly.

In addition to that, we use in vitro assays as a final product characterization step. Again, as indicated, we've got now a sealed, closed container in which the product is ready to be implanted. The next step is for the surgeon to actually place it into the patient.

In this particular case, we look at four different in vitro assays: Bioburden, that one we've discussed, the purity, the potency, and then the final product characteristics. As I've indicated before, we've used this in a way that has allowed us to follow the in-process tests. So it was used as no-go/go criteria. But here what you can see is that the final product characteristics has certain elements it has to pass before it can even be released.

So this, if you will look at these, these are actually stop criteria. If it does not -- if that in vitro assay does not give us the right answer, the product production is stopped and it is not provided to the patient.

So what I hope you've seen from this -- and

this is taking a slightly different cut, slightly focused area, where in vitro assays can be used not only in the preclinical, also in the clinical areas. But it can also be used in a manufacturing environment where you can make some real decisions in real time that allow you to put a product forward that is, in fact, going to be safe and effective.

The last thing I want to cover here real quickly comes back to something again we haven't talked much about. But if we're going to think holistically about the generation of these products, I think we have to think about the environment in which we manufacture these products.

And here, again, in vitro assays could really be greatly deployed. I won't go through those here. But anyone that's involved in any kind of environmental monitoring, in vitro assays form a foundation for assessing the environment and ensuring that you're meeting the appropriate standards.

The one thing that I bring forward here -- and it was alluded to by another speaker, and I'm sorry, I forgot who it was. But somebody talked

about the use of in vitro assays to rapidly assess media and its components.

That message was basically the same one I wanted to deliver here. As we look at all the potential areas for in vitro assay application in the production of -- and development of tissue-engineered regenerative products, the media and its components and monitoring this particularly -- not so much with ours, but there are some examples where the media elements are somewhat labile. And having a way of getting quick assessment of whether that media still has what you think it has is an important aspect. And I think in vitro methods would be quite useful with that.

One of the things I was asked to do was to talk about the production challenges and where in vitro methods may, in fact, be able to help us. So for us, the material sourcing is a significant challenge. We have an autologous product. It comes in non-sterile, so in vitro methods that would allow us to assess the bioburden in that particular sample would be quite useful.

In addition to that, one of the things that's

quite significant is process segregation. This is more of a manufacturing question than really an in vitro question. But since the manufacturing is done in vitro, I've kept it here because actually having the segregation of the process -- co-culture of cells, the ability to do that, to be able to consistently do that kind of activity, having different types of process growth environments like the cell factory that would allow for mass production of primary cells -- those kinds of things would be very, very useful types of in vitro methods to have. Again, the application really relates more to production.

One of the things that is a very, very significant challenge for us is that our lot size is one. Basically, what that means is when we've got final product, having an in vitro method that we could actually depend upon, that would give us the whole set of different endpoints that we need to assess that, could be done very quickly and would save hours of agony as we wait for results to come back.

And that's something -- again, here I would emphasize is probably an in vitro method that has

speed. So as one thinks about developing these assays, an assay that could be done quickly, colorimetric, possibly, somewhat quantitative and so on.

Then lastly as a point here, the automation of the process is something where in vitro methods can actually be quite significant. I already talked about the use of different types of cell factories that could be used to grow primary cells more effectively.

But having these different types of in vitro methods become automated would allow as you go into scale, as you start making -- go from making tens to hundreds of these to thousands of these, being able to assess these different endpoints appropriately and rapidly would be quite useful.

Just before closing, one of the questions I was asked to address was what are some potential approaches to product characterization that might benefit from new in vitro assay development. I really look forward to tomorrow's discussion. I think this is where NIST and at least industry and obviously FDA can come together because one of the big challenges we see for us -- and again, because of a lot size of one -- is

the ability to have non-destructive assessments. It's one thing to do an assessment on an entire cell population. It's quite another to do an assessment on an individual cell within a construct where you want to understand the relationship.

As we've already talked about -- I believe Buddy or one of the speakers referred to it as that cell/scaffold interaction. Those can be quite relevant. And being able to assess those on an individual cell basis in a non-destructive manner -- again, envision the process that we're confronted with -- and I would contend that any other company that's thinking about doing this type of work will have to contend with, and that is you've got a closed, contained, living product.

How can you follow that and be assured that that product is the product that you want when you can't touch it, you can't take a piece of it, you can't do something with it and you want to give that assurance that is, in fact, the product that is appropriate for that patient.

Being able to use different approaches,

imaging I put here. Imaging has been mentioned by others, so I won't spend a lot of time on it. But the ability to image the viability of that cell -- not plugging any particular product here, but General Electric, for example, has a fluorescent metabolite that plugs into the hem synthesis pathway and it will fluoresce. I understand it's FDA approved for the use of diagnosis of bladder cancer.

Those kinds of markers that could be put in to follow the cells, cause them to fluoresce, would be very useful as non-destructive methods. And I think those kinds of assays, being able to develop and explore, would be quite powerful.

Just in summary, then, as we look at the use of in vitro methods in combination product development, for Tengion and our neobladder augment product, we are regulated under the leadership of CBER in collaboration with CDRH, so we deal with both of the key agencies.

As Dr. Witten pointed out, they're involved in regulating products. We will try to meet and use in vitro methods that are appropriate for CBER and in vitro methods that are appropriate for CDRH, one on the

scaffold, one on the cell. And then as I've indicated, that construct having to meet really the needs of both.

Obviously, all of our manufacturing, and where I've really focused on for the purposes of today's discussion, fall underneath the regulated guidelines that have been put out and can comply then in both terms of assay validation as well as qualification.

One of the things that I think is quite useful, in vitro studies and in vitro assays allow us to assess the attributes of that product. The ability to be able to use the in vitro methods to look at purity, potency, functionality -- many of the previous speakers have gone through that -- this is really where it's quite impactful in the whole process.

Importantly particularly, for at least Tengion, where we can extrapolate and understand the outcome of that in vitro assay as it relates to the animal study and then, ultimately, how that in vitro assay and that animal study relate to a clinical study, those pieces allow us to be able to select and choose the appropriate in vitro assays that one can use and

whittle down all the different assays that have to be done.

Just lastly, the challenges I put forward -- and I look forward to the discussions tomorrow, some of the thinking of those folks that do think about bioanalytical methods and how we can actually use a method that will allow us to characterize these products, look at the raw material and develop these assays for better reproducibility and better assessments, for confirming that the product, in fact, is appropriate and safe and effective.

With that, I'd like to thank you and I'm happy to answer any questions.

DR. HURSH: Questions for Dr. Bertram?

MS. HUNSECKER: Hi, I'm Rosemary Hunsecker, NIBIB NIH. I forgot before to introduce myself.

When you had the sort of schema up there about what looked like sort of broad descriptions of release criteria, there was a focus, very appropriately I think, on qualifying the metabolic activity, the functional activity of the cells, and about the safety aspects of the microbiological quality of those cells.

But you didn't talk a lot about qualifying the material, the construct.

So I was wondering if that's because as a precursor to even getting started with populating these cells, there's an underlying whole other QC process that you didn't have time to discuss.

DR. BERTRAM: Yeah, and, in fact, that's exactly right. I focused largely on the biologics side. I knew Buddy was going to be talking. The scaffold side is pretty well characterized, and I didn't go into that. But, in fact, just as you said, there's a whole QC. And there's a series of specs that the scaffolds have to meet in order to even enter into the production process. All of those specs are defined. They're all clear for a biomaterial. And because of the stability of a biomaterial itself, I didn't review those.

It's also -- just to emphasize the point Buddy made, most of this stuff has gotten relatively straightforward. And so you can get pretty good characterization of most biomaterials that you can use at least in a clinical setting.

MS. HUNSECKER: But those materials, those empty bladders and whatever you call them, they have to be fabricated by you. You can't go to a catalog somewhere. You still have to manipulate those biomaterials into the structure that you need.

DR. BERTRAM: That's correct, yes.

MS. HUNSECKER: Can I have one more question? You also alluded to the fact that there's a -- you get to a point with these constructs where they're kind of ready to go from a biological perspective, but there's still a lot of safety questions you have about -- for example, the microbiology, et cetera.

So are you, and perhaps others, giving thought to stability and stasis technologies, like how do you freeze these things and keep them functional or other things that would aid in the distribution and the maintenance of these things while they get from the point where they're ready and the point where they're actually at the surgical suite?

DR. BERTRAM: Right. So there's two elements to that. One of them is -- Rosemary, you can maybe ask to have a follow-up because I'm not sure there's -- I'm

going to address your question two ways.

One is how it relates to in vitro methods and then the other one, which is I think a product development question, which I'll add. I'll answer the first one on the in vitro.

That was my point, in fact, about having assays that could be done in real time as you go forward. When dealing with a living material -- I won't call it a tissue, but it's a living material. The moment you make that measurement, as soon as that measurement's done, it has changed by definition or it wouldn't be living.

The ability then to have these very rapid assays -- so I very much agree with you. Yes, we put a lot of thought into that to figure out how we can get those assays as quickly as possible, and we're always looking for someone who's got a rapid assay.

In terms of the -- this may be the follow-up, I don't know. In terms of the product development question about freezing or transport, we actually studied quite extensively to look at what would be the best approach to provide an optimal product that the

patient could use very quickly, very effectively, and would be consistent with -- give us a regenerative outcome.

We happen to have chosen an approach of giving these fresh. Now, that was an approach chosen after doing a series of studies and was considered to be optimal. Obviously, we have built into our process a series of stop points so that if for some reason the patient can't have it implanted, things can stop. But that final product is actually shipped fresh.

MS. HUNSECKER: It's interesting. When I speak to the guys at Genzyme, they tell me they know the airline schedules for their hospitals they contributed to better than the airlines.

DR. BERTRAM: Yeah, I can pretty much identify with these Genzyme guys.

DR. HURSH: Dr. Ratner.

DR. RATNER: Yeah, Buddy Ratner here. You covered a lot of really interesting points. One of the things you said is that as you're going along, if the construct is not looking good, you would not give it back to the patient. But, of course, you have their

cells being cultured. Would you try it again, or alternatively, would you study those cells and find out what went wrong?

DR. BERTRAM: Yeah, both actually. So to your point, again, it was a little bit Rosemary's question also. I didn't get into the specific development or manufacturing, if you will, process step-by-step.

There's a series of backup or, if you will, rescue steps that come in. So if we get to that point and for whatever reason, I'll make this -- it's contaminated. I mean choose your favorite reason why it doesn't release criteria.

What happens then is we go back to the backups and we start the process over. And, again, each one of those stop-start points has got a time. So we know exactly the time. It's not only as Genzyme, they know the airline schedules. We know the schedules of the cells, what the production time takes. We can immediately deal with the patient and the physician.

Your second question slipped my mind. Sorry.

DR. RATNER: Yeah, so if things don't work,

you do have these cells. Would you examine the cells to see if there's something special about them?

DR. BERTRAM: Yeah, the short answer is yes. It's amazing as we've worked -- we've focused on the process, the process itself, because the raw material varies. The process has got to be consistent as possible. And what's amazing is we're actually finding a fair amount of consistency within the process itself.

Now, to your question do we study them, we study them to the extent that we can, that the patient has given us release. So we do not do genetic analysis. There's been no authority granted to us on that.

But to the extent that we can study them, we take every opportunity to understand what we could do different with the process so that if we encounter a patient like that again, we can adjust to that and then deploy that as a general process step.

DR. TUAN: So, Tim, that was a great talk. Rocky Tuan, NIH.

So I was really happy that you mentioned the bioburden test. And it's not only for opportunistic

infections that happen during the process, but it's because a lot of the tissue degeneration and what have you that we have, result from either sepsis or some type of infection, or either viral, bacterial, whatever.

And so inherent in your analysis, then, you have to take into consideration the bioburden that comes with the source. And to what extent do you take that particular piece of information in your go/no-go from the get-go, really? So I'm just curious on your thoughts on that.

DR. BERTRAM: You've landed exactly on the point that autologous company -- whether they get their samples supposedly from a sterile body site or not, we all know the body is not a sterile environment. It can be contaminated at the surgical site. So you've actually landed on probably what is the most significant challenge.

It goes outside of the specifics of this, but this is where having an assay that occurs at the moment of the sampling could the physician tell us that this has got a bioburden. Some states do not allow to

report certain viral infection status. Well, we don't want to be expanding and causing more severe viral infections if, in fact, the patient -- so having a rapid viral screen as a mechanism -- and there's a whole series of these.

So you're exactly right. And a lot of our effort -- and, again, it's outside of the discussion here. But a lot of our effort is looking at how we will deal with bioburden. What do we encounter? How can we clean that? Can we clean it? Is it possible? What can you do with it? Or do you just basically tell the patient no?

And so those kinds of decisions are where in vitro assays are very powerful because if you can get them done fast, then, in fact, you can refer the patient to a different therapeutic modality.

DR. TUAN: I think that's very important, yeah.

DR. BERTRAM: Yeah, yeah. Well, it's also very significant -- sorry, just one other point, if I might, if I'm going over time.

One of the significant challenges we've

got -- and it goes outside of this, but in vitro assays will help it, and that is cost of goods. The fewer times that we have to do expensive assays but the more data we can gather during the process is going to drive cost of goods down. And therein lies one of the most significant industrial challenges, if you will, business challenges, if there is using and finding in vitro assays that help us give quick answers and make quick decisions.

DR. HURSH: Okay. You've been waiting longer. Sorry.

