

Armed Forces Institute of Regenerative Medicine

Annual Report 2013
Technical Progress Reports

AFIRM OUR SCIENCE FOR THEIR HEALING





Armed Forces Institute of Regenerative Medicine

Annual Report 2013 Technical Progress Reports

This report contains technical progress reports of all currently funded Armed Forces Institute of Regenerative Medicine research projects. Nontechnical summaries of these projects are contained in the AFIRM Annual Report 2013.

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I: Introduction

Background

Nearly 6,800 U.S. military fatalities and more than 51,000 injuries have resulted from the wars in Iraq and Afghanistan.¹ The use of improvised explosive devices in these conflicts has led to a substantial increase in severe blast trauma; explosive injury mechanisms have accounted for approximately three-quarters of all combat-related injuries.² Scientific advances in body armor to protect the torso and vital organs, faster evacuation from the battlefield after injury, and major advances in trauma resuscitation save wounded warriors who would have died of their injuries in previous conflicts. However, those who survive often have seriously debilitating injuries to unprotected areas of the face, neck, head, and limbs, causing massive trauma and tissue loss.

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¹ [Http://www.defense.gov/news/casualty.pdf](http://www.defense.gov/news/casualty.pdf) as of January 15, 2014.

² Belmont, et al. *J Trauma Acute Care Surg.* 2012 Jul;73(1):3-12.



I: Introduction

The emerging field of regenerative medicine holds great potential for healing military personnel with debilitating, disfiguring, and disabling injuries. Regenerative medicine focuses on (1) restoring the structure and function of tissues and organs that have been damaged, and (2) finding methods of curing previously untreatable injuries and diseases. Scientists are applying a variety of approaches to prompt the body to regenerate cells and tissues, often using the patient's own cells combined with degradable biomaterials. Use of a patient's own cells eliminates the possibility of tissue rejection. Recent years have seen the rapid development of technologies for engineering tissues. Scientists ultimately hope to deliver advanced therapies, such as whole organs and engineered fingers and limbs, to injured members of the military as well as civilians.

Research Goals

The AFIRM is a multi-institutional, interdisciplinary network of leading universities, hospitals, and private companies working to develop advanced treatment options for severely wounded warfighters. The AFIRM was designed to accelerate the delivery of regenerative medicine therapies to treat our most severely injured Service members, but in particular those coming from our theaters of operation in

Iraq and Afghanistan. Clinical trials have begun for several AFIRM products, including advanced transplantation strategies and engineered skin replacement applications. The inclusion of military patients in these trials is the first step in delivering advanced technologies to wounded warriors.

The Five Major Research Programs of the AFIRM

Limb and Digit Salvage

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

Craniofacial Reconstruction

Massive bone and soft tissue loss to the face and head due to blast forces is a devastating injury. Researchers funded by the AFIRM Craniofacial Reconstruction program are designing and developing therapies that healthcare providers can use to return form and function of the face, head, and neck to warfighters with severe craniofacial injuries. These therapies are expected to (1) regenerate functional bone and cartilage to levels of the face; (2) restore motor and sensate competencies through muscle, vascular, and nerve regeneration; (3) mitigate scar formation; (4) prevent infection; and (5) eliminate



RCCC researcher Glenda Evans analyzes images on bone regeneration.



Rutgers graduate student Koustubh Dube works in the walk-in hood used for kilogram-scale polymer synthesis at the New Jersey Center for Biomaterials.

skin coverage deficits through tissue engineering. The creation and delivery of new polymers and tissues will preserve and regenerate bone and soft tissue capable of administering stem cells, growth factors, bone derivatives, and therapeutic drugs.

Scarless Wound Healing

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

Burn Repair

Although there have been many advances in medical care, severe burns are still associated with substantial morbidity and mortality. The AFIRM Burn Repair program is leveraging regenerative medicine

technology to (1) prevent wound infection, (2) prevent burn inflammation and injury progression, (3) speed the generation of a viable wound bed and reduce the reharvest time of autograft donor sites, (4) improve skin substitutes for burn wound grafting when autografts are not immediately available, and (5) prevent and manage scars.

Compartment Syndrome

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

History

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



I: Introduction

In 2007, USAMRMC, the Office of Naval Research (ONR), the U.S. Air Force Office of the Surgeon General, the National Institutes of Health (NIH), and the Veterans Health Administration of the Department of Veterans Affairs (VA) agreed to co-fund the new institute. These funds, along with \$10 million (M) from the 2007 War Supplemental bill, provided \$8.5M per year in funding for the AFIRM, which was deemed sufficient to proceed. A Program Announcement was released in August 2007, and seven proposals were received in October 2007. Two finalists were selected for oral presentations in December 2007. Both received scores of “excellent,” and one was selected for funding. White House staffers learned of the AFIRM and invited representatives from USAMRMC to meet and discuss the new institute.

After two meetings, and upon hearing that funding was available for only one AFIRM finalist, the DoD was tasked to provide funding for the second AFIRM finalist. Within 1 week, an additional \$8.5M per year was transferred to USAMRMC’s budget lines. Both AFIRM finalists signed USAMRMC cooperative agreements in March 2008.

Funding: A Six-Way Partnership

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

- U.S. Army
- U.S. Navy, ONR
- U.S. Air Force, Office of the Surgeon General
- Veterans Health Administration



Cell Therapy technicians at Lonza Walkersville, Inc. perform cell culture in a Lonza current Good Manufacturing Practices-compliant facility.

- Defense Health Program
- NIH

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

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

AFIRM was recently extended for a sixth year with no additional funds. A new effort, referred to as AFIRM II, will build on the success of the AFIRM. Following an open competition, the Wake Forest University School of Medicine (Wake Forest Baptist Medical Center) was selected to lead AFIRM II. Funded through a cooperative agreement with USAMRMC, ONR, NIH, Air Force Medical Service, VA Office of Research and Development, and Office of the Assistant Secretary of Defense for

Health Affairs, AFIRM II is scheduled to begin in fiscal year 2013. AFIRM II will encompass five key research areas, including extremity regeneration, craniomaxillofacial regeneration, skin regeneration, composite tissue allotransplantation and immunomodulation, and genitourinary/lower abdomen reconstruction.

Structure

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

Rutgers–Cleveland Clinic Consortium

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

The RCCC consists of the following member institutions:

- Rutgers, the State University of New Jersey/New Jersey Center for Biomaterials
- Cleveland Clinic
- Brigham and Women’s Hospital
- Carnegie Mellon University
- Case Western Reserve University
- Cooper Medical School of Rowan University

- Dartmouth Hitchcock Medical Center/Thayer School of Engineering
- Massachusetts General Hospital/Harvard Medical School
- Massachusetts Institute of Technology
- Minnesota Medical Research Foundation
- Mayo Clinic College of Medicine
- Northwestern University
- Stony Brook University
- University of Cincinnati
- University of Florida
- University of Medicine and Dentistry of New Jersey
- University of Minnesota
- University of Michigan
- University of Pennsylvania
- University of Virginia
- Vanderbilt University

Wake Forest–Pittsburgh Consortium

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

The WFPC consists of the following member institutions:

- Wake Forest Institute for Regenerative Medicine/Wake Forest University
- McGowan Institute for Regenerative Medicine/University of Pittsburgh
- Allegheny-Singer Research Institute
- Carnegie Mellon University
- Georgia Institute of Technology
- Institute for Collaborative Biotechnologies (includes University of California, Santa Barbara; Massachusetts Institute of Technology; and California Institute of Technology)



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- Johns Hopkins University School of Medicine
- Oregon Health and Science University
- Rice University
- Sanford-Burnham Medical Research Institute/
University of California, Santa Barbara
- Stanford University
- Tufts University
- University of California, Berkeley
- University of Chicago
- University of Texas Health Science Center
at Houston
- University of Wisconsin
- Vanderbilt University

Additional Collaborators to the AFIRM

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

- Allegheny-Singer Research Institute
- Armand Trousseau Hospital (France)
- Arizona Burn Center
- Arteriocyte Medical Systems, Inc.
- Avita Medical, LLC
- Axonia Medical
- Baylor All Saints Medical Center
- Baylor College of Medicine
- Baylor University Medical Center
- Biogeneral, Inc.
- Biologics Consulting Group
- Biosafe-America
- BioStat International, Inc.
- BonWrx, Inc.
- Buffalo General Hospital
- CVPPath Institute, Inc.
- Cynvenio Biosystems
- Emory University
- Evonics
- Fidia Advanced Biopolymers (Italy)
- GID Group, Inc.
- Glycosan BioSystems, Inc.
- Healthpoint Biotherapeutics, Ltd./
DFB Bioscience
- ImageIQ, Inc.
- InGeneron, Inc.
- Integra Spine/Integra LifeSciences
- Intercytex Ltd.
- Intrinsic Corp.
- Jefferson University Hospital
- Johann Wolfgang University (Germany)
- Kensey Nash Corporation
- KeraNetics, LLC
- Kinetic Concepts, Inc.
- Lexmark, Inc.
- LifeCell Corporation
- LifeNet Health
- Lonza Walkersville, Inc.
- Louisiana State University



RCCC researcher Jarred Nesbitt performs post-operative care in the animal care facility.



Christine Miller, a National Research Council postdoctoral fellow at the U.S. Army Institute of Surgical Research, prepares Ribonucleic acid (RNA) samples for sequencing to identify RNA-based therapeutics for the treatment of drug-resistant biofilm pathogens commonly associated with chronic wounds.

- Loyola University Medical Center
- Maricopa Integrated Health Systems
- Massachusetts Eye and Ear Infirmmary
- MedDRA Assistance, Inc.
- Medical University of South Carolina
- Medtronic, Inc.
- Montefiore Medical Center
- Morgridge Institute for Research
- Neodyne Biosciences
- NeoMatrix Formulations, Inc.
- New York University
- Nitinol Development Corporation
- Norman Noble, Inc.
- North Carolina State University
- NOVOTEC
- NovoPedics, Inc.
- Numia Medical
- Ohio State University
- Oregon Biomedical Engineering Institute
- Oregon Medical Laser Center
- Organogenesis, Inc.
- Orlando Regional Medical Center
- Osteotech, Inc.
- Pennington Biomedical Research Center, Louisiana State University
- PeriTec Biosciences, Ltd.
- Philadelphia University
- Proxy Biomedical
- Queensland University of Technology (Australia)
- Radboud University of Nijmegen Medical Centre (The Netherlands)
- ResearchPoint Global
- Resonetics
- Rockefeller University
- Royal Perth Hospital (Australia)
- St. Barnabas Medical Center
- San Antonio Military Medical Center – North (formerly Brooke Army Medical Center)
- Shanghai 9th People’s Hospital (China)
- SimQuest, LLC
- Spaulding Rehabilitation Hospital
- Special Operations Medical Command-Fort Bragg
- Stratatech Corporation
- Stryker Corporation
- Texas Tech University
- Tolera Therapeutics, Inc.
- Trident Biomedical, Inc.
- University Hospitals, Cleveland
- University of Alabama at Birmingham
- University of California, Davis
- University of California, Los Angeles
- University of Colorado Hospital



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RCCC researcher Joseph Morand performs peripheral nerve microsurgery under a microscope at the University of Pennsylvania.

- University of Indiana
- University of Kentucky
- University of Massachusetts, Lowell
- University of Michigan
- University of North Carolina at Chapel Hill
- University of South Florida/Tampa General Hospital
- University of Tampere (Finland)
- University of Tennessee Health Science Center
- University of Texas at Arlington
- University of Texas at Austin
- University of Texas, San Antonio
- University of Texas Southwestern Medical Center
- University of Utah
- University of Washington-Harborview Medical Center
- University of Wisconsin–Madison
- Virginia Commonwealth University

- Washington Hospital Center (Washington, DC)

Programs and Projects

Research activities are organized into programs within each consortium. While some of the consortia programs are directly comparable to the AFIRM's major research programs (e.g., RCCC's CranioMaxilloFacial Program), other consortia programs consolidate expertise (e.g., WFPC's Extremity Injury Program, which integrates experts in the areas of limb and digit salvage and compartment

syndrome). A scientist or clinician Program Leader coordinates each program, which consists of multiple projects. One or more Project Leaders directs each project, which can vary in size from a single laboratory to a collaboration spanning multiple institutions.

Consortium members evaluate all levels of the operation on an annual basis to monitor progress and guide the consortium's activities. Active project management by each consortium has reshaped the programs, leading to the termination or reduced funding of some projects and the addition of projects that are more promising for accelerated development. The consortia also engage external scientific program and product development consultants who provide advice regarding clinical trials, product development plans, and other recommendations for commercialization. Additionally, information for the public, including clinical trial opportunities, has been made available through websites developed and maintained by the consortia.

In addition to the three core groups (RCCC, WFPC, and USAISR), intramural researchers from the NIH and/or the Veterans Health Administration can

participate in the AFIRM program, although none have chosen to do so as of yet. With the approval of a program leader, these intramural researchers can lead projects.

Management and Oversight

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

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

Board of Directors

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

for the AFIRM by presiding over the IPT and the Program Synergy Group.

Integrated Project Team

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

The specific responsibilities of the IPT are to:

- Approve the annual report and program plans that are presented to the BOD.
- Ensure that all AFIRM research projects are aligned with military requirements.
- Monitor and evaluate the activities and progress of the AFIRM programs and management, and provide recommendations based on their expertise.



Bridget Ford, a postdoctoral fellow in the Extremity Trauma and Regenerative Medicine task area at USAISR, prepares adipose-derived stem cells for use in tissue engineered scaffolds for wound-healing applications.



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- Facilitate the military's evaluation and purchasing of products developed by the AFIRM.
- Assist consortia directors and management teams in internal communication within the DoD, and in understanding and meeting DoD regulation and reporting requirements relative to AFIRM performance.
- Facilitate the leveraging of AFIRM resources by coordinating with other funding agencies that support closely related research.

The IPT's Steering Group has day-to-day decision-making authority over the AFIRM and recommends major changes in research direction or funding to the voting members of the IPT. This group is chaired by the AFIRM Project Director and also includes the USAISR Commander, the Combat Casualty Care Senior Scientist, the Contracting Officer, and the Directors and Co-Directors of the RCCC and the WFPC. Among other activities, the Steering Group ensures that all AFIRM research projects are aligned with military requirements, reviews AFIRM research allocation, establishes decision points and continuation criteria, assesses project and program achievements in relation to milestones and timelines, and recommends continuation or termination of programs and individual projects to the IPT.

The IPT has additional members from the Army, Navy, Air Force, and VA (one representative from

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

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

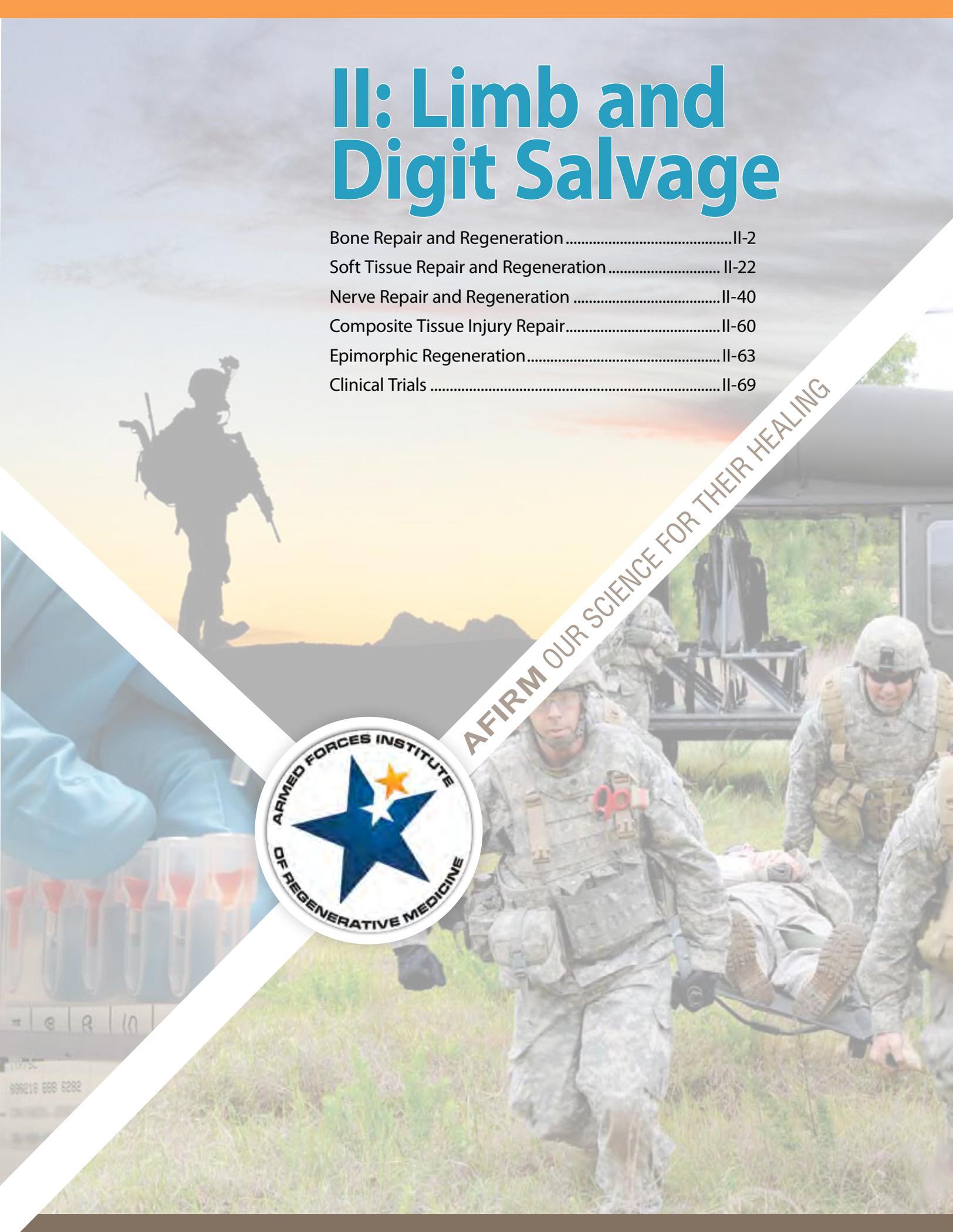
Program Synergy Group

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

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AFIRM OUR SCIENCE FOR THEIR HEALING





Bone Repair and Regeneration

Advanced 3D Scaffolds for Large Segmental Bone Defects: Non-Load Bearing Tyrosine-Derived Polycarbonate Scaffolds

Project 4.2.1a, RCCC

Team Leader(s): Ophir Ortiz, PhD and Joachim Kohn, PhD (Rutgers University)

Project Team(s): Sonja Lobo, DDS, PhD, Narayan Pallassana, PhD, Shuang Chen, BS, and Barry Cunningham, MS (Rutgers University)

Collaborator(s): Racquel LeGeros, PhD (New York University)

Therapy: Advanced regeneration of segmental bone defects

Deliverable(s): Advanced biodegradable scaffolds for segmental bone defects

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers successfully synthesized calcium phosphates (CaPs) with varying solubilities and incorporated the CaPs into the tyrosine-derived polycarbonate (TyrPC) polymer to produce composite scaffolds. They conducted in vivo assessments of these TyrPC+CaP scaffolds. They demonstrated that multiple TyrPC+CaP composite scaffolds regenerated bone as well or significantly better than commercially available bone scaffold controls in the rabbit radius critical-size defect model. The researchers identified the best performing TyrPC-based scaffold to be tested in the goat calvaria critical-size defect model during the upcoming year (see RCCC Project 4.5.1b in Chapter III).

Key Words: bone repair; bone defect; bone graft; scaffold; osteogenesis

Introduction

The repair of large bone defects and fracture non-unions in extremity injury is a key target of the AFIRM Limb and Digit Salvage Program. Treating these injuries represents an ongoing need, as high-energy blast injuries from improvised explosive devices have become increasingly common. Extremity injuries traditionally comprise the majority (58-88%) of traumatic injuries among U.S. Service members. The contemporary standards of care for bone defects and complex fractures, in both civilian and military practice, include many options for wound management, fixation and bone grafting. One potential option for bone replacement is the use of ceramic-based grafts. The AFIRM Limb and Digit Salvage Program will provide injured warriors with one or more new clinical methods that can significantly improve bone regeneration, specifically by quickly and reliably regenerating bone defects greater than 3 cm or bone non-unions. Project 4.2.1a focuses on developing advanced scaffolds to treat these large segmental bone defects.

Research Progress – Year 5

Screening of TyrPC-CaP scaffolds with and without bone marrow aspirate (BMA) in rabbit critical size defect models (S9, S10 and S13)

Three in vivo studies to evaluate the performance of TyrPC-based scaffolds containing CaP and BMA are reported in this annual report – S9, S10, and S13. The goals were two-fold: (1) to screen CaP formulations and (2) to assess the effects of adding biologics in the form of BMA.

In studies S9 and S13, the rabbit calvaria critical-size defect model was used to screen CaPs. S9 tested octacalcium phosphate (OCaP) in TyrPC scaffolds, known as TyrPC+OCaP. In S13, this formulation was tested to confirm the findings of S9, as well as to compare to other formulations of TyrPC+CaP. Study S10 focused on screening CaPs in the rabbit radius critical-size defect model. The reason for this change in model (from calvaria to radius) was to confirm the findings of the pre-screening

of S9. S9 and S10 samples were shipped to Carnegie Mellon University for surgeries by the Hollinger Lab (performed at Allegheny General Hospital). Explants were harvested at 8 weeks and shipped to ImageIQ for microCT, and the Mayo Clinic for histology/histomorphometry. S13 surgeries were performed at Rutgers. Explants were obtained at 8 weeks and shipped to ImageIQ for microCT, and the Mayo Clinic for histology/histomorphometry.

The results from this study (S9) are shown in **Figure 1**. No significant difference was noted in calvaria defects treated with TyrPC-based scaffolds containing either BMA or concentrated BMA. The microCT data of newly formed trabecular bone divided by the region of interest (BV/TV%) showed that the best performing scaffold was the predicate device, Vitoss™. The histomorphometry data was also used to determine new bone formation where the area of the defect was measured and divided by the total area of analysis (BA/TA%). These data showed that the TyrPC+CaP+BMA treatment group had the highest average new bone regeneration relative to the other groups. New bone was observed and quantified within the critical-size defects when they were treated with TyrPC+OCaP formulations. All treatment groups had osseointegration at the interface between native bone and the scaffolds.

Representative histological images stained with Trichrome for the treatment groups evaluated in the rabbit calvaria critical-size defect model (S13) are shown in **Figure 2**. These images were used for qualitative analysis of bone growth within the defect area. In addition, the blue-colored area (specific for bone) was quantified within the defects. Osseointegration at the interface between native bone and the defect was observed for all treatment groups. The best performing scaffold in this cohort was the TyrPC+OCaP.

Conclusions

CaP minerals are an essential component of the composition of native bone. In Year 5, the Rutgers team utilized CaPs to enhance the performance of TyrPC bone regeneration scaffolds. Four variations

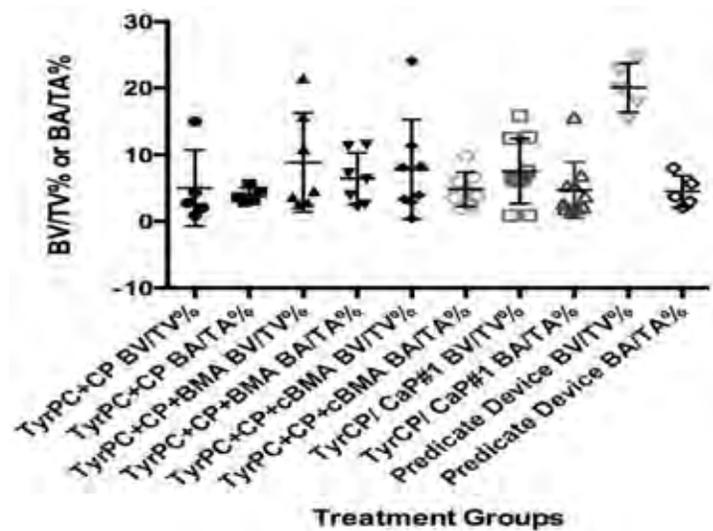


Figure 1. Graph showing the mean and standard deviation for each treatment group. BV/TV% pertains to microCT data, while BA/TA% pertains to histomorphometry data.

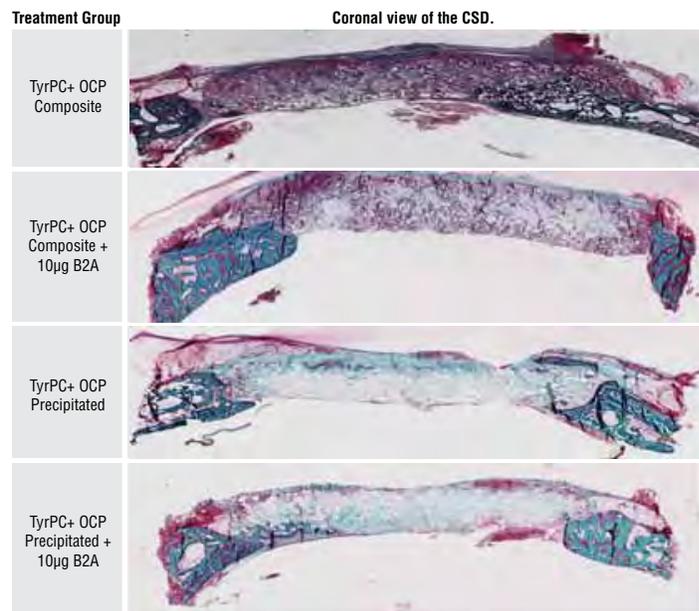


Figure 2. Representative histology images of samples from S13 (magnification= 0.63x).

of CaP mineral were produced in consultation with Dr. Racquel LeGeros at New York University. Analysis with microCT revealed that three of the four TyrPC+CaP composite scaffolds regenerated bone as well or significantly better than commercially available bone scaffold controls. The team identified TyrPC+OCaP as the best performer in the test group. This formulation will be used



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in the upcoming in vivo study in Project 4.5.1b. Concurrently, efforts on feasibility studies for product development will be accelerated to help ensure a fast and seamless transition to industry. The Rutgers-Cleveland Clinic Consortium teams are getting closer to the goal of providing a new treatment option for military and civilian patients suffering from severe bone injury.

Research Plans for the Upcoming Year

Scaffolds studied in Project 4.2.1 were screened in vivo. The data obtained from these studies has been used in conjunction with data generated from

Project 4.5.1b to downselect the treatment groups for the pivotal goat calvaria study to be done in Year 6 in Project 4.5.1b.

Planned Clinical Transitions

A Phase I clinical trial has been designed, and the team currently is seeking funding. A 510(k) application will be filed for the first generation bone regeneration scaffold. Subsequent combination products that include a soluble or tethered biologic will be pursued on the pre-market approval regulatory pathway. See Project 4.5.8 for more information on progress made on product development in Year 5.



Bone Repair and Regeneration**Advanced 3D Scaffolds for Large Segmental Bone Defects: Partial Load-Bearing Poly(Propylene Fumarate) Scaffolds****Project 4.2.1b, RCCC**

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

Project Team(s): Mahrokh Dadsetan, PhD, Alan Miller, PhD, Suzanne Segovis, MBA, James Herrick, MS, and Lichun Lu, PhD (Mayo Clinic)

Collaborator(s): Pamela Brown-Baer, PhD and Joseph Wenke, PhD, U.S. Army Institute for Surgical Research (USAISR); Raquel LeGeros, PhD (New York University)

Therapy: Treatment of segmental bone defects

Deliverable(s): Poly(propylene fumarate) (PPF) scaffolds coated on the surface with calcium phosphate (CaP) for bone regeneration using a three-dimensional solid freeform fabrication method

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL4

Key Accomplishments: The researchers enhanced the in vivo performance of PPF scaffolds using a calcium-containing surface coating, and they enhanced in vivo bone formation with controlled delivery of bone morphogenetic protein-2 (BMP-2) from collagen containing hydrogels in the scaffold pores. Histological analysis of PPF scaffolds harvested from rabbit calvarial defect was completed as well as the assessment of functional integration of scaffolds in critically sized rabbit calvarial defect. The researchers demonstrated that the combination of CaP coating and BMP-controlled delivery resulted in increasing bone volume in vivo in a dose-dependent manner for all scaffolds tested.

Key Words: bone repair; bone defect; bone graft; scaffold; osteoinduction; calcium phosphate; osteoconduction

Introduction

The conflicts in Iraq and Afghanistan have seen improvements in body protection, with a resulting increase in the frequency of extremity injuries in our wounded warfighters. Two thirds of the extremity fractures are Grades 2 and 3 (severe), and frequently have segmental skeletal defects as a component of injury. The repair of segmental bone defects and fracture non-unions is a key target of the AFIRM Limb and Digit Salvage Program. Often, the injuries also include damaged or missing soft tissues, and a compromised stable, continuous skeletal base, which is an essential platform for the healing of the injured soft tissues. Current skeletal reconstruction methods appropriately address many of these injuries. There is, however, a need for additional surgical options for those wounded warriors for whom the existing methods do not result in a healed skeletal defect. Current bone graft options include autogenous cancellous bone, allograft bone, addition of bone marrow-derived cells (with or without cell processing), bone transport, and local delivery of osteoinductive proteins. These are the

same options that are available to our wounded warriors. The novel therapies that become available to our war wounded via AFIRM will be immediately translatable to our civilian population.

The Rutgers–Cleveland Clinic Consortium solution to the unmet need of bone regeneration in segmental skeletal defects or non-unions is to develop skeletal implants that: (1) can be processed into any external size and geometry; (2) have an internal architecture that is macroporous, microporous, and interconnected; (3) have an osteoconductive CaP surface coating on all internal and external surfaces; and (4) have the property of being able to deliver a variety of biomolecules in a controlled, sustained manner that will direct the cellular processes which result in bone formation. This solution offers precise implant geometry to match that of the missing bone. The shape-specific scaffold has appropriate mechanical strength to physically prevent the encroachment of adjacent soft tissues into the reconstruction volume, to bear load immediately after implantation so that joint and muscle rehabilitation can occur during bone healing, and to effect bone



II: Limb and Digit Salvage

healing. In addition, the ability to include controlled delivery of antibiotics and osteoinductive proteins, when the clinical situation calls for them, does not exist in any of the currently available therapies.

Research Progress – Year 5

MicroCT evaluation of scaffolds harvested from rabbit calvarial model

Figure 1 shows three-dimensional representative microCT images of scaffolds and axial cross sections of scaffolds after six weeks in defects. These images, which agree with the researchers' quantitative microCT data, demonstrated an increased bone volume on scaffolds coated with Synthetic Bone Mineral (SBM) and magnesium-substituted beta-tricalcium phosphate (β -TCMP) in the presence of 50 and 100 μ g bone morphogenetic protein (BMP) in the rabbit calvarial model after six weeks implantation (detailed in the AFIRM Annual Report 2012). Scaffolds with the TCP/HA blend (biphasic CaP, BCP) revealed minimum bone formation. MicroCT depth profiling on scaffolds with different coatings and BMP levels demonstrated a bone-healing response biased towards the superior side of the defect. At the high BMP dose, a clear bias was seen towards greater bone regeneration and robust response on the inferior side (dural side) of the scaffolds.

Histology

Figure 2 compares histology of scaffolds using three different coatings of BMP loading with microCT longitudinal sections. In histology images, the purple stain represents osteoid and the green stain signifies new bone formation (Gomori's trichrome). Comparable microCT longitudinal sections are shown on the left panel. It appears that bone is formed inside the pores of the scaffold and is distributed throughout the defect volume in the high BMP groups for all three coatings.

Conclusions

The combination of CaP coating and BMP-controlled delivery was shown to result in increasing bone volume in vivo, in a dose-dependent manner for all scaffolds tested. Bone was seen to grow into the pores of the scaffold and was distributed throughout the defect volume in the high BMP groups for all three coatings.

Research Plans for the Upcoming Year

After completion of the studies in the segmental rat defect model in the upcoming year, the team will test PPF-CaP composite scaffolds in large animal models using the minipig mandible and goat tibial segmental defect models (as part of Project 4.8.1).

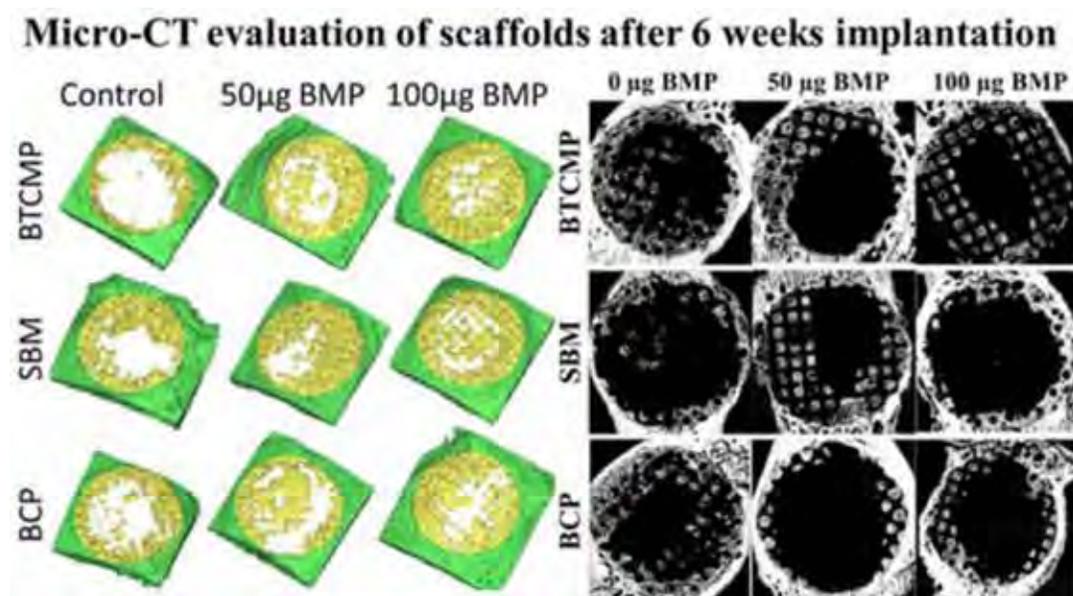


Figure 1. Three-dimensional representative microCT image of scaffolds (left panel). Axial cross sections of scaffolds after six weeks in defects (right panel).

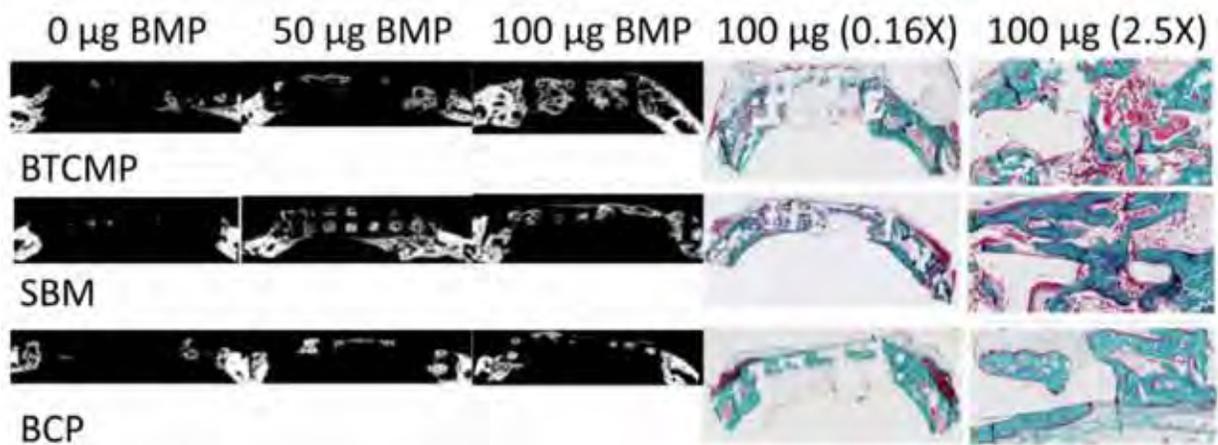


Figure 2. MicroCT and histology images of scaffolds using three different coatings of 100 µg BMP. In histology images (right panel), the purple stain represents osteoid and the green stain represents new bone formation (Gomori's trichrome). Comparable microCT longitudinal sections are shown on the left panel.

Planned Clinical Transitions

If the animal studies in the preclinical animal model are successful, the team will proceed to a first in-human pilot safety study of the scaffold implant, and perform Good Manufacturing Practices scale up, fabrication, sterilization, and pre-commercialization work for Investigational Device Exemption/510(k) submission.



Bone Repair and Regeneration

Optimizing Cell Sources for Repair of Bone Defects

Project 4.2.2, RCCC

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

Project Team(s): Viviane Luangphakdy, MS, Cynthia Boehm, BS, Hui Pan, MD, PhD, Kentaro Shinohara, PhD, MD, Tess Henderson, BS, Pownima Joshi, PhD, Tonya Caralla, PhD, Tom Patterson, PhD, and Phil Zaveri, BS (Cleveland Clinic)

Collaborator(s): Maciej Zborowski, PhD and Vince Hascall, PhD (Cleveland Clinic)

Therapy: Advanced regeneration of segmental bone defects

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

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: In January 2013, the researchers completed canine femoral multi-defect (CFMD) surgery on six dogs, using mineralized cancellous bone allograft (MCA) as a substrate. Canine marrow samples were collected during surgery and processed using two cell processing methods: selective retention (SR) and density separation (DS). Colony forming unit assay and image analysis is being used to determine the number and concentration (per unit volume) of cells and osteogenic connective tissue progenitor cells (CTP-Os) that are stably retained in the scaffold with these two cell processing methods. All bone samples have been scanned for microCT analysis, and canine marrow samples were shipped to the bone histomorphometry lab at Mayo Clinic for histological processing.

Key Words: connective tissue progenitor cells; magnetic separation; cell sourcing; hyaluronan; selective retention

Introduction

Regeneration of bone tissue requires the presence of osteogenic connective tissue progenitor cells, abbreviated “CTP-Os” (i.e., cells that are capable of making new bone). In many severe injuries, the number of CTP-Os is deficient (i.e., present in numbers that are less than optimal for bone repair). Therefore, in order to optimize bone regeneration procedures, it is very desirable to define methods that allow a surgeon to replenish the population of CTP-Os by harvesting CTP-Os from another site and transplanting them at a concentration and in an environment that optimizes their survival and their performance.

CTP-Os are found in many tissues, but particularly in bone and bone marrow. They can be harvested and processed in many ways, and many factors may improve the environment in which they are transplanted.

Project 4.2.2 addresses the need to define and competitively assess promising methods for the harvest, processing, and transplantation of CTP-Os in appropriate models, so the best possible methods

can be made available in the treatment of wounded warriors, and other victims of severe limb, spine or craniofacial trauma.

The objective of Project 4.2.2 was to compare three different methods for marrow processing: **DS** (using a centrifuge to remove red blood cells and serum), **SR** (regarding the fact that CTP-Os attach very readily to some surfaces as a means for concentration and selection), and **magnetic separation (MS)** (magnetically labeling CTP-Os and then preferentially collecting them with a magnet). All of these methods can increase CTP-O concentration. SR and MS processing can also increase CTP-O prevalence (i.e., reducing the number of non-CTPs).

Two animal models have been used to assess cell sourcing methods in AFIRM:

- The **CFMD model** was used in Years 3-4 to evaluate the performance of MS processing based on hyaluronic acid as a surface marker for CTP-Os. This is the same model that was used in Years 1-3 of AFIRM to compare and select the most effective bone scaffold materials.

- The **chronic caprine tibial defect (CCTD) model**, currently under development in AFIRM in Project 4.8.1, was also used in Years 3-4, in an attempt to assess the efficacy of SR.
- Year 5 activity was to define the in vivo performance of SR processing and DS using MCA as a substrate. MCA was identified as the current gold standard for osteoconductive bone scaffolds during Years 1-3 in Project 4.2.1. By using the CFMD model and MCA identical to that used to evaluate MS processing in Year 3, this work allows indirect but relevant comparison of performance between MS processing with SR processing and DS processing, as described in the original RCCC AFIRM proposal.

This limited but important set of analyses provides a valuable capstone to the work product of AFIRM I, and will enable important decision making in the future.

Research Progress – Year 5

Histomorphometric results of the assessment of MS in the CFMD model

Intraoperative processing of bone marrow using hyaluronan for positive selection of CTPs was shown to significantly improve bone regeneration. Histomorphometric analysis performed at the Mayo Clinic demonstrated that the area of new bone formation and vascular spaces was significantly greater when MS processing was used, compared to unprocessed bone marrow. This was associated with a decrease in the area of residual allograft and a decrease in marrow fibrosis. These findings validate that MS processing may be a viable strategy to improve clinical bone regeneration in a more stringent clinically relevant model. The CFMD model is suitable for screening, evaluating, and assessing CTP-O harvest and processing strategies.

Evaluation of bone regeneration in the CCTD model

The CCTD model has been further refined via a collaboration involving Drs. Liz Pluhar, Joan Bechtold, and Joseph Wenke of Project 4.8.1, who are charged with the task of developing and validating the CCTD model. Surgical and husbandry

protocols, and methods for quantitative outcome assessment have been greatly advanced (see Project 4.8.1 report). Project 4.2.2 work in Years 3 and 4 of AFIRM attempted to validate the CCTD model as a viable tool for advanced assessment of cell harvest and processing strategies. Large differences in the yield and quality of cells were found between the caprine proximal humerus, pelvis and sternum. Only the sternum proved to be a suitable model approximating the harvest of bone and bone marrow from humans or canines.

DS and SR in CFMD model

Canine surgeries

Six dog surgeries were conducted January 23-24, 2013. All animals recovered from surgery without surgical or anesthetic complications. One dog developed a femur fracture through one of the defect sites, which was discovered eight days after surgery. The animal was immediately euthanized and removed from the study. The euthanasia of the five remaining dogs was completed in February 2013.

Cell processing

Canine marrow samples obtained from the six animals were collected during surgery and processed using two cell processing methods: SR and DS. The cells were put in culture for colony assay analysis to determine the number and concentration (per unit volume) of cells and CTP-Os stably retained in the scaffold using these two cell processing methods.

MicroCT Analysis

All bone samples have been scanned for microCT analysis. This analysis is currently ongoing.

Histology

Following capture of high quality microCT images, on April 9, 2013, the canine bone cores were shipped to the bone histomorphometry lab at Mayo Clinic for histological processing.

Conclusions

The CFMD model used in Years 1-3 of AFIRM is an effective model for competitive comparison of scaffold and cell therapy strategies. The CFMD model was used to complete the Year 5 comparison between DS and SR processing of bone marrow-derived cells.



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Research Plans for the Upcoming Year

Cleveland Clinic will complete the competitive assessment of DS and SR processing with MCA in the CFMD model. The most effective sourcing and processing methods will be proposed for advancement from initial assessment in the CFMD model into appropriate assessment in the CCTD model, a more stringent model being developed in AFIRM Project 4.8.1 with Drs. Pluhar, Bechtold, and Wenke.

Planned Clinical Transitions

The most effective methods for cell sourcing will be evaluated alone and in combination with advanced scaffolds, surgical methods, and/or drug delivery systems to identify the most promising therapies to advance into clinical trials. Clinical trials may involve either new products or methods, and are most likely to be performed in collaboration with the Major Extremity Trauma Research Consortium.



Bone Repair and Regeneration**Point of Care Autologous Stem Cell Concentrate for Bone Defect Repair****Project 4.2.2a, RCCC**

Team Leader(s): Brian Barnes, PhD (Arteriocyte Medical Systems, Inc.)

Project Team(s): Vivek Raut, PhD (Arteriocyte Medical Systems, Inc.)

Collaborator(s): Christopher Rathbone, PhD and Joseph Wenke, PhD (U.S. Army Institute of Surgical Research); Joachim Kohn, PhD and Ophir Ortiz, PhD (Rutgers University)

Therapy: Point of care autologous bone marrow-derived stem cell concentrate

Deliverable(s): Rigorous dataset assessing bone formation in a rabbit extremity segmental critical-size defect model using bone marrow aspirate (BMA) or concentrated BMA (cBMA) combined with one of three graft materials tested.

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers performed an in vitro cell assay to test the efficacy of the Magellan® MAR01™ System. They also completed the surgeries to implant graft materials in a total of 60 rabbit segmental radial defects. Preliminary data demonstrated the efficacy of the Magellan® MAR01™ System, with a 4.4-fold enrichment in mesenchymal stem cells (MSCs), and 1.8-fold enrichment in nucleated cell concentration in the cBMA fraction, over their respective baseline values. The researchers are currently conducting radiographic, microCT, and histological assessments of the efficacy of bone graft material plus BMA in bone repair.

Key Words: bone marrow aspirate; bone graft; Magellan®; MAR01™

Introduction

Development of effective repair strategies for segmental defects has presented a persistent challenge for military trauma care. Current therapies for bone healing primarily utilize bone grafts and bone graft substitutes. Autologous bone grafting is the current gold standard in this therapy. Allograft bone, both mineralized or demineralized, obtained from cadavers has also gained popularity in recent years. Although vascularized and cancellous autograft show optimum skeletal incorporation, host morbidity limits autograft availability. Bone graft substitutes from nonhuman sources such as hydroxyapatite, tricalcium phosphate (TCP), calcium sulfate, bioactive glass, and bovine collagen also have a presence in the market. However, remodeling is not comparable to allograft. Due to the limitations of the currently available therapies, there remains an unmet need for a therapy that can regenerate bone in large gap defects. In this project, the researchers are evaluating existing methods, with the goal of developing and validating new practical clinical tools that enable surgeons to perform intraoperative harvest and processing of autogenous tissues to concentrate and select stem cells in the operating room.

The primary goal of this project is to develop a rigorous dataset demonstrating the benefits of the Magellan® MAR01™ System in bone repair. While the MAR01™ concentrate has been well characterized, no robust functional studies have been carried out in a bone repair setting. Arteriocyte hypothesized that concentrated MSC, hematopoietic stem cells and wound healing growth factors will result in rapid, more effective bone repair in combination with osteoconductive graft materials. The MAR01™ System has been well received by clinicians, but there is a clear need to demonstrate efficacy in well-understood animal models.

Research Progress - Year 1 (Project commenced in 2012)**Rabbit radial segmental defect model**

This study was conducted with approval from the U.S. Army Institute of Surgical Research Institutional Animal Care and Use Committee, and the Animal Care and Use Review Office of the U.S. Army Medical Research and Materiel Command. Study animals were cared for in accordance with the principles of the Guide for the Care and Use of Laboratory Animals.



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The Rabbit Radial Segmental Defect model was used for these studies. The researchers used 90 skeletally mature New Zealand rabbits (Charles River Laboratories) (weight 4 kg or higher). Animals received a minimum one week acclimatization period before the surgeries. Animals were randomly selected for one of six groups, or to be donors (**Table 1**). Ten animals were used for each group. BMA from two donor animals was pooled together and used to prepare graft material for all six groups. Thus, one pool of BMA was received by a total of six animals, three in the form of BMA, and three in the form of cBMA, in combination with the graft material in each group.

Preparation of graft and implantation

The researchers tested three types of bone graft materials: (1) demineralized bone matrix (DBM) material (MARO-Match™) plus gel (MARO-Fuse™), provided by Arterioocyte (Cleveland, OH); (2) collagen-beta TCP composite Mozaik™ scaffolds, provided by Integra Lifesciences (Plainsboro, NJ); and (3) tyrosine-derived polycarbonate (TyrPC) scaffolds, provided by the laboratory of Joachim Kohn at Rutgers University (Piscataway, NJ). All materials were prepared using appropriate Instruction for Use protocols. Bone grafts were prepared in six groups (Table 1).

Operative Procedure

For one batch of six treatment animals, two donor rabbits were used to harvest a total of at least 55 cc bone marrow. Each marrow draw was collected in 3 cc volume with 13% anticoagulant. After

collection, bone marrow was filtered through the MAR01™ filter to remove residual fat and clots. The researchers aliquoted 3 cc BMA for Groups 1-3 graft preparation, and 1 cc BMA was aliquoted for cell analysis. Groups 1-3 grafts were prepared immediately and implanted. Remaining bone marrow was concentrated using Magellan® MAR01™ System to 4 cc cBMA per the manufacturer's instructions. Upon this, 3 cc cBMA was aliquoted for Groups 4-6 scaffold preparation, and 1 cc cBMA was aliquoted for cell analysis. Scaffolds were prepared immediately and implanted. The sequence of implantation among Groups 1-3 and 4-6 was randomized. Post implantation, the defect site was sutured and stabilized with appropriate hardware. Animals received care for eight weeks prior to sacrifice and evaluation of the defect.

Post-operative qualitative assessment using radiographs

The defect zone was examined radiographically at four and eight weeks to ensure that there was no fracture at the site or displacement of the graft material, and to monitor bone repair. No surgical complications were reported during the training and model development. No adverse inflammatory response, infection, fracture or graft rejection was observed. Radiographs obtained post-surgery confirmed a successful grafting procedure. There was no reported discomfort or unusual observation in the rabbits post-implantation. The data collected from the radiograph assessments are currently being analyzed for new bone formation.

Table 1. Overview of treatment groups.

Scaffold type	Amount of rabbits	Components of scaffold		
		Gel (MARO-Fuse™)	BMA	cBMA
Group 1: DBM (MARO-Match™)	10	✓	✓	
Group 2: Mozaik	10		✓	
Group 3: TyrPC	10		✓	
Group 4: DBM (MARO-Match™)	10	✓		✓
Group 5: Mozaik	10			✓
Group 6: TyrPC	10			✓
Donor animals	30			

Cell analysis

BMA and cBMA cells were analyzed for white blood cells (total nucleated cells), mononuclear cells, platelet counts, and red blood cells using a hematology analyzer. A recovery yield was calculated for cBMA by dividing the sample volume by the volume of BMA, then multiplying by fold increase in the parameters. The number of MSCs and the maintenance of osteogenic potential of MSCs were characterized using a colony forming unit (CFU) assay in osteogenic culture media.

Overall, there was a 4.4-fold increase in CFU prevalence (number of CFUs in 1 million nucleated cells) in the cBMA fraction over the baseline (CFU prevalence in BMA fraction) (Figure 2A). The CFU enrichment in cBMA was consistent across all donor groups, and statistically significant compared to cBMA ($p < 0.02$). Overall, there was an 1.8-fold increase in the concentration of nucleated cells in the cBMA fraction (Figure 2B) over baseline (number of cells in the BMA fraction). These results emphasize the efficacy of Magellan® MAR01™ System in concentrating MSCs and nucleated cells. Concentration of MSCs, platelets and white blood cells could have a positive effect on the rate of bone repair.

MicroCT and histology analysis

Following sacrifice, the defect zone was explanted, and fixed in 10% neutral buffered formalin. After 48

hours, the solution was replaced with 70% ethanol to prevent demineralization until further analyses. Samples were scanned using μ CT at high resolution ($7\mu\text{m}$). The primary outcome from the μ CT analysis will be % Bone Volume. After microCT processing is complete, the specimens will be shipped to the Mayo Clinic for histological processing. The primary outcomes of histology will be: % residual scaffold, % bone, % cellularity and % void. Secondary outcomes will be: bone formation rates, osteoblast/osteoclast counts. All data will be acquired by August 2013, and final data analysis will be reported in a future annual report.

Conclusions

Arteriocyte successfully accomplished the graft implantation on all 60 treatment animals and assessed the in vitro efficacy of Magellan® MAR01™ System in concentrating BMA-derived nucleated cells and MSCs. Preliminary data demonstrated the efficacy of Magellan® MAR01™ System, with a 4.4-fold enrichment in MSCs, and 1.8-fold enrichment in nucleated cell concentration in the cBMA fraction, over their respective baseline values. Radiographic, microCT, and histological assessments of the efficacy (new bone formation) of bone graft material + BMA or cBMA in bone repair is in progress. All data will be acquired by August 2013, and final data analysis will be reported in a future annual report.

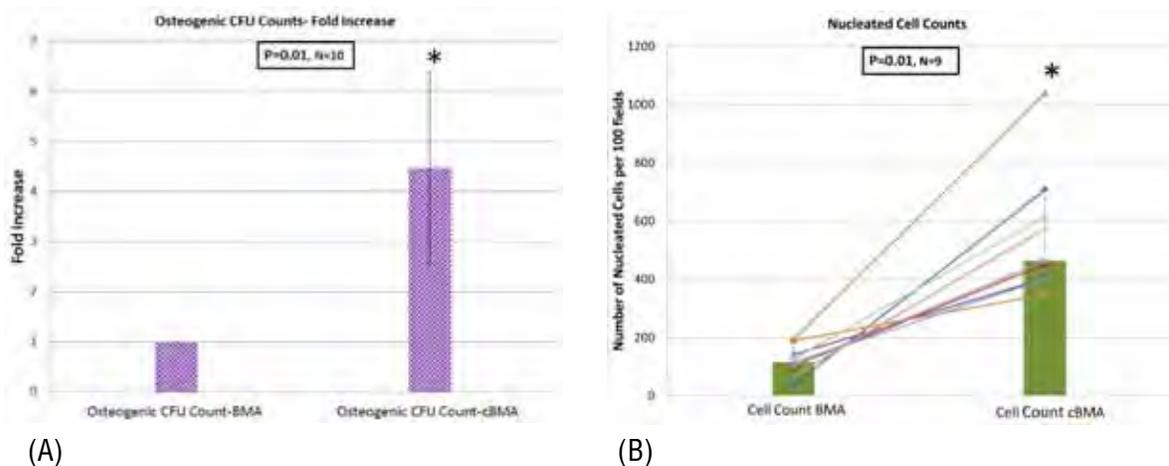


Figure 2. Efficacy of Magellan® MAR01™ System in concentrating MSCs and nucleated cells: (A) CFU prevalence in cBMA is 4.4-fold above the baseline compared to BMA. CFU enrichment was statistically significant with $P < 0.02$. Nucleated cell counts in cBMA enriched 1.8 times over the baseline over BMA. The nucleated cell concentrations were statistically significant with $P < 0.02$, and the trend among all 10 donor samples was consistent, indicating a positive enrichment with Magellan® MAR01™ System.



II: Limb and Digit Salvage

Research Plans for the Upcoming Year

The research team will complete the analysis of data in the rabbit radius defect model. Assessment of the efficacy of cBMA in improving bone repair compared to the BMA could be performed more effectively using an even more stringent model involving a larger defect, and perhaps, a soft tissue environment compromised with respect to scarring and tissue loss. Therefore, this may be more representative of the most challenging clinical situations.

Planned Clinical Transitions

The Arterioocyte Magellan® System and MAR01™ Kit are already U.S. Food and Drug Administration (FDA) approved as a Class II Device for the production of platelet rich plasma from whole blood and bone marrow to be combined with an allograft or autograft in an orthopaedic site. Arterioocyte products are manufactured in a Good Manufacturing Practices facility. Arterioocyte Magellan® is currently approved for investigation under three FDA Phase I Investigational Device Exemptions for use with other indications including regenerative therapy for critical limb ischemia, burn remediation, and compartment syndrome.

Bone Repair and Regeneration**Advancing Bone Repair using Molecular Surface Design (MSD): Biodegradable Scaffolds with Tethered Osteoinductive Biomaterials****Project 4.2.3, RCCC**

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

Project Team(s): Zheng Zhang, PhD and Sven Sommerfeld, BS (Rutgers University)

Collaborator(s): Ophir Ortiz, PhD (Rutgers University); Jeffrey Hollinger, PhD (Carnegie Mellon University)

Therapy: Advancing regeneration of bone in large bone defects and joint deformities

Deliverable(s): Scaffold with tethered osteoinductive and/or chondrogenic biomolecules

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers conducted animal experiments to assess the safety and efficacy of tethered recombinant human bone morphogenetic protein-2 (rhBMP-2) to induce bone formation in the rabbit calvaria defect model. They synthesized a library of polymers with the design criteria for cartilage formation and identified a polymer that significantly enhanced chondrogenesis of adult stem cells compared to an optimized tissue culture surface. The researchers also developed a fabrication technique to mesh the E1001(1k) polymer scaffold with the polymer to promote chondrogenesis.

Key Words: tether; rhBMP-2; bone; cartilage; scaffold; biphasic

Introduction

The Rutgers group is focused on two areas: developing the ability to regenerate long segmental bone defects and the ability to regenerate the complex tissue interface of bone and cartilage within joints. Current treatment options fail to sufficiently address these two areas. Most often, methods and materials used for large bone defects are not specifically designed or even approved for this use, but are used due to a lack of viable alternatives.

The team is seeking to advance the treatment of injured Service members by developing restorative therapeutics for osteochondral and segmental defects using an enabling technology of MSD. MSD attaches specific bioactive ligands (recombinant proteins) to the scaffold surface in order to control the cellular response and guide tissue formation. In addition to greater control and stimulation, tethering biomolecules via MSD to scaffold surfaces has the potential to greatly reduce the necessary quantity of the biomolecules, thereby decreasing the cost and increasing potential use of the device. Tethering prevents biomolecules from diffusing out of the

injury site and causing unintended side effects, such as ectopic bone formation, which occurs with rhBMP-2 eluting devices. Tightly tethered biomolecules would also be less susceptible to receptor-mediated endocytosis, prolonging the duration of activity.

Research Progress – Year 5**Biphasic scaffolds*****Chondrogenesis of human mesenchymal stem cells (hMSCs) on new polymers***

Since the Rutgers group had already found polymers that promote bone formation in Projects 4.2.1a and 4.5.1b (Chapter III), the priority in Year 5 was to identify polymers from which to fabricate the portion of the biphasic scaffolds to promote chondrogenesis. Such polymers must be adhesive to cells and flexible, a key property that is necessary to induce chondrogenesis. The researchers synthesized a small library of candidate polymers, all of which were found to support cell attachment and proliferation of hMSCs to an extent similar to standard optimized culture surfaces.



II: Limb and Digit Salvage

Next, assays were conducted to select polymers that were best for induction of chondrogenesis. In these experiments, cells were grown on the polymer surfaces and control surfaces. Chondrogenesis was assessed by production of chondrocyte glycosaminoglycans (GAGs) by staining the cells with a dye, Alcian Blue, which specifically binds with these GAGs. Expression of genes associated with chondrogenesis was also assessed by quantitative polymerase chain reaction (qPCR). Results of these assays revealed that one of the polymers was superior to the rest. **Figure 1A** shows that the selected polymer promoted widespread cell attachment. Cells seeded on the polymer quickly aggregated into masses within 24 hours and were strongly expressing GAGs by 4 days (**Figure 1B**). This process on standard surfaces typically requires one to two weeks, suggesting the benefit of the new polymer. qPCR analysis also showed an increase in expression of the early chondrogenesis marker SOX9 on the polymer surface (**Figure 1E**). These results suggest a significant effect of the polymer on the key process of chondrogenesis.

Fabrication of biphasic scaffolds

The biphasic scaffolds developed by the Rutgers group are composed of a relatively rigid layer

promoting bone regeneration, topped by a relatively soft and more hydrophilic layer promoting cartilage regeneration; discrete biological cues will be applied to each layer. These scaffolds will be evaluated for their applicability regarding the regeneration of the osteochondral interface. A polymer composition and scaffold fabrication of tyrosine-derived polycarbonates (designated as E1001[1k]) has demonstrated remarkable ability to regenerate bone in preclinical animal models. In Year 5, a fabrication technique was developed to mesh the E1001(1k) polymer scaffold with the polymer shown in **Figure 1** to promote chondrogenesis.

Figure 2 shows the behavior of these biphasic scaffolds during the hydration process. The osteogenic section (grasped in the forceps) remains rigid while the chondrogenic segment rapidly hydrates and takes on the characteristics of a semi-gel consistent with cartilage. Assessment of cell behavior in these three-dimensional scaffolds found that production of chondro-specific GAGs was upregulated in the chondrogenic segment compared to the osteogenic E1001(1K) or two-dimensional tissue culture plastic as a control (**Figure 2D**). These scaffolds remained structurally intact and maintained their mechanical properties for the target time of six weeks.

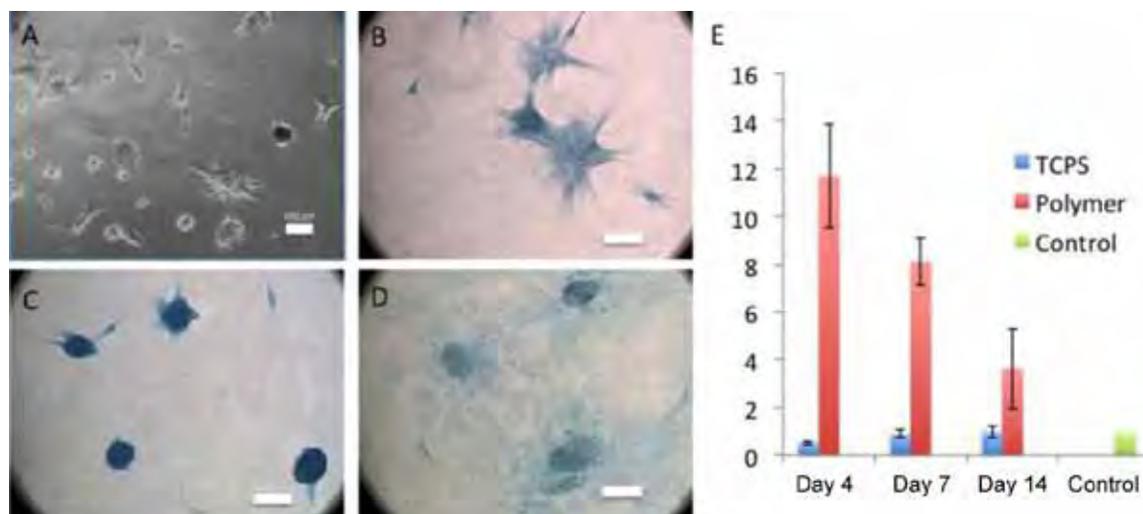


Figure 1. Chondrogenesis of hMSC on spin-coated polymer films and control surfaces tissue culture polystyrene (TCPS). (A) Phase contrast image after 1 day in culture. (B-D) Alcian Blue staining of chondro-specific GAGs on Day 4 (B), Day 7 (C) and Day 14 (D). Cell culture medium, A-C: Chondrogenic differentiation medium; D: Chondrogenic medium until day 8 then replaced with MSC growth medium. (E) Messenger ribonucleic acid levels of early chondrogenic marker SOX 9 from hMSCs cultured on polymer films as compared to TCPS. Transcript levels are expressed relative to SOX9 expression on TCPS after 24 hours.

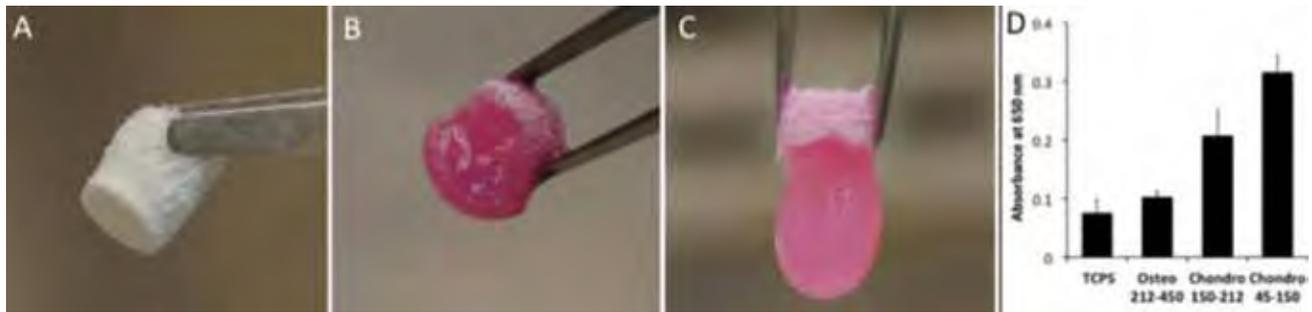


Figure 2. Morphological changes of biphasic scaffolds to cell culture. (A-C) Cylindrically cut biphasic scaffolds gripped by forceps on osteogenic segment (A) following ethylene oxide sterilization, (B) 2 days after seeding with hMSCs, and (C) 5 days after seeding with hMSCs. (D) Normalized chondrogenesis of hMSCs as assessed by Alcian Blue, with number range indicating the pore sizes present in the osteogenic layer (E1001[1k]) or the chondrogenic layer of the biphasic scaffolds.

Proliferation assays found that hMSCs continued to proliferate on the chondrogenic segment of these scaffolds, increasing by $157 \pm 11\%$ in four days.

In vivo assessment of scaffolds with tethered rhBMP-2

In Year 5, the Rutgers group tested the efficacy of scaffolds tethered with rhBMP-2 in the rabbit calvarial model. Scaffolds have recently been harvested from the rabbits, and histology and microCT will be used to gauge the efficacy of the MSD method for tethering. Final analysis of the data will be available in the upcoming year.

Conclusions

In Year 5, the Rutgers team identified pro-chondrogenic polymers and developed biphasic scaffolds for osteochondral regeneration. In vitro tests show that these scaffolds meet design criteria, and funding is being sought to proceed into animal trials. With respect to the bone regeneration scaffolds with tethered rhBMP-2, the Rutgers team used leveraged funding to conduct animal studies on the efficacy and safety of these scaffolds in a rabbit calvarial defect model. Results from this study are pending.

Research Plans for the Upcoming Year

The Rutgers team is seeking to obtain bridge funding to continue with the work on the osteochondral scaffolds, with the aim of obtaining proof-of-principle data in a relevant animal model. These biphasic scaffolds and the tethering technology have also found utility in a future project teaming with Dr. Pamela Yelick at Tufts University, again for a tissue interface. With respect to the bone regeneration scaffolds with tethered rhBMP-2, the Rutgers group has already communicated with industrial partners as the team awaits the histology from these experiments to confirm efficacy. Positive indications would lead to expansion of the technology and testing in larger animal models.

Planned Clinical Transitions

The technology of the Rutgers team, while promising, will require additional animal studies in larger animals. Should the preliminary animal data in the rabbit prove successful, the Rutgers team will consult with the U.S. Food and Drug Administration (FDA) and experienced AFIRM leadership to design the studies in the large animal models that will properly address the appropriate requirements of the FDA.



Bone Repair and Regeneration

Improved Preclinical Model for Orthopaedic Trauma

Project 4.8.1, RCCC

Team Leader(s): G. Elizabeth Pluhar, DVM, PhD (University of Minnesota); Joan Bechtold, PhD (Minnesota Medical Research Foundation); George Muschler, MD (Cleveland Clinic)

Project Team(s): Anne Nicholson, DVM, Cathy Carlson, DVM, PhD, Michelle Goulart, DVM, and Charles Seiler, BS (University of Minnesota); Viviane Luangphakdy, MS, Hui Pan, PhD, MD, Kentaro Shinohara, MD, and Cynthia Boehm, BS (Cleveland Clinic)

Collaborator(s): Joseph Wenke, PhD, Kinton Armmer, and Douglas Cortez (USAISR)

Therapy: Advanced regeneration of segmental bone defects

Deliverable(s): New large animal model of a critical bone defect

TRL Process: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers performed critical-size tibial defect surgeries on 30 goats with 12 weeks of radiographic follow-up demonstrating little to no bone formation in the defects treated with the current standards of practice, fresh autograft or morsellized allograft bone. The addition of fresh bone marrow aspirate (BMA) to the allograft did little to improve healing. The researchers observed a significant enhancement of new bone formation when they added recombinant human bone morphogenetic protein-2 (rhBMP-2) to allograft with and without BMA. Overall, the researchers' model appears to be sensitive enough to detect differences in healing among current bone grafting technologies. They created a document of the standard operating procedure for the surgeries, and produced an accompanying video of the surgical procedures.

Key Words: bone defect; caprine (goat) model; chronic; bone graft

Introduction

Large bone defects and chronic bone defects represent a difficult and, as yet, unsolved clinical challenge. The contemporary standards of care for bone defects and complex fractures (that include considerable damage of surrounding soft tissue) or fracture non-unions (have a permanent failure to heal) in civilian and military practice include many options for wound management, fixation and bone grafting. Bone grafting is a surgical procedure in which missing bone in complex fractures or fracture non-unions is replaced. Despite substantial advances in the availability of bone graft substitute materials and continuous development of new bone regeneration technologies, these wounds often fail to heal completely.

There are numerous animal models with critical-size defects in a variety of species and anatomic sites. Critical-size defects are those that do not heal without bone graft or other substitute, and the exact size of the defect varies depending on site, degree of soft tissue injury, patient age, and associated comorbidities. However, to-date, preclinical testing in

these models has resulted in complete defect healing when current standard of care treatments are used. This does not translate well to human clinical practice as these wounds often fail to heal in humans. Hence, more challenging and biologically relevant models are needed to compare improvements in the regenerative therapies that may become available to injured warriors and civilians whose injuries do not heal as readily as those in animal models.

Research Progress – Year 2 (Project commenced in 2011)

Characterization of the effect of current clinical practice standards for grafting

Assess the effect of clinical practice standards to improve the rate and extent of bone regeneration

The researchers performed critical-size tibial defect surgeries on 30 goats with 12 weeks of radiographic follow-up demonstrating little to no bone formation in the defects treated with the current standards of practice, fresh autograft or morsellized allograft bone (**Figure 1**). Approximately 20% of the defect was filled with new bone in the tibiae that were

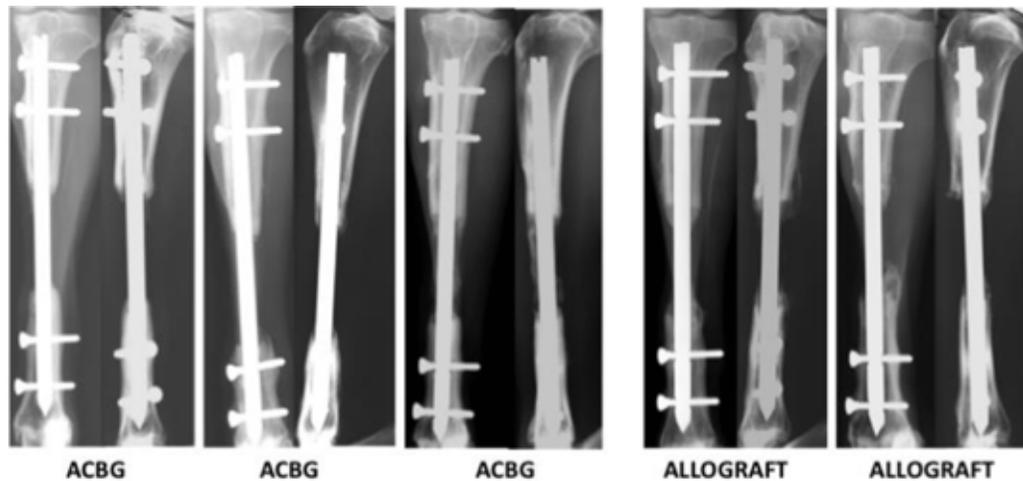


Figure 1. Radiographs (mediolateral [ML] projections) taken 12 weeks after the grafting surgery in the goats that had muscle excision, a smooth Poly(methyl methacrylate) (PMMA) spacer, and either fresh autogenous bone graft from the sternum or morsellized allograft bone placed in the defects. Note minimal new bone formation in the defects.

treated with autograft, and there was even less healing in the defects that were treated with allograft; about 7% filling with allograft alone and 12% filling with allograft mixed with BMA (**Figure 2**). None of the autograft or allograft with or without marrow-treated defects had healing defined as complete bridging of at least three of the four cortices except for a single defect with allograft and marrow. These data are summarized in **Table 1**. The radiographic assessment of bone healing shows that the model does “raise the bar” with complete healing in less than 5% of the treated defects.

Assess the effect of adding BMP-2 to the clinical practice standards

The addition of rhBMP-2 to allograft bone or allograft bone with BMA increased the amount of new bone formation in the defects (**Figure 3**). There was approximately 90% filling of the defect with new bone in all of the tibiae treated with rhBMP-2, and the majority of defects had complete bridging of bone on three or four of the cortices.

Characterize the concentration, prevalence, and biological performance of cancellous autograft and bone marrow-derived osteogenic cells in this model and their effect on bone regeneration

The work for this goal is still underway. The analysis of bone marrow from the proximal humerus and iliac crest demonstrated that the prevalence and concentration of osteogenic progenitor cells

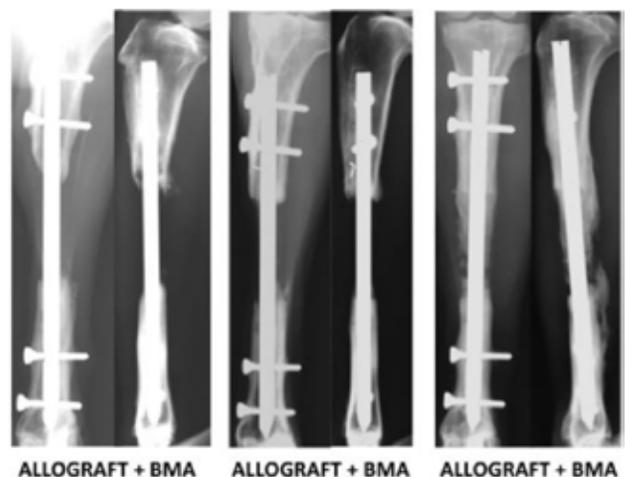


Figure 2. Radiographs (ML projections) taken 12 weeks after the grafting surgery in the goats that had muscle excision, a smooth PMMA spacer, and morsellized allograft + BMA from the sternum placed in the defects. Note new bone formation in the specimen on the left.

(11.3%, 223/ml) in goats were significantly less compared to bone marrow from humans (38.1%, 835/ml) or dogs (51.8%, 1851/ml). The BMA from the sternum had twice the number of nucleated cells/ μ l compared to the proximal humerus and ilium. The team cannot measure the effect of the prevalence of osteogenic cells on bone regeneration until the basic analysis is complete.



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Table 1. Area of bone filling the defect and number of cortices bridged by bone.

Treatment Group	New Bone Area (mm ²)	Cortices Bridged
ACBG #1	11.62	0
ACBG #2	46.2	0
ACBG #3	165.93	1
ACBG #4	194.3	1
ACBG #5	132.63	1
allograft #1	70.15	0
allograft #2	31.83	2
allograft #3	50.88	0
allograft #4	8.87	0
allograft + BMA #1	32.28	0
allograft + BMA #2	282.06	3
allograft + BMA #3	29.43	0
allograft + BMA #4	26.77	1
allograft + BMA #5	39.1	0
allograft + BMA #6	9.96	0
allograft + BMP #1	391.03	2
allograft + BMP #2	574.94	4
allograft + BMP #3	663.23	4
allograft + BMA + BMP #1	826.37	4
allograft + BMA + BMP #2	310.63	3
allograft + BMA + BMP #3	476.16	4

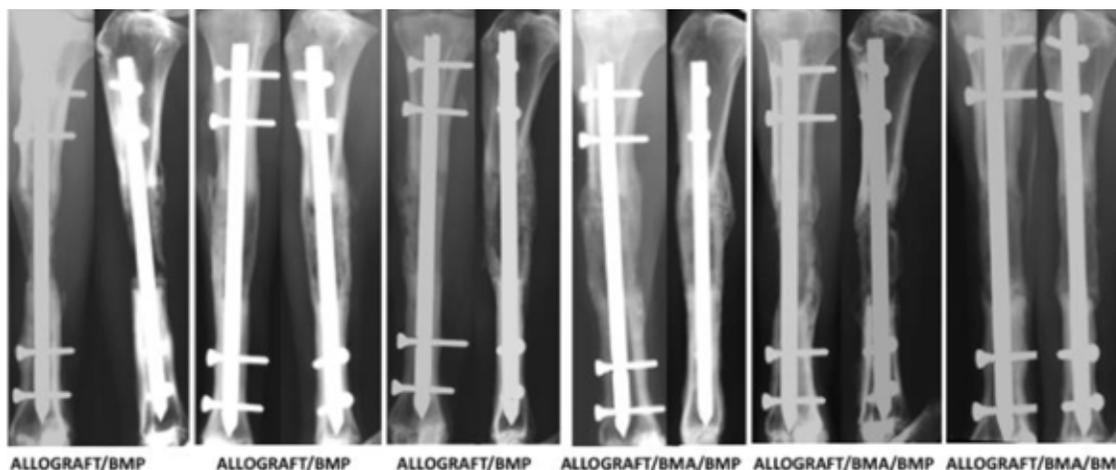


Figure 3. Radiographs (ML projections) taken 12 weeks after the grafting surgery in the goats that had muscle excision, a smooth PMMA spacer, and morsellized allograft + rhBMP-2 with and without BMA from the sternum placed in the defects. Note the robust new bone formation in all of the defects and bridging of the cortices.

Characterize the specific biological properties of the induced membrane and their effect on bone regeneration

Although there was not much variability in induced membrane weights for samples in each animal, there was some variability for weights between animals. The number of nucleated cells per gram of digested membrane tissue did not vary greatly among the specimens examined, with almost no difference for samples from the four sites in the same animal, except for a single outlier in goat 030. There was an average of 35×10^6 total nucleated cells per gram of digested induced membrane. The induced membranes were composed of fibroblast-like cells in a loose collagenous matrix with few to no inflammatory cells seen. Data collection from all centers for membrane analysis is ongoing. The analysis of the biochemical properties of the induced membranes has just begun. The team will be measuring the concentration of BMP-2, hyaluronan synthase, platelet-derived growth factor, and epidermal growth factor with ELISA kits that are species-specific for goats.

Use the validated chronic caprine tibial defect model to test new bone regenerative technologies in the AFIRM I pipeline

Systemically characterize the rate and extent of bone regeneration from the new technologies compared to the best results from Experiment #2

The project team has ordered the goats to begin working towards this goal and will initiate the

surgeries in the upcoming year. The polypropylene fumarate implants developed by Dr. Yaszemski's group at the Mayo Clinic will be evaluated with BMP added using the historical drip loading and binding method, or an infusion method using a hydrogel that will polymerize in situ.

Conclusions

The chronic caprine tibial defect model “elevates the bar” of biological challenge to a level where improvements over current standards of care or treatment can be detected. The protocol can be reproduced at different institutions by following the standard operating procedure, and all procedures are well tolerated by the goats. This model appears to be sensitive enough to detect differences in healing among current bone grafting technologies.

Research Plans for the Upcoming Year

The work to characterize the membrane induced by the PMMA spacers will continue with funding from a Congressionally Directed Medical Research Programs Peer Reviewed Orthopaedic Research Program award, “Optimization of Soft Tissue Management, Spacer Design, and Grafting Strategies for Large Segmental Bone Defects Using the Chronic Caprine Tibial Defect Model.”





Soft Tissue Repair and Regeneration

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

Project 4.3.2, RCCC

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

Project Team(s): Malika Satirraju, MS (Cleveland Clinic); Elliott Sanders, PhD (Nitinol Development Corporation); Ilia Koev, PhD (Biogeneral, Inc.); Keith Platenyk, BS (Evonics, Inc.)

Collaborator(s): Craig Bonsignore, MBA and Elliot Sanders, PhD (Nitinol Development Corporation); Keith Platenyk, BS (Evonics, Inc.); Diwalker Ramanathan, PhD (Resonetics, Inc.); Rodney White, MD and George Kopchak, BS (LA Biomed/UCLA Harborview)

Therapy: Minimally invasive treatment of arterial and venous trauma

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

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers manufactured several bioabsorbable stents from different polymers – polylactic acid (PLLA) and polydioxanone (PDO) – using an athermal laser and a fracture-resistant stent design. After a series of tests, the researchers identified appropriate dimensions to successfully allow for greater radial force and implementation. The team constructed a final delivery catheter system and initiated a second animal trial. Final prototypes were developed, and animal implants are in process.

Key Words: stent; peritoneum (tissue); polydioxanone; polylactic acid; mineral oil; blood vessel trauma; delivery system and catheter

Introduction

Over the past 15 years, there has been a transformation in vascular surgery towards minimally invasive therapy. Currently, the best minimally invasive therapy available to treat injured blood vessels is a stent lined with the synthetic material “expanded polytetrafluoroethylene.” However, the long-term success rates of these “off-label” stent grafts are sub-optimal, as they are highly susceptible to infection and failure. There is no minimally invasive stent graft specifically designed to treat blood vessel injuries taking into account the particular issues related to the trauma theatre in combat and civilian populations. This is even more important as published reports from recent conflicts indicate that blood vessel injuries to U.S. troops are five-times higher than previously thought. The competitive advantage of tissue-lined stents comes from several desirable components. In animal and human studies, the tissue lining imparts resistance to intimal hyperplasia and allows re-endothelialization. The tissue is also thin (150 μm), so it is feasible to construct a low-profile stent graft. Finally, it is well known that tissue is less susceptible to infection.

The combination of a bioabsorbable and/or fracture-resistant stent with a tissue lining produces an optimal hybrid technology. Bare metal stents alone are not an option to treat arterial injuries, as the interstices between stent struts allow for continued exsanguination. Additionally, long-term fatigue has resulted in insufficient durability. Bioabsorbable stents are similar to the above, as they have interstices that allow for continued blood flow and hemorrhage unless lined. They do have the benefit of eliminating long-term fatigue issues that have plagued stents placed in areas of high arterial movement, which lead to stent fractures and thrombosis. However, no degradable stent has been U.S. Food and Drug Administration approved for the periphery, nor has one demonstrated efficacy in patency. Additionally, no information has been published regarding the development or utility of a bioabsorbable, self-expanding stent. Recent evaluation of bioabsorbable stents in coronary arteries produced suboptimal results, and others have pursued the application of drug-eluting bioabsorbable stents. The project team’s solution is to combine tissue with the bioabsorbable stent.

Research Progress – Year 5

Meet with FDA for pre-Investigational Device Exemption (IDE) fracture-resistant nitinol stent graft design

The project team had its pre-IDE meeting with the FDA for the fracture-resistant stent design in October 2012. The meeting was a success, and the team had a follow-up meeting in March 2013. The researchers anticipate beginning the IDE in January 2014. For its bioabsorbable stent, the team will manufacture PDO and PLLA into appropriate tube designs.

Manufacture PDO and PLLA stents

The goal was to manufacture PDO and PLLA stents cut with an athermal laser (Figure 1). The team identified a source for the PDO and PLLA (Evonics, Inc.), and then identified a place to extrude the stent tubes (Biogeneral, Inc.).

The project team had two groups working on cutting the stent design, and they settled on Resonetics, Inc. Resonetics successfully cut the stent with an athermal laser in the same stent design as the Sarac group’s fracture resistant nitinol stent design (Figure 2A). This design turned out to be adequate, but the size of the tubes that are normally cut are much smaller, since the nitinol stent must be post-processed. The original stent design worked, but the thickness of the material did not allow for enough radial force (Figure 3), and was also too thin to sew the tissue.

The project team changed the design of the stent and made minor adjustments in the manufacturing process, which allowed for greater radial force (Figure 4); this made it easier to sew the tissue in order to produce its final product.



Figure 1. PDO tubes and laser-etched tubes with fracture-resistant design.



Figure 2A. Fracture-resistant nitinol stent design.



Figure 2B. Degradable stent design.

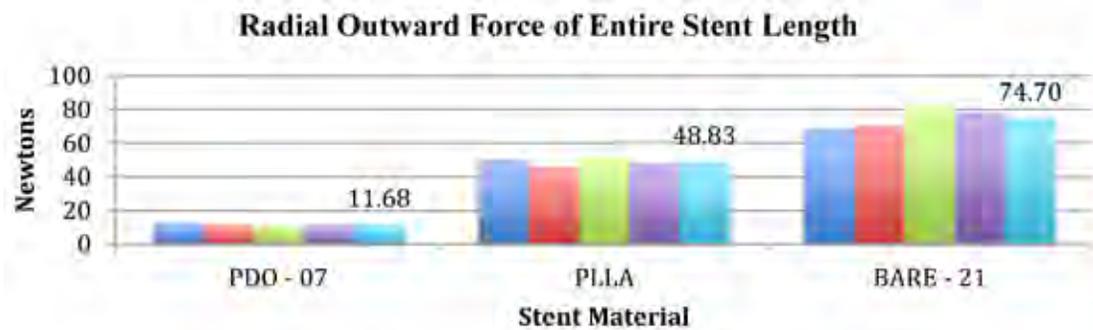


Figure 3. Radial force testing of original stent.



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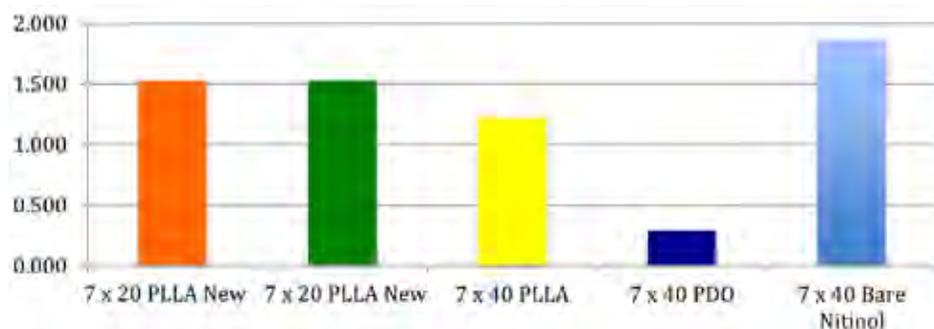


Figure 4. Radial force testing of stent with improved design.

Manufacture and sew stent grafts

The team was able to identify and manufacture appropriate dimensions for the stent graft with a thickness that allowed for enough radial force and could support sewn tissue. The new stent construction was successful, and the project team was able to easily sew the tissue onto the stents (Figure 5).



Figure 5. Newly constructed stent with sewn tissue.

Finalize delivery catheter design and in vivo tests

The delivery system (Figure 6) underwent minor modifications to remove the connectors and reduce the risk, while making it more user friendly. The project team also demonstrated that it could successfully crimp the stent. In conjunction with Peritec Biosciences, the researchers are now progressing to design verification and freeze. The team also obtained Institutional Animal Care and Use Committee, and Animal Care and Use Review



Figure 6. Delivery system prototypes.

Office approval for the evaluation of this design in the dog model in the upcoming year.

Conclusions

The project team has successfully manufactured and extruded tubes of PDO and PLLA, and cut them with an athermal laser into a fracture-resistant stent design. Final prototypes of the delivery system have been made.

Research Plans for the Upcoming Year

Animal implants will be completed, and the in vivo performance of the implants will be analyzed in the upcoming year. Depending on the animal implant results, the Sarac lab may make minor modifications to the stent design, or conduct long-term Good Laboratory Practices (GLP) studies. The team will also limit these GLP studies to one polymer, and they will complete design verification and mechanical testing.

Planned Clinical Transitions

The clinical transition will be done through Peritec Biosciences, who will obtain funding for an IDE application.



Soft Tissue Repair and Regeneration

Construction of Tissue-Engineered Blood Vessels

Project 4.3.2a, RCCC

Team Leader(s): Thomas N. Tulenko, PhD (Cooper University Hospital)

Project Team(s): Jeffrey Carpenter, MD and Shaohua Chang, PhD (Cooper University Hospital)

Collaborator(s): Paul J. DiMuzio, MD (Jefferson University Hospital)

Therapy: Vascular reconstruction

Deliverable(s): Tissue-engineered blood vessel (TEBV)

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers successfully developed a method of activating endothelial nitric-oxide synthase (eNOS) using a small molecule in adipose-derived stem cells (ASCs) previously differentiated to an eNOS+ endothelial cell (EC)-like phenotype in cell culture. The team fabricated a TEBV lined with eNOS+ ASCs as well as a cell-free TEBV. These scaffolds were successfully implanted into a dog carotid model and are being evaluated.

Key Words: tissue engineering; regenerative medicine; vascular grafts; human

Introduction

While saphenous vein grafts are now deemed successful for vascular bypass grafting, there usually is a limited donor supply, or it is destroyed in devastating combat injuries. To treat these injuries, trauma surgeons often use prosthetic (polytetrafluoroethylene [PTFE]) grafts, which have a high rate of infection and frequently fail due to thrombosis. Moreover, when PTFE grafts are used, secondary limb amputations are usually required. In addition, over half of the vein grafts fail within 10 years of surgery due to progressive intimal hyperplasia and, eventually, superimposed accelerated atherosclerosis. These observations indicate a clear need for improved arterial graft technology.

Vascular tissue engineering potentially offers a better graft than those currently available in the absence of autologous tissue. Preliminary data suggests that the expression of eNOS, the enzyme that produces nitric oxide and is therefore important to EC function, is limited in ASCs. Further studies demonstrated that for a TEBV to integrate successfully into the circulation, a fully functional EC-lined lumen is essential; this requires not only the usual EC phenotypic markers, but also a fully functional eNOS protein capable of generating NO on demand. In this project, the researchers are using human “endothelial progenitor-like” cells derived from human ASCs to form a luminal surface of

a biological tubular scaffold that can be used for segmental vascular repairs. This technology could potentially reduce the long-term risk of the scaffold failing due to infection, re-stenosis, and re-injuries.

Research Progress – Year 1 (Project commenced in 2012)

Using ASCs already differentiated into an EC-like phenotype, the Tulenko lab has developed a method to activate essential endothelial genes (CD31, von Willebrand Factor and eNOS). The team has also discovered a small molecule that can activate the expression of these genes to levels high enough to likely provide full biologic activity. These cells are currently being used to coat the lumen of TEBVs for testing in vivo (dog carotid). The researchers are also developing a cell-free TEBV, where the scaffold is coated with the small molecule in an attempt to capture and hold endothelial progenitor cells circulating in blood.

The team demonstrated that plastic treated with the small molecule increased ASC adhesion. In addition, it was shown that commercial, small intestinal submucosa (SIS) tubular scaffolds treated with the small molecule increased ASC adhesion to the scaffold (**Figure 1**). Furthermore, the superior ability of the small molecule to increase the proliferation rate of ASCs was demonstrated. These data suggest that increased rates of stem cell proliferation may result



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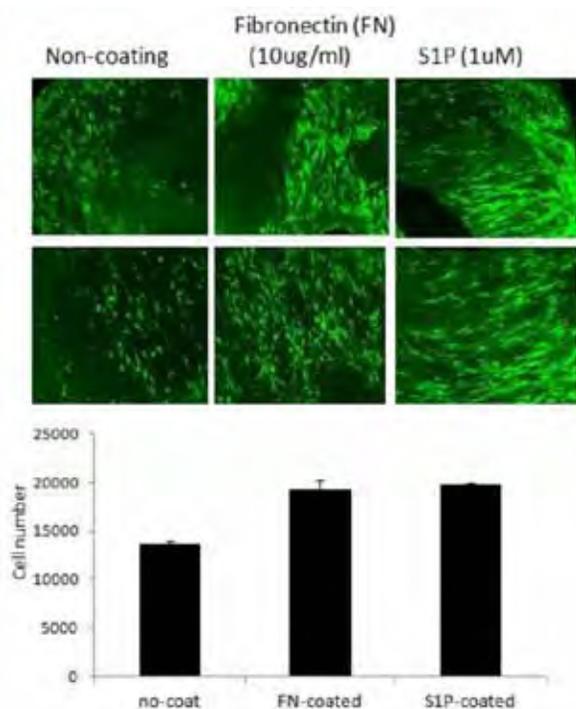


Figure 1. Increased retention of eNOS+ ASCs on SIS scaffold.

in faster luminal covering of the SIS lumen. The team hypothesized that these cells will encourage autologous cells to differentiate into endothelium and thus protect the TEBV from degradation and loss of functionality. Supporting this hypothesis is **Figure 2**, which demonstrates full patency of the SIS tubular scaffold coated with the small molecule after 28 days in vivo in the dog carotid artery.

Conclusions

Two types of TEBVs for arterial segmental repair were developed: cell-free TEBVs and cell-lined TEBVs. The cell-free TEBVs were coated with a small molecule that is believed to encourage autogenous cells to differentiate into endothelium. These scaffolds were implanted into a dog carotid model. Preliminary analysis at 28 days post-implantation suggests that the TEBV is patent and functioning as a competent arterial segment with no coagulation or intimal hyperplasia. A definitive conclusion will be made after six months post-implantation with detailed macro- and microscopic examinations. The cell-lined TEBVs are coated with ASCs that were previously differentiated into an eNOS-positive EC-like phenotype.

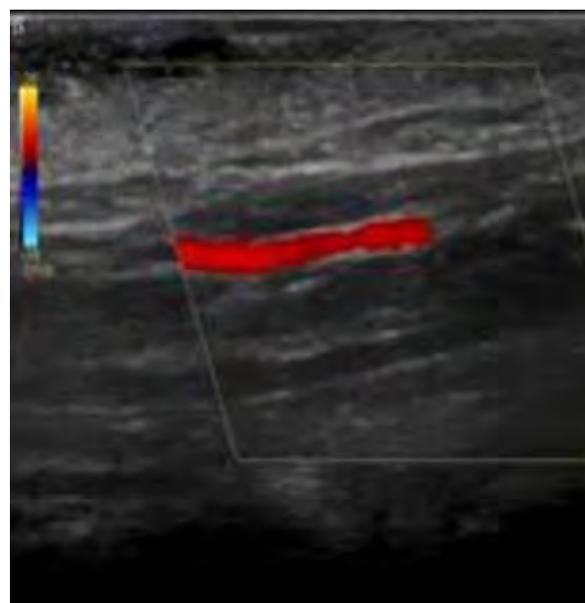


Figure 2. Ultrasound image of cell-free scaffold implanted in the dog carotid artery which is coated with the small molecule that activates eNOS in host cells adhering to the scaffold. Red color is flowing blood.

Research Plans for the Upcoming Year

In the upcoming year, the researchers will complete the in vivo evaluation of the TEBVs in the dog carotid model (6 months post-implantation). Plans beyond the upcoming year are contingent on the acquisition of funding. A National Institutes of Health grant is pending that may greatly expand the in vivo testing of the TEBVs developed in this study, both in normal rabbits and in cholesterol-fed rabbits that will generate atherosclerotic lesions. This analysis will determine whether an atherogenic profile, not unlike that seen in most people in developed countries over the course of their lifespan, will inadvertently affect the durability of the TEBVs.

Planned Clinical Transitions

If the results following testing in a second animal species (rabbit) continue to be positive, an application for testing this device in humans will be filed with the U.S. Food and Drug Administration, and a clinical protocol will be submitted for approval by the local Institutional Review Board.

