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Award Number: W81XWH-08-2-0032

TITLE: AFIRM-Wake Forest/University of Pittsburgh Consortium

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REPORT DATE: July 2012

TYPE OF REPORT: July 2012

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
July 2012	Annual	10 March 2011 – 9 March 2012

4. TITLE AND SUBTITLE

AFIRM-Wake Forest/University of Pittsburgh Consortium

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-2-0032

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wake Forest University Health Sciences
Winston-Salem, NC 27157

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Armed Forces Institute of Regenerative Medicine is focused on delivering advanced regenerative medicine technologies to help the wounded warrior improve their quality of life. We have brought together in leading scientists to develop approaches which in improving therapies to improve treat in five areas including burn treatment, craniofacial repair, scarless wound healing, treatment of compartment syndrome, and limb and digit salvage, During the past year we have continued to develop transformational technologies to aid the wounded Solider, Marines, Airmen and Sailors who have sacrificed so much for their country.

15. SUBJECT TERMS
regenerative medicine, tissue engineering, burn, scarless, wound healing, compartment syndrome, limb, digit, salvage, craniofacial

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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Wake Forest-Pittsburgh Consortium

Annual Report

2011-2012

Mission Statement

To Accelerate Regenerative Treatments for Battlefield Injuries

Letter from the AFIRM-WFPC Director

It is an honor to report on the progress made during Year Three of the Wake Forest/Pittsburgh Consortium of the Armed Forces Institute of Regenerative Medicine. We are proud of our accomplishments to date and of our vision for accelerating regenerative medicine treatments for wounded warriors.

For the past four years, we have continued to steadily advance technologies to clinical trials. For example, we are currently evaluating a new treatment for burn wounds that is an alternative to traditional grafting, an approach to hand transplantation that requires fewer immunosuppressant drugs, and a novel device to minimize scar formation after surgery. These are just a few examples of the clinical potential that is being pursued at the WFPC AFIRM consortium. Beyond the aforementioned clinical trials, we have a number of other clinical trials nearing the enrollment stage.

We remain strongly committed to the strategic plan we adopted in March of 2008 in which we established well defined guidelines to have a number of clinical deliverables over the first five years. And we have a broad pipeline of technologies in place to ensure seamless continuity for AFIRM in the next five years and beyond.

I am proud that the team we have organized is working across disciplines and institutions toward a common purpose: bringing transformational technologies to our wounded warriors. We continue to focus on strategies to leverage the synergies that exist within our teams and to even further integrate our research efforts. We have been continuing improving our processes to accelerate the translation of our technologies. With our committed program leaders, the exceptional abilities of our clinicians and researchers, and the guidance of our knowledgeable advisory panel, I believe we are well situated to continue to execute our vision for regenerative medicine as a pillar in military medical research far into the future.

Respectfully,

Anthony Atala M.D.

Principal Investigator / AFIRM

Program Summary—
Accelerating Regenerative Technologies

The use of improvised explosive devices in Iraq and Afghanistan has caused a significant increase in severe blast trauma. As of June 2012 more than 6,400 U.S. military fatalities and more than 48,600 injuries have been reported.[1] While advances in body armor, quicker evacuation from the battlefield, and advanced medical care have improved survival rates, many of the injured come home to face challenges of overcoming severe limb, head, face, and burn injuries that can take years to treat and usually result in significant lifelong impairment. The Department of Defense established the Armed Forces Institute of Regenerative Medicine (AFIRM) in 2008 with the mission of developing new products and therapies to treat severe injuries suffered by U.S. service members. There were two consortia established within AFIRM to accelerate the delivery of regenerative medicine therapies to these severely injured U.S. service members: the Wake Forest-Pittsburgh University Consortium (WFPC) and the Rutgers-Cleveland Clinic Consortium (RCCC). While these two consortia are distinct and separate entities, they have the same and complementary mission—accelerate regenerative technologies to the wounded warrior. This report provides an update on the technical progress that the WFPC has made over the last year in meeting its objectives.

Five Major Program Areas within AFIRM

Specifically, within AFIRM-WFPC there are five areas of research emphasis. These five areas are focused on developing regenerative therapies to address burn, craniofacial, and compartment syndrome related injuries, as well as limb and digit regeneration and healing without scarring.

During the fourth year of operation, WFPC has continued to advance technologies to clinical trials. Over the past two years, the effect of accelerating technology develop has resulted in some significant changes with the AFIRM-WFPC over the past year. Most notably is the shift from a project emphasis to a product emphasis in our research.

Beyond these clinical trials, a string of clinical trials nearing the enrollment phase are coming to fruition. Utilizing well-established, proven research investigators, the AFIRM has been able to expand the rehabilitative medicine knowledge base, develop models of injury, and test advanced technology products.

Craniofacial Regeneration Program

Craniofacial trauma is among the most debilitating forms of injury facing civilian and military populations due to the important aesthetic and functional role of the craniofacial complex. Blast injuries and injuries from high velocity projectiles, such as those encountered on the battlefield, present a range of therapeutic challenges and often require a staged repair. A significant need exists for the development of novel regenerative medicine approaches for the generation of both soft and hard tissues to overcome the current clinical barriers to craniofacial reconstruction. Like

[1] http://www.defense.gov/news/casualty.pdf (June 2012)

all programs within AFIRM, this program consists of several multidisciplinary, multi-institutional collaborative research teams to address the core issues associated with traumatic injuries.

Burn Program

Unquestionably, one of the most visible and life threatening injuries to military service personnel is severe burns. The current standard of care for burn injuries remains early excision and autografting, and has not fundamentally changed in over 30 years. The multi-institutional, and multi-disciplinary Burn Program's principal "thrust" is to significantly advance the operative management of burn injuries, as burn wound "closure" remains the single greatest threat to the burn-injured warfighter.

Scarless Wound Healing Program

Military trauma creates not only large wounds but also large scars. These scars are often very visible and can draw unwanted attention to the wounded warrior. In some instances the scars become so thick that they can limit movement of joints and greatly restrict the patient's ability to move. The costs associated with treatment of tissue fibrosis in the US are estimated to be over $4 billion per year. Current treatment regimens involving surgery, silicone sheeting, anti-inflammatory medications and laser/radiation have been disappointing. This is largely due to a lack of understanding of the fibrotic process. The pathophysiology of scar formation suggests the need to regulate numerous aspects of the wound environment, including cells, extracellular matrix, mechanics and biochemical signaling.

The WFPC approach encompasses a broad continuum of technologies aimed at modulating the tissue response to injury. Collectively, these projects represent a collaborative effort to address every aspect and stage of wound repair in a single research program, with the overarching aim of developing a more effective wound management paradigm. Thus the WFPC Scarless Program is composed of a synergistic combination of seven leading research groups focusing on every aspect of scarless wound healing. Industrial partners have contributed to the initiation of two clinical trials. This program utilizes complementary approaches (device, pharma, biotechnology) to balance short- and long-term objectives.

Compartment Syndrome and Limb & Digit Programs

Tissue wounds to the extremities are among the most common battlefield injuries sustained by troops during Operations Iraqi Freedom and Enduring Freedom. Particularly common traumatic injuries caused by improvised explosive devices are blast and projectile injuries. Thus there is a need to develop technologies which address both limb and digit salvage and the consequences of amputated parts. While some times the damage is obvious other times injuries are complicated by Compartment Syndrome (CS). In CS, traumatic related tissue swelling creates increased compartment pressures and this leads to ischemia and infarction of tissues. CS dramatically amplifies the battlefield injury and quickly leads to permanent muscle, nerve and vascular cell

death. Soldiers that develop CS have prolonged recovery times and rarely recover complete muscle function, and they usually do not return to active duty at the same level of performance. Most CS injuries of the extremities result in permanent disability.

This program aims to develop regenerative medicine technologies using a number of approaches from autologous stem and progenitor cells, that offer a safe and potentially effective new therapeutic avenue to amplify the body's endogenous regenerative response to injury, to hand transplants, to biomaterial approaches—all with the goal to improve the functional recovery of the injured soldier. The regenerative medicine technologies described herein have been used by AFIRM investigators and others for civilian tissue injuries safely and effectively and thus substantiate the rationale for using this approach to solve an important unmet need in the treatment of battlefield injuries.

During 2010, it became apparent that while the mission is straightforward, research and development of innovative technologies is not. In an effort to balance mission expectations with the methodical pace of research, a reorganization of the research efforts within AFIRM was performed. Since the limb and digit program and the compartment syndrome program had many similarities, these two programs (limb & digit program and compartment syndrome programs) became administratively merged into the extremities injuries program. Both programs are thematically focused on injuries to the extremities and thus will synergistically benefit from being combined. In addition, the restructured program also parallels that of the RCCC consortium.

Enabling Technologies Core

Created in 2010, a new modification to the administrative structuring of program was the creation of the Enabling Technologies Core. It was quickly realized that certain projects were developing enabling technologies which would benefit more than their originally assigned program areas. This restructuring allows for opportunities to improve interaction between programs and continue developing state-of-the-art tools and/or techniques applicable to the four program areas. The ET core functions to mature basic or platform technologies for insertion into the program areas of best fit and/or serve as a resource in multiple core areas and has opportun

Strategic Vision for Final Half of AFIRM Contract

As the midway point for AFIRM has been reached, the vision of assembling the top regenerative medicine research teams in the county to accelerate technologies has been achieved. With this success thus far, not surprisingly, there has been a shift in what AFIRM could accomplish. Most notably, there has been a different tone of project-centric management to a product centric approach to managing AFIRM. There has also been an increased emphasis on the ensuring that the regulatory and clinical portions of AFIRM are ready to accelerate the products being produced.

As AFIRM has progressed two things have become apparent. (1) While all the scientific progress of all projects can be considered excellent, the realization that not all projects will lead

to commercially viable and military relevant "products" with the final half of AFIRM. Thus there is a need to "weed and seed" new projects to bear new or improved products. (2) There are clear synergistic areas between WFPC and RCCC. On a scientific level, there have been close ties between the leadership in areas of composite tissue transplantation. In the area of communication there are efforts to create a common shared patient registry database as well as a common communication platform. From a manufacturing point of view, a common manufacturing area which uses good manufacturing practices (cGMP) has been identified. There is continuing efforts to combine the animal models to lead to better comparison of different technologies.

We are confident that by constantly improving the quality of our operation that we can improve the quality of the products we hope to deliver to the wounded warrior. The following sections provide an update on the progress made by the five program areas.

Overview of the Craniofacial Reconstruction Program

Program Leaders: Antonios G. Mikos, PhD, and Mark E. Wong, DDS

Craniofacial trauma is among the most debilitating forms of injury facing civilian and military populations due to the important aesthetic and functional role of the craniofacial complex. Blast injuries and injuries from high velocity projectiles, such as those encountered on the battlefield, present a range of therapeutic challenges and often require a staged repair. A significant need exists for the development of novel regenerative medicine approaches for the generation of both soft and hard tissues to overcome the current clinical barriers to craniofacial reconstruction. The Craniofacial Reconstruction Program comprises a multidisciplinary, multi-institutional collaborative research team to address the core issues associated with traumatic injuries to the craniofacial complex. Drawing on the strengths of each investigator on the team, an optimal set of complementary technologies have been identified to achieve hard and soft tissue regeneration in those areas designated by the AFIRM.

The foundation for the anatomy of the face is based upon the support furnished by bone and cartilage. The injury or loss of appendicular structures, such as ear and nasal cartilage tissues, is generally not life-threatening. However, deformity of highly visible facial structures significantly reduces the quality of life for an injured individual, which may lead to prolonged psychosocial problems. In addition, these structures are subjected to important directional function, channeling environmental stimuli towards the relevant special sense organs for detection while also providing distinctive facial features. Therefore, restoration of these tissues is of paramount importance.

The following technical reports provide greater insight into the progress made by the program.

Project CF-1: Engineered cartilage covered ear implants for auricular reconstruction

Team Leader(s)	James J. Yoo, MD, PhD (Wake Forest)
Project Team Members	Sang Jin Lee, PhD, John Jackson, PhD, Chang Mo Hwang, PhD, Young Min Ju, PhD, Cheil Kim, MD, PhD, Idris El-Amin, DVM, Denethia Green, BS (Wake Forest)
Collaborator(s)	Greg Sword (Porex Surgical, Stryker)
Therapy	Reconstruction of the external ear
Deliverable(s)	*Baseline:* Engineered cartilage tissue covering the commercially available alloplastic implant *Revised:* None
TRL Progress	Start of Program: TRL #3 End Year 3: TRL #4 End Year 1: TRL #3 End Year 4: TRL #4 End Year 2: TRL #4 End Year 5: TRL #4
Key Accomplishments:	In the past year, the evaluation of the structural and functional integrity of engineered cartilage covered ear implants for clinical application has continued. A study in a large animal model (rabbit) has been continually performed. The evaluation of the isolation and growth of the chondrocytes resulted in an increased production of chondrocytes.
Keywords	Auricular cartilage, chondrocytes, alloplastic ear implant, reconstruction, tissue engineering

Introduction

Traumatic injuries constitute a major cause of morbidity and mortality for the armed forces (1-4). The incidence of craniofacial injuries has been rapidly increasing due to the frequent ballistic and explosive injuries on the battlefield. Protruding tissues such as ear and nose are frequently affected in these injuries (5). 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 method for auricular reconstruction uses autologous costal cartilage as a graft material. However, autologous costal cartilage is limited in supply, provides inadequate dimensions, and is progressively absorbed after implantation (6-8). Currently, alternative approaches utilize alloplastic ear implant devices composed of silicone or polyethylene. These implants are approved by the 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 (9-13). Although alloplastic ear implants are able to effectively eliminate the morbidity associated with the costal cartilage graft, the use of these implants is often related to complications which include inflammation, infection, erosion and dislodgement (12-14). As a result, implant extrusion occurs frequently due to the limited vascularization and constant abrasion against the surrounding tissues. A common practice to

overcome these complications includes the use of a temporo-parietal tissue flap from the side of the head to cover the implant, which provides va scularized tissue cush ion against the abrasiv e implant.

In this project we have developed an engineered cartilage that entirely covers the e ar implant which would prevent implant exposure and extrus ion, while maintaining appropriate mechanical properties. Creation of cartilage tissue using soldier's own cells woul d bring benefits and minimize the morbidity associated with implant dislodgement. In this project, we plan to further refine and o ptimize the process ing system for a smooth translation into soldiers who require auricular reconstruction.

Specific Aims in the Approved Statement of Work (Year 4):

Aim 1: To demonstrate the clinical applicability of engineered cartilage ear implants
- 1.1: To demonstrate of the stru ctural and functional integrity of engineered cartilage ear implants
- 1.2: To characterize and evaluate host tissue response of engineered cartilage ear implants
- 1.3: To develop SOPs for surgical methods

Aim 2: To initiate clinical investigation of the engineered cartilage implant
- 1.1: Preparation of materials for FDA discussions
- 1.2: To control and m anage the manufacturing and quality control testin g of the engineered cartilage ear implants

Research Progress

I. Continued cell isolation and expansion using different cell sources; ear, nose, and rib cartilages (human/rabbit origins)
   - Optimized the isolation efficiency and cell growth
   - Optimized the maintenance of phenotypic and functional expression, and extracellular matrix (ECM) production

II. Developed and fabricated a novel flexible ear scaffold that mimics ear cartilage using an integrated organ printer system
   - *In vitro* evaluation of the flexible ear implants
   - *In vivo* evaluation of the flexible ear implants

III. Continued to develop SOPs for a utologous cell sourcing, expansion system , and cell delivery system (human origins).
   - SOPs for autologous cell isolation and expansion
   - SOPs for cell delivery system and its preparation

IV. Continued isolation and expansion of auri cular chondrocytes from rabbit ear for an autologous cell-based implantation
   - Isolation and expansion of auricular chondrocytes
   - Implantation of engineered cartilage covered ear constructs in rabbits
   - Characterize host tissue response and cartila ge formation of engineered cartilage covered ear implants in rabbits

1. Customized ear-shaped scaffold construction using integrated organ printer system: A computerized bioprinting system developed by our group is composed of a 3-axis stage, high precision pressure and temperature controller and four cartridges. A flexible ear-shaped scaffold fabricated by the bioprinting system. **Figure 1** shows the CAD/CAM process to obtain motion program for the scaffold fabrication using a 3-D CAD model. This motion program is a command list for operating the printing system. Sliced profiles are obtained by slicing of STL CAD model which is the most generally used for 3-D bioprinting system. This motion program is transferred to the system, and then a scaffold with a desired 3-D shape can be fabricated. Poly(ε-caprolactone) (PCL) was used as a scaffold material for ear construction. PCL printing was performed with a nozzle of 300 μm inner diameter under temperature and pressure ranges of 80~90°C and 750~800 kPa, respectively.

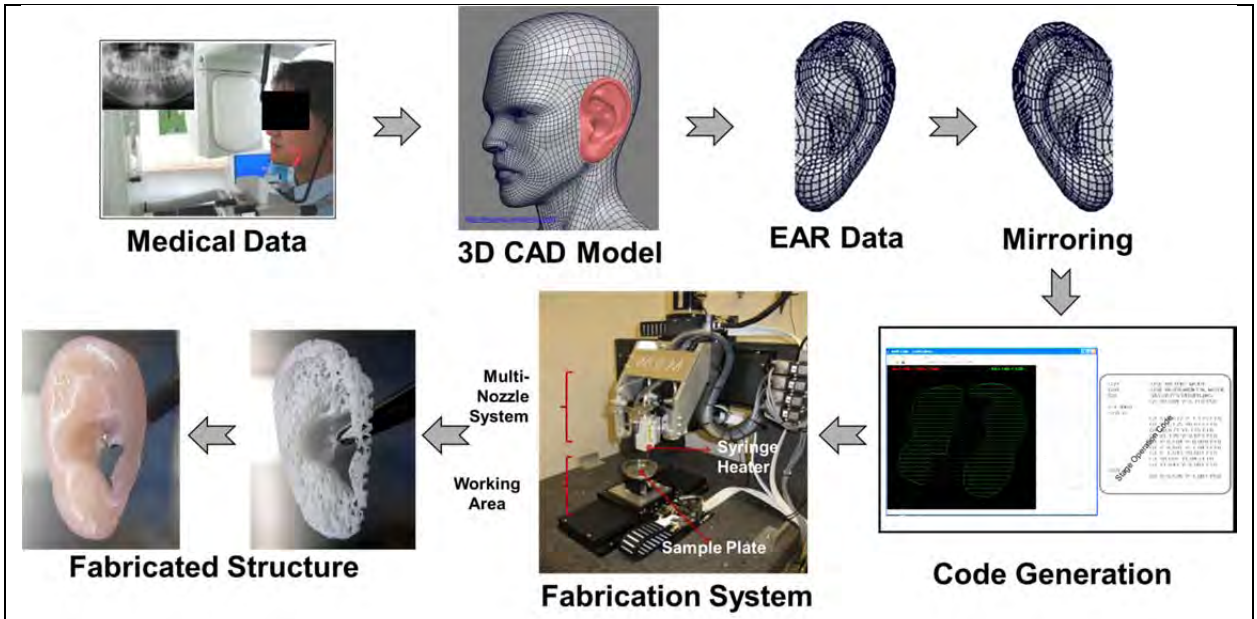

Figure 1. Customized ear-shaped scaffold construction using integrated organ printer system

The results indicate that the ear implants with primary chondrocytes (40M cells/ml) were fabricated by the integrated organ printing system in a controlled manner. The implanted ear constructs showed no evidence of skin necrosis, implant exposure or extrusion, however ear implants without cells (control) resulted in severe exposure of PCL scaffolds. In addition, this ear construct shows similar mechanical characteristics (elasticity) to those of natural ear cartilage (Figure 2A).

Histomorphological evaluations show the formation of neocartilage in the implants (Figure 2B,C). It is demonstrated that consistently presence of evenly dispersed triangular and ovoid-shaped chondrocytes with lacunae, surrounded by perichondrium. Safranin-O staining confirmed the presence of sulfated glycosaminoglycans (GAG), indicating a mature neocartilage framework

had formed. These results demonstrate that bioprinting of PCL ear scaffolds has the potential of patient specific complex ear shape fabrication with reproducible cartilage formation *in vivo*.

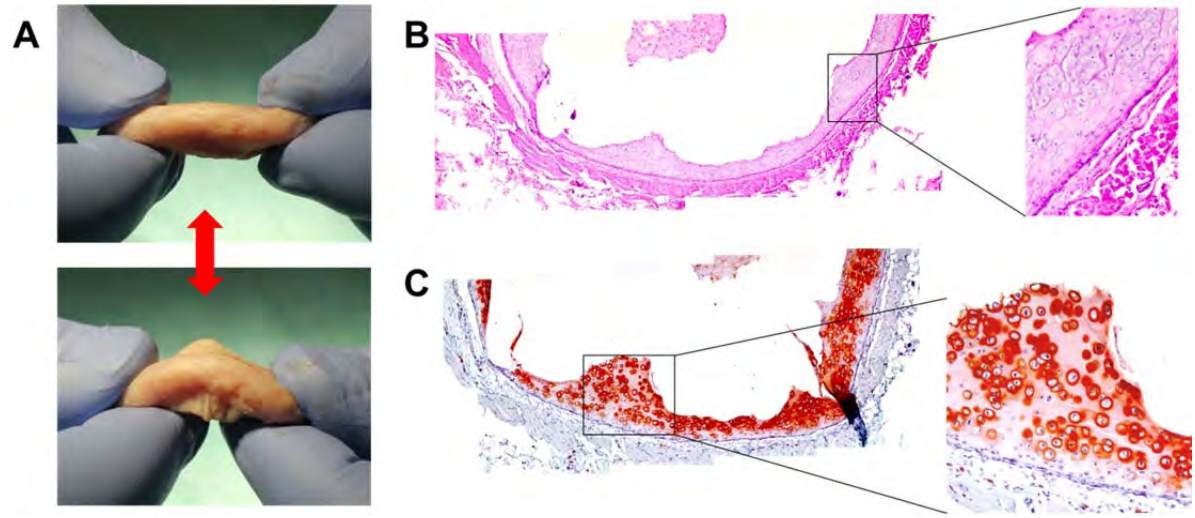

Figure 2. (A) The flexible ear scaffold can be customized for the patient's unique size and shape. (B) H&E staining and (C) Safranin-O staining of bioprinted ear scaffold after 2 month implantation *in vivo*.