DR. PARENTEAU: I have a question, and maybe you can elaborate. The criteria that you have for go or no-go are really release criteria, and you're making a rather complex construct. So somewhere along the line there had to be a validation of your process.

So did you rely on human cells in destructive testing to understand that or animal or both? Can you just elaborate a little bit on that?

DR. BERTRAM: Yeah, actually, all of the above. The process we went through to validate this started with cell lines, then went to primaries from

animals. Then actually, what we were able to do there is -- it is possible to get human tissues that are donated to various appropriate means.

We then moved into the human tissues to show that the processes would work and would give the outcomes that we wanted.

DR. PARENTEAU: So even though they're not release criteria, do you internally have a characterization of that construct, which really is your product?

DR. BERTRAM: Yeah, yeah. I didn't get into the details of what the product specs are here. It was just --

DR. PARENTEAU: It would be lovely if you could have a little mini me somewhere going along the process so that you could then gather additional data that correlates with the clinical response.

DR. BERTRAM: Well, maybe I missed your question. The mini me I thought was a second construct. What did you mean by that?

DR. PARENTEAU: A tiny -- either an in vitro methodology that made the link or like a little mini

me, a little -- that could somehow -- because to gather that data, especially during your early trials, would be so valuable to you.

DR. BERTRAM: Well, there's several different points in that. We actually did a fair amount of consideration on how to do the mini me experiments and how it could be done: tags, dual processing, a lot of different potential challenges.

The destructive testing that we did to form the foundation of this became consistent enough that we felt that we could use that.

The problem in terms of actual clinical production, as you know full well, or to commercial production, as you know full well, the surrogate is just that. It's like a biomarker as Rocky was talking. It still doesn't tell you what you're actually putting in.

And so one of the things that I'm hoping -- and particularly maybe tomorrow it'll come -- is we can develop methodology that can be used in vitro, that is, if you will, invasive without invading or destroying that. Some of these markers

that tell us about the cell, tell us about the construct, its degradation are really the kinds of things we're hoping to see develop.

DR. HURSH: That'll be the last one then.

MS. PLANT: That was really a nice talk. Anne Plant from (inaudible).

DR. HURSH: I'm not sure it's working. You may just have to shout.

MS. PLANT: I was just curious about cell sourcing also and what you do in the case of bladder cancer, and how do you evaluate cells from the patient in that case.

DR. BERTRAM: Right.

So did everybody hear the question? Not, okay.

Basically, it was about cell sourcing. What do we do about bladder cancer and how do we confirm the cells that we've got?

Actually, I did not make the purpose of this discussion right now. That is a whole separate development challenge that we've undertaken, and we're in the process of doing.

Maybe to make a very long story quite short is that through a series of preclinical studies what we've been able to do is determine what aspects of our construct are necessary to regenerate a bladder. In that understanding, what we've learned is how to deal with the cancerous urothelial cell.

I'll leave it there. I can get into more details, but the more I answer the bigger the question will become and more confused is the potential -- but basically, it's a key question. We've got a lot of development activity on that, and the short answer is that we're very close to having that tagged.

DR. HURSH: Thank you very much.

Okay. While Bernadette brings this up, I'll go ahead.

Our next speaker is Melissa Carpenter. Dr. Carpenter is the vice president of research and development at NovoCell in San Diego.

She received a PhD in neuroscience from the University of California at Irvine. She has carried out extensive research in the area of embryonic stem cells and has been associated with the companies

Cytotherapeutics, Geron and CyThera.

Her talk today will be on an in vitro characterization of a pancreatic islet construct. Welcome.

DR. CARPENTER: I'll go ahead and start. Actually, the title of my talk is about the diabetic or the islet construct. And, in fact, I guess to be more accurate or precise as the words that have been used today, I'm really going to be talking about stem cell-derived product that is to mimic islet in the context of diabetes.

So toward that end, I guess my major message today is I will be talking about cells mostly, and about stem cells, and how to use in vitro assays to characterize those cells. And the overall message is really that cells are not static, and cells generally exist in a heterogeneous situation. So the use of these assays becomes very critical when you're talking about utilizing these cells for cell therapy.

So with that in mind, what I'm going to do is start talking about islet transplantation in the context of what we're trying to accomplish here at

NovoCell. And then I'm going to talk about the characterization of different populations of cells that one would use if you were using a stem cell population and you needed to expand that population, differentiate it and then deliver it into a patient. So that will be the undifferentiated embryonic stem cells, the differentiated cells, and then marrying those cells with a capsule to protect them from the immune system. And then I'll end by talking a little bit about some of the challenges that we face going forward.

So with that said, now, I think a lot of people are familiar with the fact that islet transplantation has been occurring for quite some time. In this context, diabetic patients are implanted with primary islets that are taken from cadaveric pancreases. The islets are harvested and then placed in the liver of the patient through the intraportal vein.

The long and short of this is that if done properly and under immunosuppression, in fact, this implant will take the patient off of insulin, which is a phenomenal thing for a diabetic patient. So that's

really good news. But the bad news about this is that the patient then needs to be chronically immunosuppressed. So that's not a very good situation for the patient.

The second part that hinders this is that getting cadaveric pancreases is very, very much limiting and getting islets from those pancreases is very difficult and very artful. So this field is really very limited.

So what we've done at NovoCell is develop some technologies to get around this. The first is a polymerization or a polymer capsule, and the second will be the stem cells. So here what you see is a image of a primary islet that's encapsulated with polyethylene glycol conformal coating. And that's shown here in orange. You can see the capsule. It's about 25 to 50 microns thick, surrounds the islet and is porous so nutrients can get in and insulin presumably can get out.

What is the purpose of this is to actually protect these cells against the immune system. Now, we have used this technology with primary islets in a

small proof-of-concept clinical trial in which we implanted encapsulated cells into diabetic patients. And what we found was that, in fact, the cells appear to be protected from immune destruction. So it seems that the capsule actually is protecting. And now we're moving on to generate stem cells as our cellular product.

When you think about the kinds of sources that you can use for a cellular product for diabetes, you have a number of choices in terms of cell products. So, obviously, primary islets, I just told you there just aren't enough of them to be efficacious for enough patients. But there's also many different kinds of stem cells, and the one that we've chosen is the embryonic stem cell. And this is for a variety of reasons but largely because of the proliferative capacity of these cells. And I'll talk about that.

But for those of you who aren't familiar, embryonic stem cells actually can proliferate from extended periods of time. They've been called immortal. They've been called infinite. But the bottom line is that they can proliferate for a long

period of time and generate many, many cells, which would be sufficient to the number of patients with diabetes. And remember that diabetic patients require quite a few cells. We're talking about delivering a billion cells or more, which is quite a considerable feat.

So when you keep all this in mind, what have we decided -- what is the strategy that we've used? And here I'm going to start talking about the cells. So the strategy that we've used is we've taken embryonic stem cells, which represent a very early stage of development, and we take them and we push them along developmental biology. So the idea is to step them through differentiation in a very structured and concrete way, moving the population from an embryonic stem cell into endoderm, into pancreatic endoderm, into an endocrine cell, and then into something that's going to look like an islet cell. And we're calling it an islet cell. Really what it is -- and we'll get to this as I start going through the data -- it's an insulin producing glucose responsive cell.

So remembering that as we go through this,

cells, as I said in the beginning, don't exist in a homogenous state in your body. And in a dish, those of us who have spent a lot of time culturing cells know that most cells like to be with other cells. And as it turns out, as you march the cells through differentiation, they -- there's a mixture of cells that go along with this process.

So as you start developing your assays, you need to actually keep this in mind and try to develop assays that are going to allow you to measure the appropriate cells and the other cells in the dish that are contributing or not contributing.

So what we've done in the research lab is actually develop a protocol that takes the cells through differentiation, as I've just described, going from ES cells through, all the way down to an endocrine population. And surprisingly, this takes maybe 18 to 20 days, which I guess is about the time it takes for a salamander to regrow its limbs, which I thought matched my philosophy quite nicely.

Now, you can see here there's a whole lot of markers associated with each one of these steps, and

those are markers that are positive markers associated with each one of those steps. But what you need to remember is that you also want to be measuring negative markers at each one of these stages. So as you take the cells through your differentiation, you need to measure each step.

Now, this is very interesting research. We can make some very interesting populations in the research lab. But the next challenge is to translate this into something that's going to actually be a cellular therapy.

So what does that look like? So if we take this back into a schematic, first we have to think about what the FDA rules are. Now, this is taken straight from the FDA or the CBER website. And you can see that there's a list of tests that need to be performed or characterization that needs to be performed.

And you look at this list -- at least I did -- and I said sterility, I can do that. Purity, I can do that. Potency, I might be able to do that. Identity, these are not trivial tests. And as you

really consider a heterogeneous cell population that is changing over time, measuring these aspects with accuracy and in a reproducible fashion becomes quite challenging. And I'll take about some of these aspects as well.

Now, if we take this back to what we've actually done. And you'll see this ball and stick diagram quite a bit over my talk. So what you have here is the ES cells as they march through differentiation. And then at the end you're going to have something that looks like an islet population, encapsulate that with our polymer and then deliver it.

So what do you need to characterize here? Well, the first thing that you need to characterize is your raw material. And in this case, the raw material is cellular population. And then as you move towards your cell product, you need to characterize that as well. And it turns out that probably you're going to need populations in between. So very quickly, you can see that the need for in vitro characterization assays is actually very critical for this to be anywhere near an efficient process.

So with that in mind, again, we need to characterize the starting material, the final product and the encapsulated product. So what I'll do is I'll start by talking about characterizing the undifferentiated cells.

Now, for those of you who aren't familiar with the process of how these cells are derived, I'm going to walk through that very briefly so that you can understand why we choose the assays that we choose to do this kind of work.

So what happens when you -- the way that you generate a human embryonic stem cell is you start with a human embryo that's about five to seven days old. It's called a blastocyst. And within that embryo, you have a small cluster of cells. It's about a 100 cells. It's called the inner cell mass. Those are the cells that are going to become the embryo.

Now, these embryos are actually donated. They're donated by IVF clinics or by the patients in the IVF clinics at the time that these embryos are going to be discarded.

So we acquire the embryos. We perform this

manipulation where the inner cell mass is removed. And what happens is -- and there's a lot of different ways to actually remove the inner cell mass.

You take this and put it onto a feeder layer. And traditionally, the original work, the feeder layer was a mouse feeder layer. Most labs now that are using these cells for clinical purposes are using human feeders or are attempting to use various matrices.

And you culture these cells. So it's a co-culture. So you've got two different cells in your dish. And then over time, these cells grow up. It actually grows colonies, and you split them. And they become a cell line. So you've got a number of reagents in the dish. You've got a number of cells in the dish.

Now, what we've done is we actually -- to start our whole process of how we're going to make a cell that's going to be clinically applicable, what we ended up doing was starting at the beginning and taking these cells, and generating a cell line under clinical manufacturing conditions, which meant that we first needed to have a human feeder line that was GMP-compliant. So we outsourced that, and the testing

that was performed on that is called points-to-consider testing, which tests for adventitious viruses. So that's the first thing that needed to be accomplished.

Then we choose all of our components. The question was just asked a bit ago about reagents and raw materials. As you go through this process, the closer you control your raw materials, the simpler your testing is going to be. So we generated all of these.

I'd like to point out that your reagents don't need to be xeno-free. They need to be properly sourced.

Then we did the characterization, and I'll show you some of that. And then we generated banks. And, again, on the embryonic stem cell bank, we performed points-to-consider testing for adventitious viruses, which was our first round of safety testing, if you will, or adventitious virus testing.

So to characterize the assessment -- and I'm not going to talk about all of these things. But obviously, you want to start with some identity or purity analyses.

We've talked a bit about the morphology of

cells. And this is sometimes very difficult to quantify. Folks that are very familiar with different cell types, they can look at a microscope and say that's the cell. That's the right cell. I know that cell. Or they can say that's cell mucked up and you've done something wrong. But to quantify that is very complex, but morphology usually is quite true. You'll find that it's actually quite predictive of some of the final outcomes.

And flow cytometry has been used for surface markers. Now, when it comes down to choosing markers or biomarkers -- we had some discussion this morning about biomarkers being tied to function. And unfortunately, in many cases that I've found, we don't have functional biomarkers. We have markers that are associated with the cell that have been used historically, but they're not tied to function.

So what I would put forth is the question -- I wish I had the answer -- do you really need a functional marker, or is simply a marker that identifies the cell faithfully a good enough marker? And, again, I argue that there's going to be multiple

markers and there's going to be positive markers and negative markers.

And then obviously, you're going to want to do PCR. If appropriate, we've done some immunocytochemistry. I've already talked about adventitious viruses, our agents and viability.

The other thing that I'll spend a bit of time talking about is karyotype, and I'll come to that in a minute. Now, genetic identity is obviously very important, stability and ultimately, the function.

So let me move on and just give you one example of the characterization of markers using flow cytometry.

So in this case, you've derived a cell line and you want to make sure that the cell line has the proper marker expression, but also that it has that marker expression for an extended period of time. I'll talk more about this as I go.

Here what you see are some flat plots that showed co-expression of two markers that are typically used for human embryonic stem cells, Tra160 and Sica4. Neither one of these markers has been tied to the

function of human embryonic stem cells, although it seems that most of these cells do express them.

The blue dots that you see up in the upper right-hand corner show the cells that are double expressing both of these markers. And you can see down at the bottom of the table shows -- there's three different cell lines that I have here -- different time points over time, different passage number, if you will, and the number of cultures that we've tested.