Soft Tissue Repair and Regeneration**Functional Scaffolds for Musculoskeletal Repair and Delivery of Therapeutic Agents****Project 4.4.3a, RCCC**

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

Project Team(s): Sambit Sahoo, MD, PhD and Joseph Iannotti, MD, PhD (Cleveland Clinic)

Collaborator(s): Michael Rosen, MD (University Hospitals, Cleveland)

Therapy: Repair of large abdominal wall defects and hernias

Deliverable(s): A fiber-reinforced allograft or xenograft scaffold (dermis)

TRL Progress: Beginning, TRL 1; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers demonstrated that biologic dermal grafts could be fiber-reinforced, and then downselected the scaffold design and transitioned to a semi-automated scaffold manufacturing process to scale up scaffold generation. The team established a clinically relevant large animal (pig) model for abdominal wall repair and initiated a pilot study using two pigs. They developed a non-invasive method for post-operative monitoring of repair bulging/hernia recurrence in the pigs, and established a protocol for retrieval, mechanical testing and analysis of the pig abdominal wall.

Key Words: extracellular matrix; abdominal wall; hernia; allograft; xenograft

Introduction

Researchers have experienced major challenges in designing methods to repair abdominal wall defects and hernias that result from traumatic injuries, surgery, or chronic diseases. Abdominal wall injuries sustained during military trauma are typically large (with massive loss of tissue) and infected, and one in every three cases fails to heal. Abdominal wall hernias also complicate nearly one-third of all abdominal surgeries in the civilian population, and an estimated 350,000 hernia repairs costing a total of \$3.2 billion are performed each year in the United States alone (2006 data). Currently available synthetic and biologic grafts have demonstrated limited success. While the use of synthetic meshes has reduced hernia recurrence, these repairs often require repeat surgery as a result of complications such as mesh infection, bowel adhesion, extrusion, and fistulation. These complications can largely be avoided through the use of biologic grafts for hernia repair.

Biologic dermal grafts are commonly used in clinical practice, since they possess biomechanical properties similar to abdominal wall fascia. However, implanted dermal grafts lose mechanical strength and integrity over time, leading to complications such as bulging and hernia recurrence. Hence,

engineered improvements to biologic grafts are necessary to make them more suitable for abdominal wall repair and reconstruction. Hernia recurrence rate is very high, with 24–43% of hernia repairs resulting in repair failure and hernia recurrence. It is estimated that a 1% reduction in recurrence rate will result in a cost savings of \$32 million.

Research Progress – Year 5

The Derwin group demonstrated that the dermal grafts that were reinforced using the group's proprietary fiber reinforcement technique were significantly stronger and stiffer than native dermis grafts. Fiber reinforcement also mitigated the weakening of dermis grafts due to enzymatic degradation, and it reduced their stretching after cyclic physiologic loading. The researchers downselected the device design and used a computer-controlled stitching machine to manufacture scaled-up prototypes. They established a clinically relevant large animal (pig) model with the help of surgeon collaborators to evaluate the efficacy of the reinforced grafts in repairing a large abdominal wall hernia. The researchers also developed a non-invasive method for monitoring repair bulging/hernia recurrence in the pigs, and established protocols for pig abdominal wall retrieval, mechanical testing and data analysis. They initiated a pilot study using two pigs



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to evaluate the new surgical model and recently commenced proof-of-concept studies with the scaled-up reinforced scaffolds in the preclinical pig hernia model.

Conclusions

The project team had earlier demonstrated that biologic grafts stretch significantly with loading and after remodeling. In Year 5, the researchers showed that biologic grafts could be fiber-reinforced using their proprietary technology to improve stiffness and strength. Fiber reinforcement also reduced the weakening and stretching of graft after cyclic physiologic loading. The researchers downselected the device design and manufactured scaled-up prototypes using a semi-automated process. Notably, they initiated an animal study in pigs to evaluate the efficacy of the scaled-up reinforced grafts in repairing a large abdominal wall hernia.

Research Plans for the Upcoming Year

Regarding the proof-of-concept studies, the researchers will monitor the pigs post-operatively for repair bulging/hernia recurrence, and they will also mechanically test the repairs upon completion of the studies.

Planned Clinical Transitions

The researchers established a formal collaborative partnership with a clinical collaborator and, based on guidance from consulted clinicians, are planning a preclinical large animal study. The project team plans to continue technology validation and product development together with prospective industrial collaborators. As the device moves into product development and a regulatory path is defined, the U.S. Food and Drug Administration will be engaged to enable the execution of a clinical trial.



Soft Tissue Repair and Regeneration**Functional Scaffolds for Soft Tissue Repair and Joint Preservation****Project 4.4.3b, RCCC**

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

Project Team(s): Aaron Merriam, BS (University of Medicine and Dentistry of NJ)

Collaborator(s): Joachim Kohn, PhD and Sanjeeva Murthy, PhD (Rutgers University)

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

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

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers recovered polymer fiber/collagen/glycosaminoglycan tissue-engineered meniscus scaffolds and knee joints from 16- and 32-week functional sheep models that were previously implanted. They mechanically and histologically evaluated the recovered implants, and also histologically evaluated the joint surface of implanted and control sheep knees. The researchers made design modifications to the scaffold for long-term evaluations in a sheep model. They implanted a new batch of meniscus scaffolds into sheep for evaluation at 52 weeks.

Key Words: meniscus; scaffold; degradable polymeric fibers; collagen; regeneration; joint preservation

Introduction

This project addresses a large burden to the military and its personnel: injuries to the musculoskeletal system, specifically, the meniscus and cartilage of the knee joint. Severe damage to the knee meniscus can significantly impair the normal activity of a military member. Due to limited healing capabilities, meniscus injuries are often treated with surgical resectioning. While this treatment may lead to symptom relief, in many cases it results in the development of degenerative arthritis of the knee. The pain associated with damaged meniscal tissue as well as arthritis in the knee can have a debilitating effect on the ability of Servicemen/women to perform, inhibiting their operational capability in both the short- and long term.

The focus of this project is the development of a tissue-engineered meniscus scaffold for the treatment of moderate to severe meniscal damage. The overall goal is to develop an off-the-shelf clinical device that can be implanted at the site of a meniscal resection to help preserve the knee joint. The clinical role of the device is two-fold: (1) Provide symptom relief and rapid return of function for active military personnel, and (2) prevent the progression to degenerative knee arthritis that commonly requires costly total knee replacement surgery later in life.

Research Progress – Year 5**Full functional meniscus scaffold in a sheep implantation model**

The medial menisci of 20 sheep were removed, and 16 were replaced with a polymer fiber/collagen/glycosaminoglycan tissue-engineered meniscus scaffold (**Figure 1**). Four knees were left as meniscectomy controls. Implants were allowed to incorporate and remodel for 16 or 32 weeks before recovery. All implants have now been recovered and were found fully intact with no ruptures or fixation failures as seen with the first generation implants.

Implant Mechanical Evaluation

The recovered implants have been mechanically evaluated to assess both their strength retention and the strength of the new tissue being deposited within the construct.



Figure 1. Depiction of completed scaffold (top) and underlying polymer fiber pattern (bottom).



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The tensile strength of the second generation explants at both 16 and 32 weeks was five times higher than the normal functional loads of the native meniscus. The initial tensile stiffness of the scaffold did not significantly change the course of the study and was found to be similar to that of the native meniscus.

Confined compressive creep experiments have been performed to determine the aggregate modulus and permeability of the new tissue ingrowth. The explants are 25%–40% as stiff in compression as the native meniscus and trend towards an increased modulus over time, which may eventually reach native values at longer time points (52-week study). As expected, the permeability of the scaffolds greatly decreases after implantation, most likely as a result of the extensive tissue deposition within the matrix, with values approaching the native structure.

Implant Histological Evaluation

Excellent tissue adherence and incorporation was observed both grossly and histologically within all explants. A mild and expected inflammatory response was observed and seems to decrease from 16 to 32 weeks. The polymer fiber is found to persist in the body, with little resorption observed (Figure 2, left). Vascularization was found throughout the explants at both time points.

Knee Articular Cartilage Evaluation

Figure 2 shows micrographs of hematoxylin and eosin stained cartilage sections from the medial femoral condyles of control and experimental knees. In the native knee, the surface of the cartilage was relatively smooth and continuous, with an even arrangement of cells throughout the

cartilage (Figure 2A). In the control meniscectomy knees, moderate surface damage and hypocellularity were observed (Figure 2B). The experimental 32-week knees did show some areas of minor surface hypocellularity and minor disruption, but also had samples with no or little degeneration (Figure 2C). These observations correspond with gross observations in which control knees had severe articular damage as compared to those with the meniscus implant.

Third generation meniscus scaffold

The evaluation of the 16- and 32-week meniscus implants elucidated possible areas of improvement for the meniscus implant device. Thus, some minor modifications to the second generation scaffold have been made in preparation for a year-long sheep implantation study.

Improved Implant Design

By adjusting the molecular weight and extrusion process, Dr. Kohn's group has aided the development of a superior poly(desaminotyrosyl-tyrosine dodecyl dodecanedioate) fiber. The new polymer fiber is 80% stronger and 50% tougher than that used in the latest 16- and 32-week implantation study. In addition to the improved fiber properties, the polymer fiber pattern of the implant has also been altered to help reinforce the inner margin of the implant. This is done by adding more of the #10 and #12 patterns, which increases the number of fibers to the central inner margin of the implant.

Third Generation Scaffold in a Year-Long Sheep Implantation Study

A new batch of meniscus implants has been fabricated and implanted for a 52-week functional sheep study (8 implants, 2 controls).

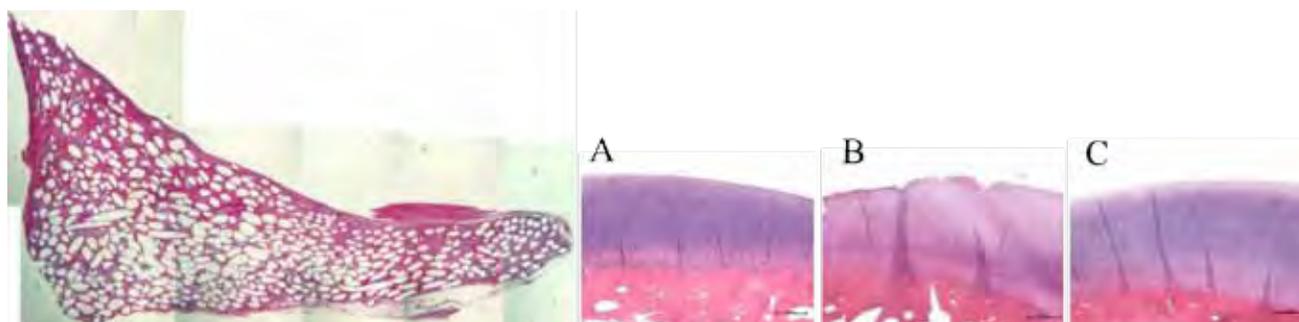


Figure 2. Left: 32-week explant stained with hematoxylin and eosin; white void spaces represent polymer fibers, pink represents new collagen deposition. A: Native femoral chondyle depicting articular cartilage and underlying bone; B: Control meniscectomy knee (32 weeks); C: 32-week implanted femoral chondyle. (Magnification rate is 20x.)

Conclusions

The researchers' tissue-engineered meniscus replacement scaffold has been shown to: (1) mimic compressive properties of the meniscus, (2) convert compressive loads into circumferential tensile loads, (3) increase the contact area in the joint space while reducing high concentrated stresses, (4) encourage cell infiltration, extracellular matrix production, and organized tissue deposition while degrading at a practical rate, and (5) have a protective effect on the articular surfaces such that degenerative changes associated with meniscectomies are reduced. The current long-term evaluation of the meniscus replacement device should sufficiently demonstrate its capabilities and bring Meniscofix one-step closer to clinical trials and U.S. Food and Drug Administration (FDA) approval.

Research Plans for the Upcoming Year

The team plans to complete the 52-week implantation study to determine the efficacy of the scaffold in a total meniscus replacement model in sheep. The neo-meniscus tissue and articular cartilage will be evaluated using biomechanical testing and histological analyses, and data will be compared to that for control unoperated knees.

Planned Clinical Transitions

Transition to clinical trials will be dependent on the results of the one-year implantation studies and planned longer-term studies through two years post-implantation. The team is in discussions with several companies who have expressed interest in partnering or co-developing the technology. They also plan to meet with representatives of the FDA to determine the best regulatory strategy for this novel medical device.





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Soft Tissue Repair and Regeneration

Oxygen-Generating Biomaterials for Large Tissue Salvage

Project 4.4.6, WFPC

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

Project Team(s): Benjamin Rowe, MD and Catherine Ward, PhD (Wake Forest University)

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

Therapy: Supply temporary oxygen to hypoxic tissue

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

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 3

Key Accomplishments: The researchers created a controllable, injectable, oxygen-generating biomaterial and tested it for sustained release of oxygen in vitro and feasibility of injection in vivo. They most recently evaluated if an oxygen-generating

compound known as sodium percarbonate (SPO) could support skeletal muscle metabolism under low-oxygen conditions using the loss of muscle contractility as a primary index of loss of metabolic activity. They identified biologically and physiologically compatible concentrations of SPO in skeletal muscle under hypoxic or ischemic conditions in vitro and in vivo, respectively. Collectively, the researchers found that SPO at a dose of 1 mg/mL represents a biocompatible oxygen-generating material capable of partially supporting skeletal muscle homeostasis under otherwise hypoxic/ischemic conditions. They also created a model of partial ischemia in the rat hindlimb and found that SPO-generated oxygen could, at least partially, maintain resting skeletal muscle metabolism in vivo in this model.

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

Introduction

Replacement or restoration of tissue loss caused by traumatic injury, congenital defects, tumor removal or severe burns is a challenge. For example, current treatment for reconstruction of large muscle loss still results in limited functional restoration and poor donor site healing. In addition, following traumatic injury or chronic peripheral vascular disease, the blood vessels supplying the tissue are compromised (or absent) and the needs of downstream organs/tissue will not be met. Restoring blood flow can take time, as natural blood vessel regrowth is a slow process, so intervention is needed to preserve tissue while the body heals itself.

Since metabolically active cells can only survive up to a few hundred micrometers away from a blood supply due to oxygen diffusion limitations, investigators have used a variety of biological approaches to promote new blood vessel growth. While such approaches are able to stimulate regrowth, the amount of time needed to establish the vascular network may be inadequate.

There are several approaches to delivering oxygen to tissues including hyperbaric oxygen therapy (HBOT), perfluorocarbons, and topically applied gaseous oxygen. HBOT uses high concentrations of oxygen gas to increase the distance oxygen can diffuse. It has also been implicated in stimulating wound-healing pathways. Advantages of the HBOT approach include current U.S. Food and Drug Administration approval for certain indications such as decompression sickness, gangrene, brain abscess, and injuries in which tissues are not getting enough oxygen. Disadvantages include expensive capital costs, and avascular tissue oxygen diffusion is still limited. Perfluorocarbons are another approach to delivering oxygen. However, the hydrophobic nature of perfluorocarbons requires the use of emulsions, frozen storage and rewarming, leading to a decline in popularity. Topical delivery of oxygen is a third approach that is available. Medical devices are available which provide transdermal sustained oxygen delivery for the treatment of chronic wounds, as well as skin grafts, burns, and frostbites. These include a small, silent, disposable, oxygen concentrator and a 43" long sterile cannula

(tube), used with any fully occlusive sterile wound dressing to continuously blanket the wound with oxygen. However, each approach has distinct advantages and disadvantages particularly in relation to the needs of the injured warrior.

The researchers' approach is to prepare injectable oxygen generating material that would allow delivery of oxygen in controlled amounts to engineered tissue scaffolds or pre-existing tissue, which may allow for improved tissue salvage and regeneration. They believe that this approach may overcome one of the major limitations in muscle salvage and tissue engineering by acting as a supplemental oxygen source in several regenerative models. The goal of this project, therefore, is to provide oxygen at a therapeutic concentration, although not necessarily replicating standard cell growing conditions used in the laboratory. The materials used are based on encapsulated solid peroxides that breakdown upon contact with water to oxygen, water and other biocompatible byproducts.

Research Progress – Year 5

Development and optimization of hind limb ischemia model to evaluate the utility of particulate oxygen generators for preservation of tissue structure and function

Skeletal muscle has a broad and plastic metabolic capacity designed to efficiently support the energy requirements of the tissue. Energy is primarily generated by oxidative metabolism at rest and during activity (i.e., exercise) in muscles. In response to low oxygen, however, the cells use a less efficient glycolytic metabolism to derive energy. However, this quickly (~4 hours) depletes the available stored energy and eventually leads to tissue death.

The researchers evaluated if an oxygen-generating compound known as SPO could support skeletal muscle metabolism under low-oxygen conditions using the loss of contractility as a primary index of loss of metabolic activity. They identified biologically and physiologically compatible concentrations of SPO in skeletal muscle under hypoxic or ischemic conditions in vitro and in vivo, respectively. Collectively, the findings of these studies demonstrated that SPO (1 mg/mL) represents a biocompatible oxygen-generating material capable of partially

supporting skeletal muscle homeostasis under otherwise hypoxic/ischemic conditions.

In response to hypoxia (low oxygen), SPO injection partially preserved intramuscular glycogen stores (* $p=0.01$ compared to uninjured muscle), which were nearly completely depleted in untreated muscles (* $p=0.0001$ compared to uninjured muscle) (**Figure 1** D-F, H; # $p=0.048$ between untreated and SPO-injected muscles), suggesting a greater use of oxidative metabolism in coordination with SPO decomposition. Additionally, 1 mg/mL SPO injection significantly attenuated the increased presence of nuclear co-localized HIF1 α ($p=0.150$ compared to uninjured muscle), which was observed in untreated muscles following hypoxia (* $p=0.001$ compared to untreated muscle) (Figure 1 A-C, G; # $p=0.0007$ between untreated and SPO-injected muscles). And lastly, free radical oxidative stress or lipid peroxidation (malonaldehyde muscle content) observed in hypoxic untreated muscle (* $p=0.023$ compared to uninjured muscle) was attenuated following 1 mg/mL SPO injection ($p=0.13$ compared to uninjured muscle) (Figure 1I; # $p=0.025$ between untreated and SPO-injected muscles). Collectively, these findings indicate that 1 mg/mL SPO partially prevented a hypoxia-induced metabolic sequela, and correspondingly preserved a greater level of contractility.

In vivo evaluation of SPO in a rat hind limb ischemia model

Upon finding that SPO-generated oxygen was capable of partially maintaining skeletal muscle resting metabolism under hypoxic conditions in vitro, the researchers sought to determine the effectiveness of SPO for the preservation of ischemic skeletal muscle tissue in vivo. To promote ischemia in the rat hindlimb, the iliac artery was ligated for 24 hours, after which time the tibialis anterior (TA) muscle (the prime dorsiflexor) was treated and assessed for signs of tissue hypoxia. As a global metabolic index, TA muscle contractility was measured via neural stimulation of the common peroneal nerve in anesthetized rats (2.0% – 2.5%) for thirty minutes (contractions performed at 100 Hz, 200 ms, 5 minute resting interval) prior to harvesting muscles for glycogen content analysis. Over the 30-minute in vivo protocol, non-ligated control muscles did not demonstrate a reduction in torque and exhibited



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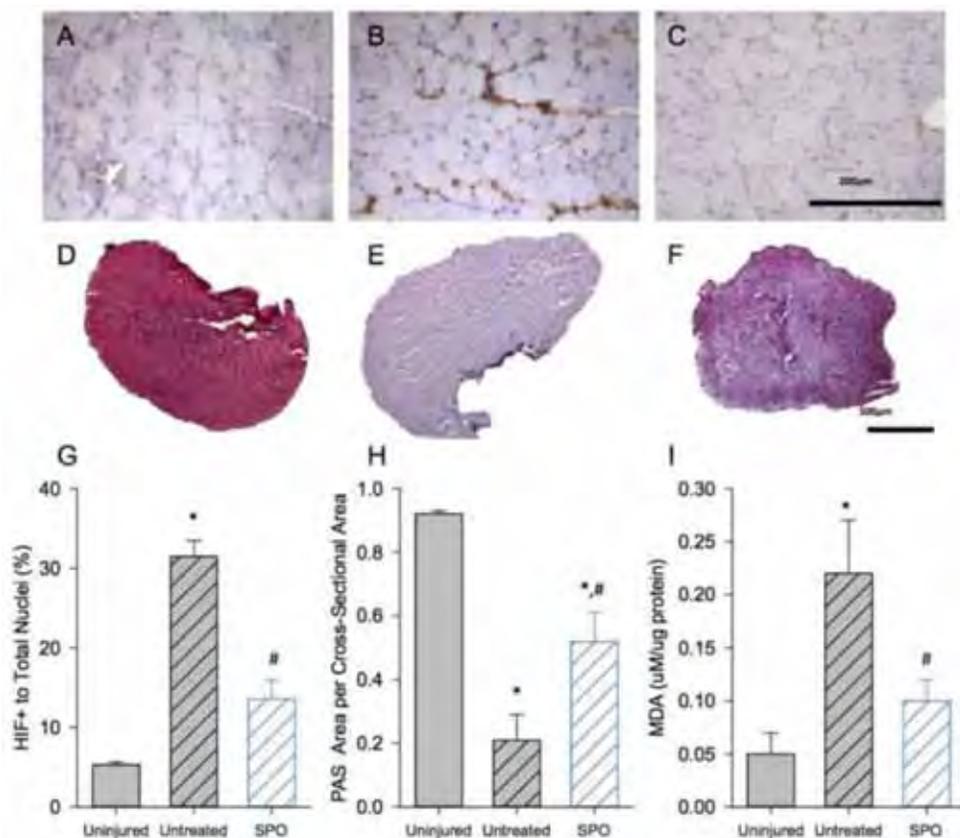


Figure 1. Hypoxia-induced HIF1 α accumulation, glycogen depletion, and oxidative stress with and without SPO. Histological assessment of extensor digitorum longus muscles with HIF1 α (A, B, C) and PAS glycogen (D, E, F) staining for uninjured (native) (A,D), untreated (B,E) and SPO (1mg/mL) (C,F). Quantitative analysis was performed with HIF1 α (G) and intramuscular glycogen (PAS; H) stained sections. Lipid peroxidation was assessed by quantifying malonaldehyde concentrations (I). * $p < 0.05$ compared to uninjured; # $p < 0.05$ compared to untreated. Values are means \pm sem; sample size = 4 – 9/group.

normal glycogen staining, indicating that with an intact blood supply, the contractile protocol does not induce fatigue (i.e., is not metabolically taxing) (Figure 2A). On the other hand, following iliac artery ligation, saline-injected TA muscles exhibited a ~90% reduction in torque within 15 minutes, and out to 30 minutes, of contractile activity that corresponded with a nearly complete depletion of intramuscular glycogen (Figure 2A). These findings are consistent with others indicating increased susceptibility to muscular fatigue with hindlimb ischemia. However, SPO injection maintained ~30% of initial torque values out to 30 minutes of testing (Figure 2A; # $p=0.044$) and maintained glycogen staining to a similar level as non-ligated muscle (Figure 2B, C-E; $p=0.57$). These findings indicate that SPO-generated oxygen can, at least partially, maintain resting skeletal muscle metabolism *in vivo* in a model of partial ischemia.

A technology that delivers oxygen to hypoxic tissues in a fashion that is independent of vascular status (i.e., in either the absence of an intact vasculature or in the face of a compromised vascular supply) would represent an important medical advance with applications regarding tissue salvage, repair and regeneration following soft tissue trauma. SPO was selected for this purpose because it theoretically produces sufficient amounts of oxygen to maintain skeletal muscle metabolism via the catalytic decomposition of hydrogen peroxide. Herein, we demonstrate that a biologically and physiologically compatible concentration of SPO partially prevented: (1) the loss of contractility, HIF1 α protein accumulation, glycogen depletion, and lipid peroxidation *in vitro* after a 30-minute incubation under hypoxic conditions (95% N₂-5% CO₂; 37°C) while muscles were relatively inactive (duty cycle < 0.001); (2) the rise in baseline tension, indicative of a loss

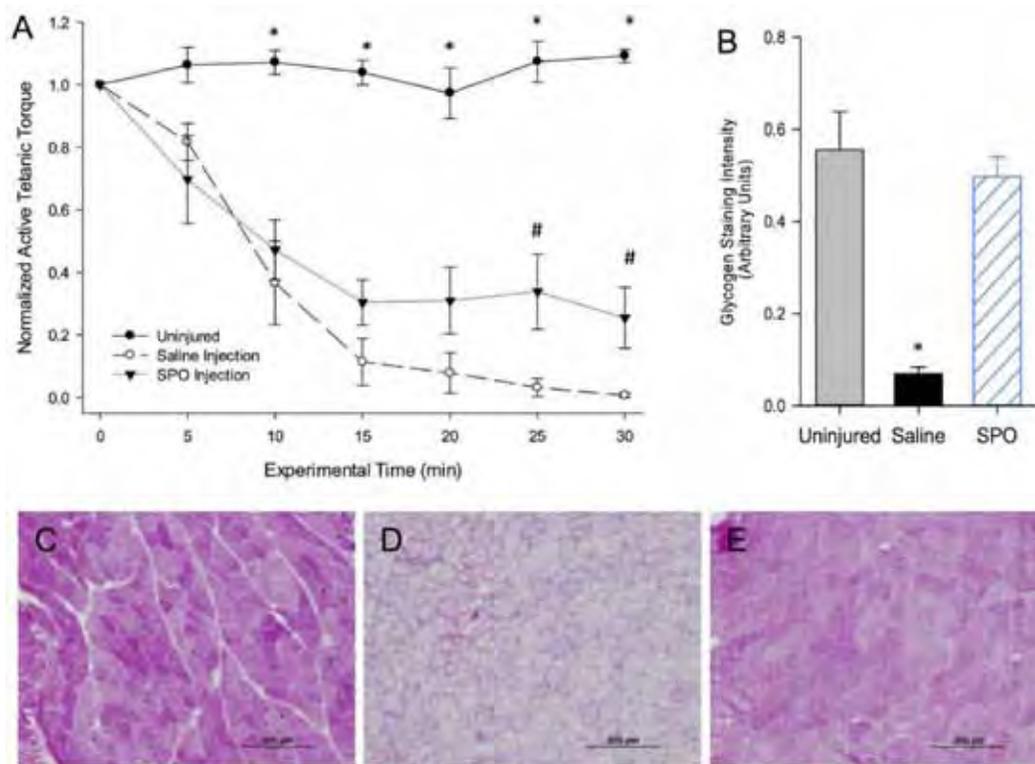


Figure 2. In vivo TA muscle contractility and glycogen depletion with and without SPO after 24 hours of hindlimb ischemia. (A) Ratio of isometric torque (Nmm) measured during repeated tetanic contractions (100 Hz pulse frequency, 0.1 ms pulse width, 200 ms train duration, 1 contraction every 5 minutes) normalized to initial torque values. Within a given time point, (*) Uninjured muscles maintained greater initial torque than all other groups and (#) SPO-injected muscles maintained greater initial torque than Saline-injected muscles ($p < 0.05$). (B) Quantification of intramuscular glycogen (Periodic Acid Schiff stain) in TA muscles from Uninjured (C), Saline- (D), and SPO-injected (E) muscles. * Saline-injected muscles have significantly less intramuscular glycogen than all other groups ($p < 0.05$). Values are means \pm sem; sample size = 3/group.

of intracellular Ca^{2+} homeostasis, in vitro during a contractile fatigue protocol (duty cycle = 0.1) performed in oxygenated conditions (95% O_2 -5% CO_2 ; 37°C); and (3) the diminution of TA muscle torque and glycogen depletion in a partial hindlimb ischemia model in vivo, again while the muscles were relatively inactive (duty cycle < 0.001). Collectively, the results of these studies support the researchers' overarching hypothesis that SPO, an oxygen-generating compound, has the capacity to support resting skeletal muscle metabolism under otherwise hypoxic conditions.

From a clinical standpoint, the utilization of SPO technology to provide an adequate source of oxygen for cells and tissues would be of extreme value in cases where oxygen diffusion limitations exacerbate tissue damage and restrict the spectrum of therapeutic possibilities. Clinical evidence suggests the

potential for oxygen to increase the efficiency of salvage of ischemic skin flaps/grafts and crush injuries in humans in studies using HBOT treatment. This has also been an effective method to reduce skeletal muscle ischemia/reperfusion injury in rats. To investigate the capacity of SPO to preserve skeletal muscle homeostasis in vivo, an optimized SPO concentration and dosage were correlated from in vitro results and applied in a partial hindlimb ischemia rat model. This model has previously been shown to reduce blood flow to the lower extremities at rest and during activity, and to diminish muscle function out to fourteen days after ligation. In this scenario, SPO injection mitigated the impact of ischemia-induced muscle tissue hypoxia after 24 hours of iliac artery ligation, as reflected by an improved maintenance of anterior crural muscle contractility and glycogen preservation, compared



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to saline-injected groups (Figure 2A). Interestingly, the amount of SPO injected into the TA muscle provided theoretically only ~22% of the oxygen required for resting skeletal muscle of this size. However, even this amount of SPO-derived supplemental O₂ appeared capable of partially preserving muscle homeostasis in this model. More comprehensive animal models and analyses should be performed to further optimize concentrations, volumes, and perhaps improved formulations of SPO.

Therefore, the researchers posit that SPO at 1 mg/mL is a biologically and physiologically compatible oxygen-generating biomaterial that, at the very least, can partially support resting metabolism and thereby prevent redox modifications that characterize the acute response to hypoxia, and may lead to otherwise irreversible damage in skeletal muscle. However, one should keep in mind that skeletal muscle has a broad metabolic range, wherein metabolic activity may rise ~6 – 10 times that of resting metabolism during intense activity. Moreover, human muscles that are comprised of mixed-fiber types with a continuum of metabolic and functional phenotypes that may present added challenges to SPO treatment. It is currently unclear what the metabolic requirements are in vivo for tissue salvage following limb polytrauma, although there are reports indicating that skeletal muscle metabolism is elevated in response to injury or trauma. In short, based on the researchers' initial observations with this novel material, in the current test systems, it is reasonable to conclude that SPO-mediated oxygen delivery (even with this first generation biomaterial formulation) would be adequate for preservation of muscle homeostasis and function following traumatic injuries that are expected to largely eliminate voluntary movement of the damaged/affected limb.

Future studies will be aimed at directly testing this hypothesis, by assessing the capacity of SPO to provide supplemental oxygen for the preservation of oxidative metabolism in the context of extremity trauma.

Conclusions

This project has been focused on developing a chemically based oxygen delivery system. As this technology matures, the laboratory has increasingly become focused on testing the feasibility of delivering oxygen for assisting in tissue preservation or salvage in vivo. The results suggest that this could be used as a readily available treatment to delay the onset of additional tissue damage resulting from compromised blood flow.

Research Plans for the Upcoming Year

The researchers will continue to conduct animal studies to evaluate the efficacy of the material. They will test the technology in multiple systems where ischemia/hypoxia may cause detrimental effects, and this approach may be most beneficial. Because organs are composed of multiple cell types, the researchers will continue to analyze several different tissue systems including skeletal muscle, bone, nerve, and skin. Optimization of the most promising tissue systems will be pursued in later years along with optimizing clinically relevant applications and delivery methods. Nonetheless, the primary focus of experiments will be to optimize the model and delivery protocol for the oxygen-generating biomaterials to demonstrate physiologically relevant improvement in skeletal muscle structure and function under clinically relevant experimental conditions.

Soft Tissue Repair and Regeneration**Mechanical Manipulation of the Wound Environment****Project 4.5.8, WFPC**

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

Project Team(s): Michael T. Longaker MD, MBA (Stanford University); Robert Langer (Massachusetts Institute of Technology)

Collaborator(s): None

Therapy: Vascularized tissue engineering

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

TRL Progress: Beginning, TRL 1; Current, TRL 3; AFIRM I Target, TRL 3

Key Accomplishments: The production of explantable microvascular beds (EMBs) using a

bioreactor was developed and refined by the Gurtner group as the basis for organ-level tissue engineering. Selective decellularization with preservation of the existing circulatory bed was optimized early in the study period, and this formed the basis for on-going studies to maximize cell engraftment consistent with organ level cellular reconstitution. Refinements in the protocol during the past year permitted cell engraftment for a replantable, vascularized tissue-engineering construct with the ability to non-invasively monitor flap and cell mass survival long-term in vivo.

Key Words: tissue engineering, explantable microvascular beds, decellularization, bioreactor

Introduction

Injured or missing extremities, failing organs, and significant burn injuries continue to place a huge burden on wounded soldiers and society. Tissue engineering holds the promise of creating replacement organs outside the human body. The focus of the Gurtner group on decellularization as a useful tool in organ-level tissue engineering has been validated recently by the decellularization of whole organs with reconstitution of cell mass by other groups. However, these groups have failed to address the two major obstacles that have hindered the development of techniques to fabricate replacement organs, and that have been the specific focus of the Gurtner group's study: (1) the inability to adequately vascularize tissue constructs in vitro, and (2) the inability to re-integrate these tissues into the systemic circulation.

The Gurtner group's focus on selective decellularization with preservation of the existing circulation has permitted the engraftment of a large biomass in vivo. More traditional tissue engineering approaches, including recent organ decellularization approaches, have attempted to engraft cells in vitro into decellularized organs without a functional circulation, thereby making transplantation

impossible. Examining the Gurtner group's novel approach to engineering constructs of organ-level complexity in more detail permits an understanding of the potential to fabricate autologous, vascularized neo-organs in vitro from expendable vascularized tissue flaps. This approach starts with the vascular system and surrounding stromal support as the scaffold, and builds tissue from the "inside-out" as compared to existing paradigms of tissue engineering. The researchers have successfully constructed a novel perfusion bioreactor system that permits the cultivation of EMBs for extended periods ex vivo. They have demonstrated extremely efficient seeding of these cultivated EMB constructs with primitive progenitor and stem cells after a decellularization process. In summary, this technology has advanced beyond "proof of principle" toward a reproducible, flexible regenerative environment based on a bioreactor system.

Research Progress – Year 4 (Project funded in 2009)

Having optimized the decellularization process, the Gurtner group has continued to perform detailed studies to optimize cell seeding to maximize cell engraftment within EMBs following replantation as the basis for organ-level tissue-engineering



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strategies. During the past year, the researchers further optimized the supportive matrix into which the EMBs are placed during perfusion on the ex vivo bioreactor system, and they established the reliable and reproducible nature of reimplantation for this approach. Notably, they achieved improvement in the control of pH changes during decellurization (data not shown).

Since the EMB is encased in a gel during the ex vivo period, the researchers examined the feasibility of using this opportunity to further augment cell delivery in vivo. To this end, they examined the capacity of a biomimetic pullulan-collagen hydrogel to create a functional biomaterial-based stem cell niche for the delivery of mesenchymal stem cells in vivo. They added N-isopropylacrylamide (NIPAM)-Chitosan to this thermoreversible hydrogel to facilitate external cell survival. Their data suggest that the pullulan-collagen hydrogels provide a functional niche capable of long-term cell viability while also up-regulating a number of pluripotency genes, which is further augmented by the addition of NIPAM-Chitosan. This level of control is essential for large volume and organ-level tissue engineering, and the researchers are not aware of another technology that provides this degree of control.

In Year 2 of the project, the Gurtner group had initially demonstrated the ability to seed human Luciferase+ adipose-derived stem cells (human ASCs) on rat EMBs, and confirmed the survival of the cells during and after perfusion on the bioreactor (**Figure 1**). Having established proof of principle using human cells, the researchers moved to examine syngeneic engraftment through the use of rat Luciferase+ ASCs into rat EMBs for re-implantation, as a direct correlate of the clinical implementation of autologous cells for tissue and neo-organ fabrication.

In parallel, an alternative approach to replace physiologic function in vivo using Minicircle deoxyribonucleic acid (DNA) was examined as a means of replacing the expression of single proteins lost through deficiency syndromes, e.g., human factor IX deficiency, through their incorporation within EMBs. The Gurtner group achieved significant progress using vascularized superficial inferior artery-based tissue flaps (a type of EMB) engineered with Minicircle DNA, which provides significantly enhanced and sustainable transgene expression, resulting in significantly higher levels of protein production than standard in vivo techniques. Despite these advances, the level of systemic protein

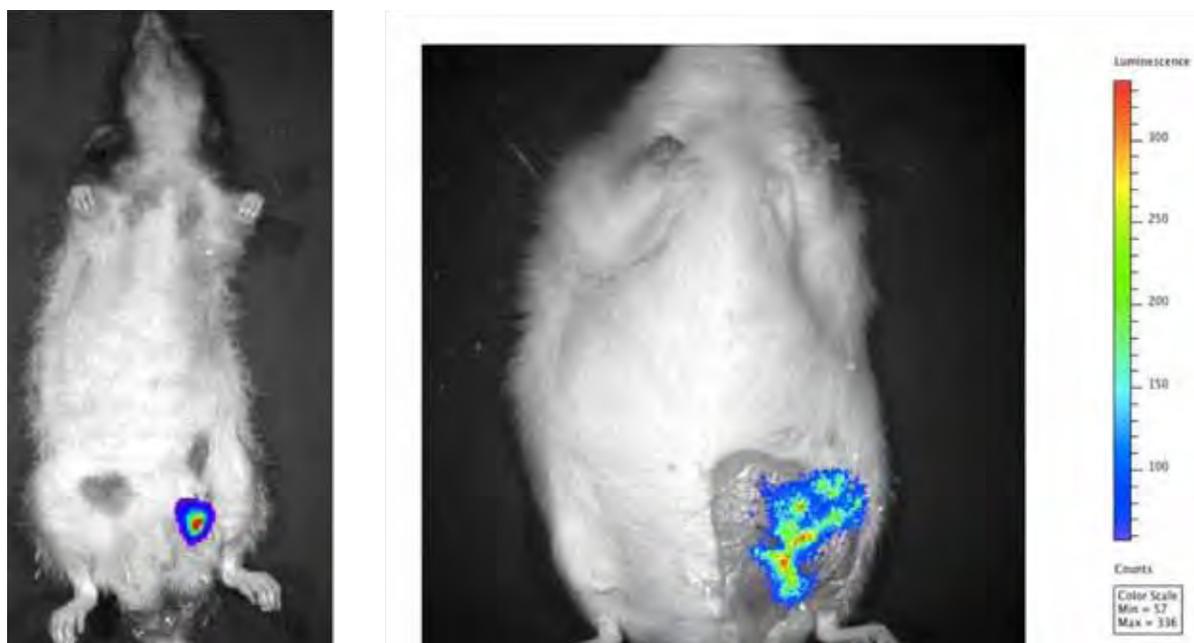


Figure 1. Bioluminescence imaging demonstrating engraftment of luciferase-positive human ASC's (left) into a nude rat, and luciferase-positive ASC's (right) into an immune competent rat. This displays non-invasive monitoring of surviving cell mass over time to assist proof of concept.

expression in this small tissue flap (i.e., relative to the size of the animal) was still lower than that required to fully reverse the effects of factor IX deficiency and, therefore, future efforts will be directed at larger adipose tissue flaps (such as the omentum) as ideal candidates for Minicircle transfection, due to their larger size, excellent vascularization and expendability. Nonetheless, this work successfully demonstrated that transfection with Minicircle DNA is a viable and safe in vivo gene delivery system that provides efficient mid-term transgene expression with minimal adverse effects. This technology appears to provide safe and sustainable systemic protein release if applied on a larger scale.

The delivery of cells in a predictable and oriented fashion remains more difficult. Organ-level tissue engineering requires a sufficiently large mass of cells to replicate the organ function, and these cells must maintain appropriate spatial relationships to facilitate neo-organ function. For these reasons, the researchers have focused on maximizing total seeded cell biomass through techniques for large-cell perfusion and engraftment. This has maximized engrafted cell volume and should pave the way for organ-level tissue engineering in the near future.

Conclusions

The Gurtner group has successfully validated and optimized their protocol for the isolation and maintenance of EMBs based on the rat superficial inferior epigastric vessels on an ex vivo bioreactor system. They have developed a decellularization protocol that achieves tissue decellularization while preserving matrix architecture and macroscopic vascular structure. The group successfully re-anastomosed the EMB into the native vascular circulation, and established the ability to track the biomass non-invasively long-term. They have successfully seeded decellularized rat tissue flaps with human and rat ASCs. The potential of this technology is tremendous, and the researchers continue to make significant strides toward refining this novel paradigm for organ-level engineering and regenerative medicine.

Future Research Plans

Further studies will seek to further improve long-term engraftment and functional cellular differentiation for organ-level tissue engineering.

Planned Clinical Transitions

N/A

Corrections/Changes Planned for Future

N/A





Nerve Repair and Regeneration

Repair of Segmental Nerve Defects: A Theragnostic System Solution for Optimal Nerve Repair - Prevention of Muscle Atrophy

Project 4.4.1, RCCC

Team Leader(s): Robert S. Langer, ScD and Daniel G. Anderson, PhD (Massachusetts Institute of Technology [MIT])

Project Team(s): Liang Guo, PhD and Ning Zhang, PhD (MIT)

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

Therapy: A theragnostic solution for promoting peripheral nerve repair by epimysial electrical stimulation and recording of denervated muscle.

Deliverable(s): Techniques for in vivo tracking of nerve regeneration and estimating number of motor units to assess reinnervation; technique for reducing denervated muscle atrophy using implanted, epimysial, stretchable microelectrode array.

TRL Progress: Beginning, TRL 1; Current, TRL 3; AFIRM I Target, TRL 3

Key Accomplishments: The researchers focused on the development and optimization of an implantable, organic, stretchable microelectrode array (OSMEA) and successfully mitigated potential issues associated with microfabrication. They systematically characterized the OSMEA's mechanical and electrical performances, and confirmed the advantages of using the conducting polymer pentaerythritol ethoxylate/polypyrrole (PEE-PPy) in making the OSMEA for the proposed application.

Key Words: peripheral nerve repair; muscle denervation atrophy; organic stretchable microelectrode array; epimysial stimulation and recording

Introduction

The course of peripheral nerve repair ranges from a few months to more than one year. Denervated muscles quickly undergo atrophy, which can significantly affect functional recovery of motor reinnervation. The common clinical method to treat muscle atrophy uses functional electrical stimulation.

However, conventional transcutaneous electrodes are not optimal for peripheral nerve repair and can induce significant tissue damage. Intramuscular electrodes are rarely used in a clinical setting to treat muscle denervation atrophy, primarily because of the invasiveness of the approach and the need for a large electrode area capable of delivering the current required to elicit contraction of the denervated muscle. Conventional epimysial (i.e., on the muscle surface) electrodes are sometimes bulky and unable to meet the stringent needs of peripheral nerve repair. Meanwhile, little information with regard to the time course of nerve regeneration and motor reinnervation has been collected. There remains a need for an effective, continuous interface that is suitable for stimulation of denervated muscle and

for the real-time in vivo monitoring of the nerve regeneration time course. Additionally, though many efforts have been devoted to this end, it is still unclear what the optimal stimulation protocols should be. Variations in experimental methods and animal models used in the literature make cross-comparisons even more difficult.

Research Progress – Year 5

In Year 5, the MIT team continued the project that sought to preserve denervated muscles during the course of nerve repair using electrical stimulation. The team has focused on the development and optimization of an implantable OSMEA for epimysial stimulation and recording. In addition, the researchers have set up a new animal experiment platform for the treatment of muscle atrophy following denervation through the use of electrical stimulation. From the beginning of the project, the OSMEA development has been performed with awareness that the device needs to be bio-compatible in order to obtain U.S. Food and Drug Administration approval in the future.

While further in-depth characterization of the OSMEA technology is still ongoing, data from up-to-date evaluations on the materials and OSMEA are very encouraging for the intended application. A conducting polymer was synthesized from PEE-PPy (Figure 1). Electrical testing of the PEE-PPy film demonstrated a conductivity of 116 S/cm at room temperature. Mechanical testing of the PEE-PPy film resulted in an ultimate tensile strain of 36% (Figure 2), and more importantly, the resistance had only a ~35% increase at the ultimate tensile strain



Figure 1. Images of a PEE-PPy composite film (2.5 cm x 2.5 cm x 15 µm) showing different smoothness on two sides: top image, the side facing the platinum electrode; bottom, the side facing the plating solution.

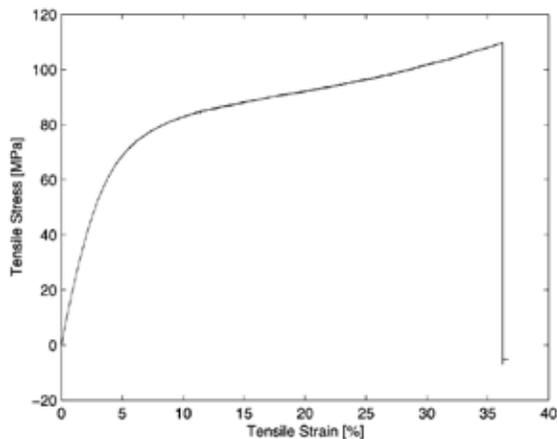


Figure 2. Stress-strain curve of a tested PEE-PPy specimen with an ultimate tensile strain of 36%.

(Figure 3). These results confirmed the electrical and mechanical advantages of using the conducting polymer PEE-PPy in making the OSMEA (Figure 4) for the proposed application. The researchers also evaluated the long-term stability of two types of the conducting polymer in phosphate-buffered saline at 37°C for one month. The team investigated the cause for the polymer's instability, and improved material designs are currently being testing. Overall, the device development effort has now successfully achieved its initial goals.

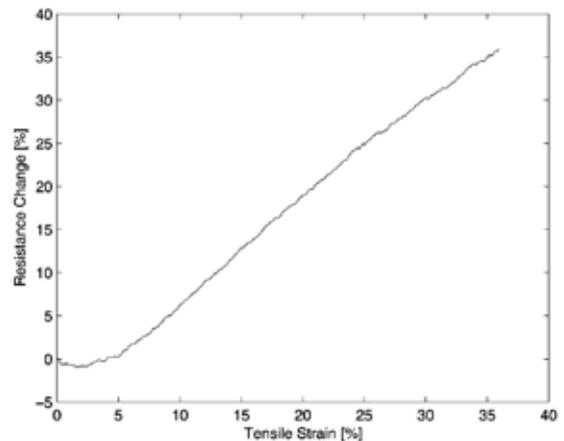


Figure 3. Resistance-strain curve of the tested PEE-PPy specimen, revealing a linear relationship with only a ~35% increase in resistance at the ultimate tensile strain.



Figure 4. Image of the OSMEA made of medical-grade polydimethylsiloxane and PEE-PPy.



II: Limb and Digit Salvage

Conclusions

The MIT team has successfully optimized the fabrication method of the OSMEA and has systematically characterized the OSMEA's mechanical and electrical performances. These results showed promise for the OSMEA as a theragnostic solution for promoting peripheral nerve repair by epimysial electrical stimulation and recording of denervated muscle. The team is currently preparing a manuscript, and animal studies will be followed shortly to develop the OSMEA for the prevention of muscle denervation atrophy during the time course of peripheral nerve repair.

The OSMEA is a platform technology that is also applicable to many other exciting neural interfacing applications, including spinal cord surface stimulation, electrocorticogram-based brain-computer interfaces, and retinal prostheses.

Research Plans for the Upcoming Year

The researchers plan to connect the entire system (OSMEA, headstage/stimulator, data acquisition system) for use in acute rat experiments. They will

test the long-term in vivo performance of OSMEA as an epimysial interface. They will also develop diagnostic and therapeutic techniques using a rat sciatic nerve transection model.

Planned Clinical Transitions

The MIT team is currently focusing on the pre-clinical animal study to prove the safety and efficacy of the proposed approach. This stage is expected to take at least three years. Once success is achieved with this stage, an Investigational Device Exemption will be planned to move forward to human clinical trials. Animal studies are needed to test biocompatibility and reliability of the devices, and to develop the diagnostic techniques and therapeutic protocols. The MIT team is using a rat sciatic nerve transection model for the development. The subcutaneous implantation study is used for in vivo biocompatibility evaluation; acute experiments are used for preliminary development of the diagnostic and therapeutic techniques; implantation experiments are used for further development and evaluation of efficacy of the techniques.



Nerve Repair and Regeneration

Repair of Segmental Nerve Defects

Project 4.4.2, RCCC

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

Project Team(s): Robert Spinner, MD, Huan Wang, MD, PhD, Diana Angius, MD, Aditya Chawla, MD, Ralph de Boer, MD, Glenda Evans, BA, Joaquin Hidalgo, MD, Andrew Knight, PhD, Adil Ladak, MD, Jarred Nesbitt, Chandan Reddy, MD, Jing Rui, MD, Brett Runge, PhD, Suzanne Segovis, MBA, Le Wang, MD, Peng Wu, MD, and Shuya Zhang, BS (Mayo Clinic)

Collaborator(s): Joachim Kohn, PhD, Basak Clements, PhD, Divya Bhatnagar, PhD, and Jennifer Chan, BS (Rutgers University); Paulina S. Hill, PhD and Hao Cheng, PhD (Massachusetts Institute of Technology)

Therapy: Treatment of peripheral nerve injuries

Deliverable(s): Tissue-engineered scaffolds suitable to repair large, motor nerve defects

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 3

Key Accomplishments: The researchers standardized a critical-size nerve defect model and delayed nerve repair model. They delineated denervation muscle atrophy time course and characteristics, and identified prognostic factors for functional recovery. They also optimized Schwann cell isolation and expansion from adult rat nerves that could later be translated to human autologous Schwann cell preparation for conditioning polycaprolactone fumarate (PCLF) conduits. It was confirmed that thicker coatings of hyaluronic acid (HA) on the braided conduits would remain intact for six weeks in vitro and that airbrushing a layer of polymer coating is a new and faster way of covering the macropores with degradable polymers. The team also demonstrated that the braided conduits can be effectively sterilized by a validated ethylene oxide (EtO) sterilization method with endotoxin levels that are within U.S. Food and Drug Administration (FDA) specifications.

Key Words: peripheral nerve; conduit; scaffold; biodegradable polymer; growth factors

Introduction

Peripheral nerve lesions cause disabilities that greatly affect the quality of life of those involved. These injuries also have significant socioeconomic impact. Nerve injuries that occurred in battlefield are frequently more complicated and often involve loss of entire segments of major nerves spanning 5-20 cm. A limb salvaged without nerve function is useless and, in most cases, painful.

Autologous nerve graft is the current clinical gold standard of repairing nerve defects. However, limited availability and donor site morbidity limits the usefulness of autografts. Alternatives to nerve autografts are needed. A number of different materials have been explored for use in aiding nerve regeneration, both natural and synthetic. Although promising, they have shown little to no efficacy for the repair of longer defects. Current commercially available nerve tubes are only approved by the FDA to be used for repair of nerve defects ranging from 15 mm to 30 mm. Clinical reports of using these tubes have mainly shown effectiveness in repairing

sensory nerves. Application in repairing motor nerves is sporadic and non-conclusive.

The Mayo group aims to develop biodegradable PCLF conduits that will promote improved nerve regeneration over current commercially available tubes both in motor function recovery and in gap length. The Rutgers team optimized the braid design, developed coatings, and incorporated bioactive molecules to improve nerve regeneration over longer gaps. This work is ongoing and will be supported by an National Institutes of Health (NIH) R01 grant.

Research Progress – Year 5

Efficacy of PCLF conduits in repairing critical-size nerve defect

Using a Lewis rat sciatic nerve 1 cm defect and 1.5 cm defect model, PCLF nerve conduits have been shown to support nerve regrowth across a critical-size defect. The efficacy of the conduits, as indicated by the extent of recovery in nerve conduction studies and in muscle weight restoration, was comparable to commercial collagen conduits.



II: Limb and Digit Salvage

Critical changes in denervation muscle atrophy and their impact on functional recovery

Using a rate delayed nerve repair model in the rat sciatic nerve, several electrophysiological and molecular parameters were identified that are relevant prognostic factors for functional outcomes and can potentially serve as treatment and/or intervention targets. These include muscle fibrillation potentials and several myogenic-related genes: myogenic regulatory factors, myogenin, nicotinic acetylcholine receptors, muscle-specific kinase, cyclin-dependent kinase inhibitor and insulin-like growth factor-I.

Isolation and expansion of Schwann cells

Schwann cells were isolated from three nerve sources of adult rats: the sciatic nerve (a major mixed nerve), sural nerve (a sensory nerve with a few motor fibers) and medial antebrachial cutaneous nerve (a pure sensory nerve). By giving a course of predegeneration of the harvested nerve before cell expansion, a high purity (over 90%) culture of Schwann cells was obtained. This optimized Schwann cell culture protocol laid the foundation for future use of autologous Schwann cells from available donor nerves for conditioning nerve conduits to enhance nerve regeneration.

Braiding nerve conduits: A technology to introduce porosity and flexibility

The Rutgers team hypothesized that the macropores of the braided conduits are detrimental for nerve regeneration, and limiting the porosity will decrease the fibrotic infiltration and allow for the formation of an epineurium around the axons. Therefore, studies were conducted in Year 5 to optimize the braided conduit design with minimized porosity while maintaining permeability, which is essential for successful nerve regeneration. In proof of principle studies, tyrosine-derived polycarbonates (TyrPC) were used.

Coating of braided conduits with secondary layers to reduce porosity

Braided conduits were fabricated from a relatively faster degrading TyrPC, E1001(1K), for optimization studies of conduit design. These are referred to as second generation conduits. During Year 5, the

Rutgers team used the single layer braided conduits as well as three coating strategies to minimize the conduit porosity. These strategies involved coating the conduits with an airbrushed layer of polymer coating and a thick layer of fibrin glue as well as coating with cross-linked HA hydrogels, which have been shown to be cell repellent and led to improved nerve regeneration in a six-week study.

Optimizing the braid design

Second generation E1001(1K) braided conduits were shown to be macroporous, similar to the first generation E0000 conduits, but had a smaller fiber diameter forming a tighter braid with a uniform pore-size distribution. These conduits were not heat treated at the end of braiding, which rendered them a tight and highly flexible structure.

In vitro degradation of conduit coatings

The in vitro integrity of the coatings was investigated in a six-week in vitro degradation study. After the six-week in vitro study, the alternating dip-coated HA coating was shown to maintain the best coverage of the pores as well as the surface of the fibers.

Optimizing the sterility method for the braided conduits

EtO was evaluated as the sterilization method for the braided conduits as opposed to ultraviolet sterilization, which was used before. The EtO-treated braided conduits were completely sterilized and maintained their architecture and flexibility with a minimal molecular weight loss. The coated braided conduits tested for endotoxins had a detection limit < 0.04 endotoxin units/device. This limit was within the endotoxin detection limit specified by FDA.

Conclusions

The Mayo team has identified a novel in-house material, PCLF, which is both biocompatible and biodegradable. PCLF nerve conduits have been shown to support robust nerve regeneration in preclinical models when used to bridge a critical-size nerve defect. The Mayo team has also validated an FDA-acceptable method, EtO, for sterilization of PCLF nerve conduits. PCLF conduits maintained stability and mechanical properties upon EtO treatment. EtO-treated PCLF tubes supported nerve regrowth when used to bridge nerve gaps. The PCLF

conduit is being moved forward towards a Phase I clinical trial to assess the safety of this polymer conduit for implantation in patients.

The Mayo team has also identified electrophysiologic, histologic, and molecular parameters of the muscles that can potentially serve as sensitive prognostic markers for functional outcomes after nerve reconstruction, and as targets of intervention for enhancing regeneration. In addition, the Mayo team has developed and optimized the protocols for isolation and expansion of Schwann cells from adult nerve sources. This laid the foundation for the group's future work to condition the PCLF nerve conduit with autologous Schwann cells to enhance its capacity for supporting nerve regeneration.

The Rutgers team found that flexible, hydrogel-coated and airbrushed braided conduits can provide a mechanically optimized nerve conduit. The Rutgers team is ready to apply this fabrication methodology to PCLF fibers. By combining such conduits with biologically active fillers or cells and peptides, it may be possible to create a nerve regeneration conduit capable of bridging long nerve gaps.

Research Plans for the Upcoming Year

The Mayo team will complete the additional pre-clinical testing requested by the FDA in response to the prior Investigational Device Exemption (IDE) submission. With support from the Office of Research Regulatory Support at Mayo Clinic, an investigator-initiated IDE will be filed with the FDA. Institutional Review Board (IRB) approval will then be obtained to conduct a Phase I clinical trial to assess the safety of PCLF conduits in patients with a 6 cm nerve defect resulting from a sural nerve biopsy (see Project 4.4.1a report).

In the upcoming year, the Mayo team will work to upscale through Good Manufacturing Practices (GMP) the manufacture of autologous human Schwann cells from a sural nerve. The group will also conduct experiments in large animals to test

the safety and efficacy of PCLF nerve conduits supplemented with Schwann cells in preparation to submit a combination product IDE to the FDA for a clinical trial.

The Rutgers group has scaled up the conduit prototype for large animal testing and long nerve gaps. This work is ongoing where the braided conduits are being used to bridge long gaps (>5 cm) in the pig model of peripheral nerve injury in collaboration with the University of Pennsylvania (see the Project 4.4.2b report). Future generations of the TyrPC braided nerve conduit will be explored via NIH R01 funding.

Planned Clinical Transitions

The Mayo Clinic has in place a quality management plan for GMP that has been manufacturing products for clinical trials with FDA approval for more than 10 years. Therefore, the team is proposing to manufacture the nerve scaffold in-house in the Mayo Clinic GMP facility. The clinical protocol, A Phase I Safety Study to Assess the Safety of Novel Biodegradable Scaffolds for Peripheral Nerve Repair, has been already reviewed by the Mayo Clinic IRB and is awaiting FDA approval of the IDE (see Project 4.4.1a report). The research committee of the Mayo Clinic Department of Neurology has reviewed and approved the clinical protocol of this trial. The Mayo Clinic Venture is apprised of this Mayo-owned intellectual property. Upon completion of the safety trial, the Mayo team will actively interact with industry entities that have already shown interest in licensing and commercializing the product.

The Rutgers group has an exclusive license with Trident Biomedical, Inc. for the library of TyrPCs, and discussions have been initiated to define required funding to advance planned animal studies. The Rutgers team is also working with two separate industrial partners for the large-scale current GMP synthesis of TyrPCs and the fabrication of the braided conduits.



Nerve Repair and Regeneration

Cells and Bioactive Molecules Delivery in Peripheral Nerve Restoration

Project 4.4.2a, RCCC

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

Project Team(s): Can Ozturk, MD, Miroslaw Lukaszuk, MD, Maria Madajka, PhD, Halil Uygur, MD, Jacek Szopinski, MD, PhD, Grzegorz Kwiecien, MD, Adam Bobkiewicz, MD, and Joanna Cwykiel, MSc (Cleveland Clinic)

Collaborator(s): N/A

Therapy: Long nerve gap repair

Deliverable(s): Method of cellular therapeutics by local administration of bone marrow stromal cells (BMSC) therapy into transplanted epineural nerve conduits

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers developed naturally occurring epineural sheath conduits, filled them with BMSC, and tested them for repairing long median nerve gap defects in 13 sheep. They demonstrated that BMSC are immunologically neutral prior to injection into the conduit; no expression of proinflammatory markers or cytokines was detected. They also observed that the epineural tube alone provides a neuro-permissive environment for nerve regeneration since it is expressing Laminin B2, the key extracellular matrix protein supporting axonal growth. The epineural conduit proved to be a new and successful surgical technique to bridge 6 cm long gaps created in the median nerve. The structure and integrity of the tested epineural sheath conduit was well preserved for up to six months after transplantation.

Key Words: epineural sheath; bone marrow stromal cells; sheep model; long nerve repair; conduit

Introduction

There has been an increase in the number of traumatic peripheral nerve injuries due to the current wars (Afghanistan and Iraq), terrorist attacks (India, Somalia, Boston Marathon), natural disasters (earthquakes, hurricanes) and industrial and motor vehicle accidents. Severe limb injuries frequently require complex bone, muscle, and nerve reconstruction. Excessive regional loss of overlying soft tissue and muscle in patients with multiple extremity injuries leads to the direct additional loss of nerve segments spanning from 5-20 cm. Furthermore, there is a limited supply and length of nerves available for grafting, especially in the multi-extremity amputations often seen in combat-related traumatic injury. Autologous nerve grafts have several drawbacks, including pain and loss of function in the donor sensory nerve distribution, size mismatch, and neuroma formation. As an alternative to nerve autografts, a number of natural and synthetic materials have been explored; however, their efficacy in repair of long nerve defects is limited. Nerve allografts require immunosuppression to prevent

allograft rejection. Thus, the multitude of side effects of immunosuppressive protocols limits their clinical application. Due to these limitations, there is a need for readily available nerve tissue material, which is crucial in the treatment of complex extremity injuries.

To meet these needs, the Siemionow team developed and tested epineural sheath conduits filled with BMSC for nerve regeneration within 6 cm nerve gaps. This conduit creates an ideal microenvironment for nerve regeneration as it not only protects against local fibrotic and inflammatory insults, but it also provides a source of growth factors crucial to effective nerve regeneration. Unlike artery and vein grafting, epineural tube harvesting also results in a lesser degree of donor site morbidity and has the potential to be an unlimited conduit resource via cadaveric harvesting. BMSC are multipotent cells that have the ability to differentiate into several cell lineages. BMSC have the potential to augment neural regeneration through the production of neurotrophic factors, anti-inflammatory effects, and differentiation into neural support cells. BMSC can

be reliably isolated and cultured for use in allogeneic models. The addition of BMSC will serve as a source of mechanical support and growth factors, and will enhance the regenerative properties of the conduit over long gaps.

There is a clinical need for the development of conduits providing regeneration over longer nerve gaps. The Siemionow team is directly addressing this need by introduction of natural epineural sheath conduits for 6 cm critical-size defects. These conduits enriched with BMSC for enhancement of nerve regeneration have a great potential for clinical application and commercialization. There is an unlimited supply of epineural sheaths that can be enriched with BMSC, and stored and used “off-the-shelf” during procedures of nerve defect repair as well as in cases of spinal decompression and trauma.

Research Progress – Year 5

Characterization of BMSC prior to injection into the empty epineural sheath tube

Sheep bone marrow was harvested using the protocol already established by the Siemionow team. BMSC were obtained after culturing bone marrow for two weeks, with regular complete media changes every three days. BMSC were fluorescently labeled for the presence of immunogenic markers and analyzed by flow cytometry. Flow cytometry data showed the lack of expression of immunogenic markers, which is an advantage for the use of these cells in allogeneic settings. To confirm immunological neutrality of the BMSC population, quantitative ELISA assay was performed. No expression of proinflammatory cytokines was detected.

Fluorescent immunostaining of neurogenic markers on the surface of epineural sheath tubes

Harvested epineural sheaths from sheep median nerve were frozen in liquid nitrogen, then fixed and fluorescently labeled for the presence of neurogenic (S-100, glial fibrillary acidic protein and Laminin B2) markers (**Figure 1**). No expression of S-100 and glial fibrillary acidic protein was observed, which indicates that all Schwann cells were removed including intermediate nerve filaments. High expression of Laminin B2 was seen, which indicates a strong protein network foundation for newly growing axons. Laminin B2 is an important and

biologically active part of the basal lamina, influencing neuronal cell differentiation, migration, adhesion as well as phenotype and survival.

Epineural sheath conduit preparation and transplantation

Empty epineural sheath tubes were created from sheep median nerves. Nerves were exposed and dissected, and all fascicle were removed by the pulled-out technique. The epineural sheath tube was then filled with fluorescently labeled BMSC, which created a conduit that was then transplanted into recipient animals. The following experimental groups were prepared: (1) autologous conduit with autologous BMSC, (2) allogeneic conduit with allogeneic BMSC, (3) saline-filled conduit, and (4) autograft technique (served as control). All experimental groups were under daily observation for six months.

Macroscopic evaluation of long nerve defect repair six months after surgery

Nine animals were euthanized on schedule, six months after transplantation of the epineural sheath conduit. No signs of inflammation, rejection, or conduit leakage were observed six months after transplantation of the conduit in any of the sacrificed animals. The structure of the conduit was preserved, and anastomosis sites were without inflammation (**Figure 2A-C**). Transplanted conduits showed elasticity and no disruption in structure. No differences in the conduit integrity were observed during post-mortem analysis comparing the allogeneic and autogenic cell-filled conduits. Macroscopic observation of the cross-section of the conduit

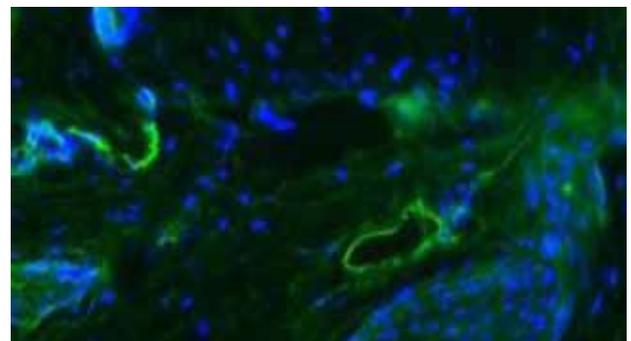


Figure 1. Fluorescent staining of epineural sheath prepared from sheep median nerve. Blue color represents nuclei (DAPI), green color represents FITC-positive signal for the presence of Laminin B2. (Magnification 200x.)



II: Limb and Digit Salvage



Figure 2A. Autograft after dissection from the left forelimb six months after transplantation. Arrows indicate proximal and distal anastomosis sites.



Figure 2B. Dissected autologous epineurial sheath tube filled with saline six months after transplantation. Arrows indicate proximal and distal anastomosis sites.



Figure 2C. Autologous epineurial sheath filled with 55×10^6 autologous BMSC six months after transplantation. Arrows indicate proximal and distal anastomosis sites.

showed structures comparable to the cross-section of the intact sciatic nerve. The integrity and shape of the cross-section of the conduit were preserved.

Somatosensory-evoked potential analysis six months after conduit transplantation

Somatosensory-evoked potential measurements were performed before euthanasia six months after transplantation of the epineurial sheath conduit. Two electrodes were placed in the distal anastomosis site of the transplanted conduit and the contralateral sensory cortex on the surface of the brain. Current stimulation in the range of 1-2 mA was applied, and electric activity response of the sensory cortex was recorded. The shortest latency was recorded for the intact median nerve and was comparable with the autograft score. The longest latency was recorded for autogenic epineurial sheath conduits filled with saline (45.45 ms). In comparison, allogeneic conduits filled with allogeneic BMSC had a shorter response (40.28 ms). This observation might suggest that there is a faster processing signal in the presence of BMSC and/or their involvement in the development of new neurofilaments.

Conclusions

The use of the epineurial sheath conduit technique is a new method for the repair of long nerve gaps. This technique does not require lifelong immunosuppression. Epineurial tubes provide a neuro-permissive environment for axonal growth and regeneration.

Research Plans for the Upcoming Year

The Siemionow team is planning to investigate different cryopreservation and lyophilization methods for the long-term storage of the epineurial sheath conduits. A prepared epineurial sheath will be dried without destroying its structure or will be treated with different cryopreservation media. Neurogenic, angiogenic and immunogenic markers will be analyzed before and after epineurial sheath tube preservation to ensure the stability and integrity of the conduit. Successful cryopreservation will allow proceeding towards the creation of human epineurial sheath conduits, which can be preserved and stored for the use in multi-trauma patients such as the military personnel. Creation of human epineurial

sheath conduits will allow nerve regeneration over the long nerve gaps. The future goal is to characterize, optimize and make a clinically applicable bio-conduit built of a naturally occurring human epineural sheath filled with human mesenchymal stem cells, and to test its regenerative potential in the clinical settings.

Planned Clinical Transitions

The researchers propose to introduce a novel method of cellular therapeutics in the form of epineural conduits supported with BMSC, applied to patients with long nerve defects to facilitate nerve regeneration. This application may be used

in patients with motor neuron disease, cerebral infarct, degenerative nerve disease and peripheral nerve injury. Local administration of BMSC may also play a significant therapeutic role in promoting functional recovery in paraplegic patients suffering from the effects of severe spinal cord injury. BMSC can be applied for enhancement of nerve regeneration as a part of autograft or allograft transplantation therapy. The clinical transition plan will be initiated after all the experimental groups have been completed, and then collected data will be analyzed. Currently, human cadaver studies are being performed to optimize the best method for the collection and preparation of epineural conduits.





Nerve Repair and Regeneration

Repair of Peripheral Nerve Injury Using Tissue-Engineered Nerve Grafts Encased in Biodegradable Nerve Guidance Tubes

Project 4.4.2b, RCCC

Team Leader(s): Douglas H. Smith, MD and D. Kacy Cullen, PhD (University of Pennsylvania)

Project Team(s): D. Kacy Cullen, PhD (University of Pennsylvania)

Collaborator(s): Harry C. Ledebur, PhD (Axonia Medical); Joachim Kohn, PhD, Divya Bhatnagar, PhD, and Basak Clements, PhD (Rutgers University); and Anthony Windebank, PhD (Mayo Clinic)

Therapy: Tissue-engineered nerve graft (TENGG) for repair of major peripheral nerve injury (PNI)

Deliverable(s): Evaluation of RCCC-AFIRM nerve guidance tubes (NGTs) and TENGGs in a rat model of PNI; selection of an NGT for encasement of TENGGs in support of future large animal testing

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers demonstrated accelerated axonal regeneration across TENGGs encased in NGTs compared to experimental AFIRM NGTs alone or commercially available (U.S. Food and Drug Administration [FDA] approved) NGTs alone. The team determined that TENGGs possessed a novel (and in many ways superior) mechanism of action compared to NGTs and autografts, which is categorized by axon-induced axon growth (not possible with an autograft), maintenance of the distal pathway (not possible with an autograft), increased Schwann cell (SC) infiltration (vs. NGTs), and the presence of pioneer axons (vs. NGTs). Overall, the mechanism of action and early-stage results further support the premise that TENGGs may enable regeneration of nerves across critical, 5+ cm, peripheral nerve lesions following major PNI.

Key Words: peripheral nerve; extremity trauma; blast injury; allogeneic; repair; restoration of function; tissue engineered nerve graft

Introduction

PNI is a major source of warfighter and civilian morbidity. Indeed, only 50% of patients achieve good to normal restoration of nerve function following surgical repair, regardless of the strategy. This stems from the inadequacy of current PNI repair strategies, where even the “gold-standard” treatment—the autograft—is largely ineffective for major PNI, which is defined as (1) loss of a large segment of nerve (i.e., >5 cm) or (2) an injury occurring closer to the spinal cord (e.g., in the shoulder or thigh) that results in extremely long distances for axon regeneration to distal targets (such as the hand or foot). Overall, despite decades of work, peripheral nerve repair has not progressed beyond the current standards, which are to use either (a) NGTs to bridge small gaps or (b) autografts for larger defects.

This research team, in conjunction with Axonia Medical, Inc., is developing novel TENGGs that have the potential to not only surpass the performance of

autografts, but also to repair currently untreatable peripheral nerve injuries. Custom TENGGs offer the following advantages: (1) a more robust and faster rate of axonal regeneration; (2) maintenance of the distal nerve sheath (the distal pathway is necessary for long distance axonal regeneration and target innervation); (3) an accelerated recovery response; (4) the fact that they are an off-the-shelf product (compared to an autograft); and finally, (5) they are an allogeneic product that does not require immunosuppression (TENGGs are immunoprivileged). Drawing from advanced biomaterials and three-dimensional (3D) fabrication methods available within the AFIRM team, this project is selecting and optimizing custom NGTs in which to encase TENGGs in order to repair segmental nerve defects. In this project, custom and commercially available NGTs were used to encase TENGGs, which the project researchers evaluated and compared using established in vivo preclinical models.

TENGs are lab-grown nervous tissue, comprised of long axonal tracts spanning two populations of neurons. The ability to generate these nerve grafts is based upon seminal discoveries regarding the process of axon growth via continuous mechanical tension or “stretch growth.” The project team replicated this natural process in a culture system through the controlled separation of two integrated populations of neurons using a proprietary process and device (Figure 1). During stretch growth, individual axons gradually coalesced with neighboring axons to form large axonal tracts to take on a highly organized parallel orientation. TENGs were subsequently created by embedding these living axonal tracts in a 3D matrix and removing them en masse for transplantation. Whereas other approaches have only been able to achieve axon lengths of 1-5 mm in culture, this platform was able to generate axons of 5-10 cm in as little as 14-21 days, with no theoretical limit as to the final axon length. To date, TENGs have been used for successful repair of major PNI as well as severe spinal cord injury in rodents.

Research Progress – Year 1 (Project commenced in 2012)

Fabrication of TENGs encased in various NGTs (abbreviated TENG-NGT)

Dorsal root ganglia neurons were isolated from fetal rats, transduced to express green fluorescent protein to permit in vivo identification, subjected to axonal “stretch-growth” to the desired length, and constructed into TENGs. After receiving custom NGTs

from the Kohn Lab at Rutgers and the Windebank Lab at Mayo, the Cullen Lab successfully encased TENGs in tyrosine-derived polycarbonate (TyrPC) and polycaprolactone fumarate NGTs, respectively. The Cullen Lab also encased TENGs in commercially available polyglycolic acid (PGA) NGT (Neurotube, Baxter/Synovis) and a collagen I NGT (NeuroFlex, Stryker).

Screen TENG-NGT combinations in acute, short nerve gap (1.0 cm) PNI model in rats

The research team’s objective was to screen the TENG-NGT combinations and establish the mechanism of action of TENGs (i.e., axon-mediated axon regeneration) versus the standard of care (autograft and NGTs) at two weeks post-repair. With all TENG-NGT transplants, surviving transplanted dorsal root ganglia and maintenance of the aligned axonal architecture was observed. Importantly, NGTs containing TENGs were actively facilitating the axon regeneration process, rather than simply being a permissive substrate for regeneration. Host axon penetration across TENG-NGTs was greatly increased compared to NGTs alone ($p < 0.001$) and was similar to autografts ($p = 0.66$). These “pioneer” axons traveled in front of the regenerative front for both TENG-NGTs and autografts, but not for NGTs. The researchers also found that SC infiltration was enhanced with TENGs, compared to NGTs ($p < 0.001$). Notably, the TyrPC NGT developed by the Kohn team at Rutgers was found to promote moderately faster acute axonal regeneration relative to PGA NGT alone (Synovis/Baxter).

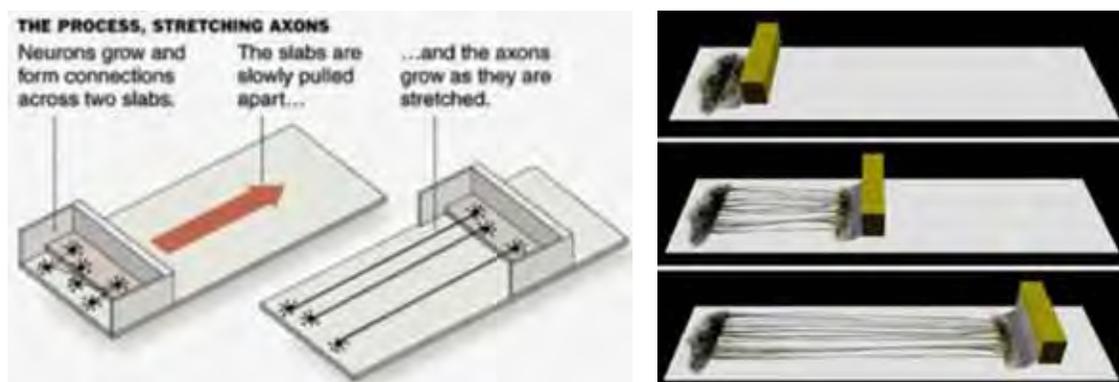


Figure 1. Demonstration of axonal stretch-growth in culture. Neurons are cultured on two overlapping membranes, which are gradually pulled apart in custom mechanobioreactors to induce stretch-growth of the spanning axons.



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In addition, the TyrPC NGT developed by the Rutgers team performed statistically similar to the FDA-approved PGA (Synovis/Baxter) or Collagen (Stryker) NGTs for TENG encasement based on acute regenerative metrics.

These analyses revealed that TENGs serve as a living scaffold to facilitate nerve regeneration via two complimentary mechanisms: (1) axon-induced axon growth: accelerated host axonal regeneration directly along TENG axons in the absence of SCs, and (2) enhanced traditional SC-mediated axonal regeneration (TENG axons increase SC infiltration and alignment, which then improves host axon regeneration). Collectively, this axon-mediated axon regeneration—unique to TENGs—compliments traditional SC-mediated axon regeneration. Although rigorous comparisons between TENGs and various NGTs alone have been made, the researchers are still evaluating different NGTs in order to identify the “best performing” one.

The TyrPC NGT alone developed by the Kohn lab at Rutgers was found to promote moderately faster acute axonal regeneration relative to PGA NGT alone (Synovis/Baxter).

The TyrPC NGT developed by Rutgers performed statistically similar to the FDA-approved PGA (Synovis/Baxter) or Collagen (Stryker) NGTs for TENG encasement based on acute regenerative metrics.

Utilize the “best performing” TENG-NGT combination in a chronic, long nerve gap (2.0 cm) PNI model in rats

The objectives of this study are to evaluate the “best performing” TENG-NGT combination and to establish the efficacy of TENGs (encased in Rutgers–Cleveland Clinic Consortium [RCCC]–AFIRM or commercially available NGTs) in repairing long defects compared to autograft and NGTs alone out to 16 weeks post-repair. Although these studies are currently ongoing, electrophysiological assessments and behavioral assessments have revealed that functional recovery (based on stimulated foot twitch, compound nerve action potential, and compound muscle action potential) was observed in some animals as early as 10 weeks following repair with TENGs or autografts, but not with NGTs alone. Histological evaluation is underway to assess the

survival of TENGs as well as axon regeneration for these longer injuries. Results will be compiled and appropriate statistical testing will be performed as animals reach terminal time points and all end point analyses are completed.

Conclusions

The project team demonstrated the superiority of TENG-NGTs to both AFIRM and FDA-approved NGTs based on acute axonal regeneration. TENG-NGTs were also shown to be equivalent to autografts based on acute axonal regeneration. Longer-term efficacy studies in the rat are currently ongoing to further evaluate the TyrPC NGT developed by Rutgers in conjunction with TENGs. Based on the acute and chronic regenerative metrics that the project team measured to date, there is no significant difference between the TyrPC NGT (Rutgers), Collagen NeuroFlex (Stryker), and PGA Neurotube (Synovis/Baxter) for TENG encasement. Evaluation of the Mayo polycaprolactone fumarate NGT is currently ongoing. If the evaluation of TENG-NGTs in chronic, long nerve gap rat PNI models indicate regenerative equivalence across RCCC–AFIRM and FDA-approved NGT composites for TENG encasement, selection of the NGT for TENG encasement will be based on advantageous mechanical properties (e.g., NGT flexibility, kink-resistance, porosity), ease of manufacture, cost and source.

The project team’s results to date (from this program and others) suggest that TENGs possess a novel, arguably superior, mechanism of action compared to NGTs alone and autografts. This mechanism of action is characterized by: (1) axon-induced axon growth (not possible with an autograft); (2) maintenance of the distal pathway (not possible with an autograft); (3) increased SC infiltration (vs. NGTs); and (4) presence of pioneer axons (vs. NGTs). A key differentiator from existing technologies and strategies, including the autograft, is this unique mechanism of action, whereby TENGs facilitate accelerated axon regeneration across the graft (via SC-independent and SC-dependent pathways), and “babysit” the distal nerve sheath to prolong its pro-regenerative ability. As a result, TENGs may not only surpass the performance of the autografts, but also offer the potential to repair currently untreatable PNIs.

Research Plans for the Upcoming Year

In conjunction with Axonia Medical and with additional support from RCCC-AFIRM, the project researchers have initiated testing of TENGs encased in commercially available and custom NGTs in a clinically relevant large animal model of PNI. In particular, the research teams of Dr. Cullen and Dr. Smith (UPenn), Dr. Kohn (Rutgers), and Axonia Medical have initiated Investigational New Drug (IND)-Enabling Nonclinical Efficacy Studies for Functional Repair of Major Nerve Trauma. Success of the proposed program and that of the researchers' advanced tissue engineering strategy will truly revolutionize PNI repair, ultimately reducing the likelihood of a lifelong debilitating disability for our wounded service members and increasing the likelihood of returning an injured warfighter to active duty.

Planned Clinical Transitions

Axonia Medical, Inc. has completed a Request for Designation with the FDA: TENGs are a combination product, consisting of a device (NGT) and a

biological component (human tissue). The FDA assigned the product to the Center for Biologics Evaluation and Research for pre-market review and regulation. Results from ongoing studies will be leveraged as part of Axonia Medical's IND application with the FDA for human clinical testing of product. These results will define the final product for human clinical testing by determining the most suitable NGT composition for TENG encasement (accomplished in conjunction with Axonia Medical's ongoing preclinical program as well as its NGT partners). This final product candidate will be scaled in conjunction with Axonia Medical's manufacturing partner, and taken into IND-enabling Good Laboratory Practices safety studies. In addition, ongoing large animal testing represents a vital element of the research team's product development efforts, and will constitute a substantive part of the research team's IND's nonclinical package. Outcomes will help define important clinical parameters in support of first in man and Phase II protocols, including subject population, product placement, site preparation, surgical practices, etc.





Nerve Repair and Regeneration

Peripheral Nerve Repair for Limb and Digit Salvage

Project 4.4.4, WFPC

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

Project Team(s): Jacqueline Bliley, MS, YenChih Lin, PhD, Mostafa Ramadan, MD, Lauren Kokai, PhD, Amir Mahan Ghaznavi, MD, Ryan Nolan, BS, Samantha Beckowski, BS, Danielle Minter, BS, Wesley Sivak, MD, PhD, Jedidiah McAtee, BS, Meghan McLaughlin, BA/BS, Doug Weber, PhD, Sun Jung Oh, BS, Gregory Williamson, Donald Crammond, PhD, Kia Washington, MD, Han Tsung-Liao, MD, PhD, Alexander Spiess, MD, and Richard Liberatore, BS (University of Pittsburgh); Marie Tupaj, PhD, James White, PhD, and Lee Tien, BS (Tufts University); Jonathan Barnwell, MD, Zhongyu John Li, MD, PhD, Mark Van Dyke, PhD, and Lauren Pace, PhD (Wake Forest University)

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

Therapy: Peripheral Nerve Repair—Combined strategy for regeneration over long (>3 cm in human) peripheral nerve gaps

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

TRL Progress: Beginning, TRL 1; Current, TRL 4; AFIRM I Target, TRL 5

Key Accomplishments: During the past year, the researchers completed an analysis of 1cm nonhuman primate (NHP) nerve gap repair studies. They established a 5 cm median nerve defect model in the NHP, using autografts and decellularized nerve allografts. They also completed an examination of polymer-based tubes containing glial cell-derived neurotrophic factor (GDNF) embedded in double wall microspheres, in a critical rat sciatic nerve defect model (1.5 cm).

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

Introduction

Approximately 1.9 million people are living with limb loss in the United States as a result of trauma, cancer, vascular problems, or congenital defects. It is well known that the presence of a copious nerve supply is a key factor in the regenerative ability among some amphibians following amputation. Peripheral nerve regeneration is a critical issue as 2.8% of trauma patients present with this type of injury. Following trauma, incomplete nerve regeneration and permanent demyelination may result, leading to lifelong disability. The application of scaffold materials can promote the clinical efficacy of nerve repair towards levels achieved with autologous nerve grafts; however, these results are often suboptimal with some loss of function or sensation still apparent. The clinical outcome remains dependent on time-to-surgery, and at present no U.S. Food and Drug Administration (FDA)-approved treatment is available for long-gap nerve repair greater than 3 cm. The specific aims of this project

are to (1) create a biodegradable nerve guidance system that delivers nerve growth and biophysical guidance to regenerating peripheral nerves, (2) move the nerve guidance system *in vivo*, and (3) utilize expertise from all three laboratories (bio-material expertise, small and large animal facilities) – Tufts University (Tufts), University of Pittsburgh (Pittsburgh), and Wake Forest University (WFIRM).

Research Progress – Year 5

In vivo implantation of novel nerve guides

In this project, the researchers have focused on developing three product types into nerve guides: silk fibroin conduits, polycaprolactone conduits, and collagen conduits. They completed a study of thermal stability and activity of chondroitinase released from silk for the reduction of scar tissue proteoglycan formation during nerve regeneration. They quantitatively characterized the mechanical properties (Young's modulus and ultimate tensile strength) of their silk nerve conduits. They prepared

and assessed the bioactivity and release kinetics of their silk microspheres encapsulating growth factors and enzymes for controlled release from silk conduits. They improved gradient design and delivery of neurotrophic factors from both polymer conduits and silk conduits. They implanted silk nerve conduits including neurotrophic factors and enzymes into a critical rat sciatic nerve defect (1.5 cm).

Table 1 summarizes the guide material/drug delivery methods that have been or are currently being tested in animal models in the Pitt and WFIRM laboratories.

The researchers have recently designed a modified nerve guide fabrication scheme to release chondroitinase and GDNF from the distal end of silk nerve conduits for in vivo rat sciatic nerve defect model studies. They completed an examination of polymer-based tubes containing GDNF embedded in double wall microspheres, in a critical rat sciatic nerve defect model (1.5 cm). They also completed a NHP study of noncritical median nerve defects

(1 cm) using keratin gel-filled collagen conduits. They also established a NHP model of critical median nerve deficits (5 cm) using autograft and decellularized nerve grafts.

The researchers have submitted a request for designation to the Office of Combination Products at the FDA for their keratin gel filler. They submitted a pre-Investigational New Drug (IND) data package to the FDA and held a pre-IND meeting with the Center for Drug Evaluation and Research/FDA to review preclinical data and clinical trial study design for their keratin gel filler.

Conclusions

During the past five years of AFIRM, all three labs have worked synergistically and made significant progress. The Tufts team utilized silk biomaterial protocols and integrating regenerative approaches towards a biodegradable nerve guidance system. Regenerative approaches include incorporating bio-physical cues (surface patterning, electrophysiology

Table 1. Nerve guide material testing

Nerve guide material	Filler	Drug delivery	Animal model	Publication
polycaprolactone (PCL) (Pittsburgh)	Keratin gel (WFIRM)	PLGA double-walled microspheres: GDNF (Pittsburgh)	Lewis rat 1.5 cm sciatic nerve defect (Pittsburgh)	Lin et al, <i>Plastic and Reconstructive Surgery</i> , 2012, 129(1):67-78.
PCL (Pittsburgh)	Keratin gel (WFIRM)	PLGA double-walled microspheres: GDNF + lithium (Pittsburgh)	Lewis rat 1.5 cm sciatic nerve defect (Pittsburgh)	Lin et al, <i>Plastic and Reconstructive Surgery</i> , 2013 (in press).
PCL (Pittsburgh)	–	PLGA double-walled microspheres (Pittsburgh)	NHP 1.0 cm median nerve defect (Pittsburgh)	Underway.
Silk (Tufts)	–	–	Lewis rat 0.8 cm sciatic nerve defect (Pittsburgh)	Ghaznavi et al, <i>American Physical Society</i> , 2011, 66(3):273.
Silk (Tufts)	–	Silk-trehalose films combined with chondroitinase delivery	Lewis rat 1.5 cm sciatic nerve defect (Pittsburgh)	Underway.
Silk (Tufts)	–	Silk gradient films combined with GDNF delivery	Lewis rat 1.5 cm sciatic nerve defect (Pittsburgh)	Lin et al, <i>American Physical Society</i> , 2011, 67(2):147-55.
Collagen Gel (WFIRM)	Keratin gel (WFIRM)	–	NHP 1.0 cm median nerve defect (WFIRM)	Pace et al, <i>Tissue Engineering</i> 2013 (under review).



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applications) and chemical cues (protein coatings, growth factor incorporation) into silk nerve guides. The Pittsburgh team then examined the guides from the Tufts team in a rat sciatic defect model. Furthermore, The Pittsburgh team was approved to utilize a NHP animal model for peripheral nerve repair by the animal committee at their university, and they have begun negotiations with both a drug company and a biomaterials company in order to manufacture Good Manufacturing Practices guides for eventual clinical trials. The Pittsburgh team has operated on both arms on the first two NHPs. Four additional primates have arrived at the University of Pittsburgh and will undergo surgery this summer. The WFIRM team completed the initial NHP nerve gap repair studies initiated in 2010. Additionally, the Wake Forest group has collaborated with the Pittsburgh team by sending keratin gels for incorporation into the drug-loaded polycaprolactone guides, and this was examined in a rat sciatic nerve defect model (and published).

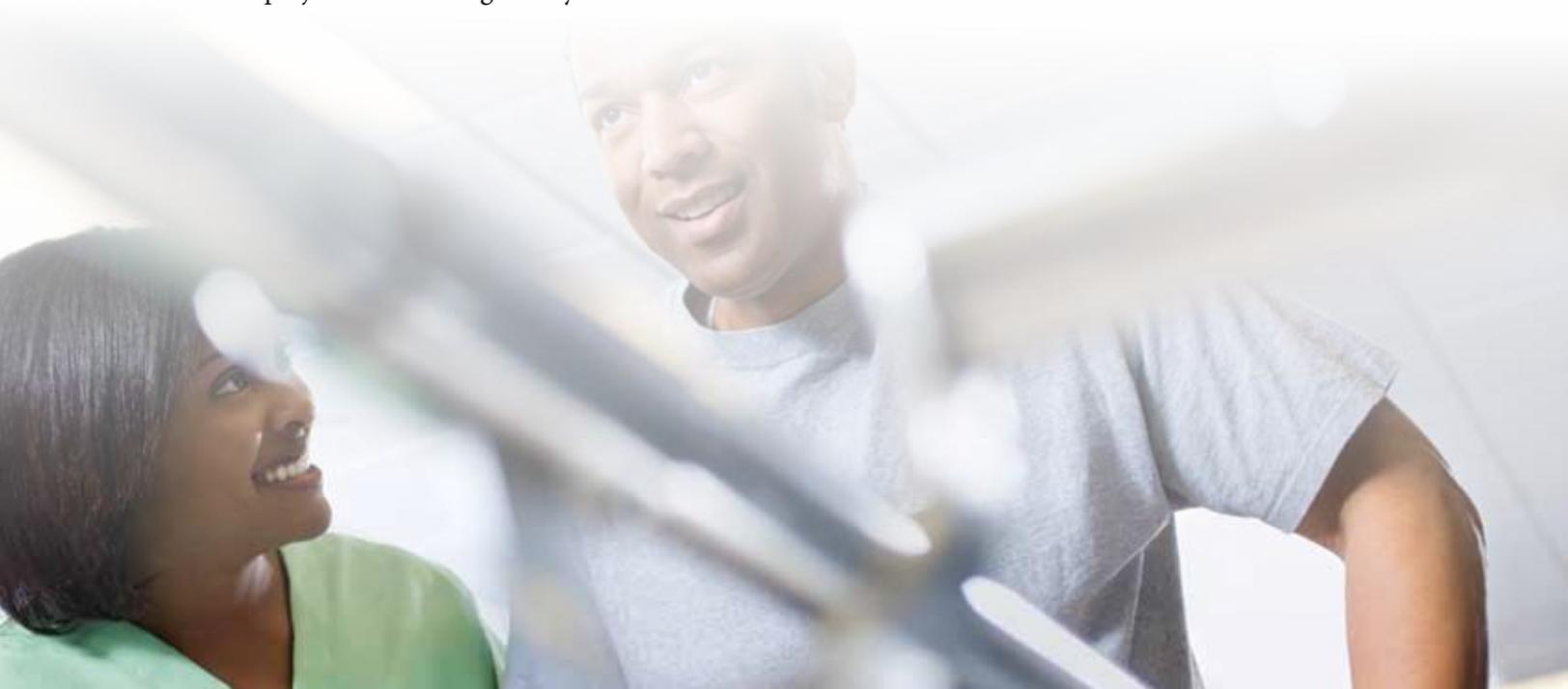
Research Plans for the Upcoming Year

The teams plan to complete the NHP median nerve gap (5 cm) studies and proceed into clinical trials. Additional silk conduits will continue to be tested in the rat sciatic nerve defect model, and a basic science pipeline will continue to provide innovative ideas to the project. An FDA regulatory consultant

has been contacted as well as several offices within the FDA (e.g., Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, and the Office of Combination Products). The nerve guide technology should proceed along the regulatory pathway as a medical device under current FDA guidelines. It is implanted in patients with the main purpose of holding the nerves and medication in place. The team is confident that TRL 5 will be reached by 2014.

Planned Clinical Transitions

After ten years of research in the area of nerve repair, the teams have completed rodent studies and have initiated a series of large animal studies (e.g., the NHP median nerve defect model). The Marra team has the freedom to operate regarding the encapsulated drug in the nerve guide, and will continue to work with the company that owns the license to GDNF to obtain their necessary data for FDA approval, and subsequent clinical trials. The first clinical trial will be a first-in-human study involving ten patients with traumatic extremity injuries. The nerve guide will be implanted at one of three potential sites (University of Pittsburgh Medical Center, Walter Reed National Military Center, or Brooke Army Medical Center at Fort Sam Houston, Texas). Continued interactions with both military and civilian surgeons will ensue.



Nerve Repair and Regeneration**Modular, Synthetic Extracellular Matrices for Regenerative Medicine****Project 4.4.5, WFPC**

Team Leader(s): Matthew Tirrell, PhD (UC Berkeley/University of Chicago)

Project Team(s): Katie Megley, BS, Won H. Suh, PhD, Nickesh Viswanathan, BS, and Seema Desai, BS (UC Berkeley); Emily Wonder, BA (University of Chicago); Brian Lin, PhD, Dan Krogstad, PhD, and Badri Ananthanarayanan, PhD (University of California, Santa Barbara)

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

Therapy: Injectable synthetic extracellular matrices for regenerative medicine

Deliverable(s): Hydrogel to fill nerve guide for peripheral nerve repair

TRL Progress: Beginning, TRL 1; Current, TRL 2; AFIRM I Target, TRL 3

Key Accomplishments: The research team demonstrated that C₁₆-W3K was not acceptable for regenerative medicine application and moved forward with a generation-two design, C₁₆-GSH. The team optimized C₁₆-GSH hydrogels using relevant in vitro models of nerve regeneration. They also performed an in vivo study using a subcutaneous implant model to determine the biocompatibility and degradation rate of C₁₆-GSH. Overall, they determined that C₁₆-GSH can support the growth of relevant cell types to nerve injury in vitro and that it is a biocompatible hydrogel.

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

Introduction

Peripheral nerve injury affects approximately 2.8% of trauma patients, many of whom encounter life-long disability. Additionally, the number of extremity injuries involving multiple tissues has increased in modern warfare with the prevalent use of improvised explosive devices. The current gold standard of treatment is an autograft where the surgeon removes pieces of a healthy nerve to repair the damaged one. This technique has inherent issues such as size limitations and donor-site morbidity. To circumvent these problems, synthetic nerve guides have been developed that show some clinical success. Proposed here is a method of supplementing the synthetic nerve guides by filling them with a bioactive peptide amphiphile gel able to recapitulate the mechanical properties of native tissue. It is hypothesized that by providing the injured nerve with an optimal physical environment, regeneration will be enhanced beyond that seen in the nerve guide alone. The aim of this work is to create an off-the-shelf treatment more efficacious than the current standard of autografting.