The final goal of ear reconstruction is aesthetic recovery and fulfills satisfaction of patients. To achieve this goal, ear scaffold should be made with consideration of symmetry between two sides. If there is one normal ear, shape of the other side ear should be same. Three-dimensional reconstruction and replication after mirroring of existing ear can provide high symmetry between two ears of patients. 3-D bioprinting can provide patient-specific three dimensional scaffolds with high symmetry in short time and can be used for optimized cartilage regeneration. We demonstrated that the computerized bio-printing technology could be applied for fabricating a clinical relevant scaffolding system for auricular. The flexible ear-shaped scaffolds were fabricated based on 3-D CAD model using CAD/CAM process and applied into in-vivo experiment after cell spraying. These results demonstrate that bioprinting of PCL ear scaffolds has the potential of patient specific complex ear shape fabrication with reproducible cartilage formation *in vivo*.

Key Research Accomplishments

- Characterized the isolation and growth of chondrocytes from ear, nose, and rib cartilage
- Developed and fabricated a novel flexible ear scaffold that mimics ear cartilage using an integrated organ printer system
- Implantation of autologous auricular chondrocyte coated ear implants in rabbits

Conclusions

Isolation and growth of human chondrocytes from several sources were performed. The identification of the optimal tissue source for covering an implant will be important for future clinical trials. A flexible 3 dimensional engineered ear implant was fabricated using a bioprinter. A transplant study using autologous chondrocyte coated ear implants in a rabbit model was initiated.

Research Plans for the Following Year

The autologous rabbit transplant study will be completed. Complete SOPs for autologous cell sourcing, expansion system, and cell delivery system of human chondrocytes. Refine the cell delivery system for clinical use.

Planned Clinical Transitions

Document Preparation and initiate communication with the FDA for a clinical trial.

Corrections/Changes Planned

No changes or corrections are planned.

Conflict of Interest Disclosure

None of the participants in this study have conflicts of interest.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	
# Post docs	4	
# grad students	1	
# undergrad students	0	
# staff members working for AFIRM	1	

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	5

*Directly supported in whole or part –time by AFIRM

Peer Reviewed Publications

1. Lee SJ, Broda C, Atala A, and Yoo JJ, Engineered cartilage covered ear implant for auricular reconstruction, Biomacromolecules, 2011;12(2):306-313

Non-Peer Reviewed Publications (Abstracts)

1. **Lee SJ**, Lee B, Hwang CM, Green D, Jeong SY, Atala A, Jackson J, Yoo JJ, Tissue Engineered Ear for Auricular Reconstruction, Advanced Technology Applications for Combat Casualty Care (ATA CCC) 2011 Conference, August 15-18, 2011, Marriott-Harbor Beach Conference Center, Fort Lauderdale, FL, USA
2. Hwang CM, Lee B, Atala A, Yoo JJ, Lee SJ, A tissue engineered cartilage covered alloplastic implant for auricular reconstruction, North Carolina Tissue Engineering & Regenerative Medicine Society 13th Annual Conference, November 4, 2011, Old Salem Visitors Center, Winston-Salem, NC, USA
3. Hwang CM, Lee BK, Green D, Jackson JD, Atala A, Yoo JJ, Lee SJ, A tissue engineered cartilage covered alloplastic implant for auricular reconstruction, The TERMIS-NA 2011 Annual Conference & Exposition, December 11-14, 2011, Hilton Americas-Houston, Houston, TX, USA
4. Hwang CM, Lee SJ, Jackson J, Atala A, Yoo JJ, A Tissue Engineered Cartilage Covered Alloplastic Implant for Auricular Reconstruction, 4th AFIRM "All Hands" Meeting, February 13-16, 2012 in TradeWinds Resort, St. Pete Beach, FL, USA
5. Kang H-W, Hwang CM, Atala A, Lee SJ, Yoo JJ, 3-D Biofabricated Ear Scaffolds for Reconstruction, 4th AFIRM "All Hands" Meeting, February 13-16, 2012 in TradeWinds Resort, St. Pete Beach, FL, USA

Project CF-2: Space Maintenance, Wound Optimization, Osseous Regeneration and Reconstruction for Craniomaxillofacial Defects

Team Leader(s)	Antonios G. Mikos, PhD (Rice University), Mark E. Wong, DDS (University of Texas Health Science Center at Houston), and F. Kurtis Kasper, PhD (Rice University)
Project Team Members	Allan M. Henslee, BS, Lucas Kinard, BS, James D. Kretlow, MD, PhD, Sarita R. Shah, BS, Patrick Spicer, BS, Limin Wang, PhD (all of Rice University); Nagi Demian, DDS, MD, Simon Young, DDS, MD, PhD (both of the University of Texas Health Science Center at Houston)
Collaborator(s)	Shanghai 9th People's Hospital, Shanghai, China and Radboud University of Nijmegen Medical Centre, Nijmegen, The Netherlands
Therapy	Staged reconstruction of large osseous defects in the craniofacial region restoring function and esthetics
Deliverable(s)	*Baseline Objective*: (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 *Revised:* None
TRL Progress	Start of Program: TRL #2 End Year 3: TRL #5 End Year 1: TRL #4 End Year 4: TRL #5 End Year 2: TRL #4
Key Accomplishments:	Porous polymethylmethacrylate-based space maintainers have been implanted in 9 patients to-date under physician directed use. IRB approval has been received for a randomized prospective clinical study awaiting funding. A study evaluating colistin-releasing porous space maintainers in an inoculated rabbit composite tissue defect model and an *in vitro* study evaluating the controlled release of various other antibiotics from polymeric microparticles were completed. An *in vitro* study evaluating the release of growth factor from an injectable hydrogel was completed and an *in vivo* study of the same hydrogel was initiated.
Keywords	craniofacial bone reconstruction; space maintenance; bone flap; controlled drug delivery; *in vivo* bioreactor

Introduction

Ballistic injuries resulting in significant soft and hard tissue loss and devitalization are commonly encountered clinical scenarios in current U.S. military combat theaters, including Operation Enduring Freedom. This project seeks to develop a method to facilitate effective staged reconstruction of large osseous defects in the craniofacial region of injured military personnel, thus restoring function and esthetics in these individuals. The purpose of this research is to optimize the results, decrease the complications and infections, and reduce the number of procedures associated with large bony reconstructions in this particular patient population through three mechanisms: 1) by the initial implantation of a biocompatible, antibiotic-releasing space maintainer within a large osseous defect during the early phases of treatment, 2) through

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

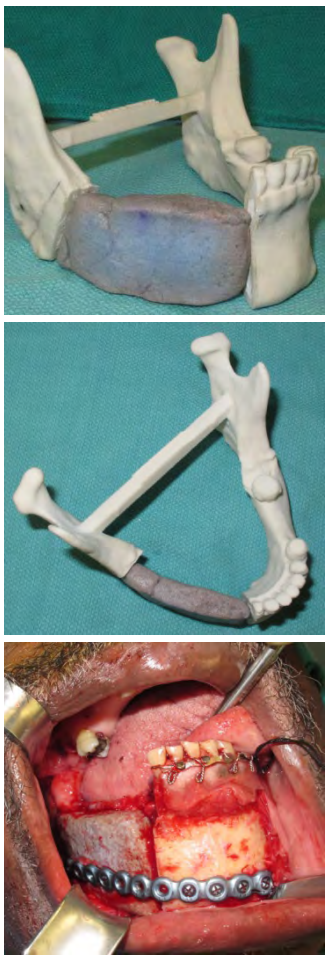

Figure 1. Porous space maintainer shown in a patient specific stereolithographic model (A and B) and placed in mandibular defect (C).

Immediate reconstruction has been shown to produce better functional as well as aesthetic out comes due to the minimization of wound contracture and maintenance of bone structure; however, immediate reconstruction with grafts or flaps fail up to 54% of the time due to infection.[1] Alloplastic materials have been used during immediate reconstruction, but commonly result in wound dehiscence or infection. Porous materials have been shown to decrease wound dehiscence through increased tissue-material interactions. Consequently, this project seeks to apply materials currently regulated for clinical use, namely, poly(methyl methacrylate) (PMMA) and a gel porogen, in novel combinations to produce porous space maintainers for the dual purpose of maintaining the bony defect space without dehiscence and releasing antibiotics in a controlled manner to mitigate wound infection.

The surgical transfer of autologous bone tissue to a bony defect site is the "gold standard" for augmentation of bone regeneration. The procedure is generally successful, however, for larger defects a vascularized flap is preferred but is typically not of the correct dimensions or shape for the defect. This project seeks to apply an existing technique pioneered in an animal model by our laboratory[2] using the body as a "bioreactor" to produce vascularized bone flaps in chambers comprising PMMA filled with clinically available bone fillers at secondary sites for transplantation to a bony defect. The vascularized bone flap technology has already seen investigational clinical use to treat bony defects in the craniofacial complex.[3]

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 an alternative site on the body generating a vascularized 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

Several important steps were made on the regulatory pathway for clearance of a porous PMMA-based space maintainer product. Toward this end, a study was completed to characterize the physicochemical properties of various formulations of porous PMMA as well as a non-porous

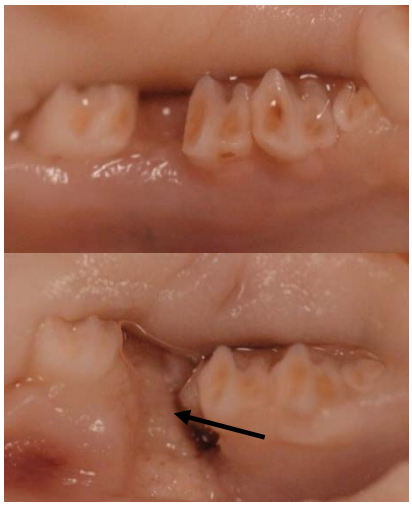

Figure 2. Photographs of (A) healed and (B) non-healed mucosal defects. Black arrow indicates exposed space maintainer.

PMMA-based bone cement formulation according to ASTM and ISO standards set forth in the FDA Guidance Document for PMMA Bone Cement. [4-6] This study found that while mechanical properties were decreased in porous materials over non-porous materials, the properties maintained are sufficient for their intended use in space maintenance applications. Moreover, the porous materials performed equally to or better than non-porous materials in other tests, such as residual monomer release and setting temperature. Additionally, a protocol was approved by the Institutional Review Board (IRB) 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 Protections Office (HRPO) approval of the protocol has been issued. However, porous PMMA-based space maintainers have been utilized in 9 patients to-date under physician-directed use. Figure 1 illustrates one such case of a porous PMMA-based space maintainer applied to fill a bone defect upon removal of a large benign pathology.

A number of studies were completed or continued in the past year investigating and optimizing the antibiotic-releasing technologies envisioned for application in the space maintenance approach. First, an *in vitro* study characterized the activity of the antibiotic colistin upon release from gelatin or poly(lactic-*co*-glycolic acid) (PLGA) microsphere carriers incorporated into porous PMMA-based space maintainers. Specifically, it was found that colistin released from either delivery vehicle (gelatin or PLGA) remained active against the target species *Acinetobacter baumannii*. Additionally, an *in vivo* study was performed to evaluate colistin-releasing porous PMMA space maintainers in a rabbit mandibular composite tissue defect model inoculated with *Acinetobacter baumannii*. This study evaluated soft tissue healing of the mucosal defect in addition to histological and safety measures. Representative images of healed and non-healed mucosal defects are shown in Figure 2. The formulation that delivered a high dose of antibiotics over an extended period of time resulted in a significantly greater number of healed mucosal defects than that which delivered a high dose with a burst release profile. Also, cultures of swabs obtained from within and around the defect showed no detectable *Acinetobacter baumannii* at the end of the study, indicating an eradication of the bacteria from the defect. Nephrotoxicity, a known adverse side effect of systemic use of colistin, was not observed in any group. To expand the applicability of this technology, an *in vitro* study was completed characterizing the release of several common antibiotics from particulate delivery systems, including vancomycin, clindamycin, cefazolin, ciprofloxacin, doxycycline and penicillin.

Finally, studies regarding degradable materials for bone augmentation were initiated. Specifically, an *in vitro* study characterizing the release of bone morphogenetic protein-2 (BMP-2) from an injectable degradable hydrogel carrier, oligo(poly(ethylene glycol) fumarate) (OPF)

was completed, showing controlled release of BMP-2 from the hydrogel. An *in vivo* study was initiated evaluating the release of BMP-2 from these OPF hydrogels for bone augmentation in a rat cranial augmentation model.

Major Task	Year 1	Year 2	Year 3	Year 4	Year 5
Develop Porous Space Maintainer					
Investigate Porous Space Maintainer *In Vivo*					
Characterize Antibiotic Release from Space Maintainers *In Vitro*					
Characterize Response to Antibiotic Release from Space Maintainers *In Vivo*					
Characterize "*In Vivo* Bioreactor" Strategies for Bone Flap Production in Large Animal					
Initiate Clinical Trial of Space Maintainer					
Initiate Clinical Trial of Antibiotic Releasing Space Maintainer					
Characterize Growth Factor Release from Hydrogels for Bone Contouring *In Vivo*					

Key Research Accomplishments

- Completed *in vitro* studies characterizing the physicochemical properties of porous PMMA-based space maintainers according to ASTM and ISO standards as outlined by FDA guidelines for PMMA-based bone cements.
- Received IRB approval for a randomized prospective clinical study of porous space maintenance versus the clinical standard in continuity defects resulting from resection of benign pathology.
- 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 mandibular defect model.
- Completed *in vitro* studies characterizing the release of several common antibiotics from carriers for inclusion into antibiotic releasing porous PMMA-based space maintainers.
- Completed *in vitro* studies characterizing the controlled release of rhBMP-2 from injectable hydrogels for bone augmentation and contouring.
- Initiated an *in vivo* study evaluating the bone augmentation capacity of injectable hydrogels releasing rhBMP-2 in a rat cranial augmentation model.

Conclusions

Considerable progress has been made over the course of the past year toward the clinical translation of porous PMMA-based space maintainers, as reflected in the continued applica tion

of the technology under physician-directed use. This progress will be continued through the initiation of a randomized, prospective clinical trial upon HRPO approval. Additional progress has been made toward the development of antibiotic-releasing implants for bony space maintenance. Specifically, an extended high-dose release of antibiotics led to more soft tissue healing and coverage in an inoculated composite tissue defect model than a burst release of antibiotics. Further, *in vitro* studies demonstrated that the antibiotic colistin remains active against *Acinetobacter baumannii* upon release from PMMA-based space maintainers. Also, expanding the potential application of antibiotic-releasing porous space maintainers, several commonly used antibiotics have been incorporated into degradable microsphere carriers for extended release, which will be continued through the application into an infected bone defect *in vivo*. Finally, the controlled delivery of BMP-2 from injectable hydrogels *in vitro* has been completed and an *in vivo* study of these hydrogels for bone augmentation has been initiated. No real or apparent conflicts of interest exist for the investigators or key study staff for this project.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	1
# Post docs	1	2
# grad students	4	1
# undergrad students	0	3
# staff members working for AFIRM	1	0

Other Project Statistics

# Honors given to AFIRM faculty	5
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	6
# Non-peer reviewed publications	6

*Directly supported in whole or part –time by AFIRM

Honors and Awards

1. 2012 Member, National Academy of Engineering (AG Mikos)
2. 2012 Member, The Academy of Medicine, Engineering and Science of Texas (AG Mikos)
3. 2012 Fellow, American Association for the Advancement of Science (AG Mikos)
4. 2012 AFIRM Fellows ISR Travel Award (AM Henslee and SR Shah)
5. 2011 Young Investigator Award, Tissue Engineering and Regenerative Medicine International Society – North America (FK Kasper)

Patents and Inventions

None

Peer-Reviewed Publications

1. Wang L, Yoon DM, Spicer PP, Henslee AM, Scott DW, Wong ME, Kasper FK and Mikos AG. Characterization of Porous Polymethylmethacrylate Space Maintainers for Craniofacial Reconstruction. Journal of Biomedical Materials Research, Part A, submitted.
2. Fong ELS, Watson BM, Kasper FK and Mikos AG. Building Bridges: Leveraging Interdisciplinary Collaborations in the Development of Biomaterials to Meet Clinical Needs. Advanced Materials, submitted.
3. Spicer PP, Kretlow JD, Henslee AM, Shi M, Young S, Demian N, Jansen JA, Wong ME, Mikos AG and Kasper FK. *In Situ* Formation of Porous Space Maintainers in a Composite Tissue Defect. Journal of Biomedical Materials Research, Part A, 100A:827-833, 2012.
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Other Publications

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2. Boyd J. "Six Rice University professors el ected AAAS fellows." Rice News. 9 Decem ber 2011, < http://news.rice.edu/2011/ 12/09/six-rice-university-professors-elected-aaas-fellows/>.
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PROJECT CF-3 TERMINATED

Project CF-4: Soft Tissue Reconstruction/Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction

Team Leader(s)	Peter Rubin (Univ. Pittsburgh), Kacey Marra (Univ. Pittsburgh), David Kaplan (Tufts), James Yoo (Wake Forest), Sang Jin Lee (Wake Forest)
Project Team Members	Evangelia Bellas, PhD (Tufts), Bruce Paniliatis, PhD (Tufts), Jodie Moreau, PhD (Tufts), Rachel Hoyer (Univ. Pittsburgh), Jolene Valentin, PhD (Univ. Pittsburgh), Nurul Aini, MD (Univ. Pittsburgh), Donna Ward, PhD (Univ. Pittsburgh), Chang Mo Hwang, PhD (Wake Forest), Sang-Hyug Park, PhD (Wake Forest), Young Min Ju, PhD (Wake Forest)
Collaborator(s)	Jeff Gimble (Louisiana State University, Pennington Research Center), Steve Badylak (Univ. Pittsburgh)
Therapy	Long-term soft tissue restoration of traumatic defects with cell based-degradable scaffolds, resulting in sustained shape and volume over time.
Deliverable(s)	*Baseline:* Objective Task 1: Engineering of vascularized connective tissue and fat pad Including the development of a 3D silk fibroin scaffold to provide sustained morphology, structure and tissue function while supporting cellular and vascular ingrowth to restore functional tissue. Baseline Objective Task 2: Development of implantable and injectable vascularized soft tissue composed of connective tissue and fat (Years 2 & 3). Including second generation scaffolds combined with stem/progenitor cells (with or without transfection with VEGF/FGF genes). Baseline Objective Task 3: Demonstration of the applicability of using implantable and injectable soft tissue composites for limb, burn, and craniofacial applications in a large animal model (Years 3 and 4). Baseline Objective Task 4: Initiation of clinical testing of soft tissue replacement for small defects (Years 4 and 5).
TRL Progress	Start of Program: TRL #2 End Year 3: TRL #4 End Year 1: TRL #3 End Year 4: TRL #5 End Year 2: TRL #4
Key Accomplishments:	In the past year, this team has conducted numerous pre-clinical studies using silk biomaterials and adipose- derived stem cells. Implanted silk scaffolds maintained volume through 18 month period in rat model. Mature adipose tissue was found in groups pre-seeded with lipoaspirate or adipose derived stem cells differentiated. Large animal studies started with scaled-up silk implants: volume maintained in 6 month study. Injectable silk foams developed and tested in small animal model.
Keywords	Adipose tissue, Adipose-derived Stem Cells, Lipoaspirate, Volume Stable, Regeneration, Silk scaffold, Soft Tissue, Connective tissue, Fibrin-based hydrogel

Introduction

Background

The retention of implanted fat and/or connective tissue grafts over time remains a challenge for soft tissue reconstruction procedures such as the repair of craniofacial defects. The restoration of traumatic soft tissue defects must start with a strategy that will restore tissue size and shape to near normal dimensions for at least one year while the body gradually remodels and regenerates the tissue with semi-normal or normal structure and function. Silk biomaterials have been employed for soft tissue reconstruction because of their tunable degradation rates and biocompatibility, their ability to be processed into various formats (sponges, gels, foams), their ability to deliver bioactive agents, and their robust mechanical properties.

Specific Aims:

Aim 1: Biomaterial-Based Scaffolds for Adipose Tissue Regeneration - An injectable scaffold will be designed to provide sustained morphology and structure for at least one year, while supporting cellular and vascular ingrowth to restore functional tissue.

1.1 Development of an injectable sustainable silk protein biomaterial scaffold that can be combined with lipoaspirate to serve as a template for soft tissue regeneration.

Aim 2: Cell-Based Scaffolds for Adipose Tissue Regeneration - The second generation scaffolds will include adipose stem/progenitor cells (hASCs; with or without transfection with vascular endothelial growth factor (VEGF)/fibroblast growth factor (FGF) genes) in combination with the scaffolds from Aim #1, to accelerate adipose tissue regeneration.

2.1 Development of quantitative analytical methods for cell characterization.

2.2 Development of an implantable sustainable silk protein biomaterial scaffold that can be cultured with hASCs, adipocytes or lipoaspirate to serve as a template for soft tissue regeneration.