This is just a small amount of the data that we've collected and the percentage of the cells in the population that express these markers. And it's generally between, say, 80 and 95 percent, which is very high. But, again, it varies. It varies over time, and it can vary between passages.

So these are -- when you consider these kinds of markers, you need to consider what your parameters are and what's going to be your acceptance criteria, if you will.

Now, the next, I think, real critical thing that you need to think about when you're talking about stem cells that are maintaining culture for extended

periods of time -- not just human ES cells but some of the other adult stem cells, if you will -- is the cytogenetic stability of the cells. And how is that measured?

Well, for the most part in human embryonic stem cell work, the karyotype is measured by a G-band. You send your cells out to a clinical cytogenetics lab, and they do a G-band for you. And this is -- these are the same labs that run amniocentesis results. It's a clinically relevant assay, if you will.

What you'll receive back -- they usually assess 20 cells. You receive a report back. The report says 20 out of 20 cells were normal or 10 out of 20 cells were normal. And then your job is there are some aneuploid cells to sort out if that is an adverse event or what that means.

Now, there's other ways to assess karyotype or the cytogenetic stability. You can look at sky banding. You can do sequence analysis. And those are considered to be perhaps more sensitive. But at the end of the day, what we don't have from any of these karyotype work is a correlation between an abnormal

karyotype and an adverse outcome.

Now, all of us by common sense, say oh, boy, an aneuploid cell population, that can't be good for you. But really, it hasn't been tied. So all the groups that I know that want to have 100 percent uploid cells, but the flip side of that is what does it mean if you don't? And these are again the kind of assays that need to be tied. These in vitro assays need to be tied to a functional outcome.

Now, the other thing -- well, I'll move on from there. So I think that measuring the cytogenetic stability of the cells is becoming increasingly important.

So as you move through the path, we assess the raw material, our starting population. But then if you're really going to use these cells for delivering a billion cells or more, then you need to run the cells through an initial expansion. And so one ES cell will become many ES cells.

And what I'm going to call this expansion, what you need to demonstrate here, I'm calling it stability. Now, stability can mean lots of different

things. And right here, what I'm referring is that the -- during this period of cell expansion where the cells are proliferating, your cells, your raw material needs to maintain a consistent composition, and that will be determined by your surface markers and so forth.

It also needs to retain a normal karyotype to show that it hasn't become aneuploid or had some adverse event. And it needs to retain its ability to properly differentiate. I'm sure most people here who have worked with cells know that if you maintain somatic cells in culture for long periods of time, they become different. They change. They lose or gain abilities.

So what we're concerned about with this population of cells, these embryonic stem cells seem to be very unique in that they retain all of the characteristics we can measure over extended periods of culture, over years of culture, over 350, 400 population doublings which is remarkable. But measuring that and measuring in a reproducible and accurate way is somewhat challenging.

Now, let's move to the second tier, the differentiated cells. So here the issue is that you've got cells that are differentiated across time, moving across time. So, obviously, you want to characterize your final product. But where do you want to stop the process and characterize? Where do you want your end process testing to be because, as you just heard from Tim, being able to stop the process at various places in tests and know whether to go forward and stop is going to be very critical and cost effective as well.

If the cells have not differentiated properly or if you've got a contamination, you need to know this earlier rather than later. So the earlier you can do your predictive outcome testing, the better in terms of your cost of goods.

The other issue here is that you need to be able deliver a fairly enriched population and how enriched that needs to be is a matter of debate really. But at the end of the day, you do need to have a source of cells that's going to give you a high yield.

And how are you going to achieve that high yield? Is that simply through your differentiation

process, that your differentiation is so robust that you end up with 80 to 90 percent of your target cell? Or do you need someplace in the middle of your differentiation to stop the process and do some sort of an enrichment step, a selection event, if you will.

When you do that, obviously, you're going to need to tie that to testing that's going to determine whether you've changed those cells. And, again, at each one of these steps, have you changed these cells in some fundamental way? And if you have, can you measure it?

So going from here, identifying this enriched population. So you've got this enriched population that you want to deliver. What do you need to know about it?

Well, you need to know the functional cells. And these are the cells that in our case are the insulin producing glucose responsive cells. So you want to know that these cells are there and in what abundance they're there.

Now, as I mentioned previously, cells exist generally in a heterogeneous format. So it's likely

that there's going to be accessory cells there. And it could be that those accessory cells are helpful. That in our case other endocrine cells or mesenchymal cells could be trophic for your target cell, and you might want them there. So the challenge is then to quantify that and determine how many you need there, and what is your range that you allow to be there for your final product.

Now, you also need to make sure that you've removed inappropriate cells. And, again, in our case it would be tumor forming cells. So stray embryonic stem cells or perhaps a cell that was cytotoxic that was delivering -- secreting enzymes, for instance, that were inappropriate to the site. So this would also be something that you would want to eliminate from your population.

Then I've got my last category, I'm calling bystander cells, which are the cells that don't appear to be doing anything bad or good, but they're there. And I want to say like fibroblasts, but I'll probably get criticized for saying fibroblasts. But the generic cell that's there that you don't know what it's doing,

it doesn't seem to be doing anything bad or good, but you've got to measure it.

Now, the next thing that you need to measure is the function of this cell product. And having an in vitro assay, something that's a predicted assay, something that would link to your ultimate potency assays, this would be a great thing to have. And what I'm going to say in the next slide is but what if you don't have it.

Now, the next thing you're going to want is in vivo animal assays. This could be done in control animals. And you're also going to want to demonstrate efficacy from these cells, ultimately. And that'll be done in your disease models.

So now I'm going to go back up to the top and I'm going to say but what if you don't have an in vitro assay. So let's say in our case we've delivered the progenitor population to animals in our scenario. So we have not delivered yet the final population that's functional.

We went to the pancreatic epithelium, this progenitor population. We delivered those cells, and

we looked at whether or not those cells would function and mature and function in an animal. And in that case, what we're going to need to do is go back and draw that back to an in vitro predictive assay.

I know I'm not supposed to show any in vivo, but I do want to make this point. But sometimes you just don't have it and you've got to do the in vivo assay and then backtrack back into in vitro because ultimately, you're not going to be able to afford to do everything in vivo.

What this graft shows is on the -- a number of animals that these cells were delivered to that were then -- and the cells were allowed to mature over time. And then the animals were assessed for their ability to respond to a glucose challenge.

In this case, what you're looking for is c-peptide release, human c-peptide release from these cells in the context of a normal animal. And what you see in these pink bars is that the basal level or the fasting level of c-peptide in the animals. And then the blue bars are -- upon challenge with glucose, you see this spike of c-peptide release which would be

consistent with insulin release. And the ratio of those two are a stimulation index, which are the numbers that are above each one of the bars. And you can see that each animal has multiple bars, and those represent different time points as the grafts mature.

So what you see here is that, yes, we've got a population of cells that we don't have a functional assay for in vitro. We've got some identity on it. But now we had to put it in vivo to see if it would function. And now we need to go back and try to determine what's going to be a proper in vitro correlate to this.

So the next thing I'm going to show is that when you do this kind of an assessment, you do need to go back and look in the animal and assess the cell population.

Here what you see is one of the grafts from one of these animals that is -- and here you see in blue are the insulin positive cells which would be the insulin secreting cells are -- the target cell we delivered was a progenitor cell. And we believe that this insulin positive cell that it made from that.

And then there are the other cells that -- other hormones, the glucagons and the somatostatin cells.

So with all this in mind, the next step is you marry this with the encapsulation procedure. Now, the question that you would want to ask, is the capsule, this polymer that we're putting around the cells -- and this is different than you've heard previously where you've got cells on top of a scaffold, and the cells are growing, and the cells are being maintained by the scaffold.

Here we're using the polymer to actually protect the cells. We know at least from the islet work that there was not, as we understood it, a biological effect between the capsules and the cells. It was simply protective effect.

But, again, once you've put this capsule on, you've changed the environment that those cells are in. You've changed what they see. You've changed the way that they interact with each other. So you need to go back and take a look at what's your viability. What's your proliferation? Are the cells still differentiating, and do they dedifferentiate? And so

they still maintain their function?

And this goes hand in hand. It asks these same questions with putting these cells into a site. Which site -- is your effect site dependent? When you put your cells into Site 1 or Site 2, they're going to have different environments. And how's that going to impact your cells and your cells within their scaffold?

So these are all aspects of what I would call a bit of a moving target. And measuring them in a consistent way is, I think, very important. Again, we're talking about cells and cells in time. It's not going to be a absolute measurement. It's not going to be, in my opinion, that your cells have to be 95 percent X or Y. You're going to need to pick ranges and ranges that are suitable.

Part of what we're doing as a small company that's building a therapy is you need to make choices that are the reasonable choices. You can't measure everything. You don't have enough money to measure everything. So you need to make strategic judgments about what your choices are going to be in terms of what you can measure and what you can't. And that

takes me back to what I said about previously, which is in vitro assays that are predictive of outcome will be really important.

That takes me to the next slide, which is -- now, it seems kind of odd for me to be talking about in vitro assays in the context of safety and tumorogenicity. But when you think about stem cells and you think about tumorogenicity and the length of the time that these kinds of assays go, it, I think, would be extremely valuable.

We were asked to comment on the kinds of assays that we think would be very helpful going forward. Something that is predictive of tumorogenicity would be extraordinarily helpful and timesaving and moneysaving.

So if you think about this in the context of a capsule as well, you need to look at your effect of dose on toxicity on cells and the polymer. In addition, your stability -- I told you that we needed to look at the stability of the cells. Well, also the stability of the polymer and those as they work together. So a number of the safety issues that we

address we're trying to evaluate in vitro as well.

So with that, I would close by saying that where are we and what do I think are some of the key issues and challenges going forward. Well, as we go through this process, or any one of us goes through this process when you're talking about cells that are used for extended periods of time and then delivered to a person, the characterization goes throughout the process. And you need to upfront generate tools and assays, and do it expediently and efficiently.

That's going to include things like your identity, your potency, your purity. You also need to watch the cells and their stability throughout your process and determine which is your go/no-go decision, what's your acceptable criteria and try to tie these assays and their outcomes to functional outcomes. And, again, the impact of marrying the device with the cells.

And with that, I would stop for questions.

DR. HURSH: Questions for Dr. Carpenter?

MS. LUMELSKY: Nadya Lumelsky from NIH. I have a couple of questions.

When normal islet sites encapsulated, it's basically a tissue which does divide. Then you derive your insulin producing cells from ES cells, you're likely to have some together with insulin producing cells some cells which continue to divide. You're likely to.

When you put this kind of cluster into a rigid scaffold -- not scaffold, capsule, they might expand. Have you thought about this issue, how long they're going to live in there and what happens to the dead cells?

DR. CARPENTER: I think about this all the time. But I point out that the new papers that have come out indicate that islet cells or beta cells might in fact divide, but they're not what I'd call an overtly proliferative population.

So what happens when you put an overtly proliferative population into these capsules? Do they explode? Do the cells necrose?

We're doing those kinds of tests in vitro. The population that we envisioned delivering is not a overly proliferative population. But, yes, the

cells -- and capsulation of cells is very difficult.

Getting cells that can survive for long periods of time in this environment is not trivial, and it is known that non-proliferative populations tend to do better in a capsule than proliferative populations. So we do envision that something that's hyper-proliferative is not an appropriate cell type.

MS. LUMELSKY: Another question. So, again, for diabetes, there is a pretty good therapy, existing therapy. Not perfect but people live for a long time.

So from that perspective having just beta-like cells in your clusters and not having alpha cells producing glucagons, which, as we know, plays a functional role in the physiology of an islet, do you think that would be sufficient and would it be better than insulin injections?

DR. CARPENTER: So this is something that we debate about. Is the best population a pure population of beta cells, or is the best population an endocrine mix?

I think you need to do the experiment head-to-head. The way that the cells actually appear

in the culture is as a group of cells in which there are glucagon expressing cells and insulin expressing cells. It looks very appropriate. So that's the way that we're actually delivering them now. But we'll have to wait and see what it's going to be actually the best population to deliver.

To your point about there are therapies out there that people inject insulin every day, the initial population that we envision going into is the more severe patients. There are patients that they are hypoglycemic unaware. They can't control their insulin. They don't read their glucose. They don't fill their glucose levels. There's some very severe patients, which this would be a very appropriate therapy for.

DR. LELKES: Melissa, this is Peter Lelkes, Drexel University, and I've two questions, please.

Number one. As I understand it, you abandoned the idea of endothelial cell presence or vascularization of your graft. So how do you think about the longevity?

In the same context, I really like your

developmental approach. However, in this approach, endothelial cells play a key role not only providing the vasculature but also providing differentiated signals very early on.

DR. CARPENTER: Yeah. So the first was one was about abandoning --

DR. LELKES: Vascularization of your implants.

DR. CARPENTER: So this may have been before my time at the company, but, in fact, the graft, the encapsulated cells that we have placed are vascularized we think. At least in the animal models, they were. So vascularization, endogenous vascularization does occur around the cells and around the encapsulated cells. So --

DR. LELKES: But what about the inter --

DR. CARPENTER: Oh, inside. Yes, so inside the capsule, it's an islet itself.