Research Progress – Year 5**Design v1: C₁₆-W3K 1****Peptide Amphiphile Monomer Design**

The initial peptide amphiphile design used by the Tirrell group included a 16 carbon fatty acid tail conjugated to a short peptide with a tryptophan residue for ultraviolet detection, alanine repeat sections for beta sheet secondary structure stabilization and lysine residues. Upon dissolution in aqueous solution, peptide amphiphile monomers self-assembled into spherical micelles. With the addition of shear force to the solution (or over long time periods), spherical micelles joined together to form a single extended worm-like micelle capable of entangling and giving rise to a hydrogel.

Materials Characterization

The transition from spherical to worm-like micelle was evident in the shear rate ramp rheological test. During the initial test, as shear rate was increased the solution initially showed no response and had the viscosity roughly of water (**Figure 1**, 1st run). At a critical point in shear rate, a sudden increase in viscosity and subsequent shear thinning was observed, indicating the formation of worm-like



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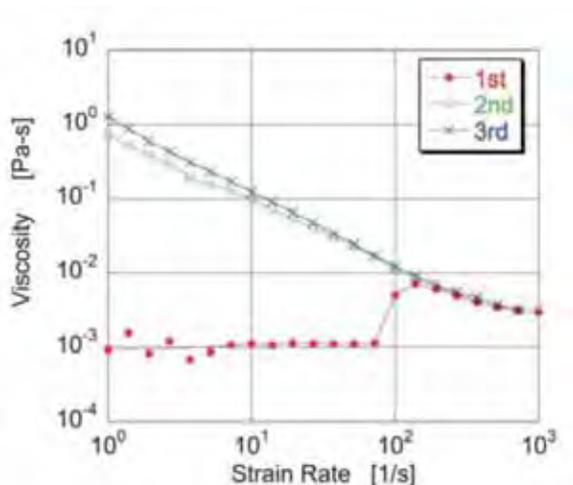


Figure 1. Shear rate test of the C_{16} -W3K solution showing a change in viscosity between the 1st, and 2nd and 3rd tests of the same sample.

entangled micelles. The 2nd and 3rd test cycles of the sample (Figure 2, 2nd and 3rd run) showed a shear thinning response with a 1000-fold increase of zero shear viscosity. Evidence of gel formation was also demonstrated with an oscillatory rheological test used to measure the modulus, or elastic resistance, of a soft material. Before the shear rate test induced change (Figure 2, open symbols), the solution had a negligible modulus, and little resistance to flow. The same sample tested after the shear rate test induced change (Figure 2, closed symbols) showed curves of a characteristic hydrogel with the storage modulus (G') greater than the loss modulus (G'') at all frequencies, and low dependence of

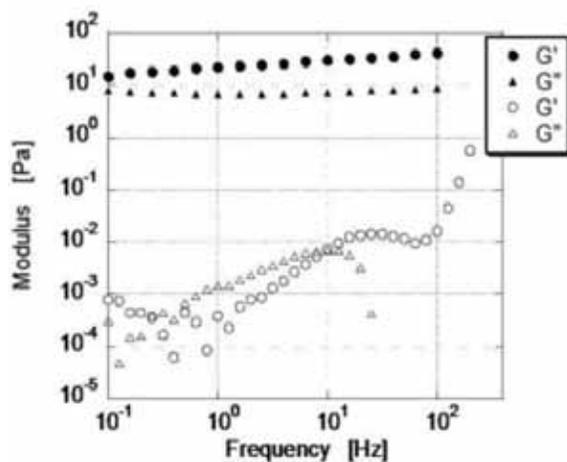


Figure 2. Oscillatory testing of C_{16} -W3K solutions before (open symbols) and after (closed symbols) the transition was induced by the simple shear test.

modulus on frequency with a 1000-fold increase in stiffness.

In vitro Development

Following materials characterization, C_{16} -W3K was tested in vitro to determine the biocompatibility of the material. Past a critical dose of 8.0 μM , C_{16} -W3K neural stem cells were negatively affected (Figure 3). The researchers determined that monomer peptide amphiphiles were disengaging from the micelle and puncturing holes in the cell membrane (data not shown). Despite efforts to mitigate toxicity issues by combining other peptide designs (C_{16} -W3E) or glycosaminoglycans (hyaluronic acid), the C_{16} -W3K was deemed not acceptable for regenerative medicine applications. The Tirrell group thus moved forward with a generation-two design, C_{16} -GSH (described below).

Design v2: C_{16} -GSH

Peptide Amphiphile (PA) Monomer Design

Using the results of the C_{16} -W3K research as a guide, the Tirrell group designed a new PA monomer able to more tightly associate in the extended micelle and thus not dissociate as a monomer and enter the cell membrane. Additionally, the Tirrell group sought to use physiological pH as the gelation trigger in the new design. To achieve this, they designed PAs in a branched architecture with histidine and serine amino acid side arms. As a result, the PAs formed weak fibers at low pH and formed strong fibers above pH 6.5. Weak fibers in solution resembled a low viscosity liquid, and strong fibers formed self-supporting hydrogels.

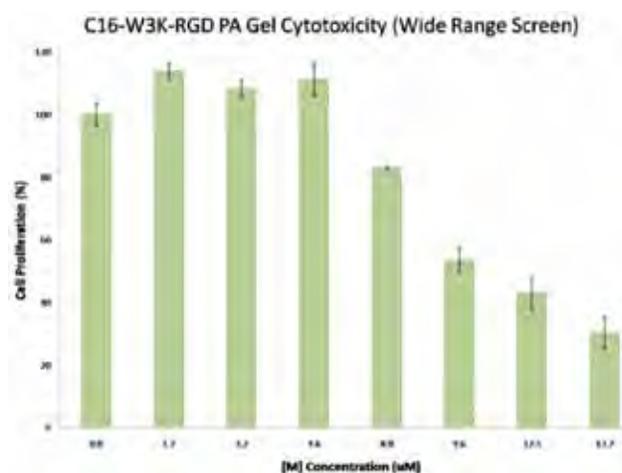


Figure 3. Neural stem cell viability after 24 hours of incubation with increasing concentrations of C_{16} -W3K.

Materials Characterization

Evidence of this switch from weak to strong fiber can be seen in the moduli of the solutions. Samples of C_{16} -GSH at varying concentration were loaded onto the rheometer at low pH and tested (Figure 4, open squares). At low pH, the solutions behaved as viscous liquids with a low storage modulus. That same sample was then raised to pH 9 by applying concentrated base, and the solution was measured again (Figure 4, closed squares). An increase of several orders of magnitude in the storage modulus occurred between pH 4 and pH 9, which indicated the formation of stiff entangled fibers that scaled with concentration. At 1% w/v in water, the PAs formed hydrogels that achieved a storage modulus (G') of approximately 10 kPa.

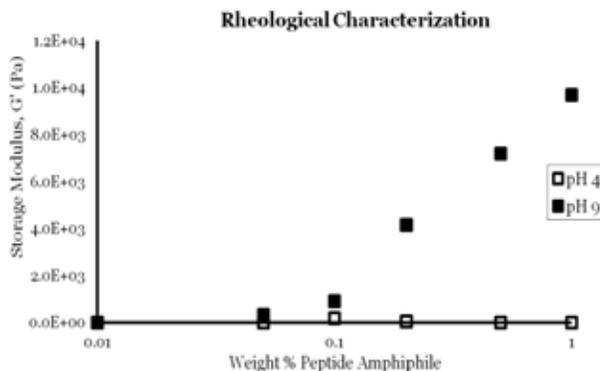


Figure 4. Tabulated results of rheological testing C_{16} -GSH hydrogels stiffened with increasing pH.

In vitro Development

The material was subsequently tested in vitro and shown to support growth of a model cell line, NIH 3T3, over a period of several days (data not shown). To optimize the hydrogel conditions for nerve repair, the research team designed a series of experiments using Schwann cells, which are considered the first responders of nerve regeneration.

Conclusions

C_{16} -W3K had interesting material properties and response to shear force, but was not biocompatible and thus not suitable for regenerative medicine applications. The research team determined that C_{16} -GSH can support the growth of relevant cell types to nerve injury in vitro. They were also able to demonstrate that C_{16} -GSH is a biocompatible hydrogel.

Future Research Plans

Going forward, the researchers will perform an in vivo nerve injury model study using C_{16} -GSH as a nerve guide filler.

Planned Clinical Transitions

There is no planned clinical translation at this time. Pre-clinical animal studies are ongoing.





Composite Tissue Injury Repair

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

Project 4.4.3, WFPC

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

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

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

Therapy: Segmental bone regeneration with possible concomitant vascular, nerve, or muscle injury

Deliverable(s): Composite limb injury animal models and spatiotemporal delivery strategies for functional limb regeneration

TRL Progress: Beginning, TRL 1; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The research team has successfully established and published composite injury models in the rat that simulate bone/nerve, bone/vascular injuries, and bone/muscle injuries. These models have been used to test nanofiber biomaterials delivery systems (patents pending) that provide spatial and temporal cues to guide improved bone, vascular, and nerve regeneration. A series of papers have been published establishing critical dose response relationships and direct clinical comparisons. A pivotal 6-month large animal (sheep) study was initiated in Year 4 and completed in May 2013.

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

Introduction

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

To address this limitation, a primary objective of this project was to establish animal models of composite tissue trauma that combine a massive segmental bone defect with peripheral nerve resection and/or femoral artery ligation. Importantly, quantitative evaluation methods such

as three-dimensional micro-computed tomography imaging, electrophysiology, biomechanics, and gait analysis have been integrated into these models to provide comparison of competing regenerative strategies. The models are being used to quantitatively evaluate spatial and temporal delivery of biological cues that direct nerve, vascular, and bone growth in a synchronized manner. In addition to regenerative technologies developed at Georgia Tech, these quantitative evaluation models are being used to test technologies developed in several other AFIRM laboratories.

Research Progress – Year 5

In a series of 23 papers over the past five years, the researchers of this project have established multiple models of composite limb injury relevant to severe military injuries to bone, muscle, blood vessels, and nerve. Biomaterial-mediated spatiotemporal delivery strategies that promote nerve, vascular, and bone regeneration have been developed and

published. These successful studies have culminated in a recent study in a sheep model at Queensland University of Technology (QUT) that replicates the human scale of injury. As shown in **Figures 1 and 2**, the new hybrid construct delivery system produces robust regeneration of massive bone defects, providing a promising new therapeutic strategy for treating severe limb injuries in both the military and civilian populations.

Conclusions

Composite multi-tissue injury models have been developed to simulate complex combat injuries and test spatial and temporal guidance strategies that take advantage of synergistic interactions among the tissues observed during development and repair. The rat model was chosen since it provides the opportunity for larger in vivo studies and is amenable to highly quantitative assessment methods (e.g., micro-computed tomography assessment of vascularization and bone formation). Variations of the composite injury model include bone/nerve injuries, bone/vascular injuries, and bone/muscle injuries. These models are available for testing regenerative strategies developed by other AFIRM investigators. The Georgia Tech team has established promising patent-pending regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of clinically approved inductive protein (bone morphogenetic protein-2 [BMP-2]). Finally, pivotal large animal sheep studies have been completed that demonstrate that the hybrid BMP delivery system can effectively regenerate human-scale (3 cm) segmental bone defects.

Research Plans for the Upcoming Year

Having completed development of an improved approach to treating acute segmental bone injuries, the researchers will next turn their attention to overcoming the challenges of chronic non-union with and without adjacent concomitant soft tissue injury or mechanical instability. They will also complete ongoing optimization studies of hydrogel composition and degradation characteristics.

Planned Clinical Transitions

Additional funds were identified for the pivotal sheep defect study at QUT, and the study was completed in May 2013. No AFIRM funds were used, but in kind, support of time was provided. The addition of the large animal study was a revision to the researchers' original plan made possible by the rapid progress of the project and successful results demonstrated in the small animal model. Completion of this study significantly improves the chances of successful transition to a human pilot study. The intellectual property will be marketed to members of the Georgia Tech Industry Partners program. The researchers have previously had success licensing patent rights to industry partners following successful large animal studies. Once proof of concept has been demonstrated in the large animal model, the research team's goal is to initiate a human clinical trial pilot study





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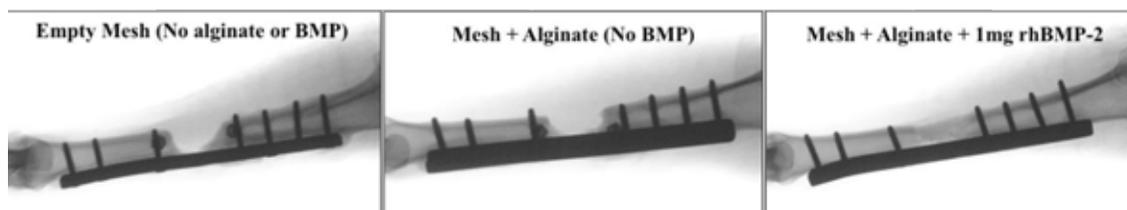


Figure 1. Sheep segmental defect study with representative 3-month X-rays showing robust bridging of the 3 cm defect in the Mesh + Hydrogel + BMP-2 group only.



Figure 2. Sheep segmental defect study showing increase in density for BMP delivery group from 2 months (left) to 3 months (middle) to 6 months (right).

Epimorphic Regeneration**Epimorphic, Non-Blastemal Approach to Digit Reconstruction****Project 4.4.1, WFPC**

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

Project Team(s): Lisa E. Carey, BS, Vineet Agrawal, MD, PhD, Scott A. Johnson, MS, Neill Turner, PhD, Li Zhang, MD, MS, and Janet Reing, MS (McGowan Institute for Regenerative Medicine, University of Pittsburgh)

Collaborator(s): Ron Stewart, PhD and Jamie Thomson, PhD, VMD (University of Wisconsin); Susan Braunhut, PhD (University of Massachusetts, Lowell); David Kaplan, PhD (Tufts University); Eileen Moss, PhD and Muthu Wijesundara, PhD (The University of Texas at Arlington)

Therapy: Treatment of limb and digit amputation with epimorphic regeneration strategies

Deliverable(s): A biologic scaffold-based strategy for inducing epimorphic regeneration in limb and digit soft tissues; a biomaterial that can facilitate functional restoration of soft tissues following traumatic injury, manufactured in multiple forms, solid sheet, gel, powder, etc.

TRL Progress: Beginning, TRL 3; Current, TRL 5; AFIRM I Target, TRL 5

Key Accomplishments: The researchers developed a rodent model of digit amputation and characterized the innate immune response to treatment with extracellular matrix (ECM), both in vitro and in vivo. They analyzed microarray data to characterize the genetic signature of ECM-induced recruitment of endogenous stem cells to the injury site and subsequent digit tissue remodeling. They demonstrated the critical role of the innate immune response in constructive tissue remodeling and the ability of the ECM to induce a constructive macrophage phenotype. In addition, the researchers refined a prototype Biomechanical Interface for Optimized Delivery of MEMS Orchestrated Mammalian Epimorphosis (BIODOME) device for controlling the microenvironment of the site of amputation, and they began to design a new prototype BIODOME for controlling the microenvironment of a soft tissue injury site in a large animal model.

Key Words: Limb and digit reconstruction, extracellular matrix, epimorphosis, stem cell recruitment, innate immune response, M2 macrophage

Introduction

Improved survival rates for soldiers following severe injury have resulted in an increase in the number of soldiers afflicted with life-altering extremity injuries, including amputations. Conventional treatment methods are inadequate to restore functional tissue in most patients and, therefore, alternative and more effective treatment options are needed. Additionally, millions of American adults and children visit doctors' offices and emergency rooms every year with limb and hand injuries. This translates to impaired quality of life for individual patients and an annual loss of \$10 billion in productivity in the United States. The causes for these injuries are primarily trauma, especially accidents with common items, including kitchen knives, doors, and power tools, as well as workplace accidents.

To address this problem, the present work investigates a method for restoration of functional tissue in

injured limbs and digits via a non-blastemal epimorphic regeneration approach. Certain non-mammalian species, such as salamanders, are capable of full regeneration of limbs through accumulation of pre-programmed stem cells at the site of amputation which form a blastema. Adult mammals are incapable of blastema formation, but non-blastemal epimorphic regeneration does occur in certain instances such as liver regeneration. Since it is possible for adult mammals to regenerate certain tissues such as liver, bone marrow and intestinal lining, it is plausible that, given the appropriate signals and the proper microenvironmental niche, additional tissues may be induced to express similar regenerative potential.

The liver, skin, bone marrow, and intestinal lining of epithelial cells are examples of tissues that exhibit non-blastemal epimorphic regeneration in adult mammals. However, virtually every other tissue



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does not have this capacity as a component of the default mechanism for wound healing. The present work, in large part, involves identifying mechanisms for inducing this non-blastemal regenerative capacity in alternative tissues. At least some of the signals to facilitate this resurrection of non-blastemal regeneration appear to reside within the ECM.

Research Progress – Year 5

Establish mouse model of digit amputation

Identify digit amputation site and treatment delivery method

Using an established mouse line (C57/BL6), the middle digit of the right hindfoot was amputated at the midpoint of the second phalanx (P2) (Figure 1). Amputation injury at this site does not result in spontaneous digit regeneration or functional remodeling. Following amputation, pepsin digested (i.e., solubilized) ECM derived from porcine urinary bladder matrix (UBM) was injected into the foot pad of the hindfoot, proximal to the amputated toe. Preliminary studies with India ink verified that the injected solution travels up the injured toe to the site of injury.

Characterize tissue remodeling response to ECM treatment of amputated digit

Using the digit amputation mouse model, the Badylak laboratory has definitively shown that endogenous multipotential stem cells are recruited to the site of injury following ECM injection (Figure 2). This accumulation of cells is termed a “multipotent cell cluster” or MCC. This induced migration of endogenous cells was caused by the local/regional administration of bioactive molecular derivatives of ECM. Studies conducted in the first year of work showed that the recruited cells express markers of multipotential progenitor cells: Sca-1, Rex-1, and Sox-2. Work in the second year demonstrated that, in the absence of treatment or amputation, fewer cells express Rex-1 and Sox-2 (Figure 2). Thus, Rex-1 and Sox-2 positive cells are specifically recruited by molecular derivatives of ECM.

Isolate and verify chemotactic fraction of ECM

In vitro testing of ECM fraction on multipotential cell chemotaxis

Solubilized UBM-ECM has been shown to be chemotactic for stem cells (Figure 3). Building upon the first year’s effort to identify the key chemotactic

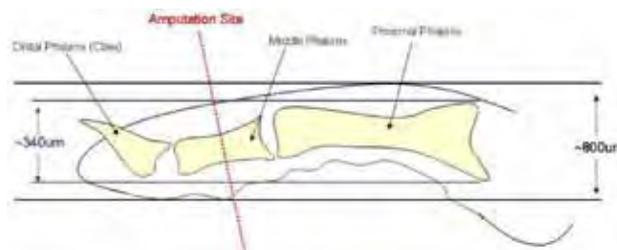


Figure 1. Site of P2 amputation in mouse model.

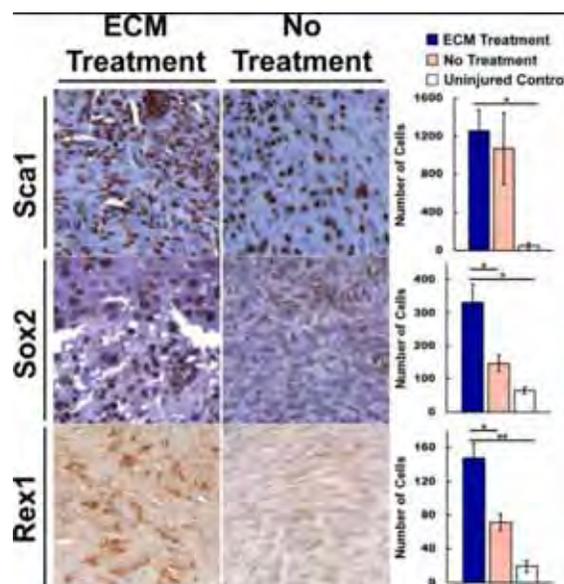


Figure 2. ECM treatment recruits multipotential progenitor cells.

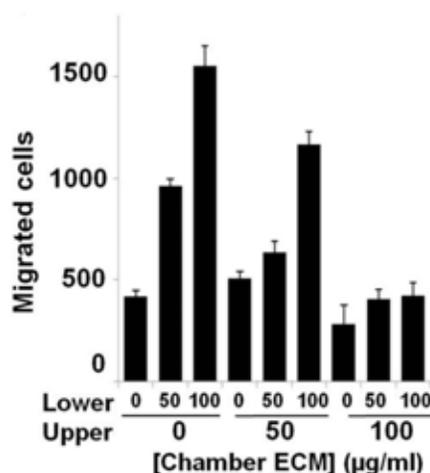


Figure 3. Molecules derived from ECM are chemotactic for human perivascular stem cells in a Boyden chamber system in vitro.

peptides from within the mix of peptides generated from enzymatic digestion, the researchers performed successive rounds of fractionation via ammonium sulfate precipitation, size exclusion chromatography and ion exchange chromatography, and they identified candidate peptides that were chemotactic for human perivascular stem cells in a Boyden chamber migration assay. The peptide fractions with the greatest chemoattractive potential were again selected and analyzed via mass spectrometry, leading to the identification of oligopeptides that were subsequently sequenced and which represent fragments of some of the parent collagen molecules.

In vivo testing of ECM fraction on multipotential cell accumulation at injury

To determine whether the isolated chemotactic peptide treatment can promote chemotaxis of multipotential cells in an *in vivo* model of digit amputation, cryptic peptide was delivered as treatment to mouse footpads after amputation through the mid P2. Analysis of the resulting tissue remodeling via flow cytometry and immunostaining identified the recruitment of Sox2+, Sca1+, multipotential cells after treatment with the isolated cryptic peptide (Figure 4).

Characterize effects of ECM degradation products on innate immune response

In vitro assessment of ECM degradation products on macrophage phenotype

Studies from the Badylak laboratory and other research groups have shown the importance of the innate immune response in tissue remodeling. During the past year, the researchers characterized the effect of ECM on macrophage polarization *in vitro*, using both human and mouse primary cells. In one study, monocytes harvested from human donors were cultured with different concentrations of UBM digested in pepsin. Cells were then labeled with immunofluorescence antibodies for markers of pro-inflammatory macrophages (M1) and pro-remodeling macrophages (M2). The markers used were CCR7 and CD206, respectively. Flow cytometry results showed that at concentrations from 100 to 1,000 $\mu\text{g/ml}$, monocytes were predominantly polarized to an M2 phenotype compared to a pepsin control (Figure 5).

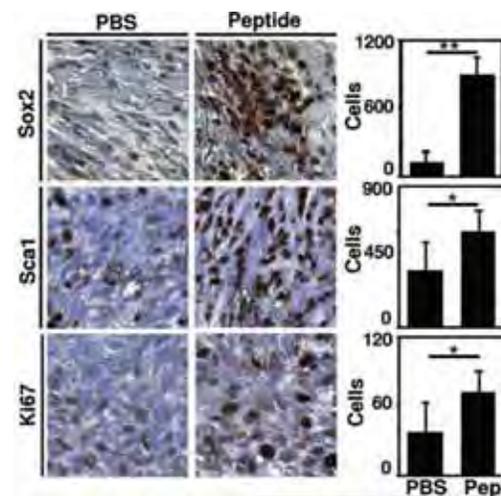


Figure 4. Histologic sections show that peptide treatment led to a greater number of Sox2, Sca1, and Ki67-positive cells at the site of amputation.

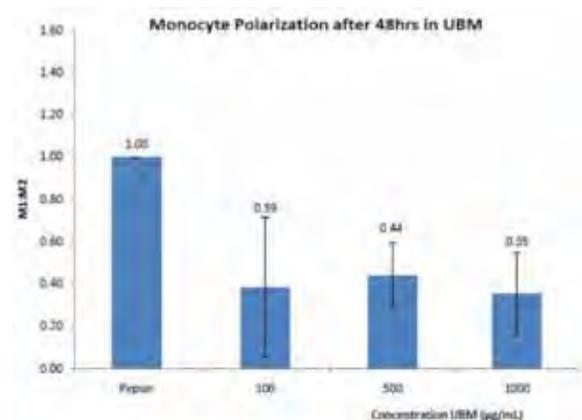


Figure 5. Human monocyte polarization expressed by M1:M2 ratio, where a value greater than one is predominantly an M1 response, lower than one is a predominantly M2 response.

Murine macrophages were harvested from bone marrow, cultured with three different types of ECM, and then stained with F4/80 (a mouse-specific macrophage marker), FIZZ1 (an M2 marker), and iNOS (an M1 marker). Preliminary imaging results show that the ECM-cultured macrophages assumed a predominantly M2 phenotype when cultured in the presence of ECM degradation products.

In vivo assessment of ECM degradation products on macrophage phenotype

The researchers' established model of digit amputation and ECM degradation product treatment was used to show that the innate immune



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response is modified *in vivo*, in response to the presence of xenogeneic ECM scaffold materials. Immunofluorescence staining of harvested frozen digit tissue showed that UBM-treated mouse digits had more macrophages present at the site of injury than non-treated control digits. A greater number of M1 macrophages as a percentage of total macrophages was present at the site of injury in non-treated control animals at Days 7 and 14 than in ECM-treated animals (Figure 6). Conversely, there was no difference in the percentage of M2 macrophages between the two groups at any time points (Figure 6). Overall, there was a higher ratio of M2 to M1 macrophages in treated digits compared to non-treated controls, at Days 7 and 14.

Genetic signature of innate immune environment at injury site, in response to ECM

A collaboration with the Thomson laboratory has led to the identification of specific transcription factors, oncogenes, and blastema genes that peak throughout the healing and regeneration of the salamander limb through deep ribonucleic acid sequencing. These newly identified genes are distinct from those that are up-regulated in response to mouse digit amputation without treatment. This work helps identify the unique genetic signature of blastema formation as well as the local cues that may be targeted to generate a similar response in mammals.

Data mining analysis of up- and down-regulated genes in mouse digits with and without ECM treatment revealed that sets of genes associated with a Type 1, pro-inflammatory immune response were down-regulated in treated toes. This response was seen at 10 and 14 days. Conversely, no differences in Type 2 genes, associated with a pro-remodeling response, were seen between treated and non-treated toes (Figure 7).

BIODOME direct delivery device for treatment and microenvironmental control

A collaboration with Dr. Eileen Moss at the University of Texas at Arlington Research Institute (UTARI), has resulted in the development of a novel prototype device designated as the BIODOME (Biomechanical Interface for Optimized Delivery of MEMS Orchestrated Mammalian Epimorphosis), which can be used to deliver soluble treatments (e.g., growth factors, ECM degradation products)

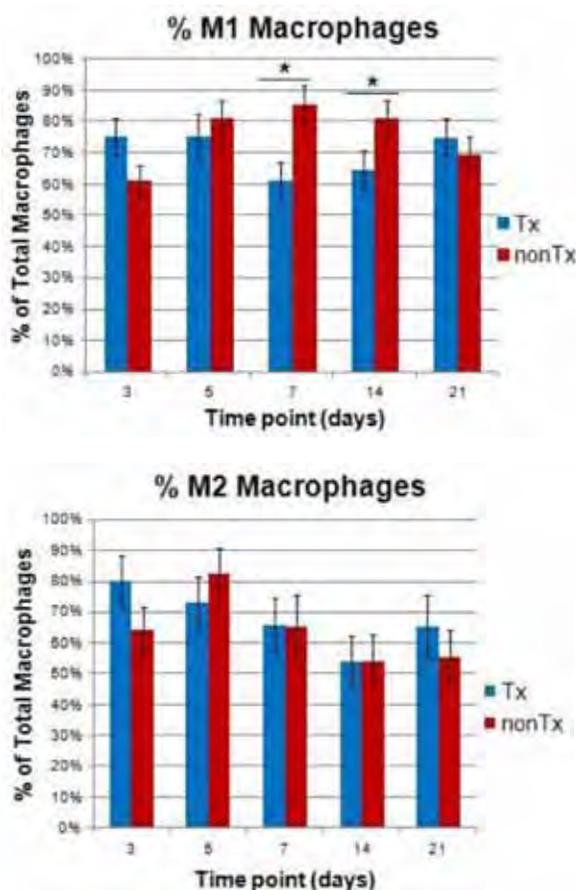


Figure 6. Quantification of M1 and M2 stained calls as a percentage of total macrophages, with and without ECM treatment.

and control variables in the microenvironment (e.g., pH, oxygen levels) in an adult mouse model of digit amputation.

Prototype design optimization

During the past year, a collaboration with researchers at UTARI yielded design improvements for the BIODOME, including better flexibility to allow for digit swelling, a smaller profile for greater mouse mobility, and shape changes which allow for easier removal and reuse, as well as better preservation of the healing digit (Figure 8). Design optimization is ongoing and will inform the future design of a BIODOME device for large animal models and, eventually, a human pilot study.

Conclusions

The Badylak group has shown the ability to recruit endogenous multipotential cells to the site of injury in a non-regenerating mammalian system (i.e., a

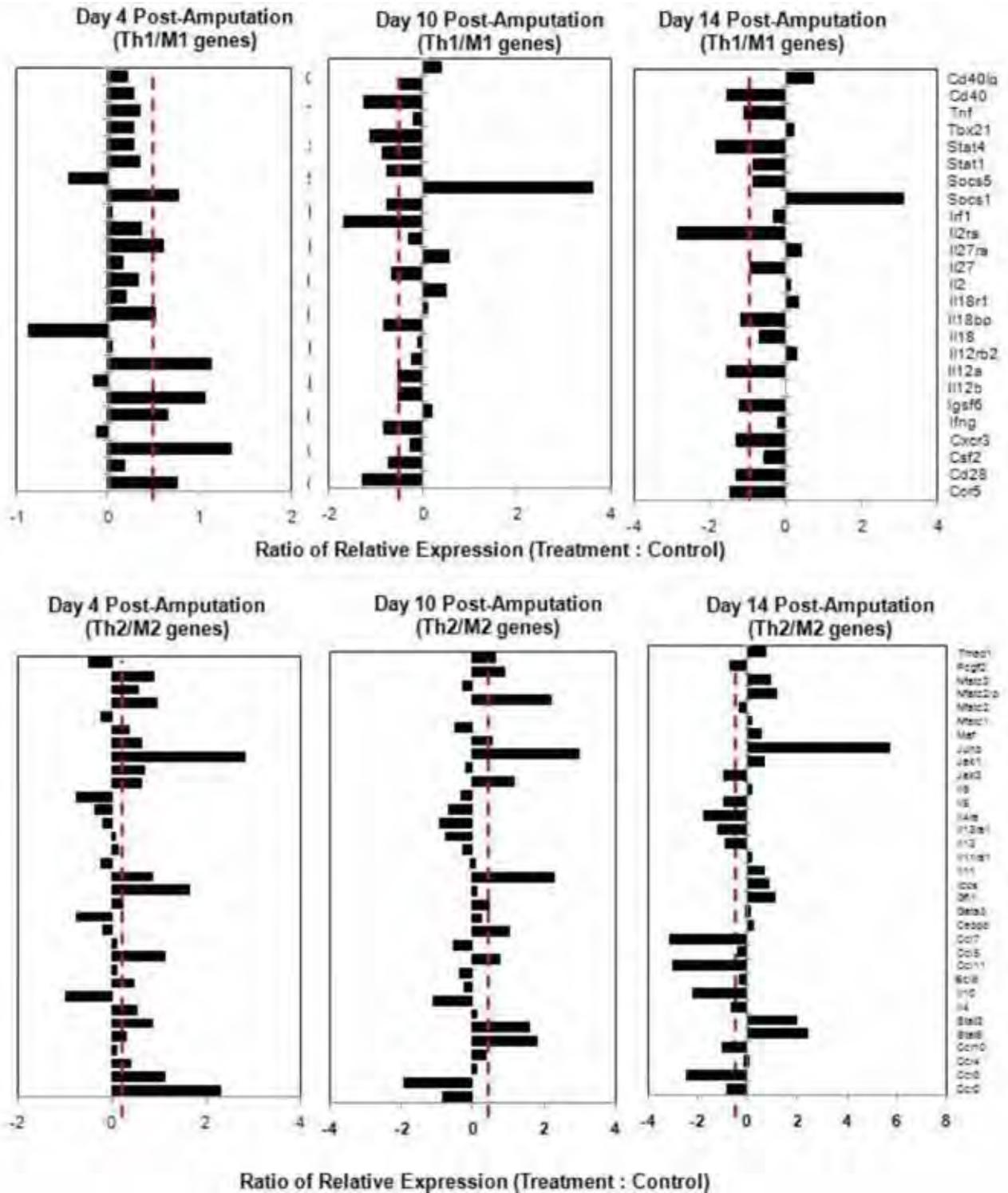


Figure 7. Microarray gene set analysis.



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Figure 8. Prototype schematic and checking fit on an amputated mouse toe.

step toward non-blastemal epimorphic regeneration). They continue to define and ultimately refine the proteins and peptides of the ECM that are involved in the recruitment of the MCC to the site of injury. The researchers also are continuing to further define the population of cells involved in the formation of the MCC and are examining the ability of those cells to differentiate into different functional tissues. The ability to specifically direct the differentiation of the MCC into functional tissue is one of the next major hurdles that they face. From these findings, it is clear that control of the “micro-environmental niche” is essential to direct complex tissue regeneration. To that end, the Badylak group is working in collaboration with researchers at UTARI to develop a prototype for a BIODOME direct-delivery device. The device will eventually be used to control microenvironmental conditions including hydration state, pH, oxygen tension, electrical potential, and other factors known to affect stem cell fate. Development and testing of the BIODOME device is ongoing through small amounts of leveraged funding.

Research Plans for the Upcoming Year

The researchers will focus on the testing and utilization of the BIODOME functional delivery device, with an objective of eventual use in human trials. Studies in mice and canines will be ongoing, as different bioactive molecules are introduced in a controlled fashion to the injury site microenvironment. Additionally, further work will be done to fractionate the ECM into constituent peptides, which will be screened to test their ability to induce or differentiate the MCC. Within the next three years, industry partners for the mass production, further development, and potential clinical testing of the bioactive peptides for treatment of limb/digit loss will be identified.

Planned Clinical Translations

The Badylak group is in a continuing collaboration with Dr. Peter Rubin at the University of Pittsburgh to evaluate a powder form of ECM for the treatment of distal digit amputations. The product being used is a commercially available U.S. Food and Drug Administration-approved form of powdered ECM. The patients who present at the University of Pittsburgh with this particular problem are evaluated, and selected individuals are treated based upon the findings of the work conducted during the past several years of research using the digit amputation model. The goal of this clinical trial is to show efficacy of the ECM treatment against the current standard of care.

In collaboration with researchers at UTARI, the laboratory is developing the prototype for a canine BIODOME treatment delivery device to be used in a preclinical efficacy trial for tissue regeneration using control of the injury site microenvironmental niche.

Clinical Trials

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

Project 4.4.2, WFPC

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

Project Team(s): Gerald Brandacher, MD, Damon S. Cooney, MD, PhD, Justin M. Sacks, MD, Jaimie T Shores, MD, Stefan Schneeberger, MD, Eric Wimmers, MD and Zuhaib Ibrahim, MD (Johns Hopkins University); Vijay S. Gorantla, MD, PhD and Joseph E. Losee, MD (University of Pittsburgh)

Collaborator(s): None

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

Deliverable(s): Phase I (Translational Studies), immunomodulatory protocol that combines systemic stem cell-based therapy with local immunosuppression in a swine heterotopic hind limb model of composite tissue allotransplantation (CTA); Phase II (Clinical Trial), reconstructive transplantation as treatment for hand or forearm loss under a novel cell-based immunomodulatory protocol.

TRL Progress: Beginning, TRL 4; Current, TRL 5 and 6; AFIRM I Target, TRL 6

Key Accomplishments: The researchers optimized a targeted immunomodulatory protocol using donor BM infusion in combination with co-stimulation blockade (Cytotoxic T Lymphocyte-Associated Antigen 4 Immunoglobulin [CTLA4Ig]) in a full major histocompatibility complex (MHC) mismatched miniature swine model. Indefinite graft survival was achieved off immunosuppression in 5 out of 8 animals that received the CTLA4Ig fusion protein-based regimen. Ten successful hand/forearm transplants were performed in six patients, including the first bilateral and first above-elbow transplant in the United States. The first clinical protocol in hand transplantation using donor BM cell therapies and tacrolimus monotherapy was implemented. All compliant patients transplanted to date are maintained on a single immunosuppressive drug at low levels, and they continue to have increased motor and sensory function of their transplanted hands, which correlates with their level of amputation, time after transplant and participation in hand therapy.

Key Words: Hand transplantation, immunosuppression, immunomodulation, swine, composite tissue allotransplantation

Introduction

Extremity trauma accounts for the majority of battlefield injuries sustained by troops during Operation Iraqi Freedom and Operation Enduring Freedom. In this context, CTA such as upper extremity and hand transplantation represents a valuable and novel treatment modality that can restore form and function better than any conventional reconstructive strategy. Despite excellent and highly encouraging functional results of such transplants, the requirement of life-long multi-drug immunosuppression hinders its widespread clinical application. These immunosuppressive medications carry the risk of potential serious adverse effects and complications. In order to favor the risk benefit balance, one has to minimize immunosuppression and improve functional recovery and quality of

life. This research proposal was aimed at minimizing and possibly eliminating the requirement for maintenance immunosuppression through targeted immunomodulation.

This project has two phases:

PHASE I (Translational Studies): A preclinical model of targeted immunomodulation in heterotopic hind limb transplantation utilizing complete MHC-mismatched miniature swine. Research trials are parallel and complementary to the clinical trial, and work in each arm will be detailed separately.

PHASE II (Clinical Trial): The overall goal is to establish hand transplantation as a treatment strategy for reconstruction of disabling combat injuries involving hand or forearm loss using a novel BM/stem-cell based protocol.



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Research Progress – Year 5

Phase I (Translational Studies)

During Years 1-2, the team determined the safety, efficacy and optimum dose of donor BM cell infusion. During Years 3-5, the BM infusion protocol was further optimized and combined with costimulatory blockade in the form of CTLA4Ig in a stringent, fully mismatched hind limb transplantation large animal model. Such a targeted immunomodulatory protocol using CTLA4Ig resulted in significantly prolonged graft survival, and when combined with donor BM, resulted in indefinite graft survival and immune tolerance without the requirement of long-term medication (**Figure 1**). This state of operational tolerance, however, was reversed by removal of the graft, indicating the essential role of vascularized BM within a CTA. Furthermore, there was persistence of donor-derived cells in various recipient tissues (chimerism), which is indicative of immune tolerance. The team did not observe any signs of chronic rejection, donor-specific antibodies, or graft versus host disease in long-term survivors undergoing treatment with this novel protocol. This represents the first translational large animal model that enables tolerance induction without the need for immunosuppression across a full MHC barrier.

Phase II (Clinical Trial)

Ten successful upper extremity transplants (hand/forearm/arm) have been performed in six patients, including the first bilateral and first above elbow transplant in the United States. The costs associated with the transplants were sponsored by

University of Pittsburgh (Patients #1-5) or Johns Hopkins (Patient #6), and were partly underwritten by AFIRM funding (Patients #1 and 6). The most recent transplant was performed in December 2012 at Johns Hopkins. The recipient was a soldier who survived four-limb amputation due to a roadside bomb explosion in Iraq in 2009. The patient sustained a right above-elbow amputation and a very proximal left forearm-level amputation. The surgery was the most extensive double-arm transplant performed to date in the U.S. The patient did not experience any immediate or significant transplant-related complications. Bone healing has been progressing, and definitive evidence of motor nerve regeneration was present in the left wrist extensors and extrinsic digit flexors by 10 and 12 weeks, respectively. The right arm demonstrated evidence of reinnervation to the same muscle groups by Week 15. A mild episode of skin rejection occurred in Week 16 without sequelae. Donor BM infusion has allowed minimization of recipient immunosuppression to a single agent of tacrolimus.

The patient is currently being transferred back to Walter Reed National Military Medical Center to continue hand therapy and rehabilitation. Four out of the five other previously transplanted patients* are being maintained on a single immunosuppressive drug at low levels. Patients demonstrated sustained improvements in motor function (range of motion, grip and pinch strength, variable intrinsic muscle recovery) and sensory return correlating with the time after transplantation, level of amputation, and participation in hand therapy. Four out of five patients regained significant function, allowing resumption of independent living.

However, Patient #1, despite excellent immunological and functional outcomes during the first years after transplant, developed significant issues coping with the requirements, responsibilities and regimens for hand transplantation, severely affecting his immunologic and functional results. The patient received counseling and support from the study team together

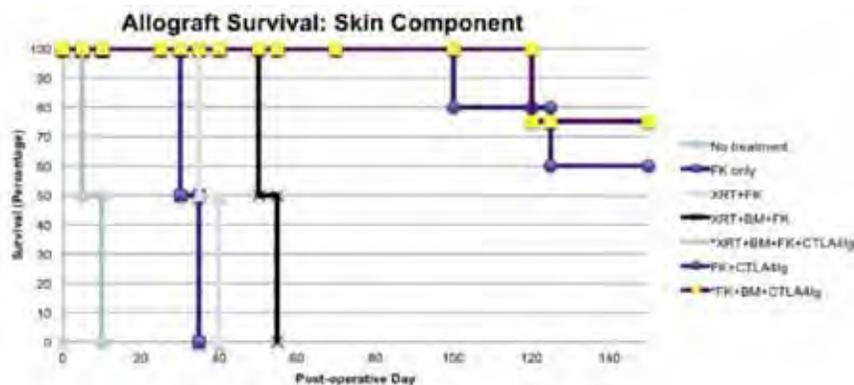


Figure 1. Translational trial: Kaplan-Meier curve demonstrating rejection free allograft survival. The combined costimulatory blockade and donor BM-based immunomodulatory protocol resulted in indefinite survival off immunosuppression in 2 out of 3 animals.

with the Wounded Warrior Regiment to invest in his transplant outcomes. These efforts, however, did not result in behavioral change, and the patient's persistent non-compliance with medication made rejection episodes difficult to control and resulted in damage to the graft despite increased immunosuppressive medication. Ultimately, a recommendation to explant his hand was made to the patient, who concurred with the decision after accepting his failure to keep up his commitments to the protocol. The allograft was explanted in March 2013 without complications. The patient did not have serious sequelae due to immunosuppression, and ultimately sustained no adverse effects related to the transplantation.

** One patient was placed on an additional agent (myfortic) for a limited time for transient increase in creatinine.*

Conclusions

This translational trial features the development of a clinically relevant heterotopic hind limb transplant model for CTA using a novel immunomodulatory protocol. The addition of CTLA4Ig enabled optimization of induction therapy, reduced maintenance immunosuppression, and indefinitely prolonged graft survival. In parallel, such immunomodulatory concepts have been applied in clinical hand transplantation using a novel BM cell-based strategy that aims to reduce maintenance immunosuppression necessary for successful CTA. All compliant patients transplanted to date are maintained on a single immunosuppressive drug at low levels and continue to have increased motor and sensory function of their transplanted hands. The success of this novel protocol will allow for greater clinical application of hand transplantation for reconstruction of upper extremity amputations.

Research Plans for the Upcoming Year

Based on the significant data obtained in Phase I (translational studies), combined BM cell-based therapies and CTLA4Ig represents a potential paradigm shift in the immunosuppression protocols in CTA. This study will form the basis of future research in optimizing cell-based immunomodulatory protocols for inducing immune tolerance in CTA.

Planned Clinical Transitions

The project goal is to promote long-term graft acceptance while minimizing the need for immunosuppressive drug therapy. An optimized strategy is planned, combining targeted immunomodulation, BM stem cell/fusion protein induction to further reduce maintenance immunosuppression and allow weaning of systemic drug therapy. This regimen might enable tolerance induction and reduce side effects related to high-dose immunosuppression, and will hopefully enable widespread clinical application of hand transplantation for the reconstruction of upper extremity amputations.

Corrections/Changes Planned for the Future

PHASE I: The combination of CTLA4Ig and donor BM infusion has resulted in excellent outcome with indefinite graft survival off immunosuppression. Since these results have remained reproducible, investigative local immunomodulatory strategies will be employed only in instances where systemic immunomodulatory strategies fail to induce immune tolerance.

PHASE II: The team requested a revision to the Statement of Work (SOW) for the original AFIRM proposal on Upper Extremity Transplantation being implemented at the University of Pittsburgh and Johns Hopkins University School of Medicine. The original SOW limited the transplant candidates to the military population. However, given the study's stringent inclusion/exclusion criteria, challenges in reaching these Wounded Warriors, and the delays encountered when the program expanded to include the site at Johns Hopkins, participant recruitment proved more challenging than anticipated. For these reasons, the team requested that the SOW be expanded to include both military and civilian upper extremity amputees. Along with the SOW revisions, the team is requesting a no-cost extension period of performance until 2014. With these changes, the team anticipates being able to satisfy participant recruitment goals of the study, thereby generating valuable data to further this AFIRM-funded clinical trial.



Clinical Trials

A Clinical Trial to Assess the Safety of a Novel Scaffold Biomaterial

Project 4.4.1a, RCCC

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

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

Collaborator(s): Ralph Carmichael JD, Daniel Pollmann JD, Erengul Carmichael, MBA, and Justin Hughes (BONWRx, Inc.)

Therapy: Treatment of peripheral nerve injuries

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

TRL Progress: Beginning, TRL 3; Current, TRL 5; AFIRM I Target, TRL 6

Key Accomplishments: The researchers validated a U.S. Food and Drug Administration (FDA)-approved sterilization method for their polycaprolactone fumarate (PCLF) conduits that involves the use of ethylene oxide (EtO) and was not associated with any cytotoxicity. They obtained Institutional Review Board (IRB) approval to initiate a feasibility clinical trial and follow 10 patients with sural nerve biopsy but without repair. They determined the final formulation and sterilization method for the PCLF tubes that will be used in the clinical trial. They drafted and submitted an initial Investigational Device Exemption (IDE) to the FDA, and completed several activities in support of a second IDE submission.

Key Words: peripheral nerve; conduit; biodegradable polymer; Good Manufacturing Practices; U.S. Food and Drug Administration; clinical trial

Introduction

Peripheral nerve lesions occur through a variety of injuries including battlefield injuries. The resulting disabilities greatly affect the quality of life of those involved. These injuries also have a significant socioeconomic impact. With an estimated 300,000 nerve injuries annually, the socioeconomic burden is great. Nerve injuries occurring in the battlefield are frequently more complicated and often involve loss of entire segments of major nerves spanning 5-20 cm. A limb salvaged without nerve function is useless and, in most cases, painful.

The current clinical gold standard of repairing nerve defects is autologous nerve graft, which has its limitations. Alternatives to nerve autografts are needed. A number of FDA-approved, commercially available nerve tubes can be used to aid repair of nerve defects ranging from 1.5-3.0 cm. These tubes have mainly shown effectiveness in repairing sensory nerves in clinical studies. Application of these nerve tubes in repairing motor nerves has been sporadic and inconclusive. The nerve tubes have shown little to no efficacy for the repair of longer defects in either civilian or military applications. Therefore, an immediate need is the development of

novel strategies that surpass the current commercially available nerve conduits for nerve repair for both military and civilian populations.

The Mayo team is developing novel biodegradable polymer nerve conduits that are suitable to repair nerve defects longer than 3 cm. They are testing these nerve conduits on a carefully defined clinical model: sural nerve biopsy that leaves a 6-cm nerve gap. Through a rigorous downselect process, they identified PCLF as a novel in-house biocompatible and biodegradable material. PCLF nerve conduits have been shown to support robust nerve regeneration in preclinical models when used to bridge a critical-size nerve defect.

Clinical Trial Status

A clinical trial to assess the feasibility of the study has been initiated to assess the technologies and measurement modalities that will be used in the proposed study. Scaffolds are not being inserted in these patients. This trial will continue (with Mayo funding), but the data will be used as preliminary data to support the submission of the IDE and IRB applications for the scaffold trial. More detailed information of work completed during the past year follows.

The Mayo team validated an FDA acceptable method, EtO, for the sterilization of PCLF nerve conduits. The PCLF conduits maintained stability and mechanical properties upon EtO treatment. The researchers determined that the EtO-treated PCLF tubes have the capacity to support nerve regeneration across a critical-size gap (1.5 cm) in a rat model. The researchers also confirmed the prototype of the mold for fabrication of human-size PCLF conduits, and fabricated the first batch of human-size PCLF conduits using routers to be used for Good Manufacturing Practices manufacturing. They drafted and filed an initial IDE with the FDA in May 2012.

The researchers obtained IRB approval on December 4, 2012, to initiate the separate feasibility clinical trial to enroll and follow 10 patients with sural nerve biopsy but without repair. They completed procedures, product drawings/specifications, and production documentation for PCLF conduits to be manufactured for clinical trial use. They performed additional studies and evaluations utilizing devices produced in compliance with production procedures. NAMSA and Centurion completed biocompatibility and sterilization validation studies on final product equivalent devices that were provided by the Mayo Clinic. The researchers also confirmed final design and specifications for device, packaging, and formulation.

The Mayo team completed several studies and reports in preparation to resubmit their IDE. Specifically, they (1) completed final signed

test reports for materials, methods, and data; (2) repeated studies with the current PCLF and EtO sterilization; (3) conducted mechanical testing, suture pull-out testing, functional testing, a mouse implantation study, residual testing, and shelf-life and aging tests; and (4) completed test reports with the data from the testing.

Most recently, the researchers provided nerve conduits for testing by third parties to support the second IDE submission. They completed final upscaling. They began to work on submitting a clinical protocol for IRB and Human Research Protection Office approval upon IDE approval. They began work on enrolling study subjects in the 10-patient feasibility trial, and they initiated a plan to complete the project with IDE submission and scaffold manufacture at the Mayo Clinic.

Future Clinical Plans

The Mayo team will complete the additional pre-clinical testing requested by the FDA in response to the prior IDE submission using AFIRM I funding. With support from the Office of Research Regulatory Support at Mayo Clinic, an investigator-initiated IDE will be filed with the FDA. IRB approval will then be obtained to conduct a Phase I clinical trial to assess the safety of PCLF conduits in patients with a 6 cm nerve defect resulting from a sural nerve biopsy. The Phase I clinical trial will be conducted using leveraged funding from the Mayo Clinic. Results of the clinical trial will be reported to the Department of Defense.



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Bone Regeneration

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

Project 4.1.2, WFPC

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Collaborator(s): Shanghai 9th People's Hospital, Shanghai, China; Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; Synthasome, Inc., San Diego, CA; Wake Forest Institute for Regenerative Medicine, Winston-Salem, NC; U.S. Army Institute of Surgical Research

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

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

TRL Progress: Beginning, TRL 2; Current, TRL 5; AFIRM I Target, TRL 5

Key Accomplishments: The researchers completed in vitro studies characterizing the physicochemical properties of porous Poly(methyl methacrylate) (PMMA)-based space maintainers as well as in vitro studies to fabricate and compare poly(lactic-co-glycolic) acid (PLGA) microparticles loaded with several common antibiotics with respect to morphology, entrapment efficiency, release kinetics, and activity against bacteria. Constructs demonstrated sustained release up to at least 28 days with retained antibiotic activity. They completed an in vivo study evaluating the efficacy of colistin-releasing PMMA-based space maintainers in mitigating an *Acinetobacter baumannii* infection in a rabbit composite tissue defect. They also initiated (independent of AFIRM funding) the investigation of an "in vivo bioreactor" approach for the generation of a vascularized bone flap for repair of mandibular defects in a sheep model.

Key Words: craniofacial bone reconstruction; space maintenance; bone flap; controlled drug delivery; in vivo bioreactor

Introduction

The overarching objective of this project is to optimize the results, decrease the complications and infections, and reduce the number of procedures associated with large bony reconstructions in the military and civilian patient populations through: (1) the initial implantation of a biocompatible, antibiotic-releasing space maintainer within a large osseous defect during the early phases of treatment, (2) the implantation of an "in vivo bioreactor" construct away from the site of injury that will allow for the generation of a vascularized bone flap to be used as donor tissue for second stage reconstructive surgeries, and (3) augmentation of the implanted

vascularized bone flap within the recipient defect site using an injectable system tailored for the delivery of growth factors needed to promote bone regeneration and wound healing until sufficient integration of the bone flap has occurred.

The overall strategy would involve the placement of an antibiotic-releasing space maintainer into a bone defect at the time of injury. Simultaneously, a bone chamber could be placed at a distal site on the body, generating a vascularised bone flap. After wound healing, the bone flap could be harvested from the chamber and placed into the defect upon removal of the space maintainer.

Research Progress – Year 5

Antibiotic-releasing porous PMMA-based space maintainers

In vitro characterization

A number of in vitro studies were completed or initiated in the past year to investigate and optimize the antibiotic-releasing technologies for use with the space-maintenance approach. The studies were conducted according to American Society for Testing and Materials and International Organization for Standardization standards as outlined by U.S. Food and Drug Administration (FDA) guidelines for PMMA-based bone cements. The researchers performed in vitro studies to evaluate the release of cefazolin, clindamycin, ciprofloxacin, colistin, doxycycline, vancomycin, and tobramycin from PLGA microspheres. These PLGA microspheres were incorporated into porous PMMA-based space maintainers and the release of antibiotics was characterized. Specifically, PMMA-based space maintainers were fabricated by hand in molds using a clinical-grade PMMA bone cement (SmartSet® HV; DePuy Orthopaedics, Warsaw, IN), comprising a powder of methylmethacrylate/methyl acrylate copolymer, benzoyl peroxide, and zirconium dioxide, and a liquid phase of methylmethacrylate, N,N-dimethyl-p-toluidine, and hydroquinone, to which a hydrogel of carboxymethylcellulose (Spectrum Chemical Manufacturing Corp., Gardena, CA) prepared at 9 weight percent in distilled water, and PLGA microspheres were added. Constructs demonstrated sustained release up to at least 28 days with retained antibiotic activity.

In vivo evaluation of porous-PMMA based space maintainers

The team evaluated colistin-releasing porous PMMA constructs in a rabbit mandibular composite tissue defect model inoculated with *Acinetobacter baumannii*. They evaluated gross soft tissue healing as well as histologic and safety measures. The porous PMMA-based space maintainers for this study were fabricated by hand in molds using a clinical-grade PMMA bone cement (SmartSet® HV), to which PLGA microspheres and/or a gelatin hydrogel (source of gelatin: SURGIFLO® Hemostatic Matrix; Ethicon, Somerville, NJ) were added. It was found that antibiotic delivered at a high dose over an extended period resulted in better

mucosal healing than defects receiving a burst dose of antibiotics. Healed and non-healed defects were observed histologically, as well as varying responses at the tissue-implant interface. Cultures of swabs taken from the defect, saliva, and blood did not grow *A. baumannii*. No rabbits in the study showed signs of nephrotoxicity, as assessed by blood urea nitrogen and creatinine levels, indicating that local delivery of colistin did not result in nephrotoxic levels of colistin systemically.

The researchers also completed an in vivo study evaluating the bone augmentation capacity of injectable hydrogels releasing BMP-2 in a rat cranial augmentation model (data not shown). In addition, they initiated an in vivo study aimed at evaluating the biocompatibility and efficacy of clindamycin- and tobramycin-loaded PLGA microspheres in a rat infected bone defect model using *S. aureus* and *Pseudomonas aeruginosa*.

Vascularized bone flap generation

In vivo generation of vascularized flap in a sheep model

The researchers collaborated with Wake Forest University, independent of AFIRM funding, in order to investigate the use of an “in vivo bioreactor”-generated vascularized flap for autologous transplantation into a sheep mandibular defect. Preliminary results indicate that a fibrovascular flap can be generated in approximation with the ribs of sheep and autologously transplanted into a defect in the angle of the mandible to regenerate bone (Figure 1).

Conclusions

Considerable progress was made over the course of the past year toward the development and clinical translation of porous PMMA-based space maintainers. In partnership with Synthasome, Inc., the research team initiated work toward developing the manufacturing process and manufacturing the product using a fully documented Good Manufacturing Practices (GMP)-compliant manufacturing process suitable for Investigational Device Exemption (IDE) submission and approval by the FDA, and interactions with the FDA were initiated. Additional progress was made toward the development of antibiotic-releasing implants for bony space maintenance in vitro, and the efficacy of the



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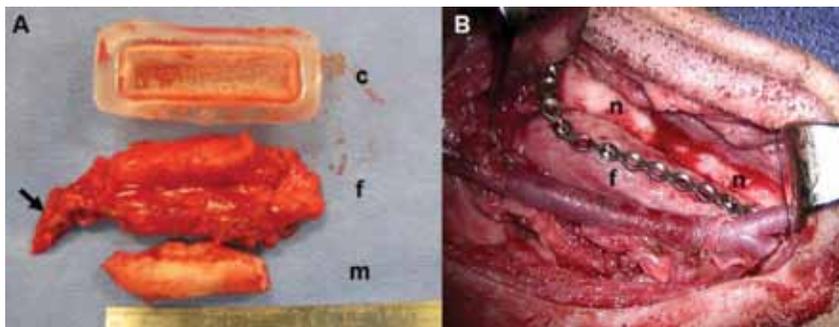


Figure 1. Representative images of the mandibular flap grown in an in vivo ovine bioreactor before (A) and after (B) implantation into an angular defect. In (A), the mandibular flap (f) is shown alongside the PMMA chamber (c) as well as the bone taken to create the mandibular angular defect (m). The arrow shows the harvested vascular pedicle, containing the intercostal artery and vein used for anastomosis into the defect site. The mandibular flap (f) is plated into the angular defect site against native bone (n), and anastomosis is made with the vessels of the neck.

approach was demonstrated in a preclinical infected rabbit mandibular defect model. The potential application of the antibiotic-releasing porous space maintainer technology was expanded to include a broad spectrum of clinically relevant antibiotics incorporated into degradable microsphere carriers for extended release. Based on the significant progress made through Years 1-5 toward the pre-clinical development of the porous PMMA-based space maintainer technology, the project stands well positioned for future determination of the safety and efficacy of the technology in a clinical study.

Future Research Plans

The research team will continue to work in partnership with Synthasome, Inc. toward the development of the GMP-compliant manufacturing process of the porous PMMA-based space maintainer technology, and ongoing interactions with the FDA will be maintained to facilitate regulatory consideration and clinical translation of the technology. Ongoing studies investigating the release of antibiotics from porous space maintainers will be continued in vitro and in preclinical animal models to facilitate timely

clinical translation of the technology. Moreover, studies will be continued toward the optimization of the “in vivo bioreactor” technology for vascularized bone flap generation.

Planned Clinical Transitions

Based upon preliminary interactions with the FDA during a pre-510(k) meeting held at the FDA in June 2012, the 510(k) mechanism is expected to be the appropriate regulatory pathway for the porous space maintainer technology, and the 510(k) application will be strengthened by a “proof of principle” clinical study, which may require an IDE. Accordingly, a protocol was submitted to and approved by the Institutional Review Board of the University of Texas Health Science Center at Houston to initiate a randomized, prospective clinical study of the porous PMMA-based space maintainer technology against clinical standards evaluating safety and efficacy. The study will commence once Human Research Protection Office approval of the protocol has been issued, which will depend upon either FDA clearance of the product or approval of an IDE.

Bone Regeneration

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Allograft Bone/Polymer Composites (4.5.1a) / Expedited Commercialization of an Injectable Bone/Allograft Composite for Open Fractures (4.5.7)

Project 4.5.1a/4.5.7, RCCC

Team Leader(s): Scott A. Guelcher, PhD (Vanderbilt University)

Project Team(s): Pamela Brown-Baer, DDS, Joseph C. Wenke, PhD, and COL Robert Hale, MD (U.S. Army Institute of Surgical Research (USAISR)); Kasia Zienkiewicz, MS and Anne Talley, BS (Vanderbilt University)

Collaborator(s): Kerem Kelvekci, PhD, Daniel Shimko, PhD, and Susan Drapeau, PhD (Medtronic)

Therapy: Bone graft therapy

Deliverable(s): Injectable, settable low viscosity (LV[®]) bone graft; injectable, settable LV bone graft augmented with recombinant human bone morphogenetic protein-2 (rhBMP-2)

TRL Progress: (4.5.1a) Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

(4.5.7) Beginning, TRL 3; Current, TRL 5; AFIRM I Target, TRL 7

Key Accomplishments: The researchers determined that LV grafts supported bone remodeling in a sheep femoral defect model and passed ISO10993 biocompatibility tests. They also found that delivery of rhBMP-2 from LV/MASTERGRAFT[®] formulations enhanced new bone formation in a rat critical-size calvarial defect model.

Key Words: injectable; settable; bone graft; rhBMP-2; rhGDF-5; rabbit

Introduction

Recent studies examining the battle injury patterns and resource impacts of injuries for Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) have highlighted the significance of injuries to the head and neck. The frequency of head and neck injuries has increased in the current conflicts in Iraq and Afghanistan in comparison to previous wars. Head and neck injuries account for 29.4% of all battle injuries sustained in OIF and OEF, required 20% of all treatment resources, had the highest mean disability rating (52%), and will command 27% of all total projected benefit costs. The high incidence of open fractures and the associated high complication rate result in a large patient population that requires improved treatments.

Vanderbilt researchers are pursuing two projects in collaboration with USAISR and Medtronic: (1) develop injectable, settable LV bone grafts for

repair of metaphyseal bone defects, and (2) develop injectable, settable LV bone grafts augmented with rhBMP-2 for regeneration of alveolar bone that supports dentition and maintains space as a compression-resistant matrix. Proof-of-concept experiments have shown that allograft/polyurethane (PUR) composite grafts regenerate new bone in rat and rabbit femoral plug defects, and in rabbit critical-size calvarial defects. Addition of rhBMP-2 as a powder prior to mixing enhances bone healing. Currently, there is no commercially available injectable load-bearing bone graft for delivery of recombinant growth factors such as rhBMP-2. LV augmented with growth factors is being developed to meet this compelling clinical need, which presents several advantages: (1) administration using minimally invasive surgical techniques, (2) mechanical strength approximating that of host mandibular bone to provide space maintenance and prevent



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soft tissue prolapse, and (3) sustained release of rhBMP-2, which is anticipated to lower the required dose. Considering that no product with all these attributes is currently available, LV augmented with growth factors is anticipated to be a disruptive technology that positively impacts the clinical management of open fractures in the face.

Research Progress – Year 5

Commercialization of injectable and settable LV bone grafts

Remodeling of LV injectable bone grafts in a sheep femoral plug defect model

The researchers injected LV grafts into 11 x 18 mm sheep femoral condyle plug defects which resulted in new bone formation and remodeling at four months (funded by Medtronic). Representative histological and microCT sections showed minimal inflammation as well as a ring of new bone that formed around the perimeter of the grafts for all treatment groups. Additional sheep will be evaluated at one- and two-year time points.

Current Good Manufacturing Practices (cGMP) manufacturing and International Organization for Standardization (ISO) 10993 testing (AFIRM supplement)

The primary technological hurdle faced by the program was the absence of a reliable supply of lysine triisocyanate. To overcome this hurdle, Medtronic contracted with a third-party manufacturer to establish a new synthetic route that would be cost-effective and could be performed safely at commercial scale. A fivefold reduction in anticipated raw material costs versus the baseline process was achieved. The other main constituent of the carrier is a degradable polyester polyol (ϵ -caprolactone-co-glycolide-co-DL-lactide). In November 2012, Medtronic contracted with a third-party manufacturer to develop a commercial scale process to manufacture the polyester polyol. Analyses of initial feasibility runs have shown close agreement with reference samples. Medtronic also completed Good Laboratory Practices biocompatibility testing according to ISO 10993 guidelines, which has demonstrated the safety of the final cured graft in a full biocompatibility panel. Remaining AFIRM funds have been budgeted for packaging development, sterilization method development, and stability testing.

Remodeling of flowable LV synthetic grafts augmented with rhBMP-2 in a critical-size rat calvarial defect model

Remodeling of injectable synthetic biocomposites incorporating rhBMP-2 in a rat calvarial defect

After reviewing the rhBMP-2 and rhGDF-5 preliminary data, Medtronic and the Vanderbilt team agreed that rhBMP-2 would likely perform better in the mandibular defects. Thus, Year 5 studies focused primarily on rhBMP-2. Two matrices were evaluated:

sucrose (S) and MasterGraft (MG, 85%:15% blend of tricalcium phosphate and hydroxyapatite). Scanning electron microscopy (SEM) images (Figure 1)

reveal pores resulting from gas blowing as well as matrix particles. Representative radiographs are presented in Figure 2 and suggest that bone

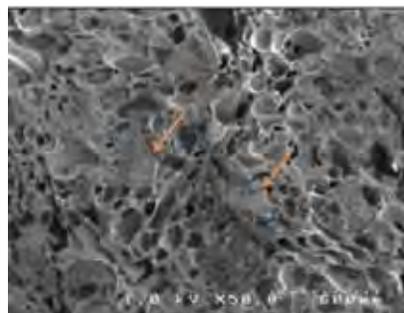
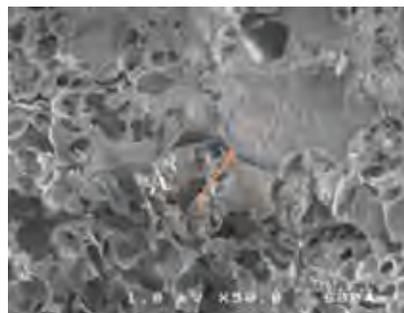


Figure 1. SEM images of PUR-MG (top) and PUR-S (bottom). Scale bar represents 600 μ m.

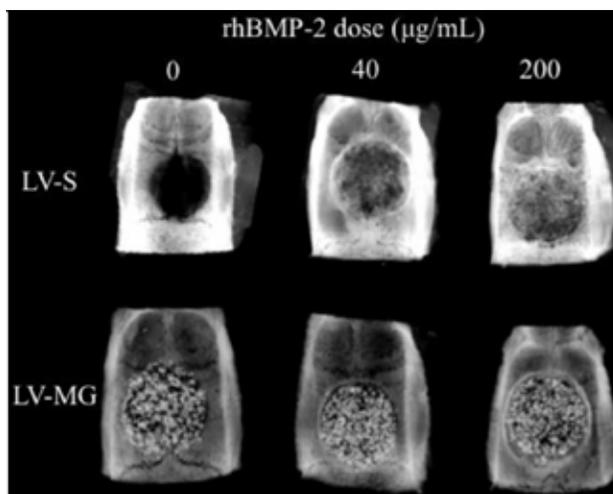


Figure 2. Representative radiographs of LV grafts augmented with rhBMP-2 at eight weeks.

healing is progressing from four to eight weeks in the presence of rhBMP-2. At eight weeks, histological sections stained with hematoxylin and eosin (H&E) revealed that most of the new bone formation in the LV-S+BMP graft is around the perimeter of the graft, while H&E sections of the LV-MG+BMP graft showed new bone formation throughout the volume of the defect. Thus, the LV-MG formulation will move forward to more extensive testing in larger animal models of craniofacial bone regeneration.

In vivo rhBMP-2 release kinetics (funded by Orthopaedic Extremity Trauma Research Program)

The researchers measured the release of rhBMP-2 in vivo by radiolabeling rhBMP-2 and injecting the biocomposites into 6x11 mm defects in bilateral rabbit femoral condyle defects. The in vitro and in vivo release kinetics showed that LV grafts supported diffusion-controlled release of rhBMP-2 for up to six weeks. The higher porosity of the LV-S grafts was anticipated to result in faster-release kinetics, but minimal differences in the rate of rhBMP-2 release were observed between the LV-S and LV-MG grafts. MicroCT images at eight weeks suggested that LV-MG+BMP supported more new bone formation than LV-S+BMP.

Conclusions

The researchers demonstrated that LV grafts remodel and heal when injected into sheep femoral condyle plug defects. cGMP raw materials used to prepare the grafts have been manufactured, and the supplier for the polyester polyol component has been identified. When augmented with recombinant human growth factors, LV grafts enhance new bone formation in a rat calvarial defect model. LV grafts augmented with rhBMP-2 will be evaluated in proof-of-concept canine and porcine models of mandibular bone regeneration with AFIRM I funding.

Future Research Plans

Injectable, settable LV bone grafts: Manufacture of cGMP prepolymer, polyester polyol, and catalyst solution will continue. Vanderbilt is working with Medtronic on characterizing the storage stability of each component and designing new approaches for improving the storage stability of the catalyst solution. Medtronic is planning to apply for additional supplemental funding to support completion of the commercialization activities as well as a clinical trial with the LV graft in patients with metaphyseal bone defects.

LV grafts augmented with rhBMP-2: Lead-candidate grafts identified in AFIRM I will be tested in pre-clinical models of ridge augmentation as a first step toward a clinical trial. Vanderbilt, USAISR, and Medtronic will also collaborate on preclinical testing of LV grafts augmented with rhBMP-2 in mandible continuity defects that are representative of injuries sustained during combat operations.

Planned Clinical Transitions

Injectable, settable LV bone grafts: The project team will contract internally with Medtronic management for expanded cross-functional support and additional capital investment. Tasks for the commercialization team will be submission of a 510(k) Premarket Notification for a bone void filler indication, execution of design transfers to contract manufacturers, process validations, and production of saleable material. Further upstream development of the LV technology will continue at Vanderbilt towards weight-bearing applications and as a carrier for growth factor and antibiotic delivery.

LV grafts augmented with rhBMP-2: LV augmented with rhBMP-2 will be evaluated in preclinical canine and nonhuman primate models of alveolar ridge augmentation. Pending 510(k) regulatory clearance of the LV bone graft and successful completion of the preclinical studies, preparation for a clinical trial in patients requiring ridge augmentation will begin.



Bone Regeneration

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Pre-Formed Tyrosine-Derived Polycarbonates

Project 4.5.1b, RCCC

Team Leader(s): Joachim Kohn, PhD and Ophir Ortiz, PhD (Rutgers University); Jeffrey Hollinger, DDS, PhD (Carnegie Mellon University)

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Collaborator(s): Amit Vasanji, PhD, Rick Rozik, and Brett Hoover, MBA, MS (ImageIQ, Inc.); Pam Brown-Baer, PhD (USAISR)

Therapy: Bone regenerative therapies for the cranio-maxillofacial (CMF) complex

Deliverable(s): Bioactive tyrosine-derived polycarbonate scaffolds for bone regeneration fabricated by salt leaching

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The Kohn team optimized the fabrication method for preparing scaffolds coated with calcium phosphate (CaP) for the rabbit

calvaria and radius defect models. The Hollinger team completed 60 percent of safety and efficacy studies of tyrosine-derived polycarbonates (TyrPC)+CaP scaffolds in the critical-size defect (CSD) rabbit calvaria and radius models. Analysis of both microCT and histology data generated from three preclinical rabbit studies confirmed the bone regenerating effects of CaP and bone morphogenetic protein-2 (BMP-2) in the CSD rabbit calvarial model. The researchers determined that treatment with ethylene oxide (EtO) is the most suitable sterilization method for TyrPC bone regeneration scaffolds. The researchers also developed and began analyzing a database containing information on microCT, histology, and histomorphometry analyses of samples from their calvaria and radius scaffold studies. They selected two TyrPC-based formulations for evaluation in a goat calvaria CSD model.

Key Words: bone regeneration; tyrosine-derived polycarbonates; biodegradable bioresorbable porous scaffold; osteoconductivity; cranio-maxillofacial; calcium phosphate coating

Introduction

The overall goal for the AFIRM Craniofacial Reconstruction projects is to produce therapeutics that will regenerate the complex bony anatomical zones of the cranio-maxillofacial (CMF) region that have been damaged and avulsed as a consequence of military trauma. This complex effort exploits versatile biomaterials and biological scaffold platforms. The approach to CMF therapy is being staged, proceeding from the least biomechanically challenging to the most challenging zones in the CMF. Consequently, the challenge of regenerating bone in the calvaria and upper face was taken on first by the Kohn/Hollinger team in Project 4.5.1b. The project's multi-year research plan depended on standardized animal models, progressing from the rabbit to the pivotal pre-clinical model, the goat.

The polymer platform focuses on TyrPC. The porogen-leached TyrPC scaffolds have shown a remarkable ability to regenerate bone in the rabbit CSD calvarial model in preliminary studies performed at Carnegie Mellon University (CMU), U.S. Army Institute of Surgical Research (USAISR), and Rutgers University during Years 2-4. Building on these results, the research team's Year 5 activities focused on completing the analysis of animal work performed in Year 4 and selecting a scaffold formulation to move into a large animal model in the upcoming year. The specific aims of Year 5 were to:

1. Complete the analysis of microCT and histological data for three rabbit calvaria pre-clinical studies performed at CMU and USAISR.
2. Assess the effects of sterilization with EtO and gamma irradiation on scaffold properties.

3. Using pre-clinical data collected in Years 1-5 as a guide, define the top two scaffold formulations with which to evaluate in the upcoming goat study.

Research Progress – Year 5

Complete the analysis of microCT and histological data for three rabbit calvaria pre-clinical studies performed at CMU and USAISR

Evaluation of TyrPC scaffolds containing CaP minerals in the rabbit calvaria CSD model (S3 data)

The researchers performed surgeries for study S3 from December 2010 through January 2011 at Allegheny General Hospital (AGH). Since the data collection was not completed until the beginning of Year 5, results for the S3 study are being reported now. Details of the surgeries can be found in the 2012 AFIRM Annual Report. This study ranged from 2 to 12 weeks, and evaluated three treatment groups: TyrPC+CaP (precipitated CaP coating), TyrPC+BMM (bone mineral mix, a precipitated CaP coating containing magnesium, zinc, and fluoride) and ChronOS (bone graft substitute [BGS]). MicroCT analysis was performed at ImageIQ, and histology/histomorphometry was conducted by the Hollinger Group at CMU. The key result was that the TyrPC+CaP had the highest average amount of bone regeneration as compared to TyrPC+BMM and commercially available ChronOS.

Evaluation of TyrPC scaffolds containing both CaP minerals and bone marrow aspirate (BMA) in the rabbit calvaria CSD model (S9 data)

Surgeries for study S9 were performed during AFIRM Year 4 at AGH, and the data collection was

completed during Year 5. Treatment groups investigated were TyrPC, TyrPC+CaP+BMA (BMA), TyrPC+CaP+cBMA (concentrated bone marrow aspirate), TyrPC+CaP composite, and Vitoss as the predicate device. The research team determined that the treatment group TyrPC+CaP+BMA had the highest average bone regeneration as compared to the other treatment groups, including commercially available Vitoss.

Evaluation of TyrPC scaffolds containing both CaP minerals and bone morphogenetic protein-2 (BMP-2) in the rabbit calvaria CSD model (S15 data)

Surgeries for study S15 were performed at the USAISR. MicroCT was performed at ImageIQ, and histology was performed at CMU by the Hollinger team. The complete set of histology images was obtained in Year 5. This study evaluated the effects of increasing the dose of BMP-2 per scaffold, (0, 10, 25, or 50 µg). The key result was that a dose of 50 µg per scaffold produced the highest amount of bone regeneration.

Sterilization study of composite scaffolds: EtO vs. gamma irradiation

The effects of sterilization on TyrPC scaffolds, which included scaffolds with either betaTCP (TyrPC+CP1) or OCaP (TyrPC+CP2) were evaluated, and data are summarized in **Table 1**. Analytical techniques used for this assessment were:

- Gel permeation chromatography (GPC)
- Nuclear magnetic resonance (NMR)
- Scanning electron microscopy (SEM)
- X-ray diffraction (XRD)

Table 1. Effect of sterilization with EtO gas or gamma irradiation on the properties of TyrPC scaffolds containing betaTCP (TyrPC+CP1) or OCaP (TyrPC+CP2).

Scaffold ID	GPC: MW retention after EtO	GPC: MW retention after gamma (25kGy)	GPC: MW retention after gamma (40kGy)	NMR: Change in chemical composition?	SEM: Change in architecture?	XRD: Change in crystallinity?
TyrPC+CP1	97%	82%	76%	No	No	No
TyrPC+CP2	93%	79%	72%	No	No	No



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GPC results showed that all three sterilization methods affected molecular weight (MW) retention. EtO produced the highest MW retention for both types of scaffolds tested. NMR analyses demonstrated that there were no chemical transformations for any combination of scaffold and sterilization technique. SEM analyses demonstrated that the overall macro structure of the scaffolds remained the same after the three exposure methods used in this study. XRD analyses demonstrated that there was no detrimental effect due to exposure to the sterilization processes on the crystallinity of both scaffolds.

The only parameter that was affected by sterilization was MW. NMR, SEM, and XRD showed that the other measured parameters, as described above, remained the same before and after sterilization. Therefore, because EtO allowed for the highest MW retention, it should be considered as the most suitable sterilization method for TyrPC scaffolds.

Selection of scaffold formulations for goat study using analysis of pre-clinical data

The researchers combined and analyzed 6-week post-surgery data collected from the preclinical studies using the rabbit CSD calvaria model performed in Years 1-5. Treatment with scaffolds made of E1001(1K), E1001(1K) with a CaP coating (E1001(1K)+CaP), E1001(1K) containing octacalcium phosphate composite (E1001(1K)+OCaP), and three commercially available scaffolds (ChronOS, Mozaik, and Vitoss) were analyzed for their effect on bone regeneration based on data generated from

microCT and histology. Analysis of the microCT data (**Figure 1A**) suggests that defects treated with TyrPC+OCaP had the highest average new bone formation as compared to other TyrPC-containing formulations. Analysis of all groups (TyrPC-containing scaffolds vs. predicate devices ChronOS and Vitoss) suggests that Vitoss had significantly more bone formation as compared to all other formulations. However, analysis of the histomorphometry data did not confirm this trend (**Figure 1B**), where no statistical significance was detected among all groups (as determined by one way ANOVA followed by the Tukey post hoc).

A second analysis of the data was performed by the team. In this case, data for the three best performers in each treatment group and the controls were plotted and analyzed (**Figure 2**). This analysis (based on microCT and histology data) showed that defects treated with TyrPC-based scaffolds had more bone formation than most of the predicate devices. MicroCT showed defects treated with TyrPC+OCaP produced the most new bone formation, while histomorphometry showed that defects treated with TyrPC+CaP produced the most new bone formation. These two groups (TyrPC+CaP and TyrPC+OCaP) will be further studied in the upcoming goat study (S16, 20 goats, 16 weeks survival).

Database development and analysis

In Year 5, the Kohn laboratory developed a database containing information about microCT, histology, and histomorphometry analyses of all rabbit

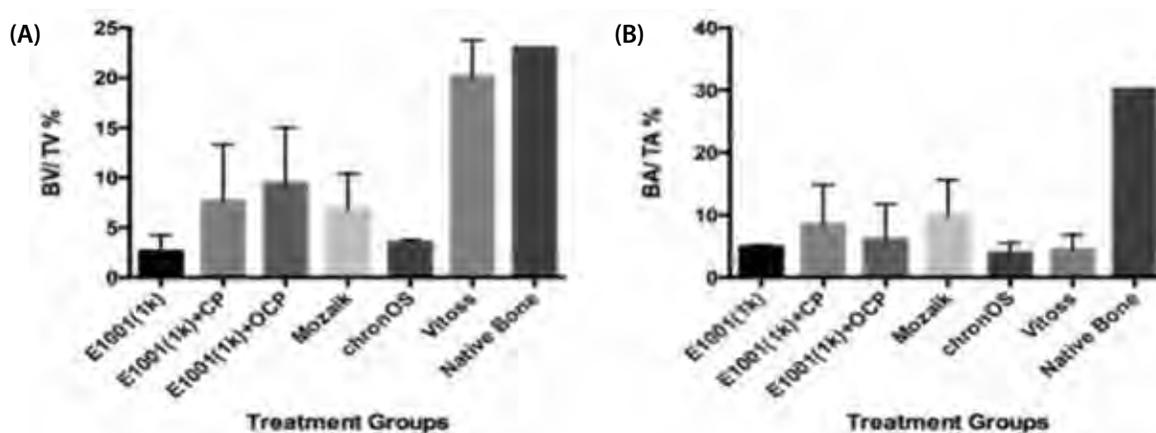


Figure 1. Cumulative six-week rabbit calvaria data of (A) microCT and (B) histomorphometry. MicroCT data show that the formulation with the highest average bone formation was Vitoss. However, histomorphometry shows that the formulation with the highest average was Mozaik, another predicate device.

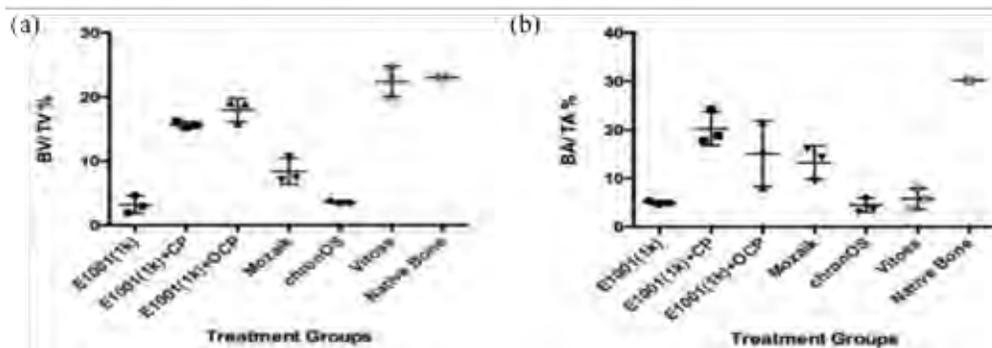


Figure 2. Cumulative 6-week rabbit calvaria data of (a) microCT and (b) histomorphometry. Plots show the three best performing samples from each treatment group, the three predicate devices, and the native bone control.

calvaria and radius scaffold studies performed during AFIRM I. The team also completed the analysis of the database, which showed that: (1) the surgical handling properties of TyrPC-based scaffolds were much better than commercially available BGS, which were friable and particulate; (2) the in vivo biocompatibility, biodegradability and osteoconductivity of TyrPC-based scaffolds were similar to commercially available BGS; and (3) the incorporation of a minimal dose of growth factor into TyrPC-based scaffolds significantly increased new bone formation. The database also helped in the identification of the best performing TyrPC-based scaffolds.

Conclusions

TyrPC-based scaffolds were sterilized using two methods that are standard in the medical device industry: EtO and gamma irradiation. NMR, GPC, SEM, and XRD analysis showed that EtO can be used to treat TyrPC-based scaffolds with the caveat that the temperature and humidity must be within a certain range.

Analysis of all microCT, histology, and histomorphometry data generated from rabbit calvaria CSD studies performed in Years 1-5 showed that:

- The surgical handling properties of TyrPC-based materials were much better than the BGS Mozaik or ChronOS, which were friable and particulate.
- The biocompatibility, biodegradability and osteoconductivity of TyrPC-based scaffolds were similar to commercially available devices in the rabbit calvaria and radius CSD models.

- The incorporation of a minimal dose of rhBMP-2 (25 μ g/scaffold) into TyrPC+CaP significantly increased new bone formation as compared to TyrPC+CaP alone, illustrating the effectiveness of adding bioactive factors into synthetic scaffolds.
- The best performing TyrPC-based scaffolds in the rabbit calvaria CSD model were: TyrPC+CaP and TyrPC+OCaP.

This project is linked to RCCC Project 4.2.1a (Chapter II) where various materials containing CaP have been formulated together with TyrPC, and the resulting scaffolds downselected to the most osteoconductive materials. In addition, this project is linked to RCCC Project 4.5.8 (later in this chapter) where methods for reproducible manufacturing of TyrPC bone regenerations scaffolds are being developed. In conjunction with Year 5 results from these two partnering projects, Project 4.5.1b is ready to implement the pivotal goat calvarial model going forward.

Future Research Plans

The researchers have obtained Institutional Animal Care and Use Committee approval for the evaluation of TyrPC-containing bone regeneration scaffolds in the goat CSD calvaria model. In the upcoming year, they will evaluate the two best performing TyrPC-based scaffolds (TyrPC+CaP and TyrPC+OCaP) in the goat model. The surgeries will be conducted at the Allegheny Singer Research Institute, and microCT and histology will be used to assess new bone formation 16 weeks post-surgery.



Bone Regeneration

Vascular Tissue Engineering

Project 4.5.6, RCCC

Team Leader(s): Daniel G. Anderson, PhD and Robert S. Langer, ScD (Massachusetts Institute of Technology [MIT])

Project Team(s): Omar F. Khan, PhD and Edmond Zaia, BS (MIT)

Collaborator(s): Michael Longaker, PhD (Stanford University)

Therapy: Vascular tissue engineering treatments using bioactive scaffolds

Deliverable(s): A polymeric scaffold that induces the growth of new blood vessels (angiogenesis) and recruits cells that stabilize blood vessels (pericytes) to quicken and enhance wound healing.

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The MIT group has demonstrated the ability to graft growth factors onto the surface of a poly(ether sulfone) (PES) vascularizing membrane. They have also demonstrated in vivo the ability to increase angiogenesis and decrease fibrosis by changing the surface geometry of the vascularizing membrane. As an alternative to protein and growth factor use, they incorporated new potentially therapeutic surface chemistries onto the membranes by grafting additional polymers to the PES surface via synthetic organic chemistry (free radical polymerization).

Key Words: vascularization; fibrosis; injury; necrosis; regeneration; immune system

Introduction

For injured military personnel and civilians, treatment of large tissue wounds requires large scaffolds, and the long-term engraftment of the engineered tissues will be highly dependent on interconnection with the surrounding host tissue to form a vascular network that is capable of blood perfusion. The MIT group's strategy is to create (A) a microenvironment that releases synthetic growth factor, or (B) a bioactive pro-angiogenic microenvironment within the scaffold system to generate a vascular network around and within the engineered tissues.

Research Progress – Year 3 (Project commenced in 2010)

Poly(ether sulfone) membrane modification protocol

PES is a material that is amenable to surface modifications and can be microfabricated into a variety of conformations suitable for biomedical applications. The research team developed and optimized protocols to modify the surface of PES to incorporate bioactive molecules (such as growth factors) or pendant polymer side chains that incorporate therapeutic functional groups.

Growth factor-modified PES membranes

To attach bioactive growth factors to the surface of the PES material, the researchers studied several intermediary linking polymers; alkyne-based linkers proved the most successful. **Figure 1** shows the modest changes to the overall topography of the PES implants after modification.

Enhancing the vascularization of PES implants by grafting new chemical groups to the implant's surface via the free radical polymerization of vinyl monomers

To complement the use of growth factors on the surface of the PES materials, the researchers also developed protocols to grow polymeric chains along the surface of the material from their vinyl monomers. These monomers polymerize to form pendent polymer chains that decorate the bulk surface and partially decorate the inside of the pores (**Figure 2**).

Surface geometry of the implant and influence on its ability to promote growth of new blood vessels (angiogenesis) and implant tolerance

The research team subcutaneously implanted PES implants with various surface geometries into normal rats for 30 days, and then examined the implants for the development of new blood vessels

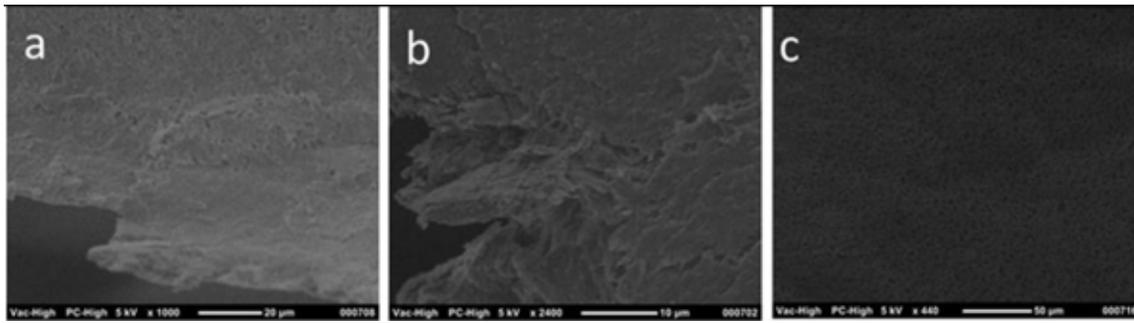


Figure 1. Scanning electron micrographs of PES polymers at various stages of treatment. (a) Unmodified PES. (b) PES with intermediate linker polymer. (c) PES with vascular endothelial growth factor (VEGF) grafted to the surface via the intermediate linker polymer. Surface treatment did not appear to drastically alter the porosity or morphology of the PES membranes.

(i.e., mature endothelial cells that express the Tie2 protein) and fibrosis (the isolation and encapsulation of implants in scar-like tissue caused by the body's assumption that the implant is dangerous). As illustrated in **Figures 3 and 4**, the researchers discovered that varying the surface geometry (in the absence of other chemical modifications) was sufficient to induce changes in angiogenesis and fibrosis.

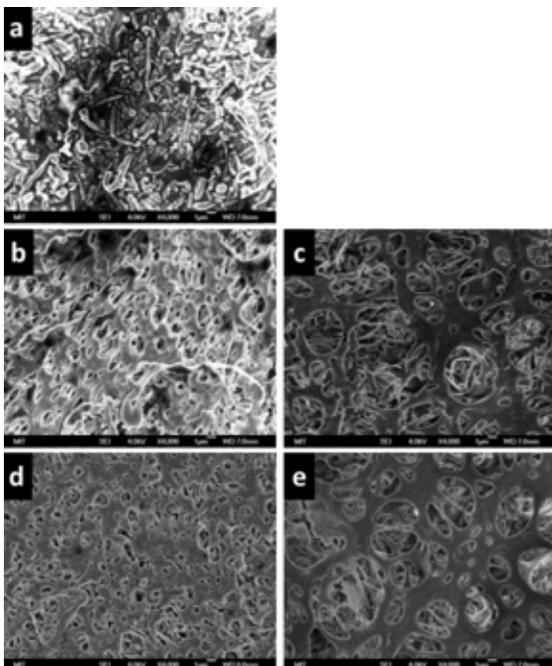


Figure 2. Scanning electron micrographs of PES polymers with different "grafting to" polymer side chains. (a) Unmodified PES. (b) Vinyl monomer 1. (c) Vinyl monomer 2. (d) Vinyl monomer 3. (e) Vinyl monomer 4. Vinyl monomers, when polymerized to the PES surface, appeared to reduce the roughness of the implant surface.

Conclusions

The MIT group identified the need to create mature, stable blood vessels, an outcome that is not necessarily achieved by immobilizing VEGF alone on a biomaterial surface. Maturity and longevity of the newly formed vasculature around the PES implant is generated by the inclusion of pericytes, a cell type that wraps around the outer diameter of the blood vessels. To recruit both pericytes and endothelial cells, the researchers identified and selected

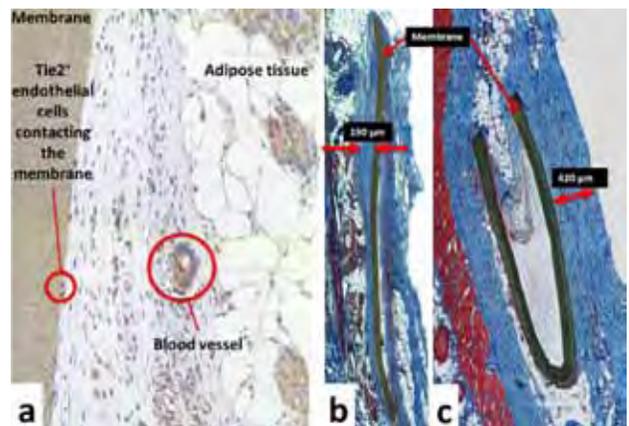


Figure 3. PES membrane implanted subcutaneously in a normal rat after 30 days. (a) Mature endothelial cells are identified by their brown Tie2 staining. Cell nuclei appear blue. Similar to the stable blood vessel circled in the image, Tie2-expressing endothelial cells are found at the tissue-implant interface. (b) Masson's trichrome stain of Geometry 1 PES implants and (c) Masson's trichrome stain of Geometry 7 PES implants. Both (b) and (c) illustrate the differences in implant fibrosis. The thickness of the fibrotic capsule was markedly reduced with Geometry 1 (190 μm vs 420 μm).



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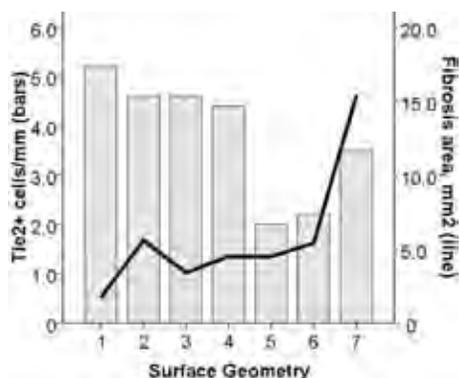


Figure 4. The PES membrane geometry affects angiogenesis and fibrosis. The chart shows quantification of the angiogenic and fibrotic response to subcutaneous PES implants of various geometries after 30 days in vivo in normal rats. The MIT team conducted experiments in duplicate. The grey bars show the number of Tie2-expressing endothelial cells (i.e., mature and stable cells) that were present per unit length of the implant's perimeter. The black line measures the area of the fibrotic capsule from a cross-section of the implant.

pro-angiogenic and anti-inflammatory growth factors for screening. They also selected PES as the next iteration of the polymeric biomaterial to use to initiate angiogenesis and modulate the immune response upon implantation. The research team demonstrated that growth factors can be immobilized onto PES and that the surface chemistry of the PES implants can be further modified by grafting new polymers to its surface via synthetic organic chemistry. They successfully established an effective grafting protocol to produce a combinatorial

library of PES-based biopolymers with a variety of chemical and growth factor surface modifications. The team demonstrated that surface geometry can also be used to tune angiogenesis and modulate the immune response, including implant fibrosis.

Future Research Plans

The modified PES materials exhibited the ability to increase angiogenesis and reduce fibrosis. These properties make the material amenable for immunomodulation applications such as cell encapsulation and transplantation. As a proof-of-concept, the material will be used to encapsulate islets to treat a diabetic mouse. Because the membrane induces angiogenesis, blood vessels will be formed on the membrane surface, and diffusion from the blood vessels across the membrane into the PES capsule's interior will sustain the transplanted cells. This protocol will reduce transplant necrosis. Similarly, insulin produced by the encapsulated cells will be able to diffuse into the adjacent blood vessels. The measurable outcome of these experiments will be the reduction in blood glucose levels after a glucose challenge.

Planned Clinical Transitions

After demonstrating that transplanted islet cells in the MIT team's PES membranes can replace the function of diseased islets in the mouse model of diabetes, results will be confirmed in rats and non-human primates. Following the success of non-human primate trials, clinical trials will be scheduled.