Aim 3: Injectable Fibrin-based Hydrogel System for Soft Tissue Regeneration

2.1 Development of an injectable hybrid hydrogel system composed of alginate particles and a fibrin hydrogel

2.2 Development of a porous injectable hydrogel system composed of rapid degradable gelatin microfibers

2.3 *In vivo* evaluation of an injectable hydrogel system with dermal fibroblasts

Previous Year's Achievements

The research group successfully combined silk based scaffolds, lipoaspirate, and stem cells to form stable injectable and implantable soft tissue constructs in a small animal model. These accomplishments are on track with the proposed milestones for this project, and the group has maintained a pathway for developing clinically useful injectable and implantable soft tissue therapies that can improve facial deformities with greater precision than current techniques.

Research Progress

Silk Sponge and Foam *in Vivo* Studies:

The long-term silk sponge *in vivo* study was completed. Silk sponges were implanted as is, with stem cells cultured *ex vivo* or with lipoaspirate into a rat for up to 18 months. Volume was maintained in all groups (Figure1a, 1c) through 18 months. Tissue was regenerated as the sponge degraded (Fig 1a, 1b). Adipose tissue was found only in seeded groups (Fig 1- Right). This study demonstrated volume retention greater than 6 months. The silk sponge, alone or with autologous

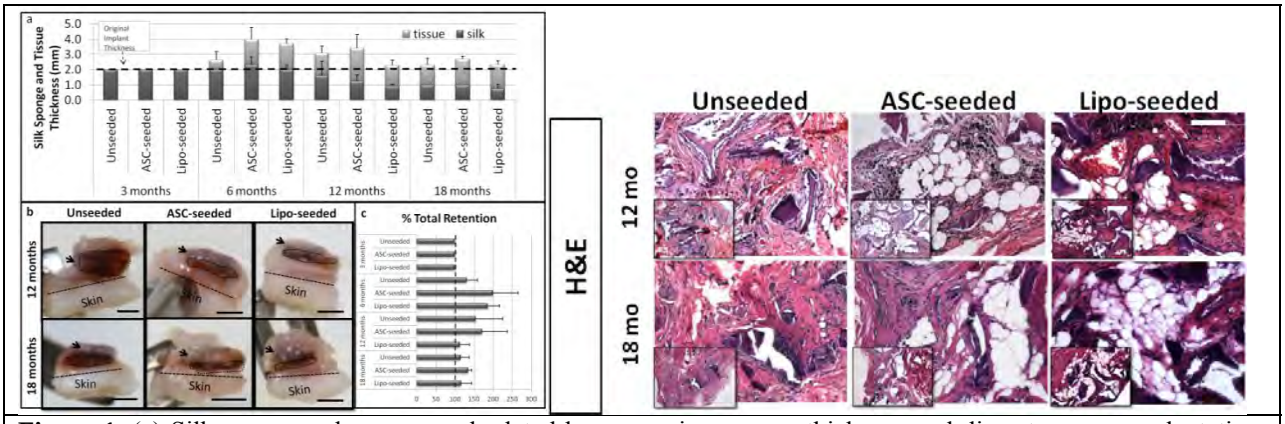

Figure 1. (a) Silk sponge volume was calculated by measuring sponge thickness and diameter upon explantation. Complete volume retention in all groups was seen. (b) Cross-sections of 12 (top row) and 18 (bottom row) month explants are shown for unseeded (left), ASC-seeded (middle) and lipo-seeded (right) groups. Square dotted lines outline the silk sponge, while rectangular dotted lines demarcate skin from subcutaneous tissue. Arrows point to regions of subcutaneous fat. Subcutaneous fat formation was greatest in lipo-seeded group (right column) and least in the unseeded group (left column). Scale bar- 2 mm. (c) Total retention (silk+regenerated tissue). Right panel- H&E images show presence of mature adipocytes within pre-seeded groups. Scale bar- 100 µm, inset- 200 µm.

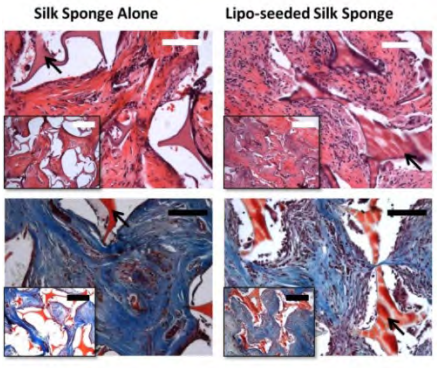

Figure 2. H&E (top), Masson's Trichrome (bottom) images of silk sponges implanted in a horse after 6 months. Silk is visible (black arrows). Tissue is well-organized and volume was retained. No mature adipocytes were present at 6 months. Scale bar- 100 µm, inset- 200 µm.

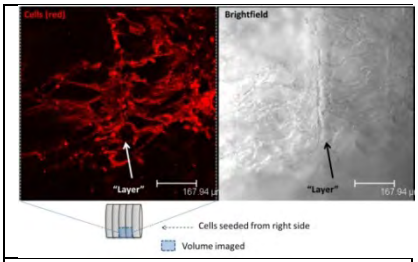

Figure 3. Cells (red) migrate through foam layers. Confocal image of fluorescently labeled cells (fluorescent- left; bright field- right) on silk foam. Side surface perpendicular to seeding surface was imaged.

lipoaspirate, was implanted into the back of a horse for 6 months, to demonstrate a scale-up/pre-clinical model. As with the small animal study, the sponges had maintained volume though the 6 month period. In both unseeded and seeded groups, the regenerated tissue was well organized collagenous connective tissue (Figure 2). Injectable silk foam was conducive to cell spreading, attachment and survival (Figure 3) *in vitro*. The foam was easily injected via a custom injection gun, through

the skin and remained palpable after 90 days *in vivo* (Figure 4-Left). The foams are well integrated with surrounding host tissue, and vasculature is seen leading to the foams at early time-points (Figure 4-Right).

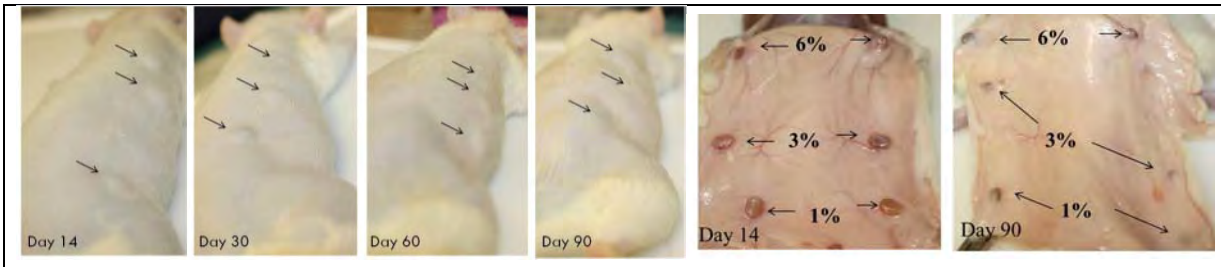

Figure 4. Left: Foams are visible and palpable through skin up through 90 days. Skin healed well after injection. Right: Injectable silk foams (1, 3, 6% silk w/v) after 14 and 90 days post injection. Vasculature leading to foams was evident at early time points. Foams are well integrated with surrounding host tissue. After 90 days, 50% of silk foam was retained.

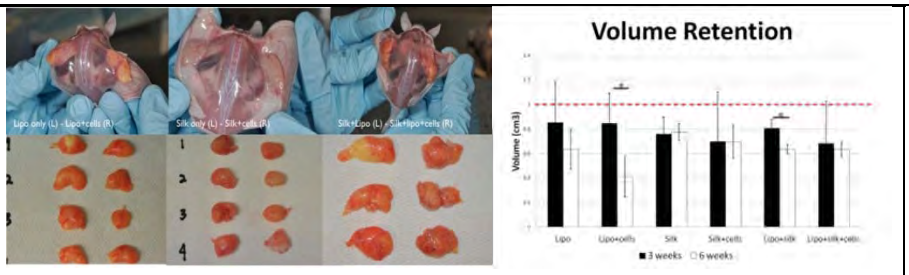

Figure 5. (a) Macroscopic images of fat grafts explanted at 6 weeks. (b) Volume retention at 3 and 6 weeks for 8% silk gels. (*P< 0.05)

Silk Gel *In Vivo* Studies: Injectable silk gel has been fabricated and tested *in vivo* in an athymic mouse model to study volume maintenance and biologic response over time. 8% silk gel was combined with lipoaspirate at 0:1, 1:0, 1:3 ratios of gel:lipoaspirate, half were combined with 4 million ASCs/ml. Macroscopically all explants showed normal fat appearance and were encapsulated (Figure 5a). No decrease in volume retention was noted for the lipoaspirate/silk gel/ASC group (Figure 5b). Grafts composed of lipoaspirate contained blood vessels and normal fat tissue (Figure 6). Cellularity was increased in groups containing ASCs. When blended with lipoaspirate, the silk gel did not appear to inhibit vascularization of the implants.

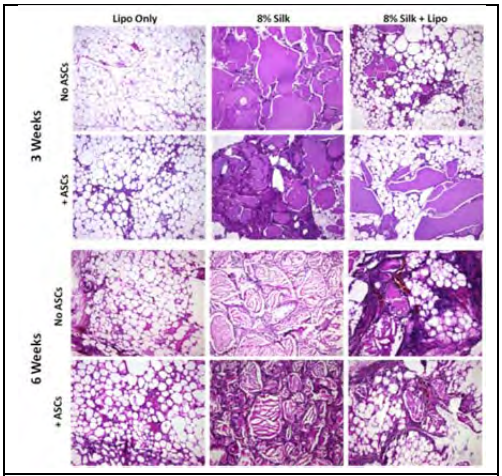

Figure 6. H&E images of explanted silk fat grafts at 3 and 6 weeks (10X).

In vivo evaluation of injectable alginate particle-embedded fibrin hydrogels

A novel hybrid hydrogel system was developed that consisted of alginate particles and fibrin matrix that maintained tissue volume over the long term. Alginate particles were fabricated by mixing 5% alginate with a 20 mM calcium solution. Dermal fibroblasts and alginate particles were embedded in fibrin hydrogels using a dual syringe mixer. *In vivo* explants showed that cells contained within fibrin-only hydrogels did not contribute to neo-tissue formation, and the fibrin

was fully degraded within a 12-week period (Figure 7). In the alginate-fibrin system, higher cellularity and vascular ingrowth were observed *in vivo* (Figure 8). This resulted in neo-tissue formation in the alginate-fibrin hydrogels. These results demonstrate that fibrin may enhance cell proliferation and accelerate formation of extracellular matrix (ECM) proteins in the alginate-fibrin system, while the alginate particles could contribute to volume retention.

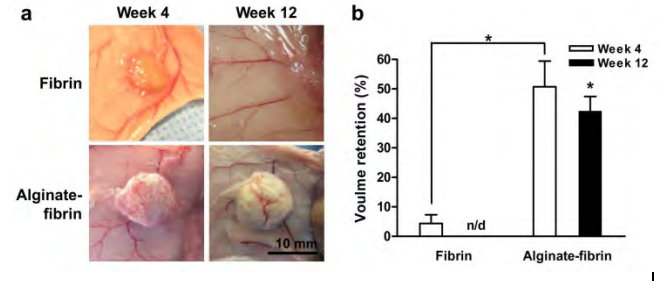

Figure 7. (a) Gross appearance and (b) volume retention (%) of cell-hydrogel constructs at 4 and 12 weeks of implantation. At 12 weeks of implantation, the cell-fibrin constructs were completely absorbed and any tissue remnant was not observed while the alginate-fibrin hydrogels maintained approximately 42.4% of original volume (n/d: not detectable).

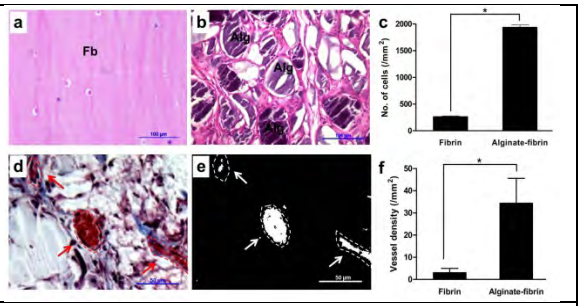

Figure 8. H&E staining of the cell-hydrogel constructs at 4 weeks of implantation: (a) fibrin and (b) fibrin/alginate. (c) The alginate-fibrin constructs showed higher cellularity when compared with the fibrin-only constructs (**P*<0.01). (d-f) Vessel density (vessels/area) in the cell-hydrogel constructs was analyzed by the autofluorescence of RBCs. (f) The vascularization in the alginate-fibrin constructs was significantly higher than that in the fibrin-only constructs (**P*<0.01).

Key Research Accomplishments

- Completed 18 month study in small animal model using silk sponge implants with lipoaspirate. First study to show long term volume stable adipose tissue regeneration.
- Initiated short term horse study (1, 3 month) with silk sponges, foams, and gels with autologous lipoaspirate, and completed 6 month horse study with silk implants combined with autologous lipoaspirate – volume stable.
- Injectable silk foams and custom injection gun developed (IP protected). Preliminary 2 week in-vitro studies demonstrate stem cells survive and migrate through foams. Foams readily absorb lipoaspirate. Preliminary in-vivo study with foams alone completed.
- Completed in-vivo silk gel studies, which indicated that fat grafts containing lipoaspirate, silk, and ASCs showed retention of the graft over time and a favorable tissue response.
- Alginate particles embedded in fibrin hydrogel improved volume retention over time while the transplanted cells produced ECM proteins *in vivo*
- Porous structure generated by gelatin microfibers enhanced cell proliferation and ECM production in the hydrogels *in vivo*

Conclusions

Research Plan for the Following Years: Small animal studies are underway in which various formats of silk injectables (hydrogel, sponges, foams) are being tested in physiologically relevant models (subcutaneous, subm uscular and intr amuscular sites). Discussions for Pre-IDE submissions initiated. Currently TRL4, anticipated TRL5 by end of year 5.

Planned Clinical Transitions: Initial clinical trials are underway via the U. Pittsbu rgh. Discussions with VCs initiated.

Corrections/Changes Planned: No major changes are anticipated as the results for long term *in vivo* shape retention and function for remodeling have been as expected.

Conflict of Interest Disclosure: None

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	5	
# Post docs	3	0
# grad students		2
# undergrad students		2
# staff members working for AFIRM		1
Other Project Statistics		
# Honors given to AFIRM faculty		
# Doctorates awarded under A FIRM support		1
# Masters degrees awarded under A FIRM support		
# Inventions disclosed		3
# Patents awarded		
# Peer reviewed publications		3
# Non-peer reviewed publications		6

*Directly supported in whole or part –time by AFIRM

Honors and Awards

1. E. Bellas (Tufts):
    a. Fellows Leadership Council Chair, Armed Forces Institute of Regenerative Medicine (2010 – Present)
    b. Craniofacial Reconstruction Session Fellow Co-Chair, AFIRM Annual Meeting (Feb. 2012)
    c. Stem Cell Sources Session Student Co-Chair, TERMIS-NA Annual Conference (Dec. 2011)
    d. Travel Award to TERMIS-NA 2010 Meeting, Armed Forces Institute of Regenerative Medicine (Jun 2011)

Patents and Inventions

1. Each of the main technologies has been patented:
    a. Basic silk foams
    b. Basic silk gels
    c. Injectable foams

Peer-Reviewed Publications

1. Choi, J., Bellas, E., Gimble, J., Vunjak-Nova kovic, G., Kaplan, D. Lipolytic Function of Adipocyte/Endothelial Co-Cultures. Tissue Engineering Part A. 2011 May; 17(9-10): 1437-44.
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Project CF-5: Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle

Team Leader(s)	George J. Christ, PhD (Wake Forest)
Project Team Members	James Yoo, M.D., Ph.D., Sang Jin Lee, Ph.D., Benjamin T. Corona, Ph.D., Masood A. Machingal, Ph.D., Venu Kesireddy, Ph.D., Catherine Ward, Ph.D.
Collaborator(s)	Tom Walters, Ph.D., USAISR; David Kaplan, Ph.D., Tufts
Therapy	Autologous bioengineered skeletal muscle implant for functional reconstruction/repair of complex craniofacial injuries
Deliverable(s)	*Baseline:* An implantable tissue engineered muscle repair (TEMR) construct capable of restoring clinically relevant force/tension following a VML injury. *Revised:* None
TRL Progress	Start of program TRL #2 End year 3: TRL #4 End year 1: TRL #3 End year 4: TRL #4 End year 2: TRL #3 End year 5: TRL #4
Key Accomplishments:	Completion of the POC findings (physiological and histological) documenting the rat tibialis anterior VML injury model as an appropriate model for the evaluation of the tissue engineered muscle repair (TEMR) technology. Completion of pilot studies to evaluate a 1st generation tunable silk scaffold, as well as development and initial evaluation of 2nd generation silk scaffold guided by the initial round of observations both *in vitro* and *in vivo*. Further characterization of human muscle progenitor cells for derivation of a clinically applicable population of cells at up to the fourth passage
Keywords	tissue engineering, skeletal muscle repair, volumetric muscle loss, bioreactor, muscle progenitor cells, myoblasts, 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 by warfighters. Our long-term goal is creation of a skeletal muscle tissue implant capable of generating clinically relevant force/tension. Engineering skeletal muscle tissues de novo with the patient's own cells would accelerate wound healing with cosmetic augmentation of the tissue defect, and thus, enhance restoration of tissue function. This proposal will continue the development of a technology to further probe the feasibility and applicability of creating contractile skeletal muscle tissues through use of a bioreactor system in conjunction with novel biomaterials/scaffolds and optimized bioreactor protocols. The initial clinical application will be

repair and restoration of craniofacial battlefield wounds.

As we are currently finishing the fourth year of work and moving into the fifth year, our efforts thus far have been focused on Specific Aims 1, 2, & 4. We are awaiting a Pre-IND conversation with the FDA to determine if a large animal study will be required prior to our planned "first in man" study. If not, we will focus the resources originally devoted to the large animal study toward completion of the preclinical work required for an IND application. We remain on track with respect to all major timelines and deliverables as originally proposed.

The Specific Aims of the originally funded proposal are:

Specific Aim 1: Technical Objective: Demonstrate "proof of concept" for engineering functional (i.e., contractile) skeletal muscle tissue for craniofacial defects (Years 1-2). Task #1.1: To generate an organized muscle tissue in vitro (Year 1).

Task #1.2: Optimize scaffold/biomaterial and bioreactor protocol for engineering contractile skeletal muscle (Year 1-2).

Task #1.3: To characterize muscle tissue engineered in vivo in a mouse model (Years 1-2)

Specific Aim 2: Technical Objective: Feasibility Study: implantation of engineered skeletal muscle in a rat skeletal muscle replacement model (Years 2-3).

Task #2.1: To demonstrate that engineered muscle tissue can be used for the functional restoration of a tissue defect in a musculoskeletal rodent injury model (Years 2-3).

Task #2.2: To characterize muscle tissue engineered in vivo (Year 2-3).

Specific Aim 3: Technical Objective: Applicability Study: Implantation of engineered skeletal muscle in a large animal (dog) model of craniofacial defects (Years 4-5).

Task #3.1: To demonstrate that engineered muscle can be used for the functional restoration of a tissue defect in a craniofacial muscle injury model (Years 4-5).

Task #3.2: Structural, functional, and biomechanical assessment of the retrieved engineered muscle tissue (Year 4-5).

Specific Aim 4: Technical Objective: To determine the feasibility of using biopsies from human patients for the engineering of functional skeletal muscle (Years 3-5).

Task #4.1: Scaffolds and bioreactor protocols determined in Task #1 will be used to engineer functional human skeletal muscle tissue in vitro. (Years 3-5).

Task #4.2: Assessment of engineered human skeletal muscle function in vitro (Years 3-5).

A detailed description of last year's accomplishments, and a synopsis of the work for the 2 years prior to that, as well as development of the tibialis anterior (TA) volumetric muscle loss (VML) injury model can be found on pages 3-10 of last year's annual report. The following pages contain a brief description of the last 12 months of progress.

Research Progress

TEMR treatment for TA VML injury study: additional histological analyses

During year four the preliminary physiological and histological analyses of the initial TA VML injury model at 3-months post-implantation time point was completed. Preparation of the functional recovery data, tissue morphology, and histology into a manuscript was begun and completion is anticipated for Q2 of year 5. Comparison of the functional recovery data for the TEMR treated TA VML injuries with the BAM-scaffold-only treated injuries showed significant variability in recovery for the TEMR-treated constructs; ranging from nearly complete functional recovery in nearly ½ of the animals (n=6), to little or no recovery in the others (n=7; total); we are still determining the most physiologically relevant expression for comparison of the extent of recovery, and expect these analyses to be completed in Q1 of year 5. Nonetheless, the observed variability in the extent of functional recovery prompted further histological investigation. Thus, a pan-macrophage stain was conducted and highlighted the presence of a significant inflammatory response in the TEMR-treated TA VML injuries with poor recovery. In addition, myosin heavy chain staining showed thicker fibers had formed within the construct- beyond the native tissue interface of the TEMR-treated TA VML injuries with near complete recovery. An experiment is currently underway to determine if diminished cellular coverage during implantation of the sheet-like TEMR constructs (due to excessive handling during implantation relative to the ease of implantation in the LD VML injury model) is responsible for the observed inflammatory response and poor degree of recovery observed with some constructs implanted in this animal model. Clearly further analyses and investigations are required, but nonetheless, these initial observations suggest that a sheet-based scaffold design may be suboptimal for more 3D VML injuries; especially when compared to the more consistent functional recovery observed for the planar sheets-type injuries characteristic of the LD VML injury model. Hence, our continued focus below on development of alternative scaffolds for treatment of the full spectrum of craniofacial VML injuries.