Now, to go to the next step, which is about the differentiated cells, if you look at these cells, there's CD31-positive cells. There's endothelial cells mixed in with this. And those go in, and the animals

that we've engrafted, we think that -- we're not sure if we're putting them or if they're emerging in the graft

But you're absolutely right that the endothelial cells are playing a part. And whether that's going to be the cells that we deliver or it's going to be the endogenous cells, that interaction is very important to the differentiation and the maintenance of the cells.

MR. EISNER: Hi, Dominic Eisner, Rules-Based Medicine. Great talk. Just a quick question on where do you see the role of, let's say, monitoring secreted factors as a more modern day sort of simple of vitro testing, maybe multiple markers?

DR. CARPENTER: So I think the question was the role of secreted factors as an in vitro test, yes? And if we could simply measure insulin secretion, or the secretion of a growth factor, or a hormone as a predicted assay, I think that would be wonderful. That would be a very clean test if it was predictive of outcome.

MR. EISNER: And even for the whole process

of the differentiation?

DR. CARPENTER: And for the whole process of differentiation was the question? Yes, if we could do in process testing that was a secreted factor that could give us go/no-go, that would make things much simpler.

DR. HURSH: We're now running enough behind that we've decided we're going to take our break now, and we're going to make it a 10-minute break. So it's 3:38. Please be back in 10 minutes, which is 3:48.

(Whereupon, a recess was taken.)

DR. HURSH: Our next speaker is going to be Scott Nyberg. Dr. Nyberg is a professor of surgery at the Mayo Clinic. He received an MD from Johns Hopkins and his PhD in biomedical engineering from the University of Minnesota. He did his residency and surgical training at the University of Minnesota and is board certified by the American Board of Surgery and the American Society of Transplant Surgeons.

His talk today will be on the in vitro characterization of an ex vivo liver construct.

DR. NYBERG: Thank you very much. I

appreciate the invitation to speak from the FDA. This is really an honor.

As said, I'm a professor of surgery. I've also got training in engineering. But this talk will come from more of a clinical perspective, which I believe is important, though I'm a strong believer in the scientific method, especially from a sort of hypothesis-driven answering how and why question perspective which is important.

My overview on my talk, essentially the ex vivo liver constructs have two main areas. There's the clinical and therapeutic use, and there's also the diagnostic, drug toxicity testing since hepatotoxicity is an important aspect of evaluating new pharmaceutical agents.

I'm going to focus on the therapeutic liver failure aspects. So I'll talk briefly about the impact of the problem, brief history and the rationale for ex vivo therapies. I think it's important to understand what it is we're trying to do with these devices. Then comment on the standards, the safety, purity, potency, consistency, and possibly the efficacy of these devices

as I was asked to do so by the organizers of today's meeting. And then comment on what assessment techniques have been used in the past, what's needed and what is in development.

As far as liver disease goes, it's the eighth most common cause of death in the United States, so roughly 40,000 deaths per year. Liver transplantation is the primary therapy for these people with end-stage liver disease. We perform a little over 6,000 liver transplants a year. So you can see, there's a significant imbalance between the supply of organs for transplant and the demand for transplantation. Thus, there's a significant need for a liver support device.

I like this slide. It puts things into perspective, the difference between chronic liver disease, which is a large problem that affects almost or over 5 million patients of which approximately 200,000 to 250,000 admissions each year along with acute liver disease.

Acute liver disease refers to any one of us in the room that's healthy and within a matter of one to two months develop liver failure. So that's a

smaller group of patients. But as you can see, 4,000 hospitalized and approximately 2,000 deaths. So it has a 50 percent mortality.

Even chronic liver disease, once you've been hospitalized, has a 20 percent mortality. So they're both large problems and significant -- especially in acute liver disease, which could affect young healthy people with a 50 percent mortality.

What are the goals of therapy? Really, the goals are to prevent the manifestations of liver failure, brain swelling being the most lethal. Approximately 30 to 40 percent of patients with liver failure will die of brain swelling similar to a traumatic injury, though this is really related to a build-up of toxins in the blood that cause the brain to swell.

Kidneys stop functioning. Normally, lungs stop functioning, and you develop a systemic response similar to sepsis that's called SIRS, and this is probably the release of cytokines from the sick injured liver that these patients also have.

So the device can serve as a bridge to

transplantation, allow time for spontaneous recovery, with the end-point, really, is what's the survival at some time point; either 30 days or one year is what's usually considered.

Why use an ex vivo liver construct to treat liver failure? I mean the simple answer is liver failure results from the loss of liver function, so provide liver cells would be the best source of liver function.

There have been a variety of non-cell based systems that have been attempted over the past 50 years. None have been shown to be efficacious to the point that they're in regular use. So there is a strong belief that liver cells would be able to provide this missing liver function because they provide synthetic activities, albumin production, growth factor production.

They regulate amino acids, fatty acids, cytokines. These are the functions of normal, primary hepatocytes. A device provides selective detoxification, both of protein and water-soluble waste molecules as opposed to an open system like plasma

exchange.

If you have liver failure in Japan where there isn't a regular supply -- abundant supply of organs for transplantation, you'll be plasma exchanged as long as they have enough plasma to do the therapy, which is fairly wasteful. That's an open system. A liver construct would be a closed system. Generally, they're considered extracorporeal, and I'll explain in a second why extracorporeal.

There's always the debate between transformed and primary hepatocytes. More recently, people are looking at stem cell therapies to grow primary cells. We heard a very good discussion on use of stem cells to create pancreatic islets. Many investigators are trying to do the same thing with stem cells to create primary hepatocytes. In fact, my lab's involved with that. But I really believe, and most people that I've talked to, although we can produce cells that make albumin and do some limited functions of hepatocytes, we're many years away from primary hepatocytes from stem cells.

So transform would mean a tumor cell line

most likely from a human to benefit from the human proteins and the human metabolic activity versus a primary cell from a animal such as the pig. So they have similar protein synthesis activities.

If you look at ureagenesis -- and I'll explain this a little further in my sort of assay discussion part of the talk -- ureagenesis is a very complex seven-step pathway that there isn't a human cell line that's ever been shown to perform all seven steps completely to detoxify ammonia to urea. You need a primary hepatocytes to do that as this point.

Similarly, most P450 activities are not fully expressed in tumor lines. So, again, a primary hepatocytes -- or transformed cells will grow which is a benefit. There's some risk of tumor spread from the device. That has to be considered as does infectious risks if you're going to use a source like pig hepatocytes.

This is 20 years ago, in fact, the first publication of a cell-based therapy to treat liver failure. The term I usually use is BAL or bioartificial liver, extracorporeal liver support

device also used. Essentially, it's a system that contains a semi-permeable membrane to separate the patient's blood from the cells which are out in this extra-capillary space of the device.

The device serves to -- or the membrane is permeable to oxygen and nutrients so the cells are maintained in a healthy state. The cells detoxify waste products, such as ammonia, which has been strongly linked to the cerebral edema I mentioned earlier and to a water soluble molecule like urea, which can be eliminated by the kidneys as it passes back through the membrane.

Bilirubin would just be a classic example of a non-polar molecule that's albumin bound that's brought to hepatocytes. It's conjugated and made water soluble. It's either excreted in the bile or passes into the blood stream and again is eliminated in the urine, and then a variety of proteins such as clotting factors and albumin.

So hepatocytes has many functions, hundreds of functions. Which one is the most important? No one really knows. I would say ammonia detoxification is

very important, but there are probably many other. Amino metabolism activities, many other functions that are essential.

There have been a variety of over the past 20 years of devices that have been developed. This is a simple diced liver system where you just take a liver and cut it into cubes. In fact, there's a system that's currently just completing preclinical studies that I think the FDA is looking at that involves dicing livers, not quite in the same squares that this shows but more in slices. But this technology or this idea is still out there.

Most investigators have looked at individual cells. Hepatocytes are anchorage-dependent cells. They need something to attach to. So the hollow fiber membrane is generally considered the first choice. The limitation with the hollow fiber membrane is you can only attach so many cells, and scale up of these devices has been a huge barrier. It's been a major limitation.

So one way to address the scale up would be to add microcarrier beads, which are the plastic round

spheres you see with the gray spots being the hepatocytes that are attached to these beads. Others have looked at collagen gels, either sandwich techniques or -- this is actually a collagen noodle that has hepatocytes entrapped in the collagen so that they're a variety of constructs.

Our group and others are interested in spheroids. And the benefit of spheroids is there's no real collagen lattice work. They create their own. You isolate the cells and put them into the system.

And hepatocytes, although they're anchorage-dependent and they've also been thought to just die when you put them in suspension, if you agitate the system appropriately, they'll spontaneously form these spheroid bodies that are analogous to normal liver lobules within a matter of hours, very quickly within six hours. I personally feel that this has a huge advantage from a scale-up point of view because now you can grow cells in a three-dimensional reservoir-like bioreactor and address the number of cells needed.

This is a close-up of a spheroid after seven

days in culture. If I had a pointer, I'd show you on the surface there these microvillae that are consistent with the basal lateral surface, and then these pores that are consistent with bile canaliculi of the apical membrane. So these are very sort of consistent with differentiated normal cells.

A paper published about two years ago used confocal microscopy, added dextran beads into the system. And you could see them permeate through these pores within the spheroids that were consistent with bile ducts. In addition, they did a bile salt assay with confocal microscopy, and you can see the bile salts being concentrated with these pores suggesting that these are polarized primary hepatocytes.

On close-up, you see smooth and rough endoplasmic reticulum. You also see these granules which are glycogen granules. And that's probably one of the best indicators, I'm told by pathologists, that these hepatocytes are happy, that they have sufficient nutrients. If you can synthesize glycogen in culture, that's probably one of the best markers that you're supplying enough nutrients.

In addition, these dark spots within the mitochondria are called matrical bodies and they're consistent or specific for hepatocyte mitochondria. And then what you're really hoping to see are bile canaliculi with a tight junction on each side that's consistent with a polarized hepatocyte.

To summarize the past 20 years, there have been 30 different devices that have been developed. Since 1987, 14 of those devices have made it to clinical trials. However, none have yet to receive FDA approval in the United States. So there's still challenges.

As I've mentioned earlier, there are a number of challenges. But I think scale up to having enough cells to treat a patient who's very sick with liver failure has been the biggest barrier, both to the extracorporeal devices and to the hepatocyte transplant therapies.

In my mind, the next generation device will look something like this. There's a membrane to separate the patient's blood from the extracorporeal circuit. The system, the extracorporeal circuit can

include such things in the circuit with charcoal resin, even dialysis.

This system is consistent with what's called MARS or albumin dialysis, which is what's used in Europe and throughout Asia. It's not yet been sort of implemented in the United States. It's approved but not yet in use. There have probably been 10,000 therapies utilized worldwide with albumin dialysis. Then if you add in ex vivo liver construct to this extracorporeal circuits, you make use of the benefits of the cells and their metabolic activities.

The system that we work with in my lab looks like this. This is just a prototype. This wouldn't be what the therapeutic system looked like. But this system as shown contains 400 grams of hepatocytes, which is approximately 400 hundred billion cells. It's a huge number of cells that are allowed to function in this suspension because of this spheroid geometry.

We've grown these cells for -- they're not really growing. We've maintained them in a quiescent state for approximately a month. So that gives you advantages of both the fully differentiated primary

cells and some longevity in culture.

On my right here, the screen is a viability stain that just shows that, that the cells stained green, which is the viable cells. And then under standard HNE, you can see what the cells look like inside the spheroid.

To address the standards of how you're going to evaluate these ex vivo constructs, again, we're interested in safety to the patient; purity of the system versus, I guess, impurity; consistency of batch to batch isolations and potency. And is there some minimum criteria that need to be met?

Then finally, you can never get away from efficacy. But I'll focus on the top four in my talk. This is sort of a general generic step-by-step release criteria for a system. Most hepatocytes are isolated from livers through a perfusion technique. It's generally a two-step technique where you digest the liver cells to get isolated cells.

I don't show it in this form. But if someone's interested in stem cells, you'd have your stem cells and eventually they would be matured into an

isolated cell and sort of enter the process at this step.

Then you form your construct. In our case, we're forming spheroids. But this is sort of your construct step, and then you treat the patient once they've met criteria.

So the safety, purity, potency, consistency steps are checked here. These are just sort of what I would recommend. I haven't discussed this with the FDA, but this is sort of the way I would see it working within ex vivo liver construct. You could assess the donor liver for -- the donor animal -- for safety, consistency. Once the cells are isolated, that's when you do your purity evaluation and your consistency evaluation.

Once you have your liver construct formed, that's another point to assess safety, potency, consistency. And then finally, when you're treating the patient, you also have to continue to look at safety. And that's the efficacy point.

To go through each of those in a little more detail, cell safety, there's this code of regulations

that people have referred to that need to be followed. And then if you're interested in a device using tumor cells, you definitely need to be thinking about the tumor risk, which I think is probably best measured on line by a loss of cells from the device.

If you're using a tumor cell line, there are a number of animal models that can be used to assess tumorogenicity, but it really depends does the cell get out of the device and get into a patient. If they're going to be transplanted later that day or the next day be immunosuppressed, that is a big deal. Or the risk is higher with a tumor cell than with a primary cell.

There's also from a patient's point of view the risk of zoonosis, which would be an infection transmitted from the cells. Many of you may be familiar with the porcine endogenous retroviruses. I think it's very appropriate to be looking for infectious risks.

As a transplant surgeon who frequently transplants human organs from one to another, you can never eliminate all of the potential risks of a transmission. You have to sort of make a case-by-case

judgment. Is the recipient who may die tomorrow or may die in a month a suitable candidate for this particular organ? Which may be from a patient with hepatitis or it may be from a patient with a past exposure. So even whether we're talking about animal organs or talking about human organs, there's always this consideration.