Bone Regeneration

Accelerating the Development of Bone Regeneration Scaffolds Based on Tyrosine-Derived Polycarbonate

Project 4.5.8, RCCC

Team Leader(s): Carmine P. Iovine, PhD and Joachim Kohn, PhD (Rutgers University)

Project Team(s): Ophir Ortiz, PhD, Ganesan Subramanian, MS, Shuang Chen, BS, (Rutgers University); Howard Schroyer, BA (Independent Contractor, Manufacturing Process Advisor)

Collaborator(s): None

Therapy: Small bone fracture repair process

Deliverable(s): Transfer a report for use by external scaffold fabrication vendors that defines a scalable and validated laboratory process for the manufacture of first generation bone regeneration scaffold for human use.

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers scaled up the tyrosine-based polycarbonate polymerization process to the kilogram (kg) scale and initiated the production of 3 kg of polymer at a third-party manufacturer. They standardized the scaffold characterization techniques and established preliminary specifications. They also standardized the bone pin injection molding process, and had more than 200 bone pins injection-molded by a third-party fabricator. The pins were submitted for evaluation by an external venue. The researchers also completed the laboratory scale-up and process validation of the base (pre-calcium phosphate coated) E1001(1K)CTL bone regeneration scaffold.

Key Words: bone regeneration; tyrosine-derived polycarbonates; fracture fixation

Introduction

There is a clinical need to improve orthopedic devices that are used to repair and regenerate bone, specifically by making bone grafts osteoconductive (i.e., promoting the attachment and growth of bone at the bone-implant interface). The development of an osteoconductive polymer, one that is based on tyrosine-derived polycarbonates, which are both osteoconductive and tissue integrative, would significantly improve the clinical performance of degradable orthopedic devices. Currently, two types of orthopedic products that may help the wounded warfighter are: (1) bone regeneration scaffolds for maxillofacial reconstruction and (2) bone regeneration scaffolds for treating defects in long bones. Two products that serve these applications, based on a tyrosine-derived polymer, are moving toward commercialization within the AFIRM. A resorbable bone pin based on a tyrosine-derived polycarbonate was developed as an intermediate step toward the overall goal of regulatory approval for a bone regeneration scaffold containing calcium phosphate (CaP) that is

based on the same tyrosine-derived polycarbonate. The bone regeneration scaffold offers an improved treatment option for both military and civilian populations. The current objective of this project is to develop a scalable process suitable for transfer to a commercial manufacturing site that is able to produce bone regeneration scaffolds for human use.

Research Progress – Year 3 (Project commenced in 2010)

Monomer and polymer material synthesis

Approximately 4 kg of tyrosine-derived monomers was produced at Rutgers University. A third-party manufacturer will use this monomer to produce ~3 kg of polymer from three separate 1 kg scale-up production runs. To date, the first of the three batches has been completed. The polymer produced will be used to support both bone pin and scaffold product development for this project, as well as for the other AFIRM bone regeneration projects (Limb and Digit Salvage Projects 4.2.1 and 4.2.3, and Craniofacial Reconstruction Project 4.5.1b).



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Bone regeneration scaffold fabrication

The research team scaled up the laboratory processes for the production of a CaP-containing tyrosine-derived polycarbonate-based regenerative bone scaffold. In this process, designated as the coating process, a base scaffold is first fabricated using a patented porogen leaching technique, and this material is then coated in situ with the CaP mineral DCPD (di-calcium phosphate di-hydrate). All process steps associated with the base (pre-coated) scaffold were scaled-up and validated. The relationship of polymer composition/properties, molding formulation, freezing rate/temperature and porogen leaching to the reproducibility of the required pore architecture were defined and codified through specifications and standard operating procedures (SOPs) for the intermediate and final products.

Bone pin fabrication

A third-party fabricator successfully injection molded a large quantity of bone pins from the same tyrosine-derived polycarbonate that is used in the bone regeneration scaffold process. Multiple lots of the bio-absorbable polymer were utilized. A mold lubricant processing aide was used at 0.5%-1.5%. Injection molding parameters were optimized during preliminary trials and 234 pins were successfully made from a 90 g of batch of polymer (each pin weighs about 200 mg). This run of bone pins, using the documented process, yielded a very consistent result from a manufacturing standpoint and a large quantity of useable parts. The mechanical properties of the bone pins produced by the optimized injection molding process are currently being evaluated by a third-party laboratory.

Conclusions

An external manufacturer has completed polymer synthesis of the first of three batches at the 1 kg scale. This ~3 kg of tyrosine-derived polycarbonate will be used for the manufacture of bone pins or bone regeneration scaffolds. The Rutgers research team is preparing to engage in Good Manufacturing Practices (GMP) certification in the near future as necessary. The process for the injection molding of the tyrosine-derived polycarbonate required to produce small bone fixation pins has been fully optimized at a third-party GMP fabricator. In addition, over 200 bone pins were successfully produced using this optimized process. The laboratory study and scale-up of the bone regeneration scaffold process based on tyrosine-derived polycarbonate is nearing completion, with all steps prior to coating with CaP completed. The researchers optimized the conditions for reproducible scaffold production to allow use of the entire molecular weight specification range of the polycarbonate. They standardized measurement techniques and established preliminary manufacturing specifications and SOPs for the base scaffold. Completion of the process studies in the next quarter will lead to the development of the scale-up transfer report to be used in discussions with potential third-party scaffold fabricators.

Future Research Plans

Future efforts will be focused on completing the process development study for the tyrosine-derived polycarbonate bone regeneration scaffold. Specifically, all the remaining process steps associated with the CaP coating as well as the sterilization and packaging of the final product will be investigated. A manufacturing transfer report will be generated, and this information will be used to engage third-party GMP scaffold fabricators in commercial discussions.

Bone Regeneration**Preclinical Animal Model Development for Bone Regeneration Studies****Project 4.5.1c, USAISR**

Team Leader(s): Pamela R. Brown Baer, DDS, COL Robert Hale, DDS (Dental and Trauma Research Detachment [DTRD], U.S. Army Institute of Surgical Research, [USAISR])

Project Team(s): COL Phillip DeNicolo, DMD MS, David T. Silliman, BS (USAISR)

Collaborator(s): Teja Guda, PhD (University of Texas at San Antonio).

Deliverable(s): Translational Animal Models for Evaluating Technologies for Clinical Mandible Reconstruction

TRL Progress: Beginning, TRL 3; Current, TRL 3/4; AFIRM I Target, TRL 5

Key Accomplishments: The researchers determined that the notch defect was not feasible as a critical-size defect model in both the rabbit mandible at 12 weeks and in the pig mandible at 16 weeks. They are now investigating a full thickness segmental defect of 2 cm in the pig mandible to develop a critical-size defect that does not heal within 20 weeks.

Key Words: Animal models, mandibular defects, bone regeneration, clinical modeling, critical-size

Introduction

Craniofacial battle injuries caused by explosive devices are characterized as open wounds and comminuted fractures. In combat casualties, the majority of the facial fractures are to the mandible. Currently, no satisfactory treatments are available to aid in reconstructing the face ravaged by explosive devices to an acceptable level, much less to natural form and function. The frequency and complexity of these injuries requires novel approaches to improve the outcome for mandibular battle wounds.

Though autologous bone grafts are considered the standard of care, novel biomaterials for bone regeneration are being developed to address the clinical challenges by combining the structural strength of biocompatible, biodegradable scaffolds with the ability to deliver growth factors and/or stem cells. Prior to clinically using these materials to restore severe mandibular battle injuries, these materials must be evaluated in clinically relevant and progressively challenging animal models. The purpose of the research was to select and validate a small and large animal model to be used to screen regenerative bone technologies prior to clinical trials.

Research Progress – Year 5**Small Preclinical Animal Model, Rabbit Mandible Notch Model**

It has been previously reported that a defect as small as 12 mm x 5 mm (three-wall) defect in the body of the rabbit mandible is critical sized over 12 weeks. An osseous defect of that size was created in the inferior border of the rabbit mandible (volume 300 mm³) and the edges of the defect were cauterized to further discourage bone regeneration and make the bone regeneration model more stringent. Six rabbits were euthanized at 6 weeks post-surgery with no cautery of the defect edges, while ten rabbits each were euthanized at 6 and 12 weeks post-surgery, with cauterization of the defect edges. The regenerated bone volumes were evaluated post-euthanasia using micro-computed tomography (micro-CT), and bone healing quality was confirmed by histological analysis.

Micro-CT analysis: Significant bone healing response

In terms of regenerated bone volume, micro-CT analysis showed that significantly less bone was regenerated at 6 and 12 weeks in the surgical sites with cautery as compared to their respective



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contralateral controls. Without cautery at 6 weeks, no difference in bone volume regenerated within the defect was detected between the surgical mandibles and their contralateral controls (**Figure 1A**).

Histomorphometric analysis: Bone formation in teeth canals and inflammation

While the histomorphometry data showed similar trends to that of the micro-CT, no significant differences between surgical groups were found (**Figure 1B**). Further examination of the histological slides revealed localized inflammation present at the interface of the apex of teeth and the superior defect border in groups when cauterization was used, at both 6 and 12 weeks (**Figure 1C**). No inflammation was seen in the non-cautery groups (**Figure 1D**). Also noted in the histology was the presence of bone growth into the root canals of the cut teeth (**Figure 1D**). Strong trends were observed that bone formation occurred into the teeth canals without cauterization, while cauterization increased inflammation and impacted the quality of healing.

A robust bone healing response was observed in the 12 x 5 mm defect size at 6 weeks. Even after inhibition of bone growth by cauterization, the regenerated bone increased from 6 to 12 weeks, reaching a level similar to non-cauterized bone at 6 weeks. Cauterization of the defect margins resulted in a delay of bone growth and increased inflammation.

The bone growing into the exposed root canals may indicate an aggressive osteogenic response from periodontal and dental stem cells due to the alteration of the local micro environment as a result of creating a non-segmental defect without teeth extraction. Contrary to multiple literature reports, this study showed the 12 x 5 mm notch defect to not be critical-sized in the rabbit mandible at 12 weeks, even with cauterization of the bone margins.

Large Preclinical Animal Model, Pig Mandible Notch Model

Anatomical investigation of pigs and goats revealed that the pig was the superior animal for mandibular regenerative studies with regard to similarity to human physiology. Though pigs have been used for these studies, a standardized experimental investigation of a mandibular defect is lacking. It was hypothesized that a mandibular defect with a volume greater than 5 cm³ with bordering periosteal stripping would not heal without intervention, creating a challenging pre-clinical large animal bone regenerative model similar to mandibular traumatic injuries observed in a military population. Subtotal osseous mandible defects were created bilaterally in five dentally mature pigs to evaluate post-operative complications, jaw stability, and the surgical site's healing potential with and without the clinical gold standard, autologous bone graft. Evaluation with

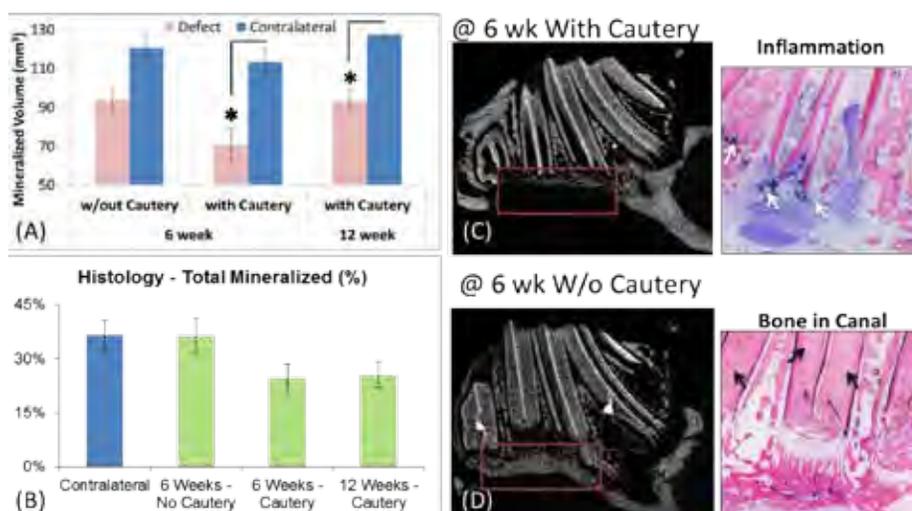


Figure 1. (A) Regenerated tissue volume in surgical defect compared to contralateral hemi-mandible from micro-CT analysis; **(B)** bone-to-tissue volume ratio in each group from histo-morphometric analysis; **(C)** representative micro-CT section in group with bone margin cauterization at 6 weeks, and histology showing signs of inflammation (white arrows); **(D)** micro-CT cross section from 6-week group without cautery, and histology inset from same group showing signs of bone growth into teeth canals (black arrows). Similar observations were made at 12 weeks.

computed tomography (CT) imaging was completed pre-surgery (0 week) and at 4 and 16 weeks post-surgery, with post euthanasia mandible harvest and micro-CT and histological assessment after survival for 16 weeks.

Micro-CT analysis: Significant bone healing response

Techniques were devised to ensure the correct region of interest was defined for analysis allowing accurate, consistent, and reproducible quantification of bone regeneration via clinical CT, micro-CT, and histology (**Figure 2A, B**). The micro-CT scans illustrate significant bone regeneration occurred in both treated and untreated defects at 16 weeks. The defects treated with the autologous bone showed significantly higher bone regeneration volume when compared to the untreated defects after 16 weeks (Figure 2C).

Reconstructions and measurements of regenerated bone volume normalized to pre-operative bone volume from 4-week and 16-week clinical CT scans indicate that all animals showed significant autograft resorption and minimal bone regeneration at 4 weeks. However, all animals also showed recovery and significant bone regeneration after 16 weeks (Figure 2D) in both the defect and autograft groups. Additionally, after 16 weeks, the defect treated with

the autologous bone graft showed a significantly higher relative regenerated bone volume when compared to the untreated defect (104% vs. 77%), corroborating micro-CT results. Although persistent sinus tract infections were observed in two pigs, this did not appear to decrease or significantly affect the bone healing response at the defect sites.

Histomorphometric analysis: Bone formation confirmed

Histological sections were prepared on undecalcified pig mandibles, and viable connected bone formation was observed in both the empty defect group as well as in the defects treated with autologous bone grafts. This was supported by visible osteoblasts and osteocytes, extensive mineralizing osteoid as well as evidence of rudimentary osteons observed in the samples at 16 weeks (**Figure 3**).

Despite the fact that the defects were larger in volume ($\approx 6 \text{ cm}^3$) than defects previously reported as critical-size defect (5 cm^3), substantial bone healing is observed at the 16-week period, even without any treatment. A systematic way of defining the defect at the 16-week time point was conducted using 64-slice clinical CT, micro-CT and histological analysis. Quantification of healing using CT and micro-CT scans allowed the group to determine that while both the empty defect and autograft

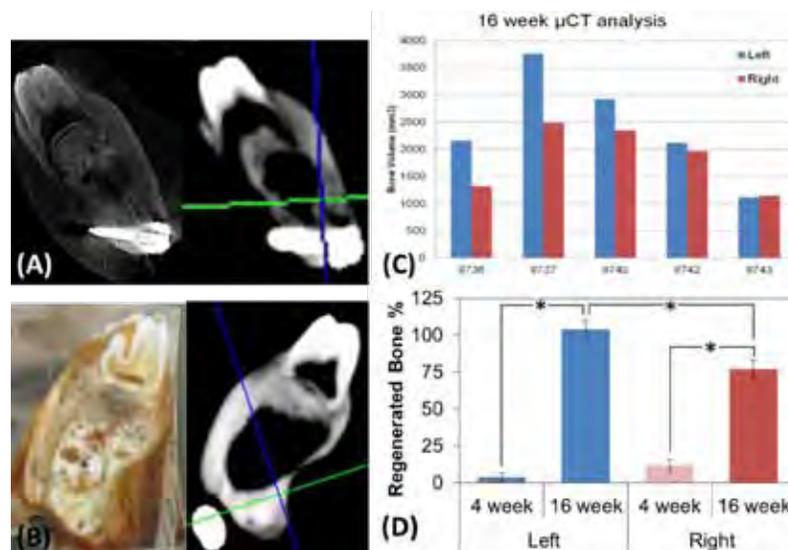


Figure 2. (A) Correspondence of features by appropriate orientation the same animal using micro-CT and clinical CT (B) Similar correlation between clinical CT and histological sections (C) micro-CT quantification of regenerated bone volume at 16 weeks in the left mandible (implanted with autologous graft) and right mandible (untreated defect site) of each of the pigs. (D) Quantity of bone volume regenerated at week 4 and week 16 post-op compared to initial bone volume in the defect at week 0 pre-op. (Significant differences at $p < 0.05$ indicated by *)



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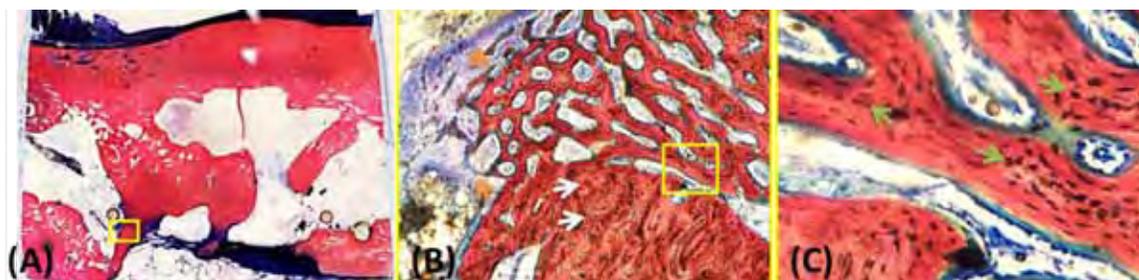


Figure 3. (A) Histological section of an untreated defect at 16 weeks stained with Sandersons rapid bone stain and counter-stained with Van Geison (yellow insets shown at higher magnification); (B) white arrows indicate rudimentary osteons and orange arrows indicate mineralizing osteoid and organized collagen; (C) green arrows indicate osteocytes.

groups showed little to no bone in the defect space at 4 weeks, a robust healing response was observed by 16 weeks post-op when the total bone regenerated in both autograft treated and untreated defects was similar to the bone volume before defect creation. Histological findings indicated viable bone formation, supported by visible osteoblasts and osteocytes, extensive mineralizing osteoid as well as evidence of rudimentary osteons. *These results indicate that the notch model cannot be recommended as a critical-size defect large animal model.*

Large Preclinical Animal Model, Pig Mandible Segmental Model

With the notch model having been demonstrated as not being critical-sized in both the rabbit mandible at 12 weeks and in the pig mandible at 16 weeks, a full thickness segmental defect of 2 cm is being investigated in the pig mandible to develop a critical-sized defect that does not heal within 20 weeks. It is anticipated that the 2 cm segmental defect in the pig mandible would comprise a challenging preclinical model to mimic the battle injuries of our wounded warriors and would allow for accurate testing and surgical planning of bone regenerative technologies. Additionally, clinical 64-slice CT scans which have been proven to correlate well with micro-CT data will be acquired at regular intervals to study the temporal response of bone healing as well as to develop a clinically viable methodology of evaluation. Data will be corroborated using micro-CT and histology at the conclusion of the study.

Animal study approved: Animal study ongoing

The pig mandible segmental defect has been approved by the Institutional Animal Care and Use Committee at USAISR DTRD as well as by the Animal Care and Use Review Office in May 2013. The study is currently underway.

Conclusions

Ultimately, validating challenging mandible injury models in animals will aid in progressing pre-clinical studies of bone regenerating biomaterials necessary to better restore mandibular battle wounds. Additionally, comparison of clinically relevant analysis techniques such as 64-slice CT with traditional preclinical methods will allow for smooth translation of biomaterial grafts into clinical studies.

Future Research Plans

USAISR DTRD will develop the pig mandible segmental defect model to establish a critical-size pre-clinical large animal model for craniomaxillofacial trauma such that novel biomaterials-based therapies for bone healing can be evaluated for efficacy and clinical therapy planning. In addition to creating a challenging animal model that mimics the battle injuries of our wounded warriors, DTRD will continue to develop novel ways to quantify bone regeneration using clinical CT studies in preclinical bone regenerative models as a therapy planning tool.

Planned Clinical Transitions

Since the study involves the development of a pre-clinical large animal model, there are no plans for clinical transition.

Soft Tissue Regeneration**Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction****Project 4.1.5, WFPC**

Team Leader(s): J. Peter Rubin, MD and Kacey Marra, PhD (University of Pittsburgh); James J. Yoo, MD, PhD and Sang Jin Lee, PhD (Wake Forest University); David Kaplan, PhD (Tufts University)

Project Team(s): Bruce Panilaitis, PhD, Evangelia Bellas, PhD, and Jodie Moreau, PhD (Tufts University); Jolene Valentin, PhD, Brian Phillips, PhD, Rachel Hoyer, BS, Nurul Aini, MD, and Donna Ward, PhD (University of Pittsburgh); Chang Mo Hwang, PhD, Young Min Ju, PhD, and Hyun Ju Lim, PhD (Wake Forest University)

Collaborator(s): Jeff Gimble (Louisiana State University, Pennington Research Center); Steve Badylak (University of Pittsburgh)

Therapy: Approach using volume stable injectable and implantable biomaterials for soft tissue regeneration

Deliverable(s): (1) Engineering of vascularized connective tissue and fat pad; (2) development of implantable and injectable vascularized soft

tissue composed of connective tissue and fat; (3) demonstration of the applicability of using implantable and injectable soft tissue composites for limb, burn, and craniofacial applications in a large animal model; and (4) initiation of clinical testing of soft tissue replacement for small defects.

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers have evaluated silk biomaterials (sponges, foams, and gels) in a large animal (horse) model with and without autologous lipoaspirate in terms of cellular response, vascularization of the graft, and volume retention. They also completed an in vivo animal study to demonstrate the feasibility of using an injectable fibrin-based hydrogel system for soft tissue regeneration.

Key Words: soft tissue, volume stable, adipose tissue, adipose-derived stem cells, silk, lipoaspirate, connective tissue, fibrin-based hydrogel

Introduction

Tissue components, such as fat, connective tissue, and muscle, are important contributors of maintaining facial tissue function. Not only do these tissues support essential functions, such as the position and optical axis of the eyes, but they provide significant coverage of the facial skeleton addressing aesthetic concerns and expression. Craniofacial injury often creates an immediate problem of preserving the volume occupied by the original soft tissue structures.

The proposed approach exploits a novel protein-scaffold system based on silk fibroin. Due to the slow degradation of three-dimensional matrices prepared from this protein, sufficient stability can be achieved to allow slow remodeling into native tissues with appropriate vascular and structural extracellular matrix (ECM) networks, while supporting mechanical and morphological requirements for the tissue.

Progress Report – Year 5***Integration of silk scaffold formats with and without lipoaspirate***

The researchers conducted a large animal study in the horse to evaluate porous silk scaffolds, porous foams, and gels with and without the addition of autologous lipoaspirate for sustained soft tissue augmentation. Scaffolds were implanted subcutaneously and harvested at 1 and 3 months to determine the progression of matrix remodeling and the order of cell recruitment within each scaffold type. The porous silk sponge was generated using two different silk processes. Aqueous sponges were cast from a water-based silk solution, and these demonstrated faster in vivo remodeling in previous evaluations. Solvent sponges were cast from silk prepared with hexafluoro-2-isopropanol that allows a higher concentration of silk to be used during casting, giving a longer degradation profile as a result. The research



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team observed different degradation rates between the two silk sponge formats, with the solvent-based sponge retaining more silk structure and subsequent tissue volume compared to aqueous silk sponges. Both scaffolds had abundant cell infiltration and vascularization, with a lipoaspirate supplement enhancing soft tissue volume more than the scaffold alone. Silk foams elicited different cellular in-growth patterns than sponges with greater cell densities around the perimeter of the foam implants. Lipoaspirate did not significantly increase soft tissue volume compared to untreated scaffold controls.

Injectable porous fibrin-based hydrogel

The team conducted in vivo studies to evaluate an injectable fibrin-based hydrogel system. They determined that the porous structure of the fibrin-based hydrogels produced by dissolvable gelatin microfibers allowed higher cell proliferation and increased cell spreading in the hydrogel. This resulted in improved tissue formation with proper vascularization in vivo, which will be important for soft tissue regeneration following craniofacial injury.

Injectable alginate-embedded fibrin-based hydrogel

The researchers observed higher cellularity and vascular in-growth in vivo in the alginate-fibrin system. This resulted in neo-tissue formation in the alginate-fibrin hydrogels. These results demonstrated that fibrin could enhance cell proliferation and accelerate formation of ECM proteins in the alginate-fibrin system, while the alginate particles could contribute to volume retention over time. This injectable hybrid system composed of degradable and non-degradable hydrogels could be a preferable approach to repair soft tissue defects.

Conclusions

The research team demonstrated the feasibility of using lipoaspirate-seeded silk scaffolds for long-term soft tissue restoration. They tested this technology in a large animal model of autologous fat grafting.

Future Research Plans

The work conducted during AFIRM I will be continued into the future: **Injectable and Implantable Soft Tissue for Craniofacial Reconstruction: Building upon AFIRM I Research to Generate Form Stable and Functional Tissue.** U.S. Army Institute of Surgical Research was added as a performance site. To generate soft pliable subcutaneous tissue, the researchers will use an injectable system containing adipose stem cells, processed adipose tissue elements, and adipose ECM scaffolds. This will be facilitated by a controlled release drug delivery system and a perfusion delivery system developed during AFIRM I. The researchers will also employ decellularized adipose tissue material manufactured by an industry partner, LifeCell. To regenerate deep structural adipose tissue, they will refine and optimize injectable (cannula deployable) silk sponge and silk foam scaffolds. This technology was developed during AFIRM I and will be optimized for clinical trials. Highly organized and innervated muscle tissue will be generated for rapid recovery of facial muscle function. A biofabrication system was developed that permits generation of organized muscle constructs possessing the neuromuscular junction. A composite function tissue will then be developed through the integration of the multiple tissue components. Silk scaffolds will follow the pre-Investigational Device Exemption U.S. Food and Drug Administration (FDA) pathways, and the integrated organ printing system will be pursued under present FDA regulations for 510(k) device.

Planned Clinical Transitions

Initial clinical trials are underway at the University of Pittsburgh, supported by the competitive AFIRM clinical trials funding programs.

Soft Tissue Regeneration**Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle****Project 4.1.6, WFPC**

Team Leader(s): George J. Christ, PhD (Wake Forest University)

Project Team(s): James Yoo, MD, PhD, Sang Jin Lee, PhD, Venu Kesireddy, PhD, Hannah Baker, BS, Chris Bergman, BS, and Manasi Vadhavkar, MS (Wake Forest Institute for Regenerative Medicine)

Collaborator(s): Tom Walters, PhD (United States Army Institute of Surgical Research); David Kaplan, PhD (Tufts University)

Therapy: Autologous bioengineered skeletal muscle implant approach for functional reconstruction/repair of complex craniofacial injuries

Deliverable(s): An implantable tissue-engineered muscle repair (TEMR) construct capable of restoring clinically relevant force/tension following volumetric muscle loss (VML) injury

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers held a pre-Investigational New Drug (IND) conversation with the U.S. Food and Drug Administration (FDA), which prompted the design of a definitive toxicology and proof of concept study in collaboration with a Contract Research Organization (CRO). They worked with the Regenerative Medicine Clinical Center (RMCC) to standardize all protocols surrounding the production of TEMR into Good Laboratory Practices (GLP)-certified standard operating procedures (SOPs) and master batch records (MBRs). Histological characterization of TEMR scaffolds made using muscle precursor cells (MPCs) sourced from human skeletal muscle biopsies illustrated formation of multinucleated and aligned myotubes after bioreactor preconditioning; moreover, characterization further demonstrated the feasibility of autologous cell sourcing applied in the TEMR technology.

Key Words: tissue engineering, skeletal muscle repair, volumetric muscle loss, bioreactor, muscle progenitor cells, myotubes

Introduction

Current management of tissue coverage and augmentation involves the use of existing host tissue to construct muscular flaps or grafts. In many instances, this approach is not feasible, delaying the rehabilitation process as well as restoration of tissue function. In fact, the inability to engineer clinically relevant functional muscle tissues remains a major hurdle to the successful skeletal muscle reconstructive procedures required to repair the complex facial injuries suffered in combat by military personnel. The long-term goal surrounding this project has been the creation of a skeletal muscle tissue implant capable of generating clinically relevant force/tension. Engineering skeletal muscle tissues de novo with the patient's own cells would accelerate wound healing with cosmetic augmentation of the tissue defect, and thus, enhance restoration of tissue function. The tissue-engineered construct consists of porcine derived bladder acellular matrix (BAM) seeded with autologous MPCs and subjected

to bioreactor cyclic stretch preconditioning. This report outlines the work completed during Year 5 of AFIRM I in which the major accomplishment was a pre-IND conversation with the FDA, which guided efforts to promote the TEMR construct technology towards a first-in-man IND study for which the envisioned application is a graft for secondary revision of cleft lip.

Research Progress – Year 5**Participation in a pre-IND conversation with the FDA**

A pre-IND meeting with the FDA occurred during the first quarter of Year 5. Feedback from reviewers has guided preparation for a definitive toxicology and proof of concept study to be carried out in cooperation with a CRO. The preparatory measures included revision of the existing surgical VML injury model to better emulate the graft size and procedure used in cleft lip and palate revision surgeries. In an in vivo study investigating regeneration



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using TEMR with the revised surgical technique, 22 rats are being studied in three groups: TEMR implants (n=8), BAM scaffold implants (n=8), and no treatment after defect creation (n=6). Eight weeks after implant, both latissimus dorsi muscles will be retrieved for analysis of muscle contractility and regeneration. A similar number of animals in identical treatment groups will also be conducted at a 4-week time point to permit comparisons with the prior VML injury model. In addition to revision of the implant procedure, the researchers have worked closely with the RMCC to standardize all protocols surrounding the production of TEMR into GLP-certified SOPs and MBRs.

Completion of an abbreviated toxicology study in the near future, at early time points (up to 1 month post-TEMR implantation), will complement the work described above, and should position the research team for filing an IND application in 2014.

Human skeletal muscle cells applied in TEMR technology

The researchers initiated feasibility studies on the collection and expansion of human pediatric MPCs. In collaboration with the RMCC, they used human skeletal muscle biopsies to source MPCs for expansion and application in the TEMR technology. Cell populations were successfully cultured to four passages, seeded onto BAM scaffolds, and subjected to bioreactor preconditioning. Histology was undertaken to examine the cell coverage and phenotype of the cells present on the scaffolds after completion of bioreactor preconditioning. Scaffolds stained for desmin contractile filaments and cell nuclei before and after preconditioning indicate preconditioning enabled improved myotube alignment and cell fusion indicative of maturing myotubes (**Figure 1**). Furthermore, the histology informed the feasibility of autologous cell sourcing in the TEMR technology.

Silk constructs show promise as a structured, degradable, and inert delivery vehicle

Collaborators at Tufts University in the Kaplan lab have fabricated three unique iterations of silk scaffolds for investigation as a scaffold material in next-generation TEMR constructs. The tunable chemical and physical properties of silk that control the construct porosity and degradation rate can be altered by varying silk concentration, freezing rate, and

the annealing processes. These tunable properties provide a platform for generating a variety of other three-dimensional (3D) geometries with the added benefit of significant drug delivery capabilities. The Wake Forest team has conducted preliminary in vivo studies on three iterations of silk scaffold without a cellular component that were implanted in the rat tibialis anterior (TA) model of VML injury. The successive iterations achieved the desired degradation rates and physical structures. While the third iteration of silk scaffold without cells (n=3) did not significantly influence functional recovery, it provided important guidance in the use of silk as a next-generation TEMR scaffold material. Work to optimize cellularity of the currently preferred iteration II constructs is ongoing in an effort to achieve aligned multinucleated myotubes characteristic of the TEMR constructs. Moving forward, the researchers envision cell-seeded, silk-based implants to be used for delivery of drugs and growth factors to aid in muscle regeneration following VML injury.

Conclusions

The work completed in Year 5 resulted in the TEMR technology making an important progression on the path to clinical translation. The major accomplishment in Year 5 was participation in a pre-IND

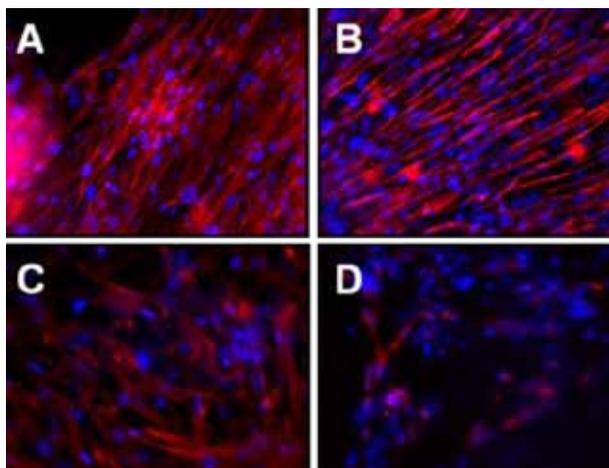


Figure 1. TEMR constructs made using human skeletal muscle cells illustrate feasibility of autologous cell sources to create de novo tissue grafts. Constructs were stained after bioreactor preconditioning (A, B 200x) or after the same time period in static culture (C, D 400x). Actin stress fibers (Phalloidin A, C) and desmin contractile filaments are stained red (anti-Desmin B, D) and cell nuclei are stained blue (DAPI).

conversation with the FDA. The researchers also initiated RMCC training for lab members in GLP standards so the team could generate GLP-certified SOPs or MBRs for all protocols involved with the production, characterization and implantation of TEMR constructs. In further efforts to move the TEMR technology forward, histological characterization of human skeletal muscle cell seeded TEMR constructs verified the presence of multinucleated, aligned myotubes, further documenting the feasibility of the use of human muscle biopsies for creation of a tissue-engineered muscle graft. A third iteration of tunable silk scaffolds was tested in the rat TA model of VML injury in the pursuit of next-generation TEMR scaffold material candidates. The characteristics required for use of silk as a 3D inert cell and drug delivery vehicle have been identified, and this will provide an important component of the development of next-generation TEMR constructs with a wider range of clinical applications.

Future Research Plans

A major goal for the future is to conduct a pilot “proof of concept” study clinical study consisting of five healthy patients with secondary unilateral cleft lip who are willing to participate in the use of bioengineered muscle constructs for surgical correction of aesthetic and functional deformities which have been incompletely corrected by current methods. In addition, the researchers will continue to develop and optimize a silk-based TEMR scaffold for both rodent TA and latissimus dorsi VML injury models to further expand the potential clinical applications of the TEMR technology. The researchers will strive to achieve FDA guidance and approval for a second-generation BAM-based TEMR scaffold for the treatment of secondary cleft lip.

Planned Clinical Transitions

The major result of the pre-IND was the design and execution of an abbreviated toxicology (up to 1 month post-TEMR implantation) and efficacy (up to 2 months post-TEMR implantation) study that will involve a CRO. Upon conclusion of these studies (shortly after completion of AFIRM I) and final consultation with FDA, the researchers will retain a CRO to assist with the design and implementation of the definitive toxicology and efficacy study required for an IND submission for the secondary revision of cleft lip. In addition, the team has revised the defect model and surgical technique for implant of TEMR in a rat latissimus dorsi in order to match the size and graft application method employed in the clinic. Results of this ongoing study will help guide the surgical technique used for the large-scale CRO-sponsored definitive toxicology and efficacy study. Furthermore, the group has worked closely with the RMCC to create SOPs and MBRs for all TEMR-related protocols and will continue to further refine these documents.

Corrections/Changes Planned for the Future

The pre-IND conversation confirmed the researchers’ decision to not conduct a large animal study using TEMR. Completion of the current study using a revised surgical model and ongoing training in GLP practices are anticipated to provide further guidance for implementation of the toxicology study and assembly of an IND application in consultation with the FDA.





Soft Tissue Regeneration

Develop Innervated, Vascularized Skeletal Muscle

Project 4.1.2, RCCC

Team Leader(s): Cathryn Sundback, ScD and Joseph Vacanti, MD (Massachusetts General Hospital)

Project Team(s): Craig Neville, PhD, Olive Mwizerwa, BS, Tom Cervantes, BS, Irina Pomerantseva, MD, PhD, and Nina Joshi, BS (Massachusetts General Hospital); Tessa Hadlock, MD and Marc Hohman, MD (Massachusetts Eye and Ear Infirmary); N. Sanjeeva Murthy, PhD and Joachim Kohn, PhD (Rutgers University)

Collaborator(s): Keith Baar, PhD (University of California, Davis); Jeffrey Widrick, PhD (Spaulding Rehabilitation Hospital); Steve Stecyk, BS (Intrinsic Corp.)

Therapy: Replacement of severely injured facial skeletal muscles

Deliverable(s): Innervated, vascularized skeletal muscle

TRL Progress: Beginning, TRL 3; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: During the past year, the researchers (1) demonstrated uniform innervation of engineered muscle constructs; (2) determined that electrical stimulation with neural-like signals increased the engineered muscle's contractile force; (3) found that engineered endothelial networks within the muscle constructs rapidly merged with the host's vasculature, and blood perfusion from the host supported the implanted construct; and (4) developed polymeric sleeves (Rutgers team) for scale-up of the muscle constructs.

Key Words: tissue-engineered skeletal muscle; vascularization; innervation; orbicularis oculi

Introduction

Traumatic injuries to the head, neck, and particularly, face have been major contributors to the mortality and morbidity of military personnel in the current conflicts in Iraq and Afghanistan. Up to 13% of all blast injury survivors have significant eye and eyelid injuries involving perforations from high-velocity projectiles. A severely damaged eyelid muscle (orbicularis oculi) prevents eyelid closure, potentially resulting in blindness. Autologous tissue transfer is the standard of care but the outcome is suboptimal. Engineered replacement orbicularis oculi will restore functionality of the eyelid, preventing blindness, as well as restore the aesthetics of the face. This therapy will improve the wounded warrior's quality of life and maximize eyelid function for return to duty. In civilian populations, eyelid reconstruction with replacement muscle would significantly benefit patients with extensive eyelid skin cancers, major facial trauma, and long-standing facial paralysis with both neural and muscular deficits.

The Massachusetts General Hospital–Rutgers team is developing a muscle construct with native muscle

morphology. Myotubes, differentiated from a co-culture of myoblasts and fibroblasts, self-assemble to form a three-dimensional (3D) muscle on the size scale of a fascicle, a cluster of skeletal muscle fibers, 500–1000 μm in diameter. The resulting construct has the complex multinucleated muscle architecture of native neonatal skeletal muscle.

The muscle constructs were designed to be rapidly perfused and scaled to a muscle the size of the orbicularis oculi. Using a vasculogenesis approach, a prevascular network was established within the engineered muscles in vitro. This vascular network readily anastomoses with the host's vasculature upon implantation. To scale the muscle size, the Rutgers team developed a highly porous scaffold in the shape of a sleeve that is used to encase the engineered muscle. These sleeves are made from a tyrosine-derived polycarbonate, which is a bioresorbable and biocompatible material.

The guiding hypothesis of this project is that the scaled-up, 3D, engineered, innervated, and prevascularized muscle can develop sufficient muscle contractility to replace orbicularis oculi function. The specific aims for Year 5 were to: (1) optimize

vascularization with the host; (2) demonstrate innervation of muscle tissue; (3) characterize muscle function; (4) engineer robust human muscle; and (5) continue scaffold development to scale up engineered muscle.

Research Progress – Year 5

Immature skeletal muscles were produced in vitro using a co-culture of primary myoblasts and fibroblasts which were seeded, proliferated, and differentiated on fibrin gel. Differentiation induced alignment and fusion of myoblasts to form long multinucleated myotubes. Contraction of the fibrin gel induced the cell sheet to roll, ultimately forming an immature muscle 10 days after differentiation. Fibrin was an appropriate scaffold material as it supported cellular proliferation and differentiation and was readily degraded during muscle construct self-assembly.

Vascularization

To induce rapid perfusion of the muscle construct, a prevascular network was established within the engineered immature muscle prior to implantation. A co-culture of endothelial cells and human mesenchymal stem cells (hMSCs) formed an endothelial network, supported and maintained by the hMSCs. Prevascularized self-assembled muscles were implanted into nude mice and explanted from one to 21 days post-implantation. Blood vessels made from human cells were observed throughout the muscle and contained red blood cells. Connection of the host and engineered vasculatures rapidly occurred. Perfusion of these engineered vessels provided vascular support to the muscle construct, which maintained viability during the critical post-implantation period.

Innervation

Functional neuromuscular junctions were formed in vitro on engineered muscle fibers with slices of rat spinal cords. Sections of spinal cords were incorporated into engineered muscle constructs during fabrication. Extensive neurite outgrowth and mature neuromuscular junctions were formed throughout a cross-section of the construct. Innervated myotubes were larger in diameter and expressed higher levels of muscle contractile proteins in comparison with uninervated myotubes.

Muscle function

3D engineered muscle was electrically stimulated using a neural-like stimulation pattern to improve muscle function. Muscle contractility was measured with standard in vitro muscle mechanics instrumentation following stimulation. The engineered muscle was responsive to extended electrical stimulation and approached fused tetanus in control and stimulated muscle constructs; a muscle recruits all motor units in a fused tetanus state, generating maximum contraction force. Electrical stimulation significantly increased the specific isometric force, relative to unstimulated controls.

Engineer human muscle

The engineering of human skeletal muscle was significantly improved by the addition of specific medium supplements and by substituting laminin for fibrin. The conditions to provide maximum benefit were first determined in 2D muscle culture and then evaluated on 3D human muscle cultures. Relative to standard culture medium, the soluble factors increased human myoblast cell proliferation and delayed muscle construct self-assembly, both of which increased the size of the resulting muscle construct.

Substitution of laminin for fibrin improved formation of human muscle constructs. When cultured with the specific medium supplements, human muscle constructs rolled more slowly when cultured on laminin in comparison with fibrin, which provided additional time for proliferation and differentiation. The resulting muscle constructs were significantly larger in size and more tightly rolled than constructs cultured on fibrin.

Scale up engineered muscle

Engineered muscle constructs will be scaled up to the size of the human orbicularis oculi by bundling self-assembled muscles. The engineered muscle may ultimately be enclosed within tyrosine-derived polycarbonate sleeves, designed and fabricated by the Kohn laboratory. Polymeric sleeves were produced using tyrosine-derived polycarbonates, but the fibers were stiff with the potential to damage soft muscle constructs. To decrease overall stiffness, the fiber parameters were optimized; filaments were then extruded and sleeves fabricated.



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Muscle constructs were easily inserted into sleeves and implanted into the fat pads of nude mice. At 2 weeks post-implantation, moderate inflammation was observed in all polymeric sleeve samples associated with the sleeve fibers. In comparison, minimal inflammation was observed in the single muscle construct control. Although moderate inflammation occurred in response to the polymeric sleeve, the bundling results are encouraging. Future studies will assess the impact of the polymeric sleeves on muscle function.

Conclusions

The main focus in Year 5 was on demonstrating uniform innervation of engineered muscle constructs and the impact of electrical stimulation on the contractility of engineered muscle constructs. Addition of nerve tissue during muscle construct fabrication led to nearly homogenous innervation of muscle fibers throughout the cross-section. Electrical stimulation with neural-like signals increased the specific contractile force of the engineered muscle constructs. In addition, new understanding was gained related to vascularization and scale-up of muscle constructs. Engineered endothelial networks within muscle constructs rapidly inosculated with the host vasculature, and perfusion from the host supported the implanted construct. Finally, polymeric sleeves were developed by the Rutgers team for scale-up of the muscle

constructs. Moderate inflammation was observed in implantation studies but further scale-up testing is warranted.

Future Research Plans

During the upcoming year, muscle constructs bundled in sleeves will be implanted into immunocompromised rodents and innervated with a host nerve. The researchers will characterize the innervation, vascularization, and muscle function of the implants. Future studies will demonstrate the use of engineered muscle constructs to replace orbicularis oculi function in immunocompetent rats or rabbits.

Planned Clinical Transitions

Given the complexity of this product, the project team will submit a Request for Designation to the U.S. Food and Drug Administration Office of Combination Products to determine appropriate regulatory path. Based on this review, they will file an Investigational New Drug or Investigational Device Exemption with the Center for Biologics Evaluation and Research or the Center for Devices and Radiological Health, respectively. They will perform pre-clinical (animal) studies in immunocompromised rats (contractile human muscle on the scale of a human orbicularis oculi) and in an immunocompetent rabbit model (autologous sphincter muscle with architecture similar to the orbicularis oculi).



Soft Tissue Regeneration**Composite Tissue Allograft Transplantation Without Lifelong Immunosuppression****Project 4.3.1c, RCCC**

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

Project Team(s): Joanna Cwykiel, MSc, Halil Uygur, MD, Grzegorz Kwiecien, MD, and Maria Madajka, PhD (Cleveland Clinic)

Collaborator(s): Jim Herrman, PhD, CEO (Tolera Therapeutics, Inc.)

Therapy: Fused human chimeric cell therapy for vascularized composite allograft (VCA) transplantation and solid organ tolerance induction

Deliverable(s): Therapeutic effect of ex vivo created donor-recipient chimeric cells on prolonged vascularized skin allograft survival

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: During the past year, the researchers studied many properties of their human chimeric cells and found that: (1) these cells do not secrete inflammatory cytokines in vitro; (2) there were few dead cells following cell fusion; and (3) the cells proliferate. The team characterized the cells' genotype and phenotype before and after culturing, and animal model testing demonstrated that the cells migrated into blood, bone marrow, and lymphoid organs.

Key Words: ex vivo created human chimeric cells; cell fusion; tolerance induction; prevention of transplant rejection; cellular therapy

Introduction

High-energy improvised explosive devices (IEDs) and fragmentation weapons are the number one explosives currently utilized during hostile attacks directed against U.S. troops, U.S. allies and civilians around the world. The distribution of injuries caused by IEDs includes: 29.4% head and neck, 5.6% thoracic, 10.7% abdominal, and 54.1% extremity. The percentage of extremity trauma is consistent with numbers seen in previous conflicts, whereas the percentages for head and neck injuries are higher. The tremendous destruction and severe trauma inflicted by the IEDs pose a serious challenge for the medical community. The most recent terrorist attack during the 2013 Boston Marathon (3 dead, almost 200 severely injured, including 10 who lost extremities) clearly shows the increasing importance of the development of new, more effective treatment solutions for both wounded warriors and civilians.

Performing successful reconstructive surgery in military and civilian patients suffering from large areas of tissue damage, in combination with the scarcity of blood supply due to the multiple lacerations, penetrating ballistic, and blast and burn injuries, is extremely challenging. Blast trauma can

cause severe limb and craniofacial injuries, large area burns, tissue devitalization, significant foreign body loading, and nearly ubiquitous bacterial colonization. Standard medical practices, as used on the civilian population, of early radical debridement, fixation of fractures with bone grafting, and wound coverage with vascularized tissue are applied. Currently, the most effective method of treatment against severe IED injuries for wounded warriors and civilian patients is VCA transplantation, which is associated with life-long toxic immunosuppressive regimens. In order to mitigate the toxic effects of immunosuppression, the development of innovative therapies is required.

Under AFIRM I, the Siemionow team successfully proved the feasibility of ex vivo fusion for the creation of human chimeric cells from cord blood cells. Introduction of cell-based therapies to the field of VCA transplantation represents a novel, regenerative medicine approach that will minimize or reduce the need for lifelong immunosuppression and will expand indications for face, hand, legs and abdominal wall transplants. While these applications will apply directly to the battlefield scenario to help traumatically wounded warriors, they will also benefit civilians.



Research Progress – Year 5

The researchers determined the phenotype and genotype of the human chimeric cells before and after culturing by flow cytometry, lymphocytotoxicity assay, and polymerase chain reaction-sequence specific oligonucleotide probe typing. They confirmed a low number of apoptotic or dead cells following the cell fusion procedure. They also confirmed the proliferative properties of the human chimeric cells by colony forming unit assay.

Evaluation of the presence of Human leukocyte antigen (HLA) class I and class II antigens on the surface of cultured human chimeric cells

The researchers cultured fused human chimeric cells for 5 days and collected the cells for evaluation of phenotypic characteristics (HLA class I and II antigens) using the lymphocytotoxicity test. The results of the test showed that culturing human chimeric cells does not affect the expression of HLA class I and II antigens on the surface of the chimeric cells.

Assessment of secretion of Th1 and Th2 cytokines by human chimeric cells following cell culturing

The researchers performed ELISA to determine the concentration of Th1 and Th2 cytokines secreted by human chimeric cells. They also performed the fusion of cord blood cells, derived from two unrelated donors (male and female). ELISA tests showed no secretion of Th1 or Th2 cytokines. The lack of Th1 and Th2 expression might indicate that human chimeric cells remain immunologically neutral in vitro.

In vivo evaluation of migratory properties of human chimeric cells in an immunocompromised rat model

Animals were euthanized 24 and 72 hours after the injection of human chimeric cells into the rat femur. Lymphoid organs such as spleen, lymph nodes, bone marrow as well as blood, liver, brain, skin and lungs were harvested and preserved. The researchers determined the presence of human chimeric cells based on human HLA class I (ABC) antibody staining by flow cytometry. They found that within the first 24 hours of injection, human chimeric cells

were capable of migrating from the injected bone to the contralateral bone, as well as to the lymphoid organs, such as spleen and lymph nodes.

Conclusions

The Siemionow team has characterized the phenotype and genotype of human chimeric cells before and after culturing for therapeutic purposes. The presence of human chimeric cells in blood, bone marrow, and lymphoid organs confirmed their migratory potential. The next step in the development of this therapy is to apply for Institutional Review Board (IRB) approval on a clinical protocol for a Phase I clinical trial.

Future Research Plans

In order to comply with the requirements for successful IRB protocol application, the Siemionow team will perform the following preclinical evaluations:

- Test the safety of human chimeric cell therapy to confirm that human chimeric cell therapy will not cause life threatening side effects in vivo.
- Test the phenotypic and genetic stability of human chimeric cells to confirm that human chimeric cells will preserve their therapeutic properties in vivo.
- Test cryopreservation methods of human chimeric cells to confirm if human chimeric cells can be stored for prolonged periods of time without losing their therapeutic properties. The phenotype and genotype stability of cryopreserved chimeric cells will be confirmed, both in vitro as well as in vivo. The storage of human chimeric cells for prolonged periods of time will allow for easy access to chimeric cells if additional doses of cellular therapy are required by the patient. In addition, a protocol for cryopreservation and banking of human chimeric cells will be established. The in vivo testing of cryopreserved human chimeric cells will confirm the engraftment, migration into lymphoid organs and bone marrow compartment, as well as the creation of stable chimeric cell lineages.

These preclinical experiments will directly support the hypothesis that human chimeric cells represent a new, safe cellular therapy, eliminating the need for

lifelong immunosuppression and thus introducing safer protocols for VCA transplantation, as well as solid organ transplants.

Planned Clinical Transitions

The introduction of cell-based therapies to the field of VCA transplantation represents a novel, regenerative medicine approach, which will minimize or reduce the need for lifelong immunosuppression.

Following confirmation of safety, stability and cryopreservation of human chimeric cells, the Siemionow team will apply for IRB approval to test the immunomodulatory effects of bone marrow-derived human fused chimeric cells as a supportive therapy for living kidney and liver donor transplantation. Tolera Therapeutics, Inc. will support the transition of chimeric cell therapy to clinical trials.





Soft Tissue Regeneration

Portable Perfusion System to Increase Preservation Time of Isolated Limbs for Transplantation

Project 4.3.1d, RCCC

Team Leader(s): Bohdan Pomahac, MD (Brigham and Women's Hospital)

Project Team(s): Bohdan Pomahac, MD and Geoffroy C. Sisk, MD (Brigham and Women's Hospital)

Collaborator(s): Numia Medical

Therapy: Composite tissue allotransplantation

Deliverable(s): Portable perfusion device for isolated upper extremity

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: After successful (delayed) execution of the award in August 2012, the Pomahac team actively sought partnerships with individuals and groups able to provide programming support for the development of an extracorporeal machine perfusion device. In April 2013, after vetting several candidates, the Pomahac team paired with Numia Medical (Newport, VT), who produced the first working prototype device. The researchers are now poised to start animal testing in a pig model.

Key Words: composite tissue allotransplantation; ischemia/reperfusion injury; extracorporeal machine perfusion; extremity injury; replantation

Introduction

Until recent years, there have been only two main-stream approaches to the treatment of traumatic upper extremity amputation. Replantation of the arm and hand is attempted in the few occasions when it is medically possible. More often, patients undergo revision amputation and are later fitted with prostheses. These can be sophisticated and functional, but they lack sensory feedback, are costly, can be difficult to don and remove, and often have an unnatural appearance. Recently, allotransplantation has introduced the ability to restore “like-with-like” by replacing the missing upper extremity with living, functional tissues. Replantation remains preferable, but allotransplantation is gaining ground as a second choice. With proper nerve reconnection/regeneration, functional and cosmetic results of arm and hand replantation and allotransplantation are superior to prostheses.

However, both replantation and allotransplantation are limited by the condition and potential degradation of the severed arm, the availability of qualified surgeons and equipment for microsurgery, and the need to reattach blood vessels within 4-6 hours of limb detachment. At present, the standard of care is to preserve severed limbs intended

for allotransplantation or replantation in a state of “cold ischemia,” on an ice slurry. Vasculature and muscle exposed to prolonged ischemia (lack of oxygen) undergo necrosis due to “reperfusion injury” (i.e., the tissue becomes damaged when blood flow returns to the limb after a period without oxygen). Rejection of the transplant also becomes more likely with longer ischemia time in transplanted limbs. This time constraint presents a bottleneck for the practice of upper extremity replantation and allotransplantation, such that candidacy for either procedure depends on the time needed to transport the patient (either the victim of traumatic amputation or the recipient of donor tissue) and the limb to a center with microsurgical capabilities. Thus, many traumatic upper extremity amputees are not considered for replantation and potential donors for upper extremity allotransplantation are rejected. The Pomahac team proposes to address this bottleneck with a device that will circulate oxygenated perfusion solution through the amputated tissues, extending the allowable ischemia time for upper extremity replantation/allotransplantation through a novel combination of engineering and physiology.

The proposed portable perfusion system will prolong the allowable ischemia time using

extracorporeal membrane oxygenation technology in a portable, easy-to-use, lightweight device. If successful, this technology will maintain the severed limb in an adequate state of oxygenation and viability for a period of time that is two to three times higher than the current standard. This will allow an increased window of time for the severed limb and patient to be transported to a center capable of microsurgical replantation or allotransplantation. Therefore, limb replantation will become available to a larger number of patients, and the donor pools for limb allotransplantation can be geographically enlarged to a radius consistent with 8- to 12-hour transport times to the nearest transplant center. The device will be portable and easy-to-operate, allowing for widespread adoption by organ banks, transplant centers and remote trauma-response medic personnel.

The Pomahac team's portable perfusion system will provide trauma first responders, small tertiary centers and transplant centers with the ability to maintain normal physiology in isolated limbs for longer durations, allowing for longer distance between sites of limb procurement and transplant

or microsurgical centers. Portable systems supporting heart and kidney transplantation have undergone clinical trials in Europe and clinical feasibility studies in the U.S., and one is U.S. Food and Drug Administration (FDA)-approved for kidney transplants. However, these systems have not been adapted for perfusion of isolated limbs in support of replantation or transplantation.

Research Progress – Year 1 (Project commenced in 2012)

Device Design/Manufacture

Design

After a study of existing extracorporeal perfusion devices used for solid organs, and subsequent lengthy discussion about the specifications that would comprise a device specifically tailored for an upper extremity application, the Pomahac team devised a schematic that demonstrated all necessary components of the device in a functioning circuit (Figure 1). This schematic was shared with Numia Medical, who used it as its template while manufacturing the prototype device.

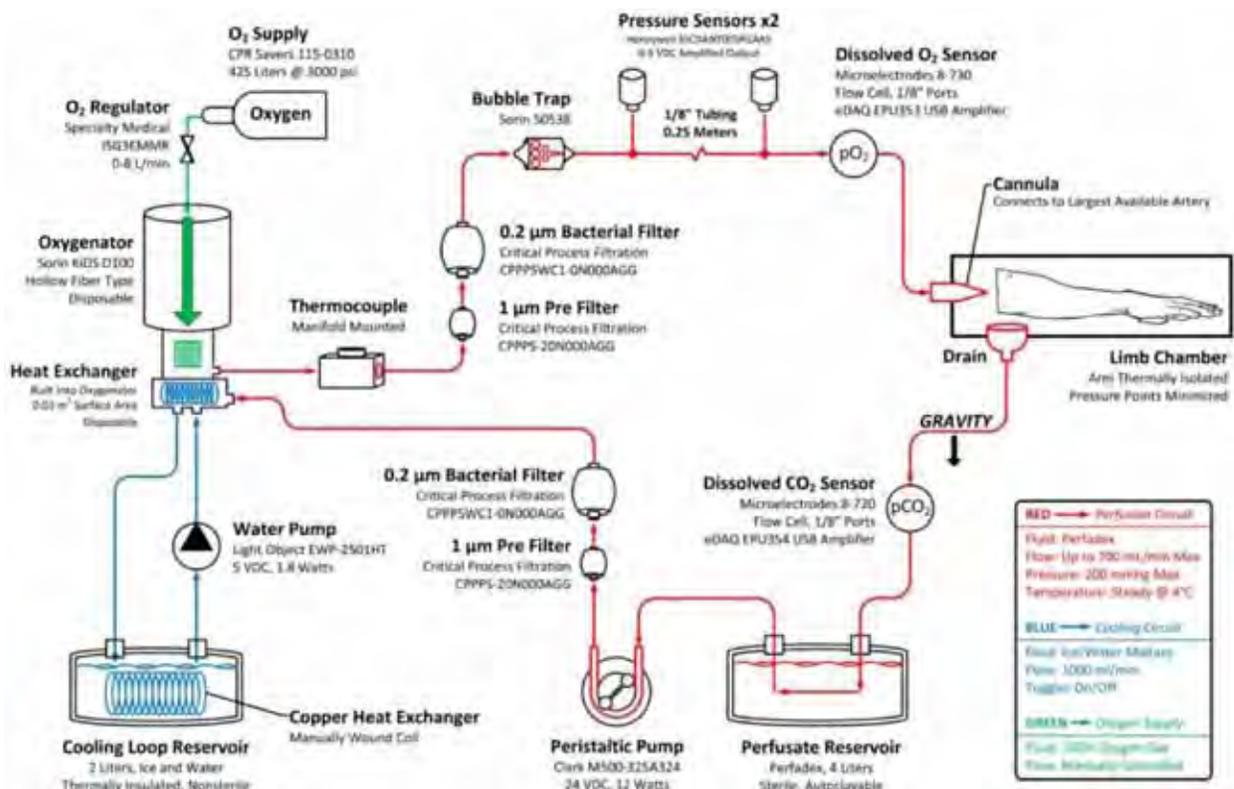


Figure 1. Schematic of the upper extremity perfusion device used in the planning stages of development.



III: Craniofacial Reconstruction

Manufacturing and programming

The first build of the upper extremity perfusion device was completed in April 2013, with electronic and mechanical infrastructure completed as overseen by the Pomahac team (Figures 2 and 3).

Cadaver and animal studies

Initial studies to optimize perfusion parameters using post-mortem cadaver tissue

The Pomahac team is now poised to begin optimizing the device with perfusion parameters (temperature, pressure, flow) that will result in maximum tissue survival. It will conduct initial trials in post-mortem tissues. The researchers will perform tissue biopsies throughout a 12-hour perfusion period and analyze tissues for evidence of cellular damage and death; no transplantation will occur in these initial studies.

Limb allotransplantation

After the Pomahac team has optimized the perfusion parameters, it plans to proceed with limb transplantation procedures in a total of five donor-recipient animal pairs using a porcine (pig) model. Transplantation of tissues will allow for a more thorough investigation of ischemia/reperfusion injury in physiologically re-perfused tissues.



Figure 2. Lift-out assembly containing oxygenator, filters, and tubing.

Conclusions

A state-of-the-art portable device that prolongs the life of an amputated limb would optimize battle-field evacuation by preserving the amputated limbs of warfighters until transfer to a microsurgical center and surgical replantation where possible. The Pomahac team has now secured the design, construction and delivery of the isolated limb perfusion device.

Future Research Plans

Using the functional prototype of the isolated limb perfusion device, the team intends to pursue animal studies in a porcine model to determine the perfusion parameters that must be used to best preserve limb tissues, based on observed chemical and histological signs of cell injury and death. The Pomahac team then intends to use these determinants to pursue a series of transplant experiments in pigs, analyzing the survival and quality of transplanted tissue after reperfusion. These studies will lay the groundwork for future studies in humans.

Planned Clinical Transitions

After optimization of the isolated limb perfusion device has been achieved, and proof of principle has been established in animals, the Pomahac team intends to pursue FDA Investigational Device Exemption approval for a transition to human studies.



Figure 3. Stainless steel and autoclave-compliant tray for transport and storage of pig limbs during testing.

Soft Tissue Regeneration**Vascularized Composite Allograft Transplantation With Topical Immunosuppression****Project 4.3.1e, RCCC**

Team Leader(s): David H. Sachs, MD and Curtis L. Cetrulo, Jr., MD (Massachusetts General Hospital); Joachim Kohn, PhD (Rutgers University)

Project Team(s): Melissa Mastroianni, MD and Christopher Mallard, BS (Massachusetts General Hospital); Ritu Goyal, PhD and Lauren Macri, PhD (Rutgers University)

Collaborator(s): N/A

Therapy: Biodegradable nanosphere-based drug delivery system to deliver topical immunosuppression for vascularized composite allograft (VCA) transplantation

Deliverable(s): Novel topical formulations of immunosuppressive drug-eluting dressings, as well as a pre-clinical protocol for the postoperative care of hand and face transplants, including the treatment of rejection episodes

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: In their first year of funding, the research team successfully formulated TyroSphere™ dressings that provided sustained release of two highly lipophilic (hydrophobic) and potent immunosuppressive agents—cyclosporin A (CsA) and tacrolimus. These drug-loaded TyroSpheres™ were stable over a range of temperatures, penetrated the dermis of cadaveric skin, and released the drug over 7 days from a gel. In addition, the researchers developed a novel clinical application of the TyroSpheres™—direct application to the wound bed prior to graft placement. The application of tacrolimus-loaded TyroSphere™ dressings both topically to the graft and directly to the wound bed significantly reduced inflammation in the skin grafts and wound beds in a baboon model.

Key Words: immunosuppression; xenoskin; hand transplants; face transplants; allograft; nanospheres; tyrosine-derived polymers

Introduction

VCA transplantation procedures offer restoration of complex anatomical and functional units, such as the hands or face, following devastating injury. Over 80 hand and 20 face transplants have been performed worldwide. Because this work involves tissues transplanted from one person to another, immunosuppression is required to prevent rejection of the transplanted tissue. The required systemic lifelong immunosuppression can cause significant adverse side effects, such as diabetes mellitus, kidney disease, and increased susceptibility to infections and certain tumors. As a result, the wide applicability of VCA has been limited.

The Transplantation Biology Research Center at Massachusetts General Hospital (MGH) has previously developed a baboon skin grafting model to investigate transplanted skin in the form of skin grafts and novel strategies for the treatment of burn

wounds. One such treatment is the use of xenoskin, derived from genetically modified swine whose cells are altered to not express the Gal protein (GalT-KO), which is responsible for hyperacute rejection of skin grafts across species. In this project, the researchers are utilizing this xenoskin as a testbed to evaluate topical immunosuppressive agents on the survival of both xenogeneic and allogeneic transplanted skin. If successful, future work will evaluate the effects of these topical immunosuppressive agents on the prolongation of VCA survival.

Research Progress – Year 1 (Project commenced in 2012)

To increase the absorption of lipophilic drugs, the Kohn laboratory has developed a nanosphere technology (referred to as TyroSpheres™) that can provide localized, sustained release of therapeutic agents that are hydrophobic, water-insoluble, and/or have poor bioavailability. The TyroSpheres™ are



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synthesized from a family of fully-degradable, ABA-type triblock copolymers made of poly(ethylene glycol), oligomers of desaminotyrosyl-tyrosine esters and suberic acid. These tyrosine-derived copolymers spontaneously self-assemble in aqueous media into TyroSpheres™ and have been shown to (1) be nontoxic in vitro and in vivo, (2) bind and deliver hydrophobic drugs, and (3) significantly enhance the permeation of hydrophobic agents into the epidermis.

Using a porcine model, the project team showed that a gel formulation containing TyroSpheres™ (TyroSpheres™-gel) increased the delivery of hydrophobic agents by maximizing contact with the skin and preventing the “run-off” effect of aqueous formulations. The researchers’ hypothesis is that this mode of administration will prolong the survival of VCAs used for transplanting the hands and face. In this project, TyroSpheres™-gel was formulated to contain either CsA or FK506—two potent, lipophilic, commonly used immunosuppressive agents.

Characterization of CsA or FK506-loaded TyroSpheres™

TyroSpheres™ in an aqueous solution or in 1%-hydroxypropyl methylcellulose (HPMC) gel were loaded with either CsA or FK506. The binding efficiencies of CsA and FK506 to the TyroSpheres™ were both 50%, irrespective of the initial loading. Based on these data, 30% initial loading was chosen

for all subsequent experiments. The release of CsA or FK506 from TyroSpheres™ and TyroSpheres™-gel was studied at 37°C for 7 days in phosphate-buffered saline (PBS). **Figure 1** shows that up to 50%-70% of the drug was released from TyroSpheres™ and more than 90% from TyroSpheres™-gel formulations after 168 hours. These release profiles satisfy the requirement for a dressing that releases over the course of 7 days.

In vitro cytotoxicity of CsA or FK506-loaded TyroSpheres™

Table 1 shows the IC₅₀ (concentration of drug that produces half of the maximal effect possible) values of the two drugs (CsA and FK506) tested on human keratinocyte (HaCaT) and human dermal fibroblast (HDF) cells. The IC₅₀ values for free and 30% loaded drugs into TyroSpheres™ were not found to be significantly different from each other, which confirms that both the free drug and drug-loaded TyroSpheres™ were not damaging to skin cells.

Solubility of CsA or FK506 in TyroSpheres™

The solubility of drugs into TyroSpheres™ was measured to be (1) 1000-fold higher for CsA and (2) ~2000-fold higher for FK506, when compared to solubility in 1X PBS. These data suggest that the TyroSpheres™ provide the opportunity to deliver high concentrations of these lipophilic agents to aqueous environments.

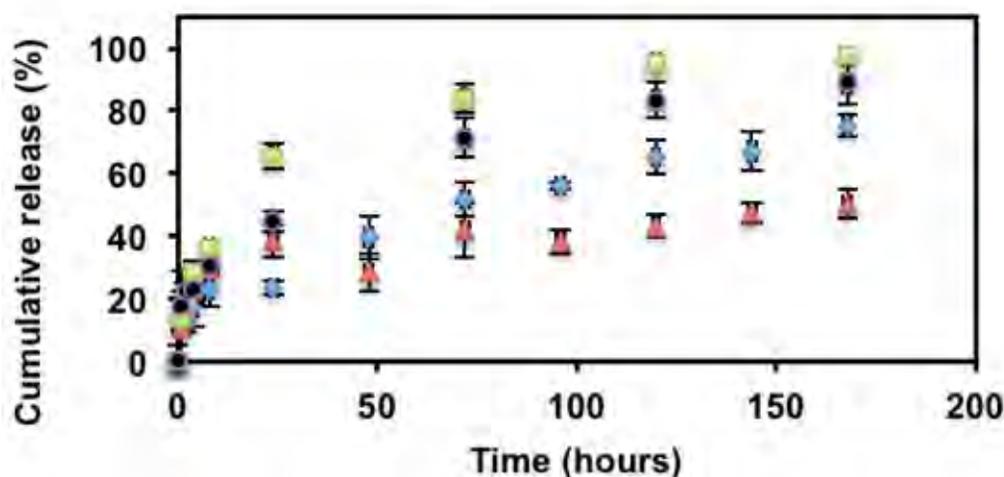


Figure 1. Release profile of 30% CsA-loaded TyroSpheres™ (blue), 30% FK506-loaded TyroSpheres™ (red), 30% CsA-loaded TyroSpheres™-gel (black) and 30% FK506-loaded TyroSpheres™-gel (green).

Table 1. IC50 values of free CsA and 30% loaded CsA and FK506 in two types of skin cells following 3 and 6 days post treatment.

Sample	HaCat (IC50±S.D. μ M)		HDF (IC50±S.D. μ M)	
	Day 3	Day 6	Day 3	Day 6
Free CsA	5.4±1.2	7.4±0.3	11.2±1.5	4.5±1.0
Free FK506	33.0±0.9	76.4±0.1	35.7±7.2	63.5±1.4
30% CsA-loaded TyroSpheres	10.0±3.1	14.9±0.2	31.0±6.8	12.9±1.3
30% FK506-loaded TyroSpheres	91.7±3.0	72.7±2.6	76.1±6.3	53.7±6.1

Stability of FK506 or CsA-loaded TyroSpheres™ in 1X PBS

The stability of FK506 or CsA-loaded TyroSpheres™ was assessed for 4 weeks at 4°, 25°, and 37°C, and compared to the stability of free FK506 or CsA (not encapsulated in TyroSpheres). Overall, encapsulated CsA (in TyroSpheres™) was significantly more stable than encapsulated FK506. At all temperatures, the stability of either encapsulated FK506 or CsA was significantly longer than that of free FK-506 or CsA (data not shown). Specifically, TyroSpheres™ protected FK506 from degradation at 4°C for 4 weeks. Some degradation was measured when stored at higher temperatures: 10% of FK506 degraded at 25°C after 3 weeks and approximately 55% degraded at 37°C after 2 weeks. TyroSpheres™ protected CsA from degradation at 4°C and 25°C up to four weeks and at 37°C up to three weeks. Significant degradation was measured at 37°C, where 50% of both encapsulated CsA and free CsA degraded after three weeks. Studies are currently ongoing to develop dry formulations of the drug-loaded TyroSpheres™ for extended stability and storage.

Penetration of 30% (w/w) CsA-loaded TyroSpheres™-gel into cadaver skin

Skin distribution studies were used to quantify the penetration of CsA into the dermal layer of cadaver skin. After 6 hours of treatment with CsA-loaded TyroSpheres™-gel (1% HPMC gel), approximately 22 μ g of CsA penetrated through the epidermis and into the dermis. No CsA was detectable in the receptor compartment, suggesting no systemic uptake. These results are similar to a previous report by Black et al., 1990, where 32 μ g of CsA was detected in the dermis with negligible systemic exposure. This result shows that the designed

formulation has a potential to be used as a topical drug delivery device in transplantation medicine where no systemic exposure is required.

In vivo evaluation of the safety and efficacy of topical administration of drug-loaded TyroSphere™ dressings in 1% (w/v) HPMC gel in a nonhuman primate model

In the MGH baboon split thickness skin graft (STSG) model, four baboons received four STSGs from a GalT-KO swine followed by four STSGs from an allogeneic donor on full thickness wound beds. These grafts were treated every other day with drug-loaded TyroSphere™ dressings until the grafts were completely rejected (providing less than 10% wound coverage).

Topical application of 30% (w/w) CsA-loaded TyroSphere™ dressings failed to significantly prolong skin graft survival

Xenografts survived a median of 12.5 days when treated topically with TyroSphere™ dressings compared to a median of 14 days when treated with a standard skin graft dressing, bacitracin™ ointment ($p=0.27$) (Table 2). A biopsy sample was examined microscopically and showed early signs of rejection in the experimentally treated xenografts, which was similar to the appearance of grafts treated with bacitracin™ only. Allografts from another nonhuman primate survived a median of 14 days when treated topically with the TyroSphere™ dressings compared to a median of 13 days when treated with standard graft dressings ($p=0.80$) (Table 2). Biopsy samples of allografts were examined microscopically, demonstrating early signs of rejection in both experimentally treated and control allografts.



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Topical application of 30% (w/w) FK506-loaded TyroSphere™ dressings also failed to significantly prolong skin graft survival

Xenografts survived a median of 12 days when treated topically with TyroSphere™ dressings compared to a median of 14 days when treated with bacitracin™ ointment (p=0.16) (Table 2). Biopsy samples of the xenografts showed microscopic evidence of inflammation present in the skin graft tissues that was similar in appearance between experimentally treated and control grafts. Allografts survived a median of 14 days when treated with the TyroSphere™ dressings compared to a median of 13 days when treated with standard skin graft dressings (p=0.80) (Table 2). As with the xenografts, biopsy samples of the allografts showed microscopic evidence of inflammation in both experimentally treated and control grafts.

In vivo evaluation of the safety and efficacy of wound bed application of powdered drug-loaded TyroSphere™ dressings and topical administration of drug-loaded TyroSphere™ dressings in 1% (w/v) HPMC gel in a nonhuman primate model

Effects of wound bed and topical application of 30% (w/w) CsA-loaded TyroSphere™ dressings on skin graft survival

Xenografts from a GalT-KO swine survived a median of 10 days when both the wound beds and the grafts were treated topically with TyroSphere™ dressings compared to a median of 14 days when treated with standard skin graft dressing, bacitracin ointment (p=0.0002) (Table 2). As seen in the previous two groups, biopsy samples of the

xenografts revealed microscopic evidence of inflammation in the skin grafts, similar to control-treated grafts. Allografts from another nonhuman primate survived a median of 11 days when treated topically with the TyroSphere™ dressings compared to a median of 14 days when treated with standard graft dressings (p=0.0009) (Table 2). Biopsy samples from the allografts revealed microscopic evidence of a dead upper layer of the skin graft, with much inflammation present in the deeper layers of the allografts. Biopsies from control-treated allografts showed only mild changes in the upper layers of the grafts, with less inflammation present in the deeper layers.

Effects of wound bed and topical application of 30% (w/w) FK506-loaded TyroSphere™ dressings on skin graft survival

Xenografts survived a median of 10 days when treated topically with TyroSphere™ dressings compared to a median of 14 days when treated with bacitracin™ ointment (p=0.0002) (Table 2). Allografts survived a median of 12 days when treated with the TyroSphere™ dressings compared to a median of 13 days when treated with standard skin graft dressings (p=0.0011) (Table 2). Biopsy samples of the xenografts showed microscopic evidence of a dying upper layer of the skin graft but very minimal inflammation in the upper layers of the xenograft. Deeper in the skin graft, there was much less inflammation present than is typically seen in control-treated grafts. This observation is extremely interesting as it suggests that treatment with FK506-loaded TyroSphere™ dressings did have an effect on the graft. Further studies are required to determine if this effect is clinically significant.

Table 2. Median skin graft survival (in days) compared to standard (bacitracin™) treated xenografts and allografts. The p-values listed are from statistical comparisons between the experimental and standard treatments.

Skin Graft Treatment	Xenografts Survival (days)	P-value	Allograft Survival (days)	P-value
Control (Bacitracin)	14	--	13	--
Topical CsA Only	12.5	0.27	14	0.80
Topical FK506 Only	12	0.16	14	0.80
Topical and Wound Bed CsA	10	0.0002*	11	0.0009*
Topical and Wound Bed FK506	10	0.0002*	12	0.0011*

* indicates statistical significance compared to control treatment

Unfortunately, treatment with CsA- or FK506-loaded TyroSphere™ did not significantly prolong graft survival compared to control treatment. One possible reason is that the dose, timing of application, and/or release kinetics evaluated in this study was suboptimal. Another possibility is that the drugs effectively reduced inflammation to the point that it prevented blood vessels from growing into the skin grafts for nutritional support, thus after so many days without nourishment, the grafts died and fell off. Microscopic evidence to support this theory is present in the last group, where drastically reduced inflammation was observed. To overcome this problem, the team proposed using a skin flap model in future experiments that will have its own primary vascular supply. Also, it is possible that local immunosuppression should not completely eliminate systemic immunosuppression, but rather should be used with a reduced dose of systemic immunosuppression. Finally, it is possible that the drugs' mechanisms of action were not suitable for topical therapy and a new drug with a different mechanism of action should be tested.

Conclusions

TyroSphere™ dressings were successfully formulated to provide sustained release of two highly lipophilic and very potent immunosuppressive agents: CsA and FK506. No significant prolongation of skin graft survival was observed in vivo with topical

or topical and wound bed application of CsA- or FK506-loaded TyroSpheres™ dressings. However, the application of FK506-loaded TyroSphere™ dressings both topically and to the wound bed significantly reduced inflammation in the skin grafts and wound beds. It is possible that these skin grafts failed due to a lack of nutrition from blood vessels that normally grow into the skin graft. To overcome this barrier, the team proposed using skin flaps in the future that will have an established major blood supply. The in vivo data confirmed the biocompatibility of the drug-loaded TyroSpheres™-gel, but the efficacy results suggest that further work is required to optimize the topical delivery of immunosuppression for the treatment of graft rejection.

Future Research Plans

Future work will focus on the use of a different model (a skin flap with an established blood supply) and different immunosuppressive agents, which have different mechanisms of action in vivo, and offer promise as topical immunosuppression dressings.

Planned Clinical Transitions

The project team will conduct safety and efficacy studies (TRL 5) in Good Laboratory Practices guidance, submit an Investigational New Drug application, and initiate the design of a clinical trial.





Soft Tissue Regeneration

Development of Human Lips for Facial Reconstruction

Project 4.5.2b, RCCC

Team Leader(s): Stephen E. Feinberg, DDS, PhD (University of Michigan)

Project Team(s): Zhang Wen, MD, Cynthia L. Marcelo, PhD, Shiuhyang Kuo, DDS, PhD, Eve Bingham, BS, Tarik Elarini, MD, and Dongie Ye, DDS (University of Michigan)

Collaborator(s): Paul Cedernas, MD and Melanie Urbanchek, PhD (University of Michigan)

Therapy: Facial reconstruction technologies for soft tissue injuries

Deliverable(s): A whole or part of a "set" of human lips

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 3

Key Accomplishments: In the first year of funding, the researchers fabricated a three-dimensional (3D) tissue structure with the key morphological features of a lip: epidermal skin, vermillion, and oral mucosa. This tissue-engineered mucosa-and-skin equivalent was manufactured using human oral and skin cells to produce similar anatomic and handling properties as native human lips. The researchers also developed an animal (rat) model for in vivo grafting of the lip construct (made of mucosa and skin components) and for testing functional muscle performance.

Key Words: tissue engineering; regenerative medicine; lips; human; autogenous

Introduction

The need for improved technology for craniofacial reconstruction is imminently demanded for battle-field injuries (BI) due to the exceptionally high prevalence of craniofacial injuries in combat. A recent study analyzed BI to the American soldiers during the Iraq/Afghanistan combats according to the type/distribution/mode of injury, utilizing the Joint Theatre Trauma Registry (JTTR) database. The JTTR was queried from October 19, 2001, to December 12, 2007, for BI and entered into the database using ICD-9 codes (International Statistical Classification of Diseases and Related Health Problems). The study identified 7,770 BI, approximately 26% of which were maxillofacial injuries. Eighty-four percent of maxillofacial injuries were caused by explosive devices; lacerations and open soft tissue wounds (including avulsion) occurred in 58% of these cases. According to the JTTR database study, the incidence of maxillofacial BI in Iraq and Afghanistan is significantly higher compared to previous American combats. Thus, the focus on soft tissue substitutes is exceptionally needed for the reconstruction of the significant number of soft tissue injuries from explosive devices.

Research Progress – Year 1 (Project commenced in 2012)

In vitro development of muco-cutaneous (M/C) constructs composed of both oral mucosa and skin keratinocytes with formation of a vermillion (Specific Aim 1)

The University of Michigan reports for the first time the fabrication of a 3D tissue structure containing, in a continuous layer, the morphological features of a lip: epidermal skin, vermillion, and oral mucosa. This tissue engineered M/C equivalent was manufactured using human oral and skin keratinocytes grown on an acellular, nonimmunogenic dermal equivalent (AlloDerm®) to produce a tissue equivalent with similar anatomic and handling properties as native human lips. Confirmation of the structural composition of the construct was performed using routine histology and immunohistochemistry (**Figures 1 and 2**) by identification of epithelial markers that are differentially expressed in separate anatomic areas of the lips. These full-thickness human lip skin equivalents can be used in surgical lip reconstruction in individuals suffering from lip loss from cancer, congenital deformations, and injuries after accidents.

In vivo development of a rat model for fabrication and rotation of prelaminated flaps with a functional stoma (Specific Aim 2)

The University of Michigan has successfully developed a rat model that can be utilized to assess the in vitro-developed M/C constructs after they are grafted onto the latissimus dorsi muscle in regard to perfusion in situ and musculature contraction of a created stoma within the M/C construct.

Conclusions

The University of Michigan reports for the first time the fabrication of a 3D tissue structure containing, in a continuous layer, the morphological features of a lip: epidermal skin, vermillion, and oral mucosa. The team has also successfully developed a rat model that can be utilized to assess the in vitro-developed M/C constructs after grafting onto the latissimus dorsi muscle in regard to perfusion in situ and musculature contraction of a created stoma within the M/C construct.

Future Research Plans

The researchers plan in the future to: (1) extend these studies to the use of athymic rats and use of a cellular M/C construct to fabricate the pre-laminated flap; (2) extrapolate these techniques to tissue

engineering of an anal sphincter; and (3) submit an Investigational New Drug (IND) application to the U.S. Food and Drug Administration to perform a human clinical trial for lip reconstruction.

Planned Clinical Transitions

An important advantage of the technique presented is that the 3D lip constructs are manufactured in serum-free medium modified to contain totally defined animal-free supplements and without the use of feeder layers. This culturing approach will allow grafting of the 3D lip constructs back into autogenous human recipients with minimal risk of cellular cross-contamination or immunological rejection. The approach presented in tissue engineering 3D lips will have several advantages for the reconstruction of craniofacial soft tissue injuries. In particular, the source of the cells to develop the 3D skin lips constructs will come from small punch biopsies from the oral mucosa and skin of the patient, thus making the construct autochthonous.

The University of Michigan has two ongoing clinical trials using a tissue-engineered oral mucosa manufactured in a similar way to the M/C constructs. The plan is to submit an amendment to the IND that is presently being used for the clinical trials to assist in initiating a clinical trial for lip reconstruction.

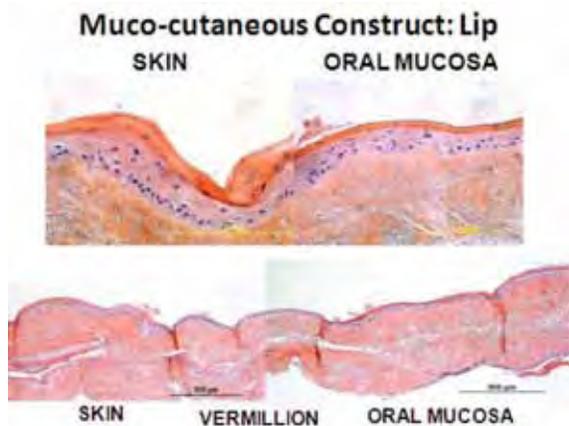


Figure 1. A histological section of a M/C construct showing (top) a different keratinizing pattern between skin (keratin) and oral keratinocytes (parakeratin) on the same dermal construct; (bottom) both skin and oral keratinocytes on the same dermal base comingling in the center to form a third distinct anatomical region, the vermillion, that contains a thinner keratinization layer than either skin or oral mucosa.

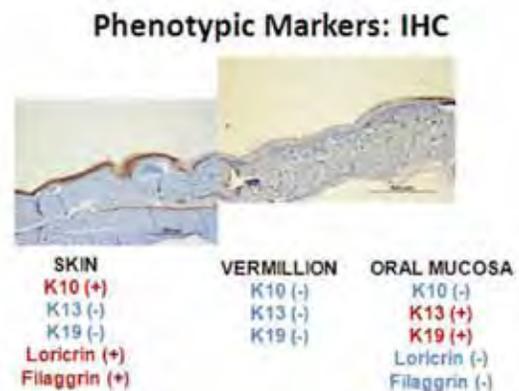


Figure 2. Immunohistochemistry of the tissue section shown in Figure 1, which reveals three distinct phenotypic regions – skin, vermillion, and oral – that correspond to the phenotype that is normally seen in a histologic section of the lip.



Cartilage Regeneration (Focus: Ear)

Engineered Cartilage Covered Ear Implants for Auricular Reconstruction

Project 4.1.1, WFPC

Team Leader(s): James J. Yoo, MD, PhD (Wake Forest University)

Project Team(s): Sang Jin Lee, PhD, John Jackson, PhD, Chang Mo Hwang, PhD, Bu-Kyu Lee, DDS, PhD, Young Min Ju, PhD, Hyun-Wook Kang, PhD, Cheil Kim, MD, PhD, Cara Clouse, DVM, Carlos Kengla, BS, and Denethia Green, BS (Wake Forest University)

Collaborator(s): Molly Purser, PhD, Richard Wysk, PhD, Rohan Shirwaiker, PhD, Ola Harrysson, PhD, and Paul Cohen, PhD (North Carolina State University)

Therapy: Reconstruction of the external ear

Deliverable(s): Engineered cartilage covered alloplastic implants; three-dimensional (3D) bioprinted ear constructs

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers optimized their isolation and expansion protocols, which yielded an increased production of human chondrocytes. They developed and fabricated a flexible and complex shaped ear scaffold using an integrated 3D organ printing system. They established a 3D bioprinting protocol for engineering ear cartilage tissue constructs. The researchers' implanted 3D bioprinted ear constructs resulted in the formation of cartilage tissue *in vivo*. They demonstrated the structural and functional integrity of the engineered cartilage covered ear implants *in vivo*.

Key Words: Auricular cartilage, chondrocytes, alloplastic ear implant, reconstruction, tissue engineering, 3D bioprinting

Introduction

Traumatic injuries constitute a major cause of morbidity and mortality for military personnel. The incidence of craniofacial injuries has rapidly increased due to the frequent ballistic and explosive injuries on the battlefield. Moreover, protruding tissues such as ear and nose are frequently affected. Although the loss of ear tissues does not pose life threatening danger, it is functionally and cosmetically debilitating, and hinders injured soldiers from returning to the society.

The standard treatment modality for external ear reconstruction uses autologous costal cartilage as a graft material; however, autologous costal cartilage is limited in supply, provides inadequate dimensions, and is progressively absorbed after implantation. Currently, alternative approaches utilize alloplastic ear implant devices composed of silicone or polyethylene. These implants are approved by the U.S. Food and Drug Administration (FDA), and they are nontoxic, cause minimal foreign body reactions, and possess adequate mechanical properties for use in non-load-bearing tissues of the craniofacial region. Although alloplastic ear implants are

able to effectively eliminate the morbidity associated with the costal cartilage graft, the use of these implants is often associated with complications, including inflammation, infection, erosion and dislodgement. As a result, implant extrusion occurs frequently due to the limited vascularization and constant abrasion against the surrounding tissues.

In this project, the research team demonstrates that 3D bioprinting system can be used for the production of a complex human ear-shaped tissue construct with living cells. More importantly, the ear-shaped constructs are able to form cartilage tissue that possesses the characteristics of human auricles when implanted *in vivo*. Creation of cartilage tissue using a soldier's own cells would bring enormous benefits and minimize the morbidity associated with implant dislodgement.

Research Progress – Year 5

3D bioprinting of customized ear implant *Customized ear-shaped scaffold construction using an integrated organ printer system*

The researchers investigated whether their 3D bioprinting system could be used to construct a

complex shaped human ear consisting of cartilage cells. Their computerized bioprinting system is composed of a 3-axis stage, high precision pressure and temperature controller, and four cartridges. They used this system to generate a human-sized tissue engineered flexible ear cartilage construct. A computed tomography image (Figure 1A) was used to develop a motion program (Figure 1B), which involved the printing of three distinct biomaterials: cell (chondrocyte)-laden composite hydrogel, polycaprolactone (PCL), and Pluronic F-127. A tissue-engineered ear construct was printed using culture expanded rabbit ear chondrocytes (Figure 1 C, D). The printed ear-shaped constructs showed $91\pm 8\%$ cell viability at 1 day after printing ($n=3$). After 5 weeks of culture, the histological staining of the ear constructs showed the production of cartilaginous matrix (Figure 1F). The cells contained in the newly

formed tissue showed similar morphological characteristics as those in native ear cartilage.

In vivo evaluation of the 3D bioprinted ear tissue constructs

To evaluate in vivo stability of the bioprinted ear constructs, the researchers implanted the printed constructs subcutaneously in mice. The implanted ear constructs showed no evidence of skin necrosis, implant exposure or extrusion. The retrieved ear constructs showed similar mechanical characteristics (elasticity) as natural ear cartilage. Histomorphological evaluation showed the formation of neocartilage within the implants. The researchers consistently observed evenly dispersed triangular and ovoid-shaped chondrocytes with lacunae, surrounded by perichondrium. Safranin-O staining confirmed the presence of sulfated glycosaminoglycans, indicating the formation of a mature neocartilage framework.

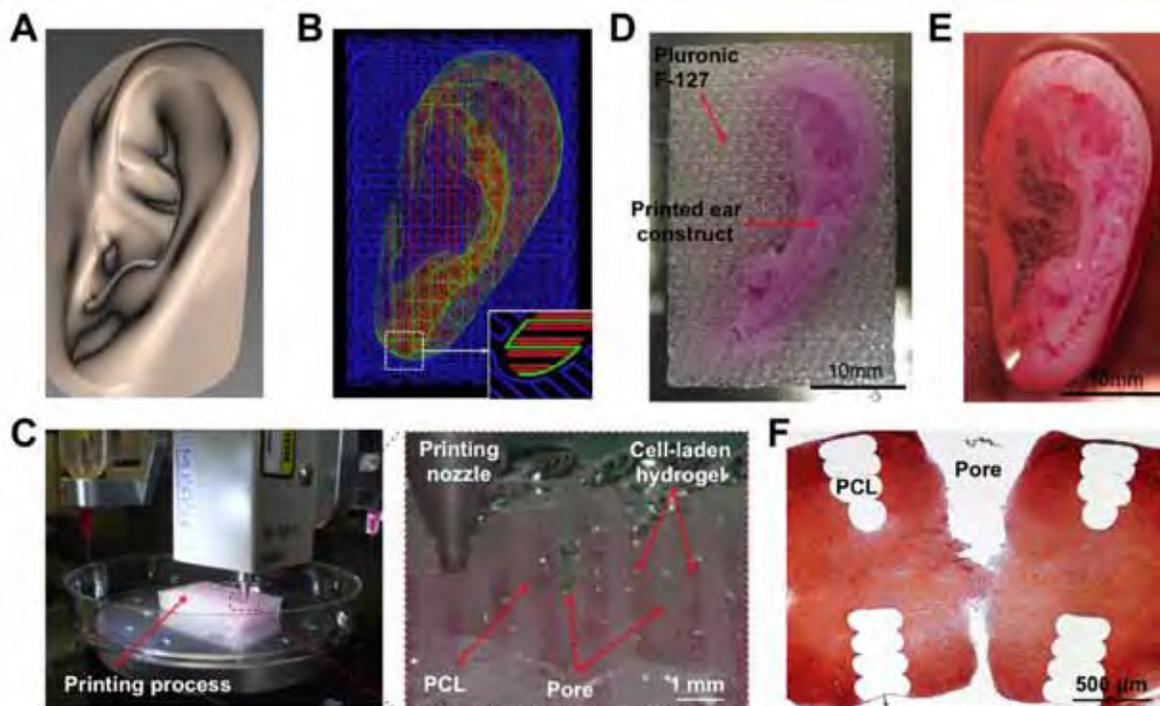


Figure 1. Ear cartilage reconstruction: (A) 3D computer-aided design (CAD) of a human ear. (B) Visualized motion program used to print 3D architecture of human ear. The motion program was generated by using 3D CAD model (A). Lines of green, blue, and red colors indicate the dispensing paths of PCL, Pluronic F-127, and cell-laden hydrogel, respectively. (C) 3D printing process using the integrated organ printing system. The image shows patterning of a layer of the construct. Photographs of the 3D printed ear cartilage construct (D) with sacrificial Pluronic F-127 and (E) after removing sacrificial material by dissolving with cold medium. (F) Safranin-O staining of the 3D printed ear cartilage construct after being cultured in chondrogenic medium for 5 weeks in vitro. The staining indicates the production of glycosaminoglycans.



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The researchers' main goal of ear reconstruction is aesthetic recovery. To achieve this goal, ear tissue constructs should be made with consideration of symmetry between the two sides. Mirroring of an existing ear can aid in achieving high symmetry between ears. As such, 3D bioprinting can readily generate patient-specific 3D tissue constructs with symmetry. In this study, the researchers demonstrated that computerized bioprinting technology can be utilized to fabricate a clinically relevant scaffolding system for auricular cartilage tissue regeneration. They fabricated flexible ear-shaped scaffolds based on a 3D CAD model using a CAD/computer-aided manufacturing process in an in vivo experiment. These results show that bioprinting of ear tissue constructs has the potential to produce patient-specific complex ear-shaped constructs with reproducible cartilage formation in vivo.