Completion of initial investigations of tunable silk constructs for VML injury repair

As outlined in the SOW, in this fourth year of funding (2011-2012) silk has been the biomaterial of focus for exploration as a potential alternative source for the TEMR construct. Specifically, we tested 2 different iterations of the novel tunable 3D silk scaffold made by the Kaplan team at Tufts. Studies were conducted both in *in vitro* and *in vivo*. Specifically, the study which began in year 3 (p. 9, 3rd Annual AFIRM Report) was completed and morphological and histological analysis guided the creation and testing of a 2nd generation silk construct (Figure 1). While some modest degree of functional recovery was observed in rats implanted with the silk-based TEMR construct in the TA VML injury, upon retrieval of these tissues, the silk constructs showed a lack of integration with the native tissue. Furthermore, the histology portrayed a lack of integration and new muscle formation within or surrounding the constructs (Figure 1; n=2). Although not shown, cell seeded silk scaffolds for this 1st generation showed little or no functional improvement (n=3). These initial findings guided the design and implementation of a 2nd

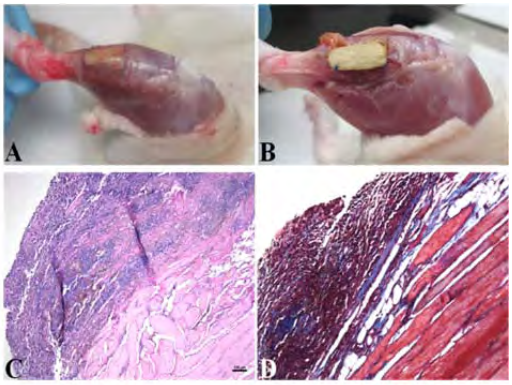

Figure 1. First generation silk scaffold morphology and histology three months after implant. External tissue morphology at the fascicular layer (A) and below the fascia at the muscular layer (B). Hematoxylin and eosin stain at 100x (C) and Masson's trichrome at 100x (D).

generation of silk scaffold. Researchers on the Kaplan team sought to increase the integration of the silk with the host tissue by increasing both the porosity and the degradation rate. These 2nd generation scaffolds were manufactured with and without the arginine-glycine-aspartic acid, RGD, cell attachment sequences. At the Christ lab, preliminary *in vitro* studies illustrated that both RGD and non-RGD coupled constructs were both capable of achieving cellular infiltration throughout the scaffold. An *in vivo* pilot study was conducted using non-RGD, non-cell seeded constructs. In contrast to the 1st generation, the 2nd generation scaffolds were more precisely fashioned to fit the contour of the surgically created VML injury- reducing the bulk and thickness of the implanted construct. Results from the initial functional testing indicated an average of ≅65% functional recovery at 8 weeks (n=2; in the absence of cell seeding). Retrieval of the tissues for morphological and histological assessment was carried out at 9 weeks post implantation. In stark contrast to observations with the 1st generation silk scaffold, there was a dramatic improvement in degradation of the 2nd generation scaffold, as revealed during explant and histological analysis at 9 weeks post-implantation (compare Figure 1 & Figure 2). As illustrated in Figure 2 there appeared to be excellent integration of the 2nd generation silk scaffold with the host tissue- even in the absence of cellular seeding at implantation. These preliminary results bode well for this 2nd generation scaffold and will be used to guide the continued development creation and implementation of future silk constructs. In summary, these constructs show promise as a scalable and translatable treatment for VML injuries.

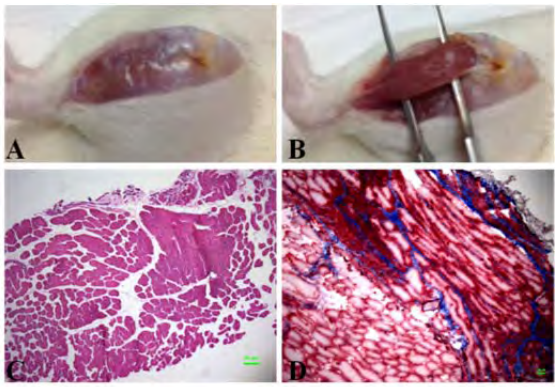

Figure 2. Second generation silk scaffold morphology and histology two months after implant. External tissue morphology at the fascicular layer (A) and below the fascia at the muscular layer (B). Hematoxylin and eosin stain at 100x (C) and Masson's trichrome at 100x (D).

Evaluation of phenotype and scalability of human muscle precursor cells.

In collaboration with the RMCC (Regenerative Medicine Clinical Center) team at WFIRM, the expansion, proliferation and phenotype of human MPCs has been characterized out to the fourth cell passage. Characterization of human muscle MPCs on BAMs caffolds and after preconditioning is presently underway.

Key Research Accomplishments:

- Completion of the POC findings (physiological and histological) documenting significant functional restoration (marginal to near complete recovery) of the rat tibialis anterior 3 months post-implantation of the tissue engineered muscle repair (TEMR) construct.
- Completed additional histologic al studies of sub-groups of animals (i.e., modest versus extensive functional recovery) in order to begin to ascertain, at least in part, the putative mechanistic basis for the large observed variability in functional recovery. These data will be used to guide further BAM-scaffold seeding protocol optimization and improved implantation techniques.
- Completion of *in vivo* pilot studies on two generations of silk scaffolds for TA VML injury that showed a ≅47 and ≅65% functional recovery at 2 months post-implantation, respectively.
- Conducted histological analysis of 1st and 2nd generation silk construct-tissue explants to decipher mechanisms of regeneration, integration with surrounding tissue, and to guide further manufacture of the novel tunable silk scaffolds.
- In collaboration with the WFIRM GMP facility has advanced the manufacturing process of this technology in human cells. In this coordinated work, human muscle progenitor cells (MPCs) were successfully expanded to a clinical ly relevant population in size and phenotype (Task #4.1). The report below provides a detailed outline of the progress made during this year.
- We have begun communication with the FDA to establish a date for a pre-IND teleconference to discuss the use of the TEMR BAM scaffold sscalable sheet-based technology for treatment of revision of primary cleft lip.

Conclusion

We have made significant progress and remain on target with respect to completion of all milestones/deliverables. The most important accomplishments this past year are initiation of contact with the FDA and demonstration of the feasability of silk as an alternative scaffold material for treatment of a spectrum of VML injuries.

a. Research Plans for the Last Year: We will use the guidance from the FDA per-IND teleconference to determine the definitive toxicology and final preclinical studies required for filing of an IND for use of the TEMR BAM scaffold technology for revision of primary cleft lip. We will continue development of the tunable silk scaffold to optimize for pre-implantation cell seeding as well as improved host tissue integration and functional recovery of the rat TA VML injury model.

b. Planned Clinical Transitions: There are 2 key development events planned for year 5. First, we are in the process of setting a date for a pre-IND teleconference with the FDA during the remaining portion of this study. Second, once that date is set we will prepare our briefing document for submission to FDA. Based on that conversation we still hope to achieve our goal of submitting an IND application within 6-9 months of completion of AFIRM I. Again, we will need to finish a definitive toxicology study prior to that submission. We anticipate that we will need additional funds in the first 6 months post-AFIRM I in order to complete

this study. With this information in hand, we will be in an excellent position to submit the IND by the end of the Q3 2013. In addition, we anticipate moving forward with a Gen II technology that includes SOPs designed to increase cellular density/phenotype during bioreactor conditioning and prior to implantation. Details of this approach can be found in a recent publication using leveraged funding from TATRC (Corona et al., 2012). These data will provide the pilot studies for our primary focus in our AFIRM 2 proposal. As noted in the prior annual report, we are hopeful that the FDA will accept the rat TA VML injury model as the definitive pre-clinical study required prior to human trials. If so, we could eliminate the canine studies planned for year 5, and accelerate the remaining toxicology, manufacturing and Gen II studies. Finally, as noted above, we have decided to pursue a first in man study for cleft lip rather than Bell's Palsy, as we believe this indication better suits our initial technology in the VML patient population described in the recent article by Grogan and Hsu (3). Nonetheless, we will continue to discuss and refine the clinical development plan internally at WFIRM, as well as in consultation/collaboration with our colleagues at ISR and within the Craniofacial program consortium (Drs. Wong and Freeman at Houston).

Corrections/changes planned for next year and rationale for changes: As noted elsewhere in the text, there is a course corrections for year 4:

- shift of the first in man application to cleft lip from Bell's Palsy.

Conflict of Interest Disclosure: N/A.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	1
# Post docs	3	
# grad students	1	
# undergrad students		
# staff members working for AFIRM		3

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	
# Inventions disclosed	
# Patents awarded	
# Peer reviewed publications	2
# Non-peer reviewed publications	1

*Directly supported in whole or part –time by AFIRM

Honors and awards: None

Patents and Inventions: None

Peer-Reviewed publications:

Machingal MA, Corona BT, Walters TJ, Kesireddy V, Koval CN, Dannahower A, Zhao W, Yoo J, Christ GJ. A Tissue Engineered Muscle Repair (TE-MR) Construct for Functional Restoration of an Irrecoverable Muscle Injury in a Murine Model. Tissue Eng Part A. 2011 May 6. [Epub ahead of print]

*Corona BT, Machingal MA, Criswell T, Vadhavkar M, Dannahower A, Bergman C, Zhao W, Christ GJ: Further development of a tissue engineered muscle repair (TEMR) construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss (VML) injury. Tissue Eng Part A. 2012 Mar 22. [Epub ahead of print] PMID: 22439962.

*-this publication is directly applicable to continued development of the AFIRM TEMR technology, but was developed using leveraged TATRC funding.

Other Publications:

1. Andersson KE and Christ GJ: Introduction to Regenerative Pharmacology. A primer on the role of pharmacological sciences in regenerative medicine. In: Regenerative Pharmacology, Cambridge University Press, KE Andersson and GJ Christ, Eds., (In press), 2012.

Overview of the Burn Program

Program Leader – James H. Holmes IV, MD

The unmet needs for improving the treatment and outcomes of burn-injured military service personnel are unquestioned and warrant immediate amelioration, as wounded warriors continue to return from abroad with devastating burns. The current standard of care for burn injuries remains early excision and auto-grafting, and has not fundamentally changed in over 30 years. The multi-national, multi-institutional, and multi-disciplinary WFPC Burn Program is poised to answer the call and deliver innovations that will undoubtedly alter the way burns are managed. The principal "thrust" of the WFPC Burn Program is aimed at significantly advancing the operative management of burn injuries, as burn wound "closure" remains the single greatest threat to the burn-injured warfighter.

Project BS-1: Tissue Engineered Skin Products- ICX-SKN

Team Leader(s)	Vincent Ronfard, Ph.D. (Healthpoint Biotherapeutics, Ltd.), Paul Kemp, Ph.D. (Intercytex Ltd),
Project Team Members	Dennis L. Carson, Ph.D., D.A.B.T., Kathi Mujynya Ludunge B.S./MBA, Sarah Ramsay, M.S. John Lovelady PhD, Jonathan Elwell, Sarah Drinkwater
Collaborator(s)	Penny Johnson PhD, Clare Lovelady PhD
Therapy	A permanent dermal skin graft replacement (ICX-SKN) which can be integrated and remodeled by the host for burns
Deliverable(s)	*Baseline:* Initiate human clinical evaluation of ICX-SKN by Q3 2013
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #2/3 End Year 1: TRL #1 End Year 4: TRL # 2/3 End Year 2: TRL #2
Key Accomplishments:	During the past 4 years Intercytex first, and then HealthPoint Biotherapeutics, Ltd with Intercytexhave developed the Tissue Engineered Skin Products- ICX-SKN. Extensive work on characterization in year 3 helped to define the product for manufacturing process control and final product specifications and this has been used in year 4 to examine the raw material requirements as a pre-requisite to developing a CMC section for the IND application which we expect to submit in 2012. As part of this we have determined that bovine serum and certain growth factors can be removed and the fibrin content reduced without adversely affecting the final product. This helps to identify which components are needed for ICX-SKN production as well as improving the safety profile of the product and significantly reducing the cost of goods of the final construct which will greatly improve the options available in the upcoming clinical trial.
Keywords	burn, matrix, skin graft replacement, human dermal fibroblasts, fibrin

Introduction

The need exists for an "off the shelf" skin replacement that is instantly available and alleviates the need to take full thickness skin grafts. Several so-called "living skin equivalents" (LSE) and "living dermal equivalents" (LDE) approved by the FDA are currently available. Although these materials, such as Apligraf, Dermagraft and Orcel, work as artificial skin grafts, in reality no current living product meets the rigorous requirements necessary to accomplish this function. Rather, the dermal component of these products is rapidly degraded in the wound environment, releasing the cells, which then contribute to wound healing by secondary intention.

In contrast to these earlier LSEs and LDEs that have used either a pre-formed collagen matrix or biodegradable synthetic mesh as the initial support system, the intent from the start of this program (ICX-SKN) was to develop a more biologically robust extra-cellular matrix by allowing the fibroblasts themselves to produce the material in-vitro. Others have shown that fibroblasts allowed to grow to super-confluency in-vitro are able to synthesize a relatively strong extra-cellular matrix, but the resulting material is extremely thin and fragile.

The intent of this program, therefore, was to extend the findings of Neidert et al (1), who have shown that cells grown within a fibrin scaffold gradually remodeled this scaffold into a cell-synthesized matrix. A freeze-drying process has been developed that consistently produces freeze-dried 10x10cm dSKN intermediates to ICX-SKN product specifications, including the ability of the matrix to sustain viable HDFs (i.e. cell friendliness), the ability to withstand manual manipulation, adequate mechanical strength and appropriate resistance to collagenase B digestion after storage. A regime of thermal treatment and primary drying steps with temperatures and hold times was created which could ultimately be transferred to a contract manufacturer with GMP accreditation to successfully freeze-dry batches of 10x 10cm pSKN intermediates. ICX-SKN has been shown to be a new generation off-the-shelf dermal replacement (2). The intended use for burn injury treatment is the simultaneous application ICX-SKN and autograft in a single step procedure.

Research Progress

A requirement before any clinical study can begin on either a Medical Device or Biological is the submission of a Chemistry, Manufacturing and Controls (CMC) dossier and part of this is the justification of the necessity of each component in the product. For this reason and for the ongoing need to minimize cost of goods of the ICX-SKN a series of alternate compositions needed to be evaluated and compared.

Simple Casting Chamber for measurement of composition alternatives

The dish prototype that has been developed in year 3 for production of first in man clinical samples (2.5 X 3.5 cm) requires 3.7ml of cast media. This means that an extensive series of cast composition change experiments would be costly in terms of raw material and would produce constructs that were larger than needed at this stage of the process. A transwell alternative was therefore assessed for this stage of the development which used 50% less starting material.

Manufacturing

Total removal of serum and TGF B
Reduction in process time (7 wks reduced to 5 wks)
Reduction in Fibrin/Thrombin (Tisseel)

We have stepwise reduced the amount of fibrinogen and thrombin with the unexpected result that the resulting constructs appear to be more robust (i.e. stronger) when compared directly to those cast at the same time to the original formulation.

Removal of freeze drying step

Currently, we have eliminated consideration of freeze-drying. We have determined that, once harvested after five-week maturation, constructs can be stored in a shipping/storage medium for up to 21 days at 2-8 ^{o}C (in dark conditions) and retain viability as determined by Alamar Blue. Further tests are ongoing to determine if this can be extended to 28 days. In addition, we will determine if viability can be maintained after storage at ambient temperature (~23^{o}C).

Shipping

Constructs were shipped from ICX (Manchester, UK) to HP (TX, USA), and based on Alamar Blue assays performed after arrival at HP the constructs remained metabolically active during shipping.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry measure the energy required to heat a sample. If the sample undergoes a phase change as happens when a collagen triple helix is denatured then this phase change can be exothermic or in the case of collagen denatuturation, endothermic. DSC analysis of the samples produced during the compositional modification studies is ongoing.

Histology of the construct

The new formulation of ICX were produced in the UK, shipped and tested in the pig in the US into excised porcine burn wounds.

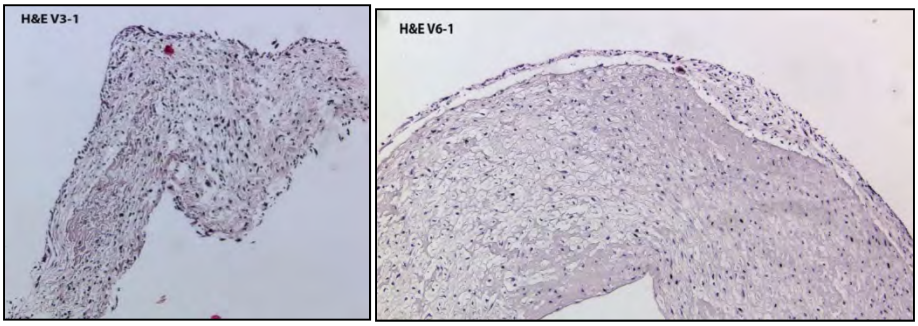

Figure 1. H &E staining of ICX V3 and V6 100X

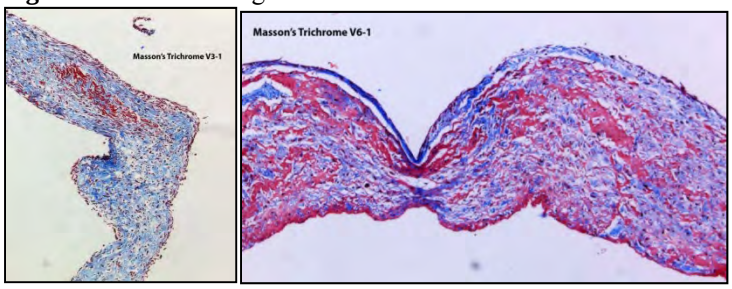

Figure 2. Masson's trichrome staining of ICX V3 and V6. 100X

Pig Study

In alignment with the approved protocol, sixteen burn wounds were generated on the dorsum of two pigs using 2 cm diameter heated brass rods. After 24 hours, the burned tissue was excised and the resultant open wounds were treated with one of five treatments. The study plan was to sample one pig after a week and the other after three weeks.

Preliminary Study Results

The burn wounding and excision process proceeded in alignment with the design and historic studies. Figure 3 shows images of a representative burn wound, an excised wound, and the excised wound with the wound edge marked in black (first row of photos). Lower panel photographs shows an ICX-SKN treated wound with the non-stick dressing and benzoin resin applied, followed by the application of moist gauze and the transparent film dressing.

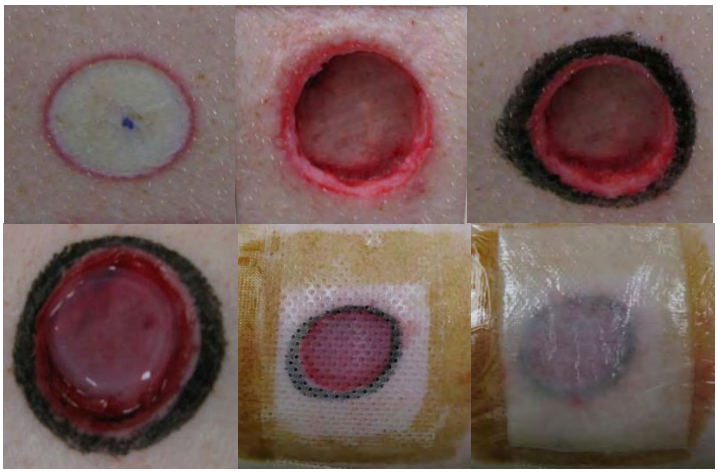

Figure 3: Wounds before treatment and after ICX-SKN treatment

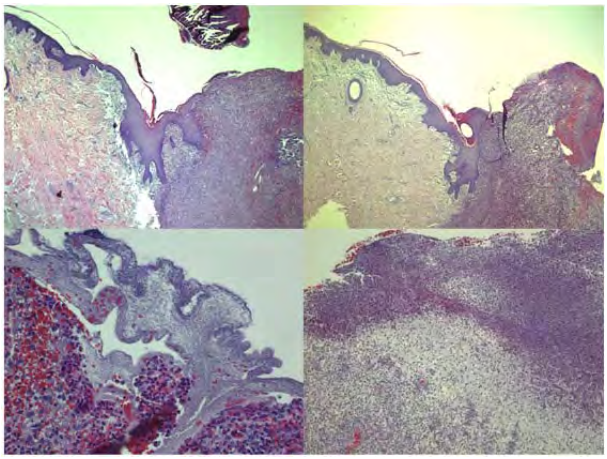

Figure 4: H&E stained tissues showing robust granulation of the wounds after a week (upper images, 40X), possible ICX-SKN remaining at the surface of one wound (lower left, 400X), and a region of lower cell density in a granulation tissue bed (lower right, 100X**)**

Augmentation of research capacity

In order to expand and advance our research capacity, in December of 2010 several scientists at our Fort Worth TX facility were trained in the manufacturing process of ICX-SKN. The future development involves manufacturing capabilities in both Fort Worth, TX and Lausanne, Switzerland.

Clinical development

It is the goal to have a pre-IND meeting with the FDA in early fall of year 2012.

Key Research Accomplishments

Important progress has been made in developing new version of ICX. These versions are less expensive to produce keeping similar biocompatibility as demonstrated by the preclinical results obtained on the pig burn model. This gives us confidence to move forward the development of this product and meet with the FDA for a pre-IND meeting. We believe the combination of cells, ECM produced matrix and human fibrin would provide an ideal dermal substrate allowing rapid vascularization and optimal take of skin autograft in one step procedure.

Conclusions

Collaboration between Intercytex and Healthpoint Biotherapeutics should allow us to achieve this goal. Moving forward, Healthpoint Biotherapeutics will continue to produce ICX-SKN at 2 different sites. The immediate need is to pick the lead candidate and evaluate it in the pig burn model at the end of the year. The lead candidate from these studies will be selected for testing in humans. As this project moves forward, interactions with the FDA will begin as plans are finalized for an IND and the start of the clinical trial in 2013.