I think you do as much safety testing as you can possibly do. I think that's very appropriate. As far as pigs have gone, there have been a number of papers published. And there really hasn't been evidence of a pathogenic virus release from pigs in humans that I know of.

Purity of cells, there are a number of markers. This is just my lab's profile of the markers we think are important. If you want to identify a hepatocyte, if they make albumin and express hepatocyte nuclear factor 4-alpha, they're essentially a hepatocyte. You can easily test that with flow cytometry.

Bile duct epithelial cells, cytokeratin 19 stain, Kupffer cells, which are the macrophages of the liver, F4/80 antigen; Ito cells, which are stellate

cells, which are an extracellular matrix producing cell within the liver if they're active -- smooth muscle actin stain, that's been correlated also with cirrhosis development in the liver and actin would be the GFAP marker. And then endothelial cells PECAM -- I think these are important to characterize.

In your animals, typically, we see 80 to 85 percent hepatocytes and the other 10 to 15 percent are the non-parenchymal cells. To establish purity in every isolation, that would be up to the agreement the company, the sponsor and the FDA if that's necessary. But it needs to be established in your source of cells.

The potency of the cells, I mean this is something that should be evaluated before you treat the patient. There needs to be some minimal criteria.

In the past, devices that have made it to clinical trials, really what they've looked at is just oxygen consumption. But the primary hepatocytes have a very sort of high need for oxygen. So it's easy to measure consumption and the viability of the cells.

When you use tumor cells, they tend to be less oxygen dependent. They're more glycolytic. They

produce lactate. They don't produce much oxygen. So that oxygen consumption isn't as helpful.

But you can list a long list of assays, albumin production, P450 assays. Personally, I think urea cycle activity is very important, and there are some new custom microarray and proteomics techniques that can be used to characterize these cells.

I'll talk briefly about the microarray since we use that in my lab. This is just an example of a hepatocyte bioreactor showing stable oxygen consumption over five days. But this is an example of what would be an important release criteria.

Viability staining, this is a hepatocyte spheroid after a month. Using confocal microscopy, most of the cells, probably 250 cells in this spheroid are viable. They stain green. The few that are dead are out here on the perimeter, the nuclei stain orange.

You can also use confocal microscopy to understand how these spheroids aggregate. This is a cadherin and adhesion surface marker that stains positively on the surface of these cells. If you add EGTA or something to remove the calcium, the spheroids

fall apart or they don't form at all. So it is a calcium-dependent process.

This is a neat stain. DAPI, you're probably familiar with, general nuclear stain. These are human hepatocytes on this side, and a tumor line on this side. The CEA stain is fairly specific for proliferating epithelial cells. So you see very few -- most of these hepatocytes are quiescent, non-replicating.

Where the C3A cell line, which is a hepatoblastoma line currently used in a clinical device in evaluation in China -- it's been evaluated here in the United States in the past. The cells are quite metabolically active with frequent mitotic activity.

This is an example of our custom microarray. Essentially, the array we've developed has 250 genes that are all liver related. This slide shows the 50 that are cytochrome P450 related.

In general, a little over 50 percent, there's very little change over a month in culture. There are some that decrease in time, some right away in the first two days. We're interested in ways to maintain

stable P450 activity.

It's unclear which one of these are the most important. 3A4 is probably very important. That's in here some place. It gradually falls off over the first two weeks. Of interest is 1A1, which is an important metabolic cytochrome for aerial benzene-like molecules, increases dramatically over the 28 days. It's also highly induced if you test it with different inducing agents like beta naphthoflavone.

I talked about the urea cycle. It's a complex cycle that occurs both inside the mitochondria and then out in the cytoplasm. Our assay we use, you add heavy ammonia and watch the heavy ammonia appear and heavy urea, and that's a very specific assay.

Many times people are just looking at urea production. Well, if you have arginine and have arginase, you can produce urea. So it's not very specific for the intact urea cycle, which I think is very important from a therapeutic point of view.

Again, with our custom microarray, we can look at the six enzymes of the urea cycle. We can see arginase actually increases quite high on day 2 and

then comes back down. Three of the enzymes are stable over a month. Two of them do drop off quickly.

This is an area of research as far as trying to develop or identify transcription factors that could be used to up-regulate those two enzymes and maintain stable ureagenesis over time.

Consistency. Just to summarize these cell markers, microarray data, even proteomics data, which I don't have experience with, but I can see this being quite a good system since liver cells are so metabolically active, to utilize proteomics as a cell system or as a bioreactor.

Looking at the viability, oxygen consumption and then any markers of detoxification, historically, adding diazepam to the system or lidocaine to the system and looking for metabolite production have been used. You want some quick assays that can be used as release criteria before the system's brought to the bedside.

And then efficacy, this is my only slide on efficacy. But clearly, you have to consider that if you're going to treat patients, have you improved their

survival either spontaneously or with liver transplant. Ideally, spontaneously so you can avoid the immunosuppression. But a live patient with a liver transplant is still better than a dead patient.

Time to recovery, how long are they in ICU, how long are they in the hospital, those are things to be considered in evaluating the system.

Then what are these extra-hepatic manifestations of liver failure that can be measured? Intracranial swelling can be measured with a bolt. In our hospital in our ICU, we routinely put bolts in the patients.

How well do their lungs work, how well do their kidneys work, all these are sort of secondary endpoints.

So a brief summary. What questions should be asked in assessing these systems for therapeutic uses? It's really simple. I mean is it safe and is it reliable and does it work? I've sort of outlined what I think are the important ways to look at this, but those are the questions.

What methods are available? There are many.

It can be microscopic, biochemical and then in the end clinical markers.

Then what methods should be developed? Personally, I think these custom microarrays, if they could be more rapid turnaround, they're very useful in assessing many functions with the liver, where you're not just looking at one activity. You're looking at many. They're very useful.

I've suggested proteomics, though that's not my area of expertise. And then other assays specific to liver functions like ureagenesis and albumin that are quite specific to the liver.

Thank you very much.

DR. HURSH: We have time for one or two questions.

DR. BERTRAM: Scott, thank you. Tim Bertram, Tengion. That was a nice talk.

Quick question for you. The microarray analysis, I'm curious. And this is an in vitro, in vivo extrapolation question.

Have you found or are you convinced that the microarray for RNA expression actually correlates with

the ultimate function of the cell? Specifically, just choose 1A1, is the expression of that gene in vitro actually giving you the 1A1 function in terms of --

DR. NYBERG: The quick answer is a very close correlation. We have a custom microarray that looks at 250 genes. In addition, the postdoc in my lab has looked at 50 of the genes within our real-time RTPCR to confirm the sort of consistency. We haven't looked at all 250, but we've looked the 50 we think are most important. In order to get the data published, you need both.

We also have functional assays. There's a fluorogenic assay where you add FOXY resorufin and look at resorufin and look at production. And there's a very close correlation, especially with the beta naphthoflavone induction. If you see a five to tenfold increase in the RNA levels, you'll see a tenfold increase in product.

So I've found them to be quite useful. I know that there's -- when you say custom microarrays, there's a certain concern that that may not be as useful. But in my experience, they've been very good.

Our facility, the Mayo GCRC, that's helped me do the research has a very reliable assay.

UNKNOWN QUESTIONER: Are these the ABI low density arrays? Are they PCR based, or are they hybridization based?

DR. NYBERG: I believe it's a hybridization based. I don't set them up, but they're on a glass slide. It's a specific custom array. It's not a Affymetrix array. It's not a large -- but these are sort of specific. You have to look for the sequences in gene bank and isolate -- or determine the appropriate sequence, and then we double-check that with real-time PCR. I'm sorry I can't explain it any better than that.

UNKNOWN QUESTIONER: There is a very -- it's just now coming out for the stem cell field now, a PCR based low density array has a very high 10^8 dynamic range, very specific. That's certainly what we're switching to.

DR. NYBERG: Yeah, if my postdoc was here, she could explain things very carefully for you.

UNKNOWN QUESTIONER: So you get these cells

by perfusion of cadaveric -- of donor livers, of cadaveric livers. Where do you get the cells from?

DR. NYBERG: Well, we have two main sources. For our clinical device, it'll be pigs. So these are pigs from the Mayo barrier facilities. So they're a germ free pig.

There's also liver specimens. We wouldn't use the liver specimens from the OR to treat a patient. But we have a regular source of human hepatocytes from my own cases and my colleagues' cases where we do liver resections, and then we perfuse the livers to get human cells. Those are for research studies in the lab.

But to treat patients in the next five years, I personally think the primary pig hepatocytes are the option. I realize that's controversial. Some people may say a human liver tumor line since there's a company that's using them already. Someday, ideally, primary human hepatocytes from some source where they've been expanded ex vivo. But I don't see that that's going to be available in the short term.

DR. HURSH: Thank you very much.

Okay. Our next talk will be from Peter

Lelkes. Dr. Lelkes is the Calhoun chair professor of cellular tissue engineering in the school of biomedical engineering science and health systems at Drexel University.

He received his PhD at the Technical University of Aachen, Germany, did postdoctoral research at the Weizmann Institute, and has held positions at the National Institutes of Health and the University of Wisconsin prior to going to Drexel in 2000.

His talk today will be the in vitro characterization of a fetal lung construct. Welcome.

DR. LELKES: Thank you very much to the organizers for inviting me and letting -- being ready at this late hour still to listen to some of our work.

What I would really like to do is since this is a rather new system, I would like to take a few minutes to introduce the system and some of the basic results and then talk about how to manipulate scaffolds and the effects of these manipulations on the cells. So we talk really about a cell/scaffold product. And at the very end, I would like to talk about

characterization parameters and some -- think about with you some assays and methods that one could introduce for lung constructs, but certainly also for other kinds of constructs.

Well, to start with, there is a definitely a clinical need for pulmonary tissue engineering. And as any good tissue engineering concept, it starts out with a unmet clinical need.

Specifically in the case of pediatric pulmonary diseases, this is a group of diseases called bronchopulmonary dysplasia, which results from pulmonary hyperplasia in which the lung is underdeveloped. And it's a major cause for neonatal mortality and morbidity, and it remains largely untreatable. As I said, the main clinical pathology is the unsufficient development of the distal lung. And this is what we will be focusing on, on the distal lung and specifically of the alveoli and the vasculature.

So our hypothesis is then that in vitro engineered pulmonary tissue constructs could, at least in clinical pediatric applications but also we believe in some adult applications, help with some of the

regeneration and palliative developments. But also as most of the other tissue-engineered constructs, as such, constructs could eventually be used in vitro as pharmacological models for drug development, screening, and also for understanding bases of pulmonary development.

Very quickly. For those of you not familiar with this lung development, alveolization is a highly choreographed morphogenetic process which happens really in the final stages of this lung development, and it starts from essentially a sac in the pseudoglandular stage.

Listed here are the times for a murine model that we have been using mostly. So embryonic day 10 to 12, 15, you have this pseudoglandular stage which then develops into the canalicular stage when you start seeing sacculation here.

Then later on, on embryonic day 17 through birth and through postnatal development, you have the terminal sac differentiation, the formation of alveoli, which is characterized by the close-up position of these sacculated lobes. And they are enrobed by blood

vessels that also go into and reach into the clefts. So this is an important functional indication for the development of the lung.

So therefore, what we tried in our first stages to develop is an experimental model system in which we've taken embryonic day 17 and a half murine fetal pulmonary cells. And after some experiments, we found that the best way of getting successful reconstruction lung-like tissue is indeed leave at that, not purify the cells any further. Rather take a gmish out of mixed cells, very importantly containing epithelial cells, endothelial cells and some other quote unquote mesenchymal cells.

We started out, as many of us do, with a Matrigel system just to show that this can work. And then once it started working, we switched quickly over to a collagen system assuming that the collagen is a rather neutral natural extracellular matrix protein, which allows then signalization between the cells and remodeling of the matrix. And then we can test for the morphogenetic induction of the sacculation and the vascularization.

Importantly, I think in our case was the proper choice of the growth media. We started out, again, as many of us do, with a base medium, which contained fetal bovine serum. And in our case, this was definitely the wrong way to go. So we then switched over to serum-free medium, which we started building up in its complexity. As a first additive, we added insulin, transferrin and selenium.

As a second stage that led to success, after we started reading more carefully developmental biological literature and determined that three of the most important morphogenetic growth factors would be a mix of fibroblast growth factors; namely, FGF-2, FGF-7 and FGF-10. So in some of the slides, you will see this abbreviated as F serum-free growth factor, a supplemented medium.

Well, to make a long story very short, if we grow the cells inside the collagen hydrogels at 10 percent fetal bovine serum, all we get is a cyst-like structure. However, if we do the very same experiment in the presence of all the growth factors, FGF-2, 7, and 10, we see this very nice sacculated

structure here, well developed, very reminiscent of the nascent alveoli.

If we now do some staining in confocal microscopy, you will see indeed that these are glandular structures that are lined not only with epithelial cells but in this case they are lined with type 2 alveoli epithelial cells, the hallmark for lung differentiation.

The marker for us, which we will propose to use as a marker for developmental later on, is characterization, as we see this Surfactant Protein C, which is indeed a marker for the maturation of these cells.