Conclusions

The research team has demonstrated that cartilage tissues can be engineered to serve as a biological cover for a commercially available ear implant. Their results indicate that a bioengineered cartilage covering the ear-shaped implants improves the interface between the implant and surrounding host tissue, thus overcoming many of the limitations associated with the use of alloplastic implants for auricular cartilage tissue reconstruction. Isolation and growth of human chondrocytes from several sources were performed. Identification of the optimal tissue source for covering an implant will

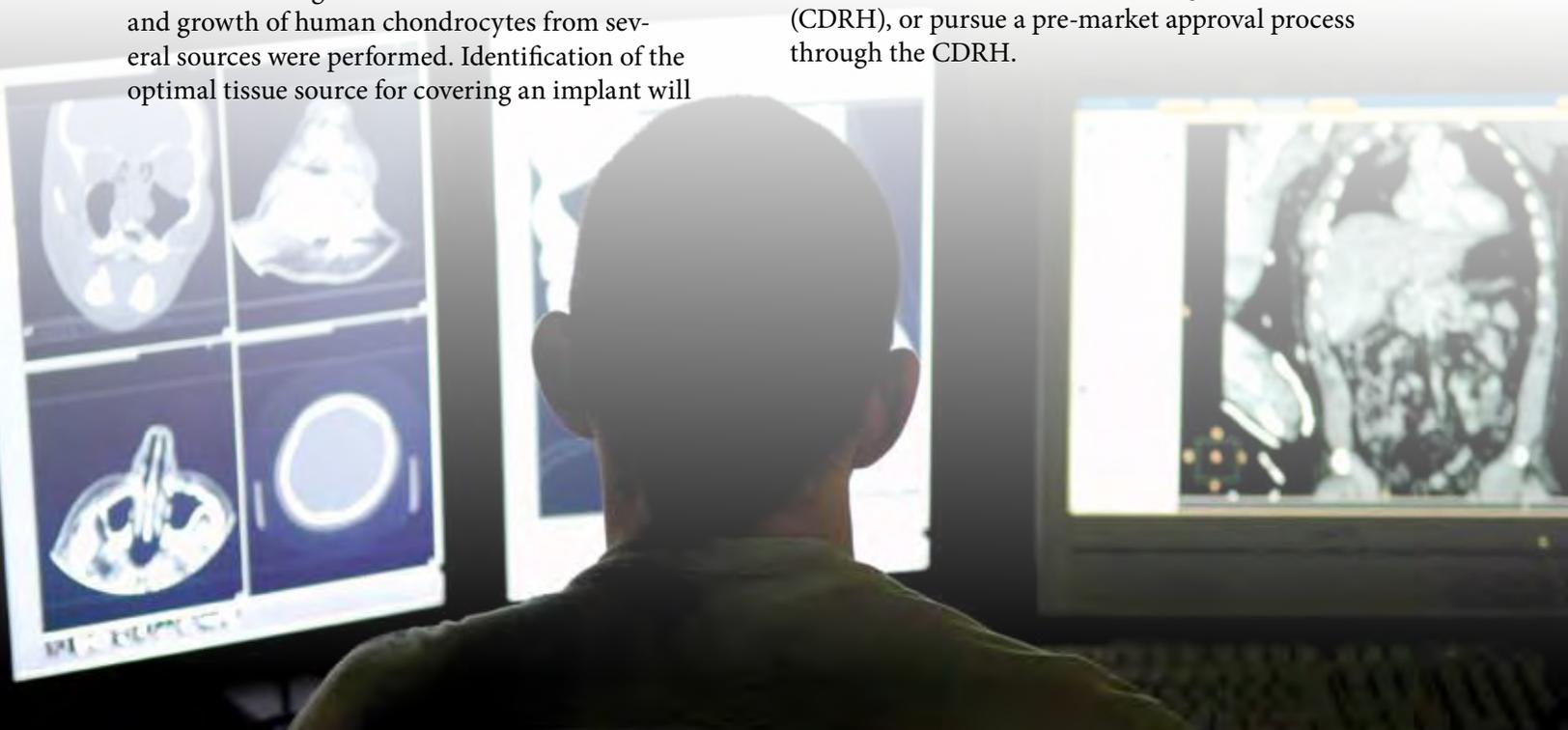
be important for future clinical applications. The researchers also successfully demonstrated that a 3D bioprinting system can be used to produce a complex human ear-shaped tissue construct with living cells. A unique aspect of this technology is the ability to use the contralateral side of the patient as a template for bioprinting a damaged or injured tissue. Overall, the research team's results demonstrate that a 3D bioprinting system may allow for the generation of clinically relevant tissue-engineered products that can immediately replace the damaged or injured tissues.

Future Research Plans

The research team will continue to optimize the customized ear implants using the 3D bioprinting system. In vivo animal studies using 3D printed ear constructs are currently being conducted.

Planned Clinical Transitions

The researchers are planning to contact the Office of Combination Products (OCP) at the FDA to confirm the classification of their 3D bioprinter and, if needed, file a request for designation with OCP in accordance with 21 Code of Federal Regulations Part 3. Their approach will be to apply for a Humanitarian Device Exemption or an Investigational Device Exemption through the Center for Devices and Radiological Health (CDRH), or pursue a pre-market approval process through the CDRH.



Cartilage Regeneration (Focus: Ear)**Engineering of a Replacement Autologous Outer Ear Using a Collagen/Titanium Platform****Project 4.5.4, RCCC**

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

Project Team(s): Mack Cheney, MD, Tessa Hadlock, MD, and Marc Hohman, MD (Massachusetts Eye and Ear Infirmary, Facial Plastic Surgery); Irina Pomerantseva, MD, PhD, Craig Neville, PhD, Nina Joshi, MS, Tom Cervantes, BS, and Anya Kimura, BA (MGH Tissue Engineering); Mark A. Randolph (MGH Plastic Surgery Research Laboratory)

Collaborator(s): Nicholas Roscioli (Kensey Nash Corp.)

Therapy: Treatment options using tissue engineered cartilage constructs for ear replacement

Deliverable(s): A permanent, implantable, engineered, living external ear replacement

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers expanded cartilage cells (chondrocytes) successfully and sufficiently without losing the cells' ability to form neocartilage. They engineered high-quality stable cartilage in both immunocompromised rodents and large immunocompetent animals, and made ear-shaped neocartilage from chondrocytes in sheep. The ear construct's size and shape were largely maintained after 12 weeks implantation in the animal models. The team developed analytical non-invasive methods for assessing three-dimensional (3D) changes, which suggest that the wire framework properties could be improved to eliminate shape changes due to wire sliding.

Key Words: tissue engineered ear; cartilage; porous collagen; chondrocytes

Introduction

Following blast injuries to the head and neck, limited options exist for reconstructing the external ear. Current clinical approaches include implantation of a cartilage framework hand-carved from the patient's own (autologous) rib cartilage; or a synthetic ear-shaped implant made of porous polyethylene (Medpor®). Both options are prone to complications, require multiple surgeries, and have unpredictable and often poor cosmetic outcomes. The MGH-engineered autologous ear replacements combine the best of current clinical approaches: the precisely defined architecture of Medpor® implants and the autologous properties of carved cartilage. The goal of this project is to expedite the development of a permanent, implantable, living external ear for the injured Service member, and to achieve cosmetic outcomes that meet patient expectations.

Research Progress – Year 5**Develop reliable cell source**

Native autologous chondrocytes remain the best source for engineered auricular cartilage. For an

adult-sized ear, 100–150 million chondrocytes are required. In a clinical setting, the source of healthy cartilage for acquiring autologous chondrocytes is restricted. A small biopsy of healthy cartilage will likely serve as the source of autologous chondrocytes for a wounded warfighter. The desired cell number can be achieved by extensive chondrocyte proliferation in vitro. However, chondrocytes de-differentiate with repeated passaging, losing their potential to form neocartilage.

Demonstrate neocartilage formation and stability in immunocompromised animals (animal and human cells) using re-differentiated chondrocytes expanded beyond 300-fold

The researchers engineered high quality contiguous cartilage from extensively expanded chondrocytes in nude rats after 6 and 12 weeks. They confirmed the neocartilage quality histologically and bio-chemically; the quality improved with increased implantation time. Chondrocytes are known to de-differentiate and lose their ability to form high quality cartilage after extensive expansion in traditional



III: Craniofacial Reconstruction

culture; however, the research team was able to effectively prevent de-differentiation.

Demonstrate neocartilage formation and stability in an autologous sheep model using re-differentiated chondrocytes expanded beyond 300-fold

The researchers engineered simple-shaped (disk) neocartilage from extensively expanded chondrocytes after 6 and 12 weeks implantation in sheep. Histologically, robust contiguous neocartilage was generated at both time points; the quality of neocartilage remained stable as evidenced by the intensity and distribution of glycosaminoglycans (GAGs) stained with toluidine blue and safranin-O and cartilage-specific collagen type II. The neocartilage quality improved with increasing implantation time based on the results of elastin staining. At 6 weeks post-implantation, the GAG content was approximately half of that in native cartilage, 84.6 ± 10.5 and 154.4 ± 8.0 $\mu\text{g}/\text{mg}$ dry weights, respectively ($p < 0.05$).

Assess shape and size retention and formation of neocartilage in the adult human ear-shaped scaffolds in vivo

Evaluate retention of original ear size and shape in sheep, achieving TRL 4 upon completion

The researchers engineered composite ear-shaped constructs with internal wire supporting sheep using extensively expanded chondrocytes. The construct length was reduced $6.3 \pm 2.1\%$ and width was reduced $5.7 \pm 1.6\%$ after 12 weeks in vivo, consistent with the previously reported data on ear-shaped constructs with embedded wire support. This is the first demonstration of ear-shaped implant engineered with extensively expanded chondrocytes in a pilot sheep study.

Modify ear scaffold design

Shape distortion is usually quantified based on a comparison of 2D contours. However, this approach is limited because it does not characterize the full 3D geometry of the ear construct. The MGH team developed a new analytical methodology to compare the 3D structure of engineered ears in a minimally invasive approach based on high-resolution computed tomography scans. The images were divided into segments, and 3D changes in each segment and the overall wire framework were measured and several parameters calculated. The areas of deformation were identified. The researchers used the results to drive a deterministic selection of wire

framework properties; this approach is useful to select possible alternative materials and corresponding range of wire diameters.

Crafting wire skeletons by hand is cumbersome and time-consuming; framework shape variability ultimately impacts the shape of the collagen ear scaffold. Furthermore, the irregularities create difficulties in the collagen molding process. The MGH team identified additional wire framework manufacturing processes: direct metal laser sintering and electron beam melting. Ear-shaped constructs with metal frameworks manufactured with these methods are being analyzed after 12 weeks implantation.

Conclusions

Chondrocytes were successfully and sufficiently expanded without loss of ability to form neocartilage. High quality stable cartilage was engineered in both immunocompromised rodents and large immunocompetent animals. Ear-shaped neocartilage was engineered from these chondrocytes in sheep. The ear construct size and shape were largely maintained after 12 weeks implantation. Analytical non-invasive methods for assessing 3D changes suggest that the wire framework properties could be improved to eliminate shape changes due to wire sliding.

Future Research Plans

Beyond AFIRM I, the MGH team will continue developing clinically relevant cell sources for the engineered ear. Long-term stability of cartilage engineered from extensively expanded chondrocytes and retention of the size and shape of ear-shaped scaffolds will be assessed in an immunocompetent animal model.

Planned Clinical Transitions

The MGH research team will submit a Request for Designation to the U.S. Food and Drug Administration to determine the regulatory path for the engineered ear. In preparation for clinical trials, researchers will perform a Good Laboratory Practices preclinical trial in sheep to demonstrate the safety and efficacy of the engineered ear. The team will develop a protocol for a pilot clinical trial, and submit it to the local Institutional Review Board.

Clinical Trials**Clinical Trial – Composite Tissue Allograft Transplantation (Face)****Project 4.3.1a, RCCC**

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

Project Team(s): Cheryl Smith, BSN, RN (Cleveland Clinic)

Collaborator(s): Col. Robert Hale, DDS (U.S. Army Institute of Surgical Research)

Therapy: Facial transplantation to replace missing components replaced by human tissues of the same quality, color and texture with lifelong immunosuppression

Deliverable(s): Composite tissue allograft transplantation procedure capable of restoring function and aesthetic appearance of the face

TRL Progress: Beginning, TRL 5; Current, TRL 6; AFIRM I Target, TRL 7

Key Accomplishments: During the past year, the research team completed five detailed evaluations on potential face transplant subjects enrolled in the study and approved one patient for listing for face transplantation. Based upon 52 months of data collected and analyzed from an individual who received a face transplant by the research team prior to the start of AFIRM, face transplantation appears to be a safe and effective, single-stage treatment procedure for subjects with severe facial deficit and deformity. Through objective testing, face transplantation has been shown to provide a return of mastication, the ability to speak clearly, smell, smile, frown, and kiss. In addition, an improved self-image has been shown. Potential serious adverse events, such as infection and rejection, which may occur with any transplant, have been successfully treated.

Key Words: Siemionow; transplant; allograft; immunosuppression

Introduction

Recent military conflicts have created a patient cohort (approximately 400 identified patients) that could benefit from vascularized composite allograft transplantation of the face, upper extremity, or other tissue. Over 150 facial trauma or burn patients may benefit from this type of procedure. This estimate includes burn patients who served in Operation Iraqi Freedom and/or Operation Enduring Freedom only. The current status of need in the civilian population is forecasted to be 272 patients. An unmet clinical need exists for a single surgical procedure (treatment) that is capable of restoring the function and aesthetic appearance of the face. Transplantation of composite face/bone allograft to reconstruct complex, war-related craniofacial defects will provide superior results in a single surgical procedure. This project addresses several critical military capability gaps: Inability to repair/replace neuromuscular tissue units of the face, replace missing or damaged composite facial features, match elasticity pigmentation to normal skin, and lack of implantable vascular scaffold.

The first U.S. face transplant was carried out at the Cleveland Clinic in December of 2008 (prior to AFIRM) under the Institutional Review Board (IRB)-approved protocol 6914: "Protocol for Composite Facial Allograft Transplant." This patient is now 52 months post-transplant and living at home independently. The graft is maintained under minimal immunosuppression, and two rejection episodes were successfully treated. Pain has decreased and the patient, through continuous testing, reports a superior aesthetic outcome in comparison to prior reconstructive procedures. In addition, important functions of facial structures have returned such as mastication, normal swallowing, breathing through the nose, and drinking from a cup. The percutaneous endoscopic gastrostomy tube was removed and the tracheostomy closed. Speech is intelligible, and the ability to smile, kiss, and express emotions through facial features has returned. Over 4 years of post-transplant outcomes have been collected, analyzed, and reported at AFIRM meetings.



Clinical Trial Status

Referrals and Screening

In Year 5, five patients were identified and screened under the inclusion/exclusion criteria of the Cleveland Clinic IRB-approved protocol (see below). The project achieved a major milestone in that one of the five screened patients was officially approved for listing for a face transplant. To increase subject screening and enrollment from civilian and military hospitals, an information sheet for physicians was created for distribution to referring physicians. IRB and Human Research Protection Office (HRPO) approval was obtained prior to distribution. The cost of a face transplant, immunosuppression, and physician office visits is covered by the grant for the life of the patient, or until funding is exhausted.

Inclusion/Exclusion Criteria

Inclusion Criteria

1. Subject must be willing to sign the informed consent and agree to all follow-up procedures, including the realistic understanding of the impact of face transplantation on their lifestyle.
2. Subject will be evaluated by a plastic surgeon to assess indications for facial transplantation based on severity and complexity of facial deformity.
3. Subject must be between the ages of 18 and 60.
4. Subject must be willing to undergo a psychiatric and social services pre-transplantation evaluation.
5. Subject must be willing to undergo major face surgery.
6. Subject must be a candidate for general anesthesia.
7. Subject must be willing to comply with post-transplant physical therapy.
8. Subject must be willing and able to receive potent drugs to induce and maintain immunosuppression and follow the infection prophylaxis protocol.
9. Subject must be willing to receive standard vaccinations such as influenza, pneumococcus, and hepatitis B.

10. Subject must be free of malignant tumors for 5 or more years, with the exception of certain skin cancers.

Exclusion Criteria

1. Subject that shows history of persistent non-compliance.
2. Psychiatric evaluation findings that may indicate non-compliance or mental instability.
3. Presence of an active infection including Human Immunodeficiency Virus, mycobacteria, hepatitis B, and hepatitis C.
4. Presence of an occult infection (e.g., dental abscess, urinary tract infection, tuberculosis, or history of systemic/occult infection within 3 months of surgery).
5. Any current chemical dependency including alcohol.
6. Subjects at high risk for the recurrence of malignancy with the exception of certain skin cancers.
7. Any diagnosis that the qualifying plastic surgeon feels would put the subject at high risk for the surgical procedure of face transplantation.
8. Subjects who do not have adequate donor site tissue available for coverage in the event of face transplant failure.

Transplant Process

Subjects are evaluated by a multidisciplinary transplant team to determine the best surgical approach for each patient. Transplantation evaluation is comprehensive, and each patient receives the following consults: transplant infectious disease, psychiatry, ethics, social work, and anesthesia. All subjects undergo extensive laboratory, diagnostic, and radiologic testing during evaluation. The Principal Investigator and multidisciplinary transplant team analyze and discuss all results, and they consult with the goal of optimal patient reconstruction. Due to the complexity and need for lifelong immunosuppression, a Participant Protection Liaison is assigned to all potential face transplant recipients with reporting requirements to the Cleveland Clinic IRB.

Conclusions

Face transplantation is a safe and effective single stage treatment procedure for subjects with severe facial deficit and deformity. Through objective testing, face transplantation has been shown to provide a return of functional deficits such as mastication, the ability to speak clearly, and the return of sensation. In addition, an improved self-image has been shown along with the return of facial mimetics, such as smile, frown, and the ability to kiss. Potential serious adverse events, such as infection and rejection that may occur with any transplant,

have been successfully treated. No serious adverse events have been reported during the screening process for face transplantation.

Future Clinical Plans

The two major goals for the upcoming year are to (1) perform one face transplant and (2) officially list a second patient for face transplantation. Patient safety and monitoring regulations will be followed according to IRB and HRPO regulations. All IRB and HRPO reporting and accreditation requirements will be met.





Clinical Trials

Clinical Trial – Anti-TCR Monoclonal Antibody (TOL101) for Prophylaxis of Acute Organ Rejection in Patients Receiving Renal Transplantation

Project 4.3.1b, RCCC

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

Project Team(s): Stuart Flechner, MD, David Goldfarb, MD, Richard Fatica, MD, Jon Myles, MD, Andres Chiesa-Vottero, MD, and Cheryl Smith (Cleveland Clinic); Tolera Therapeutics, Inc.

Collaborator(s): University of Colorado, Baylor University Medical Center, Cleveland Clinic, Medical University of South Carolina, St. Barnabas Medical Center, University of Michigan, University of Utah, University of Kentucky, and University of Virginia

Therapy: Transplant treatment that minimizes risk of infection and malignancies

Deliverable(s): TOL101, which is an anti $\alpha\beta$ T-cell receptor (TCR) murine IgMK monoclonal antibody

TRL Progress: Beginning, TRL 4; Current, TRL 7; AFIRM I Target, TRL 7

Key Accomplishments: To date, a total of 36 subjects (nine cohorts) have been enrolled in the study, and a total of 60 biopsies were sent to the Cleveland Clinic Pathology Core for analysis. A Clinical Study Report was prepared by Tolera Therapeutics, Inc. and presented to the U.S. Food and Drug Administration (FDA). Drug-related adverse events were limited to a transient rash with pruritus (one subject) in cohort 9. Twenty-seven serious adverse events have been reported, with only one (pneumonia) possibly related to the study drug. The data demonstrated that although the total number of patients who have received TOL101 to date is relatively small, the benign safety profile and easily assessed pharmacodynamic target allows a dosing regimen to be selected for Phase III testing from the Part A phase of the Phase I/II study.

Key Words: TOL101; anti-TCR monoclonal antibody; renal transplantation

Introduction

Immunosuppressive therapies fall into two general categories according to their duration of use: (1) Induction therapy with antibodies, which are administered during surgery and for a short period of time following transplant surgery in an effort to render the immune system less able to mount an initial rejection response; and (2) maintenance immunosuppression with anti-metabolites and/or calcineurin inhibitors, which, because of nephrotoxicity, are initiated in kidney transplant recipients after the graft begins functioning in an effort to prevent rejection over the long term. Steroids are generally given in high doses during surgery to help prevent the acute rejection response and are then rapidly tapered to low maintenance doses or to complete withdrawal. A more specific approach to the prevention of acute organ rejection is by targeting the $\alpha\beta$ TCR alone, sparing T cells, which may provide similar or better efficacy than the currently

used T cell-depleting antibodies while carrying fewer risks in terms of development of opportunistic infections and malignancies.

This Phase I/II clinical trial is evaluating the safety, pharmacokinetics, pharmacodynamics and preliminary efficacy of ascending doses of the selectively blocking $\alpha\beta$ TCR antibody, TOL101, in subjects undergoing their first renal transplantation. The researchers hope to demonstrate the ability of TOL101 to decrease or eliminate the need for a lifelong immunosuppressive drug regimen. The broader impact of this therapy would be to use TOL101 as an induction therapy during composite face/bone allotransplantation to reconstruct complex, war-related craniofacial defects, and provide a safer antibody compared to currently used antibodies (e.g., Campath or antithymocyte globulin), which will expose military patients to lower incidence of side effects such as opportunistic infections, cytokine release syndrome, and development

of myeloproliferative diseases. The same unmet need exists for the civilian population.

The nonclinical studies conducted with TOL101 support the potential efficacy and safety of this novel antibody in renal transplant patients. Antibody formation, which is an expected consequence of administering murine antibodies to humans, and the potential for modest cytokine release at higher doses, were the only potential risks to humans identified in the TOL101 nonclinical studies. In this clinical trial, TOL101 is being studied as part of an immunosuppressive regimen that includes tacrolimus, mycophenolate mofetil, and steroids. The hypothesis of the clinical trial is that use of TOL101 will result in a lower incidence of opportunistic infections, faster recovery of the immune system of kidney allograft recipients, and better functional outcome (confirmed by standard kidney function assays and kidney biopsies).

Cleveland Clinic Pathology & Laboratory Medicine is serving as the central core laboratory for the analysis of all of the biopsy samples for the clinical trial. The Cleveland Clinic Pathology & Laboratory Medicine may also provide services during the next phase of clinical trials (Phase III). Approximately \$10 million in funding has been leveraged from Tolera Therapeutics, Inc., which is conducting the clinical trial. The ultimate goal of this project is to develop TOL101 as an induction therapy for vascularized composite allografts (face, hand, abdominal wall) used to reconstruct complex, war-related craniofacial and extremity defects. If this trial is successful in reducing side effects of immunosuppression, TOL101 may also be used off-label in other solid organ transplant recipients.

Clinical Trial Status

To date, a total of 36 subjects (nine cohorts) have been enrolled in the study and a total of 60 biopsies were sent to the Cleveland Clinic Pathology Core for analysis. A Clinical Study Report was prepared by Tolera Therapeutics, Inc. and presented to the FDA. Drug-related adverse events were limited to a transient rash with pruritus (one subject) in cohort

nine. Twenty-seven serious adverse events have been reported, with only one (pneumonia) possibly related to the study drug. The data demonstrated that although the total number of patients who have received TOL101 to date is relatively small, the benign safety profile (with the exception of known dose and infusion-related rashes) and easily assessed pharmacodynamic target (<25 CD3+ cells/mm³) allows a dosing regimen to be selected for Phase III testing from the Part A phase of the Phase I/II study.

The Phase I/II portion of the study is closed to enrollment (see “Future Clinical Plans” for details), and the Cleveland Clinic Pathology & Laboratory Medicine will serve as Core Lab Services for the remaining active patients in cohort 9.

Conclusions

Through grant support from AFIRM I/Department of Defense, the Cleveland Clinic Pathology Core Laboratory has successfully analyzed and reported biopsy results. Completion of the Phase I/II clinical trial in kidney transplants brings this novel therapy closer to the military goal of developing new and safer therapies for use during VCA transplants, such as the hand and face.

Future Clinical Plans

The FDA has agreed to a revised clinical development plan to modify the Phase I/II study protocol by eliminating Part B and proceeding directly into an adequately powered Phase III study. Therefore, the Phase I/II study is closed to further enrollment, and Tolera Therapeutics, Inc. is planning for study site closeout visits. All sites will remain active and able to respond to data queries through the database lock. Tolera Therapeutics, Inc. and Cleveland Clinic Pathology Core Laboratory will continue to monitor and complete biopsy reports on participants in this study. The receipt and analysis of biopsies from eight subjects enrolled in cohort 9 have completed dosing and are in the follow-up phase of the study.



Clinical Trials

Clinical Trial – Use of Tissue Engineered Human Oral Mucosa for Large Soft-Tissue Intra-Oral Defects

Project 4.5.2, RCCC

Team Leader(s): Stephen E. Feinberg, DDS, PhD (University of Michigan)

Project Team(s): Cynthia L. Marcelo, PhD, Hiroto Kato, DDS, PhD, Myra Kim (MK), ScD, Kevin Weatherwax, BA, Joseph I. Helman, DMD, Brent Ward, DDS, MD, James Washington, BS, Eve Bingham, BS, Shiuhyang Kuo, DDS, PhD, Kevin Tremper, PhD, MD, Blake Roessler, MD, William Giannobile, DDS, DMSc, and Mark Vartanian (University of Michigan); Jeffrey O. Hollinger, DDS, PhD (Carnegie Mellon University)

Collaborator(s): LifeCell Corporation, a subsidiary of Kinetic Concepts, Inc.

Therapy: Repair of intraoral soft tissue defects

Deliverable(s): Ex Vivo Produced Oral Mucosa Equivalent (EVPOME)

TRL Progress: Beginning, TRL 5; Current, TRL 5; AFIRM I Target, TRL 6

Key Accomplishments: In the first year of funding, the researchers have (1) developed a manufacturing process to fabricate large-sized EVPOMEs for the clinical trial, (2) successfully fabricated a large EVPOME device in a current Good Manufacturing Practices (cGMP) facility (3) received U.S. Food and Drug Administration (FDA) approval of an amendment to an already existing Investigational New Drug (IND) application, (4) obtained approval from the University of Michigan’s Institutional Review Board (IRB) for the clinical protocol, and (5) submitted the clinical protocol to the Human Research Protection Office (HRPO).

Key Words: tissue engineering; regenerative medicine; oral mucosa; human; autogenous

Introduction

Unmet need for military personnel: The need for improved technology for craniofacial reconstruction is imminently demanded for battlefield injuries due to the exceptionally high prevalence of craniofacial injuries in combat. Thus, the focus on soft tissue substitutes is exceptionally needed for the reconstruction of the significant number of soft tissue injuries from explosive devices.

Unmet need for the civilian population: One of the most difficult regions of the face to reconstruct after avulsion is the oral region, specifically the lips, due to the complex natural architecture of the lost tissue (compromising skin, oral mucosa, and muscle tissue) that is very difficult to replace.

This project addresses several critical military capability gaps for treatment of craniofacial injury: (1) Inability to restore complete functionality and aesthetics of the face (primary), (2) the need to use regenerative medicine and tissue engineering to replace missing or damaged composite facial features, and (3) the inability to repair/replace neuromuscular tissue units of the face.

The major goal of this project is to use a tissue engineering/regenerative medicine approach, in conjunction with the surgical technique of prelamination, to create a prevascularized composite soft tissue flap. The project involves the initiation and completion of a clinical trial titled “A randomized, parallel-group (autogenous EVPOME vs. AlloDerm® without incorporation of keratinocytes) study in subjects reconstructed with large defect mandibular resection in need of vestibuloplasty for dental rehabilitation.” The successful completion of this study will allow the investigators to utilize the EVPOME for major areas of intra-oral reconstruction, and as a platform technology for the tissue engineering of a set of human lips.

Clinical Trial Status (Project commenced in 2012)

Manufacturing of large 5 cm x 3 cm EVPOMEs

Materials and components design and fabrication of EVPOME

Since there was no commercial cultureware available to manufacture the 5 cm x 3 cm size of

EVPOME (**Figure 1**) that was necessary for this clinical trial, the team successfully designed and developed the necessary cultureware.

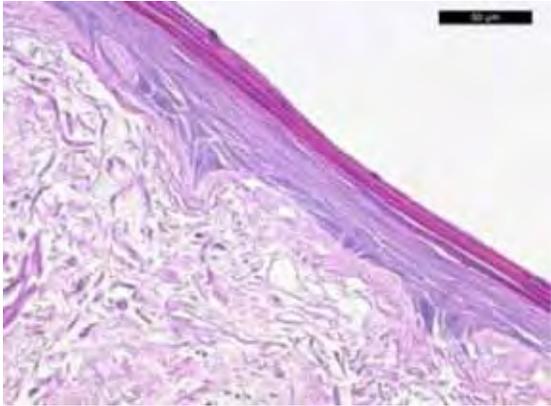


Figure 1. Histology of the large 5 cm x 3 cm EVPOME shows a well developed cellular layer on the dermal matrix, AlloDerm®.

Optimization of culture conditions

The research team optimized two critical parameters for the development of EVPOME. First, they used a MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in conjunction with histology to optimize the necessary cell seed density. Then, they determined the optimal thickness of AlloDerm® for use in the study.

In their first year of funding, the Feinberg team successfully fabricated a large EVPOME device in a cGMP facility based at the University of Michigan.

Submission of regulatory documents (IND, IRB)

Submission of amendments to an existing IND to Center for Biologics Evaluation and Research (CBER)/FDA

Dr. Feinberg has had several conversations with the FDA, and it was agreed upon that it would be more appropriate to submit an amendment to the presently active IND #10118 than to submit a new IND.

The amendment was submitted in March 2013 and approval was received on May 29, 2013.

Approval of clinical protocol by University of Michigan IRB (IRBMED)

The clinical protocol was successfully approved by the University of Michigan IRBMED. The approved protocol was subsequently submitted to the U.S. Army Medical Research and Materiel Command HRPO.

Conclusions

The Feinberg team successfully fabricated a large EVPOME device in a cGMP facility based at the University of Michigan. The IND amendment was approved by CBER and the clinical protocol was approved by the University of Michigan's IRB. Once the clinical protocol is approved by the HRPO, enrollment of subjects will begin.

Future Clinical Plans

Future plans include: (1) Coordination of this project with the Principal Investigator's other U.S. Department of Defense Phase IIA clinical trial entitled "A randomized, parallel-group [autogenous EVPOME] vs. palatal oral mucosa safety and efficacy study in subjects requiring additional keratinized oral mucosa for dental rehabilitation with endosseous dental implants" that is being conducted under IND #10118; and (2) submission of an IND to the FDA to perform a human clinical trial for lip reconstruction based on this project and the study under IND #10118.

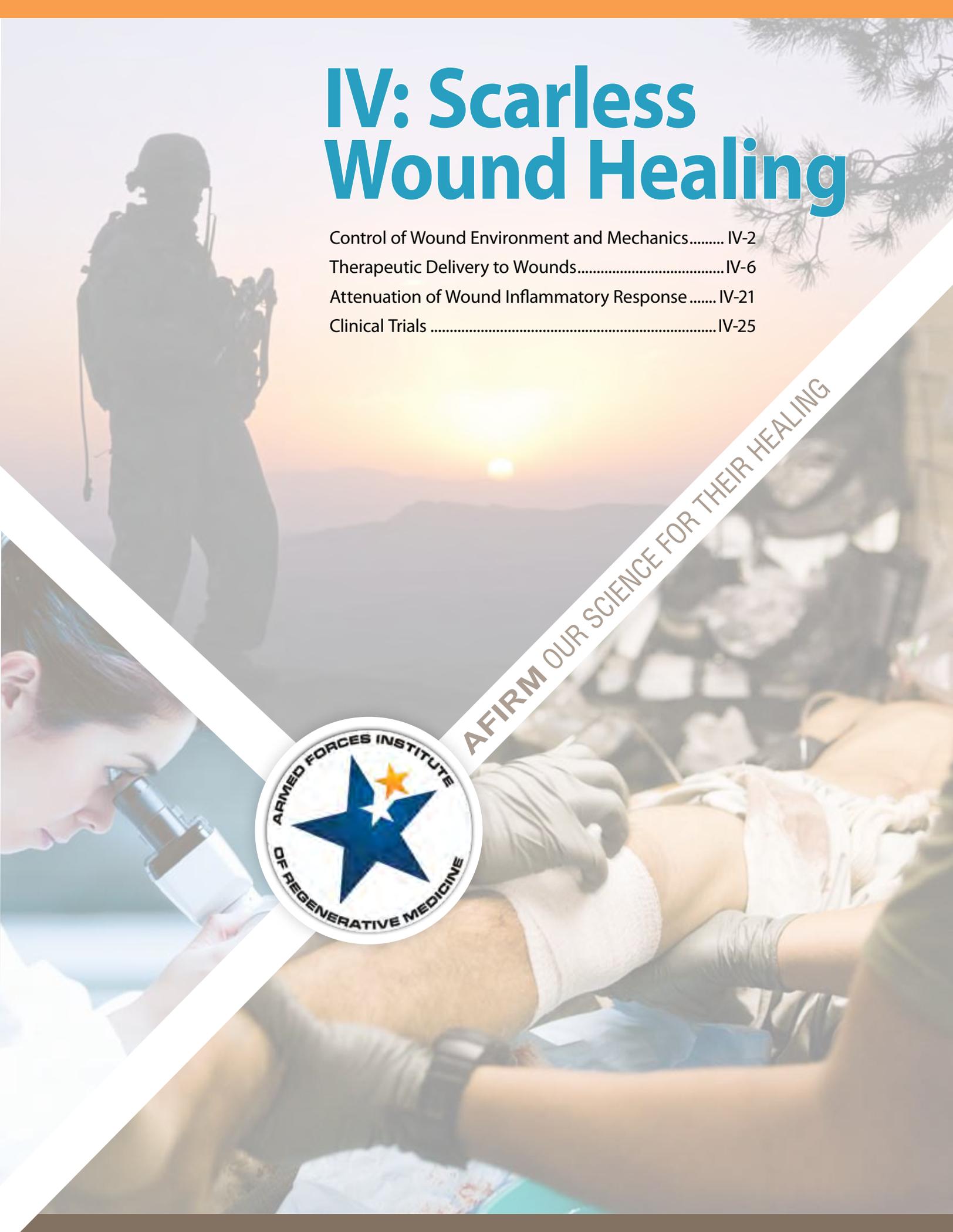
Planned Commercialization Transitions

The technology in development is similar to the development of orphan drugs, in that the target market area is very specific: post-traumatic (explosives, burns, gunshot and motor vehicle accidents) and post-oncologic surgery.

IV: Scarless Wound Healing

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AFIRM OUR SCIENCE FOR THEIR HEALING





IV: Scarless Wound Healing

Control of Wound Environment and Mechanics

Mechanical Manipulation of the Wound Environment

Project 4.5.1, WFPC

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

Project Team(s): Anthony Oro, MD, PhD, Michael T. Longaker, MD, MBA and Reinhold Dauskardt, PhD (Stanford University)

Collaborator(s): Neodyne Biosciences; Biomaterials and Advanced Drug Delivery Center at Stanford University

Therapy: Control of wound environment to minimize scarring

Deliverable(s): Battlefield-ready, region-specific devices capable of stress-shielding mechanical forces to minimize scar formation

TRL Progress: Beginning, TRL 4; Current, TRL 7; AFIRM I Target, TRL 7

Key Accomplishments: The researchers have shown that the molecular target focal adhesion kinase (FAK) is a critical regulator in mechanotransduction, specifically demonstrating the importance of fibroblast FAK expression in dermal scarring and skin fibrosis. During the past year, they evaluated the role of keratinocyte-specific FAK on wound healing progression and extracellular matrix composition. They identified a signaling pathway, mediated by FAK activation, leading to increased epidermal levels of matrix metalloproteinase 9 (MMP9). They also critically analyzed the role of epidermal FAK in wound healing, via a murine model of keratinocyte FAK deletion. They demonstrated that keratinocyte FAK influences dermal regeneration and homeostasis.

Key Words: Hypertrophic scarring, wound healing, wound device, mechanotransduction, fibrosis

Introduction

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. Multiple factors are known to influence wound repair (such as inflammation, oxygen tension, and ischemia) but therapeutic modalities aimed at these targets have been largely unsuccessful. The current inability to adequately control scar formation therefore generates an unmet need to develop novel therapeutic interventions targeting this gap.

Mechanical force has long been recognized to influence cellular behavior in vitro, and clinical observations based on Langer's lines and hypertrophic scarring corroborate this phenomenon in vivo. These findings prompted the current study to examine the role of mechanical stress in scar formation and to develop a novel device to actively control wound environment mechanics to mitigate fibrosis. Ultimately, the researchers aim to create battlefield-ready, region-specific devices for different wounded areas of the body, capable of precision stress-shielding of mechanical forces to minimize scar formation. The technology currently being developed enables precision stress-shielding

of area-specific wound forces through a portable, ready-to-use, simple pressure adhesive dressing that can be readily employed on the battlefield immediately following injury. A Phase I/II clinical trial has shown significant improvement in scar formation. Neodyne Biosciences Inc. is in the process of conducting Phase III trials, which will recruit a larger patient population.

Furthermore, the Gurtner group has been investigating the role of molecular pathways underlying mechanical force and tension in skin, ultimately leading to scar formation. They have identified the FAK as a central element in regulating hypertrophic scar formation through activation of inflammation and fibrosis. They are developing therapeutic interventions targeting FAK, which may ameliorate scar formation.

Research Progress – Year 5

The Gurtner group has completed all key aspects of their AFIRM I proposal on the mechanical manipulation of the wound environment to improve scarring.

The researchers initiated experiments during the past year to further explore the function of

epidermal FAK in the process of wound healing. They developed a specific FAK knock out (KO) mouse, which was then subjected to an excisional wound healing model to investigate the relevance of epithelial FAK pathways *in vivo*. Interestingly, significant defects in the wound healing process could be observed in the FAK KO group. Furthermore, FAK KO wounds were also significantly weaker compared to control wounds and more brittle based on higher fracture strain levels relative to ultimate tensile strength (Figure 1). In order to analyze the effect on extracellular matrix composition, the

researchers performed histologic analyses, which revealed a decreased dermal thickness associated with diminished levels of collagens 1 and 3. Paradoxically, the transcriptional levels of these collagens were elevated, suggesting an increased matrix turn-over (Figure 2).

To investigate the increased matrix turn-over, the Gurtner group examined MMPs, which are known mediators of collagen degradation and have been implicated in wound healing. Interestingly, MMP9 expression was significantly upregulated in FAK KO keratinocytes, which was further confirmed

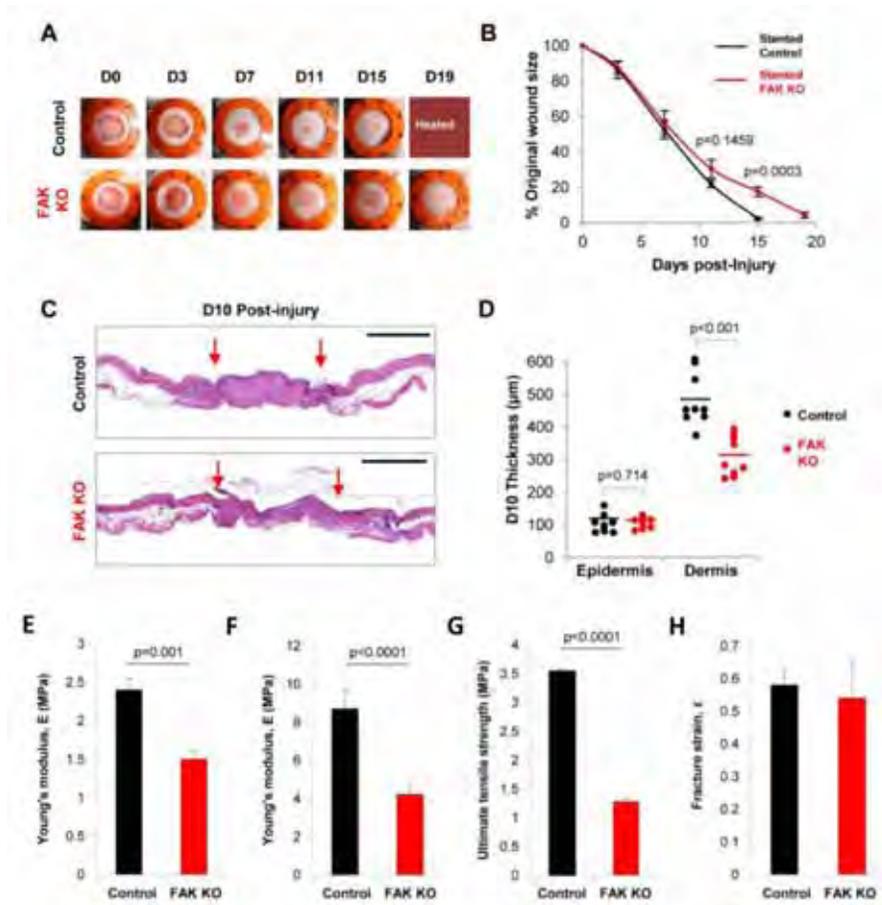


Figure 1. Gross and histomorphometric analysis of stented FAK KO excisional wounds. (A) Digital photographs of stented full thickness excisional wounds. (B) Quantification of wound closure for stented wounds. (C) Representative H&E micrographs of stented excisional wounds showing a gross decrease in thickness of FAK KO wounds. Red arrows indicate wound margins. (D) Quantification of epidermal and dermal wound thickness at post-injury day 10. $n = 9$ wounds. (E) FAK KO wounds are significantly less stiff than control wounds at physiologic strain levels (10-20%) and (F) suprphysiologic strain levels (30-50%). (G) FAK KO wounds have a significantly lower ultimate tensile strength than control wounds but (H) similar fracture strain levels. The brittleness of a material is characterized by a fracture strain level closer to the ultimate tensile strength. Data are means \pm one SEM. Scale bar 1mm. * $p < 0.001$. Statistical analyses for all presented data were performed using Student's unpaired t-test or one-way ANOVA for multiple comparisons (MATLAB, Natick, MA).



IV: Scarless Wound Healing

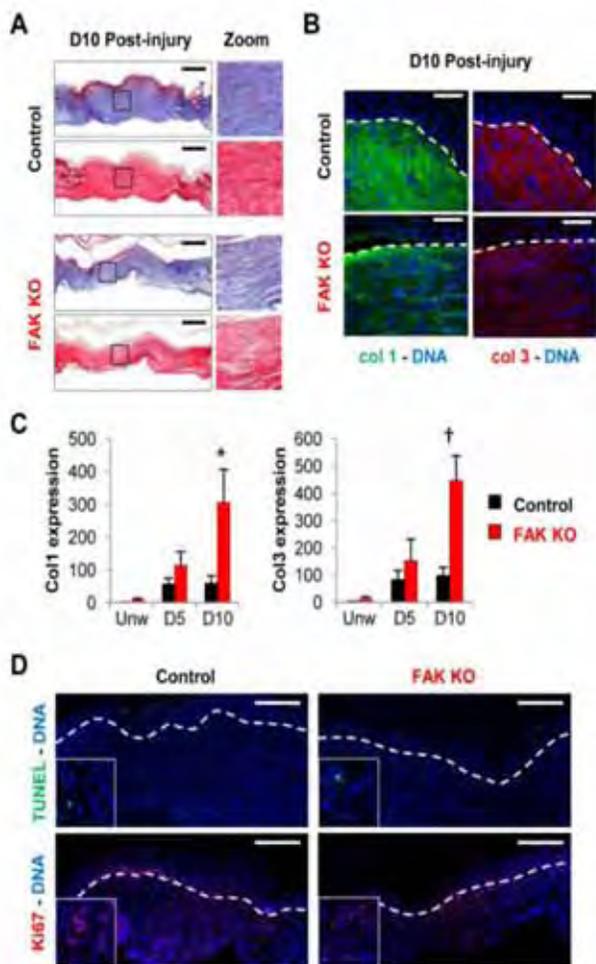


Figure 2. Analysis of wound matrix properties. (A) FAK KO wounds demonstrate decreased matrix density based on trichrome and picosirius red staining. Box indicates zoom area. Scale bar 400 μ m. (B) Representative images of collagen 1- and 3-specific immunolocalization showing a decrease in collagen with FAK KO. Dashed white line indicates basement membrane. Scale bar 50 μ m. (C) Quantification of collagen 1 and 3 gene expression on quantitative polymerase chain reaction (qPCR) showing a paradoxical increase in collagen expression with FAK KO. Data are means \pm one SEM. $n = 5$. "Unw" is unwounded skin. * $p < 0.05$; † $p < 0.01$. (D) Representative images of apoptotic terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and the proliferation marker Ki67 at Day 10 post-injury, demonstrating that the above decrease in matrix thickness was not due to differences in cell apoptosis or decreased proliferation. Bottom left inset shows high power field. Dashed white line indicates basement membrane. Scale bar 200 μ m.

using immunohistochemical analysis (**Figure 3**). To obtain a more profound insight into the signaling pathways that link increased MMP9 with keratinocyte FAK, the researchers analyzed the activation of several intracellular kinases that are commonly involved in such processes, and demonstrated increased phosphorylation of the stress-activated protein kinase p38 in FAK KO keratinocytes. This finding suggested that FAK was a negative regulator of p38. To confirm that p38 activation was associated with MMP9 secretion, blockage of p38 was performed and found to effectively attenuate MMP9 secretion in both control and FAK KO keratinocytes (**Figure 4**). Together, these data provide a novel pathway linking mechanical force to wound remodeling.

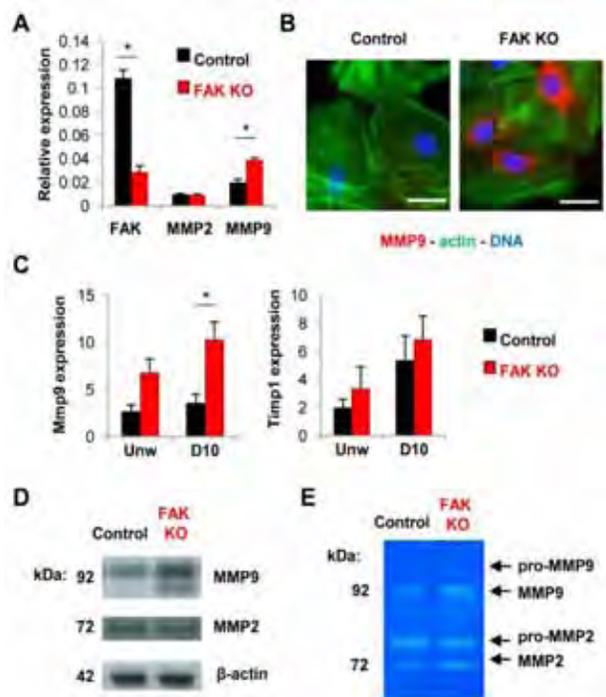


Figure 3. FAK KO wounds are characterized by increased MMP9 levels and activity. (A) qPCR analysis in keratinocytes in vitro. (B) MMP9 immunofluorescence in keratinocytes. $n = 4$. (C) qPCR analysis of wound MMP9 and Timp1 gene expression. $n = 5$. "Unw" is unwounded skin. (D) Representative immunoblots of matrix enzymes in Day 10 wounds. $n = 4$. (E) Gelatin zymograms demonstrating increased MMP9 activity in FAK KO wounds. $n = 3$. Data are means \pm one SEM. Scale bar 10 μ m in C. * $p < 0.05$.

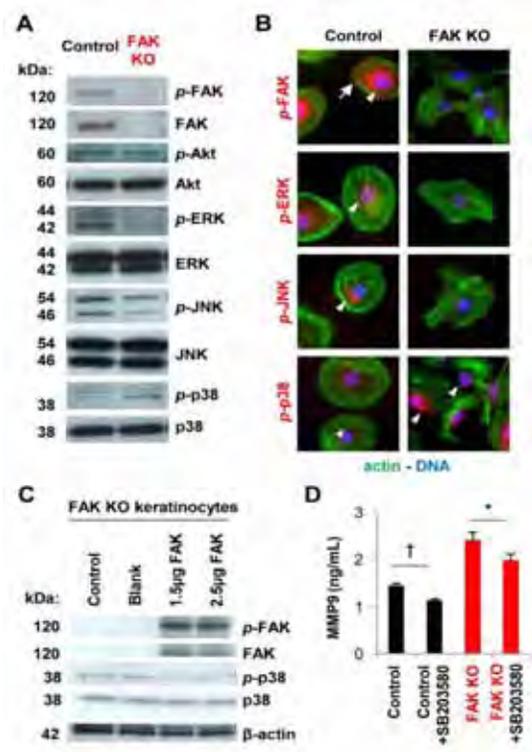


Figure 4. Intracellular pathways driving aberrant MMP9 secretion from FAK KO keratinocytes. (A) Representative immunoblots demonstrating increased p38 phosphorylation in FAK KO keratinocytes. $n=3$. (B) Representative immunofluorescence images of mitogen activated protein kinase activation (red color) confirming increased p38 activation in FAK KO keratinocytes. Arrows point to membrane-associated signal; arrowheads point to cytoplasmic signal. (C) Restoration of FAK in FAK KO keratinocytes attenuates p38 activation. (D) Administration of p38 inhibitor ($10\mu\text{M}$ SB203580) blocked MMP9 secretion. Data are means \pm one SEM. $n=3$. * $p<0.05$; † $p<0.01$.

Overall, this work provides a comprehensive analysis of the biomolecular interactions that occur during scarring and wound healing. Specifically, FAK has been established as a central protein in mechanosensation, activating a cascade of intricate intracellular downstream signaling in multiple cell types. Moreover, the rigorous characterization of keratinocyte FAK signaling during wound healing helps to frame the previous findings on fibroblast FAK, and will enable the development of targeted strategies towards improving scarring and wound healing. Subsequent work concentrating on the utilization of small molecules targeting FAK will build on this foundation.

Conclusions

During the past 5 years of AFIRM I funding, the Gurtner group has successfully developed an adhesive stress-shielding device, which has been shown to significantly improve scarring in clinical trials. In understanding the molecular biology underlying scar formation and dysfunctional wound healing, the researchers have identified FAK as a key mediator in mechanotransduction.

Future Research Plans

Going forward, the researchers aim to develop therapeutic approaches to specifically target FAK to improve tissue regeneration.

Planned Clinical Transitions

A Phase III clinical trial is currently being conducted with significant interim data for the polymeric stress-shielding device (see Clinical Trials section, Project 4.5.9). Additionally, the group will be engaging in U.S. Food and Drug Administration approval for small molecule FAK inhibition, and an appropriate regulatory package will be provided to gain allowance to afford for the initiation of a Phase I clinical trial.



Therapeutic Delivery to Wounds

Therapy to Limit Injury (TLI) and Promote Non-Scar Healing After Burns and Severe Battle Trauma

Project 4.6.3, RCCC

Team Leader(s): Thomas Mustoe, MD (Northwestern University)

Project Team(s): Seok Jong Hong, PhD, Sheng-Xian Jia, MD, PhD, Ping Xie, PhD, and Marina Vracar-Grabar, MSc (Northwestern University)

Collaborator(s): Richard Clark, MD (Stony Brook University)

Therapy: Curcumin to enhance healing and attenuate scarring

Deliverable(s): Intravenous (IV) treatment with curcumin

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers confirmed the effectiveness of curcumin in reducing necrosis and supporting the survival of skin flaps in a porcine model. They determined that the most effective time for treatment was at the time of surgery with a single 3 μ m dose. The effect was confirmed 7 and 28 days after surgery. The researchers found that treatment with curcumin 4 or 24 hours after the surgery had no statistically significant effect in rescuing injury when compared to vehicle control.

Key Words: burn; scar; curcumin; rabbit ear model; pig skin flap

Introduction

There is no effective therapy to limit ischemia/reperfusion injury in marginally viable tissue following major trauma. Curcumin is known as an anti-inflammatory, antioxidant and antimicrobial agent. While many treatments tested to date have not shown great benefit for ischemic or ischemia-reperfusion injury, medical-grade curcumin administered as an IV treatment at the time of injury (or soon afterward) can potentially prevent further injury progression and support scarless healing. Over the past 5 years, the Mustoe team has obtained proof of concept of curcumin's efficacy in wound healing: curcumin supports wound epithelialization and formation of granulation tissue following a single IV treatment in the hypertrophic scar rabbit ear wound model, an ischemic model, and the rabbit ear ischemia reperfusion model.

Given the positive results achieved by the researchers with curcumin in their rabbit wound healing models, they extended the project to large animals. They used the pig skin flap model to create 8 flaps (4 on each flank), which were raised on its dorsal base and sutured in place, leading to a distal zone of

necrosis. By measuring flap survival at 7 days post flap elevation, with curcumin given systemically at the time of surgery, this model simulates the clinical application.

Research Progress – Year 5

During the final year of the AFIRM I project, the project team's goal was to further explore the timing and dosing of curcumin IV therapy in a large animal model, the porcine skin flap model. The researchers administered curcumin at the time of surgery, and at 4 and 24 hours post-surgery, and explored the reduction in post-surgery necrotic injury. This model was designed to simulate the clinical application by giving curcumin systemically at the time of surgery and then measuring flap survival at 7 days after surgery.

The researchers found that the most effective IV treatment was a single 3 μ m dose given at the time of surgery (**Figure 1**). This effect was confirmed at 7 and 28 days after surgery. Treatment with IV curcumin at 4 and 24 hours after the surgery had no statistically significant injury-reducing effect in comparison to vehicle control (**Figure 2**), although there was an improvement over no treatment.

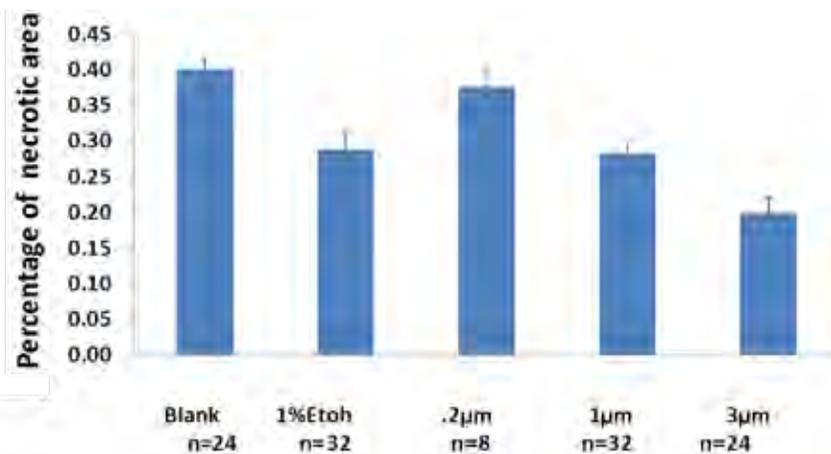


Figure 1. Quantitative image analysis of the excised pig skin flaps or template-traced necrotic areas 7 days after surgery. Percentage of the necrotic area was determined as the ratio of the necrotic (black) area to the total skin flap area (14 x 4 cm). A single IV curcumin treatment (3 µm) at the time of surgery reduced the size of the necrotic (black) area. A statistically significant effect was achieved in animals treated with 3 µm curcumin in comparison to vehicle treated controls. There was a dose response trend. (n=number of flaps. T-test $p < 0.01$, 1% Et-OH vs. 3 µm C. and T-test $p < 0.01$, 1 µm vs. 3 µm)

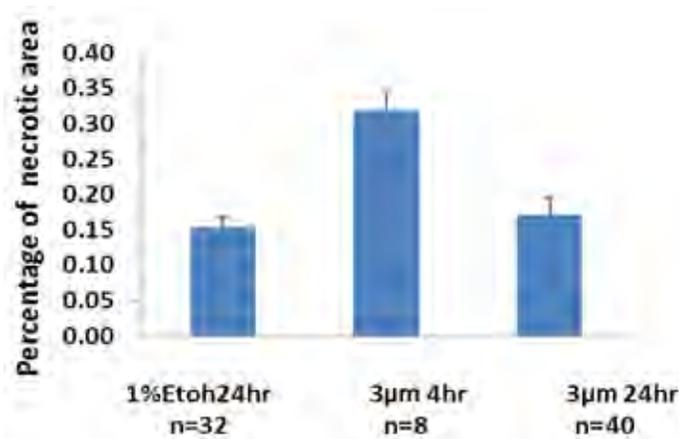


Figure 2. Quantitative image analysis of the excised pig skin flaps or the template-traced necrotic areas 7 days after surgery. The percentage of the necrotic area was determined as the ratio of the necrotic (black) area to the total skin flap area (14 x 4 cm). IV curcumin was administered either 4 hours or 24 hours after surgery. There was no statistically significant post-injury rescuing effect with 3 µm curcumin administered 4 hours or 24 hours post-surgery. (N=number of flaps)

However, the vehicle control (ethanol) also showed some effects, so no statistical significance was achieved for treatment started after injury.

The researchers followed skin flap survival for 28 days. They analyzed the necrotic area of each flap and presented results as a ratio of the necrotic area to the full skin flap area. Their results confirmed that systemically administered curcumin at a dose of 3 µm had a significant effect on supporting tissue

survival, over the course of 28 days (Figure 3). Quantification of the skin necrosis 7 days after surgery indicated a dose-dependent effect of curcumin.

Conclusions

The research team confirmed the effectiveness of IV curcumin in reducing necrosis and supporting survival of the skin flaps in their porcine model. Taken together with the results obtained in the rabbit



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ear wound models in previous years, IV curcumin therapy can promote wound healing and has an injury-reducing effect.

Future Research Plans

Although this project will not continue with AFIRM funding support, the research will continue if an appropriate partner is identified and an alternative funding source is secured.

Planned Clinical Transitions

The transition to Good Manufacturing Practices is expected to be straightforward based on discussions with manufacturers of curcumin. NeoMatrix Formulations, Inc., in collaboration with an industrial partner, is interested in negotiating an option to license the technology from Stony Brook University, which has filed a patent on the IV use of curcumin. Once this is accomplished, a funding source will be sought.

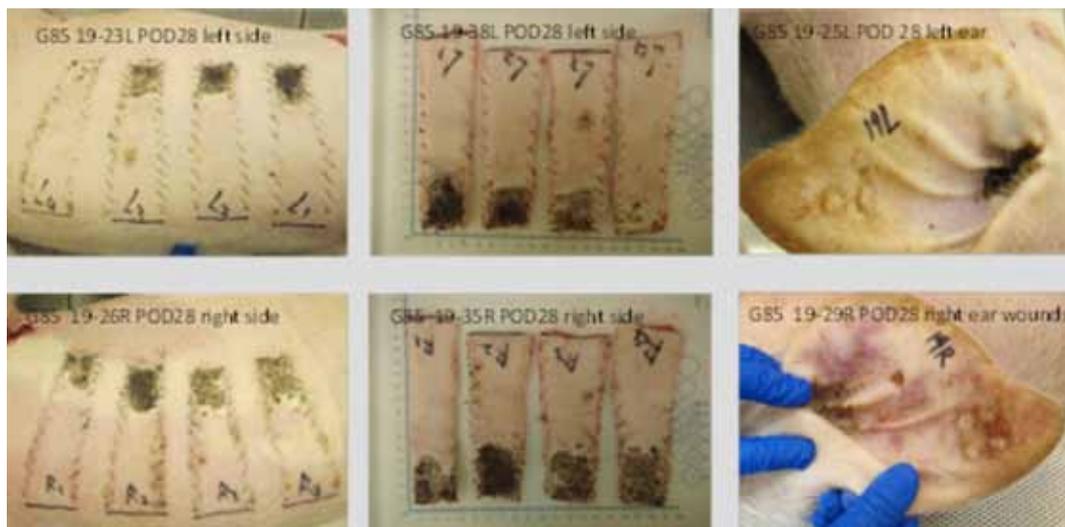
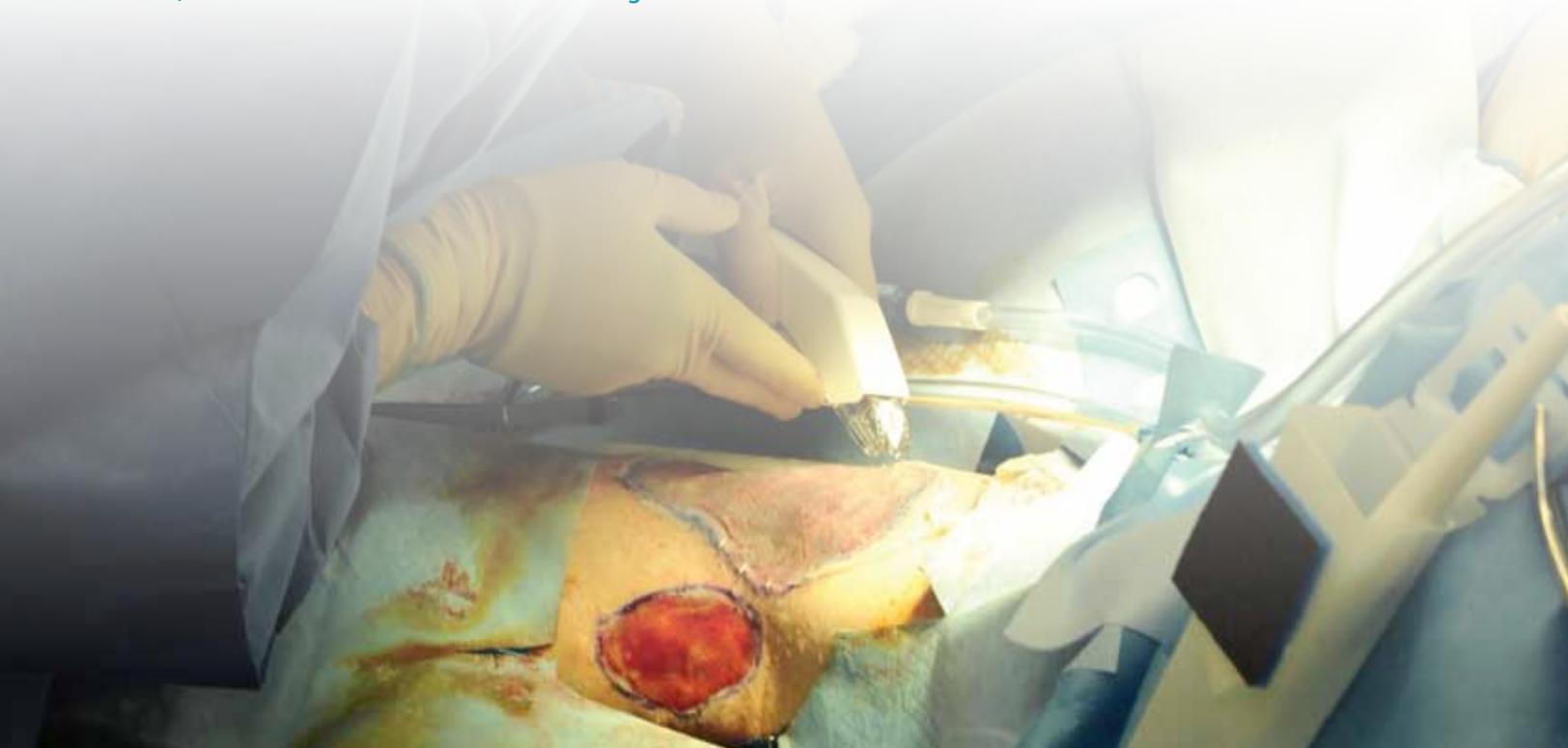


Figure 3. Survival of the flaps 28 days after surgery indicates the injury-reducing effect of IV curcumin ($3 \mu\text{m}$ dose). The ear wounds healed without forming scar tissue.



Therapeutic Delivery to Wounds**Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management****Project 4.7.1, RCCC**

Team Leader(s): Adam J. Katz, MD (University of Florida)

Project Team(s): Ning Yang, PhD and Hulan Shang, MS (University of Florida)

Collaborator(s): Glycosan/BioSystems, Inc.; The GID Group, Inc.; Lifecell, Inc.; Integra Lifesciences

Therapy: Tissue repair and wound healing therapy using Autologous Wound Paste (AWP).

Deliverable(s): A novel and flexible AWP platform for the treatment of full thickness cutaneous wounds due to blast injury, amenable to a “point-of-care” treatment paradigm.

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers initiated testing and characterization of a novel stromal vascular fraction (SVF) cell isolation device. They established fundamental quality control (e.g., endotoxin; cell dispersion) and potency assays (e.g., paracrine secretion; endothelial cell [EC] migration) for the wound paste formulation. They also compared subtle but important variations in the specific formulation of the dermal wound paste (DWP) in the context of established assays. Finally, they demonstrated evidence that DWP secretes numerous bioactive factors that can impact angiogenesis and cell chemotaxis.

Key Words: adipose stromal cells; autologous cell therapies; stromal vascular fraction; wound healing; wound contraction

Introduction

Essentially all battlefield wounds involve some component of skin and/or soft-tissue injury; in many cases, the tissue loss/damage can be quite extensive. The reasons for this, according to recent literature, are because explosive mechanisms (e.g., improvised explosive devices, mortars, rocket-propelled grenades, landmines) account for nearly 80% of all combat casualties in Operation Iraqi Freedom/Operation Enduring Freedom. These types of injuries tend to involve multiple body areas simultaneously (on average 4 wounds per soldier), with over 80% involving the extremities, or the head and neck.

Regardless of etiology, extensive full thickness wounds are associated with significant cutaneous scar formation, even with the implementation of the most advanced reconstructive techniques. Such scarring can result in significant deformity and/or functional loss, due to the normal physiological process of wound contraction – even in the setting of definitive skin graft or flap treatment. Wound contraction, in turn, can lead to the distortion of

surrounding tissues and structures, producing scar contractures that cause aesthetic deformity and functional limitations.

The reconstructive principles for challenging wounds are the same whether the mechanism of injury is traumatic or surgical, and whether the injury/defect involves the extremities, the trunk, or the face. Often these reconstructive considerations are at odds with each other, forcing treatment decisions that optimize certain aspects of patient care at the expense of others. Sometimes, as in the case of wounded Servicemen and -women involved in recent and current conflicts, treatment options are severely limited simply by a lack of available donor tissue. In all cases, reconstruction/repair requires some degree of donor morbidity associated with the harvest of skin grafts or flaps—sometimes resulting in significant “donor disease” that is itself disfiguring and/or disabling.

The working hypothesis of the Katz team is that constructs composed of autologous adipose-derived SVF cells, human acellular dermal matrix (ADM), and glycosaminoglycan hydrogels can mediate the



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effective replacement of missing dermis in a more expedient timeframe and without the donor morbidity associated with current available strategies.

Research Progress – Year 5

Characterization of SVF cell output using the GID SVF-1 disposable cell isolation unit

The GID Group, Inc. has developed a disposable unit for the rapid isolation of cells from adipose tissue. The GID SVF-1™ is a sterile single-use disposable tissue canister used for harvesting, filtering, separating, concentrating, and transferring autologous tissue components for reintroduction to the same patient during a single surgical procedure. At present, the GID SVF-1™ is CE mark certified, but it is not yet available in the United States. The Katz team initiated testing of the GID SVF-1 during this past year. Cell output was analyzed for yield, viability, and endotoxin levels.

Cell viability within DWP over a 14-day culture period

DWP was formulated using fresh human adipose-derived SVF cells, hyaluronic acid, ADM and human serum (HuS). Formulations involved the use of either cell stabilization solution (CSS), or CSS with 5% HuS. The paste was shaped into standardized discs and then cultured up to 14 days. Cell viability was measured at Day 1, Day 7 and Day 14 using Alamar Blue Assay (see **Table 1**). After 2 weeks of culture, the DWP discs were fixed, sectioned and stained using histochemical and immunohistochemical methods. Conditioned media from the numerous samples was saved at Day 1, Day 7 and Day 14, and used for cell migration assays and a Luminex assay. The research team evaluated three different ADM types during the course of these studies.

DWP discs were formulated and cultured for 14 days in “Augmented Culture Medium” (ACM),

which is a Dulbecco’s Modified Eagle Medium (DMEM)/F12-based laboratory-formulated medium with 1% HuS, antibiotics and various growth factors. Culture medium was replaced at Day 7. To mimic the extreme in vivo environment of a wound, the team also prepared a simplified culture medium (SCM) consisting only of DMEM/F12 with 5% HuS and antibiotic (but without the various growth factors included in ACM). Cell viability within DWP was measured under these two culture conditions. In addition, three different dermal matrices were studied within this DWP system, all of which are commercially available and U.S. Food and Drug Administration (FDA) approved for clinical use.

A 2-week culture period was chosen because this is roughly the time period required for engraftment and revascularization of an acellular dermal scaffold in vivo. Based on AlamarBlue testing, relative cell viability increased at Day 7 and Day 14 in both formulations (CSS++ and CSS 5% HuS) and culture conditions (ACM vs. SCM). At Day 7, cell number increased 140% in augmented culture conditions (n=11) (CSS++ & ACM) and 152% in simplified culture condition (n=6) (CSS5%HuS and SCM) compared to culture at Day 1. At Day 14, cell number increased 180% in augmented culture condition (CSS++ and ACM) and 177% in simplified culture conditions (CSS5%HuS and SCM) relative to Day 1. These results demonstrate that DWP formulations made with ADM matrix #1 supports cell viability and proliferation for up to 14 days – even in simplified (spartan) culture conditions (SCM).

Cell viability within DWP formulated with ADM matrix #2 and cultured in augmented culture medium

DWP was formulated using ADM matrix #2 and cultured in ACM. Average cell number was noted to increase by 176% and 308% at Day 7 and Day 14, respectively.

Table 1. Cell viability within DWP formulated with ADM Matrix #1 and cultured in augmented and simplified culture media

Culture condition	Day 1	Day 7	Day 14
CSS++ & ACM (n=11)	100%	140%	180%
CSS5%HuS & SCM (n=6)	100%	152%	177%

Cell viability within DWP formulated with ADM matrix #3 and cultured in SCM

DWP was formulated (CSS5%HuS) with ADM matrix #3 and cultured in SCM. Average cell number increased 153% and 204% at Day 7 and Day 14, respectively.

Histology and immunohistochemistry

DWP samples were fixed and paraffin embedded after they were cultured for 14 days. Samples were then sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E) and CD31.

Histology

H&E staining of DWP formulated with each of the three types of ADM matrix demonstrated that SVF cells were distributed evenly in all groups. In some samples, cells established a monolayer or multilayer formation on the surface of the paste samples. Based on these findings, the researchers determined that seeded SVF cells can proliferate within and upon the DWP.

CD31 staining

CD31 (PECAM-1) immunohistochemistry was used to identify ECs. CD31-positive staining indicated (and confirmed) that ECs exist within fresh SVF cell isolations, and that such cells survive the 2-week culture period within the DWP. In some instances, the CD31+ECs could be seen to form lumen-like structures within the paste formulation, highly suggestive of neo-capillary structures (**Figure 1**).

Proliferation staining

Some samples were stained for Ki67, a marker of cell proliferation. Results demonstrate that some cells within the DWP remain active in the proliferative state (**Figure 2**).

ECs migration assay

The researchers collected conditioned DWP media at Day 0, Day 7 and Day 14 for EC migration assays using transwell inserts. DWP-conditioned media was placed in the bottom part of the well and ECs were cultured on the upper surface of the transwell insert. After a designated incubation time, the undersurface of the transwell insert was fixed,

and the cells that had migrated toward the conditioned medium in the lower chamber were stained with crystal violet, and counted using Image J. The number of migrated ECs was significantly higher in DWP-conditioned medium groups compared to control groups that contained conditioned medium from acellular DWP. In addition, the number of migrated cells was increased in Day 14 conditioned medium compared to Day 7 conditioned medium (see **Figure 3**). These results demonstrate that SVF cells within the DWP may secrete bioactive factors that are chemotactic to human ECs. These findings support the team's hypothesis that SVF cells may accelerate the engraftment and revascularization of acellular dermal matrices.

Luminex results

The Katz team also investigated the type and amount of growth factors secreted into culture media by DWP formulations. This was performed using the Luminex[®] xMAP technique. This is a multiplex bead-based immunoassay platform that simultaneously measures multiple analytes

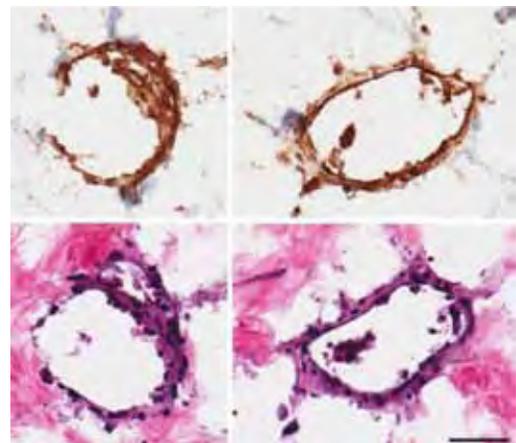


Figure 1. CD31 staining and H&E staining of lumen-like structures within DWP. Bar=50 μm . (ADM #1)



Figure 2. Cell proliferation as demonstrated by Ki67 staining (red) in DWP formulated with ADM Matrix #1. Bar=100 μm . (Blue/DAPI: cell nuclei; Red/Ki67: proliferating cells)



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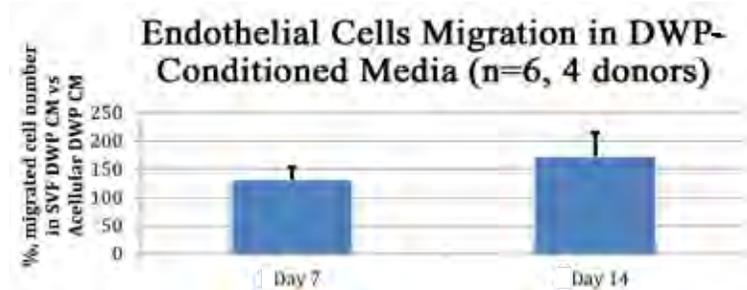


Figure 3. Endothelial cell migration in DWP-conditioned media. Migrated ECs in SVF-DWP-conditioned media compare with migrated ECs in acellular-DWP-conditioned media. Values above 100% present more migrated ECs in SVF-DWP-conditioned media than in acellular-DWP-conditioned media.

(proteins) by exciting a sample with a laser, and subsequently analyzing the wavelength of emitted light. The researchers investigated growth factors that are known to be involved in the processes of tissue regeneration and wound healing, such as angiogenesis, inflammation and immune-modulation, cell differentiation, etc. The graph in **Figure 4** shows the relative change in growth factor levels during Week 2 of culture relative to Week 1.

Conclusions

Despite delays inherent in the relocation process of the Katz team, a number of milestones were achieved. First, the researchers initiated a collection of formal standard operating procedures guiding the research and development process for DWP. This will prove to be critical for future regulatory

submissions. Second, the team initiated testing and characterization of the GID cell isolation device. Third, the team made excellent progress in the development and validation of quality control and potency assays, with which the reproducibility and bioactivity of wound paste can be measured. Finally, subtle but important variations in the specific formulation of DWP were tested and compared to one another. These studies will ultimately help guide the specific formulation and assays that will be used to complete pre-clinical animal studies, and ultimately be applied to human clinical trials.

Future Research Plans

During the next year, the research team will focus efforts on completing pre-clinical animal studies that are necessary for supporting an Investigational New Drug (IND) application filing, as determined by a previous pre-IND meeting with the FDA.

Planned Clinical Transitions

Upon completion of studies necessary to support an IND filing (or as completion of such nears), the research team will pursue funding for Phase I clinical trial testing. Dr. Katz continues to foster relationships with potential commercial partners.

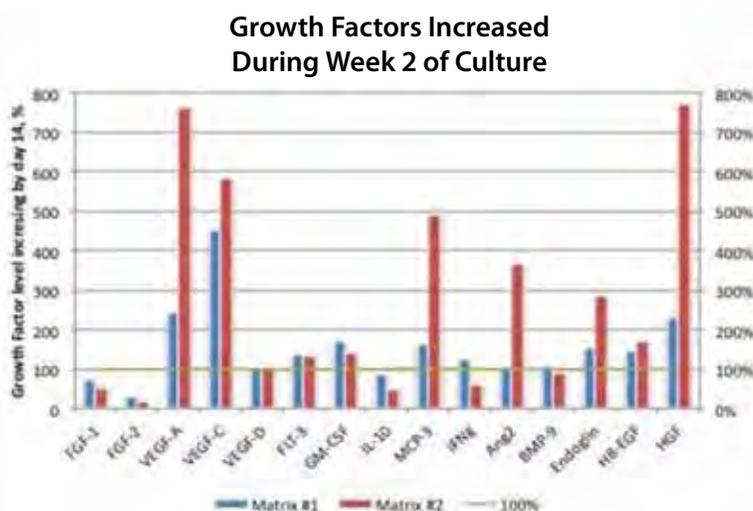


Figure 4. Relative change in growth factor levels from DWP conditioned medium in Week 2 of culture relative to Week 1 of culture. Values below 100% represent a relative decrease in Week 2.

Therapeutic Delivery to Wounds**Regenerative Bandage for Battlefield Wounds****Project 4.5.2, WFPC**

Team Leader(s): Geoffrey C. Gurtner, MD and Michael T. Longaker MD, MBA (Stanford University)

Project Team(s): Anthony Oro, MD, PhD (Stanford University)

Collaborator(s): None

Therapy: Improved wound healing and reduced scarring

Deliverable(s): Regenerative bandage that promotes fetal-like wound healing instead of scarring

TRL Progress: Beginning, TRL 1; Current, TRL 5; AFIRM I Target, TRL 5

Key Accomplishments: During the past year, the research team demonstrated the utility of mesenchymal stem cell (MSC)-seeded bioscaffolds for the accelerated healing and appendage formation of humanized murine excisional wounds, as compared to direct MSC injection and no treatment controls. They also evaluated the effect of hydrogel seeding on MSC survival within humanized excisional wounds and found an increase in cell viability and engraftment among hydrogel-seeded cells as compared to direct injection. In addition, they determined the beneficial effect of hydrogel seeding on MSC secretion of angiogenic cytokines in vitro is recapitulated in the in vivo wound environment.

Key Words: dermal matrix, wound healing, fetal skin

Introduction

Soldiers returning from Iraq and Afghanistan have sustained significant trauma to the head, neck, face, and limbs. Appropriately timed interventions are critical for the treatment of traumatic wounds, with wound coverage being especially important for the reduction of inflammation and infection, processes that can lead to wound contractions and disability. Even with timely intervention, however, the elimination of scarring and regeneration of underlying soft tissue remains a challenge. Novel approaches are needed that focus on minimizing the inflammatory and fibrotic cascade in the initial days following injury, as the modulation of these processes will ultimately help promote tissue regeneration.

Currently, there are several commercially available products used for wound coverage and skin engineering applications based on human or pig skin. While these decellularized matrices have demonstrated some efficacy compared to no treatment at all, they can be costly, and their clinical results are often suboptimal with regard to their ultimate cosmetic and functional outcomes.

The research team's proposed approach begins immediately post-injury, with application of a regenerative bandage consisting of a biomimetic matrix and human progenitor cells designed to maintain an acute wound in a pro-regenerative state of "suspended animation," and prevent the onset of scarring, fibrosis, and infection. Utilizing their knowledge of wound healing, scarless repair and burn therapies, the researchers hope to preserve wounds in a "fresh state" through application of this cytokine and cell-rich regenerative bandage, and thereby optimize the results of definitive therapy provided back in the United States.

To achieve these aims, the Gurtner/Longaker group has developed hygroscopic dressings mimicking unwounded dermal micropatterning. This engineered construct was found to significantly improve cutaneous wound healing in a mouse model and demonstrated potent immunomodulatory properties that enhanced wound vascularization. Moreover, these constructs can be seeded with various cell types in order to deliver both cytokines and cells to the wound environment. The researchers are now focused on quantifying the regenerative potential of this template for the efficient delivery of cells to the wound.



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Research Progress – Year 5

The Gurtner/Longaker team has completed all key aspects of their AFIRM I proposal on the generation of a regenerative bandage for battlefield wounds. They first developed a biomimetic pullulan-hydrogel bioscaffold specifically designed to mimic the extracellular characteristics of unwounded skin. During this process, they optimized scaffold pore size and interconnections through the use of a salt-induced phase inversion technique and iterative component concentration analyses. This optimized hydrogel architecture was shown to recapitulate the reticular distribution of human dermal matrix while maintaining flexible properties essential for skin applications. Moreover, *in vivo* experiments with unseeded hydrogels demonstrated that these constructs promoted murine wound healing via enhancement of stromal cell recruitment and formation of vascularized granulation tissue.

The next set of experiments focused on determining the cell-biocompatibility of scaffolds, which were shown to retain their open porous architecture throughout processing and viably sustain human fibroblasts, as well as murine bone-marrow derived MSCs and endothelial cells *in vitro*. Further supportive of the successful recapitulation of a functional cell niche, bioscaffolds were also shown to improve the delivery and engraftment of stem cells to high-oxidative-stress ischemic tissues *in vivo*.

Encouraged by this functionality, the regenerative potential of cell-seeded bioscaffolds was next determined through a side-by-side phenotypic comparison of hydrogel seeded and two-dimensional culture-grown murine bone marrow-derived MSCs. Implicating a critical influence of the wound healing capacity of these cells, hydrogels were found to induce MSC expression of a variety of angiogenic cytokines, as well as upregulate the production of pluripotency and self-renewal associated transcription factors. Confirming the utility of this bioscaffold for *in vivo* tissue regeneration applications, MSC-hydrogel constructs were found to significantly accelerate healing of murine excisional wounds as compared to direct MSC injection and no treatment controls (**Figure 1**), as well as promote native skin appendage formation.

As a partial explanation for this enhanced functionality, bioluminescence imaging and

fluorescence-activated cell sorting analysis of luciferase+/green fluorescent protein (GFP)+ MSCs indicated that stem cells delivered within the hydrogel remained viable longer and demonstrated enhanced engraftment efficiency as compared to direct injection (**Figure 2**). Additionally, wounds that were treated with MSC-seeded hydrogels demonstrated significantly increased levels of vascular endothelial growth factor and other angiogenic and tissue remodeling cytokines (**Figure 3**), ultimately leading to increased neovascularization. Furthermore, hydrogel delivered MSCs retained their full differentiation potential, and were shown to form fibroblasts, pericytes and endothelial cells following delivery.

Overall, this work comprehensively demonstrates that this novel biomimetic hydrogel provides a functional niche capable of augmenting stem cell regenerative potential and accelerating wound healing.

Conclusions

The Gurtner/Longaker team has developed a novel regenerative bandage composed of stem cells seeded on a biocompatible scaffold that demonstrated the regenerative potential of this construct. Building upon developmental and construct optimization work from previous years, recent *in vivo* studies confirmed the hydrogel creates an environment for MSCs that increases cell survival and vasculogenic cytokine expression. The technologies developed herein lay the foundation for future work aimed at determining the optimal cell type and seeding methodology for this bandage, and ultimately evaluating the potential of this construct in the clinical setting.

Future Research Plans

In the next phase of this project, the researchers will utilize microfluidic single-cell transcriptional analysis to identify the optimal cell source for this bandage, with an ultimate test being the efficacy of this optimized construct in a clinical setting.

Planned Clinical Transitions

Following a refinement period over the next few years, the safety and regenerative capacity of the hydrogel-based regenerative bandage will be tested in a Phase I clinical trial. Phase II-III clinical testing will follow.

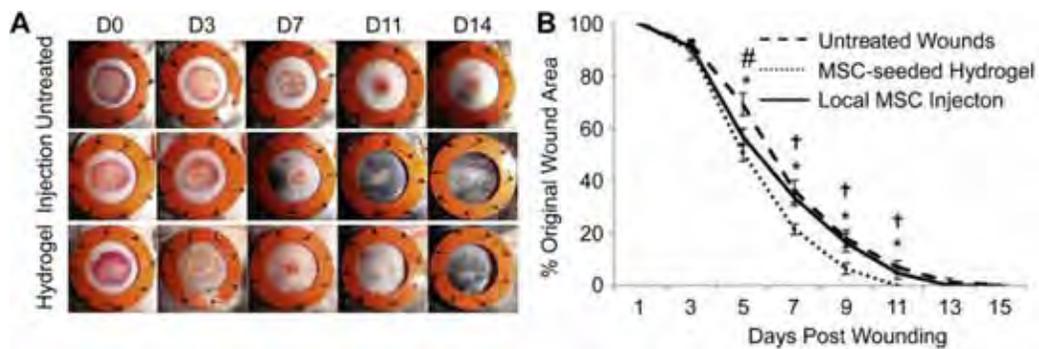


Figure 1. Effects of MSC delivery method on wound closure. (A): Gross photos. (B): Wound closure curves demonstrate significantly accelerated healing in wounds treated with MSC-seeded hydrogels. * $p < 0.05$ untreated vs. MSC-seeded scaffold, # $p < 0.05$ untreated vs. local MSC injection, † $p < 0.05$ local MSC injection vs. MSC-seeded scaffold. Statistical analyses of all presented data performed using a student's unpaired t test.

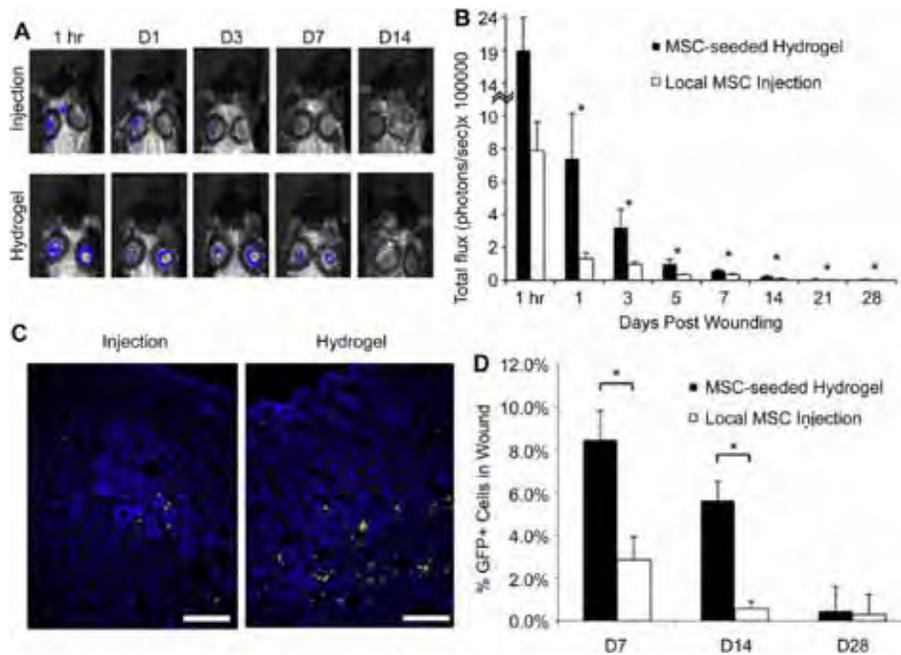


Figure 2. In vivo MSC viability and engraftment. (A): Bioluminescence imaging was used to follow luciferase(+) MSCs delivered into wounds. (B): Quantification of total flux. (C): Immunofluorescent images of GFP(+) MSCs within day 14 wounds. Scale bar 100 mm (D): Flow cytometric quantification of GFP(+) cells in wounds. * $p < 0.05$.

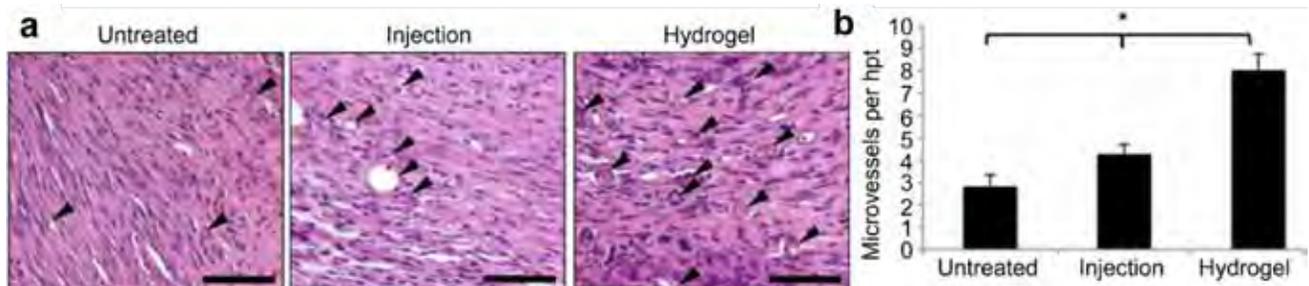


Figure 3. Evaluation of wound angiogenic cytokine levels at Day 3 post-wounding. * $p < 0.05$.



Therapeutic Delivery to Wounds

Scarless Wound Healing through Nanoparticle-mediated Molecular Therapies

Project 4.5.5, WFPC

Team Leader(s): Sandeep Kathju, MD, PhD (University of Pittsburgh)

Project Team(s): Latha Satish, PhD; and Phillip Gallo, PhD (University of Pittsburgh)

Collaborator(s): N/A

Therapy: 1. Formulation containing small interfering ribonucleic acid (siRNAs) that can be applied to wounds to mitigate scar formation. 2. Probiotic therapy to burn wounds to inhibit pathogenic infection and reduce scar.

Deliverable(s): 1. An agent that can be introduced into healing wounds through non-viral means to mitigate scar. 2. A protocol for the use of probiotic bacteria in healing wounds to suppress invasive infection and reduce scar.

TRL Progress: Beginning, TRL 1; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers established new protocols that will allow them to inhibit chaperonin containing T-complex polypeptide subunit eta (CCT-eta) more stably and for a longer period of time in a healing wound milieu. They demonstrated that inhibition of CCT-eta can inhibit fibrosis in an animal model of incisional wound healing. They identified novel strains of bacteria that may be used as probiotic therapy for other pathogens. Finally, they demonstrated that probiotic therapy can rescue an organism from burn wound-induced sepsis and death, and can inhibit burn wound-induced scarring.

Key Words: Scarless wound healing, nanoparticles, siRNA, probiotics, burns

Introduction

In adult humans (and all postnatal mammals), wound healing is invariably accompanied by the formation of scar. Scar can become problematic in any part of the body, but certain anatomical areas are particularly vulnerable. The extremities, especially the forearm and hand, are sites where the formation of scar following injury is particularly problematic. Essential to the proper functioning of these structures is uncompromised range of motion, which is frequently severely limited by scarring after trauma, even to the point of absolute immobility and severe and deforming contracture. The scar in question can arise from penetrating or blunt trauma; from direct injury to muscle/tendon or indirectly from injury to underlying bone or overlying skin. Even if injured tissues are suitable for direct surgical repair, the ultimate functional outcome can still be poor due solely to resulting scar. Burn injuries are also particularly prone to extensive and crippling scarring. On any body surface, but particularly in the extremities, skin burn injuries can limit the function of deeper

normal structures by the restrictive scar envelope they induce.

Recognizing the major limitations scar places on the success of their efforts, surgeons have long sought adjunctive measures that can improve surgical outcome. Many agents have been tried with only limited and sporadic success. To date, there remains no generally reliable way to prevent the formation of scar in the aftermath of injury.

In contrast to adult mammals, fetal mammals are able to heal their wounds without scarring. This “scarless wound healing” is an innate property of fetal tissues, not a benefit of the protected uterine environment. We have compared the patterns of gene expression in healing fetal wounds versus adult wounds in animal models, and noted that the gene for the CCT-eta is increased in scar-forming adult wounds but decreased in scarlessly healing fetal wounds. We then demonstrated that artificially reducing CCT-eta levels in fibroblasts, the cells responsible for scar formation, can inhibit both their ability to move and their ability to contract. We hypothesized that decreasing CCT-eta

in adult wounds would result in a beneficial effect on scar formation, with less scar deposition and contraction.

In the case of burn injuries specifically, another contributing factor to the crippling scar that can ensue is infection of the burn wound. This infection can cause an exaggerated inflammatory response, both locally at the burn site and also throughout the whole animal/person. Burn wound infection remains the leading cause of death after burn injury; *Pseudomonas aeruginosa* is a frequently encountered bacterium that infects burn wounds. We hypothesized that pre-treating burn wounds with *Lactobacillus*, a bacteria that is generally harmless in humans, as a probiotic therapy would counteract a *Pseudomonas* infection and inhibit the morbidity that can arise after burn injury. Probiotic therapy is the application of living bacteria for the benefit of the host organism.

Research Progress – Year 5

CCT-eta inhibition in an animal wound model

To determine if reducing CCT-eta could mitigate scar formation, we tested an inhibitor of CCT-eta in an animal wound model. Incisional wounds were made on the backs of rabbits (under anesthesia); some wounds were allowed to heal spontaneously, while others were treated with a CCT-eta inhibitor. After 4–5 weeks, these healed wounds were chemically and mechanically examined for their scar-related properties. The researchers found that treatment with a CCT-eta inhibitor resulted in marked beneficial effects for the healed wounds, reducing the parameters of scar formation while at the same time increasing the strength of the wounds compared to control untreated wounds. These encouraging results suggest that this strategy may prove fruitful in arriving at a novel therapy to prevent scarring.

Probiotic therapy in a model of burn wound infection

To test whether probiotic therapy would help lessen scar after burn injury, an animal model of burn wound infection was established. Limited full thickness burns were placed on rabbits (under anesthesia). These burn wounds were then treated with either *Lactobacillus* alone, *Pseudomonas* alone, or *Lactobacillus* with *Pseudomonas*. After approximately 4 weeks, healed burn wounds were examined for their scar-related properties. Application of *Lactobacillus* with the *Pseudomonas* resulted in improved wound healing compared to *Pseudomonas* alone, and appeared to decrease both the length and intensity of *Pseudomonas* infection.

Probiotic therapy in a model of burn wound-induced sepsis

Because sepsis (the spread of infection to the bloodstream potentially causing death) is the most feared complication of burn wound infection, the researchers also examined the effect of probiotic therapy with *Lactobacillus* in an animal model of burn wound sepsis. Mice had full thickness burns placed on their backs (under anesthesia). These burns were then injected with either *Lactobacillus* alone, *Pseudomonas* alone, or *Lactobacillus* with *Pseudomonas*. Mice treated with *Lactobacillus* alone all survived. Mice treated with *Pseudomonas* alone showed a >90% mortality, demonstrating that sepsis and death was resulting from the infected burn wound. Probiotic therapy with *Lactobacillus* in addition to the *Pseudomonas* reduced the mortality to <10%. This dramatic difference suggests that probiotic therapy may be a useful means of treating the problem of burn wound infection.

Conclusions

Scar formation remains a major cause of morbidity in both the military and civilian populations; as yet, there is no generally applicable agent proven to inhibit scarring. Both our CCT-eta inhibitor and our use of probiotic therapy demonstrate efficacy in pre-clinical systems and show great potential to achieve clinical translatability.



Therapeutic Delivery to Wounds

Peptide-mediated Delivery of Therapeutic Compounds into Injured Tissues during Secondary Intervention

Project 4.5.6, WFPC

Team Leader(s): Erkki Ruoslahti, M.D., Ph.D. (University of California, Santa Barbara [UCSB] and Sanford-Burnham Medical Research Institute)

Project Team(s): Tero Järvinen, M.D., Ph.D. (UCSB, Sanford-Burnham Medical Research Institute and University of Tampere, Finland); Shweta Sharma, Ph.D. and Gary Braun, Ph.D. (UCSB, Sanford-Burnham Medical Research Institute)

Collaborator(s): None

Therapy: Systemic drug targeting to injured tissues/preventing scarring/enhancing tissue regeneration

Deliverable(s): Systemic and local wound targeting with peptides that penetrate into wound and scar tissue.

TRL Progress: Beginning, TRL 1; Current, TRL 5/3; AFIRM I Target, TRL 5/3

Key Accomplishments: The researchers established an effective technology for the enhanced delivery of therapeutic compounds into injured tissues. They demonstrated the general applicability and effectiveness of the peptide-mediated methodology in delivering therapeutics to wounds. They found that a man-made protein consisting of the wound-homing peptide CARSKNKDC (CAR) and the anti-fibrotic protein decorin suppresses scar formation in mice with skin wounds. They also showed that a wound-homing peptide can deliver co-administered drugs not coupled to the peptide to injured tissue. Finally, they made the surprising discovery that the CAR peptide alone promotes wound healing, creating another drug candidate.