Planned Clinical Transitions

The project plans include evaluating the ICX-SKN in humans. An IND will be assembled and filed with FDA. Thus, once the lead candidate is selected, the development of the IND will begin. This will require the completion of an appropriate preclinical package along with a clinical protocol. In addition, the protocol will have to be approved through the appropriate IRB for the clinical site(s).

Corrections/Changes Planned

Healthpoint Biotherapeutics purchased the ICX-SKN assets in February 2010 and has kept the program moving forward. The technology transfer and validation of the master cell banks has been accomplished. Since that date all research and collaborative effort between Intercytex and

Healthpoint Biotherapeutics has been performed without DoD funding. This represents one year and 6 months of company commitment. In May 2011, Healthpoint Biotherapeutics received the notification of the AFIRM grant agreement. This recent grant approval will provide the necessary resources to accelerate the development of ICX-SKN as a novel treatment for burn injuries incurred by those in the military and the public in general. The uncertainty of the grant approval and the delay of the response drastically reduced the progress of the project. This is the main reason Healthpoint Biotherapeutics is asking for approval of a new timeline with the proposed start of the clinical trial in Q2 2013 (see new timeline below).

Conflict of Interest Disclosure

None

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2. Boyd M, Flasza M, Johnson PA, Roberts JS, Kemp P. Integration and persistence of an investigational human living skin equivalent (ICX-SKN) in human surgical wounds. Regen Med. 2007 Jul;2(4):363-70.

Project BS-3: 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

Team Leader(s)	**Team Leader(s):** Jörg C. Gerlach, M.D., Ph.D. (University of Pittsburgh)
Project Team Members	Patrick Over, Matthew Young, and Roger Esteban, Ph.D. (University of Pittsburgh)
Collaborator(s)	James Holmes, M.D. (Wake Forest) and Steven Wolf, M.D. (USAISR)
Therapy	Skin stem cell delivery for cell-based treatment of burn wounds
Deliverable(s)	*Baseline:* (1) Optimized cell isolation and spraying methodologies and (2) Next generation skin gun FDA-appr oved spray device that can deposit fetal skin stem cells onto wound surfaces
	Revised:
TRL Progress	Start of Program: TRL 1 End Year 3: TRL 3 End Year 1: TRL 1 End Year 4: TRL 4 End Year 2: TRL 2
Key Accomplishments:	The research group has established an antibody m arker characterization panel for fetal and adult epid ermal cells and demonstra ted the differentiation stage of various skin cells and stem cells, as previou sly proposed. Fetal epiderm al and dermal progenitor isolation, *in-vitro* culture, expansion and cell banking are initiated acc ording to plan. The group also s ubmitted the docum ents necessary for the spray device FDA 510K approval.
Keywords	Skin stem cells, burn wounds, human fetal tissue, progenitor cells, cell spray method

Introduction

The survival of combat victims with larger related burns is of ten limited, since large burn areas reduce the availability of healthy donor skin areas for split-s kin 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 treatm ents for burns, other than m esh grafting, do not speed up epidermal reepithelialization time and do not 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 th e wound and varies from conservative therapies in smaller second-degree burns to split-skin m esh-grafting, cadaveric, and artificial skin sheet coverage in third-degree burns. Mesh grafting is the gold standard, but donor area is lim ited and it also g enerates an add itional wound, both of wh ich are p roblematic in larg er combat-related burns. Ratios between donor area sizes, com pared to treatable mesh-graft size, are typically 1:3, and only in large area cases are up to 1:8. The results beco me worse with the inc rease of this ratio. Regenerative medicine research provides cell-based therapies that are design ed to improve

wound healing by offering viable cells that accele rate regeneration and re duce 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 epider mal stem cells f rom the basal layer of the epidermis to be successful. In full thic kness third-degree wounds, additionally derm al mesenchymal stem cells are to be considered to compensate for the dermis loss.

The Gerlach group has developed sk in-cell isolation techniques fo r epidermal and dermal stem cells from adult and fetal tissues, along with ot her methods and devices to im prove single cell spray grafting. Applications us ing autologous cells for partial- thickness burns w ere reported both in Europe and the United St ates. In addition, the group e xplores cell spray grafting of dermal cells to address third-degree burn therapy. Adult- and fetal-derived epidermal and dermal progenitor cells were com pared and off-the-shel f product develop ment was initiated. In this report, the group provides progre ss data on fetal and adult epid ermal and dermal cell studies *in vitro*.

Research Progress

1. Adult skin progenitor cells.

This year the group focused on characterizing epid ermal progenitors from adult (autologous) cell isolations for cell spray deposit ion. The group also investigat ed dermal cell isolation and performed progenitor characteriza tion, cell culture behavior and stemness studies, in order to address third-degree burn therapy development.

1.1. Adult epidermal keratinocytes

The group has defined a panel of markers for different keratinocyte differentiation stages in order to identify the isolated cell quality. This panel includes two basal keratinocyte stem cell markers (CK15 and α6integrin), two early differe ntiation stages that (CK5 and CK13), and one late differentiation stage m arker (LOR). The re sults were com pared to skin biopsies by immunofluorescence assays. The combination of CK15 and α6integrin in stacked layer confocal immune microscopy confirms the undulated basal layer and revealed the irregular distribution of basal keratinocyte over the inner surface of the epidermis (*stratum basale*).

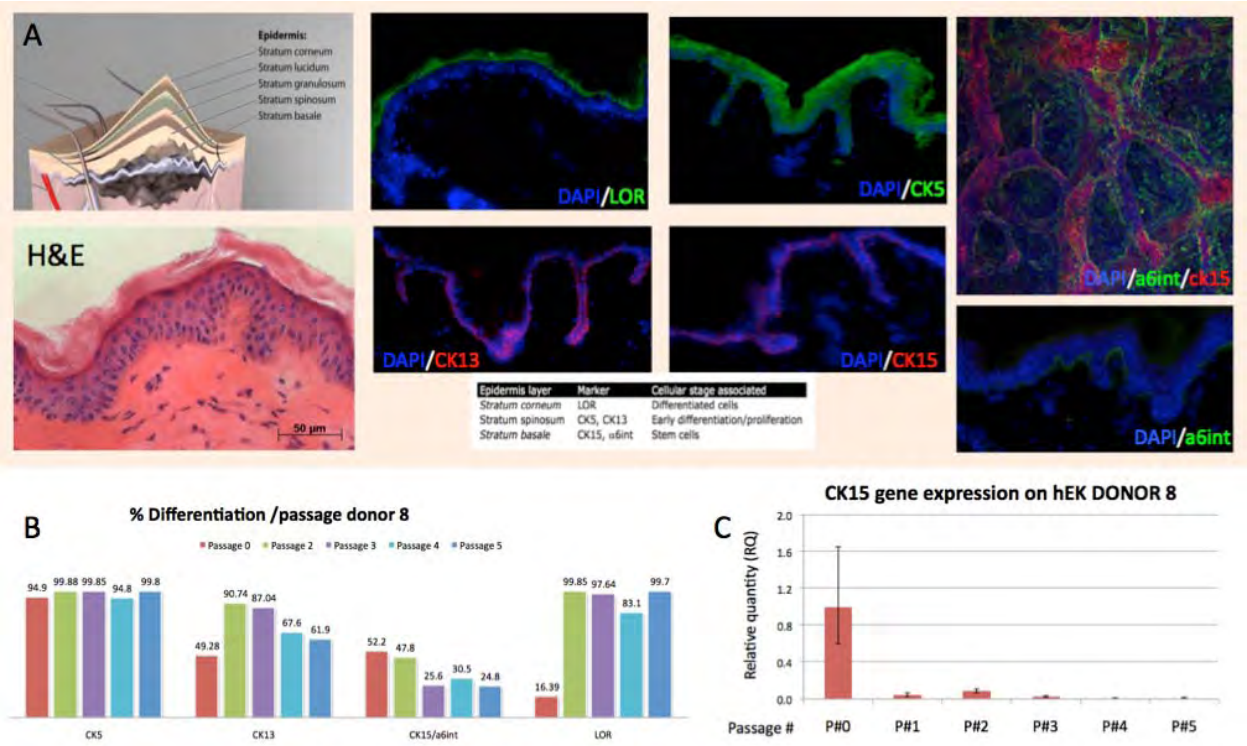

Figure 1. Antibody markers for keratinocyte diferentiation stage (A). Flow cytometry results on differentiation markers for 5 passages on donor 8 (B). Donor 8 gene expression results for basal keratinocyte marker CK15 during 5 passages.

Further studies using the differentiation stage markers on cells in culture showed the effects of *in vitro* techniques on cell differentiation. The group has compared isolated keratinocytes from biopsies with cultured cells during several passages. Using the differentiation stage markers in a combination with flow cytometry (Figure 1.B) and real-time PCR (Figure 1.C) a progressive differentiation along the passages with an increase of the differentiation markers (CK5, CK13, LOR) was described. It was also shown that the *in vitro* culture effect decreases the stemness marker expression (CK15/α6int) depending on culture passages and time. These results have implications for the cell-banking activities and the choice of adult versus fetal derived progenitors.

This analysis can also be used to establish a quality control database for predicting autologous cell isolation results in an on-site setting that are thought to depend on the patients age, lifestyle (ethanol), previous diseases (diabetes), and medication.

1.2. Adult dermal fibroblasts

Previously, the group investigated properties of dermal fibroblasts for their potential use in clinical studies on full-thickness burns. The group isolated progenitor cells from dermis during epidermal cell spray-grafting procedures at the UPMC Mercy Hospital Burn Center. Previously

developed techniques were used. The group also studied derm al progenitor isolation from full thickness skin obtained from aesthetic surgery donations in collaboration with Peter Rubin, M.D.

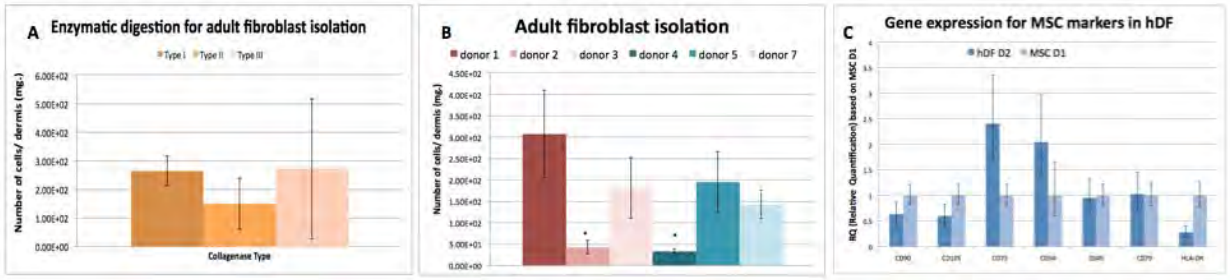

Figure 2. Number of fibroblasts isolated after collagenase treatment per mg. of dermis (A). Number of fibroblasts isolated per mg. of dermis (B). Analysis of MSC markers for gene expression performed on adult dermal fibroblasts.

The group concluded that there is no significant difference am ong 3 tested collagenase types (Figure 1.A). The am ount of dermis to be dige sted in re lation to the volume of e nzyme used turned out to be im portant. As can be observed in Figure 2.B, higher am ounts of dermis liberate lower amount of cells (m arked with asterisk) in com parable amounts of solution. Thus, the dermis thickness and the enzyme volume will impact the cell isolation results.

The group started derm al cell isolations mim icking an on-site procedure during clinical epidermal cell iso lations. In order to standardize the ex periments according to the regular practices performed in the hospi tal, the group used a derm atome with 8/1,000 of an inch to obtain the sam e thickness of skin from aesthetic specimen donations as being used for burn patients. These results confirm previous work in Berlin, Germany and can be used to advance autologous dermal reconstruction for third-degree burn wounds.

2. Fetal skin progenitor cells.

This 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.

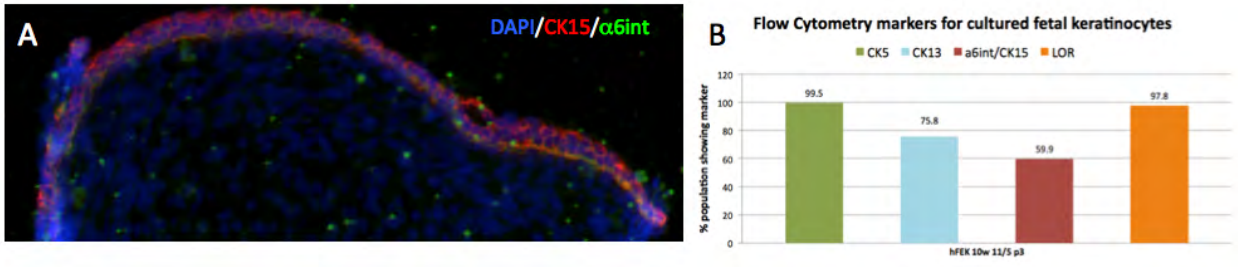

Figure 3. Immunofluorescence staining on a 10 weeks fetal human biopsy for markers a6int and CK15 (A). Flow cytometry results for differentiation stage to detect the effect of differentiation during in vitro culture cell expansion.

2.1. Fetal epidermal keratinocytes

The group analyzed stemness antibody markers (a6int and CK15) to determinate the differentiation degree of fetal keratinocytes (Figure 3.A) in tissue biopsies before *in vitro* cell growth.

This information was needed in order to implement isolated cell flow cytometry and gene expression analyses. Cells were seeded in *in-vitro* culture Petri dishes for expansion to obtain sufficient cell numbers for analyses and cell banking. After three passages, the effect of *in vitro* culture was analyzed on fetal keratinocytes. The cultured keratinocytes population showed around 60% of the stemness markers a6int and CK15. Interestingly, the amount of differentiation markers (e.g. CK13, C5 and LOR) is high as well after the *in vitro* culture.

2.2. Fetal dermal cells

The group collected more data about the culture behavior of dermal cells in order to get more information about their progenitor potential, which is of interest for wound healing in full-thickness burns. The results suggest that isolated fetal tissue derived "fibroblasts" include a population that shows the same markers (Figure 4.A) as Mesenchymal Stromal Cells (MSC), as published by the International Society for Cellular Therapy, ISCT (Dominici et al., 2006) [2] Nevertheless, not the entire population showed MSC markers.

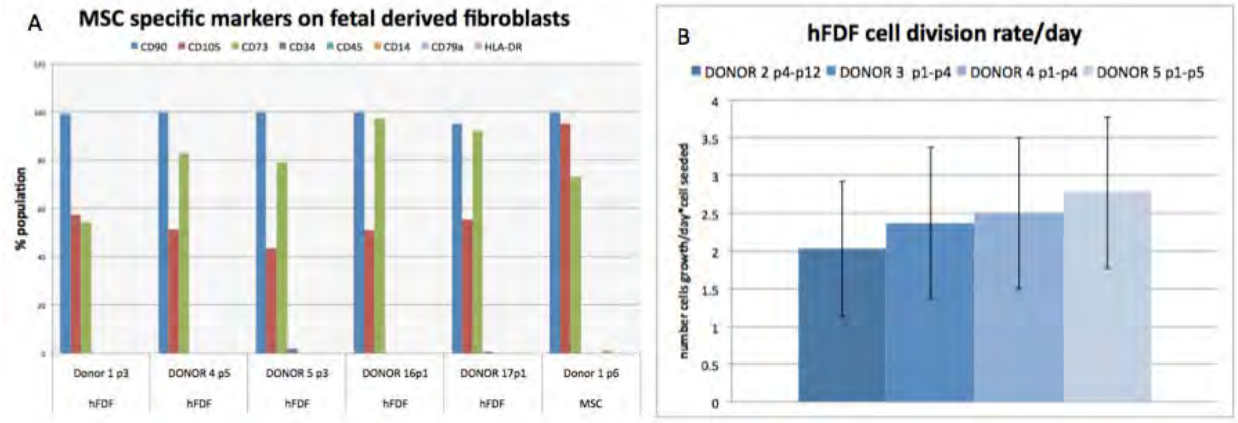

Figure 4. Results of flow cytometry data showing Mesenchymal Stromal Cell specific markers on different populations of fetal derived fibroblasts. (A). Cell division rate for different donors.

The group started isolating cells from different donors, expanding them *in-vitro* and cryo-preserving them after several passages in liquid nitrogen tanks for a dermal cell banking. These cells showed a reproducible and sufficient cell expansion rate (Figure 4.B) making them suitable as an off-the-shelf product.

[2] Dominici M, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; (8) 4:315-317).

3. Skin cell spray deposition device for grafting, "Skin Gun" prototype

The group has tested successfully the new prototype of SCS Berlin for experimental cell spray deposition. Parallel work in the framework of Innovative Practice in the UPMC Mercy Hospital Burn Unit demonstrated clinical feasibility, practicability, and reproducibility with superior results of the application over the ReCell sprayer of Avita. The group also initiated a FDA 510K process approval. With the help of Mr. Bold of SCS Berlin, the technical documentation, functional evaluation and a risk analysis could be performed; and with the help of the regulatory affair consultant Patsy Trisler, all documents for the process could be compiled.

Key Research Accomplishments

- Established and tested an antibody panel for adult as well as fetal epidermal cell differentiation evaluations.
- Established methods for and determined the differentiation stage on isolated adult and fetal keratinocytes using flow cytometry and gene expression.
- Established isolation methods for adult dermal cells in parallel to epidermal cells in an on-site setting during a regular procedure for cell based therapy for skin wound healing.
- Determined the quality of fetal keratinocytes and dermal cells after cell growth, during a cell expansion process to enable cell banking.
- Submitted documentation and application to the FDA for a "Skin Gun" 510K approval.

Conclusions

The group concludes that the use of primary adult keratinocytes that are isolated in an on-site setting exhibit a higher number of basal keratinocytes as well as colony forming capability, and thus providing a higher potential for wound healing over *in vitro* cultured/expanded keratinocytes. For the use of autologous cells, we conclude that on-site cell isolation and spraying is superior to the use of expanded cells (such as with the Genzyme Protocol). The group also demonstrated a high content of epidermal progenitors, suggesting that its own method is competing well with the ReCell method. Additionally, for epidermal cells, the group also established an autologous dermal cell isolation technique, that may address third-degree burn healing.

The results on fetal tissue-derived cells suggest that fetal epidermal and dermal progenitor cells cultured *in vitro* do not represent pure stem cells populations. While the dermal progenitor expansion and cell banking results are very promising, the fetal epidermal progenitor expansion is not yet satisfying. Some further method refinement and studies are required to obtain sufficient data for a decision on using either fetal or adult cells for the anticipated final work on product development for cell-based wound healing therapies.

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	1	0
# Post docs	1	0
# grad students	0	0
# undergrad students	0	0
# staff members working for AFIRM	2	0

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	1
# Patents awarded	1
# Peer reviewed publications	3
# Non-peer reviewed publications	3

*Directly supported in whole or part –time by AFIRM

Honors and Awards: None this reporting period

Patents and Inventions: Skin Gun device of SCS patent pending

Peer-Reviewed Publications

1. Gerlach JC, Johnen C, Ottom an C, Bräutigam K, Plettig J, Belfekroun C, Münch S, Hartmann B. Method for autol ogous single skin cell isolati on for regenerative cell spray transplantation with non-cultured cells. Int J Artif Organs. 2011; 34(3): 271-9.
2. Gerlach J, Johnen C, McCoy E, Bräutigam K, Plettig J, Corcos A. A utologous skin cell spray-transplantation for a deep derm al burn patient in an ambulant trea tment room setting. Burns 2011; (37) 4:e19-e23
3. Chista Johnen; Cinzia Chinnici; Fabio Triolo ; Jörn Plettig; Kirten Bräuigam; Giandomenico Amico; Matthew Young; Patric k Over; Roger Esteban-Vives; Morris Turner; Robert Thompson; Eva Schm elzer, Ph.D., Prof.; Katrin Zeilinger; Peter Rubin; Giovanni Vizzini; Pier-Giulio Conaldi; Bruno Gridelli; Jörg C. Gerlach, M.D., Ph.D. Phenotypical characterization of 6 - 2 1 week gestational age hum an dermis and epiderm al cell isolation methods for in vitro studies on epidermal progenitors. Submitted to Burns 2012

Other Publications

1. Esteban-Vives R, Young M, Chinicci C, Plettig J , Johnen C, Triolo F, Turner M, Tho mpson R, Over P, Rubin P, Am ico G, Ge rlach J. De livery of Stem Cells to a Burn W ound via a Spray Device - Exploring Human Fetal Skin Progenitor Cells for Regenerative Medicine Cell Based Therapy. All-Hands meeting Poster 2011.
2. Esteban-Vives R, Young M, Chinicci C, Plettig J , Johnen C, Triolo F, Turner M, Tho mpson R, Over P, Rubin P, Am ico G, Gerlach J. Deli very of Epidermal and Dermal Stem Cells to a Burn Wound - Exploring Human Fetal Skin Progenitor Cells for Regenerative Medicine Cell Based Therapy. All-Hands meeting Poster 2012.
3. Esteban-Vives R, Young M, Chinicci C, Plettig J , Johnen C, Triolo F, Turner M, Tho mpson R, Over P, Rubin P, Am ico G, Gerlach J. Deli very of Epidermal and Dermal Stem Cells to a Burn Wound. University of Pittsb urgh. Postdoctoral meeting. Poster 2012. Best Poster Award.