So not only did we observe the epithelial morphogenesis, but if we now stain the construct, double stain them for a marker for epithelial cells, which is here in red, and then looked at a marker for endothelial cells for which we used a isolectin B4, which is characteristic for murine and rodent endothelial cells, you can see indeed that you have the formation of the vascular capillary-like network, which is closely surrounding the alveoli-like structure of

the epithelial cells.

If you look now further with a confocal microscopy, what you can see -- and this time the staining is the other way around. You have the vasculature, the endothelial cells stained in red, and they are enrobing a lumen containing cyst-like structure, the epithelial structure.

Furthermore, what we found -- and it's interesting -- I don't have time to go into the data. This has just been published. Definitely in the vicinity of those alveoli-like structures, we find truly lumenized microvessels, all this in vitro. Here you see the epithelial cells, only the nuclei are stained. And here you see the endothelial cells, and you see the clear lumen in those capillary networks.

Further, as I mentioned to you before, one of the hallmarks of alveolization through the perinatal period is the formation of the vasculature that goes into the cleft-like structures. So here's a staining of such epithelial cyst-like structures. And you see the clefts here. And when we double stain now for endothelial cells, you see that there's the network of

endothelial cells. And indeed, they're growing morphogenetically correctly into these structures.

I don't have time to talk about the importance of the extracellular matrix proteins that are produced at this time that guide both the morphogenesis of the epithelial and the endothelial cell component. There's organotypic and highly restricted deposition of, for example, tenascin and laminen type 5 that guide the morphogenesis of the epithelial component and of the epithelial cell branching here into the clefts respectively.

As was mentioned before -- I believe, Buddy, you talked about this, about the decellularized tissues as a tissue-specific biomimetic scaffold. We also did some work, and this is a typical scanning electromicrograph of the ultrastructure of lung tissue after decellularization.

What you can see is that this is formed out of a honeycomb-like network of nanofibrous structures. So we thought, of course, being in a school of biomedical engineering and dealing with scaffolds, we'll do the same. So we took, as many of us do, the

process of electrospinning and generated matrices. And as a first attempt -- like, again, many of us did -- we used some of those biodegradable polymers, PLAA or PLGA as we heard before.

This is the results. The result was pretty unimpressive, to say the least. Namely, we did indeed generate mass fibers, and for controls we took also porous scaffolds, and we could seed them with the mix of the fetal pulmonary cells.

But even under the very best conditions in which we -- in the hydrogels obtained tissue specific differentiation and morphogenesis, all we got -- even in the best of all cases with the right medium, with the right growth factors, all we got was an overgrowth of cells of the mesenchymal line, of mesenchymal cells specifically. And we lost the expression of the marker for type 2 epithelial cells, SPC, as shown here in the R2 PCRs.

So when you do the experiments in Matrigel, we get very nice expression of SPC and vimentin. In the presence of PLLA and PLGA, we only see the expression of vimentin; no more SPC.

So the question, of course, is to be asked what could be the reason then? And I believe there are two reasons for this. Number one is I think we are barking up the wrong tree in terms of the matrix biomechanics because scaffolds made out of these synthetic polymers are far beyond what is naturally found in the lung. They are in the megapascals, whereas, as I'm going to show you, we should be somewhere in the hundreds of pascals and not megapascals or kilo pascals.

Number one and number two, as you will see from the next set of slides, I think it's also the lack of integrin or cell specific receptor activation that does not allow the specific development of -- or the specific differentiation of these lung derived cells.

To prove this point, what we did is -- here is a histology slide from the University of Wisconsin. And what it shows is that elastin is a major component of the lung and it is important for alveolization during embryonic development.

So what we then did -- recognizing this, we electrospun fibers out of elastin. And this is two

hours post-seeding. You can see in this lousy image. I apologize for that. But what you can see is you see the fibers and you see at the same time individual fetal pulmonary cells dispersed within the system.

Now, when we in this system grow the cells in the presence of 10 percent fetal bovine system, the result is as negative as before. So we get blobs of epithelial cells and surrounded -- staining green surrounded by endothelial cells and we have many other cells. You can see also the fiber somewhere here in the background. But we don't get organotypic morphogenesis.

By contrast, when we take the system and put now elastin as a scaffold, we take the appropriate serum-free growth factor supplemented medium. After two days, we get the cysts. After seven days, we get very impressive branching morphogenesis of the alveoli.

Then if we look at this system now after double staining as before, the green are the epithelial structures forming the cyst-like with the cleft structures. And then you see it totally enrobed by a network of microcapillaries. And, of course, the cells

continue to express SPC. So they also differentiate as quoting from the morphogenesis.

So there's lots of challenges ahead, and I'm not going to talk about any of them except for within the framework of this conference -- how do we standardize the scaffolds for this process? What can we do for that? And, again, how do we get to understand how to standardize this is to play around with parameters and to see what works and what does not work.

So, for example, we started looking at the effects of collagen concentration. And if you look 48 hours post-seeding, all our experiments have been done in 1 and a half milligrams per ml of collagen type one. If we increase the concentration of collagen, the formation of these alveoli-like structures is inhibited. So concentration seems to be an important parameter.

If we now look at SEM images of these gels, we can see that the fiber sizes decrease dramatically if we go from a .75 mgs per ml all the way to 3 mgs per ml. Similarly, what we have learned before, and others

before us have produced substantial evidence, is by playing around with the pH during the polymerization stage, you can modulate both the fiber sizes, as shown here. This is pH 5 and here at pH 10. So the fiber size is about half at pH 10 than it as at 5.

But more importantly, you can modulate the viscoelastic properties of these gels. You can modulate the viscoelastic properties both with the collagen concentration as well as with the pH. And I put here some comparable data together.

So, for example, in a collagen concentration of .75, we have a shear modulus of 2.8 pascals. So that's the dynamic shear modulus. This is very close to liquid, but it's still a gel. If we go to 3 mgs per ml, we have a shear modulus of about 200 pascal. Remember, it's pascal, not kilo or not megapascals. It's a very low end.

Similarly, when we play around with the pH at pH 4 and a half, we have a shear modulus of 4. And then this goes up to about 270 when we increase the pH to 10. So both concentration and the pH in which the gel is formed are critical for the success of

alveolization. And let me show you an example for that.

So at pH 4.5, or at the low concentration, we only get cyst formation. We get very nice circulation at pH 7 or at the concentration of 1 and a half mgs per ml. And at 3 mgs per ml, we have inhibited again the formation of these tissue-specific circulated structures.

How can we try to -- besides knowing the concentration, how can we try now to characterize these parameters in a noninvasive method?

In my surrounding, there are a number of colleagues. And I'm going to talk about their work that we are doing in part in collaboration. And this is work by Elizabeth Papazoglou, who's interested in noninvasive optical characterization of materials.

So she has been using diffuse reflectance scattering, looking at collagen gels and can establish a very nice correlation between collagen concentration on one side, or gluterate concentration, which means crosslinking, and the diffuse signal that she obtains from diffuse reflectance scattering. So a very nice

noninvasive method for characterizing scaffolds that you generate under various conditions.

Similarly, this is an example not from collagen but from alginate hydrogels that were crosslinked with different amounts of calcium chloride. If you look at small angle light scattering, if you look at the vertical polarization component, there's not much of a difference. However, if you look at the horizontal polarization, there's significant difference indicating structural differences between the two materials crosslinked at different calcium chloride concentrations.

Finally, as one of the optical methods, new methods, I would like to introduce to you the elliptically polarized light imaging system that was developed by Todd Doehring, who's a young colleague. And this is a system that he has been using to characterize collagen fibrils, specifically in this case in either collagen gels, obviously, or in cardiac valves that are retrieved either from healthy patients for some -- from cadavers or from in pathological conditions. And he sees significant differences. I'm

not going to go into the details.

But this is an elegant method to optically, noninvasively characterize fibril structures. And then one of the advantages, that he takes and he uses this system for three-dimensional reconstruction, in this case of a medial meniscus based on the system, of this elliptical polarized system that he has developed.

So with that, let's think about some proposed standardization. This is what I was asked to talk about. So what is our objective really for this?

From my vantage point, what I would like to do is to generate an integrated approach in which I correlate scaffold properties, A, through nondestructive testing of mechanical properties and then also the use nondestructive optical and mechanical testing, and then also use these tests of the macro scale as well as on the micro scale, and then compare these results with the functionality in terms, for example, in vitro of cell seeding capabilities and the biocompatibility and the capability; in the specific case of the lung, to induce the tissue specific differentiation of both vascular components and

epithelial components.

So macroscopically -- and I think I can go through relatively quickly because a number of previous speakers spoke about this. Again, I, like Buddy, don't have any financial interest in either Instron or in Bose, but we use these instruments.

So I think it's important to look at the stress relaxation modulus, both in compression and tension, because these materials are nonlinearly viscoelastic. Look -- the rheological characterization slide, like, for example, with the common plate rheometer, both a constant stress and a constant strain.

Optically, we have a whole slew of techniques available such as diffuse reflectance spectroscopy, near-infrared, for example, in the scattering mode, which gives very nice penetration into the scaffolds. Optical coherence tomography, what I mentioned before, EPLIS, the elliptical polarization and the small angle light scattering.

Of course, microscopically, we, as others, use confocal laser imaging, atomic force microscopy,

SEM and TEM. And the new method that has been developed already at the Max Planck in Stuttgart -- and one of our most youngest colleagues is bringing this system to Drexel -- is called SEM FIB. That's scanning electron microscope focused ion beam, which allows you to measure mechanical properties of individual nanofibers in situ.

Advance material characterization for physical characterization, I think it's very important to look at the materials before and after scaffold fabrication and check for process-related modifications. We should be using FTIR, Fourier transform infrared, so that we can pick up any functional groups that may have been modified, Raman spectroscopy both regular and enhanced Raman. And in terms of functionality, obviously, cytotoxicity, we can make the best scaffold. But if they are toxic to the cells, forget about it.

Cytocompatibility in vitro is limited, I believe to adhesion, to penetration, a very important aspect because in many scaffolds you get things to grow on the surface but it's difficult to get them really

into the scaffold. Proliferation of the cells once they are there. And importantly, functionally do these scaffolds allow differentiating capacities?

Cell sources, we have, of course a problem starting out with the fact that I think you have to have like in many other systems a heterogeneous cell source. It has to contain both the endothelial and epithelial and mesenchymal component. And for our case, it certainly cannot be the SPC, the fetal pulmonary cells. I don't think many people would like to give parts of their lung in order to build another lung.

So therefore, our focus and that I guess of many other labs, is the targeted differentiation of stem or progenitor cells. Important question to standardize is what is the right ratio of these cells? How do we vary the ratio of the cells? How does this affect the final product, and what are the seeding densities?

In terms of the scaffolds that we can use, hydrogels, we have shown this. I think we can also use the proper kind of nanofibers and scaffolds. We have

to take into account the matrix biomechanics and stiffness, and specifically, in terms of the activation of integrins and other receptors to the proper use of natural versus synthetic scaffolds.

In terms of growth factors, we have to understand that the growth factors that we added are multifaceted and they have to be morphogenic and differentiative. We cannot just work with one single agent. And as based on some of our in vivo studies, which I don't have any time to talk about, we need to incorporate slow controlled, slow release capacities in these scaffolds and they have to be also standardized.

Important is the capability of the system once we have the right growth factors to be able to remodel the extracellular matrix. This is one of the advantages of starting out with a collagen system and to document the temporal synthesis of specific ECM molecules. And I mentioned tenascin and laminen 5.

I didn't have time to talk about the control of spatially restricted deposition around the nascent alveolar forming units and the nascent capillaries.

Finally, what would be our markers? I

believe structurally the formation of these sacculated alveoli is a very good marker for cell differentiation, and it is a good marker for both endothelial and epithelial components. Functionally, I believe surfactant synthesis and release will be good indicator for obtaining a differentiated tissue.

Then in terms of pediatric lung tissue engineering, of course, the goal is to integrate it into the host. But another goal should be that you integrate an in vitro built construct into the host with the hope and the understanding that it can mature in vivo and then repair the diseased underdeveloped lung.

One question that I would like to raise as a final question and one question that I think is germane to everyone here: when is the best moment to go from in vitro to in vivo? So how long do you -- how do you marinate your constructs in vitro before you apply it? Is it at one point too late? Have you over-differentiated your system?

So with this question, I just want to say thank you very much to the members in my lab. And

specifically I would like to point out, as NIH and our other government organizations like to see nowadays, interdisciplinary research. So this is really interdisciplinary research in which the clinical relevance and component came from Chris Finck, a very talented pediatric surgeon. And then much of the work was carried out by bioengineering graduate students. And we had a lot of input from Peter Jones, a lung developmental biologist. So I think this combination has helped us to develop a good concept.

Thank you very much.

DR. HURSH: I think we might have time for one question. Are there any questions?

DR. PARENTEAU: I have a question. How would these constructs be introduced into the young lung? Can you just explain how -- you know you're going to have this --

DR. LELKES: I'm not the surgeon, obviously. But I just can tell you we are doing animal experiments right now for this. So we are trying to do exactly this, and so far I think we have partial success. So our learning curve is increasing. So we haven't killed

all the animals.

UNKNOWN QUESTIONER: I'll just talk loud.

DR. LELKES: It's not a problem.

UNKNOWN QUESTIONER: When you were using the elastin material and you were looking at differences in the (inaudible) in both natural and synthetic polymers having a very profound effect on cellular responses. So you had a very profound response, difference in your concentrations. Have you looked at this particular effect? (Inaudible).