Key Words: Wound angiogenesis, homing peptides, anti-scarring, TGF- β

Introduction

As our body heals damaged tissue, it often does not regenerate the original tissue but replaces it with fibrotic scar tissue. The advantage of this process is speed, but the scar tissue fails to perform the functions of the original tissue. Another problem is that this healing process often overshoots, creating an excess of scar tissue, which can seriously limit normal function, such as the movement of a limb. Moreover, scar formation caused by a chronic injury can turn the bulk of an organ into scar tissue, creating life-threatening conditions such as liver cirrhosis and lung fibrosis. Thus, there is a great need for treatments that would control scar formation while enhancing the restoration of normal tissue after battlefield and civilian injuries. Current options in reducing scar formation are limited to local treatments, which are not very effective, and are impractical in multiple injuries. Clearly, systemic approaches to tissue repair would be valuable. Systemic delivery has its own problems: as the drug used will spread in the whole body, it may cause side effects. Also, more of the drug is needed than

with a local treatment, which drives up cost. In this project, the researchers have been developing a systemic treatment that is based on a new anti-scarring agent and also addresses the aforementioned general problems of systemic therapy.

Research Progress – Year 5

Targeting wounds

Homing peptide strategy

The research team's strategy to achieve effective systemic delivery of therapeutic agents into injured tissues is to use targeting compounds as guidance devices. These compounds have the ability to attach to the blood vessels in injured areas as they circulate in the blood through these vessels, but they do not bind to vessels in normal tissues. The binding to the vessels concentrates the compound in the wound, and if a drug is attached to the compound, more of the drug will accumulate in the wound than would go there otherwise. This makes it possible to use lesser amounts of a drug, reducing both side effects and cost.

The researchers' targeting compounds are peptides (small pieces of protein), which are selected from huge collections of peptides based on their ability to bind ("home") to wound tissue. They have focused on a wound-homing peptide that appears to be more efficient than other peptides in homing to early wounds. This peptide—referred to as the CAR peptide—consists of only 9 amino acids (a typical protein has 500-1000 amino acids).

Broad applicability of the technology

The research team's first task under this grant was to establish a general technology for the targeted delivery of therapeutics into injured tissues. They have shown that the CAR peptide can deliver into wounds payloads ranging from drug-like small molecules to proteins and the much bigger nanoparticles. They used skin wounds in mice for these studies. In the same model, the researchers showed that CAR homes to wound tissue before new blood vessels form in it, expanding the use of the technology. Using mice with lungs injured by high blood pressure in lung vessels or fibrosis, they showed that CAR also homes to tissues injured in ways other than wounding. Surprising results additionally showed that CAR promotes the delivery into wounds and other injured tissues of drugs that have been given together with the peptide but not chemically linked to the peptide. This finding provides another, potentially highly useful application of the technology. Collectively, these results establish general applicability and effectiveness of the targeting technology. The next section provides an example of this.

CAR-decorin anti-scarring agent

Design of a new anti-scarring agent, CAR-decorin

The research team's second task was to perform pre-clinical studies on an injury-seeking anti-scarring agent they designed to help advance it into clinical trials.

Prior to this grant, the researchers had discovered a new protein, decorin, which they found could prevent scarring. A biotech company at that time failed to bring decorin into the clinic, partly because it was expensive to manufacture the protein. The researchers felt that this product would still be valuable, as no good alternatives have emerged in the

intervening years. Armed with their new targeting technology, they believed that they were in a good position to create a successful decorin-based treatment for scarring conditions.

Efficacy of CAR-decorin

The research team designed a wound-targeted version of decorin by coupling it to the CAR peptide. They showed that the joint compound, CAR-decorin, accumulated in wounds more effectively than decorin alone. They found CAR-decorin to be more active than decorin in suppressing the activity of the main scar-causing growth factor than decorin. Thus, as they had hoped, they had a highly active variation of decorin that was effective at much lower doses than the original decorin. Thus, as originally projected, the researchers have created a new systemically acting anti-scarring compound and demonstrated its activity in a preclinical wound model. The company that has licensed the CAR and CAR-decorin technologies will now take the product further along the path of clinical development. The researchers are mainly interested in the mechanism whereby CAR penetrates into injured tissues.

CAR peptide promotes wound healing

While working on the CAR peptide and CAR-decorin, the researchers noticed that wounded mice treated with repeated injections of the CAR peptide without any drug attached to it promoted wound healing. Thus, they now have a way of preventing scarring and at the same time promoting the healing of wounds.

Conclusions

The researchers have established a peptide-based delivery system for drugs and biologics to wounds, and more broadly, to tissues damaged by injury or a disease process. The delivery can be accomplished either by covalently linking the payload to be delivered to the peptide, or by administering the two together, without chemical linking. The co-administration method is expected to greatly facilitate the introduction of the approach to the clinic because it is not necessary to create new chemical entities by coupling drugs to the peptide. The researchers have also been successful with the second aim of the original application by showing that a recombinant protein constructed by fusing a wound-homing peptide to an anti-fibrotic protein



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prevents scar formation in mouse skin wounds. The fusion with the homing peptide boosted the activity of the anti-fibrotic protein, not only by its expected activity in increasing the accumulation in wound tissue, but by greatly augmenting the activity of the main scar-causing protein in wounds. The researchers have also made the surprising discovery that their wound-homing peptide possesses an inherent

biological activity: it accelerates wound healing. Finally, this technology has been transferred to a biotech company for clinical development.

Planned Clinical Transitions

The technology has been licensed to a biotech company for further pre-clinical studies and introduction into the clinic.



Attenuation of Wound Inflammatory Response**Multi-functional Bioscaffolds for Promoting Scarless Wound Healing****Project 4.5.3, WFPC**

Team Leader(s): Newell R. Washburn, PhD (Carnegie Mellon University [CMU])

Project Team(s): Emily Friedrich (CMU)

Collaborator(s): Robert J. Christy, PhD (U.S. Army Institute of Surgical Research [USAISR])

Therapy: Cytokine-neutralizing gels

Deliverable(s): Gels that locally neutralize pro-inflammatory cytokines

Objective: Gels that inhibit burn progression through localized modulation of inflammation

TRL Progress: Beginning, TRL 1; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers found that a conjugation of anti-tumor necrosis factor- α (TNF- α) to hyaluronic acid (HA) led to a significant reduction in secondary necrosis in a burn injury progression model.

Key Words: antibody, cytokine, burns, inflammation, polymer

Introduction

The trajectory of burn wound healing is a complex process starting with necrosis due to the thermal injury, followed by a two-stage inflammatory process, delayed cell death, formation of granulation tissue, and remodeling. The complications from partial or full-thickness burns are broad ranging, including compromised protection by the epidermis and loss of resident leukocytes and lymphocytes, edema, reduced host defenses to bacterial colonization, multiple organ failure, and loss of connective tissue cells that would normally contribute to the repair response. Burned tissue has been modeled as having three concentric zones: (1) irreversibly damaged tissue in the zone of coagulation; (2) hypoperfused tissue in the zone of stasis; and (3) edematous tissue in the zone of hyperemia. The central necrotic zone often progresses into surrounding zones, which increases the likelihood of hypertrophic scarring and patient morbidity. Deleterious physiological responses following thermal injuries are driven by inflammatory responses. Currently, there are no approved therapies for preventing burn progression and secondary necrosis.

Research Progress – Year 5**Identify formulations that are active in reducing secondary necrosis*****Comparing formulations***

The Washburn group explored whether topical application of antibodies targeting TNF- α or interleukin-6, both purchased from R&D Systems and species-matched to rat to ensure strong binding in vivo, conjugated to HA could reduce the extension of necrosis by modulating inflammation locally in a partial thickness rat burn model. Partial thickness to deep partial thickness burn injuries present significant challenges in healing, as these burns often progress following the initial thermal insult, resulting in necrotic expansion and increased likelihood of secondary complications. Necrotic expansion is driven by a microenvironment with elevated levels of pro-inflammatory mediators, and local neutralization of these using antibody conjugates could reduce burn progression.

Comparing conjugated (anti-TNF- α)-HA to mixtures of the same

The Washburn group, in collaboration with Dr. Christy's group at USAISR, tested mixtures of anti-TNF- α and HA with conjugated (anti-TNF- α)-HA to determine whether conjugation was important for activities in a burn injury model. The burn progression results confirm that conjugation



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is critical for the potent anti-inflammatory effects (e.g., reduction in secondary necrosis) observed with these materials (Figure 1).

Conclusions

The researchers determined that the conjugation of anti-TNF- α and HA led to a significant reduction in secondary necrosis in a burn injury progression model.

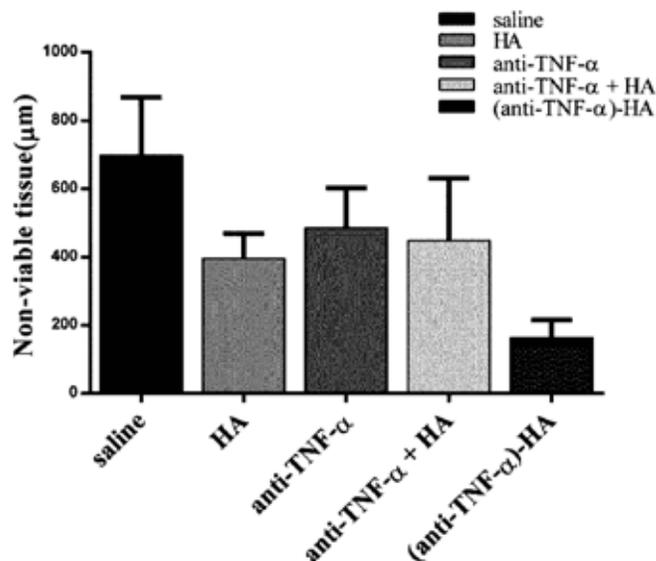


Figure 1. Thickness of necrotic tissue on Day 7. The (anti-TNF- α)-HA conjugate-treated burn sites had significantly thinner necrotic zones than the sites treated with other compositions.



Attenuation of Wound Inflammatory Response**Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration****Project 4.5.4, WFPC**

Team Leader(s): Patricia A. Hebda, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine [MIRM])

Project Team(s): Joseph E. Dohar, MD and Tianbing Yang, PhD (University of Pittsburgh, MIRM)

Collaborator(s): None

Therapy: Attenuate local inflammatory responses to reduce scarring and promote healing

Deliverable(s): Proof of main concepts

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The research team completed cell therapy studies using regenerative phenotype donor cells. The researchers revised their combination drug therapy protocol to use U.S. Food and Drug Administration-approved pharmaceutical agents. Several important differences in wound modulation were found to be shared by regenerative fibroblasts, supporting the promise of using a patient's own cells for regenerative (scarless) wound therapies. Wound therapy, with drugs and donor cells, was optimized for translational studies to come.

Key Words: scarless healing, inflammation, fibrosis, cell therapy

Introduction

Two critical technical challenges of inducing regenerative healing are the suppression of inflammation and the control of fibroblast recruitment from the surrounding host tissue. Inflammation triggers the release of mediators that result in scarring. Wounds on the battlefield can be major, requiring amputation, reconstruction, or other medical interventions, or minor, able to be managed primarily, or somewhere in between. But regardless of the extent and complexity of the wound, there is always the concern that the wound will heal with scar, rather than regeneration—with potential impairment of tissue function. In the burn environment, scarring can become hypertrophic, which further limits function and impairs cosmesis.

Research Progress – Year 5**Combination anti-inflammatory, anti-fibrotic pharmacologic therapy**

Early, short-term treatment with anti-inflammatory agents can attenuate the wound inflammatory response with downstream effects on healing. The finding that treated wounds have different collagen type deposition with higher regain of tensile strength supports the research team's hypothesis that controlled modulation of inflammation can decrease fibrosis without impeding the healing

process, and demonstrates that further pursuit of this approach is warranted.

Cell therapy using donor cells with regenerative phenotypes

Phenotypic and functional features of four different donor cell types were assessed to explore the cellular and molecular mechanisms contributing to quality of healing/scarring—adult skin fibroblasts, embryonic day 15 (E15) skin fibroblasts, E18 skin fibroblasts and adipose-derived stem cells (ASCs). Cells with regenerative phenotypes (E15 fibroblasts and ASCs) produced faster healing with a more mature and better organized collagen matrix. Functional features of the donor cells correlated with healing outcomes, suggesting that not all donor cells are equally beneficial.

Applying cell therapy together with pharmacologic therapy

The investigators evaluated the synergistic effects of cell therapy and anti-inflammatory treatment (with COX-2 inhibitors), and both showed significant improvements in wound healing. However, no synergistic effects were observed using the two approaches together in the rat model; that is, there was no further enhancement to the rate of healing by combining cell therapy with a COX-2 inhibitor. The results may be different in other animal models



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and humans, but the main finding is that the anti-inflammatory treatment does not interfere with cell therapy and still contributes to reduced fibrosis.

Conclusions

The research team has established that early, short-term treatment with anti-inflammatory agents attenuates the wound inflammatory response with downstream effects on healing. The finding that treated wounds have different collagen type deposition with higher regain of tensile strength supports the hypothesis that controlled modulation of inflammation can decrease fibrosis without impeding the healing process and demonstrates that further pursuit of this approach is warranted.

Future Research Plans

The next phase of the work is aimed toward two well-established components of wound healing that are linked to scar formation—the inflammatory response and the fibroblast phenotype. By the informed regulation of these wound healing components, the course of wound healing may be

redirected toward a more regenerative outcome. The goal is to design and optimize the combination pharmacologic therapy that provides a wound environment for rapid, regenerative healing with minimal scar formation, based on previous and emergent results achieved during AFIRM I.

Planned Clinical Transitions

Funding is needed to continue the work into the translational phase, after which time there should be a well-established foundation to support the adoption of this combination anti-scarring topical treatment for scar prevention in a number of indications including combat-related burns and trauma. This intervention could be readily available in field hospitals and has potential value as an adjunctive treatment with cell- and tissue-based wound therapies.

Corrections/Changes Planned for the Future

The research team will focus on the pharmacological therapy and consider combination with cell or tissue therapy as an adjunctive, supportive measure.



Clinical Trials

Clinical Trial: Autologous Fat Transfer for Scar Prevention and Remodeling (AFT-SPAR)

Project 4.7.3, RCCC

Team Leader(s): Adam J. Katz, MD (University of Florida [UF])

Project Team(s): Justine M. Pierson, BS (University of Florida); Lauren Macri, PhD (Rutgers University); Jane Reese, MBA, MS and Emily Collins, MS (Case Western Reserve University)

Collaborator(s): David Drake, MD and Catherine Ratliff, PhD (University of Virginia [UVA]); Colonel Robert Hale, DDS, Rodney Chan, MD and Gale Mankoff, RN (U.S. Army Institute of Surgical Research [USAISR]/ Brooke Army Medical Center [BAMC])

Therapy: Scar prevention and management

Deliverable(s): Autologous fat transfer for scar prevention and remodeling (AFT-SPAR): A new indication for an existing surgical procedure

TRL Progress: Beginning, TRL 5; Current, TRL 6; AFIRM I Target, TRL 7

Key Accomplishments: To date, the researchers have enrolled and treated a total of 10 patients. The key accomplishments for the past year were the successful transition of the clinical trial to the UF, and the finalization of all administrative and protocol-related logistics at both UF and USAISR/BAMC, such that the trial is open to enrollment at two new sites.

Key Words: adipose tissue; fat grafting; scar

Introduction

Severe blast and burn injuries are associated with extensive cutaneous scar formation, even with the implementation of the most advanced reconstructive techniques. Such scarring can result in significant deformity and/or functional loss. From a tissue-level perspective, many scar beds are devoid of a subcutaneous layer (hypodermis). This deficit is often overlooked or minimized as part of a patient's reconstruction and recovery, yet it presents a significant and important challenge and therapeutic target. The "creation" of a hypodermis (subcutaneous) layer beneath a tight, immobile scar bed (whether through AFT or by the de novo regeneration/engineered replacement of such tissue) has the potential to affect both the appearance and quality of a scar, and therefore to improve the patient's quality of life.

Current available therapies, such as semi-occlusive dressings (e.g., silicone), pressure (garment) therapy, and/or local steroid injections, are limited in their use and/or impact. There is clearly a need for more effective methods and therapeutics for scar prevention and management. Moreover, AFT could be used in tandem with one or more of these existing treatments.

Recent reports suggest that adipose tissue and the mesenchymal stem cells it contains may not only enhance the healing of difficult wounds, but beneficially affect the scarring process as well. AFT represents the simplest and most fundamental approach to exploring this concept, with the potential to provide an immediate positive impact.

AFT is a single-stage procedure that involves the removal of adipose tissue from one site of a patient followed by the immediate and autologous transplantation/infiltration of this tissue into a different site of the same patient. AFT is a routine surgical procedure, albeit with the clinical intent of bulking and/or filling contour deformities, and has been proven to be safe (>50,000 procedures were performed last year in the U.S. alone). With evidence-based delineation of efficacious and reproducible treatment parameters and techniques, AFT-SPAR could emerge as a simple yet effective approach to improving the lives of wounded soldiers. The results of this trial may change the manner in which burned and other scarred patients are routinely cared for, supporting the use of a relatively simple procedure to decrease the cosmetic, functional, and/or psychological consequences patients experience



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in association with scarring. However, this approach has yet to be evaluated within the setting of controlled studies; evidence-based treatment parameters need to be established.

Clinical Trial Status

During this past year, Dr. Katz relocated from UVA to UF. As part of, and during this relocation, the original UVA site remained open for subject enrollment, with Dr. David Drake as the site primary investigator, and Catherine Ratliff continuing as the site study coordinator. During the year, approximately six new subjects were screened for the study at the UVA site. Unfortunately, none of these subjects proceeded to subsequent trial enrollment. This low rate of enrollment remains the major limiting factor to the expedient completion of the study. Primary reasons given by subjects for choosing not to enroll in the study relate to concerns associated with time commitment and missing more work, “surgical fatigue,” and concern that the treatment site would look abnormally different than control (sham) site at the conclusion of the study.

The UVA site is scheduled to close as a treatment site as of July 2013. During its time as the primary site, UVA enrolled and treated a total of 10 patients—seven in the late treatment cohort and three in the early treatment cohort. The late treatment cohort has advanced to the highest treatment dose per protocol. The early treatment protocol will begin the second level treatment dose with the next enrolled subject. All data acquisition has proceeded smoothly to this point and there have been no adverse events, but it is still too early to draw any conclusions from the data. All 10 of these patients have completed the entire 12-month follow-up period.

While UVA will be closing as a treatment site, this past year saw the slow, but eventual opening of two new clinical trial sites: UF and USAISR/BAMC. These large milestones included many smaller tasks, including the preparation and approval of new

budgets, new statements of work, the preparation, submission and approval of Institutional Review Board protocols and informed consents, the completion of financial contracts between institutions and the Department of Defense/Rutgers, and the submission and approval of protocols and consent forms by the Human Research Protection Office. In addition, new equipment had to be purchased, shipped, learned, and tested. Updates to standard operating procedures and manual operating protocol had to be written and circulated. Multiple modifications to protocols and consents had to be submitted at each of the three institutions.

Throughout the year, the team held regularly scheduled telephone conferences with pertinent team members and supporting staff. At the time of this writing, the team is happy to report that UF and USAISR/BAMC are both open for enrollment, and both have initiated the pre-screening of several potential subjects.

As mentioned above, active challenges relate primarily to efforts to enhance the enrollment of patients into the study. This is being addressed by providing some monetary incentive to potential subjects, as well as by looking for opportunities to treat patients within the context of the trial in conjunction with their going to surgery for other indications.

Future Clinical Plans

Future efforts will now be focused on the enrollment of patients at UF and USAISR/BAMC.

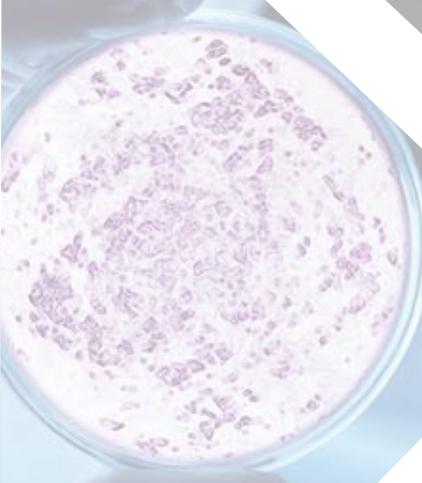
Planned Commercialization Transitions

There are multiple companies that currently market fat tissue transplantation devices and systems. If the evidence from this trial supports the use of AFT for scar remodeling, commercialization will not be a problem.

V: Burn Repair

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Intravenous Treatment of Burn Injury

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

Project 4.6.1, RCCC

Team Leader(s): Fubao Lin, PhD and Richard Clark, MD (Stony Brook University)

Project Team(s): Adam J. Singer, MD and Laurie Crawford, BS (Stony Brook University)

Collaborator(s): Marcia Tonnesen, MD (Stony Brook University)

Therapy: Therapy for burn injury progression

Deliverable(s): Intravenous administration of fibronectin (FN)-derived peptide, P12

TRL Progress: Beginning, TRL 1; Current, TRL 4; AFIRM I Target, TRL 5

Key Accomplishments: The researchers demonstrated that P12 is a cryptic peptide and is exposed by the interaction of fibroblasts with FN when cells are seeded.

Key Words: burn; injury progression; P12

Introduction

Battlefield polytrauma secondary to blasts and explosions is increasingly common, affects multiple sites, and is complex. Many of these injuries, but particularly burns, are subject to progressive tissue damage over subsequent days that is likely secondary to repetitive reperfusion injury. Progressive extension of burns and other battlefield injuries can have a devastating effect. Over the course of a few days to one week, deep partial thickness burns can become full-thickness burns leading in the short term to increased tissue loss, longer healing time, and greater morbidity and mortality. In the long term, increased scarring, wound contractures and poor quality of life become major issues. Therapies to improve blood flow, such as non-steroidal anti-inflammatory agents and anti-coagulants (heparin) have not shown substantial benefit in preventing burn injury progression. Hence, despite many studies, therapy to limit burn injury progression remains an unmet need.

Over the past year, the researchers strove to further determine the safety and efficacy of the therapeutic agent P12 and its derivative, and establish systemic dosing details and treatment validation to prevent burn injury progression, reduce inflammation, induce healing and inhibit scarring with P12 or

P12 derivative intravenous infusion. Additionally, a new project (4.6.1a) was funded this year to develop P12 or its derivative as a first-in-class therapeutic for burn injury progression. The major deliverable would be the requisite Investigational New Drug (IND)-enabling data and resultant final protocol for clinical evaluation of P12 as a therapeutic agent to limit burn injury progression. Details on the relevant in vivo studies can be found within the Project 4.6.1a progress report.

Research Progress – Year 5

P12 is a cryptic peptide derived from the FN first type III (FNIII1) repeat

Previously, the research team demonstrated that FN or FN growth factor binding domains enhanced cell survival only when they were bound to the cell culture surface. They showed no effects on FN-null cell or adult human dermal fibroblast survival when they were added in solution even in the presence of platelet-derived growth factor-BB (PDGF-BB). The researchers identified a 14-mer peptide, named P12, in the FNIII1 repeat that exhibited growth factor binding activity. They found that P12 supported FN-null cell or human fibroblast survival under both surface-bound and in-solution conditions in the presence of PDGF-BB. This led the investigators

to hypothesize that P12 is a cryptic peptide embedded in FN that is exposed when cells spread and migrate over FN, which applies enough tension to open surface-bound FNIII1. While FN is in solution, no such tension is applied and P12 is not exposed.

To confirm this hypothesis, FN, P12, or FNIII1-derivative P12 parent peptides, Ana1-38 (38-mer) and P1(25-mer), were coupled to the Glutathione S-transferase-tagged FN central cell binding domain, which had been pre-coated on 96-well plates. The results demonstrated that only FN was detectable when a polyclonal antibody to intact FN was used. When a polyclonal antibody against P12 was used, both P12 and P12 parent peptides, Ana1-38 and P1, were detectable, while intact FN was not (Figure 1). These results demonstrate that P12 is a cryptic peptide embedded in FN.

To further confirm this conclusion, human fibroblasts were seeded on 48-well cell culture plates in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) until they were confluent. Cells were removed with 20 mM EDTA alone (Figure 2A, B) or in the presence of P12 polyclonal antibody (Figure 2C). The results showed that FN was detectable with antibody to intact FN (Figure 2A). P12 was detectable only when antibody to P12 was present during cell removal. After removing cells, P12 was not detectable even if FN was present. These results further confirmed that P12 is a cryptic peptide embedded in FN. It is consistent with the findings of FNIII1 unfolding to a mechanically stable intermediate about four times the length of the native folded state. FN molecule unfolding within intact fibers under cell-derived force has been reported.

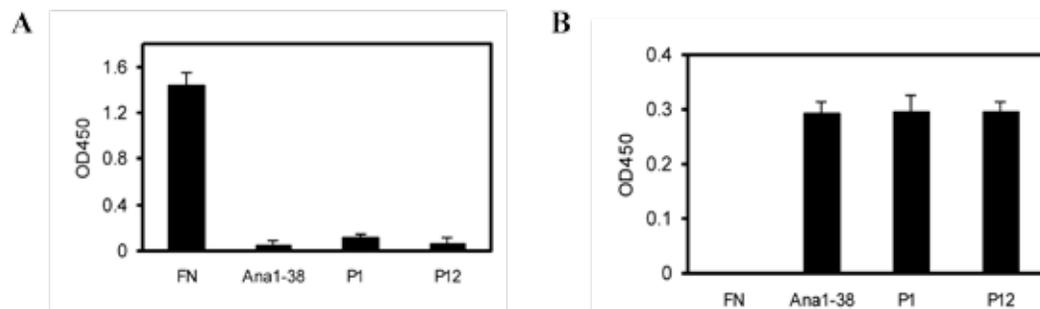


Figure 1. Detection of FN or P12 by enzyme-linked immunosorbent assay. The same molar amount of FN or FN-derived peptides were coated on 96-well plates. After blocking with 2% bovine serum albumin, FN or P12 was probed with specific polyclonal antibody against intact FN (A) or P12 peptide (B), followed by incubation with horseradish peroxidase-labeled 2nd antibody. The relative amount of bound antibody was detected by colorimetric assay and optical density was measured at 450 nm. Mean values \pm SE, $n=6$.



Figure 2. Detection of FN or P12 by fluorescence images. Human fibroblasts were seeded on 48-well cell culture plates in DMEM with 10% FBS until they were confluent. Cells were removed with 20 mM EDTA alone (A, B) or in the presence of P12 polyclonal antibody (C). After washing, FN or P12 were probed with polyclonal antibody specifically against intact FN (A) or P12 (B, C), followed by incubation with biotin-labeled 2nd antibody and avidin-labeled Qdot 625. Fluorescence images were taken with EVOS microscope (AMG).



Conclusions

It is known that FN is depleted in the blood and tissue after burns. The researchers have shown that the FN-derived peptide P12 in concert with growth factors sustains tissue cell survival. Current in vivo results demonstrate that an infusion of P12 limits burn injury progression and speeds re-epithelialization (see Project 4.6.1a for details). The researchers hope that this therapy will reduce the morbidity and mortality of burn injury in the wounded warrior, and allow them to return to active service sooner than otherwise would be possible.

Future Research Plans

The research team will continue to investigate the mechanism of P12 bioactivity at the molecular level in both endothelial cells and fibroblasts (supported by a National Institutes of Health R21 grant).

Planned Clinical Transitions

The researchers will continue to execute pre-IND studies of P12 treatment to prevent burn injury progression. These studies are being supported by AFIRM through a subcontract awarded to NeoMatrix Formulations, Inc. (refer to Project 4.6.1a for more details). NeoMatrix Formulations, Inc. intends to file an IND based on the activities supported by this award by the end of 2015.



Intravenous Treatment of Burn Injury**Pre-IND Studies for a Novel Cell Survival Peptide that Limits Burn Injury Progression****Project 4.6.1a, RCCC**

Team Leader(s): Richard Clark, MD (NeoMatrix Formulations, Inc.)

Project Team(s): Fubao Lin, PhD and Laurie Crawford, BS (NeoMatrix Formulations, Inc.)

Collaborator(s): Adam J. Singer, MD (Stony Brook University)

Therapy: Therapy for burn injury progression

Deliverable(s): Intravenous (IV) administration of fibronectin (FN)-derived peptide, P12

TRL Progress: Beginning, TRL 4; Current, TRL 4; AFIRM I Target, TRL 5

Key Accomplishments: The researchers demonstrated that cyclic P12 (cP12) significantly enhances re-epithelialization of burn injury in the swine vertical burn injury model.

Key Words: burn; injury progression; P12

Introduction

Battlefield polytrauma secondary to blasts and explosions is increasingly common, affects multiple sites, and is complex. Many of these injuries, but particularly burns, are subject to progressive tissue damage over subsequent days with conversion of partial-thickness burns to full-thickness burns. In the U.S. civilian population each year, approximately 500,000 patients with burns present to emergency departments. Of 40,000 annual hospital admissions, 25,000 burn victims are admitted to specialized burn centers. Progressive extension of burns can have a devastating effect. Over the course of a few days to one week, deep partial thickness burns can become full-thickness burns, which in the short term leads to increased tissue loss, longer healing time, and greater morbidity and mortality. In the long term, increased scarring, wound contractures and poor quality of life become major issues. While the exact mechanism(s) leading to conversion of the zone of ischemia to full-blown necrosis is unclear, several processes, including oxidant and cytokine stress resulting from inflammation as well as ischemia/reperfusion, probably play a role. Therapies to improve blood flow, such as non-steroidal anti-inflammatory agents and anti-coagulants (heparin) have not shown substantial benefit in preventing burn injury progression. Therefore, therapy to limit burn injury progression is an unmet need.

P12 is a recently discovered 14-residue peptide that is cryptic within the immunoglobulin sandwich type of β -pleated sheet of FN first type III repeat. FN, a 500 kDa glycoprotein, circulates in the blood and is produced and deposited by tissue cells in the provisional extracellular matrix (ECM) during tissue formation. As a critical component of the provisional ECM, FN plays a vital role in embryogenesis, morphogenesis and wound healing, but is deficient in burn patients' wounds and blood. Unlike FN, P12 in solution promotes mesenchymal cell growth, proliferation and migration intrinsically and synergistically with a variety of growth factors including platelet-derived growth factor-BB (PDGF-BB) and vascular endothelial cell growth factor (VEGF). Furthermore, P12 protects adult human dermal fibroblasts and human dermal microvascular endothelial cells from cell death induced by oxidative and cytokine stress and/or nutrient withdrawal in the presence of PDGF-BB or VEGF, respectively. Most importantly, P12 limits burn injury progression in rat and porcine burn models, and mitigates scarring and scar contraction in a vertical burn injury progression pig model.

The overall objective of this project is to develop a novel, cell survival peptide (P12) as a first-in-class therapeutic for burn injury progression. The major deliverable is the requisite Investigational New Drug (IND)-enabling data and resultant final protocol for clinical evaluation of P12 as a therapeutic agent to limit burn injury progression. Successful



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completion of this project would translate in vitro and preclinical knowledge of the peptide's activity to a potential therapy that would transform the way we care for burns. NeoMatrix Formulations, Inc. has already obtained orphan drug designation for the peptide, thus creating access to potential funding through the U.S. Food and Drug Administration as well as non-government sources to support clinical trials and commercialization.

Research Progress – Year 1 (Project began in 2012)

Porcine vertical burn injury progression model

In order to determine the optimal dose and treatment times for systemically administered P12 or P12 derivative, the researchers used female Yorkshire swine (~20 kg) for their burn injury model. While under general anesthesia, 20 burns were created on the back of each animal using a 2.5 x 2.5 cm x 7.5 cm aluminum bar. The bar was heated in a water bath to 800C and then applied perpendicular to the skin for a period of 20 seconds. The researchers infused cP12 or buffer 1 hour post-burn. Full thickness 6 mm punch biopsies were taken from all burns at time points as indicated in **Figure 1**, leaving a perimeter of approximately 5 mm from the burn edge to eliminate an edge effect. The biopsies were bisected and fixed in formalin.

Burn injury progression infused with or without cP12

To study the effects of cP12 on vertical burn injury progression, the research team infused swine with

cP12 in Lactated Ringers solution at 0.1, 0.3, or 1.0 mg/kg, or Lactated Ringers solution alone as a control at 1 hour post-burn. The results demonstrated that cP12 at 0.1 and 0.3 mg/kg significantly enhanced re-epithelialization of burn injury at Day 7 and Day 10 (**Figure 2**). The highest dose of cP12 (1 mg/kg) showed little effect on re-epithelialization of burn injury, which the researchers feel may be related to a potential side effect of vasoactive constriction during infusion.

Conclusions

In the related Project 4.1.2, the researchers have shown that the FN-derived peptide P12 in concert with growth factors sustains tissue cell survival. The current in vivo results from this project demonstrate that an infusion of P12 limits burn injury progression and speeds re-epithelialization. The researchers hope that this therapy will reduce the morbidity and mortality of burn injury in the wounded warrior, and in addition allow them to return to active service sooner than otherwise possible.

Future Research Plans

Preclinical trials will continue with the support of the U.S. Army Medical Research and Materiel Command through AFIRM I.

Planned Clinical Transitions

The research team will continue ongoing pre-IND studies of P12 treatment to prevent burn injury progression. NeoMatrix Formulations, Inc. intends to file an IND based on the activities supported by this award by the end of 2015.

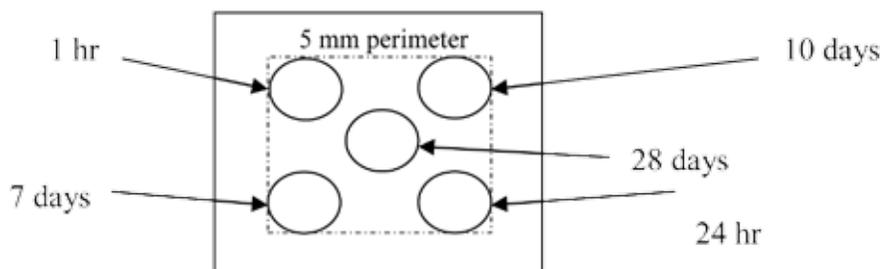


Figure 1. 2.5 x 2.5 cm burn with edge perimeter and biopsy locations in porcine vertical burn injury progression mode. A full-thickness 6 mm punch biopsy was taken at the time point as indicated. The samples were bisected and fixed in formalin. Samples collected at 7 and 10 days post burn were analyzed for re-epithelialization. Samples collected at 28 days post burn were analyzed for scar depth. Samples taken at 1 and 24 hours were processed and stored.

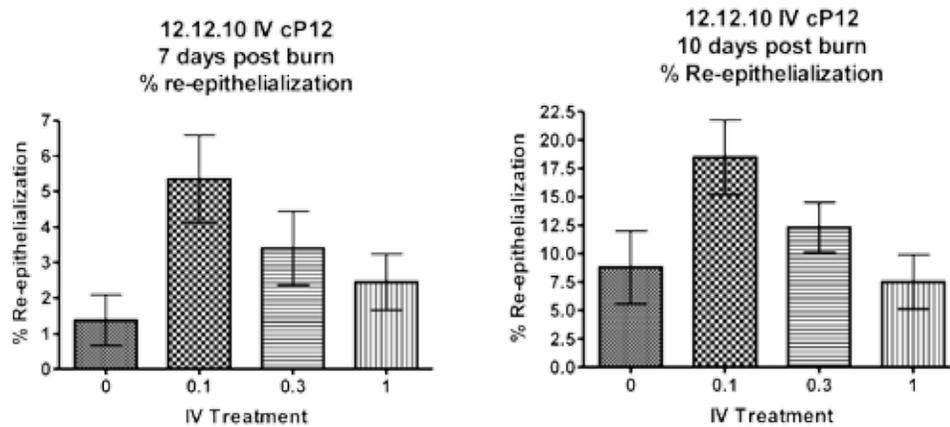


Figure 2. cP12 enhances re-epithelialization of burn injury. Buffer or varying doses of cP12 (0.1, 0.3, 1.0 mg/kg) were infused 1 hour after burns. Biopsy were taken at Day 7 and Day 10, bisected, and fixed in formalin. All biopsies were alcohol-dehydrated, xylene-cleared, paraffin embedded, sectioned at 5 μ m, stained with Hematoxylin and Eosin, and evaluated by a board-certified dermatopathologist for re-epithelialization. Statistical analysis was performed using 1-way Analysis of Variance (Kruskal-Wallis Test and Dunn's Multiple Comparison Test, n=20). Future studies will test treatments at lower dosages.





Topical Treatment of Burn Injury

Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues

Project 4.2.3, WFPC

Team Leader(s): Mark Van Dyke, PhD (Wake Forest School of Medicine [WFSM])

Project Team(s): Deepika Poranki, PhD, Carmen Gaines, PhD, and Olga Roberts, PhD (WFSM)

Collaborator(s): James Holmes, MD, Joseph Molnar, MD, PhD, Justin Saul, PhD, Mark Lively, PhD, and Roche de Guzman, PhD (WFSM); Luke Burnett, PhD (KeraNetics, LLC); Michelle Merrill (Wake Forest University)

Therapy: Wound dressing for burn therapy

Deliverable(s): Keratin biomaterial-based burn treatment development and preclinical testing

TRL Progress: Beginning, TRL 3; Current, TRL 5; AFIRM I Target, TRL 5

Key Accomplishments: The researchers have completed a mechanistic study and a pivotal preclinical study in swine. The swine study showed promising results for faster wound closure and no scarring. The U.S. Food and Drug Administration (FDA) 510(k) pathway is being actively pursued, but will not allow claims of tissue salvage (i.e., enhanced cell survival after thermal injury). Tissue salvage claims can be pursued with additional preclinical testing that the FDA will require for such an indication.

Key Words: Burn, keratin, biomaterial, hydrogel, skin

Introduction

For burns requiring hospitalization, the standard of care often involves a period of wound care and observation until wound demarcation is complete and the burn surgeon can make a determination as to the need for excision of dead skin and grafting. This “wait and see” period is based on the process of conversion, where damaged cells “convert” from thermally stressed to dead tissue. This is also referred to as burn wound progression. While the specific structures and cellular interactions remain to be fully understood, keratin biomaterials offer a platform for biomedical applications wherein materials can be tuned to elicit behaviors of interest (e.g., protection after thermal stress, wound healing).

During the tenure of this project, the researchers utilized their previously developed swine burn model to conduct a pivotal, preclinical trial. They also completed a mechanistic study to help define the primary mode of action of the keratin biomaterial. All of this work was conducted with AFIRM funding.

Research Progress – Year 5

Pivotal Swine Burn Study

This study was conducted using a swine burn model previously developed by the project team.

Twenty-eight (28) female Yorkshire swine were used under a protocol approved by the Wake Forest Institutional Animal Care and Use Committee and the Animal Care and Use Review Office. The animals were randomly divided into the two arms of the study, one in which treatment would be administered within 60 minutes of burning and another in which treatment was delayed for 10 hours. In each arm, 14 animals were randomized and 12 burn wounds were created on each animal with heated cylindrical brass blocks, 6 on each side of the dorsal mid-line between the shoulder and hip under general anesthesia. The brass blocks were heated in an 80:20 (volume:volume) boiling PEG:water solution. Once the boiling liquid was heated to 105-115°C, the brass blocks were used to create burns on the pig by contact with the skin for 20 seconds. The wounds were randomized into 1 of 4 treatment groups: silver sulfadiazine (SSD), Coloplast™ (a collagen-based wound gel), keratose hydrogel used in previous pilot studies, and the KeraStat™ Burn formulation.

For each treatment, at each time point in both arms of the study, six replicate wounds were created using two animals. The researchers used 3 cc of SSD, keratin or Coloplast on each wound, and all wounds were covered with a Telfa pad, Ioban™ dressing, protective plastic shield, and a nylon jacket. Every 3 days, the dressings were removed, and the wounds

were cleaned and debrided with saline-soaked gauze. Digital photos were taken with a color wheel and ruler in view for digital image processing. On Days 1, 3, 6, 9, 12, 15, and 30 post-surgery, two animals were euthanized and tissue was collected. The skin tissue sections were analyzed histologically using Gomori trichrome, hematoxylin and eosin, and von Willebrand factor (blood vessels) staining, and results were quantified using typical morphometric techniques. Digital photos taken at dressing changes were measured for wound area.

Wound area measurements showed that keratin-treated wounds were significantly smaller at Days 9, 12, and 15 compared to SSD cream. Wounds were also tattooed so that contracture could be measured. No appreciable wound contracture was noted in any of the treatment groups. Histomorphometric analysis showed two distinct phases of healing. In one phase, there was a modest rate of re-epithelialization due to the changing nature of the wound bed. A second, more rapid phase was marked by the appearance of granulation tissue. Re-epithelialization data for both phases appeared to show that keratin treatment provided benefit in the early phase of treatment whereas SSD did not, and all treatments appeared to provide benefit in the later phase of healing (although SSD was discontinued after Day 15). Although all wounds healed in this model, the average days to wound closure was the shortest in the KeraStat Burn treatment group. A non-healing, critical-size burn model was proposed for more rigorous testing of the optimized keratin hydrogel (KeraStat Burn), but funding to develop and utilize this model was not awarded.

Heat Shock Mechanism Study

Heat shock experiments have been concluded using the in vitro model (previously reported) in which gene microarray analysis was used to investigate the regulation of cell death pathways in keratin-treated mouse dermal fibroblasts. Briefly, mouse dermal fibroblasts were isolated from adult CD1 mouse ear pads and grown to near confluence. Cultures were treated at 44°C for 150 minutes to induce necrosis and stress. Following this “heat shock,” cells were maintained for 6 hours under normal culture conditions, and then non-adherent cells were removed and treatments were applied to the remaining adherent cells. Cells were harvested at

12, 18, and 24 hours, and ribonucleic acid extraction was performed for polymerase chain reaction (PCR) microarray analysis. Complementary deoxyribonucleic acid (DNA) synthesis was performed and then a PCR microarray mouse cell death pathway finder was used to identify the expression of genes that are involved in cell death.

The researchers compared post-treatment (12, 18, and 24 hours) to the pre-treatment control (6 hours). They calculated the ratio of gene expression (fold change) for gamma keratose treatment to fibroblast media treatment (i.e., fold change value for keratose treatment divided by the fold change value for fibroblast media control treatment). There were four main findings from the experiments:

1. As expected, heat treatment of mouse dermal fibroblasts upregulated cell death pathway-related genes.
2. Gene expression was more consistent (i.e., smaller p values) in cells treated with gamma keratose compared to cells treated with fibroblast growth media at the early time point post thermal stress (12 hours).
3. A single gamma keratose treatment at 0.1 mg/mL appeared to influence gene expression at 12 and 18 hours post-thermal stress (6 and 12 hours post-treatment, respectively), but this effect was diminished by 24 hours.
4. In general, treating these thermally stressed cells with gamma keratose substantially diminished gene upregulation compared to treatment with fresh fibroblast growth media.

To further explain the first point, real-time PCR array data analysis showed that there was an overall upregulation of the genes involved in cell death pathways (necrosis, autophagy, apoptosis) 6 hours post thermal stress (i.e., 12-hour time point), and the cell viability data from the in vitro thermal stress model correlates to this point. After 6 hours post thermal stress, there was a drop in the cell viability from 100% to 53.7% ± 4.35.

To further explain the second point, at 12 hours post thermal stress, gamma keratose-treated cells showed a statistically significant ($p < 0.05$) difference in expression of 29 genes compared to the pre-treatment control (6 hours). Whereas in the growth media-treated cells, although there was high



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expression (fold change) of the genes compared to the pre-treatment control, no statistical significance was observed ($p > 0.05$) due to the variability in the sample replicates for this treatment group.

As mentioned in point three, treating the thermally stressed cells once with 0.1 mg/mL gamma keratose was able to maintain cell viability by regulating the cell death-related genes at 12 and 18 hours. At 12 hours, 29 genes had a significant ($p < 0.05$) gene expression in gamma keratose-treated cells compared to the pre-treatment control (6 hours). Of these, 19 genes were highly expressed by the growth media-treated cells. By 18 hours, only 18 genes were significantly ($p < 0.05$) expressed in gamma keratose-treated cells, whereas 34 genes were significantly ($p < 0.05$) expressed in growth media-treated cells compared to the pre-treatment control (6 hours). The regulation of cell death-related genes by gamma keratose strongly correlates with the in vitro thermal stress model cell viability results. In the in vitro model at 12 hours post thermal stress, gamma keratose-treated cells were able to maintain cell viability at $37.8\% \pm 0.85$ compared to growth

media-treated cells at $24.41\% \pm 2.20$, and the difference was statistically significant ($p < 0.05$). At 18 hours, gamma keratose-treated cells were able to maintain cell viability at $39.83\% \pm 5.54$, whereas in growth media-treated cells, additional cell death was observed with an average percent cell viability of only $20.18\% \pm 4.58$. This difference was statistically significant ($p < 0.01$). By 24 hours, there was no significant differences in the cell viability, and the gene expression was similar between the two treatment groups (**Table 1**).

To further explain the fourth point, at 12 hours, 10 genes in gamma keratose-treated cells showed a statistically significant difference ($p < 0.01$) compared to the pre-treatment control. Among these 10 genes, two genes involved in autophagy (Ulk1) and both apoptosis and autophagy (Casp 3) were upregulated, 8 genes involved in necrosis (Parp2, Pvr), autophagy (Atg5, Map1lc3a, Atg7), apoptosis (proapoptotic[Fas1]) and both apoptosis and autophagy (Bax, Akt1) were downregulated in the gamma keratose-treated group compared to the growth media-treated cells (**Table 2**).

Table 1. Gene expression of gamma keratose treated cells at 24 hours

Genes showing significant difference to the pre-treatment control (i.e., p -value < 0.01) for gamma keratose-treated cells at 24 hours were compared to growth media-treated cells. Green color represents the upregulated genes and brown color represents downregulated genes. The fifth column represents the ratio of up/downregulation (gamma keratose/growth media; i.e., < 1 indicates growth media is more upregulated).

Gene Symbol	Gamma keratose-treated cells p-value < 0.01	Growth media-treated cells fold change	Gamma keratose-treated cells fold change	Gamma keratose-treated cells fold change/Growth media-treated cells fold change	Pathway Involved
Esr1	0.0000	0.1003	0.0416	0.4148	Autophagy
Ctsb	0.0002	0.4640	0.4456	0.9603	Autophagy
Gadd45a	0.0003	0.4604	0.3283	0.7131	Pro Apoptotic
Tnfrsf11b	0.0012	0.3350	0.2434	0.7266	Anti Apoptotic
Galnt5	0.0025	0.2445	0.1254	0.5129	Necrosis
Bcl2l1	0.0036	2.9325	3.0940	1.0551	Apoptosis and Autophagy
Birc2	0.0046	4.2163	3.2102	0.7614	Pro Apoptotic
Rab25	0.0062	6.9642	5.5078	0.7909	Necrosis
S100a7a	0.0081	7.8709	8.1772	1.0389	Necrosis
Abl1	0.0088	3.7581	5.2269	1.3908	Pro Apoptotic
Casp1	0.0089	2.4365	2.5534	1.0480	Pro Apoptotic

Table 2. Gene expression of gamma keratose treated cells at 12 hours
Genes showing significant difference to the pre-treatment control (i.e., p-value <0.01) for gamma keratose-treated cells at 12 hours were compared to growth media-treated cells. Green color represents the upregulated genes and brown color represents downregulated genes. The fifth column represents the ratio of up/downregulation (gamma keratose/growth media; i.e., <1 indicates growth media is more upregulated).

Gene Symbol	Gamma keratose-treated cells p-value <0.01	Growth media-treated cells fold change	Gamma keratose-treated cells fold change	Gamma keratose-treated cells fold change/Growth media-treated cells fold change	Pathway Involved
FasI	0.0002	38.1630	8.4802	0.2222	Pro-Apoptotic
Parp2	0.0008	9.5073	2.6155	0.2751	Necrosis
Atg5	0.0023	15.4901	2.4423	0.1577	Autophagy
Bax	0.0030	4.6507	2.8363	0.6099	Apoptosis and Autophagy
Pvr	0.0044	5.8728	2.1364	0.3638	Necrosis
Map1lc3a	0.0044	6.8016	2.9062	0.4273	Autophagy
Atg7	0.0057	41.1923	2.6280	0.0638	Autophagy
Ulk1	0.0059	3.7571	8.0148	2.1332	Autophagy
Akt1	0.0079	21.7459	2.8546	0.1313	Apoptosis and Autophagy
Casp3	0.0086	0.4917	3.0328	6.1680	Apoptosis and Autophagy

This study has been submitted for publication and is currently under review.

Regulatory and Commercialization

While the definitive preclinical testing in the swine model showed promising results, more testing of the tissue salvage capabilities of the keratin biomaterials is required in a non-healing, critical-size swine burn model. Funding for the development and use of such a model was requested of AFIRM but not awarded. Clinical testing of this aspect was discussed with FDA but not allowed under the 510(k) clearance pathway being pursued by the product development team, as no predicate device exists with similar label indications. A product label allowing treatment of third degree burns and claims of tissue salvage would require a full pre-market approval pathway, a non-healing critical-size preclinical burn study as mentioned above, and multiple clinical trials.

Conclusions

The team has completed preclinical efficacy and safety testing required for an Investigational Device Exemption (IDE) application to the FDA and progressed through the pre-IDE process. FDA has responded favorably, and the team is currently preparing a formal IDE application and finalizing manufacturing operations under Quality System Regulation 21 Code of Federal Regulations 820. The definitive preclinical testing in the swine model showed promising results, but more testing of the tissue salvage capabilities of keratin biomaterials is required in a critical-size swine burn model (i.e., one that does not spontaneously heal and would require grafting to completely close).

Planned Clinical Translations

Pre-IDE activities with FDA have already been completed. The commercial product being developed from this research, KeraStat Burn, is being considered for clinical trial evaluation under an IDE currently in preparation by KeraNetics.



Topical Treatment of Burn Injury

Polymeric, Antimicrobial, Absorbent Wound Dressing Providing Sustained Release of Iodine

Project 4.6.4, RCCC

Team Leader(s): Carmine P. Iovine, MS (Rutgers University)

Project Team(s): Richard Clark, MD and Adam Singer, MD (Stony Brook University); Joachim Kohn, PhD and Ganesan Subramanian, MS (Rutgers University)

Collaborator(s): N/A

Therapy: Treatment of infected traumatic or thermal, heavily exudating wounds

Deliverable(s): Absorbent, conformable, non-adherent, self-supporting foam-based wound dressing containing 2%-15% by weight of bound molecular iodine, which is released in contact with wound in an on-demand, sustained manner over a period of several days.

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers coupled the pre-polymer process with a mold/cure/cut fabrication sequence that produces a uniform, well-defined product on a very consistent basis. This process has been scaled up in the lab, and the researchers have shown that it is reproducible and controllable at this laboratory scale. They completed in vitro characterization of the antimicrobial scope and efficacy of the starch-polyurethane copolymer iodophor vs. predicate devices. These studies verified the broad anti-bacterial and anti-fungal properties and sustainable slow release benefits of the starch polymer approach. Room temperature stability studies on the packaged device are very encouraging with six months of data indicating no change in product functionality.

Key Words: iodine; polymeric iodophor; wound dressing; antimicrobial; starch

Introduction

Polymeric iodophors are stable non-covalent complexes of certain polymers with molecular iodine. Although water-soluble polymeric iodophors have an advantage over “tincture”-based iodine, they still release to the wound site too quickly because of their solubility. Often, this quick release characteristic provides a much higher dose of iodine than is required for the intended antimicrobial action. The iodine is used up by side reactions with body fluids, thus depleting the reservoir prematurely and allowing bacteria to re-colonize the wound site. From a clinical point of view, wound dressings based on water-soluble polymeric iodophors can only contain low levels of iodine and the dressings must be changed very frequently in use.

By utilizing a water-insoluble polymeric iodophor as the carrier for iodine, the concentration of free iodine in the solution can be maintained at a low level, thus avoiding the issues associated with water-soluble iodophors, including the premature burst-like release of the iodine. Many approaches

to insoluble polymer iodophors have been reported. These include, for example, various iodine impregnated nylon fiber structures as wound dressings. Such materials are not very absorbent, they release iodine in minutes, and their iodine loading capacity is low. Broad spectrum, antimicrobial dressing materials based on modified polyvinylalcohol (PVA), where this known water-soluble polymeric iodophor is made insoluble by reaction with aldehydes at substitution levels approaching 30 mole percent, have also been reported and tested. These materials can typically carry up to 4%-8% iodine by weight, and this is released over a period of hours. However, the modified PVA is unstable in contact with heavily exuding wounds, which results in a slow release of the modification agent over time. This increases the water solubility of the iodophor and accelerates the release of iodine. Insoluble polyurethane antimicrobial foams and films have also been described in the literature. These materials are stable but can only release iodine over a period of several hours. The iodine is weakly bound to the

polymer carrier as a charge transfer complex, and this accounts for the relatively short duration release characteristics.

These various limitations of iodine-bearing wound dressings point to the need for an insoluble anti-microbial polymeric iodophor that is stable, highly absorbent, can be loaded with high levels of strongly bound iodine, and can release the iodine in a controlled and sustained manner over a period of several days.

Research Progress – Year 5

Previously, the research team developed a dressing that provides sustained delivery of iodine over a prolonged period of time (days) at an effective daily dose level by using starch as an iodophor. Starch binds strongly with molecular iodine, and combining the starch polymer with a polyester polyurethane foam matrix makes the starch-iodine combination insoluble. This allows a sustained release of the iodine while providing an absorbent and highly conformable wound dressing material.

Shelf-life study

The research team investigated the shelf stability of the packaged polymeric wound dressing at room temperature. The objective of the study was to evaluate and justify the recommended shelf life, storage conditions and desired packaging for the proposed product. The scope of this study includes the monitoring and documentation of any changes to: the baseline iodine content of the packaged device; the product packaging; and the iodine release profile. Five separate batches of base foam were prepared, sliced into approximately 10 individual 3 mm thick x 100 mm diameter pieces, and iodinated in the standard manner. The baseline iodine content was determined to be 5% by weight. Each circular patch was halved and packed in an 8" x 4" heat sealable poly-aluminum sachet. The samples were stored at 25°C ± 2°C for the study period of 12 months. During each month of the study, one set of samples was removed and measured for percent of iodine content, release profile, and package integrity. After 4 months at room temperature, the iodine content of the dressing showed a slight downward trend indicating an overall decrease of approximately 10% in the base iodine content. Package integrity was maintained in excellent condition with no

sign of iodine evaporation. The overall sustained iodine hourly release rate remained substantially unchanged for all 4 monthly data points. This study is ongoing.

In vitro efficacy

The researchers tested the iodinated wound dressing prototype against a range of gram-negative and gram-positive bacterial species using both the Kirby-Bauer test and a modified test which incorporates a pre-leaching protocol in order to highlight the long-term sustainable release attribute. In the Kirby-Bauer test, an anti-microbial impregnated disk of contact wound dressing is placed onto an agar plate inoculated with a lawn of bacteria. The anti-microbial payload diffuses into the agar surrounding the disk, which kills the bacteria and creates a "zone of inhibition" where bacterial growth is precluded. The zone of inhibition is measured after 24 hours of contact. The Kirby-Bauer test was performed against the following bacterial species, using standard aseptic technique:

Staphylococcus aureus sub aureus
Rosenbach (ATCC #25923)

Staphylococcus saprophyticus sub saprophyticus (ATCC #35552)

Bacillus cereus (ATCC #2)

Pseudomonas aeruginosa Schroeter (ATCC #10145)

Enterobacter cloacae sub cloacae Jordan (ATCC #13047).

Efficacy of the iodinated wound dressing against these species was measured in comparison to two commercially available wound dressings: Iodoflex® Cadexomer Iodine pads, 0.9% iodine w/w (Smith & Nephew), and Silverlon® antimicrobial silver burn contact dressing (Argentum Medical, LLC). The iodinated wound dressing proved effective against all five bacterial species, with greater efficacy against the three gram-positive species. Although the wound dressing demonstrated a similar pattern of action as did the Iodoflex® predicate, the average zone of bacterial inhibition was approximately 5-10 mm smaller in diameter. This can be explained by the extended-release profile of the iodinated wound dressing, in contrast to the burst-release profile of the Iodoflex® pad.



From these results, one can conclude that the iodinated wound dressing shows comparable functionality to the two commercially available products after 24 hours. Kirby-Bauer experiments using “pre-leached” sample disks were also performed. The iodinated wound dressing maintained its antibacterial properties against three of the five tested species of bacteria for at least 96 hours (72 hours of pre-leaching, plus 24 hours treatment), and against four of the five species for 48 hours (24 hours of pre-leaching, plus 24 hours treatment). The Iodoflex® pad released its entire iodine load after 24 hours of pre-leaching, and demonstrated no subsequent antibacterial activity. The Silverlon® dressing showed a low level of antimicrobial activity against all species after up to 72 hours of pre-leaching, although its efficacy was roughly half that of the non-preleached product.

Conclusions

The research team coupled the pre-polymer process with a mold/cure/cut fabrication sequence and produced a uniform well-defined product on a very

consistent basis. They completed scale-up of the process in the lab, and demonstrated that it is very reproducible and controllable at this laboratory scale. The team completed in vitro characterization of the range of anti-microbial activity and efficacy of the starch-polyurethane copolymer iodophor vs. other technologies and predicate devices. These studies verified the broad anti-bacterial and anti-fungal properties and sustainable slow release benefits of the starch inter-polymer approach. Room temperature stability studies on the packaged device are very encouraging with 6 months of data showing no change in product functionality.

Future Research Plans

The 12-month shelf-life study will be completed in the upcoming year. The next step of this research is to evaluate the efficacy of the iodine-releasing wound dressing in an infected animal model.

Planned Clinical Transitions

Discussions with potential commercialization partners will be pursued.



Topical Treatment of Burn Injury**Topical P12 Therapy to Limit Burn Injury Progression and Improve Healing****Project 4.6.5, RCCC**

Team Leader(s): Lauren Macri, PhD (Rutgers University); Richard Clark, MD (Stony Brook University)

Project Team(s): Ritu Goyal, PhD and Veena Bolikal (Rutgers University); Adam Singer, MD, Fubao Lin, PhD, Atulya Prasad, MS and Laurie Crawford (Stony Brook University)

Collaborator(s): N/A

Therapy: Topical therapy to limit burn progression and accelerate healing

Deliverable(s): Product 1: A controlled release formulation of fibronectin (FN)-derived peptide (Drug delivery system); Product 2: A tissue engineered construct with tethered FN-derived peptide (Tethered drug system)

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers demonstrated the linear release of cyclized peptide (cP12) from ultrafast (<24 hours) and fast (<4 days)-eroding cP12-loaded electrospun fiber mats. They determined a suitable sterilization method for cP12-loaded fiber mats: electron-beam irradiation with 25 kGy. They developed a porcine partial-thickness burn model suitable for evaluating topical therapies. They demonstrated the in vivo safety of the cP12-loaded fiber mats.

Finally, they established positive controls using the FN-cell binding domain (FNIII8-11) for fibroblast attachment, spreading and adhesion, which will be used to compare the performance of peptide-tethered coated surfaces.

Key Words: burns; drug delivery; peptide; tyrosine-derived polycarbonate; biodegradable; hydrogel; burn progression; electrospinning

Introduction

Burn injuries can be extremely painful, debilitating, and complex to treat. One reason is that burn injuries are not localized to the initial site of trauma, but progress (or extend) in size horizontally and/or vertically, often converting partial-thickness burns into non-healing full-thickness burns. By reducing or preventing burn injury progression, researchers could (1) minimize tissue loss and the need for grafting, (2) shorten healing and hospitalization time, (3) lower rates of morbidity and mortality, and (4) decrease scarring and contracture. Although many therapeutic agents have shown promise in small animal models, these beneficial effects have not been translated into U.S. Food and Drug Administration (FDA)-approved therapies. Thus, a major clinical challenge exists to develop novel therapies that can inhibit burn progression, accelerate healing, and/or limit scar formation.

P12, a 14-mer peptide derived from FN, has been identified by the Clark team at Stony Brook University, and shows significant promise in the treatment of burns. Since it is possible that naked peptides are susceptible to proteolytic degradation,

the team modified P12 via cyclization (cP12) to increase its stability in the burn wound. Both peptides (P12 and cP12) were shown to provide in vitro protection against oxidative and cytokine stress of adult human dermal fibroblasts (AHDfFs), and their intravenous administration reduced burn injury progression in the rat and/or porcine burn models (see Projects 4.6.1 and 4.6.1a for details).

The overall objective of this project is to deliver P12 or cP12 topically to the burn wound to inhibit burn progression, accelerate healing, and/or limit scar formation. One product from this study will be a controlled-release formulation of FN-derived peptide, while another product will be a tissue engineered construct with tethered FN-derived peptide.

Research Progress – Year 5**Fabrication and characterization of fiber mats for delivery of cP12 (cP12-loaded fiber mats)**

Two tyrosine-derived polycarbonate compositions were selected in earlier years for the ultrafast and fast delivery of therapeutic agents to burns. In Year 5, these two polymers were electrospun to



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incorporate cP12. The researchers characterized electrospun fiber mats for fiber morphology, cP12 loading, thermal properties, and release kinetics. This report focuses only on the results obtained with the ultrafast-eroding polymer, E5003.5(2K). The plots of cP12 released from fibers over time were linear, irrespective of cP12 loading (**Figure 1**). The fit to the zero order release model suggests that release from E5003.5(2K) fibers is predominantly controlled by polymer erosion and is minimally dependent on the payload concentration.

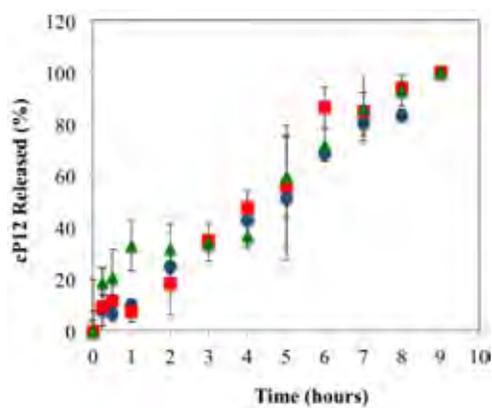


Figure 1. The effect of cP12 loading composition on in vitro cumulative release of cP12 profiled into phosphate buffered saline (pH 7.4) at 37°C from E5003.5(2K) fiber mats: (▲) 5%, (■) 0.4, (●) 0.06 wt% loading. The results are presented as mean \pm standard deviation (n = 4).

Development of a suitable sterilization method for cP12-loaded fiber mats

Sterilization of the product is mandatory for in vivo application. In Year 5, the Macri team at Rutgers University evaluated the effects of irradiation (gamma or electron beam) sterilization at two doses (25 or 45 kGy) on cP12-loaded E5003.5(2K) fiber mats that contained a high dose (1 wt%) of phenolic antioxidants (butylated hydroxytoluene or α -tocopherol [Vitamin E]). Phenolic antioxidants are commonly used to protect polymers from degradation. The results from this study suggest that electron beam irradiation at 25 kGy is a suitable sterilization method for this formulation.

Development of a porcine excised vertical burn progression model

The Clark team at Stony Brook University is using the validated vertical progression burn model in

Projects 4.6.1 and 4.6.1a to evaluate the effects of the intravenous administration of potential burn injury inhibitors (i.e., P12, cP12, and other FN derivatives). However, a modification to the protocol is required in order to evaluate the topical administration of these potential inhibitors. The research team used immediate burn excision to allow direct contact between the cP12-loaded fiber mats and the wound bed. The goal of the study was to identify an exposure time that reproducibly produced a mid-dermal, partial-thickness burn that ultimately healed with a contracted, discolored scar. The primary outcome of the study was the measurement of scar formation 28 days after injury (representative photographs are shown in **Figure 2**). It was determined that exposure of porcine skin to a preheated aluminum bar at 70°C for 30 seconds resulted in a mid-dermal, partial-thickness burn that ultimately healed with a contracted, discolored scar, regardless of immediate tangential excision.

Evaluation of in vivo performance of cP12-loaded fiber mats in porcine partial-thickness burn model

The researchers believe that electrospun tyrosine-derived fiber mats offer the potential for topical clinical therapies requiring ultrafast or fast delivery of the therapeutic agent (cP12). An exploratory in vivo porcine study was performed to evaluate the effects of topical cP12 on healing and scarring. Mid-dermal burns were tangentially excised and were either untreated or treated with fiber mats containing one of three doses of cP12. Non-excised, untreated mid-dermal burns were used as a control.

Unfortunately, treatment with cP12-loaded fiber mats did not significantly affect healing and scarring compared to untreated excised burns. The data collected and analyzed under the current study conditions suggests that topical treatment with cP12-loaded ultrafast-eroding fiber mats does not accelerate burn healing. One possible reason theorized by the researchers is that the doses and/or release kinetics evaluated in this study were suboptimal. Second, high Mw polymer degradation products may interfere with the bioavailability and bioactivity of cP12. Third, it is possible that cP12 non-specifically adsorbs to various molecules, cells, or structures in the wound that may interfere with its therapeutic effects. For example, P12 has been



Figure 2. Representative photographs of burns healed 28 days post-injury primary. Wound contracture is noted with non-excised and tangentially excised 70/30 burns. Burn wounds were created using an aluminum bar pre-heated to 70°C and applied to the skin for either 20 or 30 seconds (abbreviated as 70/20 or 70/30, respectively). Half of the burns were tangentially excised (Ex70/20 or Ex70/30) within 30 minutes of burning. Partial-thickness excisional wounds were used as controls (n=8).

shown to “stick” to red blood cells in a collaborative study. Fourth, even though current studies had focused on the use of cyclization to increase P12’s stability in the highly proteolytic burn environment, this approach may not add sufficient stability for this application. Studies are in progress to determine cP12’s susceptibility to proteolytic degradation. Going forward, the research team plans to reformulate cP12 in a gel matrix, rather than the polymeric fiber mats, to eliminate the potential for polymer interference.

Finding FN-derived peptides that enhance wound healing

The Clark team at Stony Brook is developing a denatured albumin thin-based technology to screen candidate peptides that can support optimal adult human dermal fibroblast function in vitro and in vivo. This is an alternative product concept to the drug-delivering fiber mats for enhancing wound healing. This approach provides bioactive molecules

and peptides that are tethered (bound to the surface) to an albumin film, where the concentration and presentation of the bioactive molecules and peptides can be spatially and temporally controlled. The research team believes that this approach can be used to fine-tune cellular responses that relate to healing.

Spectroscopic ellipsometry to determine thickness of albumin thin-film

A spectroscopic ellipsometer was used to determine the thickness of the thin-film. Ellipsometry measures the complex reflectance ratio (ρ) as a function of the amplitude component (ψ) and the phase difference (Δ). Using Cauchy fits for Δ values for an albumin thin-film, the group measured a thickness of approximately 1.89 ± 1.44 nm. Thus, denatured, reduced albumin does not form a layer of single molecules. However, Δ values at lower wavelengths appear to be less than 0° , as can be seen in **Figure 3**.

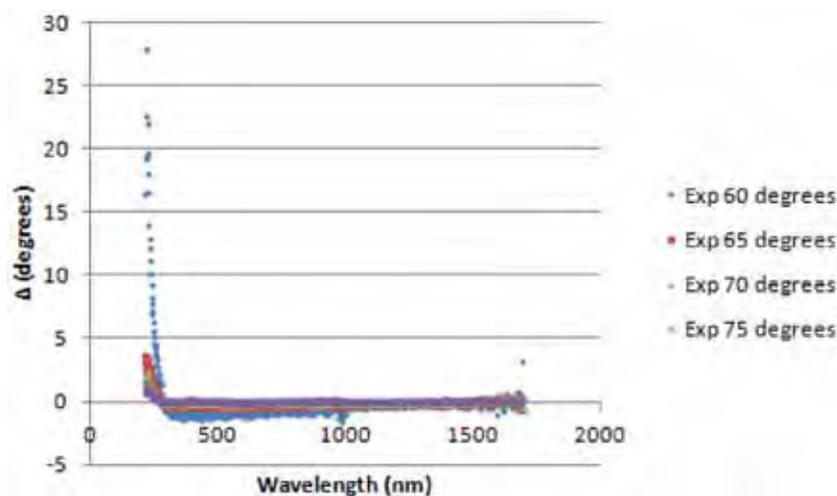


Figure 3. Representative plot of Δ vs. wavelength (nm) for an albumin thin-film determined using spectroscopic ellipsometry.



ELISA assay to quantify FN domains tethered to albumin thin-film

The Clark laboratory determined the amount of PEGylated FN domain that could be tethered to the albumin thin-film. Quantification of tethered domain was measured using an ELISA assay. The data show that absorbance first increased linearly with increasing concentration of PEGylated domain used, followed by saturation at high doses (>10 μ g/mL). By allowing the acrylate (Polyethylene Glycol Diacrylate [PEGDA])-thiol (albumin thin-film) binding reaction to occur for increasing duration, greater amounts of PEGDA₂₀₀₀-cysFNIII_{8-11,15} were detected.

AHDF attachment at four hours to GST-FNIII₈₋₁₁ and PEGDA-cysFNIII_{8-11,15}

The attachment of AHDF to various doses of FN cell-binding domain tethered to the albumin thin-film and adsorbed on tissue culture grade polystyrene was determined after four hours of incubation (Figure 4). These data suggest the AHDF attachment is comparable on GST-FNIII₈₋₁₁ and PEGDA-cysFNIII_{8-11,15} coated plates.

Conclusions

Two tyrosine-derived polycarbonate terpolymers were developed as ultrafast (<24 hours) and fast (<4 days)-releasing, fiber-based drug delivery matrices for cP12. A suitable sterilization method for these drug delivery matrices was determined. The project

team validated a porcine partial-thickness burn model that was suitable for evaluating the performance of topical burn therapies. The in vivo data confirmed the biocompatibility of cP12-loaded E5003.5(2K) fiber mats, but the efficacy results suggest that further work is required to optimize the topical delivery of cP12 for the treatment of localized burns. The researchers also obtained data suggesting that AHDF attachment is comparable on GST-FNIII₈₋₁₁ and PEGDA-cysFNIII_{8-11,15} coated plates.

Future Research Plans

Going forward, the research team will focus on the delivery of cP12 from gel matrix technologies, rather than from tyrosine-derived polycarbonates, with hopes to eliminate the potential for polymer degradation product interference with cP12 bioavailability and bioactivity.

National Institutes of Health funding will be used to determine the optimal dose of peptide required for the topical treatment of skin trauma.

Planned Clinical Transitions

The project team will conduct safety and efficacy studies (TRL 5) regarding Good Laboratory Practices guidance, submit an Investigational New Drug application, and initiate the design of a clinical trial.

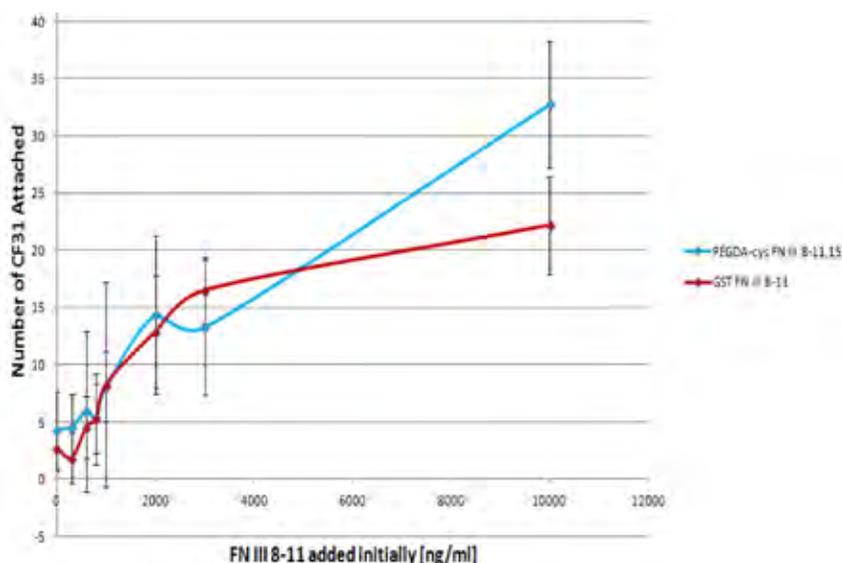


Figure 4. Comparison of AHDF attachment to various doses of PEGDA-cysFNIII_{8-11,15} tethered to albumin thin-film and GST-FNIII₈₋₁₁ adsorbed on tissue culture polystyrene.

Wound Healing and Scar Prevention

Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device: Exploring Human Skin Progenitor Cells for Regenerative Medicine Cell-Based Therapy Using Cell Spray Deposition

Project 4.2.2, WFPC

Team Leader(s): Jörg C. Gerlach, MD, PhD (University of Pittsburgh)

Project Team(s): Patrick Over, Matthew Young, and Roger Esteban, PhD (University of Pittsburgh)

Collaborator(s): James Holmes, MD (Wake Forest) and Steven Wolf, MD (U.S. Army Institute of Surgical Research)

Therapy: Skin stem cell delivery for cell-based treatment of burn wounds

Deliverable(s): (1) Optimized cell isolation and spraying methodologies and (2) next generation skin gun U.S. Food and Drug Administration (FDA)-approved spray device that can deposit fetal skin stem cells onto wound surfaces

TRL Progress: Beginning, TRL 1; Current, TRL 4; AFIRM I Target, TRL 5

Key Accomplishments: The research group has established an antibody marker panel for stemness characterization of fetal as well as adult epidermal and dermal cells. Freshly isolated adult epidermal and dermal progenitor cells can now be provided for full-thickness burn wound healing. Fetal dermal-derived fibroblasts exhibited higher wound healing activity than bone-marrow mesenchymal stromal cells. Overall, in vitro cultured fetal dermal-derived stem cells show a high potential for full thickness and larger burn wound healing as an anticipated off-the-shelf product.

Key Words: Skin stem cells, burn wounds, human fetal tissue, progenitor cells, cell spray method, human fetal dermal fibroblast (hFDF), human fetal epidermal keratinocyte, human dermal fibroblast, human epidermal keratinocyte.

Introduction

The survival of combat victims with larger related burns is often limited, since large burn areas reduce the availability of healthy donor skin areas for split-skin mesh grafting. The human body responds to skin burn injuries, involving the basal epidermal layer, with regeneration that often results in fibrosis and scarring. Current treatments for burns, other than mesh grafting, do not speed up epidermal re-epithelialization time nor reduce complications, such as infections, which contribute to significant scarring, functional impairment, and undesirable esthetic outcomes.

The therapy depends on the size and depth of the wound and varies from conservative therapies in smaller second-degree burns to split-skin mesh grafting, cadaveric, and artificial skin sheet coverage in third-degree burns. Mesh grafting is the gold

standard of burn therapies, but donor area is limited and it also generates an additional wound, both of which are problematic in larger combat-related burns. Ratios between donor area sizes, compared to treatable mesh-graft size, are typically 1:3; only in large area cases are ratios up to 1:8. The results are less successful with larger ratios. Regenerative medicine research provides cell-based therapies that are designed to improve wound healing by offering viable cells that accelerate regeneration and reduce complications. The use of isolated single cells enables split ratios larger than 1:20. In partial thickness second-degree burns, cell grafting has to consider epidermal stem cells from the basal layer of the epidermis to be successful. In full-thickness third-degree wounds, additional dermal mesenchymal stem cells are to be considered to compensate for dermis loss.



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The Gerlach group has developed a skin cell isolation technique for epidermal and dermal stem cells from adult and fetal tissues, along with other methods and devices to improve single cell spray grafting. Applications using autologous cells for partial-thickness burns were reported both in Europe and the United States. In addition, the group explores cell spray grafting of dermal cells to address third-degree burn therapy. Adult- and fetal-derived epidermal and dermal progenitor cells were compared, and off-the-shelf product development was initiated. In this report, the group provides progress data on fetal and adult epidermal and dermal cell studies in vitro.

Research Progress – Year 5

Epidermal progenitor cells

During the past year, the group continued working on epidermal progenitor characterization from adult cell isolations for cell spray deposition and fetal-derived epidermal cells.

Adult epidermal keratinocytes. The differentiation stage of the isolated keratinocytes is important

because of the importance of seeding stem cells onto the wound to increase the overall regeneration process. Therefore, all of the experiments were performed with isolated cells under the same conditions that are commonly used for burn wound healing during the cell spray therapy procedure.

Cell size is one of the key factors in determining the differentiation stage of the isolated cells. Keratinocytes increase in cell size and inner cytoplasmic complexity by accumulating vesicles of lipids and keratins during the natural cell regeneration process that occurs during skin turnover (**Figure 1A**). The researchers used cell size and cytoplasmic complexity in combination with molecular differentiation markers as a molecular approach to experimentally stray the cells and obtain the information needed to determine the stemness of the isolated cell population.

The group defined a panel of markers for keratinocyte differentiation stage determination in combination with cell size analysis. This panel included three basal keratinocyte stem cell markers (K5, CK15, and $\alpha 6$ -integrin [$\alpha 6$ int]), and two differentiation stages

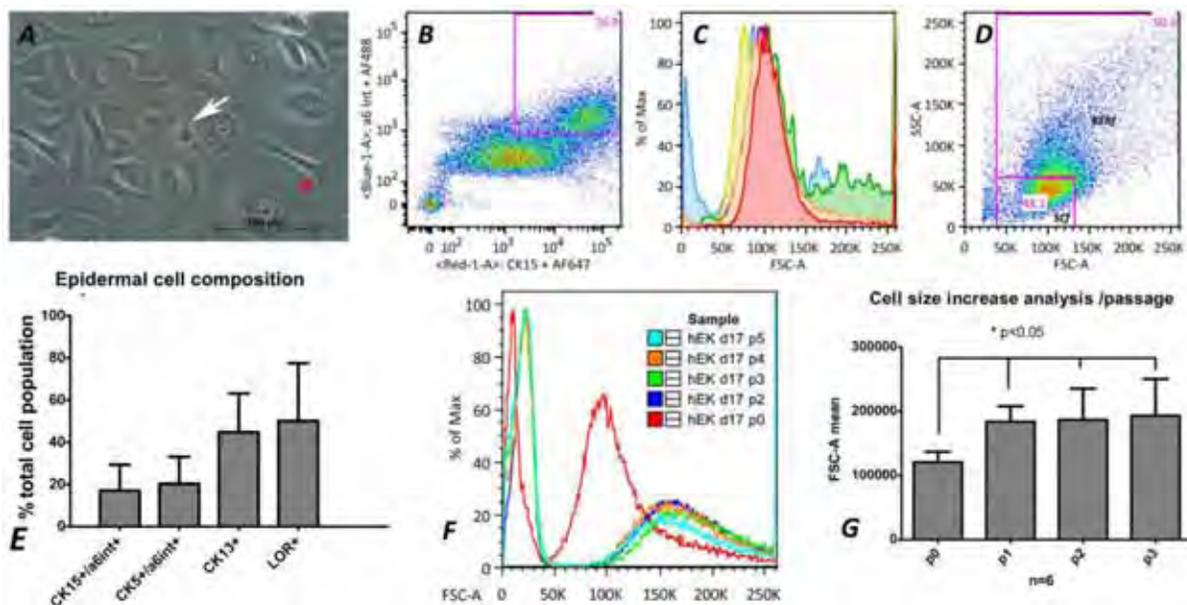


Figure 1. (A) Cell size can be used for differentiation stage discrimination parameter; (B) Gating criteria based on size (FSC-A) and K15 highest intensity; (C) K15+ $\alpha 6$ int+size comparison (n=6); (D) Gate strategy showing two Keratinocyte populations based on size; (E) Epidermal cell composition by flow cytometry showing differentiation stage; (F) Forward scatter plot area (FSC-A) showing how in vitro cultured keratinocytes increased cell size after passaging for donor 17; (G) Graph showing forward scatter plot area (FSC-A) summary for all donors (n=6). The gating strategy was used to analyze cells derived from six skin biopsies by flow cytometry (A, B, C and D). Cells were analyzed just after the isolation process and after a few weeks in the in vitro culture process.

(CK13, LOR). A gating strategy was established by a combination of results from cell size, cytoplasmic complexity and the highest expressed CK15/ α 6int cell marker (Figure 1B, C). Flow cytometry size analysis revealed that approximately 50% of the isolated cells had a size corresponding to the non-differentiated cell stage, which meant they could contain stem cells (Figure 1D). Further analysis showed that about 20%–25% of the isolated cells contained the stem cell markers α 6int, K5, and K15 (Figure 1E).

To explore the possible use of isolated adult stem cells, keratinocytes for in vitro culture and expansion cells were cultured for 6 weeks. These cells were analyzed weekly, and the results showed in vitro cultured keratinocytes increased in cell size as a consequence of the in vitro process (Figure 1F, G).

These results have implications for the cell-banking activities having a bad impact on adult isolated keratinocytes. However, repeated experiments on the cell isolation process per skin area allowed us to collect valuable information to be used on single cell spray therapy (Figure 2).

The analysis can also be used to establish a quality control database for predicting autologous cell isolation results in an onsite setting that is thought to depend on the patient's age, lifestyle (drug abuse, alcoholism), previous diseases (diabetes), and medication. The group also collected information on the quality and number of cells from epidermal cell isolation obtained from plastic surgery skin donations and patient biopsies. This information was collected to predict the biopsy area needed for

cell harvesting depending on the burned area to be treated (Figure 2).

Fetal epidermal keratinocytes. The group analyzed stemness antibody markers (α 6int, CK5, and CK15) and the increase in cell size to determine the degree of differentiation of fetal keratinocytes after passage. The group concluded that fetal-derived epidermal cells might pose serious problems when they are cultured in vitro for a longer period of time. The cells entered into terminal differentiation, after some passages, making cell banking more challenging.

Dermal progenitor cells

During the past year, the group also focused on enhancing the cell growth of isolated epidermal and dermal progenitors in vitro, and established regular analyses on cell culture behavior in order to characterize their stemness during in vitro expansion.

Adult dermal fibroblasts. The group investigated dermal cell isolation and performed progenitor characterization, cell culture behavior, and stemness studies to address third-degree burn therapy development. Adult dermal fibroblasts were isolated, attached onto a plastic surface, and grown for 2 weeks. Preliminary gene expression analysis revealed that these dermal-derived cells exhibited bone marrow mesenchymal stromal cell (BM-MSC) markers, indicating that certain cell populations could be BM-MSC-like cells. The initial results, on paracrine effectors detection on dermal-derived fibroblasts, did not show IL-1A along with very low interleukin 6 (IL-6) gene expression compared to BM-MSCs, which meant they could have a low inflammatory response. In contrast to fetal tissue-derived cells, the autologous dermal fibroblasts isolation method, when applied to full-thickness wound therapy, has the advantage of providing dermal cells into the wound without the danger of rejection.

Fetal dermal cells. Studies involving hFDF cells were introduced to find a source of cells to be used with burn wound healing when the patient has a large burn area, or when there are not enough cells available to treat the wound. The hFDF cells were obtained from fetus limbs at 8–10 gestational weeks. These cells showed an elongated, spindle-shaped morphology (fibroblast-like) with a higher division

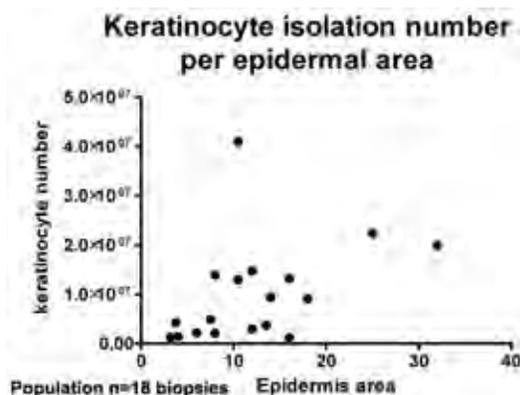


Figure 2. Keratinocytes number obtained in relation to the epidermis area (cm²).



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rate than adult fibroblast cells. Previous results suggest that isolated hFDF cells include a population that exhibits the same markers as BM-MSCs, as published by the International Society for Cellular Therapy. The research team collected more data about the culture behavior of hFDF cells to obtain further information about their progenitor potential, which is of interest for wound healing in full-thickness burns. They also studied the possibility of isolating, expanding, and freezing hFDF cells as an off-the-shelf product. Cells were isolated and expanded using micromanipulation and in vitro culture techniques for further in vitro expansion (Figure 3).

The group studied the stemness stability of the hFDF cells after three weeks of in vitro cultured population by gene expression. Preliminary gene expression results revealed that the cells started

differentiating and increasing levels of CD45 and CD34, but most of the cell population remained BM-MSC-like (Figure 4A). In vitro cultured hFDF cells were still able to differentiate into adipogenic, osteogenic, and chondrogenic lineages under an appropriate chemical induction (Figure 4B).

These BM-MSC-like cells were expanded in vitro for 3 weeks, obtaining 1.9×10^9 cells (around 2,000 vials with 1 million cells each). This expansion rate makes it possible to use these cells as an off-the-shelf product. Nevertheless, not the entire hFDF cell population showed BM-MSC markers after 3 weeks of in vitro culture. Differentiation is a natural process that occurs during the in vitro process just as it happens in the body during tissue formation. Although the differentiation was natural, hFDF still showed BM-MSC-like stem cell properties (Figure 4B) that could be of interest in enhancing



Figure 3. Cell clone generation procedure. (A) In vitro culture cell isolation under sterile conditions using equipment originally developed for embryonic stem cell techniques; (B) Stem cell knife used for removal of a single cell. (x5 magnification); (C) Incubation process at 37°C with 5% CO₂; (D) Liquid nitrogen tank that contains frozen cells at -180°C; (E) Off-the-shelf cells could be used whenever necessary with a combination of the skin gun.

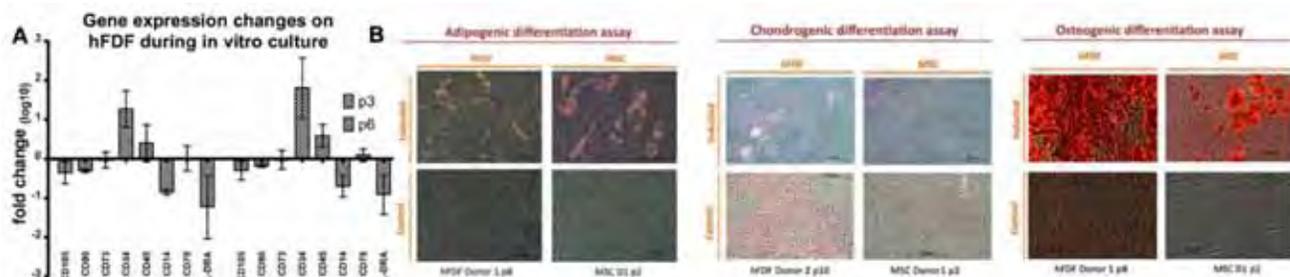


Figure 4. (A) Gene expression pattern changes of different markers during in vitro culture process of hFDF (n=3) after being cultured for 3 and 6 weeks (passage 3 and 6). Gene expression is represented using log₁₀, and values were normalized using BM-MSC gene expression. B-actin was used as a housekeeping gene; (B) Adipogenic, chondrogenic and osteogenic differentiation assay comparing hFDF to BM-MSC cells. Osteogenic differentiation was induced by B-Glycerophosphate and stained with Alizarin S red for calcium deposition. Chondrogenic differentiation was induced by TGF-β₃ and stained with Alcian Blue for proteoglycans. Adipogenic differentiation was induced by IBMX (3-isobutyl-1-methyl-xanthine) and stained with Oil O red for fatty acids.

the wound healing process. On the other hand, dermal cells can produce some paracrine effectors that are involved in the recruitment of epidermal cells or pro-inflammatory response after an injury.

To avoid quick cell differentiation, a cell-sorting step was added before further expansion. After the initial hFDF isolation, cells were cultured for 1 week to obtain enough cells for sorting. The cells were then sorted for MSC markers using the flow cytometry technology. Only the hFDF cell population that expressed the BM-MSC markers was selected for further in vitro expansion (Figure 5).

Dermal cells can produce some paracrine effectors that are involved in the recruitment of epidermal cells or pro-inflammatory response after an injury. Therefore, the researchers studied the hFDF paracrine effector production and their role during the wound healing process. This study was focused on the quantification of IL-6 and hepatocyte growth factor (HGF) produced by hFDF compared to BM-MSC lineages that showed positive effects on

wound healing. Interestingly, hFDF showed lower IL-6 levels after in vitro cell culture than BM-MSCs (Figure 6B). IL-6 is involved in an inflammatory response that is linked to scar formation as well. Reduction of IL-6 levels into the wound is an interesting goal for satisfactory wound healing. On the other hand, hFDF released more HGF than BM-MSCs (Figure 6A). HGF is involved in the regulation of wound healing as a paracrine growth factor and can enhance the wound healing by angiogenesis. hFDF cells showed a low immunogenic response based on the Class II histocompatibility antigen DR alpha chain (HLA-DRA) compared to BM-MSCs, making them suitable for allogeneic transplantation (Figure 6C).

The research team started isolating hFDF cells from different donors by sorting for BM-MSC markers, expanding them in vitro, and cryopreserving them in liquid nitrogen tanks for a dermal cell bank. In these preliminary studies, the cells showed a reproducible expansion rate, suggesting that they

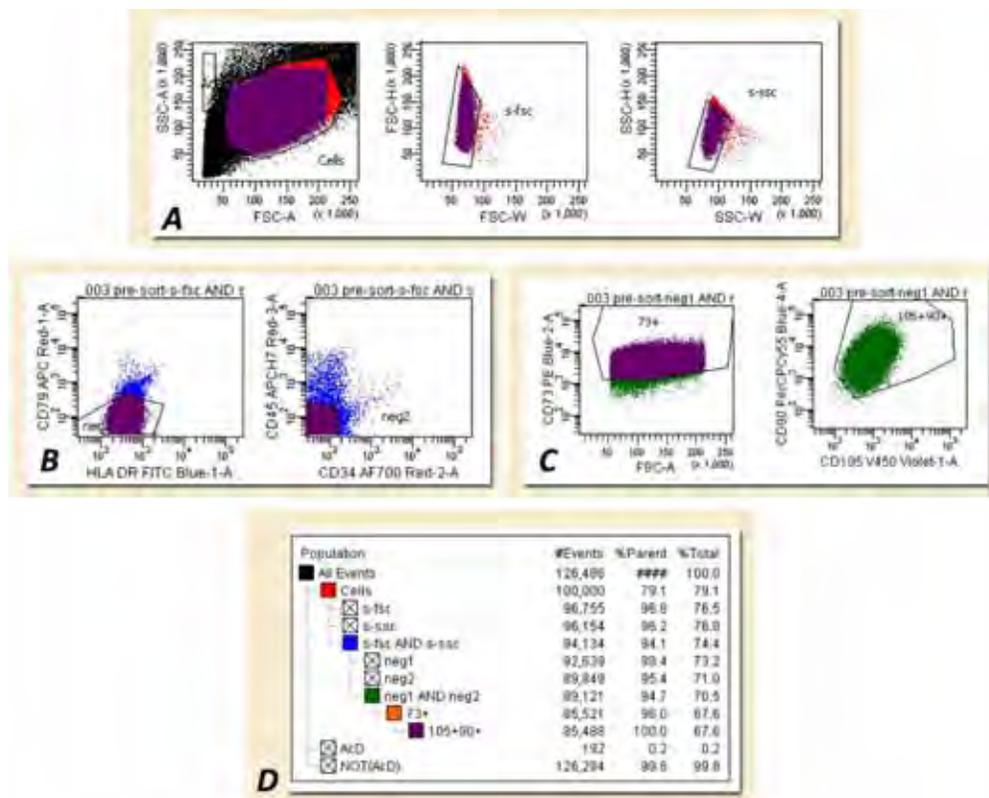


Figure 5. Flow cytometry sorting strategy. (A) Population selection based on size (FSC-A); (B) Negative cell gate selection excluding all cells CD34, CD45, CD79, HLA-DRA based on isotypes and compensation beads; (C) CD73+, CD105+, CD90+ cell selection gate; (D) Summary of fetal dermal derived cell composition used on sorting.



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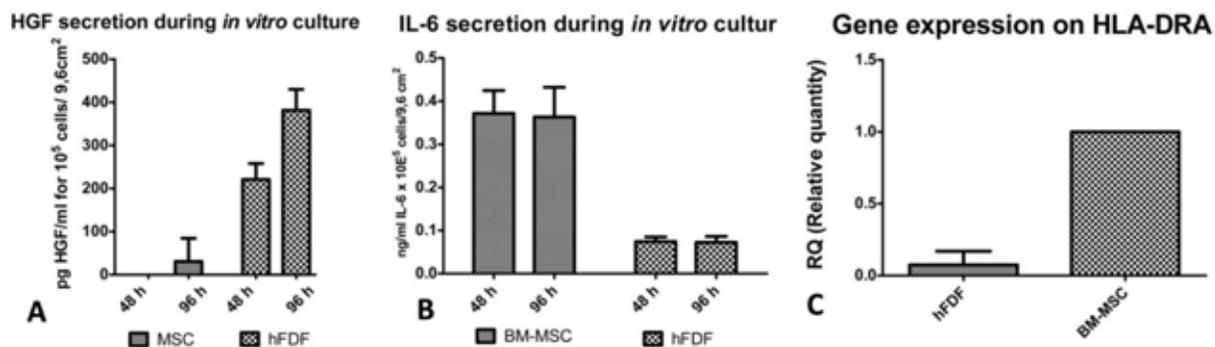


Figure 6. Paracrine effector production comparison. (A) HGF secretion comparison between BM-MSCs and hFDF cells during in vitro cell culture after 48 and 96 hours; (B) Pro-inflammatory interleukin 6 response; (C) Class II HLA-DRA gene expression comparison between fetal-derived fibroblasts (n=6) and BM-MSc (n=4).

could potentially be used as an off-the-shelf product. However, statistical confirmation and further animal tests, immunocompatibility T-cell experiments, and tumorigenicity assays are required prior to considering the cells for clinical trials.

Droplet dispersion efficiency evaluation test for skin gun prototype

The research team successfully tested a new prototype of the Stem Cell Systems “skin gun” for experimental cell spray deposition. Parallel work, in the framework of an innovative practice approach as introduced at the University of Pittsburgh Medical Center Mercy Hospital’s Burn Unit, has demonstrated clinical feasibility, practicability, and reproducibility with superior results of the application over the ReCell® sprayer by Avita Biomedical.

The research team initiated an FDA 510(k) process approval. For the application, the researchers performed a spray pattern analysis comparing the skin gun with a claimed predicate device as part of the documentation needed for the 510(k) approval. The skin gun is an electronically controlled device that delivers small drops of a particular solution (e.g., saline solution) over a surface by pushing liquid from a syringe through a needle into an air stream. The spray pattern can be adjusted by changing the air stream flow and liquid medium flow settings. The device uses four settings for both air and medium flow parameters (Table 1).