Project BS-4: Novel Keratin Biomaterials That Support the Survival of Damaged Cells and Tissues

Team Leader(s)	Mark Van Dyke, PhD, Assistant Professor, Wake Forest School of Medicine
Project Team Members	Deepika Poranki, MS (Graduate Student); Carmen Gaines, PhD (Postdoctoral Fellow), Olga Roberts, PhD (Research Associate Professor)
Collaborator(s)	Jimmy Holmes, MD (Faculty, WFSM), Joseph Molnar, MD, PhD (Faculty, WFSM), Justin Saul, PhD (Faculty, WFSM), Mark Lively, PhD (Faculty, WFSM), Luke Burnett, PhD (Scientist, KeraNetics LLC), Rochede Guzman (Postdoctoral Fellow, WFSM), Michelle Merrill (Undergraduate Student, WFU)
Therapy	Burn
Deliverable(s)	*Baseline:* Keratin biomaterial-based burn treatment development and preclinical testing *Revised:* None
TRL Progress	Start of Program: TRL #3 End Year 3: TRL #4 End Year 1: TRL #3 End Year 4: TRL #5 End Year 2: TRL #3
Key Accomplishments:	The previously developed swine burn model was utilized for a definitive preclinical study. Analyses of tissue samples and other data is complete and the results are being written up in several manuscripts for publication. Data has been submitted to CDRH/FDA and a pre-IDE meeting was held. Agreement was reached that the KeraStat™ Burn will be regulated as a device and is eligible for 510(k) clearance. Manufacturing under Quality System Regulation (QSR) guidelines has been implemented and validated at a partner company, KeraNetics LLC. A formal IDE application is being prepared for submission in 3Q2012.
Keywords	burn, keratin, biomaterial, hydrogel, swine, pig, gel, dressing, wound, skin, TBSA, tissue salvage

Introduction

Burns are one of the most catastrophic, traumatic injuries to treat. In the United States, approximately 2.4 million burn injuries are reported each year. Burns also account for 5% to 20% of conventional war causalities. The cost of burn treatment products worldwide in 2008 was $2.1 billion dollars.[1] This number is for treatment products only and does not take into account other patient care costs, which reach into the tens of billions of dollars annually. For burns requiring hospitalization, the standard of care often involves a period of wound care and observation until wound demarcation is complete and the burn surgeon can make a determination as to the need for excision of dead skin and grafting. This "wait and see" period is based on the process of conversion, where damaged cells "convert" from thermally stressed to dead tissue, and has been characterized according to Jackson's thermal wound theory, which states that burn injuries have 3 zones: Zone of Coagulation – a necrotic zone where the original injury occurred;

Zone of Stasis – area which surrounds the necrotic zone and has potentially viable cells that will survive if proper treatment is given; and Zone of Hyperemia - where the tissue recovers within 7-10 days if further infection does not occur.[2] In many patients the zone of stasis converts to dead tissue and must be removed, both because it is a necessary step in closing the wound and because the apoptotic cells produce a number of biochemical factors that elicit untoward systemic effects that can lead to death of the patient days after the initial burn injury (e.g. multiple organ failure). A high correlation between total body surface area (TBSA) burned and mortality has been firmly established with some authors reporting that every 1 percent increase in TBSA leads to a 5-10 percent increase in mortality.[3,4] Importantly, there are currently no available treatments that act primarily in the zone of stasis and decrease conversion, thereby reducing TBSA. The standard of care in burn treatment is to cover the wound to avoid infection, and there are numerous products on the market that do so quite acceptably. Silver-containing dressings and creams such as those containing silver sulfadiazine are highly utilized. However, since TBSA correlates so strongly with mortality, it follows that any treatment that effectively reduces TBSA will save lives and reduce the cost of burn care.

Keratin biomaterials have typically been described in the literature in terms of the procedures and processes used to extract them. Extracts of hair, wool and feathers were often "characterized" as heterogeneous mixtures, with estimates of more than 100 homologs being potentially present in wool extracts, for example.[5] More recently, human hair fibers have been described as consisting of 17 type I and type II alpha keratins that exist as obligate heterodimers,[6] as well as 85 matrix proteins termed keratin-associated proteins or KAPs.[7] Dr. Van Dyke's group at Wake Forest School of Medicine has spent the past eight years developing and patenting methods to isolate and purify these keratins to the level of sub-types that possess structural properties common to each. In so doing, several characteristics of the materials emerge that are not manifest in the more heterogeneous fractions. For example, alpha keratins can be separated using techniques such as dialysis and chromatography to the level of K81/K31 and K81/K33 dimers. These dimers have been shown to have the capability to form stable tetramers in solution,[8] a property that likely contributes to the strong self-assembly characteristics of these samples. Importantly, our recent work has shown that peptide by-products of the oxidation/extraction process, which have been identified using proteomic techniques, have the capacity to rescue cells from thermal damage by suppressing the up regulation of cell death signaling genes.

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, etc.). Control over this platform exists in the ability to reproducibly manufacture keratins on a large scale under QSR standards and in the intrinsic ability of keratins to self-assemble into predictable secondary and tertiary structures.[11,12] For the first time, novel keratin biomaterials can be produced in large quantities that self-assemble into matrices that can promote specific cell behaviors such as survival after thermal injury.

Development of a keratin biomaterial hydrogel-based wound dressing, KeraStat™ Burn, has been performed over the past eight years at the Wake Forest University School of Medicine (WFUSM) by Dr. Mark Van Dyke in collaboration over the past four years with KeraNetics

LLC. Prior investigations by Dr. Van Dyke have included the general biocompatibility of keratins, cell growth experiments in various culture systems, resuscitation fluids in small and large animals, bone regeneration in rodents, and both chemical and thermal burns in rodents and swine, respectively. Much of this research has demonstrated the cell and tissue compatibility of the oxidized form of keratin, keratose, which is the base material of the KeraStat Burn product. Numerous publications have demonstrated the finding that keratin biomaterials are compatible with cells and tissue, and can facilitate regeneration.[13]

Keratose has also been shown to be biocompatible by ISO 10993 safety tests conducted at Toxikon Corporation, a Contract Research Organization (CRO). These tests included MTT Cytotoxicity, Intracutaneous Injection, Klingman Maximization, Systemic Injection, Reverse Mutation Assay, Rabbit Pyrogen and Total Bioburden. In addition, Dr. Van Dyke's lab has conducted two pilot animal studies to demonstrate the feasibility of using keratin biomaterials for burn treatment: A mouse chemical burn model (**Figure 1**) and thermal burns in swine (**Figure 2**). These data demonstrate the feasibility of using a keratose hydrogel dressing to provide coverage to tissue and stabilize wound size (i.e. stop burn conversion and suppress an increase in TBSA). These data suggest that KeraStat Burn is able to facilitate the survival of tissue in Jackson's zone of stasis following burn injury. The goal of this Armed Forces Institute of Regenerative Medicine (AFIRM) project is to translate and commercialize the KeraStat Burn product so that it can be used for treatment of burned soldiers and civilians. To that end, the following aims are being pursued:

Specific Aim 1. To investigate the thermoprotective characteristics of keratin biomaterials *in vitro*(Years 1 and 2)

Specific Aim 2. To test the thermoprotective characteristics of keratin biomaterials in a pig burn injury model (Years 3 and 4)

Specific Aim 3. To conduct the first clinical investigation of a keratin biomaterial treatment for burn injury (Years 5 and 6; not currently funded)

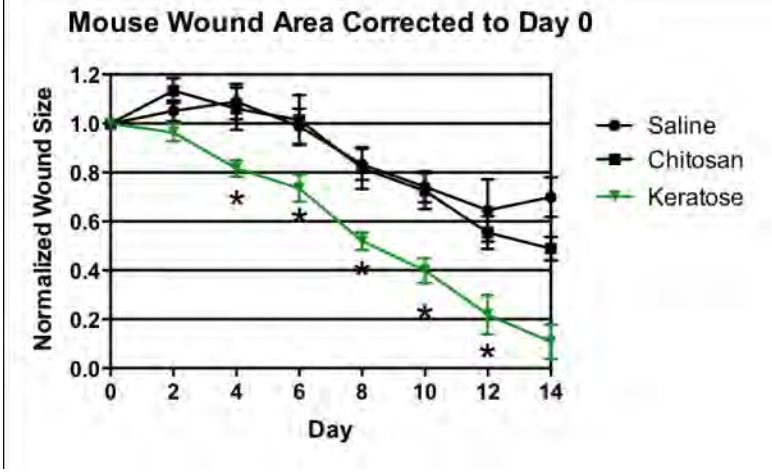

Figure 1. Mouse chemical burn wound area. Wound surface area for each treatment group was normalized to day 0. Keratose treated wounds did not grow in size and were completely healed by day 16. Other treatment groups showed an increase in initial wound size and required longer to heal. There was a statistically significant difference between keratose and both controls at all time points between day 4 and 16 (data is truncated for clarity).

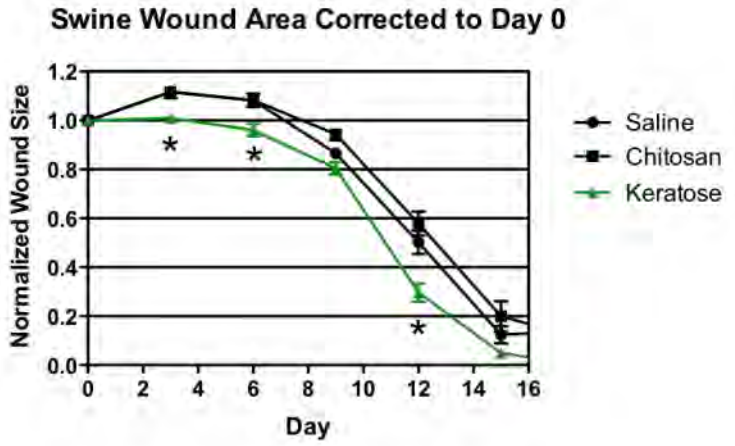

Figure 2. Swine thermal burn wound area. Wound surface area for each treatment group was normalized to day 0. Keratose treated wounds did not grow in size and were completely healed by day 18. Other treatment groups showed an increase in initial wound size and required longer to heal. There was a statistically significant difference between keratose and both controls at days 3, 6, and 12 (data is truncated for clarity).

Research Progress

The goals of this project are to investigate the underlying mechanism of keratin's apparent capacity to preserve injured tissue following burn injury (this phenomenon was discovered in earlier pilot studies in rodents and swine), optimize a keratin biomaterial system for burn treatment, and test this technology in a swine model of burn injury. In the past year of the project, we utilized our previously developed swine burn model[14] to conduct a pivotal, preclinical trial. We also continued mechanistic studies to help define the primary mode of action of the keratin biomaterial. The research experiments related to these studies are more fully described in the sub-sections below.

Pivotal Swine Burn Study

28 female Yorkshire swine were used under a protocol approved by the Wake Forest ACUC and the ACURO. The animals were randomly divided into the two arms of the study, one in which treatment would be administered within 60 minutes of burning and another in which treatment was delayed for 10 hours. In each arm 14 animals were randomized and 12 burn wounds were created with heated cylindrical brass blocks, 6 on each side of the dorsal mid-line between the shoulder and hip under general anesthesia. The brass blocks were heated in an 80:20 boiling PEG:water solution. Once the boiling liquid was heated to 105-115 ^{o}C, the brass blocks were used to create burns on the pig by contact with the skin for 20s. The wounds were randomized into one of 4 treatment groups: Saline-soaked gauze, Coloplast (a collagen-based wound gel), keratin treatment 1, and keratin treatment 2. Keratin treatment 1 represents the form of keratose hydrogel used in previous pilot studies and keratin treatment 2 represents the KeraStat Burn formulation. For each treatment, at each time point in both arms of the study, 6 replicate wounds were created using 2 animals. 3cc of keratin or Coloplast was used on each wound and all wounds were covered with a Telfa pad, Ioban dressing, a protective plastic shield, and a nylon jacket. Every three days the dressings were removed and the wounds 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 pos t-surgery, two animals were euthanized and tissue was collected for histological analysis.

The skin tissue sections were analyzed histologically using Gomori trichrome, H&E, and von Willebrand factor (blood vessels) staining and 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 silver sulfadiazine cream (SSD, **Figure 3A**). Wounds were also tattooed so that contracture could be measured. No appreciable wound contracture was noted in any of the treatment groups (**Figure 3B**). Histomorphometric analysis showed two distinct phases of healing. One in which there was a modest rate of re-epithelialization due to the changing nature of the wound bed, and a second more rapid phase that was marked by the appearance of granulation tissue. Re-epithelialization data for both phases and are shown in **Figures 4 and 5**, respectively, and rates calculated from these curves are shown in **Tables 1 and 2**, respectively. Rates of re-epithelialization were extrapolated from days 3 through 15 in order to calculated the estimated mean days to full wound closure for each treatment group (**Table 3**).

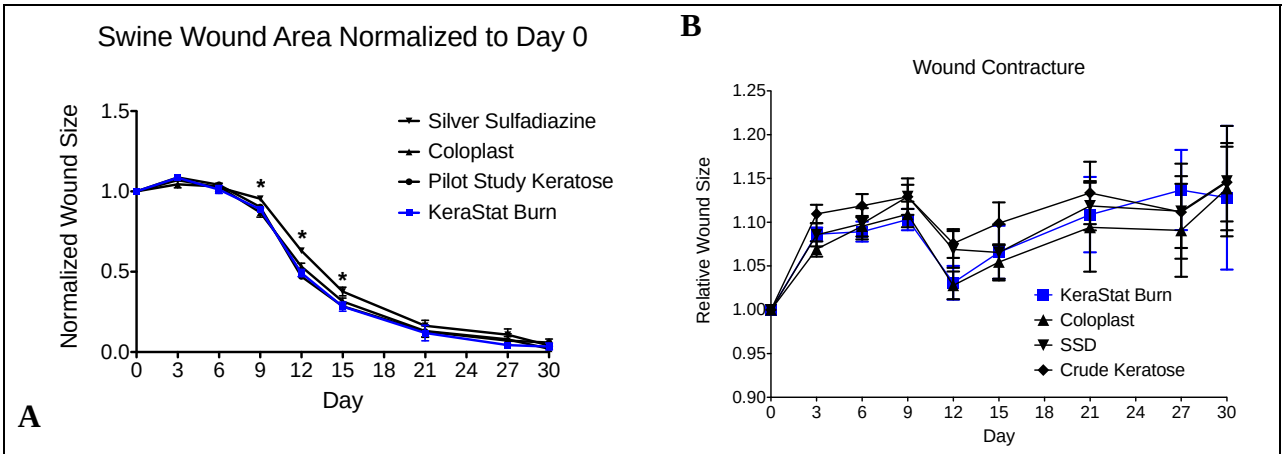

Figure 3. Morphometric analysis of digital images. Digital image analysis showed that wound area was lowest in the KeraStat Burn treatment group at days 9, 12, and 15 (A). Measurements of wound contracture showed that no treatment resulted in appreciable scarring (B).

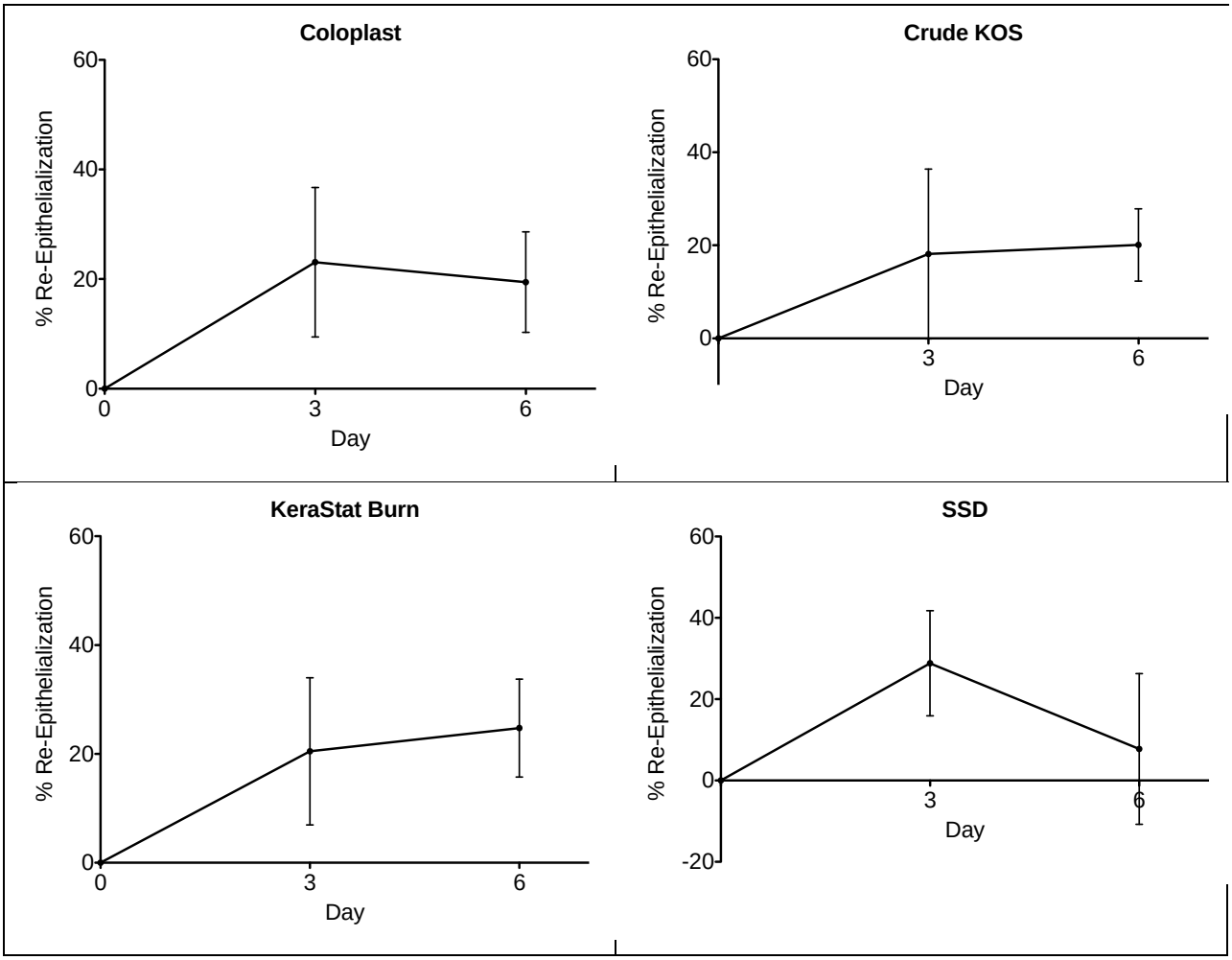

Figure 4. Initial rates of re-epithelialization in second degree burns. Burns treated with keratin biomaterials demonstrated a general upward trend in re-epithelialization at early stages of treatment, whereas Coloplast and SSD generally trended downward. Healing was generally inconsistent across replicates as indicated by the large error bars. There was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, $p>0.05$).

Table 1. Initial rates of re-epithelialization between days 0 and 6.

Treatment	Rate (% Re-Epithelialization/Day)	R Squared	% Difference vs. SSD
Coloplast	3.4 +/- 1.1	0.40	+160%
Crude KOS	3.4 +/- 1.2	0.35	+160%
KeraStat Burn	4.1 +/- 0.96	0.54	+220%
SSD	1.3 +/- 1.7	0.03	--

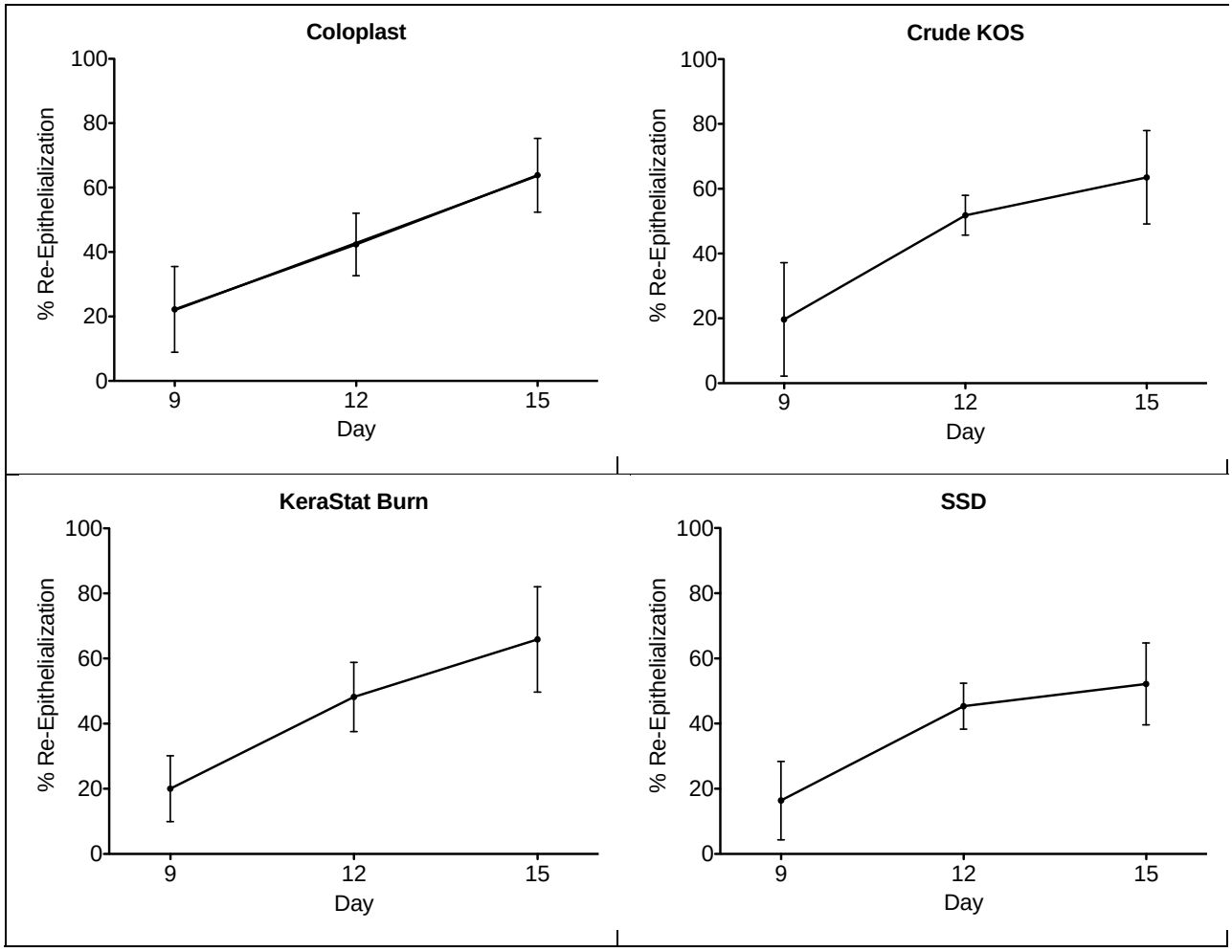

Figure 5. Rates of re-epithelialization in second degree burns during late stages of healing. All treat ments demonstrated an upward trend in healing and all, with the exception of 3 wounds in the SSD group, were completely re-epithelialized by day 30. Healing was generally more consistent across replicates as indicated by the relatively smaller error bars compared to ea rly stages of healing (see Figure X). There was no statistically significa nt difference between treatment groups (n=6, mean +/- standard deviation, p>0.05).