DR. LELKES: Thoughts about this, the answer's no. The problem is it's a very complex system. And what we have concentrated on first is not so very much the issue of porosity but the issue of mechanical properties; the issue of the mechanical properties. And we can try to reduce it, specifically when we work on hydrogels, which in the hydrogel state you don't really have the fibers as they are shown in the SEM. But in the hydrogel, it's more the viscoelastic continuum that determines the stiffness of the hydrogel.

I think what we can show is there's a good

correlation between stiffness -- let's put it like this. There's a bell-shaped curve between stiffness, and you have one optimal stiffness that can generate alveolar forming units in others, which are not as effective.

DR. HURSH: Okay. Our last speaker is Dr. Kim Benton. Dr. Benton received her PhD from the University of Alabama at Birmingham and performed postdoctoral research at the University of Pittsburgh.

She then joined the Division of Cell and Gene Therapy at the Center for Biologics. And she was the chief of Cell Therapies Branch prior to her current position as the deputy director of the Division of Cell and Gene Therapies, where her duties focus on the oversight and review of regulatory activities within the division and policy development there.

DR. BENTON: I get the enviable position of going last and trying to incorporate a lot of what you've heard today, and give an elaboration on the regulatory view of cell/scaffold characterizations. I'm building on what Dr. Witten said today.

As I've listened to all the really great

talks today, I've heard a lot of foreshadowing of the points that I'm planning to make. So I'll try to recap those and move us along.

So as Deb said in her introduction, I am from CBER, so my default is to use the biologics terminology. And so another thing I want to do in my talk today is try to point out some of the different terms that are used that basically mean the same thing, and we've heard several different words today that, for example, discuss potency.

So that's one of the things that I hope to translate and then to translate from these different regulatory languages into scientific language. And my main goal is to convey this point: That addressing scientific questions and meeting regulatory requirements are not separate and competing goals, but these are overlapping elements of successful product development.

So successful product development, to move a product into the clinic and move it through clinical trials up to approval or licensure, you must demonstrate that the product is safe, pure, potent,

clinically effective and stable. And to do this, you must have full characterization of your product using appropriate methods and meeting specifications, limits of acceptability. To do this, you need to demonstrate that you have control of your manufacturing process so that you can consistently produce a quality product.

So I'm going to move now into explaining some of the regulatory terminology, the regulatory requirements. For biologics, each lot of product, before it's released to be administered or implanted into the patient, must be tested for safety, sterility, purity, potency and identify.

And several of the speakers this afternoon have brought up that safety and sterility aren't the main focus of this talk but they're very important. Of course, they're critical to product safety. And there's still a challenge about the need for rapid methods. But my talk is going to focus on purity, identity and potency.

This is a very similar slide. It's just pointing out one difference in the device regulations. So for cellular devices, same requirements, safety,

sterility, purity, identity and word performance. So the word "performance" is used instead of potency, but it's really the same meaning of getting at the function of the product. I've also heard the term "fitness" for use today. Function, biological activity. It's really what is the product doing and how do you assess that.

So the common goal of any product that's regulated either as a biologic or a device -- so any cell/scaffold product, the goal is characterization at the physical, structural, chemical and functional levels.

Before we talk about -- or to recap some of the points that have been brought up today is that we need to first remember about the complexity and the dynamic nature and the heterogeneous nature of these products. Before you can move to characterization, you really have to challenge this and figure out how you're going to meet -- you have to lay out the challenges and figure out how you're going to try to address them.

So these products are some of the most complex. They consist of multiple components that on their own as single entities draw some very major

testing challenges, such as living cells and viable tissues. The scaffold component, I think we've heard some opinions today that there's many more techniques that are perhaps more standard and acceptable. But since there are so many different scaffold materials that may be used, this has to be factored in.

Then, of course, there's the issue that when these cell or tissue components are brought together, this is dynamic combination. They interact both in vitro before you implant and, of course, when they're put into the patient. So the question to answer is how can you test a product in vitro to predict its in vivo performance?

Another challenge that Dr. Bertram brought up is, first, that some products are made for a specific patient, typically with autologous cells, and you have a lot size of one. One production run is yielding one construct that's going to treat one patient. And you can't destroy that one product.

So you need non-destructive tests. And another potential is the production of a surrogate product made at the same time under the same

conditions, perhaps at a smaller scale. But the purpose of that surrogate product is to use for testing.

Of course, the other issue that we've heard many times today already is that these products are intended to remodel after implantation and there'll be a great degree of variability in patients' capacity for this remodeling. But still it's how can you develop your product and plan to assess it again that you can test the product to be able to predict its in vivo potential.

So we have a lot of challenges. These products are dynamic, complex and heterogeneous so we have to find a way to gain control of them and to be ale to move them forward in product development.

So the first step in achieving this is by the design of your manufacturing process. Once you've determined what your product is going to be, you need a rational scientific approach to how you're going to produce that product. And that starts with your starting materials; your cells, your scaffold selection, critical reagents in the procedure, the

procedures you're going to follow to construct this product, the controls you'll have in place.

Then you have to be able to assess that you have produced the intended product. You have to have an arsenal of test methods that you can use in process on the components and on the various in process stages during the construction and, of course, on the final product. When you have enough characterization data, you are going to set the acceptance criteria that your product for clinical use must meet within limits to be accepted for clinical use.

So having a controlled process and a well characterized product is an important element in two other concepts that I'll get to later. Some of them have already been briefly mentioned today, product stability and product comparability, which some of you may think of as bioequivalence. And also having a well characterized product and controlled product is very important to help you have confidence when you move forward in interpreting your clinical data.

So starting with where do you start with testing a cell/scaffold component, there was a question

earlier today that, yes, you have to start with testing of your individual components, your scaffold component and your cellular components. So you have your component testing. You incorporate in process testing, depending on your manufacturing process and testing of the final construct.

So going to the scaffold -- actually, I think I'm going to almost completely skip this slide because we've had really great discussions today of the scaffold testing, your material components, biomechanics, surface characterization, biocompatibility. There's nothing I can add to here but just to put that placeholder in.

Moving onto the cells, it's very important to acknowledge and consider what your cell or tissue source is going to be and realizing that a living cell is going to introduce a high degree of variability right from the start. And that will be especially true for autologous products when you don't have a cell line that you're continually producing from the same bank.

So before you even get -- when you're in the conceptual stage, think about what you can control

about your starting cells or tissue. Where and how are you going to biopsy, how are you going to get it to wherever it's collected to your manufacturing site, and are there ways that you can assess that starting material to know from the beginning whether it's going to be a successful production run or not.

Moving on to in-process testing of cells, again, when you do this, where it's most appropriate to incorporate it will depend on your product. We've seen some examples today -- clearly, you always would test a cell bank if you have a cell bank -- stages, if you are expanding your cell population, or differentiating, or any other critical steps that would be at the end of that step would be a good place; also, at the time of seeding the cells onto the scaffold, so the last chance you have to use methodologies that are dedicated towards cellular suspensions. And in-process microbiological testing, sterility microplasma is also very important to incorporate into your plan.

So the characterization of cells that can be performed at in-process stages, this is a general list: morphology, phenotype, cell number, viability,

purity and potency are -- again, I put another alternative definition, biological activity.

Then the final product must be tested. The final construct must be tested prior to implantation. A critical element is microbiological testing, sterility endotoxin. So this must be performed.

But, again, with a 3D construct, you need to determine what's the most appropriate sample that'll give you the best results, best indication that your construct is indeed sterile. So that may be the container media prior to packaging in the shipping media or the shipping media. And characterization of the final product could include physical dimensions, volume, weight, appearance, the consistency of the cell and tissue growth on the scaffold, if the cells are intended to deposit extracellular matrix or other components; how can you characterize that on the scaffold and, of course, potency or biological activity.

So I'm going to move now and explain the biologics terminology of identity, purity and potency. So going first, identity testing in the regs is

performed to test the product. It's a test that's specific for your designated product. And the results of that will distinguish that product from any other product you may make in the same manufacturing facility and to confirm that the product in the container is indeed what is stated on that label on the container.

So if anyone picks your product up off the shelf, identity testing could confirm that it is that. Identity testing could include physical or chemical characteristics testing, macroscopic or microscopic. A simple example for a cellular product might be morphology or cell surface markers.

Product purity testing must be performed to show that there are no extraneous materials in the final construct, and your purity testing includes pyrogenicity or your endotoxin. That's a safety test. Also, developing your -- it might be called a purity/impurity profile. The phenotypic analysis of the cell types that are present, a quantitative assessment of each, cell viability and residuals that may carry over from the manufacturing process could include media components such as antibiotics, growth

factors, activating agents or chemical agents.

So some of this would need to be incorporated into final product testing. An alternative strategy is to validate that you have indeed removed these materials during your procedures.

So moving on to product potency, potency is the biggest challenge for biologics, moving them from the development of product assessment tests. So I'm going to spend about four or five slides going over that concept.

So potency is interpreted to mean the specific ability or capacity of the product to affect a given result. And so an ideal potency assay would measure a relevant biological function of your product. What that doesn't mean, though, is to affect a given result is interpreted as a biological activity. The regs don't say that you have to exactly measure clinical efficacy. So there's some flexibility there.

Also, in the biologics regs, tests for potency could be in vitro or in vivo or both, specifically designed for your product to indicate its potency. So there's no mandate as to which aspect of

your product that you measure for potency or how you measure it. So there's some flexibility, and clearly, that's needed for the wide range of products that exist.

So the goal of potency, biological activity, performance, any term you want to use, is to demonstrate that each production run, each lot that you've produced has the same biological activity, again, within the limits that you specify.

It's also a very critical measure of product consistency. So in each manufacturing run, from lot to lot, or if you have autologous product, patient to patient, that you have manufacturing consistency; again, that you're within these limits of biological activity that you've established.

Potency is a necessary component of assessing product stability. And, again, knowing the biological activity of your product and that it's been consistent through your manufacturing helps. Again, you interpret your data from your clinical trial.

So deciding what to measure for potency and how you're going to measure it is a very important

consideration that needs to be given thought early, and serious thought. I've listed some important attributes of a potency assay. So when you're considering what assays you might use, see do they fit these criteria.

As I mentioned earlier, do they indicate a biological activity specific or relevant to the product? The data should provide a -- the data should be quantitative. You should get a quantitative readout from your results and that the results would be available for determination of the quality of that lot to release it for implantation. So that goes back to the issue that Tim and others raised that you need faster results.

To make the decision to release the product for patient use, again, that's based on meeting the acceptance or rejection criteria that you have predefined. You should include appropriate reference materials, if available, or other appropriate controls in your assay again to make sure you have a valid assay each time you run.

The assay must be validated for licensure. I don't have time to go into that today, but there are

guidances and ICH documents, and Sally Seaver could talk about validation with you, if you'd like. And the potency assays should indicate the stability of the product.

So what are some approaches to picking a potency assay, some possible strategies to deal with this challenge? Well, a direct measurement of a biological activity, again, would be ideal. But that always won't be available, or it may not be available to yield results in the time frame you'd need to release your product.

So another possible strategy is measuring a surrogate characteristic that you have demonstrated to correlate with biological activity and that this correlation has been made with sufficient statistically sound data. That can come from any combination of your preclinical, proof-of-concept data, in vivo, animal or clinical data, or in vitro cellular or biochemical data.

There's a possibility, a very real possibility, that a single assay on its own may not be able to meet all of the attributes that a potency assay

needs. That's with the current technologies. So another possibility is using multiple assays, which we call the assay matrix approach. And this, as it says, using multiple assays that the combined results of which constitute a potency measurement.

So an example of a potency matrix approach for a cell/scaffold product could include characterization of the cells in process, particularly at the time of seeding on the scaffold. So you have quite a number of tests that could be included at that stage.

Then testing of the final cell/scaffold product, again, what can you know about the cells on the scaffold, what they've produced if they're intended to produce extracellular matrix or growth factor, or if we're talking about encapsulate islets, can they produce -- can you measure insulin production, just some examples. Measurement of metabolites, we heard Dr. Bertram discuss that strategy in his product and, of course, physical properties of the cell/scaffold construct. Again, these alternative assays and the collection of assays need to be correlated to the

intended biological activity, again, through in vitro, preclinical or clinical data.

So this is a potential approach, or these are potential approaches. And I wanted to note earlier in my talk and I forgot. But I and the other members of the organizing committee wanted to make sure that we conveyed that the FDA isn't claiming that we have all the answers for the appropriate tests and the appropriate specifications for the interesting field of cell/scaffold products. And that's, of course, why we're here to learn from you and to have a discussion of the latest technology.

So one example that we heard very eloquently discussed earlier today, of places that there's still room for improvement and scientific development of other tests, is just in the field of testing the cells alone. Biomarkers for different cell types for different uses, functional biomarkers, cell survival, in vitro differentiation, their behavior, other aspects, could be genomic, proteomic or other techniques.

I wanted to briefly go over a couple more

concepts. I'll try to be fast but not confusing. Stability is -- you must demonstrate product safety, identity, purity and potency are maintained through the expected use of your product to establish a dating period -- again, this is regulatory terminology you might hear instead -- expiration date, shelf life, how long is your product good for, however you want to say it.

But that's what you're trying to establish with stability to data. To establish the appropriate storage and shipping conditions and as I think -- maybe Dr. Bertram and Melissa may have pointed out that having this data and analyzing stability at different points in your manufacturing process can give you some flexibility, can give you some time where you can store the product at different stages. Cryopreservation of cells is a good one, but there may be other intermediate holding steps that you can define.