The predicate device used for comparison purposes was an experimental setup simulating the Syringe Xpresse® Assist Device from Aesthetic Science Corporation (Figure 7B). This device delivers

droplets over a surface by forwarding liquid through a needle from the syringe barrel that is pushed by liquid displacement actuation from a compressed can. For the spraying pattern assays, the researchers used an experimental setup made from assembled Luer-Lock syringes (Becton Dickinson, Franklin Lakes, NJ) with Luer-Lock connectors made by Baxter (Deerfield, IL) and adjusted the liquid to set it up in a way that the liquid flows occurred at 2 ml per minute, by the use of a Rotameter (Krone, Duisburg, Germany).

The skin gun was adjusted using a combination of air and liquid flows (Figure 7C) to spray liquid onto a plastic surface (Figure 7D). The spray action, recorded at a 300 millisecond time frame, was processed and analyzed to find the differences between devices and liquid flow settings (Figure 7D, E).

A student’s t-test statistical analysis revealed a significant difference on droplet size generation comparing the skin gun spraying device to the Syringe Xpresse® device (Figure 8). At high airflow and lowest medium airflow, the skin gun device droplets generated were smaller than the Syringe Xpresse®

Table 1. Skin Gun adjustable spray pattern settings

Air Flow (ml/min)	Medium Flow (ml/min)
2120	2
2380	2.5
2740	3
3185	3.6

setup device. The error bars show that the syringe device has more variety on droplet size formation than the skin gun. The electronically controlled skin gun generates more homogeneous droplets. The researchers found that the spray patterns using comparable medium flow settings are similar in both devices. However, the droplet sizes generated depend on the medium and airflow combination.

Conclusions

The research team has presented fetal tissue-derived epidermal progenitor cells for potential cell banking and provision of a burn therapy that is based on an off-the-shelf product. They also have presented results on fetal tissue-derived dermal progenitor

cells with a high potential for cell banking providing a new approach for burn therapy that, together with the use of epidermal cells, will lead to clinical studies on advancing cell grafting for third-degree burn therapy.

Initial immunocompatibility gene expression data results on major histocompatibility complex class II (that is related to rejection) of the fetal dermal-derived cells for cell grafting as an off-the-shelf product are promising. Additionally, adult dermal progenitors were identified, which will enhance autologous onsite cell grafting and should lead to clinical studies on advancing adult autologous skin cell grafting into the area of third-degree burn therapy.

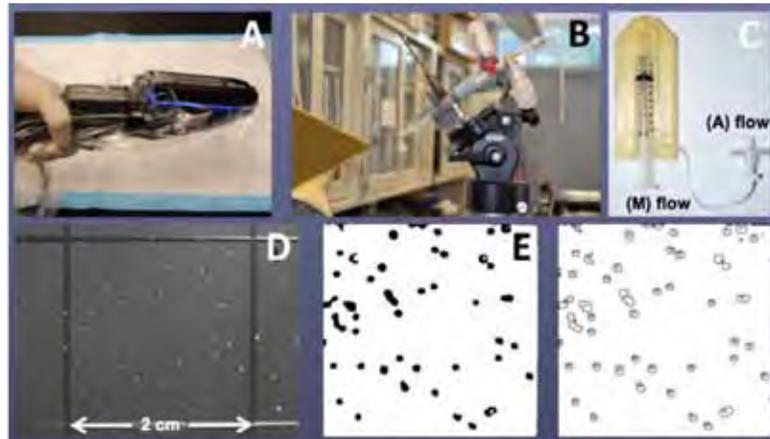


Figure 7. Droplet generation and surface distribution analysis. (A) Electronically controlled spraying device (skin gun); (B) Air pressure-controlled syringe (Predicate device); (C) The spray process was recorded using a high-speed resolution camera (Lumenera Corp.); (D) Raw picture from the sprayed area; (E) The images were processed using VirtualDub software video analysis, Photoshop CS3, Image J. Spray 4 cm² for 300 milliseconds at different air/medium flow settings.

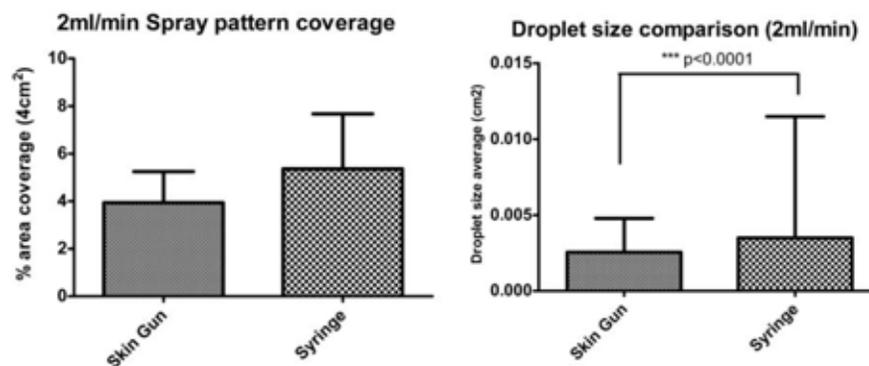


Figure 8. Spray pattern coverage (4 cm²) analysis (left) and droplet area formation between skin gun and predicate device (right). The comparison experiment was performed using 2 ml per minute medium pressure for both devices. The air pressure used in skin gun was 3185 ml per minute.



Wound Healing and Scar Prevention

In Situ Bioprinting of Skin for Battlefield Burn Injuries

Project 4.2.5, WFPC

Team Leader(s): James J. Yoo, PhD (Wake Forest Institute for Regenerative Medicine [WFIRM])

Project Team(s): John D. Jackson, PhD, Jaehyun Kim, PhD, Mohammad Albanna, PhD, Eduardo Alvaro, Weixin Zhao, MD, Hyun-Wook Kang, PhD, and Dennis Dice, Josh Tan (WFIRM)

Collaborator(s): None

Therapy: Burn repair using bioprinter-delivered skin cells

Deliverable(s): In situ bioprinted skin

TRL Progress: Beginning, TRL 2; Current, TRL 2; AFIRM I Target, TRL 3

Key Accomplishments: The researchers used a bioprinter to develop a delivery system to deploy skin cells to a wound in a spatial-specific manner. They developed isolation and expansion techniques for skin keratinocytes and dermal fibroblasts. They developed a porcine full-thickness burn wound model. They found that bioprinting of autologous skin cells enhanced the healing time of full-thickness excisional wounds as well as third-degree burn wounds.

Key Words: Wound, burn, skin, bioprinting, autologous transplantation, allogeneic transplantation

Introduction

Burn injuries typically account for 5%–20% of combat casualties. However, the changing nature of war, especially the rapid increase in the use of improvised explosive devices in combat zones, has drastically affected the number and types of burn injuries. Most battlefield burns are massive injuries and require grafts for coverage and repair, since any loss of full-thickness skin of more than 4 cm in diameter will not heal without treatment. The mortality rate increases significantly with an increase in the total body surface area burned; therefore, soldiers with extensive burns must be treated immediately for an increased chance of survival. Current treatment options such as autografts and commercially available skin products are limited in size, and some require a lengthy preparation time, making them unusable in severe cases that require prompt and aggressive measures to maintain the lives of wounded soldiers. Therefore, additional strategies are needed to address these issues.

A new approach that permits immediate burn wound stabilization with functional recovery is needed. To address the present limitations, a novel delivery system is required that would allow onsite in situ repair of battlefield burn injuries using tissue-engineered skin grafts produced with a portable skin printing system. The unique advantages of the

proposed in situ skin bioprinting system include: 1) The ability to treat massive burns immediately after stabilization of the wound in the battlefield as skin cells and matrices can be accurately delivered onto the injured sites. This would result in a skin fabrication procedure ranging from minutes to a few hours, depending on the size and type of burn. 2) The ability to deliver several dermal cell types and matrices simultaneously onto target sites to generate anatomically and functionally adequate dermal tissues. The amount and ratio of cells and matrices as well as the thickness of the skin layers can be precisely controlled by means of the drop-on-demand mechanism of bioprinters. The delivery of major skin tissue elements onto the injured site would allow for a rapid restoration of the skin and may minimize scarring and enhance cosmetic recovery.

Research Progress – Year 5

Skin Printer Designs

A bioprinter was developed using a pressure-based cartridge system for cell delivery. A wound scanning system was incorporated into the bioprinter to obtain a three-dimensional image of the wound for accurate placement of the cells in the wound.

In situ bioprinting of skin

The researchers used a nude mouse full-thickness skin wound model to demonstrate the feasibility of

using a bioprinter to repair skin wounds. Human fibroblasts and keratinocytes were printed on the wound and evaluated over a period of 6 weeks. The group found the time to wound closure to be significantly reduced (**Figure 1**). This experiment demonstrates the feasibility of in situ bioprinting for rapid care of skin wounds by demonstrating that two different skin cell types could be directly delivered onto a wound. The researchers have demonstrated that these skin cells remain viable and survive after printing. In addition, printed skin cells are able to form normal skin tissue and integrate with the surrounding skin.

Large animal model (Excisional and Burn)

This study was performed in a porcine wound model because of the similarity of porcine skin to human skin. Skin fibroblasts and keratinocytes were isolated from the dorsum of porcine skin through a partial thickness (0.015 inch) skin biopsy. Both cell types were cultured for 10 days until they reached confluence. Four (4) full-thickness excisional wounds (10x10 cm each) were created on the backs of the pigs. Each wound received a different treatment (no treatment, matrix only, allogeneic cells and autologous cells). The matrix consisted of fibrinogen, type I collagen and thrombin for cross-linking.

Figures 2 and 3 show the gross images of wound healing over the 8 weeks of the study. Wounds receiving autologous treatments showed almost complete healing in 3 weeks compared to other treatments, which required 6 weeks to heal. Wounds with autologous cells also showed an accelerated wound coverage (re-epithelialization) and had almost 95% wound re-epithelialization by the third week of the study. Wound contracture was minimal for autologous treatments throughout the study (<20% of the original wound size) compared to other treatments, which showed a progressive increase in contraction that exceeded 40% of the original wound size. Wounds receiving allogeneic cells did not show notable differences with respect to wound size, re-epithelialization and contracture when compared to the controls (untreated and matrix only). Histological analyses showed a complete formation of epidermis and dermis layers within the first 2 weeks of study in the autologous treatments. However, other treatments showed

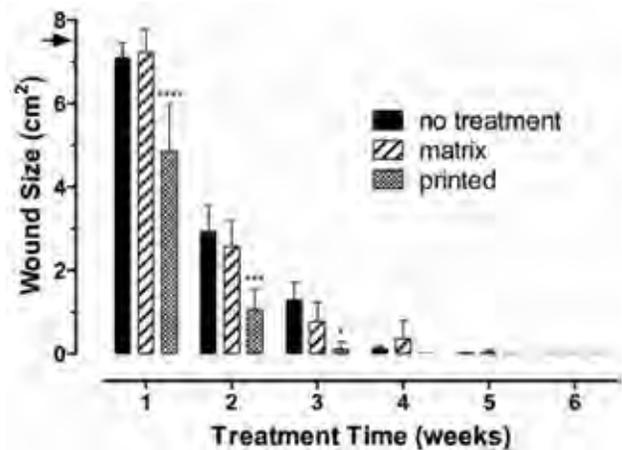


Figure 1. Analysis of wound sizes over 6 weeks. The initial wound area was 7.5 cm² (L x W: 3 x 2.5 cm). Printed skin constructs show a significantly reduced time to wound closure when compared to untreated and fibrin/collagen controls. Wound sizes were analyzed with one-way Analysis of Variance. ****p < 0.0001, n = 12; ***p < 0.001, n = 8; *p < 0.05, n = 8. 4, 5, 6 weeks: n = 4, no statistical difference detected by ANOVA (p = 0.2).

formation of epidermis and dermis layers by Week 6 of the study.

A porcine burn model was developed. Four (4) third-degree burn wounds (10x10 cm each) were created on the backs of the pigs. Two (2) days post-burn, the wounds underwent debridement and were then treated by bioprinting the skin cells. Each wound received a different treatment (no treatment, matrix only, allogeneic cells and keratinocytes). Skin fibroblasts and keratinocytes were isolated from the dorsum of porcine skin through a partial-thickness (0.015 inch) skin biopsy. Both cell types were cultured for 10 days until they reached confluence and were then used to treat the burns wounds. The matrix consisted of fibrinogen, type I collagen and thrombin for cross-linking. **Figure 4** shows the gross images of wound healing over the 7 weeks of the study.

Conclusions

Two (2) skin cell types (dermal fibroblast and keratinocytes) were bioprinted directly onto full-thickness excisional wound models. The delivered cells were able to repair skin defects. The use of autologous cells resulted in a more rapid healing of the wound with less contracture, when compared to using allogeneic cells. By 2 weeks post-bioprinting,

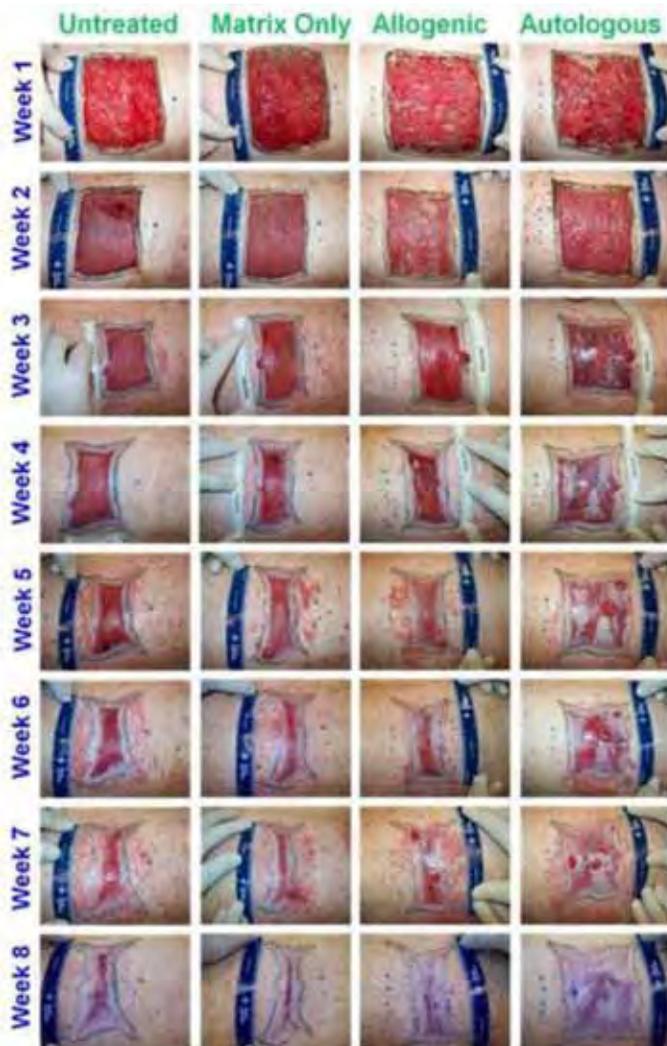


Figure 2. Gross images of progression of healing of full-thickness wound over the 8 weeks of the study.

the autologous cells formed an epithelial layer that covered up to 90% of the wound. The groups consisting of no treatment, matrix treatment only, and allogeneic skin cells had a delayed healing rate (up to 6 weeks post-bioprinting).

A third-degree burn model was successfully created in the pig. The delivery of autologous keratinocytes and dermal fibroblast shortened the healing time of the burn wounds to 2–3 weeks, whereas the no-treatment group, matrix-only group, and allogeneic cell group were delayed up to 6–8 weeks post-bioprinting. This preclinical study suggests that the use of skin bioprinting is a viable approach for rapid coverage of extensive skin wounds such as burn.

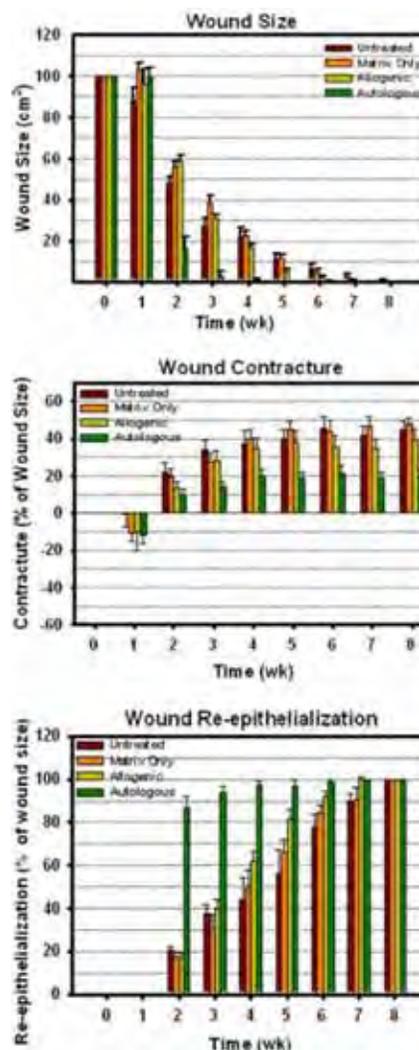


Figure 3. Wound size, contracture and re-epithelialization of untreated, matrix-only, allogenic and autologous treatments over 8 weeks of study.

Future Research Plans

This work will continue under other funding sources.

Planned Clinical Transitions

The Office of Combination Products (OCP) will be contacted to confirm the classification of the bio-printer and, if needed, file a Request for Designation with OCP in accordance with 21 Code of Federal Regulations Part 3. The combination product with a PMOA (primary mode of action) attributable to a device will apply to the Center for Devices and Radiological Health, and a biologic PMOA to the Center for Biologics Evaluation and Research.

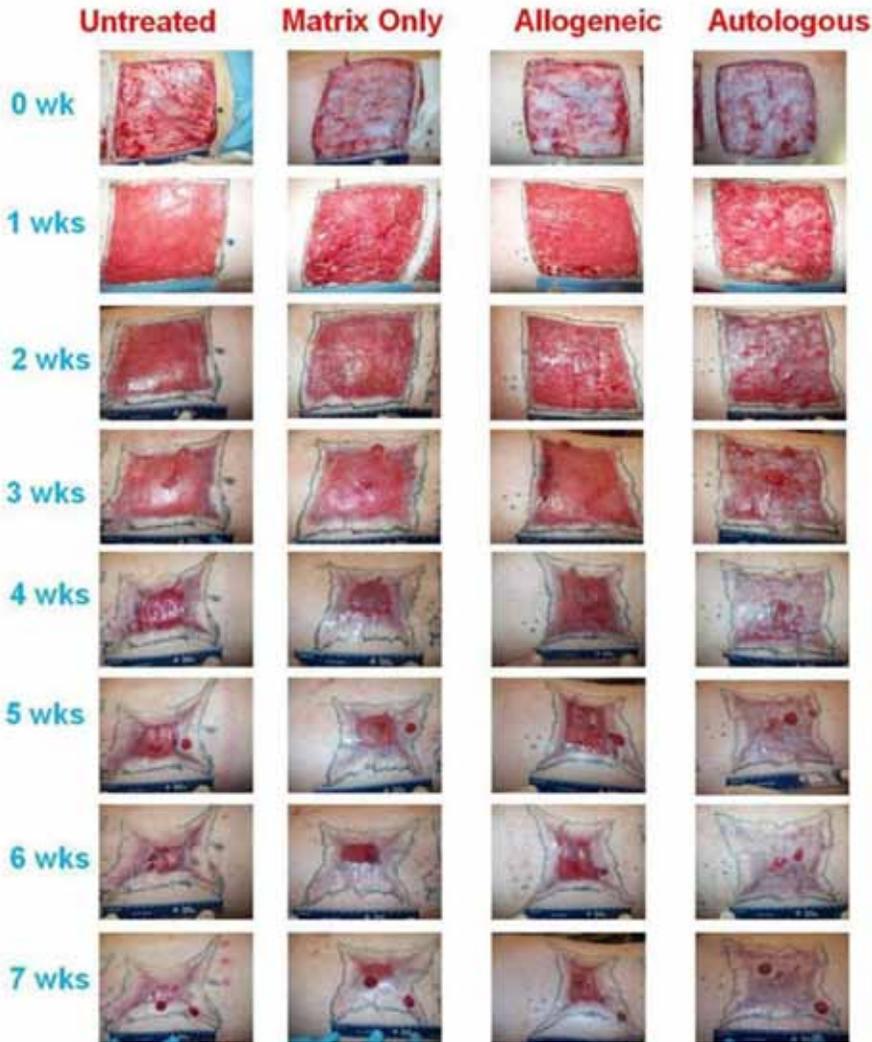
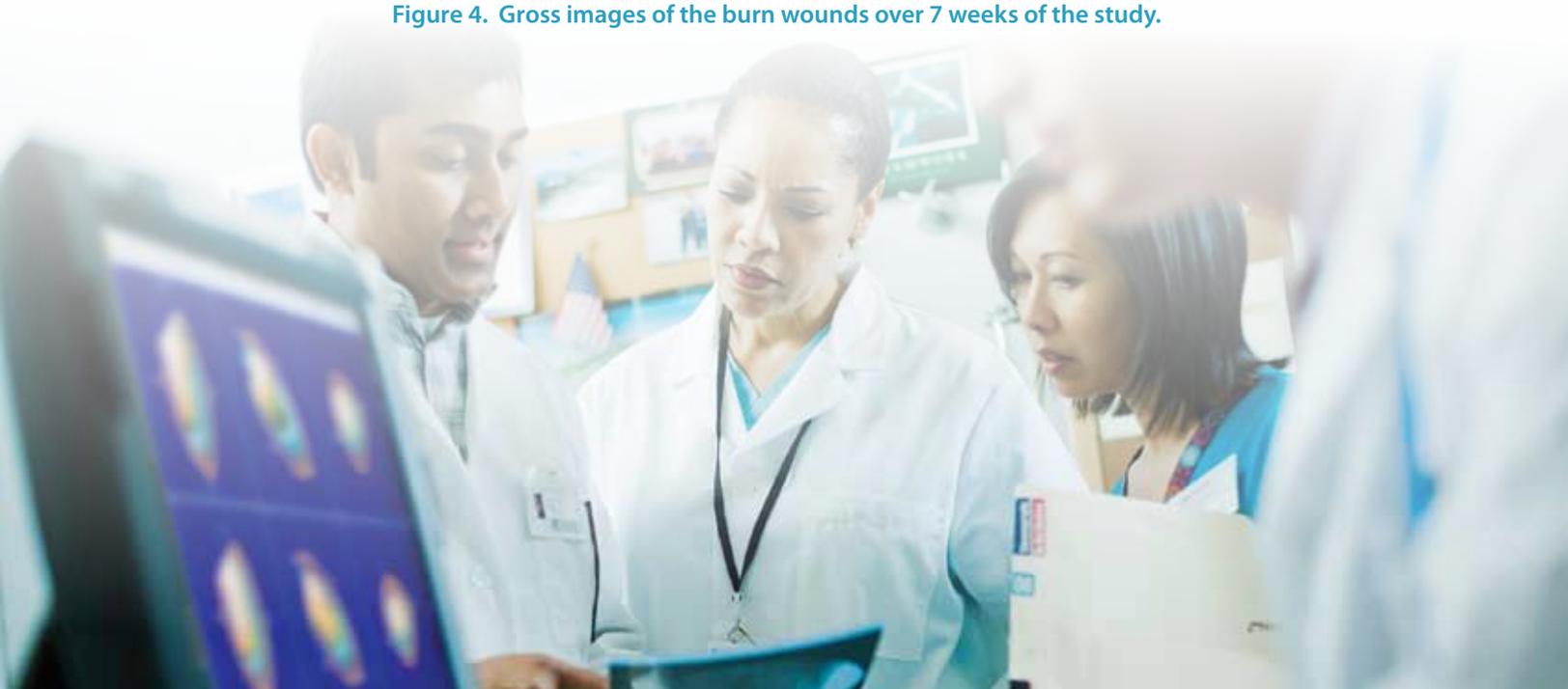


Figure 4. Gross images of the burn wounds over 7 weeks of the study.





Skin Products / Substitutes

Amniotic Fluid-Derived and Placenta-Derived Stem Cells for Burn

Project 4.2.6, WFPC

Team Leader(s): John D. Jackson, PhD, Mark Furth, (Wake Forest Institute for Regenerative Medicine [WFIRM])

Project Team(s): Jaehyun Kim, PhD, Chad Markert, PhD, Sean Murphy, PhD, Aleksander Skardal, PhD, Emily Moorefield, MS (WFIRM)

Collaborator(s): Shay Soker, PhD; James J. Yoo, MD, PhD, WFIRM

Therapy: Use of stem cells for the treatment of burn

Deliverable(s): To use multipotent stem cells from perinatal sources (amniotic fluid-derived and placenta-derived) to develop an improved “off-the-shelf”

bioengineered skin product for the treatment of extensive burns

TRL Progress: Beginning, TRL 1; Current, TRL 2; AFIRM I Target, TRL 3

Key Accomplishments: The researchers established a nude mouse full-thickness wound model and bioprinted amniotic fluid-derived stem (AFS) cells into the wound. They demonstrated enhanced wound healing as well as increased neovascularization using AFS cells. They also combined AFS cells with mature keratinocytes (KC) to enhance wound healing.

Key Words: stem cells, trophic factors, wounds

Introduction

Blast injuries not only cause penetrating injuries due to shrapnel but also induce massive burn wounds on individuals. Burn injury is a common source of morbidity and mortality in the battlefield, comprising 10%–30% of all casualties. Burn injuries tend to involve large surface areas of the skin. These types of wounds are difficult to treat due to limited donor skin sites and morbidity of the donor site. The optimal treatment of the injured combatant should be performed near the battlefield. The best available treatment of burn injuries is the use of autologous donor skin. However, the amount of donor skin that can be harvested may be limited in large area burns. Allogeneic donor skin is used but is not a permanent treatment option since the graft is ultimately rejected and must be replaced. Other commercial products for burn treatment include acellular products such as Alloderm®, Integra®, Biobane®, and cellular products such as Apligraf® and Epicel®. Each product has disadvantages ranging from required replacement of the acellular membranes to the production of fragile epithelial surfaces from the cellular products.

The use of stem cells offers a potential solution to the problems encountered with currently available treatment for extensive burns. Stem cells such as AFS cells can be expanded to large numbers and

banked for future use, and they have immunomodulatory properties. These attributes make AFS cells an ideal candidate as an “off-the-shelf” product for treatment of extensive burns in a battlefield situation.

Research Progress – Year 5

AFS cells possess properties found also in mesenchymal stem cells (MSCs), such as multipotent differentiation, immunomodulatory activity and the lack of significant immunogenicity. MSCs have shown therapeutic potential for the repair and regeneration of tissues damaged by injury or disease. In particular, MSC treatment of acute and chronic wounds results in accelerated wound closure, increased epithelialization, granulation tissue and angiogenesis. Given that AFS cells can be obtained less invasively than MSCs and show greater proliferative capacity in culture, the researchers investigated wound healing augmentation in a similar manner as MSCs.

The researchers measured and quantified wound size, contraction, and re-epithelialization. At both time points, wound closure was faster in AFS cell- and MSC-treated groups compared to the gel-only (control) group. Cell-treated groups showed greater levels of contraction at Week 1 (**Figure 1**). However, re-epithelialization levels were greater in AFS- and

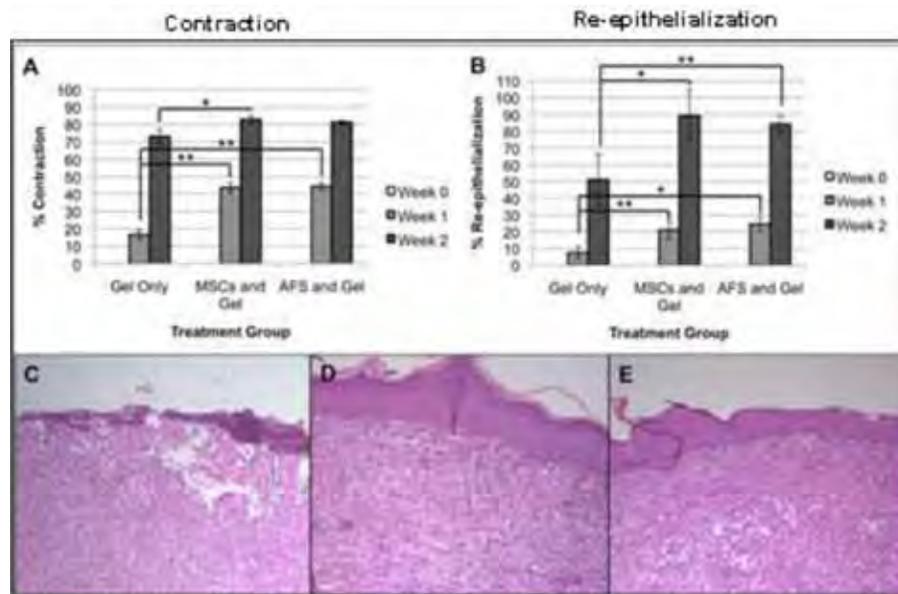


Figure 1. Wound contraction and epithelialization after treatment with AFS cells and MSCs.

MSC-treated groups compared to the gel-only group (Figure 1). More blood vessels were seen in the AFS cell and MSC groups compared to the gel-only group. At 1 week post treatment, microvessel density and blood vessel diameter were greater in the AFS cell and MSC groups when compared to the gel-only group; however, at 2 weeks post treatment, AFS cell treatment had greater vessel diameters than both the MSC and gel-only groups (Figure 2). A cell tracking study showed the number of AFS cells and MSCs decreased in the regenerating wound, which demonstrated that the stem cells did not integrate into the epithelium (Figure 3). The data show that AFS cells enhanced healing of the full-thickness skin wound as well as increased neovascularization activity in the wound. Because the AFS cells decreased over time, the increase in wound healing activity may be due to trophic factors produced by the stem cells early in the healing process.

Combination of AFS cells and KC

AFS cells and porcine KC were bioprinted onto full-thickness wounds on the backs of nude mice. The cells were printed in two layers with the AFS cells on the bottom layer and the KC on the top layer (Figure 4). In addition, each cell type was bioprinted alone as comparative groups. Wound healing was rapid with wound closure occurring by Day 14 in all groups. The number of AFS cells began to decrease by Day 7 post-bioprinting and was undetectable by Day 14 post-bioprinting. This finding

confirmed the earlier results of the transient nature of the AFS cells and MSCs post-bioprinting.

Conclusions

Deposition of AFS cells in a fibrin-collagen in a nude mouse full-thickness skin wound model increased wound closure rates, increased percentage of epithelialization, and increased levels of neovascular/angiogenic activity in the regenerating skin. The AFS cell numbers decreased over time, suggesting that AFS cells delivered trophic factors important in wound healing. These results suggest that AFS cells bioprinted as a cell therapy may be a potentially powerful tool for burn and wound healing treatments.

Future Research Plans

This work will continue under other funding sources.

Planned Clinical Transitions

The Office of Combination Products (OCP) will be contacted to confirm the classification of the bioprinter if used in the study; if needed, a Request for Designation will be filed with OCP in accordance with 21 Code of Federal Regulations Part 3. The combination product PMOA (primary mode of action) attributable to a device will apply to the Center for Devices and Radiological Health, and a biologic PMOA to the Center for Biologics Evaluation and Research.

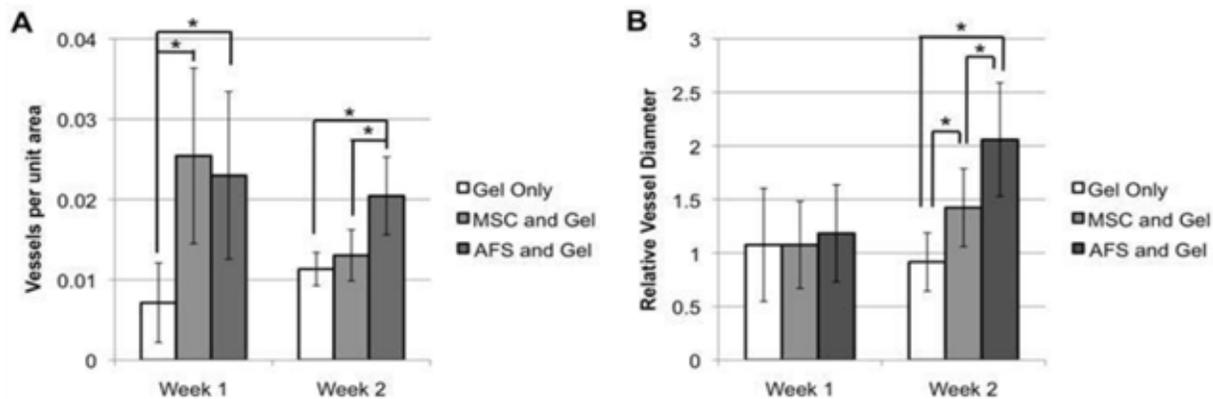


Figure 2. Microvessel density and vessel diameter after treatment with stem cells.

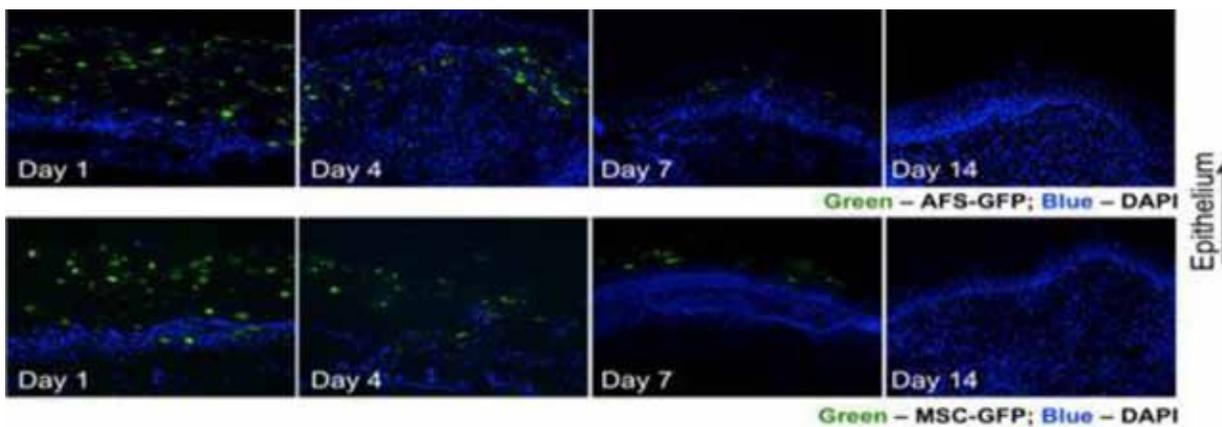


Figure 3. Stem cell tracking after bioprinting onto wound.

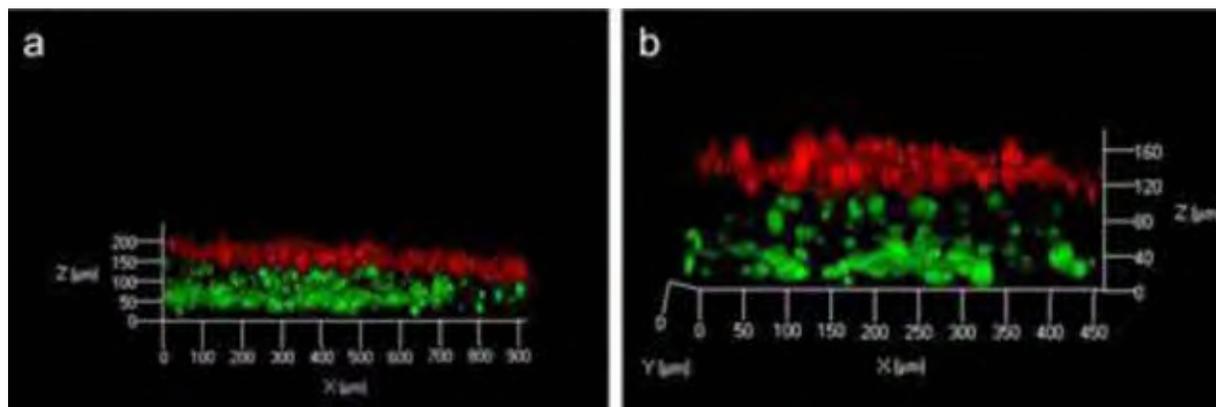


Figure 4. Tracking of GFP-lenti-labeled AFS (green) and CM-Dil-labeled KC (red) in wounds at the 4-day timepoint. (A) Confocal microscopy showing cell distribution along the z-axis (10X objective lens). (B) Confocal microscopy showing cell distribution along the z-axis (20X objective lens).

Skin Products / Substitutes

In Vitro Expanded Living Skin for Reparative Procedures

Project 4.2.8, WFPC

Team Leader(s): Sang Jin Lee, PhD, James J. Yoo, MD, PhD, and James H. Holmes, MD (Wake Forest)

Project Team(s): John Jackson, PhD, Weixin Zhao, MD, Hyun-Wook Kang, PhD, Peter Masso, BS, Peter Prim, BS, and James Kim, BS (Wake Forest)

Collaborator(s): Richard A. Wysk, PhD, Rohan A. Shirwaiker, MS, Ola Harrysson, PhD, and Paul H. Cohen, PhD (North Carolina State University)

Therapy: Treatment of burn injuries

Deliverable(s): Autologous skin grafts

TRL Progress: Beginning, TRL 4; Current, TRL 5; AFIRM I Target, TRL 5

Key Accomplishments: The researchers have developed an in vitro tissue expander system that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. The expander

system provides an accurate expansion rate for yielding target skin dimensions over a defined time period. The researchers are defining parameters that maximize the surface dimensions of skin for the treatment of battlefield burns. They are developing an effective tissue gripper system which critically affects the expansion of skin grafts. They have completed the design and built a new generation of a uniaxial bioreactor system. They have devised a strategy to perform a clinical trial, which requires processes that include toxicity testing under Good Laboratory Practices, establishment of standard operating procedures, the initiation of communication with the U.S. Food and Drug Administration (FDA), and submission of Institutional Review Board (IRB) and FDA applications.

Key Words: Autologous skin grafts, in vitro skin expander, bioreactor, burn repair

Introduction

Many reparative procedures due to battlefield trauma and burn may require additional skin for coverage. The standard of care for skin defect replacement is the use of autologous skin grafts. However, donor-site tissue availability is a major obstacle to the successful replacement of skin defects. Because of this limitation, other approaches are commonly employed to cover skin defects. These include commercially available skin products based on biomaterials and tissue engineering, allografts, and xenografts. However, these approaches also have limitations, such as the need for concomitant autograft, insufficient mechanical properties, high cost, lack of permanence, potential for infectious disease transmission, and inadequate biocompatibility.

Alternatively, subcutaneous tissue expanders or meshed split thickness skin grafts (STSGs) are used clinically to generate larger segments of autologous skin, when donor-site tissue is limited. Subcutaneous tissue expanders are balloon implants that are sequentially filled with incremental

volumes of saline to increase the amount of overlying skin. The physico-mechanical stress of the tissue expander results in biologic creep, greater mitotic activity of cells, and increased vascularity, which ultimately leads to expanded skin. Subsequently, the expanded skin can be used as a tissue flap or harvested for use as a skin graft. However, the use of a subcutaneous tissue expander is associated with an additional surgical procedure(s), which increases donor site and overall morbidity. In addition, this technique requires a lengthy wait time (on the order of months) to obtain sufficient tissue for intervention. Moreover, the discomfort associated with the increasing expander volume and the frequent tissue fibrosis remain as major limitations.

Alternatively, meshed STSGs are obtained using a graft mesher that cuts the skin into a mesh pattern, which results in greater surface dimensions before application on the wound bed. However, meshed STSGs are not considered ideal for many applications, because they leave large gaps of the open wound, which requires a longer healing time and results in a cross-hatched or cobblestone pattern of



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healed skin as scar tissue fills the gaps. Thus, the overall goal of this project is to provide wounded soldiers with large dimensions of autologous skin for reparative procedures.

Research Progress – Year 5

In vitro skin expansion bioreactor system – design considerations

Various aspects are considered in the bioreactor design for clinical application. The in vitro skin

bioreactor system must be operated in a closed system to prevent potential contamination/infection and to minimize the frequency of manipulation. For a clinical quality bioreactor system, the system was designed to fit into a biosafety cabinet and a 37°C incubator for control of contamination and temperature. One stepper motor, positioning sensor, electric thermometer and force sensor were used for the construction of a fully automatic system (Figures 1 and 2).

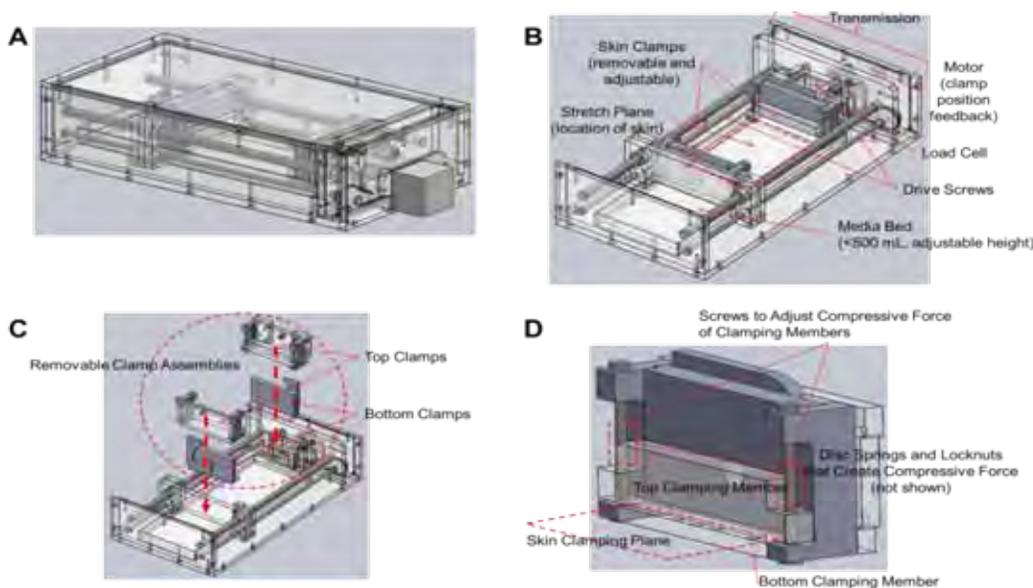


Figure 1. Clinical applicable uniaxial skin expander: (A) Fully assembled unit, (B) open (lid and sides hidden), (C) removable clamp assemblies, and (D) adjustable clamps.

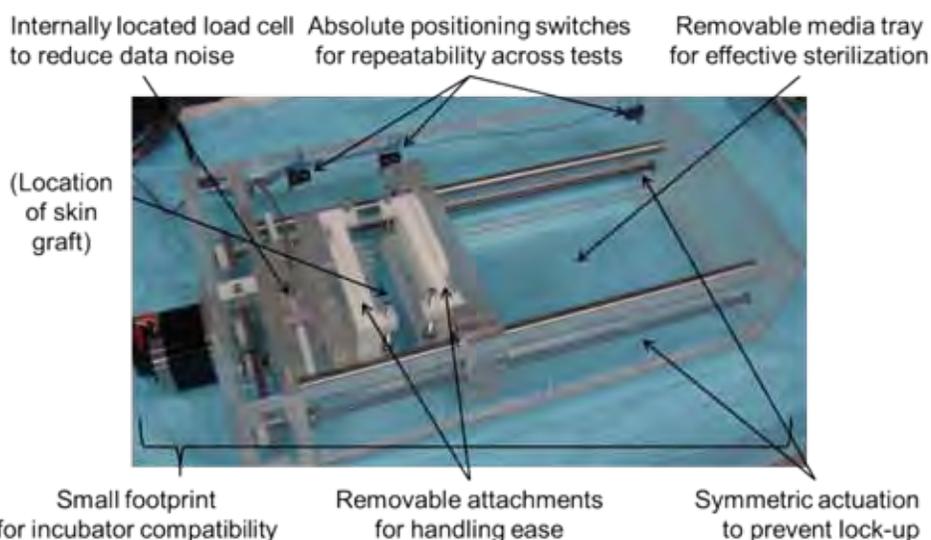


Figure 2. Clinical applicable uniaxial skin expander with fully assembled units (prototype).

Demonstration of the in vitro skin bioreactor use

To determine the feasibility of increasing the dimensions of the skin surface using the uniaxial skin bioreactor, a porcine STSG was placed in a computer-controlled expander system that incrementally exerted physical tensile stress. The expansion parameters, which included frequency, tensile strain and time, were tested for optimal expansion outcome. The researchers observed that STSG was increased over a period of 8 days in the bioreactor system (**Figure 3**). They confirmed tissue viability and structural integrity after the skin bioreactor (data not shown).

Conclusions

The researchers demonstrated that skin grafts (human and porcine) could be incrementally expanded in vitro using a computer-controlled skin bioreactor system. Cell/tissue viability was maintained and dermal structural integrity was preserved during the skin bioreactor. Thus, this technology provides an opportunity to generate large amounts of living skin grafts for burn repair and may overcome the current limitations. In vivo large animal studies using an expanded skin graft are currently being investigated to determine clinical applicability.

Future Research Plans

A skin sample will be expanded to increase 150% of the total surface area within 2 weeks in the bioreactor. The amount of expansion depends on the properties of the initial skin graft. The researchers will develop working parameters for the skin bioreactor expander system that will maximize skin expansion while maintaining tissue viability. These parameters include duration of expansion, frequency of incremental stretching, rate of expansion, and percent

expansion. Tissue viability and structural changes will be assessed using histo- and immunohistochemistry and mechanical testing.

Planned Clinical Transitions

Because the skin expansion uses equipment without any cellular components, it is defined as a device and will be under regulation of the devices section of the FDA. The researchers are working towards obtaining an Investigational Device Exemption by the FDA for approval of a prospective, multi-center, non-randomized, uncontrolled pilot study (Feasibility/Phase I). IRB approval is currently being sought. In addition to in-house expertise, external regulatory consultant(s) have been engaged to provide guidance, review documentation and provide regulatory representation at FDA meetings. Documentation in support of regulatory submissions will be prepared internally by institute staff. The researchers will approach the FDA's Office of Combination Products to determine if a Request for Designation is required. The media used in the skin expansion is similar to that used to grow cells for the production of biological molecules; in those cases, it is considered a material used during the manufacturing process and not part of the final product.

Corrections/Changes Planned for the Future

Based on conversations with an FDA consultant, the researchers will perform a preclinical large animal study, which is essential for FDA approval for the clinical trial. The revised Specific Aims of this proposal are to (1) optimize expansion parameters for maximizing surface dimensions of skin, (2) conduct validation studies of the expanded skin grafts in a preclinical burn animal model, and (3) prepare materials for FDA approval for a clinical trial.



Figure 3. Skin expansion procedure using an in vitro skin expander. Gradual expansion of skin graft under a constant load.



Skin Products / Substitutes

Burn Repair with Autologous Engineered Skin Substitutes

Project 4.7.2, RCCC

Team Leader(s): Steven Boyce, PhD (University of Cincinnati); Richard Clark, MD (Stony Brook University)

Project Team(s): Dorothy Supp, PhD (University of Cincinnati)

Collaborator(s): N/A

Therapy: Advanced therapy for extensive and deep burns

Deliverable(s): Autologous Engineered Skin Substitutes (ESS)

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 4

Key Accomplishments: The researchers developed protocols to translate research procedures into a testable therapy. They demonstrated complete restoration of skin color in autologous ESS with pigment (ESS-P). Their preliminary data showed that melanin expression can be downregulated to promote cryopreservation of human melanocytes to allow multiple applications of ESS-P.

Key Words: burns; engineered skin; pigmentation

Introduction

Military personnel. From 2003-2007, the burn unit at Fort Sam Houston (U.S. Army Institute of Surgical Research) had 1497 hospitalizations, including 656 military, of which 540 were related to the conflict in Iraq. Victims of large burns do not have sufficient donor skin to complete grafting. With each skin harvest, healing time increases as epithelial sources (glands, follicles) are removed, leaving wounds and donor sites susceptible to microbial contamination. Sepsis, which develops in part from microbial contamination and invasion in wounds, accounts for 75% of deaths from burn injuries, and is often associated with multiple organ failure. Other major aspects of recovery from burns, including immune function, positive nitrogen balance and physical therapy, all depend on completion of wound closure. A significant source of long-term morbidity is scarring at both the donor sites of skin grafts, and in wounds grafted with meshed and widely expanded skin grafts. Conversely, it is well known that grafting of wounds with sheet grafts suppresses scar formation.

Civilian population. Mortality and morbidity from burns, trauma, and other skin loss injuries remain significant medical and socio-economic problems estimated to cost more than \$500 million annually in treatment costs alone, in addition to lost productivity. Burns in the civilian population cause

approximately 15,000 hospital days in the U.S. annually, and full-thickness burns require treatment by excisional debridement and split-thickness skin grafting.

Autologous ESS. Classified as a medical device, autologous ESS consist of a lyophilized scaffold of collagen and chondroitin sulfate, populated with cultured dermal fibroblasts and epidermal keratinocytes, which organize into an analog of skin tissue. The device develops an epidermal barrier and basement membrane, and releases high levels of angiogenic growth factors, including but not limited to, vascular endothelial growth factor, basic Fibroblast Growth Factor, and Transforming Growth Factor-beta 1. In addition, both keratinocytes and fibroblasts in culture are known to release inflammatory mediators, which promote transient development of fibro-vascular tissue.

Specific Aims for Year 5

Aim 1: Analysis of pigmented ESS (ESS-P) to promote transition to TRL 4.

Aim 2: Tumorigenicity testing of cultured hM.

Aim 3: Discussions with the AFIRM leadership to promote the transition of this project to TRL5.

Research Progress – Year 5

Transplantation of ESS-P to full-thickness wounds in athymic mice.

ESS with no added hM (Figure 1A), 3×10^2 hM/cm² added (not shown), or 3×10^3 hM/cm² added (Figure 1B) were grafted onto full-thickness wounds in athymic mice. Pigment restoration is density-dependent by 12 weeks after grafting where almost no skin color is restored (Figure 1A), partial pigmentation develops (not shown), or complete skin color is restored (Figure 1B). Quantification of percentage pigmentation (n=7-10) is shown in Figure 1C, and pigment intensity in mexameter units (n=3-10) is shown in Figure 1D. Pigment intensity in ESS-P with 3×10^3 hM/cm² is not statistically different from normal skin.

Conclusions

Overall, Project 4.7.2 continues to progress toward transition to a clinical trial with verification of end points for safety and efficacy, and establishment of laboratory protocols that are anticipated to be compatible with the clinical care of burn patients. The researchers have shown that protocols for melanocyte propagation can be compatible with schedules for treatment of acute burn patients. They have restored skin color by the addition of autologous melanocytes to ESS. Finally, the tumorigenicity testing they have performed to date suggests that transplantation of cultured melanocytes is safe, and results are ready for review by the U.S. Food and Drug Administration (FDA) to initiate planning for a clinical trial. These accomplishments are anticipated to enable the development of clinical trials of autologous ESS-P.

Future Research Plans

Restoration of skin color by transplantation of hM has been accomplished during AFIRM-I, and a set of standard operating procedures will be developed going forward to determine any variability in uniformity of pigment distribution among ESS-P

containing hM from different skin donors or body sites. Also, the intensity of skin pigmentation will be determined and regulated pharmacologically in healed ESS-P compared to the original donor skin.

Planned Clinical Transitions

During AFIRM-I, the University of Cincinnati served as the regulatory sponsor of previous clinical trials, but it has decided to discontinue its service in that role. Therefore, identification of a regulatory sponsor for ESS-P is a basic requirement for transition to the clinic. Technology for ESS-P has been licensed to a corporate partner for commercialization, and that partner is a highly capable candidate to serve as a regulatory sponsor. If the partner elects not to sponsor the regulatory protocol, another corporate partner may be recruited, or the project Principal Investigator would be willing to serve as a sponsor-investigator. Transition to clean room manufacturing of ESS-P as an investigative therapeutic is needed to meet the standards for current Good Manufacturing Practices, and clinical protocols must be designed and submitted to local Institutional Review Boards, and to the Human Research Protections Office at the U.S. Army Medical Research and Materiel Command. These requirements introduce needs for regulatory oversight, data monitoring, a Data Safety Monitoring Board, and a fully compliant database for performance of data collection and reporting. Together, these requirements will constitute the regulatory protocols for a clinical trial of ESS-P. As a first step in the process toward a clinical trial, a Request for Designation will be prepared with advice from the translational research office at Wake Forest Institute for Regenerative Medicine. Determination by FDA of the regulatory identity of ESS-P as a device, biologic, drug or combination product will facilitate development of the technical, clinical and regulatory protocols needed to prepare for and perform clinical trials directed toward delivery of an advanced therapy to reduce morbidity and mortality for our nation's wounded warriors.



V: Burn Repair

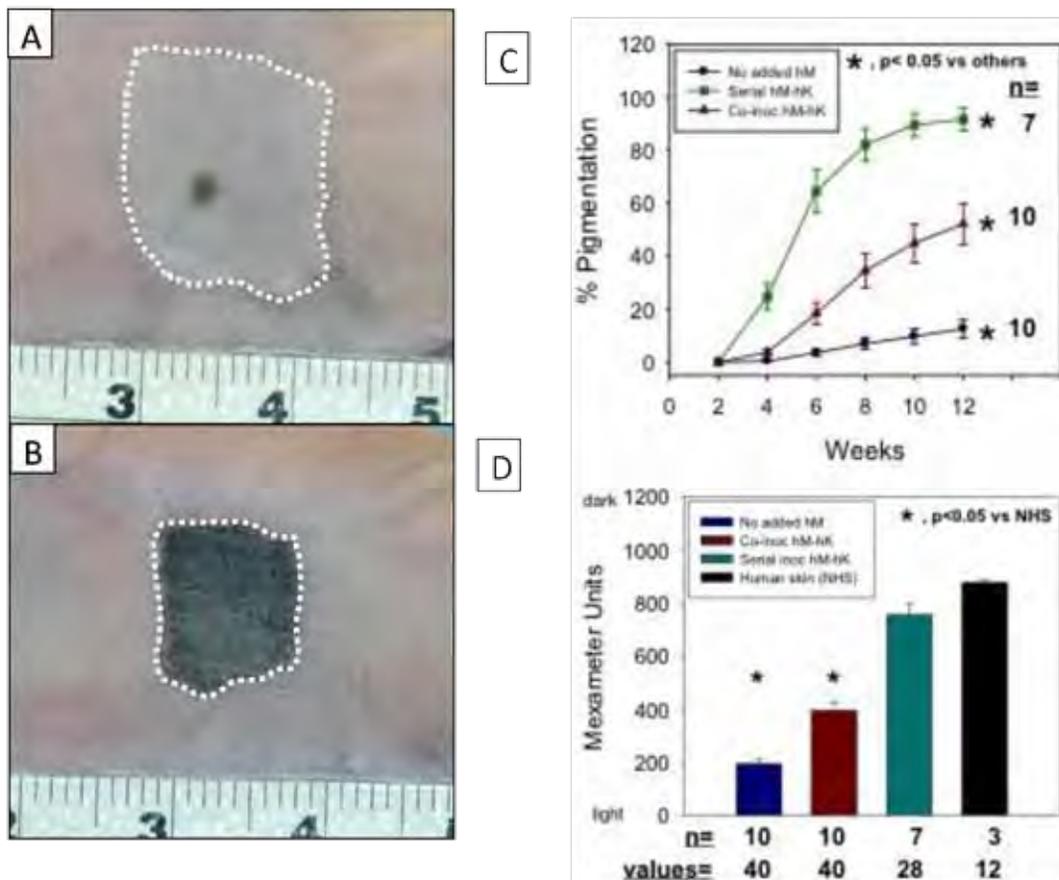


Figure 1. Examples of human ESS in vivo on athymic mice (left column, dotted lines) at 12 weeks after grafting (left column) from a recent experiment. (A) ESS with no added hM develops little or no pigmentation. (B) ESS-P with $3e3$ hM/cm² added develops more uniform pigmentation. (A, B: Scales in cm.) (C) Mean percent pigmented area in healed ESS-P increases significantly to greater than 90% after addition of hM by serial inoculation. (D) Comparison of the black skin sample used to generate the ESS-P shows no difference in mean pigment intensity. This result suggests that ESS-P restores normal skin color.



Skin Products / Substitutes

Autologous Human Debrided Adipose-Derived Stem Cells for Wound Repair in Traumatic Burn Injuries

Project 4.6.8, USAISR

Team Leader(s): Robert J. Christy, PhD (U.S. Army Institute of Surgical Research [USAISR])

Project Team(s): Postdoctoral: Shanmugasundaram Natesan, PhD; Technical: Nicole Wrice (USAISR)

Collaborator(s): Laura Suggs, PhD, University of Texas, Austin, Michael Coleman, InGeneron, Inc. Houston, TX; LTC Andre Cap, MD, PhD, Blood Research, USAISR; COL Anthony Johnson, MD, Eye Trauma, USAISR; Anthony Durkin, University of California, Irvine; Gerald Wilmark, United States Air Force (USAF)

Therapy: Burn injury therapy

Deliverable(s): Dermal equivalent using autologous stem cells

Objective: Use autologous adipose-derived stem cells (ASCs) to improve existing technology in burn wound repair and scar mitigation

TRL Progress: Beginning, TRL 3; Current, TRL 4; AFIRM I Target, TRL 5

Key Accomplishments: The researchers developed technology to decellularize the amniotic membrane for epithelial wound covering and epithelial differentiated ASC sheets. They developed PEGylated plasma-based biomaterial products to be used for wound healing and development of skin equivalents. They also developed platelet-free plasma for use instead of purified fibrin for vascularized biomaterial matrix. Finally, they developed a porcine model to determine the validity of skin constructs in deep partial-thickness burns.

Key Words: human adipose-derived stem cells, epithelialization, plasma, amniotic membrane

Introduction

Severe thermal injury accounts for approximately 5% of all combat casualties and continues to be a significant source of morbidity. In particular, as the total body surface area (TBSA) burn increases, mortality increases proportionately as a result of the inability to achieve skin closure. Currently, large body surface area burns pose significant therapeutic challenges with implications for early hemodynamic instability and sepsis, as well as later complications of scarring, contracture, and long-term disability. From a clinical standpoint, the extent and severity of burn injury determines the need for tissue-grafting or tissue substitutes. Though autografting remains the treatment of choice for excised burn wounds, this option may be severely limited in patients with extensive burns because of limited donor-site availability. Furthermore, repeat harvesting of split-thickness skin grafts from the same donor area can result in morbidity, loss of dermal thickness, excessive scarring, and increased pain.

When the extent of a burn exceeds the ability to perform a single-stage autograft, many burn centers

have adopted the use of an allograft as a temporizing measure while they await further availability of autografts. Although a wide variety of skin substitutes have been developed, the time required for revascularization and for cell expansion has limited the clinical utility of this option. The addition of cellular elements to dermal scaffolds have demonstrated superiority with respect to revascularization, but the lack of an available and convenient autologous cell source from a severely burned patient has not made this practical. The requirements for an ideal substitute include minimal donor site morbidity, availability, and the ability to reconstitute the different functions and layers of skin. Products based on autologous cultured keratinocytes and fibroblasts are more likely to contribute to actual skin restitution. Still, challenges involved with these products are the need for culture expansion and the extensive cultivation time. Moreover, such products require donor biopsy of normal skin to obtain the requisite cell types when normal tissue accessibility has proportionately decreased with percentage increase in TBSA.



Alternatively, developing a tissue-engineered skin substitute using stem cells proves to be a potential option to regenerate skin for the treatment of extensively burned patients. In particular, ASCs have been shown to possess immense potential to regenerate skin because of its substantial plasticity to differentiate into multiple cell lineages. Unfortunately, after severe burn injury, the source(s) of adipose tissue can be limited because of the availability of uninjured viable tissue and the fear of causing additional morbidity from subcutaneous liposuction. Because tangential debridement of skin often leads to debridement of some viable tissue, the **Christy team** has shown that stem cells can be isolated in adequate quantities from the adipose layer of discarded burn skin (dsASCs). Furthermore, these cells are able to integrate within the excision wound bed of an athymic rat. Unlike other cells types, such as fibroblasts, keratinocytes, and endothelial cells, which definitively require culture expansion, dsASCs can be isolated in proportionally large numbers from patients with an increasing percentage of TBSA burn. These stem cells can then be used along with collagen and fibrin-based scaffolds to develop epithelial, dermal-vascular, and hypodermal layers, which can then be used to develop a complete full-thickness skin equivalent (**Figure 1**).

Research Progress – Year 5

Development of a Sterile Amniotic Membrane (AM) Tissue Graft for Burn and Eye Trauma using Supercritical CO₂

The current methods used to prepare sterile AM allografts have limitations. For example, although rigorous donor screening practices and aseptic techniques are strictly followed, the disadvantage of cryopreservation of AM in glycerol is that the tissue is not completely sterile and the risk of infection by microorganism contamination exists. Furthermore, any toxic effects of the antibiotics used to disinfect the AM tissues have not been investigated. Other research has shown that sterilization with gamma irradiation results in destruction of the amniotic epithelium and dissolution of the compact and fibroblast connective tissue layers.

An alternative to traditional tissue processing techniques involves the use of supercritical carbon dioxide. The term “supercritical fluid” denotes a substance that, at temperature and pressure conditions above its critical point, simultaneously exhibits diffusion properties of a gas with densities and dissolution properties comparable to those of liquids. The diffusivity, density and viscosity of supercritical fluids can easily be manipulated with small changes in temperature and pressure, thus making supercritical fluids applicable to many industrial and laboratory processes.

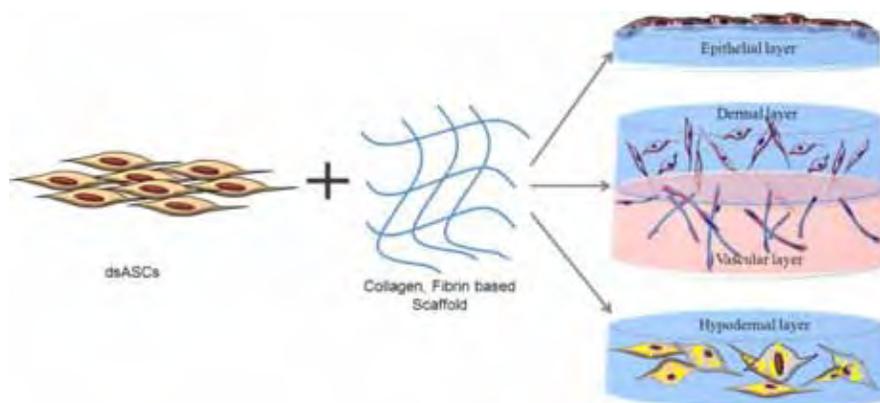


Figure 1. Schematic representation of the development of different layers of skin substitute using dsASCs and hydrogel-based matrices. Epithelial and hypodermal constructs are developed using collagen hydrogel and the vascularized dermal construct using collagen-PEG-fibrin-based bilayered hydrogel.

The researchers conducted tissue sterility experiments in accordance with industry sterilization validation guidelines. Briefly, they inoculated AM tissue pieces with 10^6 Clostridium sporogenes and processed the tissue using a Nova 2200™ SCCO₂ sterilizer (NovaSterilis, Lansing, NY) for 10 or 30 minutes with the addition of 0, 0.5, 1, or 2 ml peracetic acid (PAA). Results of the tissue sterility testing experiments (**Table 1**) revealed that a minimal treatment time of 10 minutes SCCO₂ exposure combined with 2 ml PAA would provide a sterile amniotic membrane product optimal.

Table 1. Results of the tissue sterility testing experiments

Amount of PAA (ml)	Duration of exposure to SCCO ₂ (min)	Clostridium sporogenes
0	10	Fail
0	30	Fail
0.5	10	Fail
0.5	30	Fail
1	10	Fail
1	30	Pass
2	10	Pass
2	30	Pass

This work indicates that the use of SCCO₂ presents a novel and appealing alternative to traditional AM tissue graft processing methods. This study provides evidence that AM tissues can be processed using optimized SCCO₂ methodologies in order to obtain a tissue graft that is not only sterile but possesses the physical and biochemical properties that are most similar to that of native tissue. Furthermore, we have shown that our membrane preparation is biocompatible and provides an excellent scaffold for stem cell attachment and proliferation. This amniotic membrane allograft preparation has great potential for use in wound healing and tissue engineering applications.

Blood Plasma-Based Hydrogel for Tissue Regeneration and Wound Healing

USAISR researchers previously demonstrated several unique features of PEGylated fibrinogen that make it advantageous in wound healing over other

hydrogel dressings. PEGylated fibrinogen exhibits traits of both synthetic hydrogels and natural materials. Specifically, 1) the presence of PEG provides a highly hydrated (>90% water) moist environment for managing exudates, 2) the presence of fibrinogen confers biodegradability to the material; however, our prior results show that it is significantly more stable in vitro than fibrinogen alone, and 3) the inherent biologic activity of fibrinogen encourages the natural healing process in hosts by stimulating tissue and blood vessel in-growth. This matrix system is therefore able to be responsive to cell-mediated remodeling while allowing for handling and storage under a variety of conditions.

Recently, the Christy team investigated identifying a more clinically relevant (practical) source of fibrinogen to meet the clinical need of wound-bed vascularization. In the current practice of medicine, plasma-based clot/fibrin plugs are used to treat various wound conditions. Unfortunately, these clots form a tight network of fibrin that does not allow cellular infiltration. Therefore, the plasma plugs are a poor product for regenerative purposes. Considering plasma as a viable source of fibrinogen, they recently investigated if it is able to develop a stable hydrogel matrix by using our fibrinogen PEGylation process. The researchers obtained fresh frozen plasma from a blood bank and plasma freshly isolated blood. In preliminary experiments a PEGylated-plasma mixture initiated gelation using thrombin or CaCl₂. The rationale for using CaCl₂ as an initiator of gelation was to determine if the endogenous thrombin present in plasma was sufficient to form stable hydrogels. **Figure 2** shows the representative image of PEGylated and unPEGylated plasma hydrogels. It was evident that unlike the native plasma clot, PEGylation yielded a more stable hydrogel matrix.

Rheological Measurements. The strength of the PEGylated plasma hydrogels was determined using a small-strain oscillatory shear rheometer (AR-G2, TA Instruments Ltd.) with parallel plate geometry. Samples were prepared as described above with various concentrations of both CaCl₂ (11mM-27mM) and thrombin (5U-12.5U) in a 6-well cell culture insert. For analysis, gels were placed on the bottom plate of the rheometer, and the top plate was lowered until the gel contacted the entire surface.



V: Burn Repair



Figure 2. Photograph depicts the rigidity and clarity conferred to a platelet-free plasma (PFP) gel (left) versus native PFP (right).

Frequency and strain sweeps were performed to determine the linear viscoelastic region of the gels. After a short pre-shear period, storage modulus (G') was measured at constant frequency (15 rad/s) and

strain percentage (1%). Plasma samples from various healthy donors ($n=4$) with age range of 20-50 years were analyzed. Results are presented in Figure 3.

In addition, the ultrastructural characteristics of PEGylated plasma hydrogels prepared with both CaCl_2 and thrombin were analyzed using scanning electron microscopy (SEM) technique. For SEM analysis, the gels dried as mentioned previously and then sputter-coated with a thin layer (10 nm) of gold and palladium (Anatech; Union City, CA) before examined on a Zeiss Sigma VP 40 (Zeiss-Leica, Thornwood, NY) scanning electron microscope. Figure 4 shows the SEM of images of various hydrogels. It can be observed that the porosity of hydrogels increased with increase in concentrations of CaCl_2 and thrombin. Thicker fibers were observed with lesser concentration of calcium chloride and thrombin. It was also observed that

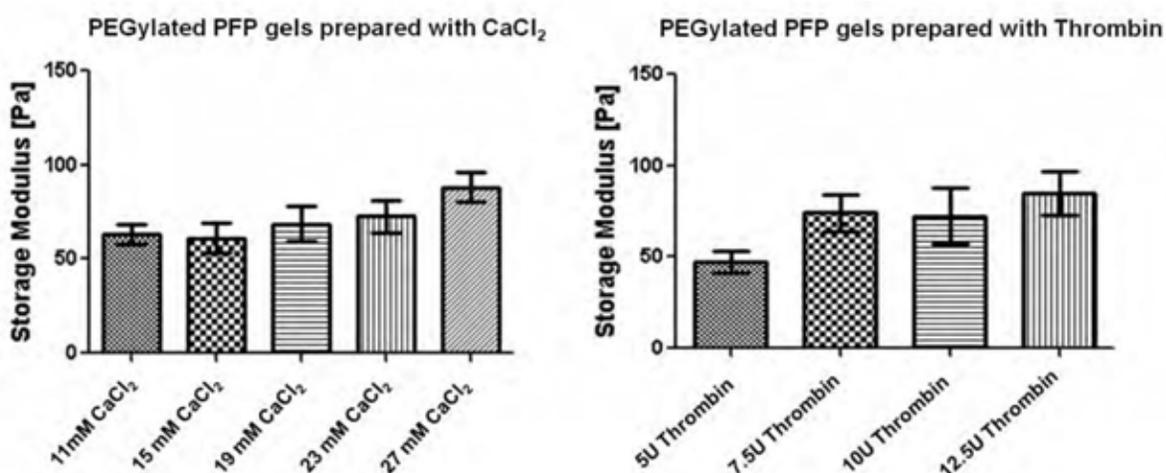


Figure 3. Rheological measurements of PEGylated plasma hydrogels prepared with different concentrations of CaCl_2 or thrombin.

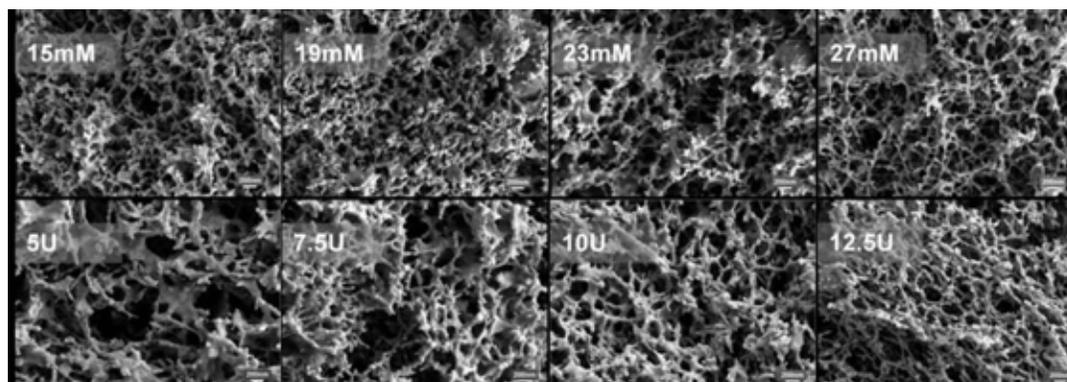


Figure 4. SEM images of PEGylated plasma hydrogels prepared with different concentration of CaCl_2 or thrombin.

thrombin-based gels showed more distinct differences between different concentration with unconnected areas of fiber network which decreased with higher concentrations.

Conclusions

The use of SCCO₂ for the preparation of tissues for transplantation and other clinical applications is only beginning to be realized. SCCO₂ makes for an attractive solvent for tissue processing for many reasons. First, SCCO₂ has low viscosity and high diffusion coefficients which allows for the penetration of solid, microporous matrices, such as tissue ECM. In addition, CO₂ has relatively low critical coordinates (i.e., 93.2–98.7 atm and 35–39 °C) making it ideal for delicate biological tissues. The low temperature of the process and the stability of CO₂ allow unwanted compounds, such as blood and lipids, to be extracted without compromising the physiological properties and mechanical integrity of the tissue. Furthermore, SCCO₂ is relatively non-toxic, thus allowing for improved biocompatibility upon implantation.

The researchers have successfully developed a novel method to modify human plasma using PEGylation technology to confer various desirable biological properties to a hydrogel scaffold for use in the development of our skin equivalent and applications of restorative and plastic surgery fields. This, and derivatives of this product, can be used alone or in combination with cells or other biologic factors to promote healing in both acute and chronic scenarios, or to temporarily or permanently (depending on type of modification) reconstruct a skin tissue. Most importantly, the simple addition of CaCl₂ alone can gel PEGylated plasma mixture (~20–30 min.) without the addition of exogenous thrombin. This provides a product that does not require exogenous thrombin (4°C storage, high cost) to prepare.

Future Research Plans

The researchers anticipate: A) Generating a PFP product from human or porcine sources using either a point-of-care plasma isolation device and/or blood plasma from a blood blank; B) chemically modifying blood plasma using PEG to form a stable hydrogel scaffold, rather than a clot; C) using the endogenous thrombin found in plasma to initiate polymerization of fibrinogen; D) using the PEG-plasma hydrogel, alone or in combination with SVF/ASCs, and evaluating the engraftment and wound healing potential in burn and excision wound models. The PEGylated plasma with SVF/ASCs can then be combined with currently available skin allografts, decellularized ECMs, epithelial sheet membranes, etc., to develop a point-of-care vascularized skin equivalent.

Planned Clinical Transitions

The researchers plan to continue to use and optimize the InGeneron, Inc. point-of-care device for the isolation of ASCs. This will be used for the isolation of cells from debrided burn patient tissue, as well as from surgically isolated adipose tissue obtained from abdominoplasty (from the approved Institutional Review Board protocol using current standard of care lipoaspiration surgical techniques). The researchers will continue to develop a more clinically relevant porcine burn model and will use this model for the development of a large (up to 20%) TBSA burn, which will provide a stringent test for their and other AFIRM-related skin equivalent products. Finally, they are continuing to address and use U.S. Food and Drug Administration-approved products that are commercially available.



Clinical Trials

A Multicenter Comparative Study of the ReCell® Device and Autologous Split-thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries

Project 4.2.7, WFPC

Team Leader(s): James H. Holmes IV, MD, Director, Wake Forest Baptist Medical Center Burn Center, Associate Professor of Surgery, Wake Forest School of Medicine (WFSM) (Winston-Salem, NC)

Project Team(s): Joseph Molnar, MD (WFSM); Rajiv Sood, MD (University of Indiana); William Hickerson, MD (University of Tennessee Health Science Center); Bruce Cairns, MD (University of North Carolina at Chapel Hill); Kevin Foster, MD (Maricopa Integrated Health Systems); David Mazingo, MD (University of Florida); Marion Jordan, MD (Washington Hospital Center, DC); Booker T. King, MD (U.S. Army Institute of Surgical Research); David Smith, MD (Tampa General/ University of South Florida); Michael Feldman, MD (Virginia Commonwealth University); Tina Palmieri, MD (University of California, Davis); John Griswold, MD (Texas Tech University Health Science Center); Marc Jeschke, MD (Sunnybrook Health Sciences Centre, Toronto)

Collaborator(s): Fiona Wood, MD (Royal Perth Hospital, Perth, Australia); William Dolphin, PhD (Avita Medical Ltd); Andrew Quick (Avita Medical Americas LLC); Annette Fagnant (MedDRA Assistance Inc.); Susanne Panzera (BioStat International, Inc.); Maureen Lyden (BioStat International, Inc.)

Therapy: Application of autologous skin cell suspension, prepared at bedside, for treatment of second-degree burn injuries. The skin cells are separated from a small, split-thickness skin sample using the ReCell® Autologous Cell Harvesting Device (Avita Medical Ltd, Cambridge UK)

Product: ReCell Autologous Cell Harvesting Device

TRL Progress: Beginning, TRL 7; Current, TRL 7; AFIRM I Target, TRL 8

Key Accomplishments: A total of 21 subjects have been enrolled and treated during the past year, bringing total enrollment to date to 85 (of 106) subjects. Thirty-six (36) subjects have been followed through 52 weeks. Eight (8) sites are actively enrolling subjects. Enrollment has been discontinued at sites with limited performance in terms of enrollment in order to focus the use of resources. Permission from the U.S. Food and Drug Administration (FDA) and Ethics approval has been secured for the addition of the Sunnybrook Health Sciences Centre to the study. Avita Medical is engaged in dialog with the FDA about potential changes toward expediting completion of the study and product approval.

Key Words: ReCell, cell spray, skin grafting, burns

Introduction

As the largest organ in the body, the skin performs a range of vital protective, immunologic neurosensory, thermoregulatory and homeostatic functions. Therefore, any wound involving thermal, electrical or chemical burn, trauma, abrasion or laceration may seriously compromise the participation, performance, health and, ultimately, life of the patient. In addition to the acute, short-term effects of inadequate wound management, the long-term effects of wounds and wound scars include pain, restriction of movement, occupational limitations, disfigurement and potential psychological impairment leading to lifelong disabilities, underemployment, and

failure to fully reintegrate into society. The rapid and effective management of wounds of an injured warfighter is, therefore, a critical factor in the determination of wound outcome and consequential morbidity and mortality.

Unmet need

There is an unmet clinical need for a field-deployable therapy resulting in regenerated skin for definitive closure with limited scarring of partial-thickness burn injuries that mitigates risks and morbidity associated with conventional grafting and the long waiting period and possible graft failure associated with cultured epithelial autograft (CEA). The minimal donor site needed for the fully self-contained

ReCell device has the potential to allow for a shift toward early treatment of indeterminate-depth burns, leveraging additional advantages of early, dermal-preserving excision. Since the donor site is small (roughly 2 cm by 2 cm, and 0.008 inch in thickness), the typical waiting period to be certain of the depth of injury before harvesting substantial conventional mesh graft donor skin is not warranted when ReCell is an option for treatment.

Military capability gaps

This project addresses gaps in the area of treatment of skin injury. The primary gap addressed by this project is “Excessive duration of healing (one day per percent burn minimum in-patient hospitalization; prolonged inpatient and outpatient rehabilitation; multiple reconstructive surgeries).” Use of ReCell is intended to allow for drastic reduction in requirement for donor skin, and associated reduction in complications for patients. Further, the resulting regenerated skin is not anticipated to require any further reconstruction as it should be comparable to the surrounding uninjured skin, which addresses two additional gaps: “Inability to control scar formation during the first 1–2 years post-burn” and “Matching elasticity pigmentation to normal skin.” Although patients in a combat zone or elsewhere with massive or greater than 40% total body surface area injuries are not directly being studied in this trial, from a clinical perspective, the data may be generalized toward an understanding of clinical benefit for these patient populations.

Analysis of competitive technologies

The main competitive technology for ReCell for treating burn patients is the split-thickness skin graft. This has been the standard of care for over 80 years and is used as either a full sheet or meshed graft depending on the size of the area to be covered and the availability of donor sites. The CEA is another competing technology that has been used to treat burn patients since the early 1990s. CEA is a lab-based procedure involving culturing of keratinocytes isolated from the patient’s harvested skin to grow sheets of new skin. The process requires special equipment and personnel, and takes up to 14–21 days to develop the autograft sheets. CEAs are typically used for large burns, greater than 30% total body surface area, when there are limited donor sites. There are still questions regarding final patient

outcome and scar quality associated with CEAs, and the patient must undergo separate procedures for donor harvesting and cultured autografting.

Solution

The ReCell Device is based on previous work of Wood and Stoner (Wounds 2003; 15:16-22) and the recognition that application of autologous skin cells could offer long-term wound closure in a clinically advantageous time frame while optimizing the patient’s outcome. The device is designed to provide a simple, safe technique for the harvesting of skin cells for enhancement of epidermal repair. The initial step involves harvesting a thin, split-thickness skin sample, followed by enzymatic and mechanical disaggregation to harvest the cells of the epidermis, dermis and epidermal–dermal junction. The separated cells and associated signaling factors are combined into a suspension containing a mixed population of live keratinocytes, melanocytes and papillary fibroblasts. The suspension is then sprayed onto the prepared wound bed. The cells migrate over the surface, providing epidermal reconstruction with site-matched characteristics of color and texture. The applied cells are incorporated into the developing epidermis. The speed of re-epithelialization is very important as the “sealing” of the skin surface limits the inflammation that has been implicated as the pivotal factor in hypertrophic scar formation. By providing a source of viable and metabolically responsive cells onto the wound surface, the ReCell technology may facilitate rapid wound healing while minimizing donor site morbidity and potentially eliminating or minimizing scar formation.

Clinical Trial Status

To date, 85 subjects have been enrolled (target = 106), with 21 enrolled in the past 12 months. Thirty-six (36) subjects have been followed through 1 year post treatment. Twelve (12) sites have enrolled at least 1 subject, with 8 sites currently actively enrolling subjects. The Annual Investigational Device Exemption report for the study was accepted without questions by the FDA.

In terms of securing approval to market the ReCell device, 510(k), De Novo and premarket approval (PMA) pathways have been considered. In short, the optimal pathway appears to be via PMA. The other pathways present considerable obstacles in terms



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of achieving agreement on either the appropriate predicate device(s) in the instance of 510(k), or the appropriate special regulatory controls guidance that would be developed for the De Novo class of products (to be applied not just to ReCell but other products using ReCell as a predicate). Use of the PMA pathway for ReCell precludes other products from establishing ReCell as a predicate device.

Conclusions

Clinical trial outcomes will be made available once 90 subjects have been followed through 16 weeks, and the data are monitored (onsite source-verified), analyzed and reported to the FDA.

Future Clinical Plans

Additional sites are being considered for participation in the study to increase the rate of enrollment. The Sunnybrook Health Science Centre will be the next site to join the effort.

Planned Commercialization Transitions

Avita Medical currently manufactures ReCell in southern California and distributes the product outside the United States via Australian, European and Chinese approvals to market. At the appropriate time, the company will make necessary plans for distribution and support in the U.S. market. Based on outcomes seen outside the U.S., Avita Medical has a keen interest in pursuing a pediatric trial (and indication), most likely for treatment of scald injuries.



Clinical Trials

Stratatech Technology for Burns

Project 4.2.9, WFPC

Team Leader(s): James Holmes IV, MD (Wake Forest)

Project Team(s): LTC Booker King, MD (U.S. Army Institute of Surgical Research [USAISR]); Michael Schurr, MD (University of Colorado Hospital); Lee Faucher, MD (University of Wisconsin Hospital and Clinics); Kevin Foster, MD (Arizona Burn Center); and Steven Wolf, MD (University of Texas Southwestern)

Collaborator(s): B. Lynn Allen-Hoffmann, PhD, Allen Comer, PhD, and Mary Lokuta, PhD (Stratatech Corporation); Leslie Jones (Research Point)

Therapy: A readily available, viable, full-thickness, allogeneic human skin substitute (StrataGraft® skin tissue) that provides immediate wound coverage and secretion of growth factors, cytokines, and antimicrobial peptides to promote the healing of severe burns and other complex skin defects.

Product: StrataGraft skin tissue

TRL Progress: Beginning, TRL 6; Current, TRL 6; AFIRM I Target, TRL 6

Key Accomplishments: The research team has obtained all necessary regulatory approvals for a dose-escalation clinical trial and has performed site initiation visits at each of the 6 clinical sites. The researchers have maintained a continuous production stream of StrataGraft skin tissue for the clinical trial at a current Good Manufacturing Practices (cGMP)-compliant biomanufacturing facility. Two (2) cohorts of 10 subjects each have been fully enrolled. The second patient cohort was treated with a larger area of StrataGraft tissue. None of the sites in either cohort 1 or cohort 2 required autografting of any percentage of the StrataGraft treated site by Day 28. There has been no evidence of safety concerns or immunological responses to the StrataGraft tissue. Allogeneic deoxyribonucleic acid (DNA) from StrataGraft tissue was not detected in any of the subjects after 3 months. These data suggest that StrataGraft provides immediate wound closure and is replaced as the patient's own cells heal the wound.

Key Words: StrataGraft skin tissue, burn, skin grafting, regenerative medicine

Introduction

Skin loss due to severe burns and trauma is a life-threatening condition affecting deployed military personnel as well as the general civilian population. These injuries frequently result in lifelong functional and cosmetic impairment that affect the quality of life for injured warfighters and their families. The severity of skin damage often exists as a gradient from superficial and deep partial thickness (DPT), to full-thickness skin loss in the same patient. The standard of care for both full-thickness and DPT burns is surgical excision followed by coverage with autologous skin grafts. In large burns, the area of healthy skin is often limiting, and must be used to cover areas of full-thickness injury. As a result, DPT areas must be temporarily managed with other means before definitive coverage with autograft. The delay in definitive wound closure extends hospitalization, increases the risk of complications, and contributes to increased healthcare costs. In addition, autografting generates painful donor wounds which are prone to infection and scarring, and never become wholly normal skin

in terms of thickness, elasticity, and strength. Therefore, alternatives to autografting the DPT component of severe burns are needed to expedite the healing of large burns and reduce or eliminate the morbidities associated with donor-site wounds.

Stratatech Corporation has developed StrataGraft tissue as a readily-available allogeneic skin substitute to promote the healing of complex skin defects due to burns and trauma. StrataGraft tissue is a living, meshable, suturable, human skin substitute that reproduces many of the structural and biological properties of normal human skin. StrataGraft tissue is composed of human keratinocytes and dermal fibroblasts organized into a full-thickness tissue with epidermal barrier function comparable to intact human skin. In addition to providing immediate wound coverage with robust barrier function, numerous antimicrobial peptides, growth factors, and cytokines secreted by the viable cells of StrataGraft tissue are anticipated to accelerate wound healing and reduce infection, thereby facilitating wound closure and cosmetic outcome.



StrataGraft tissue is being evaluated as a readily available, universal skin substitute to promote the healing of severe burns and other complex skin defects. Stratatech has completed a Phase I/IIa clinical trial in 15 patients with severe burns and other complex skin defects, designed to assess the safety and early efficacy of exposure to escalating amounts (44 to 220 cm²) of StrataGraft tissue. Subjects in this study had full-thickness skin defects requiring sequential debridement and coverage with a temporary biological dressing prior to autografting. Equivalent halves of the treatment site on each subject were treated with StrataGraft tissue or cadaver allograft for 1 week. In three cohorts of five patients each, the amount of StrataGraft tissue applied was increased sequentially from 0.3% total body surface area (TBSA) to 1.0% TBSA, and then to 1.5% TBSA. After 1 week, the allograft tissues were removed and the wound bed was evaluated. The wound was autografted when judged ready based on visual clinical assessment by the clinical investigator. Autograft take was assessed 2 weeks after autograft placement. StrataGraft tissue exhibited a good safety profile and was well tolerated with no evidence of acute immune responses. There were no deaths or treatment-related adverse events, and no subjects discontinued the study. There was no increase in the frequency or types of AE as the TBSA treated with StrataGraft tissue increased across the three cohorts.

Clinical Trial Status

This AFIRM project is designed to conduct a clinical trial to examine the safety and efficacy of StrataGraft skin tissue in promoting the healing of DPT burns without the need for autografting. Primary endpoints are the percent area of the StrataGraft treatment site requiring autografting by 28 days, and wound closure at 3 months. Additional assessments are designed to monitor adverse events, local or systemic toxicity, immunological responses to allogeneic cells of StrataGraft tissue, and persistence of the cells from StrataGraft tissue.

During the past year, the research team has made excellent progress toward completion of a multi-center clinical trial to evaluate the safety and efficacy of StrataGraft tissue as an alternative to autografting of DPT burns. Six (6) clinical sites are participating in this study: Wake Forest Baptist Medical Center, the U.S. Army Institute for Surgical

Research, University of Colorado Hospital, the University of Wisconsin Hospital and Clinics, the Arizona Burn Center at Maricopa Medical Center, and Parkland Health and Hospital System. Key documents for this study, including the clinical protocol, informed consent form, and investigator's brochure, have been reviewed and approved by the Institutional Review Board (IRB) at each of these sites. Each of the IRB packages was also reviewed and approved by Human Research Protection Office (HRPO).

Stratatech has maintained a continuous production stream of StrataGraft skin tissue for the clinical trial at Waisman Biomanufacturing, a cGMP-compliant contract manufacturing facility. All StrataGraft tissue lots produced for this trial have met all lot release criteria. ResearchPoint Global is providing clinical trial monitoring services for this trial. Activities completed during the previous year include site monitoring, data collection, and statistical analysis.

The clinical results to date have exceeded expectations. This dose-escalation study involved enrollment of two cohorts of ten subjects each. The first cohort of 10 subjects was treated with up to 220 cm² of StrataGraft tissue. All subjects in the initial dose cohort met the primary safety and efficacy endpoints for the study. All burns treated with StrataGraft tissue healed without the need for autografting, and all of these wounds remained closed after 3 months. Follow-up evaluations to 12 months have been performed on all subjects from the first cohort. Following an interim analysis of data from the first subject cohort, the Wake Forest School of Medicine I-Data and Safety Monitoring Board unequivocally recommended progression to the second patient cohort. The study protocol was revised and submitted to the Investigational New Drug (IND) application that Stratatech Corporation has established with the FDA Center for Biologics Evaluation and Research for clinical evaluation of StrataGraft skin tissue in complex skin defects.

Subjects in the second cohort were treated with up to 440 cm² of StrataGraft tissue. Enrollment in the second dose cohort was completed in August 2012. None of the sites in either cohort 1 or cohort 2 required autografting of any percentage of the StrataGraft treated site by Day 28 (**Figure 1**),

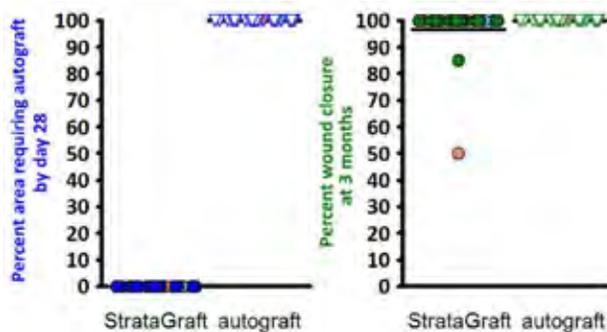


Figure 1. Autografting and wound closure assessments for subjects in the first two cohorts. The left panel shows the percentage of the initial wound areas treated with either StrataGraft tissue or autograft that required autografting by Day 28. No wound sites treated with StrataGraft required autografting by Day 28. The right panel shows the percentage of the initial wound areas treated with either StrataGraft tissue or autograft that was closed at 3 months.

although one subject in cohort 2 had loss of both StrataGraft and autograft study sites as well as other non-study sites which were deemed not ready for autograft placement by Day 28. StrataGraft treatment sites were completely closed at 3 months in 18 of 19 per-protocol subjects from cohorts 1 and 2 (Figure 1).

Because the StrataGraft-treated wounds did not need to be autografted, study subjects reported less pain at the donor site that had been prospectively identified for coverage of the StrataGraft-treated site, if needed (Figure 2). Follow-up evaluations to 12 months will be completed in the next project year.

There have been no safety concerns and no serious adverse events deemed product-related in any of the initial subjects. There has been no evidence of an acute immune response to StrataGraft tissue. Allogeneic DNA from StrataGraft tissue was not detected in tissue samples from the StrataGraft-treated site after 3 months. Unexpectedly, evaluation of scarring and cosmesis after 3 months revealed that, in some cases, the sites treated with StrataGraft were smoother, more supple, and less raised than the autograft control sites (Figure 3). Although these exciting observations must be confirmed by long-term follow-up, the results to date have exceeded expectations and suggest that a single application of StrataGraft tissue may be

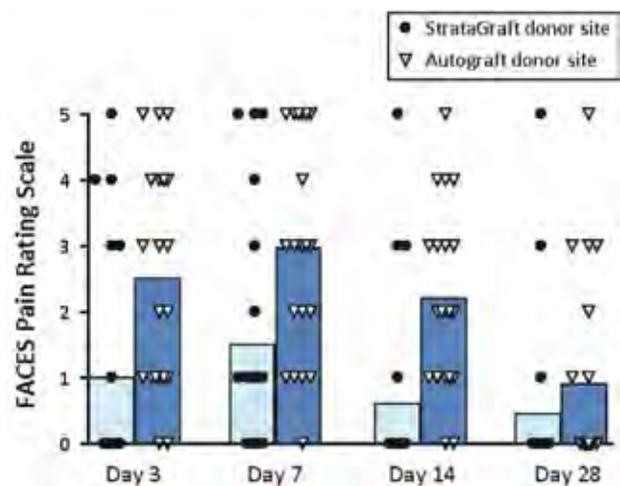


Figure 2. Donor site pain assessments through Day 28 for subjects in cohorts 1 and 2. Patient assessment of pain at the prospectively identified donor sites was documented after 3, 7, 14, and 28 days using the Wong-Baker FACES Pain Rating Scale (FPRS). Data were calculated by converting the categorical descriptors of the FPRS into corresponding numerical scores (0 = no hurt to 5 = hurts worst).



Figure 3. Wound site appearance after 3 months in a representative subject who was treated for burns to the backs of both upper arms. One arm was treated with StrataGraft tissue (left) and the other arm received an autograft (right).

able to promote the healing of DPT burns without autografting.

Conclusions

In summary, progress made during the past project year has allowed completion of patient enrollment in the first two cohorts of a clinical trial to evaluate the safety and efficacy of StrataGraft tissue as



a universal skin replacement for treatment of DPT burns. To date, the results of this trial have exceeded expectations. Continued clinical evaluation of StrataGraft skin tissue in the current clinical study will provide a strong body of data to establish the safety and efficacy of StrataGraft skin tissue as an alternative to autografting for DPT burns.

Clinical Trial Plans for the Next Year

Research plans for the coming year includes follow-up assessments of safety and efficacy outcomes, and preparation of study report.

Planned Clinical Translations

Work accomplished during the past project year has set the stage for completion of patient follow-up and monitoring in Year 6. Results of this study will inform the design of a Phase III registration study in patients with complex skin defects.

Corrections/changes planned for next year and rationale for changes

During the course of this AFIRM project, Stratatech has independently identified and funded product development advancements which are essential to

extend product shelf life, reduce costs, and streamline the production process for successful commercialization. Stratatech has focused on and has been developing long-term storage methods for the Company's tissue-engineered therapeutics. Long-term storage is critically important for broad use of StrataGraft tissue in both military and civilian settings. With these goals in mind, Stratatech has developed cryopreservation methods for current format StrataGraft tissue (44 cm², circular, collagen dermal equivalent). Cryopreserved current-format StrataGraft maintains tissue viability and other key structural and biological properties for at least 5 months. Stratatech has submitted a chemistry, manufacturing, and control amendment to the StrataGraft IND and plans to enroll a third cohort of ten patients into the STRATA2011 study to determine whether cryopreserved current-format StrataGraft tissue is comparable to refrigerated StrataGraft tissue used in the first two patient cohorts in its ability to promote healing of DPT burns without the need for autografting. The revised clinical protocol has been submitted to each of the sites participating in this study. Subject enrollment in this third patient cohort will begin as soon as IRB and HRPO approvals have been received.



Clinical Trials**Clinical Trial: Expedited Availability of Autologous Engineered Human Skin for Treatment of Burned Soldiers – Lonza Walkersville, Inc.****Project 4.7.4, RCCC**

Team Leader(s): David Smith, MBA, Christine Nigida, MS, and Theresa D’Souza, PhD (Lonza Walkersville, Inc.)