Table 2. Rates of re-epithelialization between days 9 and 15.

Treatment	Rate (% Re-Epithelialization/Day)	R Squared	% Difference vs. SSD
Coloplast	6.9 +/- 1.1	0.72	17%
Crude KOS	7.3 +/- 1.4	0.64	24%
KeraStat Burn	7.6 +/- 1.2	0.72	29%
SSD	5.9 +/- 1.2	0.62	--

Table 3. Days to wound closure.

Treatment	Mean Days to Wound Closure
Coloplast	24.2
Crude KOS	19.5
KeraStat Burn	19.2
SSD	23.4

Heat Shock Mechanism Study

Additional heat shock experiments have been conducted 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 post-natal pups and grown to near confluence. Cultures were treated at 44 ^{o}C for 150 minutes to induce necrosis and stress. After 6 hours under normal culture conditions, non-adherent cells were removed and treatments applied. Cells were harvested at 12, 18, and 24 hours and RNA extraction was performed, followed by PCR microarray analysis. cDNA synthesis was performed with the RT ^{2} First Strand Kit (SABioscience s) using an AB Applied Biosystems 96 well thermocycler following the protocol specified by the manufacturer. After the cDNA synthesis was performed, RT^{2} Profiler™ PCR microarray mouse cell death pathway finder (SABiosciences) was used to determine the expression of genes that are involved in cell death. RT^{2} Sybr green ROX qPCR master mix (SABiosciences) was used to perform the gene array experiment using an AB Applied Biosystems 7300 Real time PCR system following the protocol specified by the manufacturer. RT^{2} Profiler PCR microarray mouse cell death pathway finder analyzes the real-time expression of 86 genes related to cell death pathways, which includes apoptosis, necrosis and autophagy.

Data was analyzed using the Auto Ct settings provided by the 7300 Real Time PCR system software. Further data analysis was done using RT ^{2} profiler PCR array web based software provided by the manufacturer that follows ΔΔCt method of calculation. This provided a comparison of post-treatment (12hr, 18hr, and 24hr) to the pre-treatment control (6hr). To compare the relative up or down regulation between the gamma keratose and fibroblast growth media treatment groups, the ratio of gene expression (fold change) for gamma keratose treatment to fibroblast media treatment was calculated. Using this calculation, if the ratio is less than 1, it indicates that the gene expression is higher in fibroblast media treated cells, if it is equal to 1, the expression is the same in both the treatments, and if it is greater than 1, the expression is greater in the gamma keratose treated cells. Compared to the control group, the gamma keratose treatment at 12 hours showed a significant down regulation ($p<0.05$) in 29 out of 86 genes. In spite of the high differential gene expression (fold change) in the fibroblast media treated cells, the difference was not statistically significant compared to the pre-treatment control. Among the 29 genes that showed significant difference with gamma keratose treatment compared to the pre-treatment control, 19 genes had higher expression in the fibroblast media treated cells, 4 genes had a similar expression between both the treatment groups, and 6 genes (Htt, Mcl1, Ulk1, Atg16l1, Irgm1, Casp3) had higher expression in gamma keratose treated cells. Out of these 6

genes that had higher expression in gamma keratose cells, 4 genes are involved in autophagy, 1 gene is involved in both autophagy and apoptosis, and 1 gene is anti-apoptotic (Table 3). There were more subtle changes in gene expression at 18 and 24 hours.

The main findings from these experiments include:

1. As expected, heat treatment of mouse dermal fibroblasts up regulates cell death pathway related genes
2. A single gamma keratose treatment at 0.01mg/mL appeared to influence gene expression at 12 and 18 hours post-injury (6 and 12 hours post-treatment, respectively), but this effect was diminished by 24 hours
3. In general, treating these thermally stressed cells with gamma keratose substantially diminishes the gene up regulation compared to treatment with fresh fibroblast growth media
4. In general, gene expression was more consistent (i.e. smaller p values) in cells treated with gamma keratose compared to cells treated with fibroblast growth media

Key Research Accomplishments

- Completed a pivotal preclinical burn trial in swine
- Completed a mechanistic study that demonstrates the effect of a specific fraction of keratin, gamma keratose, on cell death pathways following thermal injury
- These data will be included, along with previously reported data and information, in the full IDE application to CDRH/FDA to be submitted 3Q2012

Conclusions

The AFIRM keratin biomaterials burn project is currently on budget and ahead of the original schedule. We have successfully completed all of the preclinical efficacy testing required for an IDE application to the FDA. Other commercialization activities and preclinical testing includes preparation of clinical batches of KeraStat Burn and final quality control and toxicity testing of these batches. These activities will be conducted by our commercialization partner, KeraNetics LLC during year 5 of the project.

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14. Gaines C, Poranki D, Du W, Clark RAF, Van Dyke M. Development of a porcine deep partial thickness burn model. Burns 2012 (accepted)

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	3
# Post docs	1	1
# grad students	1	0
# undergrad students	1	0
# staff members working for AFIRM	1	1

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Honors and Awards: None this reporting period

Patents and Inventions: None this reporting period

Peer-Reviewed Publications

1. Gaines C, Poranki D, Du W , Clark RAF, Va n Dyke M. Developm ent of a porcine deep partial thickness burn model. Burns 2012 (accepted)

Other Publications

1. Poranki D. Development of a pivotal pre-clin ical porcine deep partial thickness burn m odel and testing of a keratin biom aterial hydrogel product. Tissue Engineering & Regenerative Medicine North America (TERMIS-NA). December 2011.
2. Poranki D. Development of a pivotal pre-clin ical porcine deep partial thickness burn m odel and testing of a keratin biom aterial hydrogel product. Unites States Army Institute for Surgical Sciences (USAISR). December 2011.
3. Poranki D, Gaines C, Van Dyke ME. De velopment of a porcine deep partial (2 ^{0}) thickness burn model. North Carolina Tissue Engineer ing & Regenerative Medicine (NCTERM). November 2011
4. Poranki D, Gaines C, Van Dyke ME. Keratin Biomaterial for Burn Wound Healing. 10th Annual Charlotte Biotechnology Conference. October 2011

5. Gaines C, Poranki D, Van Dyke ME. Optim ized Deep Partial Thickness Swine Burn Model for the Evaluation of Keratin Hydrogel Treatm ents. Materials Research Science. Decem ber 2011

6. Gaines C, Poranki D, Van Dyke ME. Optimized Wound Creation in a Deep Partial Thickness Porcine Burn Model. Biom edical Engineering Society Annual Meeting (BMES). October 2011

7. Poranki D, Gaines C, Van Dyke ME. De velopment of a porcine deep partial (2 ^{0}) thickness burn model. AFIRM All Hands Meeting. January 2011

Proposals

1. Studies examining the use of KeraStat™ Burn to prevent or reduce hypertrophic scar formation in burns (Congressionally Directed Medical Research Pr ogram, 3 years; PI: Burnett L)
2. Keratin-based treatment (KeraStat™ Burn) f or cutaneous radiation in jury (CRI) (NIAID/NIH, SBIR phase I, 1 year, PI: Burnett L)
3. Design, synthesis and studies of siderom ycins, novel antibiotics, and delivery system s that specifically target infections in wounded so ldiers (Congressionally Directed Medical Research Program, 4 years; PI: Miller M) AWARDED MAY 2012

PROJECT BS-5 TERMINATED

Project BS-6: In Situ Bioprinting of Skin for Battlefield Burn Injuries

Team Leader(s)	James J. Yoo, MD, Ph.D. Wake Forest University
Project Team Members	Mohammad Albanna, Sean Murphy, Weixin Zhao, Idris El-Amin, Dennis Dice, Josh Tan
Collaborator(s)	
Therapy	Burn Repair
Deliverable(s)	*Baseline:* Bioprinted Skin *Revised:*
TRL Progress	Start of Program: TRL 1 End Year 3: TRL 3 End Year 1: TRL 2 End Year 4 TRL 4 End Year 2: TRL 3
Key Accomplishments:	The bioprinting of keratinocytes and fibroblast into a full thickness incisional wound in a porcine model has been completed. The development of a 3rd degree burn model in the pig has been initiated
Keywords	Wound, burn, skin, bioprinting, autologous cell therapy, allogeneic cell therapy

Introduction

Severe burn injuries are a major cause of mortality and morbidity in civilians and military personnel. Conventional skin grafts are often limited in providing immediate wound coverage for large wounds. To overcome this limitation, we developed a skin bio printer that accurately delivers skin cells and biomaterials to rapidly cover large wounds. In this study we investigated whether the skin bioprinter could be used for the repair of large full thickness wounds in a porcine model.

Research Progress

Skin fibroblasts and keratinocytes were isolated from the dorsum of porcine skin through a partial thickness skin biopsy of (0.015 inch). We have developed an improved cell isolation and culturing protocol of fibroblast and keratinocytes to improve the cell yield and viability in cultures. Both cells were cultured for 10 days until they reach confluence. Four full thickness excisional wounds of 10x10 cm each were created on the back of pig model (n=6). Autologous and allogenic fibroblasts and keratinocytes, suspended in fibrinogen/ collagen solution, were printed directly on two wounds. Fibroblasts were printed first and crosslinked with thrombin to form a gel layer, followed by delivering keratinocytes over the fibroblast layer. The remaining two wound groups received fibrinogen/collagen gel without cells and left untreated as controls. The animals were followed for up to 5 weeks and analyzed for wound healing, re-epithelialization and contracture.

Wounds treated with autologous cells showed complete healing by 3 weeks, where as the other treatment groups showed healing in 6 weeks (**Figure 1**). Wounds treated with autologous cells also showed an accelerated wound re-epithelialization and had almost 95% wound re-epithelialization by the third week of study (**Figure 2**). Wound contracture was minimal for autologous treatments throughout the study (<20% of the original wound size) compared to the

other treatments, which showed a progressiv e increase in contraction that exceeded 40% of the original wound size. Wounds treated with allogenic cells did not show notable differences with respect to wound size, re-epithelia lization and contracture when compared to controls (untreated and matrix only). Histological analyses showed a complete formation of epiderm is and derm is layers within the first two w eeks of study in the autologous treatments. However, other treatments showed a formation of epidermis and dermis layer by the week 6 of the study (**Figure 3**). These results dem onstrate the ability to regenerate skin within two weeks using autologous cells with minimal contraction and accelerated wound re-epithelialization.

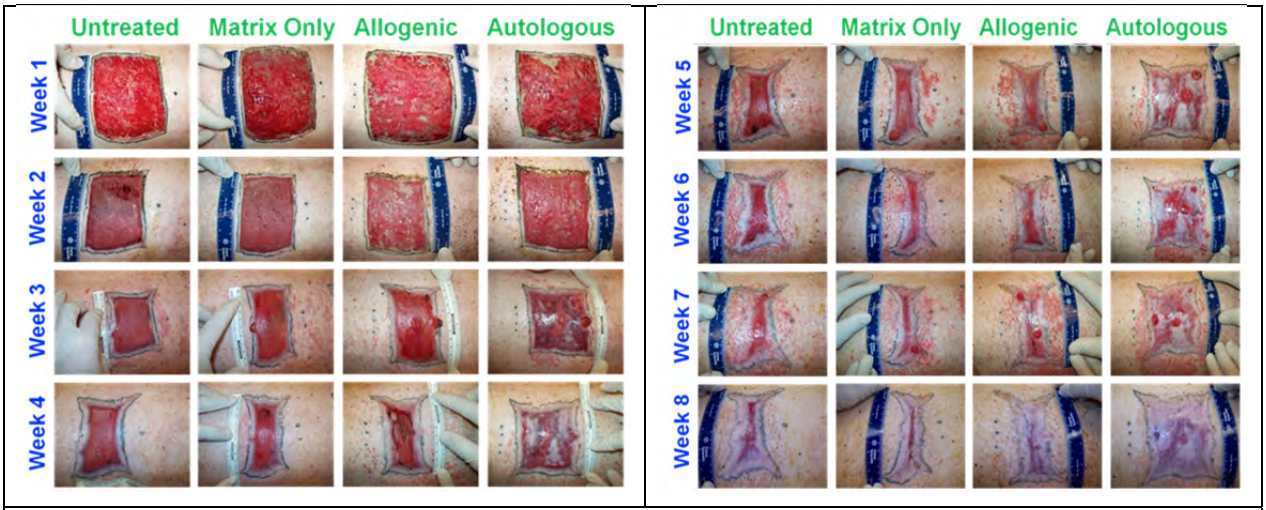

Figure 1. Gross images of wound healing over 8 weeks of study. Wounds treated with autologous cells showed complete healing by 3 weeks, where as the other treatment groups showed healing in 6 weeks.

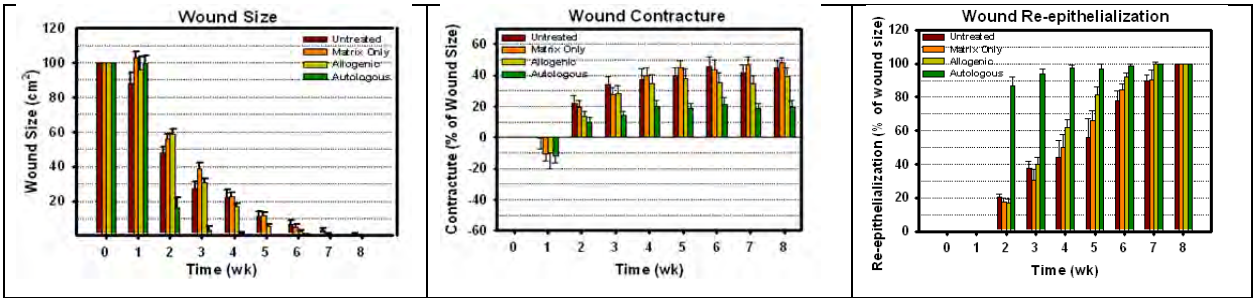

Figure 2. Wound size, contracture and re-epithelialization of untreated, matrix only, Allogenic and autologous treatments over 8 weeks of study. Wounds treated with autologous cells also showed an accel erated wound re-epithelialization and had almost 95% wound re-epithelialization by the third week of study.

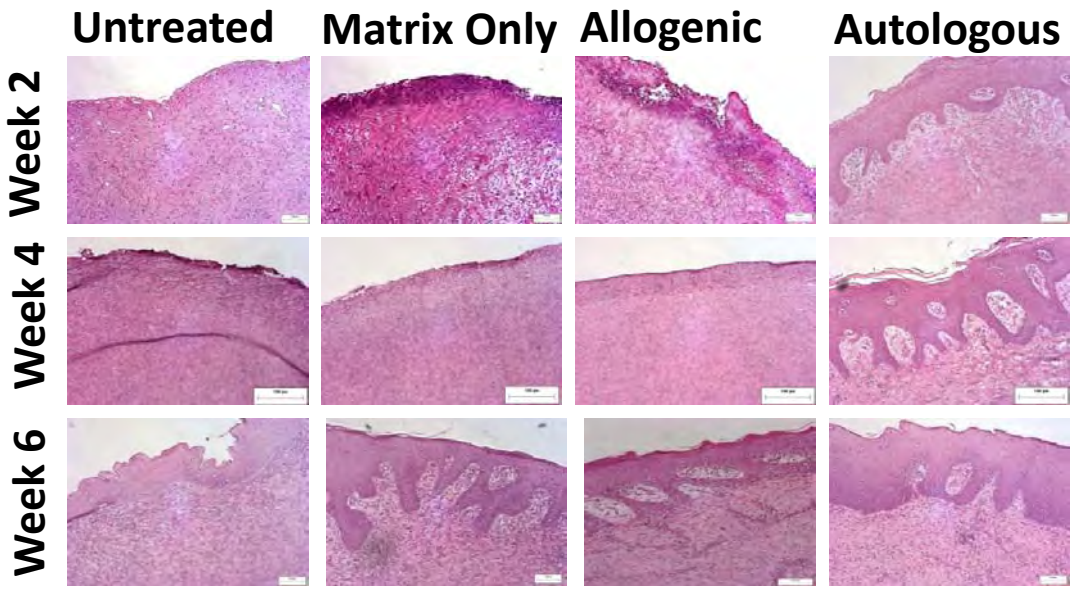

Figure 3. H&E staining of skin biopsies taken from treatments at week 2, 4 and 6 of the study**.** Complete formation of epidermis and dermis layers was observed within the first two weeks of study in the autologous treatments. However, other treatments showed a formation of epidermis and dermis layer by the week 6 of study.

Key Research Accomplishments

- Faster wound healing (3 weeks), smaller wound contracture and higher re-epithelialization rate of autologous treatments compared to other treatments
- Complete wound healing of all other treatments by 6 weeks post-injury
- Formation of epidermis and dermis in wounds receiving bioprinted autologous cells at 2 weeks post-injury
- Delayed formation (week 6) of epidermis and dermis in untreated, matrix only, and allogenic treatments

Conclusions

The delivery of skin keratinocytes and fibroblast cells and biomaterials directly on the wound using the bioprinter was successful and effectively covered the wound defect. Delivery of autologous cells accelerated wound healing. This preclinical study suggests that the use of skin bioprinting is an alternative approach for rapid coverage of extensive skin wounds such as burn rapidly.

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Research Plans for the Following Year

A 3rd degree full thickness porcine burn model will be established and evaluation of bioprinted cells in the enhancement of wound healing will be completed. Keratinocytes and fibroblast will be bioprinted into the burn and the time to wound healing will be determined as well as wound contracture and wound epithelialization.

Planned Clinical Transitions

Although no clinical trials are currently planned under this AFIRM project, the data collected from the porcine burn model will be critical in transitioning to a future clinical trial.

Corrections/changes Planned for Next Year

No changes are planned for the project in the next year.

Conflict of Interest Disclosure

The research team has no conflict of interest to disclose.

Honors given to AFIRM faculty

Best Poster Award

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	2	
# Post docs	1	1
# grad students	0	
# undergrad students	0	
# staff members working for AFIRM	1	

Other Project Statistics	
# Honors given to AFIRM faculty	1=
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	0
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Non-Peer reviewed publications (Abstracts)

1. Albanna MZ, Murphy S, Zhao W, El-Amin IB, Tan J, Dice DD, Kang HW, Jackson JD, Atala A, Yoo JJ. In situ bioprinting of skin cells for skin regeneration. NC TERMS Annual Meeting, Winston-Salem, NC, 2011.
2. Albanna MZ, Murphy S, Zhao W , El-Amin IB, Tan J, Jackson JD, Atala A, Yoo JJ. In Situ Bioprinting of Skin Cells for Skin Regene ration of Burn Wounds. WFIRM Retreat 2012, Pinehurst, NC
3. Albanna MZ, Murphy S, Zhao W , El Amin IB, Tan J, D ice DD, Kang HW , Jackson JD, Atala A, Yoo JJ. In Situ Bioprinting of Skin for Reconstruction, 2012 AUA Annual Meeting, Atlanta, Georgia. (Best Poster Award)

Project BS-7: A Comparative Study of the ReCell Device and Autologous Split-Thickness Meshed Skin Grafting in the Treatment of Acute Burn Injuries

Team Leader(s)	James Holmes, MD, Director, WFBMC Burn Center, Associate Professor of Surgery, Wake Forest School of Medicine (Winston-Salem, NC)
Project Team Members	Joseph Molnar, MD (Wake Forest School of Medicine); Rajiv Sood, MD (Univ of Indiana); William Hickerson, MD (Univ of Tennessee Health Science Center); Bruce Cairns, MD (Univ of North Carolina at Chapel Hill); Kevin Foster, MD (Maricopa Integrated Health Systems); David Mozingo, MD (Univ of Florida); Marion Jordan, MD (Washington Hospital Center, DC); Richard L. Gamelli, MD (Loyola Univ Medical Center); Booker T. King, MD (US Army Institute of Surgical Research); David Smith, MD (Tampa General/USF); Michael Feldman, MD (VA Commonwealth Univ); Tina Palmieri, MD (UC Davis); John Griswold, MD (Texas Tech Univ Health Science Center)
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	Burn Repair: Transplantation of autologous epidermal cells for treatment of second-degree burn injuries. The autologous epidermal cells are separated from a small split-thickness skin biopsy using the ReCell® Autologous Cell Harvesting (ACH) System (Avita Medical Ltd, Cambridge UK *Revised Objective:* The primary objective remains to obtain FDA marketing approval for the ReCell ACH System. However, the clinical study design was modified slightly in order to meet FDA/CBER requirements. Specifically, for the regulatory endpoint, the study has been expanded from 60 patients with 6 week follow-up to 106 patients with 4 month follow-up and the primary study hypothesis has been revised
TRL Progress	Start of Program: TRL 1 End Year 3: TRL 7 End Year 1: TRL 7 End Year 4 TRL 7 End Year 2: TRL 7
Key Accomplishments:	32 subjects have been enrolled and treated since 1-Jun 2011, bringing total enrollment to date to 63 (of 106) subjects 19 subjects have been followed through the 52-week endpoint 12 sites are actively enrolling subjects The number of sites recruiting subjects has increased from 9 to 13
Keywords	ReCell system, cell spray, skin grafting, burns

Introduction

The skin, as the largest organ in the body, performs a range of vital protective, immunologic neurosensory, thermoregulatory and homeostatic functions. Therefore, any wound involving thermal, electrical or chemical burn, trauma, abrasion or laceration may seriously compromise the participation, performance, health and ultimately, 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, under-employment, and failure to fully reintegrate into society. The rapid and effective management of wounds of an injured war-fighter is, therefore, a critical factor in the determination of wound outcome and consequential morbidity and mortality.