But to assess stability, you must have appropriate tests with the capacity to detect product degradation.

Product comparability or bioequivalence, you need to accept -- we all need to accept that manufacturing changes are highly likely in the development of a product. And to continue the interpretation of the clinical data you've collected with the product made before and after the change, you're going to have to demonstrate what we call -- or assess product comparability and ensure that there's no effect of the change on the safety, purity, potency or clinical effectiveness before and after the change.

You may say you don't plan to make a change in your manufacturing once you've locked down your procedure. But change may be forced upon you when a critical reagent is no longer manufactured or some other, such as you've lost your cell bank or cell banks are just naturally depleted, or you may change your manufacturing site, equipment, et cetera.

Another related point that I wanted to make goes on to -- that ties in with the capacity of tests that can measure effects of changes on your product. Changes in the process that could lead to changes in your product was highlighted earlier. If your

preclinical study is going to use an animal construct analogous to a human construct, that may be the best model, but you should have a way to demonstrate to us the comparability, the similarity of your animal construct and your human construct for the clinic.

So moving to sum up. So going back to the goal of reconciling regulatory requirements of real world needs, the regulations require that each lot be tested prior to release for implantation. There are undisputed limitations, the availability and quantity of samples, timing for products that have limited stability. So how quickly can you get results back?

Just the complex nature of these products affects the type of test that you can perform and that are meaningful. Some approaches: again, component and in-process testing, less traditional samples, such as running a surrogate or a construct media. These are just some examples.

But, of course, new technologies, rapid technologies that are non-destructive and yield a lot of information, a lot of bang for the buck. What gives you the most information on a small sample in the

fastest time? And, of course, the matrix approach is always a possibility, using complementary assays to meet the goals of identity, purity and potency. And also to have in your toolbox these assays that you may not use for release of the product but that are good assays that you can use for stability, comparability and making the correlation for the other assays that you will use for release. So it's good to have a toolbox that's bigger than just your release assays.

So this workshop is a great opportunity for the field to discuss the link between assay development and product development, and assay development should at a minimum keep pace with product development.

There are examples in biologic products where assay development has lagged behind clinical development. Outcome may not have been as desired, and we can't rule out that not having adequate characterization of the product didn't affect manufacturing decisions they made, design of the clinical trial, conduct of the trial.

So at this relatively early stage in the cell/scaffold field, there's an opportunity to not

repeat that problem. And so full product characterization and developing the technologies you need early and getting them in, testing your product early will help you design the appropriate manufacturing process, design the best preclinical tests to yield the most data and, of course, to design clinical studies that will get you to the goal of data for licensure.

So I want to thank you for your attention. I've listed a few references. Dr. Witten had these also. Particularly for the potency issue, I wanted to point out that we had an advisory committee meeting almost two years ago and we have the transcript of that on our Web. If you go to the advisory committee section, you can pull that up and look at that transcript. Thanks.

DR. HURSH: So we may have time for one question. Any questions for Dr. Benton? Oh, there's never questions for the FDA.

Okay. So now we're going to have the roundtable discussion, and we're going to do a little shuffling.

Also, I'm told that these mics out in the audience are not working at all and the transcriber cannot hear anything that's being said from the audience. So if you want to ask questions, you're going to have to come up to one of these mics and it's probably going to be easiest to get up to that one. So I hate to put you guys in the audience in the spot, but that's sort of what we got to do now.

So I'm going to turn this over to Dr. Durfor.

DR. DURFOR: The hour is late, and so I'm going to keep this fairly brief. I think we've all had a lot of good information today, and I want to use this very brief discussion period to sort of solidify some of the comments we've heard.

I think it's going to be easy to do because very simply I started off with ten questions and by the end of the afternoon I came down to two very specific questions. So I'm going to just get comment on that, if I could, and make sure we've got it where we want it to be.

So here's the first question I would ask our presenters and our attendees to comment on. And that

is what new important questions arise when cells and scaffolds are combined into a single construct?

Now, we've given you a list here. And so what would be helpful in your comment and in your discussion is two things. One, other issues, what's not on that list. And two -- and this gets back to my comments very early on when I talked about sometimes things we think are important as we go through product development become less important. And I used the nucleotide contamination as an example.

So I would ask our presenters and our attendees as well to look at this list and think about what happens when you put the two cells and scaffolds together, what's different, and what's important, and what's maybe less important.

DR. PARENTEAU: I'll take it. I think one of the things is that when you're doing your cell banks, and you're getting your cells and they're in vitro by themselves in culture, your goals there are very different. And the biological set point of those cells are -- they're proliferating, and they may be in hopefully a regenerative mode.

But when you put them in the construct, you again want them to change. And sometimes I think people feel that, well, I'm going to have this highly proliferative cell and then I'm just going to put it in the construct and it's not going to grow. But the whole issue of it not growing means something, and you want it to be meaningful.

So I think you have to realize that by at that point, certainly in cartilage, you want differentiation to occur. And certainly in skin, we wanted a lot of differentiation to occur.

So I think you have to consider that you're talking about an apple and an orange almost even though you're talking about the same cell type. When you introduce it into the scaffold, you want to direct that. You don't want to just leave it up to lack of growth or something like that.

DR. BERTRAM: Yeah, a couple of things come to mind on this. So I'm focusing on your point, what may not be up here. It's embedded in some of these, but just to add a couple of things.

I think what changes, and it's something we

grapple with, is the cell-cell interaction. And so since there's a two-cell production we're making, that when you put that smooth muscle cell together with the urothelial cell, there are other examples today where possibly if you put a mixture of cells into a capsule or what have you -- so cell-cell interaction.

The second point I don't see up here is this cell/scaffold interaction. Some of that can be characterized in vitro, but as a cell biologist looking at how cells respond to stimuli, they respond completely different when they interact with each other versus the scaffold.

Then what you do is you create actually a third variable, which is the cell-cell cell/scaffold. So now you've got two different cells talking to each other, and they're both being influenced by the scaffold, which creates an intriguing dynamic in a living construct, as Nancy said.

The last point I would bring up -- and it's something -- it's almost -- it may be irrelevant, but I think we're approaching an experimental design challenge that is pushing the edge of science beyond

just new technologies. In science we are taught the three scientific principles. But you're always trying to isolate a variable so that you can get it down to being able to make sure that your measurement, in fact, is interpretable.

The challenge, I think, with these cell/scaffold products is that the variables -- Kim actually alluded to this. The variables become so great and the ability to sort through it. I think some of the analyses that we're doing with the principal component analysis, some of the gene expression profiling and other technologies are opening our mind to analyses.

But one thing not mentioned here is actually the interpretation of in vitro data in which there is multiple variables that are actually moving simultaneously. And there are mechanisms to do that. Some manufacturing analyses look at that, but that's the last point to suggest to you.

DR. DURFOR: You've hit a really -- oh, Buddy, please.

DR. RATNER: If you'd like to make a

statement on this, I'm going to sort of change the subject a little bit on what Tim was talking, so.

DR. DURFOR: Well, I think it's an important observation. And this is, the data overload is not uncommon. And so knowing how to look at the many answers you get and many questions you're asking, how do you begin to prioritize?

DR. RATNER: I'd just like to make a point. It's actually an in vivo observation, but maybe it should help focus some of our thinking on in vitro tests.

If you take typically just -- let's call it a raw or non-cell-seeded scaffold, and implant it, you'll get sort of a fibrotic mess. Now, if you just implant the cells, they'll rarely stay in the site you want. They'll just be dissipated.

When you pre-seed the scaffold with the cells and culture it for a while, and then implant it, you typically get a very nice integration. It's often androgenetic integration. They're, of course, beginning the functionality of the tissue you're trying to create.

So somehow the combination of the scaffold and the cells, there's a whole new biological response in vivo. And that is probably associated with the cells producing the correct extracellular matrix that turns the whole thing on.

But somehow that really directly speaks to your question up here: What happens when you put the two together? It's a very different biological system from either of them, and somehow our in vitro assays should start addressing what -- or let's say maybe people need to do some very conscious correlation of the developmental processes and how that reacts -- the development, let's just say analysis of the extracellular matrix and the morphology and differentiation status of the cells. That could be done in vitro and then correlate that with the in vivo response to understand when the scaffold is at the right point that it induces wonderful reaction we see called tissue engineering.

DR. TUAN: I just want to echo what Buddy just said. There were some comments earlier about whether we're ever able to come up with a product that

perhaps has a higher level of performance than what we've lost, huge challenge there.

But on the other hand, I think we should be totally open to the possibility that what we are putting together could have novel properties. And our bioassays or markers and what have you ought to be able to accommodate that because otherwise we will miss it.

Just as Buddy said, when you take the cells out of their context and you put it into either a synthetic or a semi-synthetic matrix, you're making them do different things. Again, I'm trying to look at kind of developmental biology. To some sense these cells were programmed. They will make an organ tissue and what have you, following certain guidelines. But we're disrupting that. So we should be prepared for surprises. I think whatever assays we have should have that capability. I just wanted to throw that one out.

DR. DURFOR: Thank you.

Are there other comments? If not, I'm going to go -- oh, please.

MR. ROWE: So we have these biomarkers that we think are -- that we associate to be a certain level

of differentiation specific, but the cell population is still very, very heterogeneous.

My concern about that has been that just because you're measuring the marker doesn't mean this tissue was doing what you think it's doing. It could be made someplace else, and you're just measuring it.

So we've really made a major effort to try to develop biological markers that are based on tissue-specific reporters, GFP reporters that only come on when that cell reaches a certain level of differentiation. And that's, I think, proving very helpful to try to look at the microheterogeneity of a differentiating system, dedifferentiating, redifferentiating. So there's certainly in the stem cell field, that's starting to get a lot of traction.

I guess I had hoped to hear more of that approach because I think that building a menagerie of, let's say, mice that report out your differentiation cascade that you're interested in, that you're trying to redevelop, would be a very useful reagent for whatever model system that you had. And not only could you now assess its state when it's in its proliferative

phase and later on becomes in its differentiated phase, because now reporters come for the differentiation that you're hoping. But you can also in so many of these models -- you never know who actually contributed the cell, whether it was the cell that you seeded it with or whether or not it created an environment so that the host came in and did that.

Well, that's another situation where if your host is marked with one color and the donor's marked with another color, you can figure who contributed that differentiated cell.

So I guess there are these principles of developmental biology that are being used fairly regularly now in developmental biology. I think I would like to see more discussion of that -- marrying those two. We're saying we want to do developmental biology along with the engineering. And certainly, with the growth factor stuff, that was very nicely illustrated.

But I think that these tools that the developmental biologists are using now to look at develop would have worked even better. They would be

really great for this. So I was just hoping to hear more.

DR. DURFOR: That's why there's a two-day conference, and tomorrow hopefully we'll bring it to you.

I'm going to put up this last question, if I could then, and just have as a way for each of you give your summary, give your comment. This is what it's all about for us in terms of that, and each of your presentations were very clear on that.

But this is just an opportunity to come back and hit the high points of what you think are the key questions you would like to see asked for a cell/scaffold product just before it goes in humans. And since each of you gave a presentation, should you feel like you have an opportunity to hit a high point, so please feel free.

MS. SEAVER: Sally Seaver, Seaver Associates.

Having grown up with this biotech field and done a lot of things, what I'm really pleased to see is some really great new characterization techniques which everybody wants to focus on the scaffold or the cells.

But as you guys know, especially the FDA, sometimes it's the old-fashioned things that really stop you in the clinics. And what I'm not impressed with is our ability to just detect microbes better of the starting materials and something else. And I think if NSF and NIST are here with their funding agents, these are areas that we still need a lot of work on, especially because scaffolds actually hide microbes very well.

So, for instance, if you have a carbon filter and it gets contaminated, you can treat it with bleach and it is still contaminated. And realize your scaffoldings are getting better and better.

The other thing, too, that we've learned through all our years in biotech land in large production -- and I don't think we've seen it in cell therapy because the total overall production's not that big -- is that we have unexpected adventitious agents that fall out of the sky.

I think it would be lovely to again get NIST and NSF to fund people who are good at making rapid or developing generalized techniques so it doesn't have to

be individual companies or individual things to -- when the latest weird virus that we never thought would infect a particular type does occasionally affect it, we can react very rapidly and not shut down this whole field because we're too scared to go forward.

For those of you who aren't aware of this, this was -- if you're in CHO cells, no one realized that they were because -- let me finish the end of my thought. Because no one's talking about -- they're talking about doing periods of culture, but because of these rapid releases, no one wants to check for virus potentially infecting at the end.

It doesn't all come in from fetal calf serum. It comes in from probably ratty old sucrose or some common nutrient in a media thing. And what people found out, for instance, is in CHO cells, minute virus of mouse has proliferated. And they tested all their rare reagents to exhaustion and couldn't do it. But probably it could well have been one mouse at the bottom of a grain bin that contaminated some amino acid or sugar.

I mean that's life. We live with it, and

people now have very rapid tests for putting things on. But given that this field is a lot of small companies, it'd be nice if these techniques were developed so they were in general useful, so you just don't put a company out of business because they were unlucky.

DR. DURFOR: Are there other comments at this time? If not, I will encourage you all to arrive tomorrow morning early. We're starting off at 8:00, and Dr. Heineken is giving us a presentation about the coordination of tissue engineering and efforts throughout the federal government. And I think that's something that all of us can benefit hearing from. So I will see you tomorrow at 8:00.

(Whereupon, the meeting was concluded.)