Project Team(s): Stanton Gerson, MD (Case Western Reserve University); LTC Booker T. King, MD (U.S. Army Institute of Surgical Research [USAISR]); Nicole Gibran, MD (University of Washington–Harborview Medical Center)

Collaborator(s): USAISR and Harborview Medical Center

Therapy: Treatment of deep partial- and full-thickness burns ($\geq 50\%$ total body surface area) using a skin substitute.

Deliverable(s): Clinical trial of engineered skin substitute (ESS)

TRL Progress: Beginning, TRL 5; Current, TRL 6; AFIRM I Target, TRL 6

Key Accomplishments: The researchers received an Orphan Drug Product Designation (#11-3562) for the ESS on June 1, 2012. They submitted an Investigational New Drug (IND) application to the U.S. Food and Drug Administration (FDA) on July 26, 2012. They received a “Clinical Hold Letter” for the ESS on September 21, 2012. They have completed all but one item in the clinical hold letter and are providing all completed actions to the FDA for additional review.

Key Words: burns; skin substitutes; full-thickness; combination products; clinical trials

Introduction

Mortality and morbidity from burns, trauma, and other skin-loss injuries remain significant medical and socio-economic problems, estimated to cost more than \$1 billion annually including medical treatment and lost productivity. Victims of large burns do not possess sufficient donor skin to complete grafting without multiple harvesting of donor sites at 7- to 10-day intervals. With each harvest, healing time increases as epithelial sources (glands, follicles) are removed, leaving wounds and donor sites susceptible to infection. Sepsis, which develops in part from microbial contamination and invasion in wounds, accounts for 75% of deaths from burn injuries, and is often associated with multiple organ failure. Other major aspects of recovery from burns, including immune function, positive nitrogen balance and physical therapy, all depend on complete wound closure. A significant source of long-term morbidity is the development of scars in two areas: (1) the original burn wounds, which have been grafted with meshed and widely expanded

skin grafts, and (2) other body areas where skin was harvested to cover the burns (i.e., donor sites of skin grafts). Conversely, it is well known that grafting of wounds within a sheet format suppresses scar formation.

Prompt and effective wound closure remains a challenge in recovery from extensive, deep burn injuries. To address this limitation, the researchers have developed an autologous, ESS (**Figure 1**). They licensed this technology from the laboratory of



Figure 1. ESS generates a functional skin barrier promoting permanent wound closure and healing.



V: Burn Repair

Dr. Stephen Boyce at the University of Cincinnati, and they completed the technology transfer and product development. ESS may allow reductions in morbidity and mortality for soldiers who are casualties of combat-related burn injuries. Stated simply, autologous ESS may offer a life-saving alternative therapy to patients (both military and civilian) with catastrophic burn injuries.

In this study, the researchers are conducting a Phase I clinical trial on the use of an autologous ESS for the treatment of patients with burns affecting greater than 50% total body surface area.

Clinical Trial Status

To date, there are no clinical results. The research team submitted the IND application to the FDA on July 26, 2012. They were notified of “Clinical Hold” for ESS on August 23, 2012, and received a “Clinical Hold Letter” on September 21, 2012. The research team has completed all but one item in the clinical hold letter and is providing all completed actions and the in-progress data to the FDA for review. Any results and data will not be released until the clinical hold is lifted and after the clinical trial is underway. Once the trial begins, the researchers aim to enroll 10 patients at two clinical sites: Brooke Army Medical Center and Harborview Medical Center (Seattle, WA).

Conclusions

No conclusions or outcomes can be made until the clinical trial has been initiated.

Future Research Plans

In the upcoming year, the research team will develop rapid release assays and potency assays, evaluate the matrix by assessing its physical attributes, prepare for large-scale matrix manufacturing, and determine commercial packaging. In addition, the researchers are looking to increase their ESS manufacturing capability should there be a national emergency with multiple casualties or a national emergency that might include multiple treatment sites. Expansion is being explored through two paths: increasing output per lot and establishing additional manufacturing sites.

Planned Clinical Transitions

There are two phased trials planned, the first being the 10-subject trial for safety, plus two training subjects per trial site. This trial will be started under AFIRM I but is expected to be completed at some point after the period of performance is over. The larger Phase II trial is expected to be completed after AFIRM I and will include up to 40 subjects.



Clinical Trials

Enhanced Detection of Pathogens in Burn Wounds Using a Novel Multi-primer PCR/Mass Spectrometric Assay

Project 4.5.5a, WFPC

Team Leader(s): Sandeep Kathju, MD, PhD (University of Pittsburgh)

Project Team(s): Latha Satish, PhD and Phillip Gallo, PhD (University of Pittsburgh)

Collaborator(s): Sid Miller, MD and Larry Jones, MD (Ohio State University)

Therapy: No therapeutic intervention; trial is designed to evaluate the utility of the Ibis PLEX-ID™ system in the diagnosis of burn wound infection

Deliverable(s): (Ibis PLEX-ID coupled polymerase chain reaction [PCR]-ESI-mass spectrometry assay)

TRL Progress: Beginning, TRL 5; Current, TRL 5; AFIRM I Target, TRL 6

Key Accomplishments: The researchers have begun the process of patient accrual for the clinical trial and currently have up to 52 discrete patients enrolled. They are optimizing the sample extraction and preparation techniques, and they have demonstrated that sample extraction and analysis are proceeding within acceptable technical parameters.

Key Words: burn, wound, infection, pathogen, biofilm, PCR

Introduction

Unmet need

Historically, invasive burn wound infection was a devastating complication; prior to the advent of topical antimicrobial agents in the mid-1960s, mortality from burn wound-induced infection was extremely high. Recent paradigms of burn wound management have embraced the concept of early excision and grafting, and with reduced time to surgical treatment of burn wounds has also come reduced mortality, reduced surgical blood loss, and reduced length of hospitalization. Despite these advances, however, burn wounds and infection following burn injury continue to inflict significant morbidity and mortality on patients. The most cited reason for mortality in burn injuries remains infection, noted as the most common complication in burn wound patients of all age groups by the American Burn Association. In the military (as in the civilian) population, infection remains the most common complication after burn injury, and is the most commonly cited reason for mortality.

The diagnosis of an actual burn wound infection in general remains largely a clinical one, depending on such signs as surrounding cellulitis, purulent

drainage, and skin graft failure, and aided by cultural microbiology obtained from swabs, biopsies, or operative specimens. Recently, however, it has been recognized that numerous bacteria (and fungi) exist within burn wounds in the form of biofilms. Biofilms are organized communities of microorganisms encased in a matrix of extracellular polymeric substance. Biofilm bacteria are typically attached to tissue- or foreign-body surfaces, and display a highly divergent behavior from their planktonic (free floating) counterparts: they are highly resistant to conventional antibiotics, are similarly highly resistant to natural host immune responses, and very importantly for this project, are often highly recalcitrant to propagation in standard microbiological culture. In numerous clinical infectious scenarios (e.g., otitis media, prosthetic joint infection, etc.) biofilm bacteria are routinely undetected by standard microbiological techniques, but can be demonstrated by molecular biological techniques. In a recent study, Kennedy et al. used both light and electron microscopy to examine a series of burn wounds for biofilms, and found rich evidence of biofilm formation on burn wound surfaces (not eschar) consisting of mixed species of bacteria as well as some fungal elements.



This observation indicates that in many cases the true bacterial quotient of a burn wound will be unappreciated or underappreciated by standard culture, and that identification of the pathogens involved in burn wound infection may also be inadequate if microbiological culture is the sole determinant employed. This has significant implications for the management of burn wounds and suspected wound infections.

Analysis of competitive technologies

Most molecular assays for bacteria rely on PCR amplification and sequencing of the 16S ribosomal RNA genes, since these are specific to prokaryotes and can affect a broad survey of the bacterial contents of a specimen; alternatively, pathogen-specific assays can be used. The latter are limiting since they presume the identity of the infecting organism, and will be unable to detect other bacteria even if they are abundantly present. The former typically require highly specialized sequencing equipment and protocols that are not widely available, and are not likely to be of practical clinical applicability in the near future. Neither will yield any information on the presence or absence of antibiotic-resistant genes unless specifically queried.

Solution

The recent advent of a novel coupled PCR-mass spectrometric technology, the Ibis T5000™ (Abbott Laboratories, Chicago, IL), now called PLEX-ID, offers multiple potential advantages for molecular detection of biofilm-based and other infections compared to all previous molecular assays. The Ibis assay simultaneously tests for > 3000 species including virtually all known pathogens in a multiplex fashion, eliminating the need for an a priori choice as to the likely infecting organism. For known pathogens, the Ibis is capable of yielding information down to the species level, and can simultaneously assay for antibiotic resistance markers. Moreover, it is semi-quantitative (providing information on the number of bacterial genomes present) and allows for a rapid turnaround with clinically useful results available in as little as 6 hours after sample presentation.

The Ibis eliminates reliance on bacterial culture and allows for a simultaneous universal survey of pathogens without presuppositions as to their

nature while still allowing for speciation of the identified organisms. It is sensitive and rapid, with results possible in as little as 6 hours after sample presentation, and provides semi-quantitative data and a molecular antibiogram. It is automated, with a user-friendly readout that includes a confidence value as an internal quality control. It can be used to detect viral and fungal organisms as well, although this would entail the use of additional primer sets (which are available). The manufacturer (Abbott Laboratories) is currently streamlining the Ibis technology even further for future clinical applicability, thus this technology has the potential to become a widespread diagnostic aid to practicing clinicians in a way that previous broad-spectrum molecular assays have not.

The Ibis platform has never been applied to burn wound infection before this trial.

Clinical Trial Status And Results

The funds for this trial were made available at the end of calendar year 2012, with the project start date backdated to August 2012. Accordingly, the practical trial period has not yet completed a single year.

Patient Accrual

Substantial progress has been made at the principal study site (University of Pittsburgh). Approval for the study (and several subsequent modifications) has been obtained from the Institutional Review Board, and a mechanism has been established whereby samples from burn-injured patients can be collected and stored for subsequent analysis. Application has been made to the Human Research Protection Office (HRPO) for similar approval, which is anticipated soon. No grant monies have been spent on the acquisition or processing of these samples pending HRPO approval, but specimens from 52 patients have been collected in the course of their routine clinical care, in accordance with trial protocol.

Sample Extraction and Preparation

To prepare for the study, the researchers have developed a protocol to extract total DNA (bacterial and eukaryotic) from burn wound samples, using archival burn wound specimens. They have established that they can typically obtain sufficient DNA from relatively small specimen sizes, and are actively

working on a way to simultaneously extract sufficient quantities of RNA separated from the DNA. They have subjected several test DNA samples to the full Ibis analysis and obtained technically acceptable results. Importantly, control DNA samples isolated by this protocol, in which no infecting organism would be expected to occur, did prove negative on the Ibis assay, indicating no contamination in our extraction and preparation process. The researchers will apply this protocol to the study samples once HRPO approval has been granted.

Conclusions

There are no conclusions yet as the trial is ongoing and the entirety of data gathering from the collected (and future) specimens is pending HRPO approval.

Future Clinical Trial Plans

The researchers plan to carry out the data analysis portion of the trial as described in the trial protocol.

Planned Commercialization Transitions

The Ibis PLEX-ID technology is already under commercial development by Abbott Laboratories.

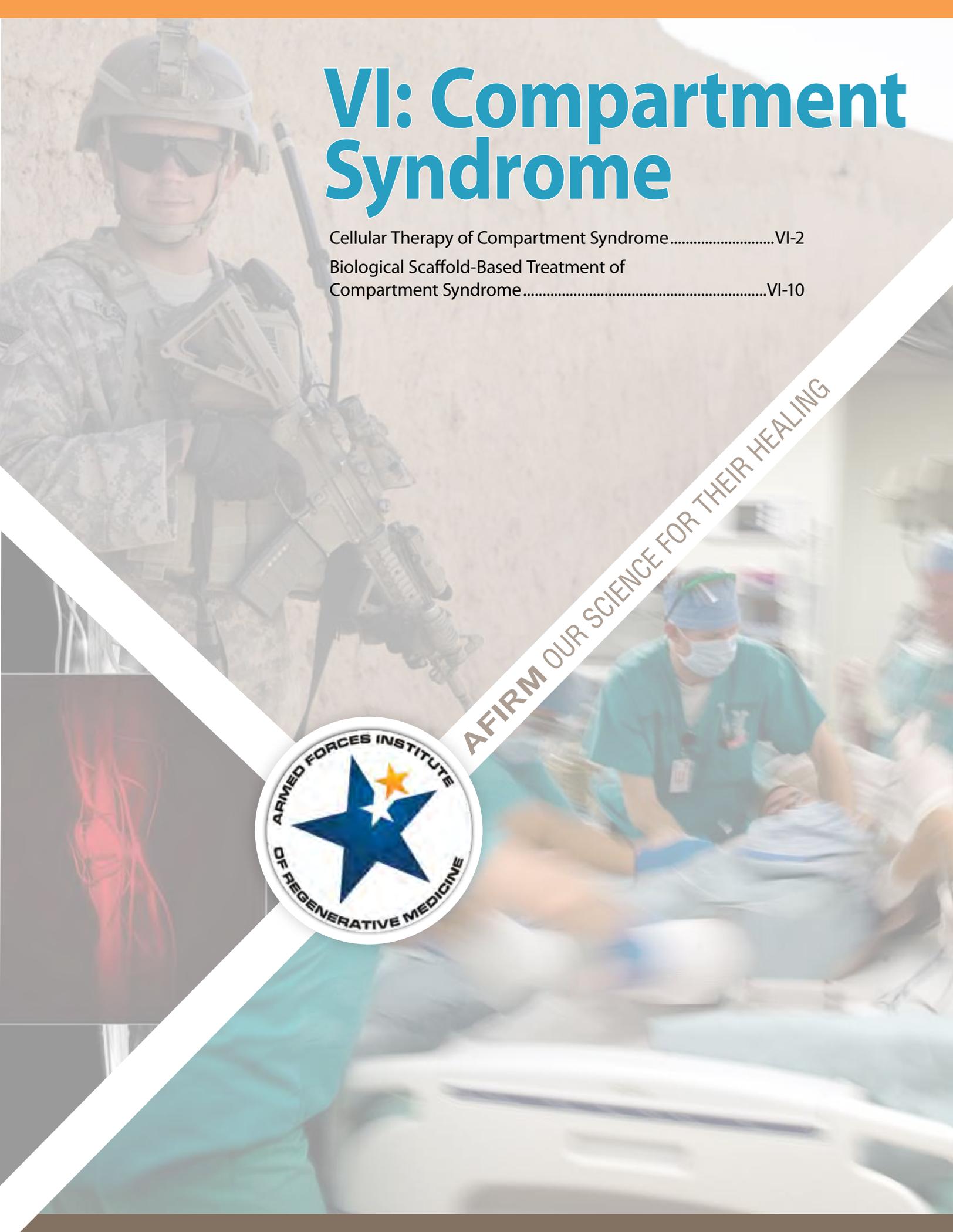


VI: Compartment Syndrome

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Biological Scaffold-Based Treatment of
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AFIRM OUR SCIENCE FOR THEIR HEALING





VI: Compartment Syndrome

Cellular Therapy of Compartment Syndrome

Cellular Therapy for Treatment and Consequences of Compartment Syndrome

Project 4.3.1, WFPC

Team Leader(s): Johnny Huard, PhD (McGowan Institute for Regenerative Medicine (MIRM), University of Pittsburgh); Shay Soker, PhD (Wake Forest Institute for Regenerative Medicine [WFIRM])

Project Team(s): Tracy Criswell, PhD (WFIRM); Burhan Gharaibeh, PhD, Nick Oyster, BS, Makoto Kobayashi, MD, Minakshi Poddar, MS, and Johannes Schnependahl, MD (MIRM)

Collaborator(s): William Wagner, PhD, and Stephen Badylak, DVM, PhD, MD (MIRM)

Therapy: Cellular therapies for treatment of Compartment Syndrome (CS)

Deliverable(s): Muscle tissue regeneration by delivering muscle stem and progenitor cells together with angiogenic and anti-fibrosis factors

TRL Progress: Beginning, TRL 1; Current, TRL 4/5; AFIRM I Target, TRL 4/5

Key Accomplishments: Using a neonatal pressure cuff, the researchers in the laboratories of Drs. Huard and Soker developed novel animal models of CS

injury. They characterized the anatomic and functional changes in the muscular, vascular and neural components of the hind limb muscles following compression-induced damage in a reproducible rat injury model that mimics CS observed in clinical patients. The researchers also demonstrated that co-seeding of endothelial cells (ECs) with muscle precursor cells (MPCs) led to enhanced vascularization, innervation and skeletal muscle tissue formation in vivo. MPCs were used in cell therapy of CS and showed formation of donor-derived, innervated myofibers. Cell culture conditions were developed for long-term expansion of MPCs (up to passage 25) without loss of myogenic regeneration capabilities. The researchers demonstrated insufficient engraftment of human MPCs after transplantation into an injured rat muscle, which highlights the need for better culturing/ screening protocols.

Key Words: Compartment Syndrome, skeletal muscle, stem cells, cell therapy, muscle precursor cells, fibrosis, Losartan, fasciotomy

Introduction

Compartment Syndrome of the limbs is a serious condition characterized by an increased pressure within the muscle compartments, which are enclosed by a non-expandable thick membrane called fascia. CS can be caused by a multitude of injuries that include trauma, interruption of venous outflow, contusion, fractures, and blast injuries that affect the musculoskeletal system; if left untreated, CS can lead to amputation or even death. In general, musculoskeletal injuries strongly impact the U.S. Army in terms of human suffering, direct and indirect monetary costs, loss of time for work or training and, perhaps most importantly, military readiness. These injuries account for a large number of disabled soldiers. In spite of the devastating consequences of CS, no successful therapies are available. The standard treatment is fasciotomy (cutting the fascia to relieve the tension) immediately after the diagnosis of CS.

In this project, the researchers aimed to create a small animal (athymic rat) model of CS to allow for the future testing of therapies to treat CS sequelae. Large animal models have the drawback of immune rejection during xenotransplantation, whereas the athymic rat model represents a viable cellular therapy model without the need for immunosuppressants. The researchers' model is being developed based on their expertise in contusion and laceration injury models in rodents and the use of murine skeletal MPC therapies.

Research Progress – Year 5

CS Injury Model Development – Early trials to create modFel at Dr. Huard's laboratory *Human CS-injured muscle biopsies*

The researchers obtained muscle biopsies from patients diagnosed with CS by trauma surgeons at the University of Pittsburgh Medical Center. They characterized these biopsies and found variability

among patients. Data from these biopsies were used to guide the researchers in the development of their rat CS model.

The use of neonatal cuff creates a CS-like injury in rats

Using a neonatal cuff (Figure 1) creates an injury that presents the clinical symptoms of CS and is more consistent than an external compression device, leading to an intracompartmental pressure (ICP) ≤ 30 millimeter of mercury diastolic blood pressure and the loss of pink coloration of footpad. The ICP measurements were taken by implanting a wireless transmitter and recording pressures every 10 seconds (Figure 2). The average ICP over the 3-hour injury period was recorded.

Progression of skeletal muscle damage following CS injury

Histological examination of transverse sections of tibialis anterior (TA) muscles revealed the severity of the injury at 3 days, and the rapid and near-complete endogenous repair of the skeletal muscle over time. All tissues examined contained a number of myofibers with normal morphology. The normal fibers were found on the periphery of the sections at 3 days, and these were present in greater numbers towards the center of the tissue at 7, 14 and 28 days post-injury. The inflammatory and necrotic areas of the injured TA showed widespread necrosis of the myofibers. Inflammatory cells, regenerating myofibers and angiogenic cells were examined to determine the type of cells present in the nucleated infiltrate, and when these cells were seen in skeletal muscle undergoing regeneration.

Incomplete recovery of muscle function may be attributed to nerve damage

Average muscle force at different time points was significantly lower in injured legs compared to controls. Four weeks after injury the function of injured muscles had recovered to 59% of the controls, and the difference in peak isometric torque between the two groups was not significant ($p=0.079$). The incomplete functional recovery but near complete return of skeletal muscle to normal morphology indicates nerve damage. The researchers began to investigate the distribution of nerve endings in the motor unit, but further work is needed to differentiate damage done to the muscle versus nerve after CS injury.

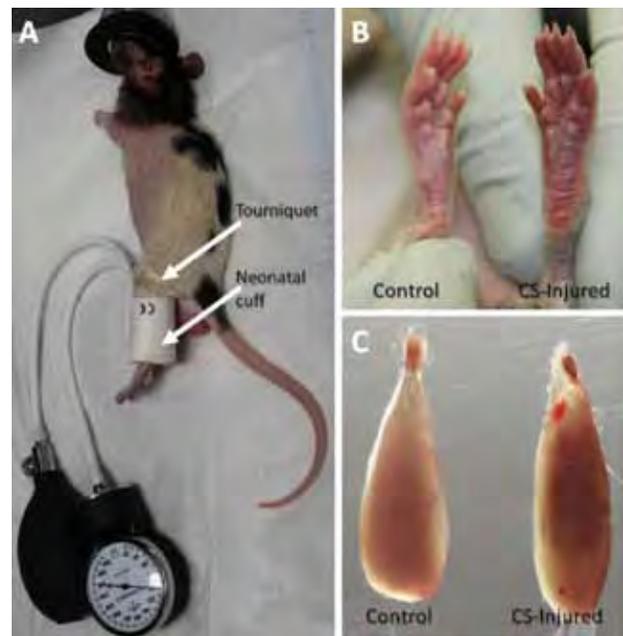


Figure 1. The use of neonatal cuff creates a CS-like injury in rats.



Figure 2. Use of probe to record ICP while creating CS injury in athymic rat.



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Development and characterization of a rat model of CS at Dr. Soker's lab

Lewis rats (8 weeks old) were used to create this CS model using neonatal blood pressure cuffs to apply external compression on the hindlimb. Histological and functional analyses were performed to document the degeneration and regeneration of the TA muscle. Complex injury to the muscle, vasculature and neural components of the tissue was noted, similar to the etiology of CS in human patients. Tissue edema and necrosis were detected within the first 4 days after injury, followed by a regenerative period resulting in mature regenerated myofibers and recovery of function by 35 days after injury.

MPC injections as cell therapy for CS

Effective cell therapy for CS is dependent on MPC survival, myo-differentiation and integration with the host tissue. MPC therapy for the treatment of CS was tested in the rat model of CS developed by the Soker team. Cells were injected 4 days after injury directly into the injured TA muscle, and muscles were removed 14 or 28 days after injury for analyses. Injected cells were integrated into the regenerating skeletal muscle (**Figure 3**), but no significant difference in muscle function was found between sham (treated with phosphate-buffered saline) and MPC-treated groups at either Day 14 or 28 after injury.

Adult rat skeletal muscle injury model

Data derived from this CS model demonstrated the extraordinary ability for self-regeneration in rats, a property that can mask the effects of cell therapy. Aging animals have an attenuated regenerative capacity that is closer to the timeline of clinical human muscle regeneration after injury, which affords a better opportunity to investigate the effects of MPC therapy on functional recovery. Older animals demonstrated a decreased regenerative capacity as compared to young rats, evidenced by increased collagen deposition and a decrease in functional recovery. Significant functional improvement was found in MPC injected muscles of adult rats 14 days after injury, similar to the functional recovery seen in the young rats. These data demonstrated the utility of using older animals as a model for studying the effects of cell therapy.

2.2.3 Vascularization of tissue engineered skeletal muscle

Using fluorescently labeled ECs and MPCs, the Soker team developed an in vitro culture system that allowed for the visualization of MPC differentiation and the neovascularization of tissue-engineered skeletal muscle tissue. Cultures of MPCs and/or ECs were seeded on an acellular bladder scaffold, implanted into the subcutaneous space of nude mice, and explanted after 8 weeks. Implanted MPCs formed muscle tissue on the scaffolds, and human umbilical vein endothelial cells were found to be integrated into mature vasculature. The combination of MPCs and ECs resulted in increased vasculature, tissue formation, and innervation of tissue engineered muscle when grown on the bladder acellular matrices scaffold in vivo as compared to MPCs alone.

Human muscle precursor cells (hMPC)

Cell therapy for CS in human patients will most likely be dependent on allogeneic sources of MPCs. The Soker team documented variability between several hMPC samples obtained from the Wake Forest Institute for Regenerative Medicine Good Manufacturing Practices facility in myotube formation and muscle differentiation marker expression

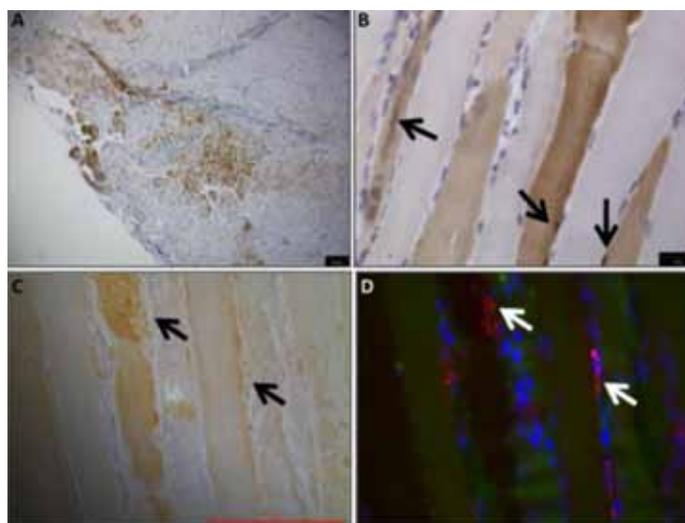


Figure 3. Location of transplanted MPCs. Green fluorescent protein⁺ myofibers were found in injured tissue 28 days post injury (A). Injected MPCs differentiated and fused into myofibers integrated with host tissue (B). Innervation of Green fluorescent protein⁺ myofibers (C) was shown using an antibody for neurofilament protein (D). Red, NF200; green, muscle autofluorescence; blue, DAPI-nuclei.

in vitro. Further research has demonstrated the inability of the hMPCs to expand, differentiate and engraft in vivo when transplanted into a CS-injured limb of immunodeficient rats. These data highlight the need for an in vitro culture protocol that can maintain the myogenic capacity of MPCs for use as cell therapy treatment for human patients with CS injuries.

Optimization of MPC maintenance and expansion in vitro and in vivo

The ex vivo expansion of MPCs is problematic since they quickly lose their growth and differentiation potential. The Soker team investigated different culture conditions to optimize the growth of these cells. Further experiments utilizing this methodology demonstrated that the myogenic differentiation capability of mouse MPCs could be maintained up to at least passage 25 (**Figure 4**). The MPCs obtained from 1 mouse limb, up to passage 11, yielded over 10×10^6 cells, which are more cells than needed for clinical purposes. The ability of MPCs to engraft into muscle in vivo is a true test of myogenicity. Green fluorescent protein (GFP)-labeled MPCs cultured in vitro up to passage 10 were able to engraft within a regenerating muscle tissue in vivo, when injected into injured TA muscles of mice. These

results provide a starting point for the development of a clinical protocol for the use of a defined culture media for the growth and expansion of hMPCs.

Losartan study

Earlier in this AFIRM project, the researchers showed that treatment with the angiotensin II receptor blocker, Losartan, effectively reduced the amount of fibrosis developed in injured skeletal muscle, as well as increased the function of affected limbs relative to controls. They most recently completed a two-person case study which showed that Losartan is an effective treatment for grade II hamstring injuries.

Conclusions

The researchers have shown that CS injury can be reliably created in a rat model. They characterized the extent of injury in this model. They showed that damage in the CS-injured rat muscle was not highly uniform – the muscle periphery was more resistant to damage than the center of the muscle. In the rodent model, the compliance of the muscle fascia does not allow a sustained increase in pressure, and this may have caused the inconsistency in the damage, which was reduced significantly by using an external compression. Still, when using

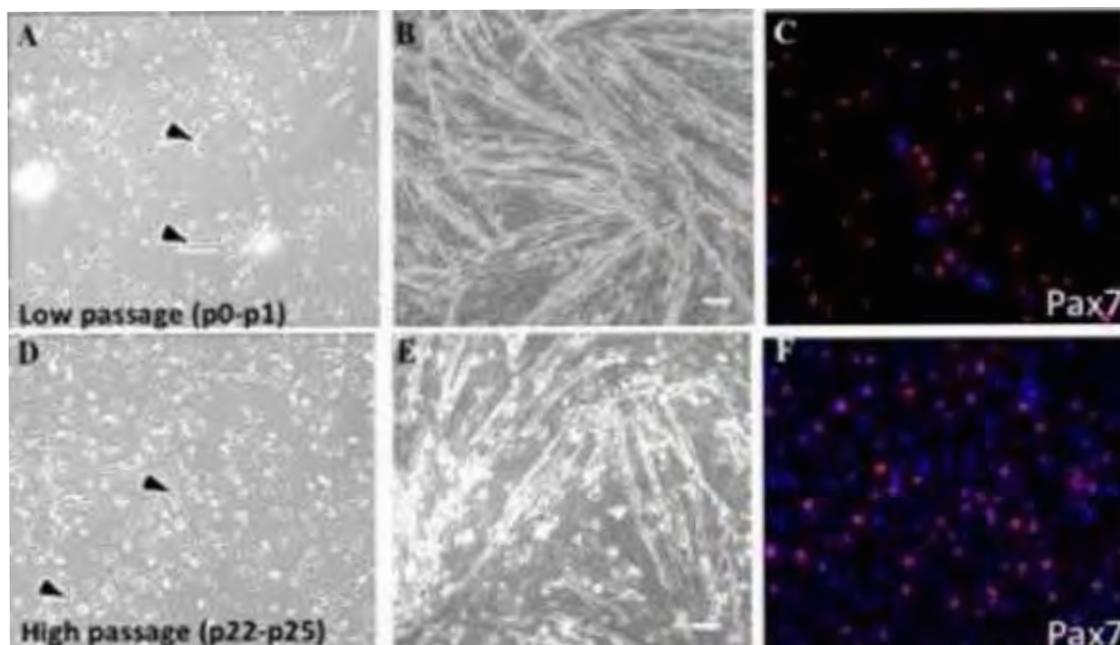


Figure 4. Expansion of MPCs in vitro to high passage number. Low (p0-p1) and high (p22-p25) numbers of cells demonstrated similar growth potential (A, D), differentiation capability (B, E) and Pax7 expression (C, F).



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young rodents to study CS, there is a need to create significant histological damage to allow detection and assessment of therapeutic interventions that can enhance muscle repair. In short, the CS-like injury model developed by the Huard team can aid in the development of novel therapies that can be used in concert with fasciotomies to manage CS injury in humans. Special attention needs to be taken when sampling injured muscles from CS patients for histopathological assessment.

Future Research Plans

The researchers plan to optimize human muscle stem and precursor cell transplantation into CS-injured muscle to enhance its repair by identifying the ideal delivery method, number of cells, and time after injury for the cell injection. They will optimize the timing and dosing of delivery of MPCs transduced to express angiogenic factors in injured skeletal muscle. They will perform MPC cell therapy with the further addition of vascular endothelial growth factor, along with MPCs, for the enhancement of vascularization during the regenerative process. They aim to develop culture conditions for the expansion of human MPCs, similar to conditions used for rodent MPCs, and validate their myogenic capacity in vivo. The researchers will continue using

Losartan to reduce fibrosis and optimize the ideal dose and time after injury to begin Losartan treatment. They plan to conduct combinatorial therapeutic strategies in the CS animal model, including delivering MPCs with and without angiogenic factors, Losartan, and Platelet Rich Plasma fractions. They will perform MPC cell therapy (using cells from human and mouse [GFP-labeled] origin) in an aged animal model, which has a diminished regenerative capability. They will also obtain more data on clinical translation of Losartan studies in muscle injury military and civilian patients. A larger, double-blinded, multi-center, clinical study is planned and, if funded, should provide such data.

Planned Clinical Transitions

This study provided the basis for future clinical work to use Losartan to treat muscle-injury patients. A larger, double-blinded, multi-center, clinical study is planned. The researchers have submitted a proposal for AFIRM II. If funded, they will obtain the relevant Institutional Review Board approvals. Dr. Soker will initiate discussion with the U.S. Food and Drug Administration in order to obtain an Investigational New Drug application status for the in vitro expanded MPCs for clinical use.



Cellular Therapy of Compartment Syndrome**Use of Bone Marrow-Derived Stem Cells for Treatment of Compartment Syndrome****Project 4.3.2, WFPC**

Team Leader(s): Kenton Gregory, MD (Oregon Health & Science University, Oregon Biomedical Engineering Institute)

Project Team(s): Bo Zheng, MD, Cynthia Gregory, PhD, Michael Rutten, PhD, Hua Xie, MD, PhD, Rose Merten, BS, Bryan Laraway, BS, Annabeth Rose, BS, James Hunt, BS, Carrie Charlton, BS, Cher Hawkey, BS, and Teresa Malarkey, CVT, LAT (Oregon Health & Science University)

Collaborator(s): OHSU Center for Regenerative Medicine, Portland, OR; USAISR, Houston, TX; Special Operations Medical Command-Fort Bragg, NC; Biosafe-America Biologics Consulting Group, Houston, TX; Torston Tonn, MD, Johann Wolfgang University, Frankfurt, Germany

Therapy: Autologous Bone Marrow Stem Cell Treatment for Compartment Syndrome (CS)

Deliverable(s): Develop a large-animal CS model and evaluate safety and efficacy of stem cell treatments to regenerate muscle and nerve tissue after extremity wounds complicated by CS

TRL Progress: Beginning, TRL 2; Current, TRL 4; AFIRM I Target, TRL 5

Key Accomplishments: The researchers developed a combat-relevant extremity CS injury in a mature large-animal swine model that results in long-term muscle and nerve disability if untreated. They completed a pivotal proof-of-concept preclinical study in Sinclair mini-swine to evaluate the deployment strategy of autologous bone marrow mononuclear cells (BM-MNCs) to treat extremity injuries complicated by CS. The researchers were able to demonstrate long-term engraftment of cells, significant improvement in muscle and nerve function, and improved gait analysis at 3-months post injury compared to control animals. No adverse events or complications were associated with any cell or sham treatments. The researchers will soon complete a long-term randomized (6-month) safety and efficacy swine study comparing BM-MNC treatment to sham controls as a prelude to clinical trials. Finally, the researchers created and used a protocol for the enzymatic digestion of intact skeletal muscle to generate a pure population of CM-Dil-labeled BM-MNCs and show that these cells are capable of multi-lineage (muscle, nerve, and vascular) differentiation.

Key Words: Extremity Compartment Syndrome; autologous bone marrow derived stem cells, muscle regeneration

Introduction

Extremity injuries are common in both the military and civilian populations. These injuries are often complicated by CS, where secondary edema and swelling increases compartment pressure that stops blood flow, resulting in ischemia and infarction of muscle and nerve tissue. The current standard of care is to perform a fasciotomy to relieve compartment pressure. Emergent fasciotomy can abrogate the ischemic process but creates additional injury. In many cases, the extracellular matrix remains intact, but cell death has occurred to an extent that endogenous regenerative capacity is exceeded, leaving a dysfunctional, atrophic limb. A potential therapy for CS could be treatment with autologous BM-MNCs to regenerate muscle, nerve, and blood vessel cells.

The goal of this project is to improve the endogenous cellular regenerative response by local treatment with autologous bone marrow stem and progenitor cells. The Gregory team hypothesizes that treatment of CS with autologous BM-MNCs, harvested one week after CS injury, will enhance tissue regeneration and clinical functional recovery.

Research Progress – Year 5**Feasibility study**

The Gregory team evaluated adult Sinclair mini-swine as a more relevant large animal CS model than the juvenile swine used in earlier studies to more accurately model injuries in adult troops. Juvenile domestic swine have more robust healing profiles than adult animals, and their growth curve is not relevant or practical for long-term studies.



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The researchers evaluated the induction of CS injury using intra-compartment infusions of autologous normal saline or autologous plasma. While saline infusions were less costly, the CS injuries with saline did not result in long-term dysfunction in the tibialis anterior (TA) muscle and were abandoned. Rehabilitation protocols for the injured swine, consistent with those performed after human extremity injury, were perfected so that post injury treatment would be clinically relevant. Stem cell injection techniques were optimized to reduce loss during injection and maximize long-term stem cell retention and engraftment. At 11 weeks post injection, engrafted CM-DiI cells were detectable throughout the injured muscle of the treatment animals. The Sinclair mini-swine proved to be a superior adult large-animal model for pivotal randomized studies.

Preclinical cell dose study

The Gregory team completed a cell dose study under grant DMAD-W81XWH-09-1-0688 to determine the therapeutic cell concentration to be used for this project's preclinical multi-dose study. A prospective, randomized, blinded, sham-controlled study comparing treatment of CS with autologous BM-MNCs versus control (cell media only) was performed in their chronic (3-month) Sinclair mini-swine model. The researchers created severe extremity injury with CS by infusing autologous plasma into the left hind leg TA muscle compartment to elevate the compartment pressure (>120 mm Hg) for six hours, after which they performed a fasciotomy. One week post CS injury, they harvested bone marrow and performed BM-MNC isolation using a Sepax (Biosafe Inc.) device. They labeled the cells with CM-DiI, and then injected the cells throughout the injured muscle. They injected total cell dosages of 0 (cell-media only), 50 million or 100 million cells (10 swine per group, total n=30). Muscle and nerve function data were recorded at five time points: pre-injury, post-injury (prior to any cell therapy), and at Weeks 1, 6, and 12 post-injury. Gait analysis data was acquired at 11 time points: pre-injury, at days 1-3, Weeks 1-6, and at the time of sacrifice (Week 12). The high cell dose concentration (100 million cells) was selected as the superior dose to be used in the AFIRM program multi-dose study.

Preclinical multi-dose study

A prospective, randomized, blinded, sham-controlled chronic (3-month) study using autologous BM-MNCs to treat CS was performed in adult Sinclair mini-swine. CS injury was induced by autologous plasma infusion to maintain TA muscle compartment pressure >120 millimeter of mercury for 6 hours, after which a fasciotomy was performed. On treatment days, BM-MNCs were isolated and injected into the injured muscle. Swine were treated either once (Week 1, n=10), twice (Weeks 1 and 2, n=8) or three times (Weeks 1, 2 and 4, n=8) to determine an optimal treatment regimen.

At Week 12, absolute muscle torque was significantly greater ($p=0.027$) in two-dose cell-treatment animals (4.3 ± 1.1 Nm) compared to control animals (3.0 ± 1.1 Nm). Also, absolute muscle torque when normalized to muscle weight was significantly greater for both one-dose (110 ± 29 Nm/kg, $p=0.036$) and two-dose (107 ± 16 Nm/kg, $p=0.032$) cell-treatment animals compared to control animals (78 ± 30 Nm/kg) (Figure 1). Nerve conduction and muscle function studies followed the same healing trend: control and treatment animals

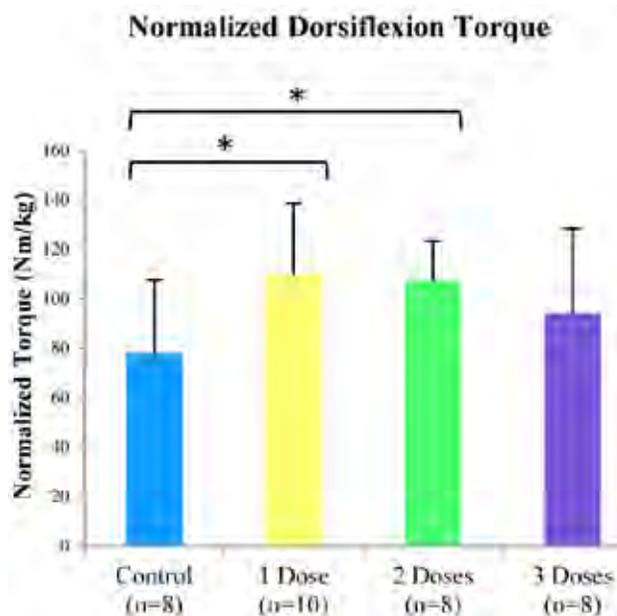


Figure 1. Absolute muscle torque normalized to muscle weight. Significant difference shown between one-dose (110 ± 29 Nm/kg, $*p=0.036$) and two-dose (107 ± 16 Nm/kg, $*p=0.032$) cell-treatment animals compared to control animals (78 ± 30 Nm/kg).

exhibited similar ongoing healing through Week 6, but then only the treatment animals continued to heal further through to the Week 12 final assessment. At Week 12, the nerve conduction velocity was significantly greater ($p=0.045$) in the two-dose cell-treatment animals (56.9 ± 4.4 m/s) compared to control animals (47.5 ± 5.5 m/s). Finally, gait analysis of hind-limb force symmetry showed significant improvement in walking ($p=0.01$) for two-dose cell treatment animals (1.07 ± 0.09) compared to control animals (0.91 ± 0.12).

Evidence of successful engraftment of injected BM-MNCs, demonstrated by the presence of CM-DiI-labeled cells in muscle tissue sections, was found at 11 weeks post-BM-MNC injection (see 2012 AFIRM Annual Report – Technical Progress Reports, page VI-9, Figure 5). To assess further the fate of the CM-DiI labeled cells that were injected into the injured muscle, a 1-cm³ portion was taken from the injured muscle and enzymatically digested. The enzymatically digested cells were then sorted into CM-DiI-positive and -negative cells. In single-antibody staining experiments, CM-DiI-labeled cells expressed the neural markers S-100, Glial fibrillary acidic protein, O4, and beta-III tubulin. They also expressed the blood-associated von Willebrand factor and muscle-associated α -smooth muscle actin.

Safety and efficacy study

With leveraged funding from grant DMAD-W81XWH-09-1-0688, the researchers performed a prospective, randomized, blinded, sham-controlled study comparing treatment of CS with autologous BM-MNCs versus control in a chronic (6-month) swine model ($n=20$) to determine long-term safety and efficacy. Severe extremity injury with CS was induced as previously described. One week post CS injury, the researchers harvested bone marrow, isolated the BM-MNCs, and labeled the cells with CM-DiI. The injured TA muscle of each swine was then treated with either a single dose of autologous BM-MNCs (100×10^6 BM-MNCs) or an equal volume of Hanks Balanced Salt Solution (control animals). Muscle and nerve function data were recorded at six time points: pre-injury, post-injury (prior to any cell therapy), and at Weeks 1, 6, 12 and

24 post-injury. Gait analysis data were acquired at nine time points: pre-injury, day 1, Weeks 1-6, 12 and at the time of sacrifice (Week 24). The animals were euthanized at Week 24, and the TA muscle and underlying nerve of the injured limb were harvested and sent for histological and immunohistochemistry analyses.

To date, 20 swine have been enrolled in the 6-month safety and efficacy study, although 4 swine have been excluded and will need to be replaced. One swine was excluded due to the inability to maintain compartment pressure above 120 mm Hg for 6 hours on the day of injury. The second swine was found dead due to asphyxiation from food lodged in its proximal larynx. The other two swine were excluded due to Sepax software failure (now corrected) that occurred in transitioning from Sepax I to Sepax II machines. To date, no adverse events or complications were associated with any cell or sham treatments.

Conclusions

Treatment of severe TA CS muscle injury with autologous BM-MNCs (a source of autologous stem and progenitor cells) in a large-animal model resulted in significantly improved muscle and nerve function in BM-MNC-treated animals compared to control animals. While control animals stopped improving clinically 6 weeks after injury, BM-MNC-treated animals continued to improve clinically through the 12-week study endpoint. Significant gait improvement was observed at 3 months in the cell-treated animals compared to control animals. No adverse events or complications were associated with any cell treatments. This research demonstrates the potential of a safe, new treatment for severe extremity injury that offers injured troops an improved functional recovery.

Future Research Plans

The Gregory team will finish their 6-month adult swine safety and efficacy study in the near future.

Planned Clinical Transitions

The Gregory team will begin a Phase I human clinical trial, working closely with the commercial partner Biosafe Group SA in AFIRM II.



Biological Scaffold-Based Treatment of Compartment Syndrome

Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy

Project 4.3.3, WFPC

Team Leader(s): William R. Wagner, PhD (University of Pittsburgh)

Project Team(s): Keisuke Takanari, MD, PhD, Ryotaro Hashizume, MD, PhD, Yi Hong, PhD, Nicholas J. Amoroso, PhD, Tomo Yoshizumi, MD, Hongbin Jian, MD, Antonio D'Amore, PhD, Kazuhiro Nonaka, PhD, and Christopher L. Dearth, PhD (University of Pittsburgh)

Collaborator(s): Stephen F. Badylak, DVM, MD, PhD and Johnny Huard, PhD (University of Pittsburgh)

Therapy: Treatment of abdominal Compartment Syndrome; development of fascial repair technology

Deliverable(s): A composite material that has shown effectiveness in the repair of abdominal wall defects in animal models

TRL Progress: Beginning, TRL 1; Current, TRL 3; AFIRM I Target, TRL 3

Key Accomplishments: A rodent model evaluating the success of muscle-derived stem cell (MDSC) microintegrated poly(ester urethane)urea (PEUU) was completed. Following the promising small animal model results, the PEUU/phosphate-buffered saline (PBS) sandwich and biohybrid scaffolds were evaluated in a more clinically relevant porcine model. Constructs showed significant ingrown tissue and were mechanically robust. Cellularized dermal extracellular matrix (dECM) gel hybrid scaffolds were fabricated and characterized in vitro. MDSCs were observed to survive the process, and preparations are underway for initial in vivo testing.

Key Words: Scaffold, abdominal wall, Compartment Syndrome, elastic, extracellular matrix

Introduction

The aim of this project is to generate an elastic scaffold based on degradable PEUU fibers, blended with extracellular matrix (ECM) digest, and ultimately with MDSCs to improve outcomes in abdominal wall reconstruction and other sites of fascial repair. The most common techniques for the reconstruction of abdominal wall defects following fasciotomy utilize synthetic mesh devices that suffer from limited biocompatibility and elasticity, and that are associated with patient discomfort, device-centered infection and recurrent herniation.

The product under development, which combines electrospun PEUU with ECM, derives elasticity from PEUU and improved biocompatibility from the ECM components. Being completely degradable, this approach ultimately results in the creation of native tissue to repair the fascia. Furthermore, the Wagner group is seeking to integrate

MDSCs into the product to further facilitate the regenerative process.

Research Progress – Year 5

Reconstructed rat abdominal wall using MDSC microintegrated electrospun elastomeric scaffolds

The researchers are investigating concurrent electrospinning and electrospraying to microintegrate green fluorescent protein (GFP) transgenic MDSCs into the scaffolds. They performed allogenic transplantation with the MDSC-integrated material. They created control scaffolds utilizing concurrent electrospray with sterile culture medium (wet PEUU) as well as simple electrospinning (dry PEUU) and commercially available expanded polytetrafluoroethylene (ePTFE). After four and eight weeks, GFP-positive cells were observed in the scaffold (5.9% and 5.1% of cells, respectively). Representative cross-sections showed high cell

infiltration by eight weeks in wet PEUU, while dry PEUU and ePTFE scaffolds had minimal infiltration. The cell integrated scaffold had higher vascularity at both time points compared to the control wet PEUU scaffold. No vascularity was observed within either dry PEUU or ePTFE. Under equal biaxial mechanical loading, the elastomeric electrospun constructs were observed to be compliant and mechanically anisotropic. The wet and MDSC microintegrated scaffolds were found to behave most similarly to native tissue.

A blended solution of PEUU and dECM as an acellular device for abdominal wall repair

An acellular device for use in a critical care setting such as decompressive abdominal fasciotomy would be attractive from a regulatory and practical standpoint for “off-the-shelf” use. For these reasons, the Wagner team, in collaboration with the Badylak team, produced an electrospun construct from a blended solution of PEUU and dECM using a single stream processing method. The resultant scaffold possessed high elasticity and flexibility with good surgical handling and mechanical characteristics that could be manipulated by altering the PEUU/dECM ratio. In vivo implantation revealed greater thickness and smooth muscle α -actin expression than a PEUU control. Although both construct types had limited cell infiltration and were, in general, stiffer and isotropic compared with native tissue, the single-stream electrospinning approach was found to be a simple processing option for creating mechanically robust material at substantial ECM mass fractions.

Development of PEUU/dECM biohybrid scaffolds with improved mechanics

The two-stream electrospinning/electrospray approach to produce a biohybrid device comprised of polyurethane, and a bioactive gel was employed again here, but with an important design modification to improve the mechanical properties. The outer layers of the scaffold were generated by electrospaying saline concurrently with PEUU electrospinning at the beginning and end of the processing period. For the PEUU fiber/dECM gel hybrid layer, porcine dECM gel solution was electrospayed instead of saline, while PEUU was electrospun. This created a “sandwich” scaffold

architecture. Control scaffolds were created using the same fabrication technique, except lacking the outer supportive layers.

Assessments were performed using a rat full-thickness abdominal wall defect model. A lateral wall defect (1 x 2.5 cm) was repaired by either the sandwich or control scaffolds. Histological assessments showed both scaffold types to have good cellular infiltration at both time points; however, the control group thinned markedly by eight weeks. At the 8-week explant time point, the sandwich group was found to have maintained its thickness and was more compliant with a similar mechanical anisotropy to that observed in native tissue.

PEUU and dECM biohybrid scaffolds evaluated in a porcine model

The most successful materials evaluated in the rat full thickness abdominal wall defect were chosen for a large animal evaluation. Biohybrid and sandwich scaffolds were fabricated as previously described. Control PEUU/PBS scaffolds were generated by concurrently electrospinning and electrospaying PEUU and saline.

Devices were evaluated in a partial thickness abdominal wall defect in Yorkshire pigs over eight weeks. Histological assessment showed that all scaffold types had pronounced cellular infiltration and ECM elaboration. In scaffolds where PEUU was concurrently electrospayed with saline (PEUU/PBS and sandwich scaffolds), residual PEUU was easily identifiable as unstained ribbons within the explant. These regions were not observed in PEUU/dECM hybrid scaffolds. Under equi-biaxial tension, constructs were mechanically robust and similar in mechanical response to native abdominal wall tissue.

Development of an MDSC integrated dECM/PEUU hybrid patch

The Wagner team made progress in the development and in vitro optimization of an MDSC integrated dECM gel hybrid patch. Cells were observed to survive the electrospaying process and were found throughout the thickness of each construct following fabrication. These constructs will be evaluated in vivo in a rat abdominal wall defect model.



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Conclusions

New material processing techniques were developed to produce mechanically robust biocompatible and bioactive constructs for treatment of abdominal Compartment Syndrome. Concurrent electrospraying a saline solution with electrospinning PEUU was found to disrupt the compact electrospun microstructure and permit cell infiltration *in vivo*. This methodology was improved by incorporating a dECM gel solution into the electrospray to create a PEUU/dECM biohybrid construct. Evaluation in a rat abdominal wall defect demonstrated that such constructs were capable of producing pronounced cellularity and *de novo* ECM elaboration. Reinforcing surface layers were added to this material in order to improve mechanical integrity.

Constructs developed in this manner were found to be superior to currently clinically available materials in terms of cellularity, ECM content, and tensile mechanical properties.

Future Research Plans

Histological evaluation and quantification of the porcine study will be completed, and the data will be compiled in a manuscript and submitted for publication. Further development and animal testing of the MDSC integrated PEUU/dECM hybrid construct will be completed.

Planned Clinical Transitions

Currently, there are no clinical transitions planned.



Biological Scaffold-Based Treatment of Compartment Syndrome**Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome****Project 4.3.4, WFPC**

Team Leader(s): Stephen F. Badylak, DVM, PhD, MD (McGowan Institute for Regenerative Medicine [MIRM])

Project Team(s): Christopher L. Dearth, PhD and Scott Johnson, MS (MIRM); Matthew T. Wolf, BS (Department of Bioengineering, University of Pittsburgh)

Collaborator(s): Johnny Huard, PhD (MIRM); Kenton Gregory, MD (Oregon Medical Laser Center, St. Vincent Medical Center)

Therapy: Treatment for peripheral compartment syndrome (PCS)

Deliverable(s): A methodology describing the use of autologous extracellular matrix (ECM) for the treatment of PCS.

TRL Progress: Beginning, TRL 1; Current, TRL 5; AFIRM I Target, TRL 5

Key Accomplishments: The researchers completed the evaluation of in situ decellularization as a treatment of PCS in the rabbit. They found that autologous compartment ECM elicits improved remodeling outcomes following PCS compared to the current standard of care. The researchers also developed a murine model of volumetric muscle loss (VML) to investigate the mechanisms of ECM remodeling in massive skeletal muscle injury. In addition, they applied their optimized in situ decellularization method to a porcine model of PCS; these studies will be completed in the upcoming year.

Key Words: Peripheral compartment syndrome (CS), extracellular matrix, bone marrow derived mononuclear cells, volumetric muscle loss, decellularization

Introduction

PCS represents a serious complication of traumatic extremity injury. Swelling within a confined space (i.e. within a muscle compartment) is associated with increased intracompartmental pressure (ICP). The increase in pressure severely reduces blood flow, resulting in necrosis of all tissues within the compartment (e.g., muscle, nerves). The standard of care for PCS is opening the compartment (fasciotomy) to relieve the pressure, followed by removal of necrotic tissue. The Badylak team has investigated a method for utilizing the biologic properties of ECM as a scaffold for constructive remodeling into functional tissue. The present work extends this concept by investigating methods of deriving autologous compartment ECM from in situ decellularization of the necrotic tissue (with autologous bone marrow mononuclear cells [BM-MNCs]) following development of PCS and resultant tissue loss.

Research Progress – Year 5**PCS model development in the rabbit anterior tibial compartment**

The researchers maintained increased ICP for 90 minutes by continuous saline infusion and muscle crush to replicate the traumatic injuries that accompany PCS. They maintained ICP above 150 mm Hg (**Figure 1A-B**) and observed necrosis on histologic evaluation (**Figure 1C**), concomitant with the rhabdomyolysis markers of hypocalcemia (**Figure 2A**) and increased creatine phosphokinase (**Figure 2B**).

Ex vivo compartment decellularization and characterization

Autologous compartment ECM was created by flushing compartment tissues with saline, 0.1% peracetic acid (PAA), and 2% sodium deoxycholate (DOC) (**Figure 3A**). These treatments, and in particular the combination of all three, resulted



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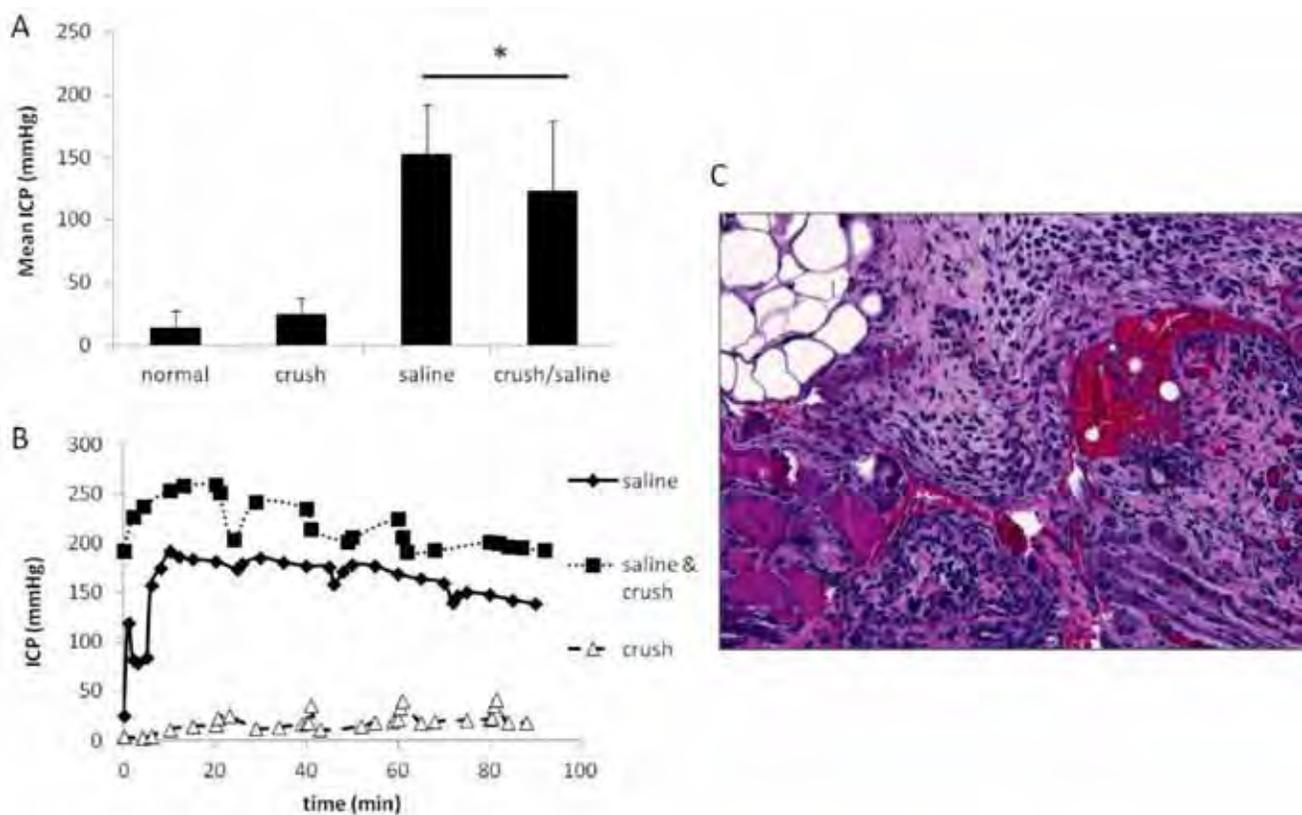


Figure 1. (A) Mean ICPs in normal, crush only, saline only, and saline with crush techniques (* $p < 0.01$). (B) Representative ICPs during CS induction. (C) Hematoxylin and Eosin demonstrating myonecrosis, edema, hemorrhage, and inflammatory cell infiltrate in the saline with crush group 7 days after the induction procedure (magnification rate is 200x).

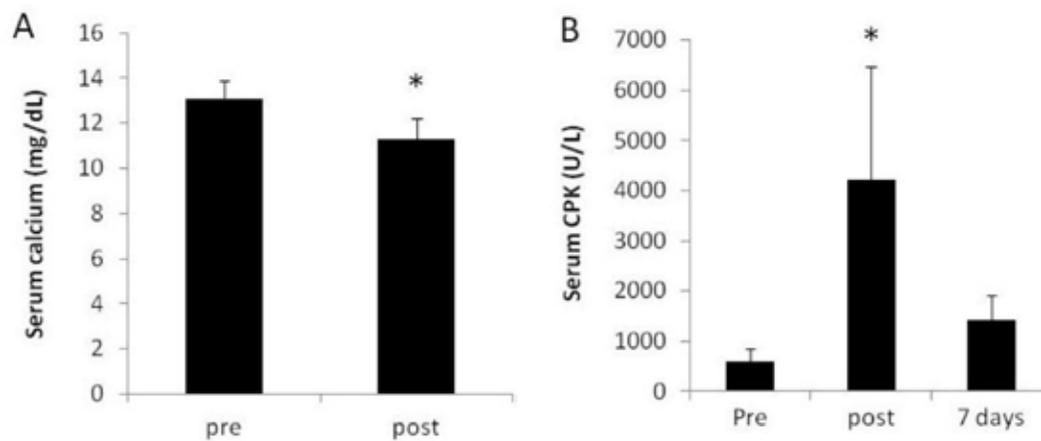


Figure 2. (A) Serum calcium levels pre- and post-induction procedure (* $p < 0.05$). (B) Serum creatinine phosphokinase levels pre, post and 7 days after the induction surgery (* $p < 0.05$).

in disruption of skeletal muscle architecture and a decrease in cell nuclei (Figure 3B) with a reduction in total deoxyribonucleic acid (DNA) content (Figure 3C). The compartment ECM created by these methods also had decreased cellular proteins such as beta-actin, myosin and heat shock protein 60 (HSP60) (Figure 4A). However, there was retention of growth factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and basic Fibroblast Growth Factor (bFGF) (Figure 4 A-C).

Compartment tissue decellularization methods with BM-MNC injection in a rabbit model of PCS

In situ compartment decellularization was performed after fasciotomy and debridement with the same groups as described above, in addition to the injection of autologous BM-MNCs. At 1 and 3 months after treatment, inflammatory cells and new muscle cells with characteristic centrally located nuclei were found in the defect area only in the group treated with saline/PAA/DOC. After 6 months, numerous large islands of skeletal muscle were present in the saline/PAA/DOC group (Figure 5). BM-MNCs labeled with Qdots were visible within the remodeling tissue after 1 and 3 months.

Translation of compartment tissue decellularization treatment with BM-MNC injection in a porcine model of PCS

The optimized in situ decellularization method using saline, PAA, and DOC in conjunction with BM-MNCs was applied to a porcine model of PCS. ICPs over 200 millimeter of mercury were successfully maintained in the pig, which was followed by PCS treatment and in situ decellularization. These studies are still in progress and will be completed by the end of the project period. The results of the pig model will provide the foundation for a Phase I clinical trial.

A murine model of VML to determine the mechanisms of ECM remodeling

A VML injury was induced in the mouse quadriceps muscle by a full-thickness resection of the tensor fascia lata and partial-thickness resection of the rectus femoris, which consistently forms scar tissue after 56 days (Figure 6). Furthermore, this injury model is remodeled after small intestinal submucosa ECM (SIS-ECM) implantation with the presence of a dense mononuclear cell infiltrate and striated muscle cells expressing desmin. The accumulation of muscle resident perivascular stem cells (PVSCs) within the ECM is associated with downstream

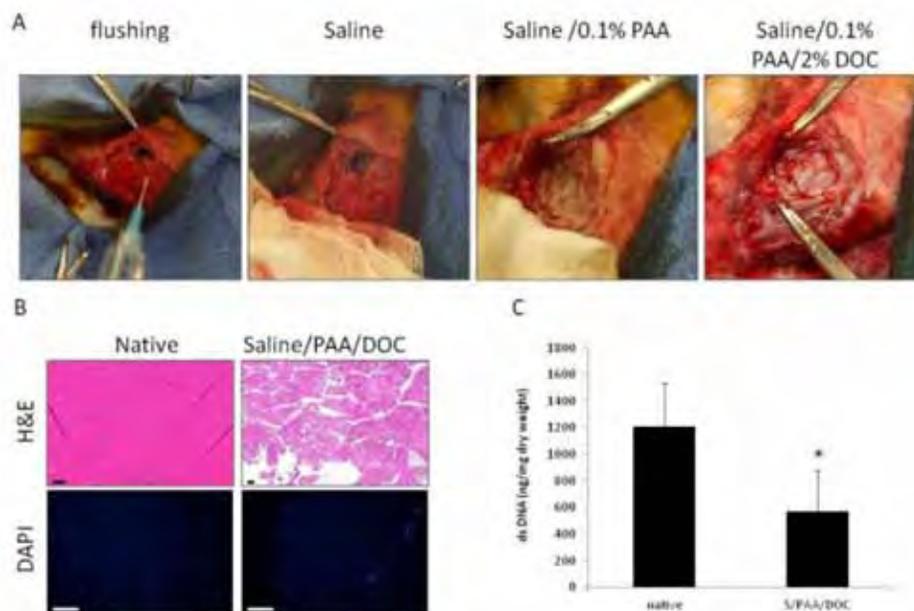


Figure 3. (A) Creation of autologous compartment ECM by flushing of the compartment with saline, 0.1% PAA and 2% DOC. (B) Hematoxylin and Eosin and DAPI images following treatment show disruption of the tissue architecture and decrease in cell nuclei. (C) DNA content is decreased after saline/PAA/DOC.



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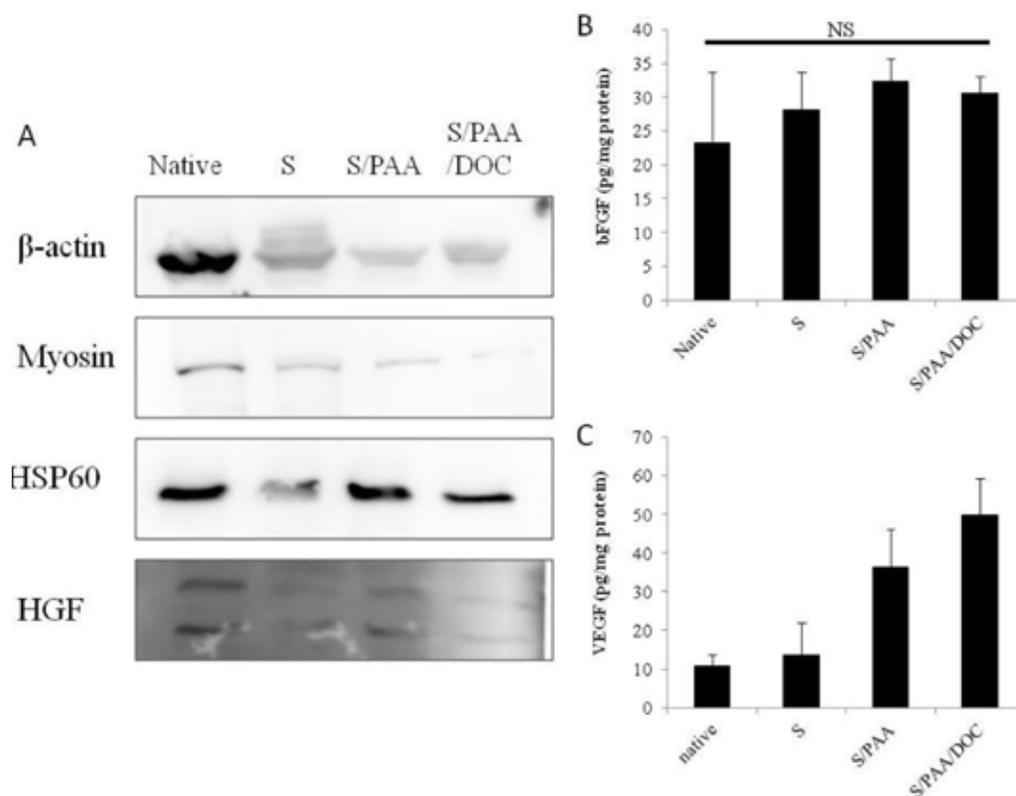


Figure 4. (A) Western Blot analysis of each treatment indicates a decrease in cellular proteins β -actin, myosin and HSP60, but retention of the growth factor HGF. (B) Decellularized compartment tissue retains the growth factor VEGF. (C) Decellularized compartment tissue retains the growth factor bFGF.

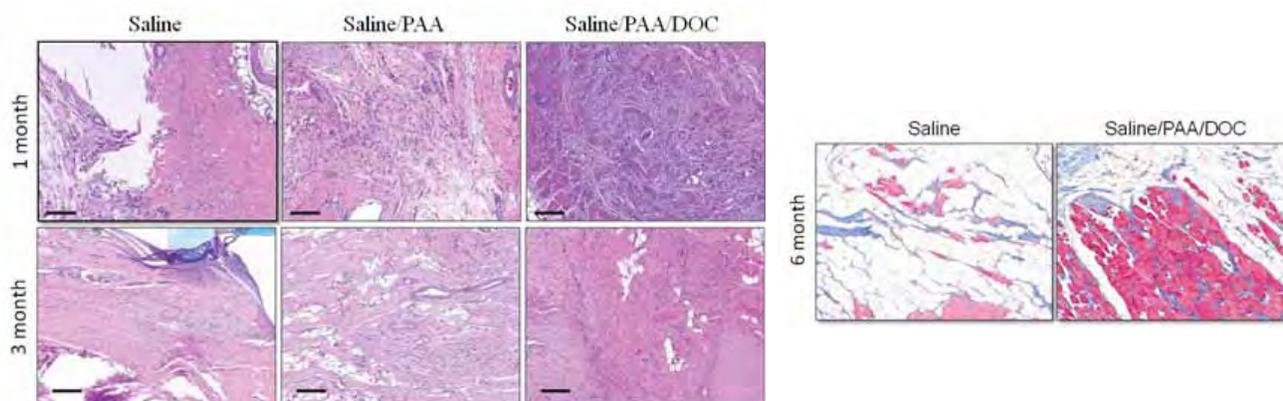


Figure 5. In situ compartment decellularization with saline, PAA, and DOC with BM-MNC injection promoted myogenesis in as early as 1 month, and this continued to 3 months (Hematoxylin and Eosin). Saline and saline/PAA treated tissue showed greater relative amounts of fibrous tissue within the defect. After 6 months, large islands of skeletal muscle were present in saline/PAA/DOC-treated compartment tissue (Masson's Trichrome).

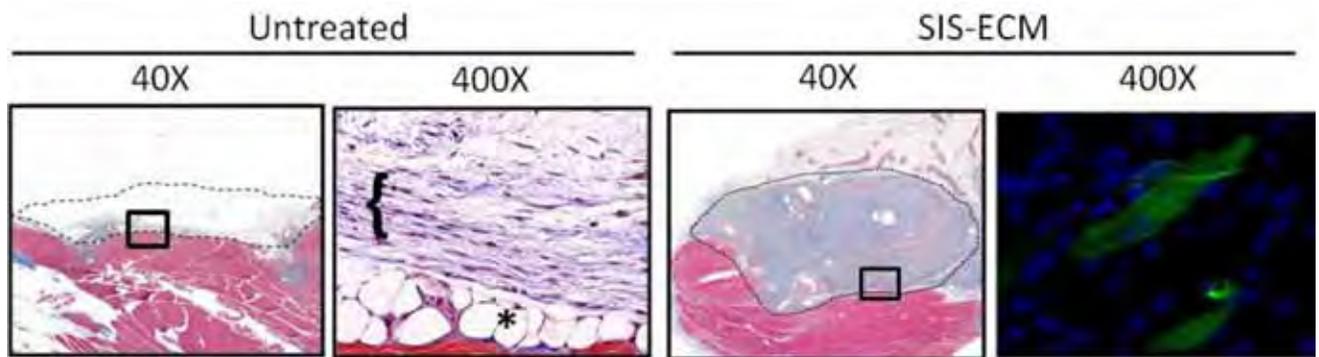


Figure 6. At 56 days post injury, a volumetric defect is visible (dotted line). Higher magnification shows the defect partially remodeled with collagenous connective tissue (†) and adipose tissue (*), and no new muscle formation. Defect sites treated with SIS-ECM maintain robust cellularity after 56 days. Immunolabeling shows striated desmin-positive cells (green) populating the area of ECM implantation.

constructive tissue remodeling with formation of site-appropriate islands of skeletal muscle tissue (Figure 7).

Conclusions

The researchers have shown that tissue loss as the result of PCS can be effectively treated using in situ decellularized autologous compartment ECM in conjunction with an autologous BM-MNC injection. Investigating ECM remodeling in a murine model of VML revealed that PVSCs participate in remodeling and may promote the increased myogenesis and reduced scarring compared to a fasciotomy and debridement PCS treatment alone.

Future Research Plans

The researchers plan to evaluate autologous ECM as a treatment for PCS in a porcine model. They will determine new muscle formation, innervation, and vascularization using histologic methods to provide the basis for clinical implementation. The murine model of VML will be used to elucidate the mechanisms of ECM remodeling following skeletal muscle injury. Stem cell and inflammatory cell (macrophage) participation will be correlated to both histologic and functional outcomes such as electromyography.

Planned Clinical Transitions

The use of autologously derived ECM as a treatment of PCS will be regulated as a treatment procedure under Investigational Device Exemption regulatory

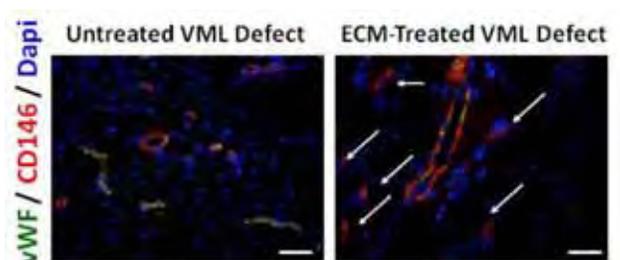


Figure 7. 14 days post-injury, untreated defect sites showed CD146-positive PVSCs predominantly in a vascular location associated with von Willebrand factor (vWF)-positive endothelium. ECM-treated defects showed PVSCs outside their normal anatomic location in the midst of the degrading ECM, suggesting participation in remodeling.

pathways; chemicals utilized in this method (e.g., DOC) have already demonstrated safety in other clinical applications. The competitive advantage of the described treatment would represent the first effective method of forming new, vascularized, innervated and functional skeletal muscle tissue after PCS. Clinical application would occur for both military and civilian populations that have PCS following extremity trauma. These preclinical studies provide evidence of safety and efficacy testing. However, considering the invasiveness and departure from standard of care, a larger and more robust preclinical study in a large animal model will likely be necessary to get approval for a Phase I study in humans.



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Biological Scaffold-Based Treatment of Compartment Syndrome

Material-Induced Host Cell Recruitment for Muscle Regeneration

Project 4.3.5, WFPC

Team Leader(s): Sang Jin Lee, PhD (Wake Forest University)

Project Team(s): James J. Yoo, MD, PhD, In Kap Ko, PhD, Young Min Ju, PhD, NaJung Kim, PhD, Cara Clouse, DVM, Sang Mi Park, BS (Wake Forest University)

Collaborator(s): Shay Soker, PhD (Wake Forest University)

Therapy: Regeneration of injured muscle tissue using host stem cells

Deliverable(s): Development of a target-specific scaffolding system that facilitates muscle tissue regeneration

TRL Progress: Beginning, TRL 2; Current, TRL 3; AFIRM I Target, TRL 4

Key Accomplishments: The researchers developed several novel injectable and implantable scaffolding systems that effectively release myogenic-inducing factors for the in vivo demonstration of host muscle satellite/progenitor cell differentiation as well as new muscle tissue formation. One scaffold system uses myogenic factor-immobilized gelatin-based microparticles, while another uses myogenic factor-immobilized decellularized muscle matrix.

The researchers demonstrated host stem cell mobilization and new muscle tissue regeneration in vivo using these target-specific scaffolding systems.

Key Words: Biomaterials, myogenic-inducing factor, host stem cell mobilization, Compartment Syndrome (CS), in situ muscle regeneration, small RNA

Introduction

CS is a common traumatic injury that results in muscle, nerve and vessel damage due to increased pressure within a confined space in the body. Although CS can affect any limb or muscle compartment, including the abdomen, it frequently occurs after trauma to the lower leg such as fracture. The standard treatment is fasciotomy, which is considered as the definitive and only treatment for acute CS. Although this procedure is able to relieve immediate concerns, muscle weakness and atrophy are continued sequelae. Various management approaches have been introduced which include physical therapy, muscle transplantation and myoblast cell therapy. However, none has entirely addressed the problems associated with the long-term consequences of CS in wounded soldiers.

In this project, the researchers aim to utilize stem or progenitor cells residing in the host to regenerate muscle tissue through the use of a target-specific scaffolding system. This approach is based on the demonstration that almost every tissue in the body contains some type of stem or progenitor cell. The putative healing mechanisms and classic foreign body reaction to implanted biomaterials have also been characterized. However, these two

mechanisms would seem to be in conflict with one another, particularly with respect to functional outcome. While small, localized day-to-day injuries can be repaired by the body's stem and progenitor cell machinery, large traumatic injury overwhelms this system and survival mechanisms take over. This process often creates a deficit of functional recovery. The specific aims of this project are to investigate this possibility using an animal model to initiate cell mobilization, recruitment, and differentiation in vivo, and to demonstrate in situ muscle tissue regeneration using a target-specific scaffolding system.

Research Progress – Year 5

Development of novel biomaterial systems for in situ muscle tissue regeneration

Development of injectable device using myogenic factor-released gelatin microparticles

The researchers developed several scaffold delivery systems that can recruit host muscle stem/progenitor cells and induce myogenic differentiation within the injured muscle region. To effectively deliver specific myogenic factors released from the system, they developed gelatin-based microparticles that were used to immobilize insulin-like growth factor-I (IGF-I) via electrostatic interactions with heparin

(Figure 1A). This system provides sustained release of IGF-I from the gelatin microparticles (Figure 1B).

The researchers then performed an in vivo study in which a large muscle defect was created by excising approximately 30-40% of the tibialis anterior (TA) muscle of Sprague-Dawley rats. They injected IGF-I-immobilized gelatin microparticles into the muscle defect region (Figure 1C). Histological and immunohistochemical evaluations of the retrieved tissue showed that IGF-I released from the gelatin microparticles effectively promoted myogenic cell migration and regenerated newly formed muscle tissue in situ (Figure 1D-E). By 1 and 4 weeks post-injection, host muscle progenitor cells expressing Pax 7 had accumulated within the muscle defect region, and the presence of abundant host vasculature was evident. Hematoxylin and Eosin and immunohistochemical staining for myosin heavy chain indicated a gradual buildup of newly formed muscle fiber bundles around the IGF-I-immobilized gelatin microparticles 4 weeks after injection.

Development of implantable device using myogenic factor-released decellularized matrices

The researchers also developed an implantable scaffolding system fabricated with decellularized muscle tissue (Figure 1A*). They successfully functionalized these decellularized scaffolds to deliver bioactive molecules by conjugating stromal cell-derived factor (SDF)-1 α and IGF-I, with heparin molecules, to the decellularized tissue scaffolds. SDF-1 α and IGF-I were electrostatically immobilized to the decellularized muscle scaffolds via electrostatic interactions with the heparin molecules. This functionalized decellularized muscle scaffold was able to release SDF-1 α and IGF-I locally in a controlled manner (Figure 1B*). The heparin-conjugated decellularized muscle scaffolds containing SDF-1 α and IGF-I were implanted into surgically created TA muscle defects in rats (Figure 1C*). The animals received systemic administrations of substance P (SP, a neurotropic factor that can enrich stem cell populations in blood circulation) at 5 days after implantation, and they were followed for 4 weeks. The researchers found that this multiple-factor delivery system

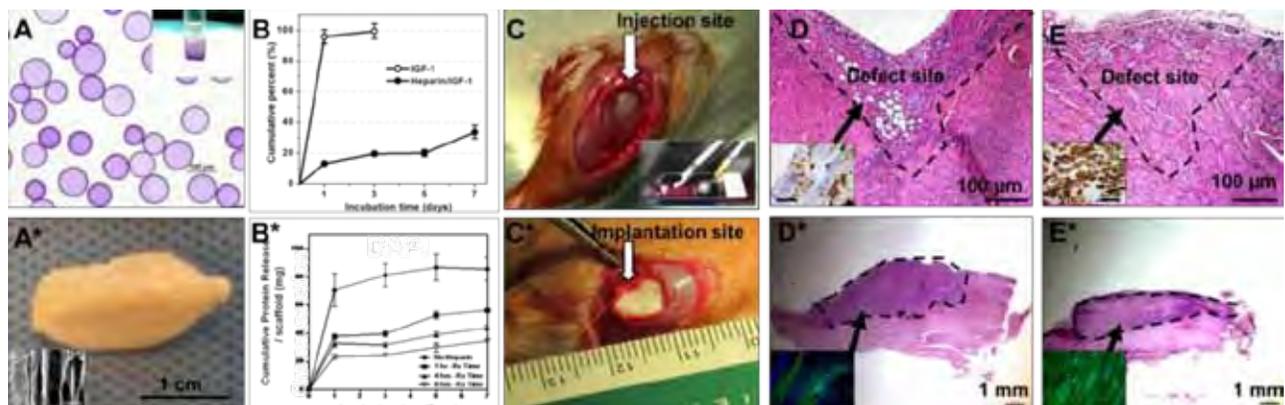


Figure 1. Rat muscle excision models: (A) Heparin-conjugated gelatin microparticles as confirmed by Toluidine Blue staining. **(B)** Release profiles of IGF-I from heparin-conjugated gelatin microparticles. **(C)** IGF-I-immobilized gelatin microparticles were injected into TA muscle defects in rats. **(D,E)** Hematoxylin and Eosin and immunohistochemical staining for anti-myosin heavy chain of the injected gelatin microparticles at 4 weeks post-surgery ($\times 100$). **(E)** IGF-I delivery through heparin-conjugated gelatin microparticles effectively promoted muscle regeneration when compared with **(D)** the controls (scaffolds that did not deliver any bioactive factors).

(A*) Heparin-conjugated decellularized muscle scaffold. **(B*)** Release profiles of IGF-I from heparin-conjugated decellularized muscle scaffolds. **(C*)** SDF-1 α /IGF-I-immobilized tissue scaffolds were implanted into the TA muscle defects in rats. **(D*,E*)** Hematoxylin and Eosin and immunohistochemical staining for anti-myosin heavy chain in the implanted decellularized tissue scaffolds at 4 weeks post-surgery ($\times 100$). **(E*)** Delivery of multiple factors (SDF-1 α /IGF-I + SP injection) effectively promoted muscle regeneration when compared with **(D*)** the scaffolds that did not deliver bioactive factors.



VI: Compartment Syndrome

enhanced the recruitment of muscle satellite cells and circulating mesenchymal stem cells into the implanted scaffold, and facilitated the formation of well-aligned myofibers (Figure 1E*), compared to controls (Figure 1D*). These findings suggest that incorporation of multiple regulatory signals into a scaffolding system may be a promising approach to achieving more efficient and effective muscle regeneration in situ.

Conclusions

This study suggests that it may be possible to use the body's biologic and environmental resources for in situ muscle tissue regeneration. The researchers demonstrate that cells expressing muscle satellite/progenitor cell markers can be mobilized into an implanted biomaterial and that these cells are capable of differentiating into muscle cells. Therefore, it may be possible to enrich the infiltrate with specific cell types and control their fate, provided the proper substrate-mediated signaling can be imparted into the scaffold. Thus, in situ regeneration of functional muscle tissue through host cell recruitment may be possible.

Future Research Plans

The researchers will continue to optimize and validate the injectable/implantable systems that can deploy multiple bioactive molecules in a controlled manner. To evaluate the clinical feasibility of the delivery of multiple bioactive molecules from the systems in vivo, they will use two rabbit muscle injury models, including models of muscle atrophy and volumetric muscle loss.

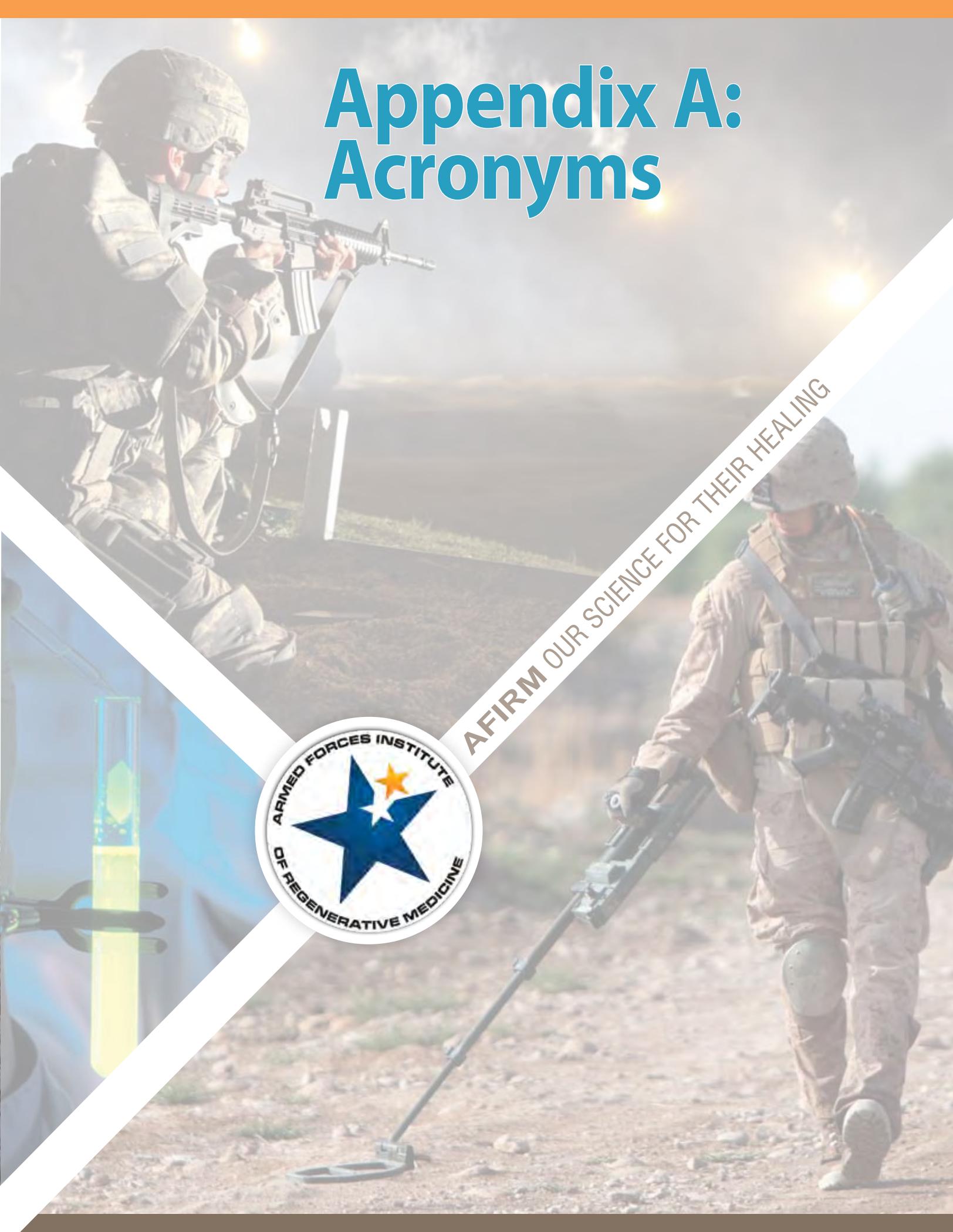
Planned Clinical Transitions

The researchers' technologies require a combination product (device + drug) approach for HCT/Ps which will meet the criteria in 21 Code of Federal Regulations 1271. Experimentation for each of the therapeutic approaches is following Good Laboratory Practices guidelines, so that all data will be fully usable for U.S. Food and Drug Administration (FDA) filings. The researchers will hold consultations with the FDA to discuss the concept for the clinical trial and the data they generate upon which the clinical plans are based.



Appendix A: Acronyms

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Appendix A: Acronyms

3D	three-dimensional	cGMP	current Good Manufacturing Practices
ACM	Augmented Culture Medium	CK15	cytokeratin 15 (epidermal stem cell marker)
ADM.....	acellular dermal matrix	cm.....	centimeter
AFIRM.....	Armed Forces Institute of Regenerative Medicine	CM-Dil	3H-Indolium,5-[[[4-(chloromethyl) }benzoyl]amino]methyl]-2-[3-(1,3-dihydro-3, 3-dimethyl-1-octadecyl-2H-indol-2-ylidene) -1-propenyl]-3,3-dimethyl-1-octadecyl-, chloride
AFS	amniotic fluid-derived stem	CMF	cranio-maxillofacial
AFT-SPAR	Autologous fat transfer for scar prevention and remodeling	CMU.....	Carnegie Mellon University
AGH	Allegheny General Hospital	CaP	calcium phosphate
AHDF.....	adult human dermal fibroblast	CRMRP	Clinical and Rehabilitative Medicine Research Program
AM	Amniotic Membrane	CRO	Contract Research Organization
ASC.....	adipose-derived stem cell	CS	Compartment Syndrome
AWP.....	Autologous Wound Paste	CsA	cyclosporin A
BAM.....	bladder acellular matrix	CSD.....	critical-size defect
BAMC.....	Brooke Army Medical Center	CSS.....	cell stabilization solution
bFGF	basic Fibroblast Growth Factor	CT	Computed Tomography
BGS.....	bone graft substitute	CTA	composite tissue allotransplantation
BI	battlefield injuries	CTLA4Ig.....	Cytotoxic T Lymphocyte-Associated Antigen 4 Immunoglobulin
BIODOME.....	Biomechanical Interface for Optimized Delivery of MEMS Orchestrated Mammalian Epimorphosis	CTP-O	connective tissue progenitor cells
BM	bone marrow	DAPI.....	4'6-diamidino-2-phenylindole
BMA.....	bone marrow aspirate	DBM.....	demineralized bone matrix
BM-MNC.....	bone marrow mononuclear cell	DMEM.....	Dulbecco's Modified Eagle Medium
BM-MSC	bone marrow mesenchymal stromal cell	dECM	dermal extracellular matrix
BMP	bone morphogenetic protein	DNA	deoxyribonucleic acid
BMP-2.....	bone morphogenetic protein-2	DOC	sodium deoxycholate
BMSC.....	bone marrow stromal cells	DoD.....	Department of Defense
BOD	Board of Directors	DPT.....	deep partial thickness
CAD.....	computer-aided design	DS.....	density separation
CAR.....	peptide CARSKNKDC	DWP	dermal wound paste
CBER.....	Center for Biologics Evaluation and Research	DTRD	Dental and Trauma Research Detachment
cBMA	concentrated BMA	E15.....	embryonic day 15
CCTD	chronic caprine tibial defect	EC	endothelial cell
CCT-eta....	chaperonin containing T-complex polypeptide	ECM	extracellular matrix
CEA.....	cultured epithelial autograft	EMB	explantable microvascular bed
CDRH.....	Center for Devices and Radiological Health	eNOS.....	endothelial nitric-oxide synthase
CFMD.....	canine femoral multi-defect	ePTFE	expanded polytetrafluoroethylene
CFR.....	Code of Federal Regulations	ESS	Engineered Skin Substitutes
CFU.....	colony forming unit		

EtO	ethylene oxide	KC	keratinocytes
EVPOME	Ex Vivo Produced Oral Mucosa Equivalent	kg	kilogram
FAK	focal adhesion kinase	KO	knock out
FBS	fetal bovine serum	LV®	low viscosity
FDA	U.S. Food and Drug Administration	M	million
FN	fibronectin	MBR	master batch record
GAG	glycosaminoglycan	M/C	muco-cutaneous
GDNF	glial cell-derived neurotrophic factor	MCA	mineralized cancellous bone allograft
GFP	green fluorescent protein	MCC	multipotent cell cluster
GLP	Good Laboratory Practices	MDSC	muscle-derived stem cell
GMP	Good Manufacturing Practices	MGH	Massachusetts General Hospital
GPC	gel permeation chromatography	MHC	major histocompatibility complex
H&E	hematoxylin and eosin	MIRM	McGowan Institute for Regenerative Medicine
HA	hyaluronic acid	MIT	Massachusetts Institute of Technology
HaCaT	human keratinocyte	ML	mediolateral
HBOT	hyperbaric oxygen therapy	MMP	matrix metalloproteinase
HDF	human dermal fibroblast	MMP9	matrix metalloproteinase 9
hFDF	human fetal dermal fibroblast	MPC	muscle precursor cell
HGF	hepatocyte growth factor	MS	magnetic separation
HLA-DRA	Class II histocompatibility antigen DR alpha chain	MSC	mesenchymal stem cell
hMPC	human muscle precursor cell	MSD	molecular surface design
hMSC	human mesenchymal stem cell	MW	molecular weight
HPMC	hydroxypropyl methylcellulose	NGT	nerve guidance tube
HRPO	Human Research Protection Office	NHP	nonhuman primate
HSP60	heat shock protein 60	NIH	National Institutes of Health
HuS	human serum	NIPAM	N-isopropylacrylamide
I/R	ischemia/reperfusion	NMR	nuclear magnetic resonance
ICP	intracompartmental pressure	OCaP	octacalcium phosphate
IDE	Investigational Device Exemption	OCP	Office of Combination Products
IED	improvised explosive device	OEF	Operation Enduring Freedom
IGF-I	insulin-like growth factor-I	OIF	Operation Iraqi Freedom
IL-6	Interleukin 6	ONR	Office of Naval Research
IND	Investigational New Drug	OSMEA	organic, stretchable microelectrode array
IPT	Integrated Project Team	P2	second phalanx
IRB	Institutional Review Board	PA	Peptide Amphiphile
ISO	International Organization for Standardization	PAA	peracetic acid
IV	Intravenous	PBS	phosphate-buffered saline
JTTR	Joint Theatre Trauma Registry	PCL	polycaprolactone
		PCLF	polycaprolactone fumarate



Appendix A: Acronyms

PCR.....	polymerase chain reaction	STSG	split thickness skin grafts
PCS.....	peripheral compartment syndrome	TA	tibialis anterior
PDGF.....	platelet-derived growth factor	TBSA.....	total body surface area
PDO	polydioxanone	TCP	tricalcium phosphate
PEE-PPy	polymer pentaerythritol ethoxylate/polypyrrole	TCPS.....	tissue culture polystyrene
PEGDA	Polyethylene Glycol Diacrylate	TCR.....	T-cell receptor
PEUU	poly(ester urethane)urea	TEBV.....	tissue-engineered blood vessel
PES	poly(ether sulfone)	TEMR.....	tissue-engineered muscle repair
PGA.....	polyglycolic acid	TENG.....	tissue-engineered nerve graft
PLGA.....	poly(lactic-co-glycolic) acid	TNF- α	anti-tumor necrosis factor- α
PLLA.....	polylactic acid	TRL	technology readiness level
PMA.....	premarket approval	TUNEL.....	terminal deoxynucleotidyl transferase dUTP nick end labeling
PMMA.....	poly(methyl methacrylate)	TyrPC	tyrosine-derived polycarbonate
PMO.....	Project Management Office	UBM.....	urinary bladder matrix
PNI.....	peripheral nerve injury	UCSB	University of California, Santa Barbara
PPF	poly(propylene fumarate)	UF	University of Florida
PTFE	polytetrafluoroethylene	USAISR.....	U.S. Army Institute of Surgical Research
PVA.....	polyvinylalcohol	USAMRMC.....	U.S. Army Medical Research and Materiel Command
PVSC	perivascular stem cell	UTARI	University of Texas at Arlington Research Institute
QUT.....	Queensland University of Technology	UTHSC	University of Texas Health Science Center at Houston
qPCR.....	quantitative polymerase chain reaction	UVA.....	University of Virginia
RCCC	Rutgers–Cleveland Clinic Consortium	VA	Department of Veterans Affairs
rhBMP-2	recombinant human bone morphogenetic protein-2	VCA.....	vascularized composite allograft
RNA	Ribonucleic acid	VEGF.....	vascular endothelial growth factor
SBM.....	Synthetic Bone Mineral	VML	volumetric muscle loss
SC.....	Schwann cell	vWF	von Willebrand Factor
SCM.....	simplified culture medium	WFIRM.....	Wake Forest Institute for Regenerative Medicine
SEM	scanning electron microscopy	WFPC.....	Wake Forest–Pittsburgh Consortium
siRNA.....	small interfering ribonucleic acid	WFSM.....	Wake Forest School of Medicine
SIS	small intestinal submucosa	WRNMMC.....	Walter Reed National Military Medical Center
SIS-ECM	small intestinal submucosa ECM	XRD	X-ray diffraction
SOP.....	Standard Operating Procedure	β -TCMP.....	beta-tricalcium phosphate
SOW.....	Statement of Work	μ M	micron
SPO.....	sodium percarbonate		
SR.....	selective retention		
SSD.....	silver sulfadiazine		

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