The ReCell Device is based on previous work of Wood & Stoner [1] and the recognition that autologous transplantation of epidermal 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 biopsy, 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[2]. 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 Device technology may facilitate rapid wound healing while minimizing donor site morbidity and potentially eliminating or minimizing scar formation.

The aims of this research program are to collect clinical data to demonstrate the safety and effectiveness of the ReCell Device compared with the standard of care, split thickness meshed grafts (STMG), for treatment of second degree burn wounds. The results from this study will be used to support a premarket application to the FDA for the ReCell Device. For the regulatory application, the hypotheses to be supported are: 1) non-inferiority with the primary efficacy endpoint defined as recipient site wound closure at the Week 4 follow-up visit of the ReCell-treated area as compared to that of the STMG-treated area, and 2) superiority in the healing of the ReCell donor site as compared to the STMG donor site at Week 1. However, in accordance with the AFIRM grant, subjects will be followed for up to 52 weeks following randomization to collect additional data pertaining to wound healing appearance/scar formation. The target enrollment in this study for evaluation of the regulatory hypotheses is 106 subjects (adjusted upward by 15% to account for potential withdrawals or non-evaluable subjects). This number of accrued subjects is also sufficient to assess the longer-term outcomes of scar formation consistent with the AFIRM grant objectives.

Subject recruitment is ongoing, with routine reminders to investigators, including regular study newsletters. To date, sixty-three study participants have been treated at twelve different burn centers in the US.

Research Progress

For prior year's progress, please see pages 426-429 of the 2011 Annual Report which can be found at http://www.afirm.mil/assets/documents/annual_report_2011.pdf,

During the previous year, 6 additional clinical sites were approved, trained and began recruitment, resulting in the treatment of 12 of the 32 subjects for the past 12 months. In late 2011, a clinical trial subject recruitment professional was retained to interview coordinators and investigators at selected sites. Ongoing review and assessment of screening logs is conducted. While no single obvious issue impedes enrollment, the level of communications with investigators has been increased via regular contact. Investigators present at the Southern Region Burn Conference (December 2011) were engaged one-on-one to discuss recruitment and for additional photography and other miscellaneous training – other investigators were updated via individual online web-meetings. Newsletters containing recruitment and other tips were distributed in February, March and May. The February Newsletter introduced a pocket reference card for the study patient selection as well as subject recruitment flyers and posters, which have been deployed as each site secures IRB approval of the material (5 sites to date). Improved surgeons' ReCell product quick reference guides and coordinators' treatment visit checklists were also distributed in February. There has been a singular focus on the recruitment of study participants, however the average rate across 13 sites remains just 2-3 per month.

The Annual IDE report for the study was accepted without questions by the FDA.

Key Research Accomplishments

- 63 subjects enrolled (total) – 32 enrolled in the past twelve months
- 19 subjects followed through 1-year post-treatment
- 12 actively enrolling sites
- 13 sites cleared to enroll subjects

Conclusions

Informal review of the (non-blinded) site investigators' assessments of donor sites and burn wound healing (the study co-primary endpoints) for the first 23 subjects indicates that the results appear to be on track for demonstration of the effectiveness of ReCell, however 3 subjects have experienced treatment-area healing at 8 weeks instead of the anticipated 4 weeks due to some form of re-injury on the ReCell-treated burn. Since this has not occurred in the control-treated burn areas, FDA will likely inquire about the robustness of ReCell vs meshed split-thickness skin graft.

Research Plans for the Following Years

The research plans for the next 2 years include completing trial enrollment goals (106 subjects) and initiate progress to program status level TRL #8.

Planned Clinical Transitions

The clinical program will be transitioning from an execution phase to a final reporting phase with completion of subject accrual anticipated over the course of the next twelve to twenty-four months. Avita Medical continues to be an industry collaborator on this program.

Corrections/Changes Planned

The program timeline has been extended due to program delays as a result of the challenges of enrolling subjects in the rigorous protocol approved by FDA/CBER.

Conflict of Interest Disclosure

None

References

1. Wood F. Clinical potential of autologous epithelial suspension. Wounds 2003; 15:16-22.
2. Stoner M, Wood F. Cultured epithelial autograft "take" confirmed by presence of cytokeratin 9. J. Invest. Derm. 1999; 112:391-392.

Project BS-8: *In vitro* Expanded Living Skin for Reparative Procedures

Team Leader(s)	Sang Jin Lee, PhD, James J. Yoo, MD, PhD, James H. Holmes (Wake Forest)
Project Team Members	John Jackson, PhD, Hyun-Wook Kang, PhD, Peter Masso, BS, Peter Prim, BS, Justin Werker, BS, Abner Mhashilkar, PhD (Wake Forest)
Collaborator(s)	None
Therapy	Treatment of burn injuries
Deliverable(s)	*Baseline:* Autologous skin grafts *Revised:* None
TRL Progress	Start of Program: TRL #4 End Year 3: TRL #5 End Year 1: TRL #4 End Year 4: TRL #5 End Year 2: TRL #5
Key Accomplishments:	We 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 utilizes a computer-controlled bioreactor capable of providing an accurate expansion rate for yielding target skin dimensions over a defined time period. We are currently defining parameters that would maximize the surface dimensions of skin for the treatment of battlefield burns. In the past year, we have focused on the development of an effective tissue gripper system which critically affects the expansion of skin grafts. A variety of gripper systems has been identified and incorporated in the system which is being designed and examined. Successful development of a gripper system would decrease tissue damage and reduce localized stress associated with the grippers resulting in fewer tears. In addition, we have completed the design and built a new generation of uniaxial bioreactor system. We have come up with a strategy to perform a clinical trial, which requires processes that include a toxicity testing under Good Laboratory Practice (GLP), establishment of Standard Operating Procedures (SOPs), the initiation of communication with the FDA, and IRB and FDA application submission.
Keywords	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 (1-5). However, donor-site tissue availability is a major obstacle to the successful replacement of skin defects (4,5). 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 (6-17). However, these approaches also have limitations, such as the need for concomitant autograft, insufficient mechanical properties, high cost, lack of permanence, potential for infectious disease transmission, and inadequate

biocompatibility. Nevertheless, many commercial skin products are being used as acceptable skin substitutes when autologous donor tissue is unavailable.

Alternatively, subcutaneous tissue expanders or meshed split-thickness skin grafts (STSG) are used clinically to generate larger segments of autologous skin, when donor-site tissue is limited (4,5). 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 (3,18,19). 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 remains as major limitations (20). Alternatively, meshed STSG 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 (1,2,4). However, meshed STSG are not considered ideal for many applications, because they leave large gaps of the open wound, which requires a longer healing time and results in a cross-hatched or cobblestone pattern of healed skin as scar tissue fills the gaps (1,4).

The overall goal of this project is to provide wounded soldiers with large dimensions of autologous skin for reparative procedures.

Aim 1: To optimize expansion parameters for maximizing surface dimensions of human skin

Aim 2: Establish Standard Operating Procedures (SOPs) for skin expansion parameters and delivery

Aim 3: To determine the applicability in wounded soldiers through a clinical trial

Research Progress

I. Continued optimization of expansion protocol for human skin
   - Optimized the expansion parameters; magnitude, frequency, slope, and expansion and resting time
   - Characterized the expanded skin matrices; histology (H&E and Masson's Trichrome) and immunohistochemistry for PCNA and TUNEL staining

II. Continued the modification of skin expansion bioreactor system
   - The prior grip system was mechanical and damaged the tissue during expansion; therefore, we are redesigning the grip system using micro-needles.
   - A new gripper system was designed and fabricated by Allied Automation to test skin in planar tension by straining the tissue (Figure 1). The system contains 16 individual arms with micro-needles on each arm to effectively grip the skin. The system is also designed to sustain compressive force that would translate the force within the gripper to stretch the skin sample to a strain area of 100% of the original skin sample area.

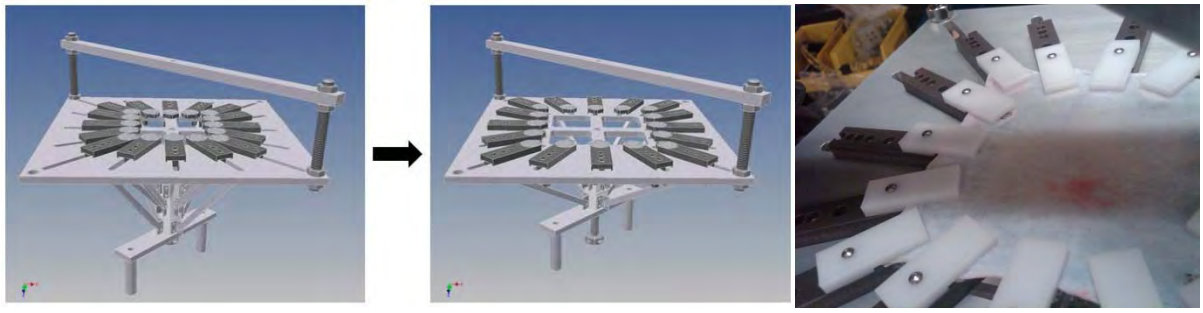

Figure 1. (Left) A new gripper system and expansion mechanism and (Right) gross appearance of gripper system with a porcine skin graft.

- Improved the software to control the skin bioreactor; recording expansion protocol, measuring tension, controlling temperature, and oxygen and carbon dioxide level

III. Continued optimization of human skin culture system
- Optimized human skin culture system with different culture medium components
- Evaluated human skin samples in culture at 7 days and 14 days: histology (H&E and Masson's Trichrome) and immunohistochemistry for PCNA and TUNEL staining

IV. Continued development of a new generation of skin expander (Figure 2)
- Optimization of skin expansion parameters (ongoing)

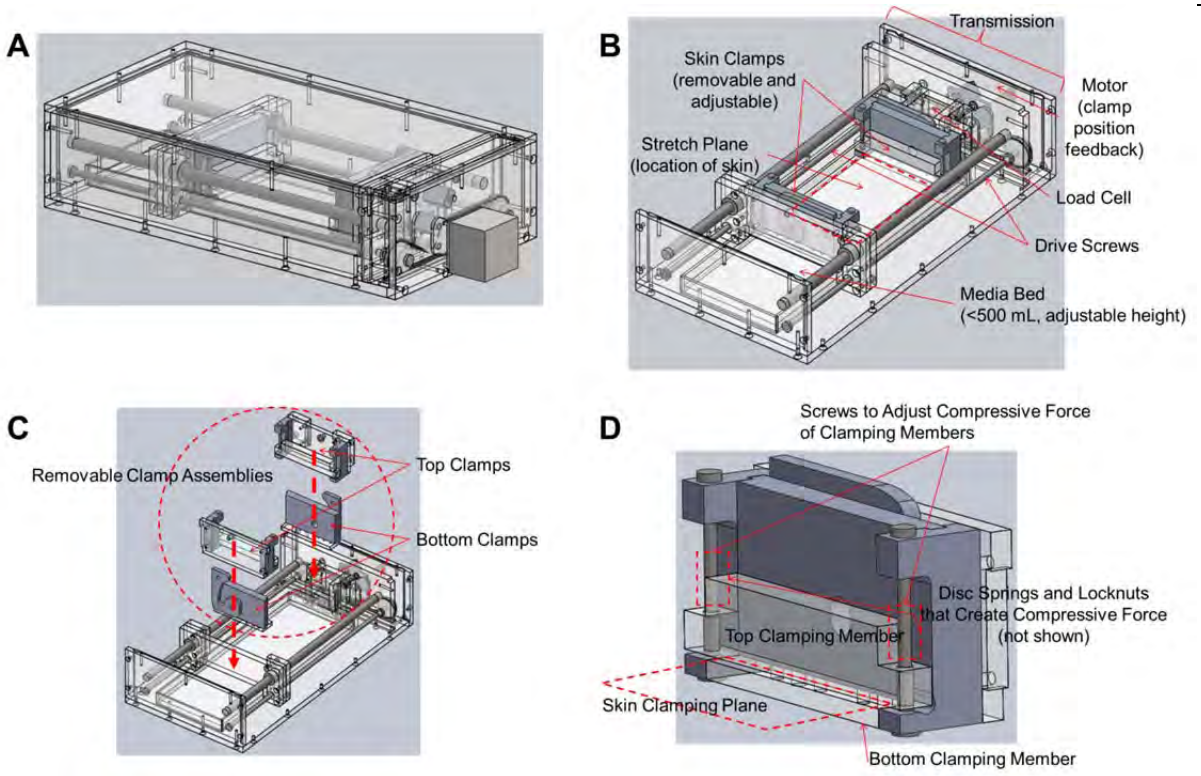

Figure 2. New generation of uniaxial skin expander: (A) Full assembled unit, (B) open (lid and sides hidden), removable clamp assemblies, and (D) adjustable clamps.

V. Continued development of standard operating procedures (SOP) for skin expansion
VI. Preparation of documents for pre-pre-IDE/IND

Skin matrices were placed in a sterile bior eactor for expansion. The edges of samples were clamped at multiple areas in the biaxial bioreactor. After placement, the initial dim ensions were measured using a sterile ruler to obtain a base line value. Subsequently, 300 m l of DMEM with 10% FBS a nd supplements (1% penicillin and st reptomycin and 1% antim ycotic) was added. Continuous flow of medium into the expansion ch amber was maintained with a peristaltic pump. The bioreactor was m onitored and kept at 37° C with a heated water jacket and 5% CO_2 was circulated through the unit for th e entire duration of expansion. Co mputer software was used to monitor the temperature, position and load of the skin throughout stretching. We have developed various protocols to m aximize the surface dim ensions of skin grafts. After stretchin g, the final dimensions of the expanded skin were m easured. The skin sam ple was rem oved from the bioreactor in preparation for skin grafting.

Various features were considered in the process of designing a bioreactor for clinical application. For example, the skin bioreactor system must be operated as a closed system to prevent potential contamination/infection and to m inimize the frequency of manipulation. A sk in bioreactor system has been designed and built for clinical trial. Medical grad e materials such as stain less steel and Teflon were used. Two stepper m otors, a positioning sensor, an electric therm ometer and a force sensor were used for the construction of this fully automatic system. In addition, two circulating pumps were im plemented for automatic medium exchange and tem perature control. The expansion site is com pletely isolated from the outside environm ent to m inimize the possibility of contamination.

Key Research Accomplishments

- Completion of a bioreactor desi gn for use in c linical trials, with construction underway (prototype)
- Development of a new gripper system to hold skin within the bioreactor
- Development of a new generation of uniaxial skin bioreactor
- Optimization of expansion parameters for human skin grafts (ongoing)
- Standard Operating Procedures (SOPs) for skin expansion are being developed (ongoing)
- Preparation of documents for pre-IDE/IND (ongoing)

Conclusions

Research Plans for the Following Years

- Materials characterization and biocompatibility testing
- Pilot study of human skin expansion in prototype device

- Assembly of study reports and information for submission Wake Forest IRB
- Pre-IDE/IND meeting with FDA and IDE/IND submission
- Construction of a clinical applicable skin expander system
- IRB and FDA approval for clinical trial
- Clinical trial (Phase I)

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 Food and Drug Administration (FDA). We are currently working towards obtaining investigational device exemption (IDE) by the FDA for approval of a prospective, multicenter, non-randomized, uncontrolled pilot study (Feasibility/Phase 1). IRB approval is currently being sought.

Correction/Changes Planned

Work described in this award could not be completed within the award period due to personnel changes that led to the delay in recruiting individuals with appropriate engineering skills. In addition, challenges in designing a gripper system that minimizes tissue damage have delayed the progress of this project. Fortunately, we have identified and partnered with an engineering firm that has the capacity to address the engineering challenges. As such, we have built several gripper prototypes and tested the effectiveness. We now have a new skin expander that is being constructed for testing and validation. We would like to request an additional 12 months of no cost extension to complete the work described in this project.

Conflict of Interest Disclosure

None

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	2
# Post docs	1	0
# grad students	1	0
# undergrad students	0	0
# staff members working for AFIRM	1	0

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under A FIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	0
# Non-peer reviewed publications	2

*Directly supported in whole or part –time by AFIRM

Non-peer Reviewed Publications (Abstracts)

1. Kang H-W, Choi J, Scarpinato P, Green D, Atala A, Holmes J, Yoo JJ, **Lee SJ**, Optimized Parameters of *In Vitro* Skin Expansion Bioreactor System for Burn Injuries, 2nd A nnual Translational Regenerative Medicine Fo rum, April 4-6, 2011, The Ronald R eagan Building and International Trade Center, Washington, DC, USA
2. Kang H-W, Werker J, Jackson J, Atala A, Holmes J, Yoo JJ, Lee SJ, Optim ized

Parameters of In Vitro Skin Expansion Bior eactor System for Burn Inju ries, 4th AFIRM "All Hands" Meeting, F ebruary 13-16, 2012 in TradeW inds Resort, St. Pete Beach, FL, USA

Project BS-9: Stratatech Technology for Burns

Team Leader(s)	James Holmes IV, MD (Wake Forest)
Project Team Members	LTC Booker King, MD (USAISR), Michael Schurr, MD (University of Colorado Hospital), Lee Faucher, MD (University of Wisconsin Hospital and Clinics), Kevin Foster, MD (Arizona Burn Center), Steven Wolf, MD (University of Texas Southwestern)
Collaborator(s)	B. Lynn Allen-Hoffmann, PhD (Stratatech Corporation), Allen Comer, PhD (Stratatech), Mary Lokuta, PhD (Stratatech), Leslie Jones (ResearchPoint)
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
Deliverable(s)	*Baseline:* To perform a human clinical trial to assess the safety and efficacy of StrataGraft skin tissue as an alternative to autografting for promoting the healing of deep partial-thickness burns *Revised:* No revisions
TRL Progress	Start of Program: N/A; End Year 3: TRL #6 End Year 1: N/A; End Year 4: TRL #6 End Year 2: N/A
Key Accomplishments:	The research team has obtained all regulatory approvals for the study from the FDA, clinical site IRBs, and USAMRMC Office of Research Protections (ORP)/Human Research Protections Office (HRPO) and has performed site initiation visits at five of the six clinical sites. The research team has maintained a continuous production stream of StrataGraft skin tissue for the clinical trial at a cGMP-compliant biomanufacturing facility. The first cohort of ten subjects has been fully enrolled. None of the subjects treated to date required autografting of the StrataGraft treatment site by day 28 and there has been no evidence of safety concerns or immunological responses to the StrataGraft tissue. Allogeneic DNA from the cells comprising StrataGraft was not seen at three months. These data suggest that StrataGraft facilitates wound closure and is replaced as the patient's own cells close the wound. Based on data from the first patient cohort, the WFSM Institutional Data and Safety Monitoring Board (I-DSMB) unequivocally recommended progression to the second cohort and treatment with larger areas of StrataGraft. Enrollment in the second cohort was initiated in April, 2012 and is ongoing
Keywords	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 war fighters and their families.[1] The severity of skin damage often exists as a gradient from superficial and deep partial-thickness, to full-thickness skin loss in the same patient. The standard of care for both full-thickness and deep partial-thickness 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, areas of deep partial-thickness injury 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 deep partial-thickness component of severe burns would 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 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 one 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 one week, the allograft tissues were removed and the wound bed was evaluated. The wound was autografted when the wound was judged ready. Autograft take was assessed two weeks after autograft placement.

StrataGraft tissue exhibited a good safety profile and was well tolerated with no evidence of acute immune responses.[2, 3] 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.

Stratatech's AFIRM project is designed to conduct a clinical trial to examine the safety and efficacy of StrataGraft skin tissue as a permanent skin replacement to promote the healing of deep partial-thickness burns without the need for autografting. Primary endpoints are: the need for autografting of the study wound 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.

Research Progress

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 deep partial-thickness burns. Following acceptance of the study design by the FDA review team, the study protocol was submitted to the investigational new drug (IND) application that Stratatech Corporation has established with the FDA Center for Biologics Evaluation and Research (CBER) for clinical evaluation of StrataGraft skin tissue in complex skin defects. Six clinical sites have been identified for this study: Wake Forest University Baptist Medical Center, the US Army Institute for Surgical Research (USAISR), 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 the IRB at each of these sites. IRB and HRPO approval for this study have been obtained at five of the six sites; approval at USAISR is anticipated shortly.

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, a clinical research organization in Austin, TX, is providing clinical trial monitoring services for this trial. Activities completed during the previous year include development of the safety monitoring plan, study database, statistical analysis plan, and data management plan. Study-specific procedures and documents have been prepared and distributed to the clinical sites.

The clinical results to date have exceeded Stratatech's expectations. Enrollment in the first dose cohort was initiated in September 2011 and was completed in February 2012. The initial group of 10 study subjects was treated with up to 220 cm^2 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 three months (**Figure 1**). Because the StrataGraft-treated wounds did not need to

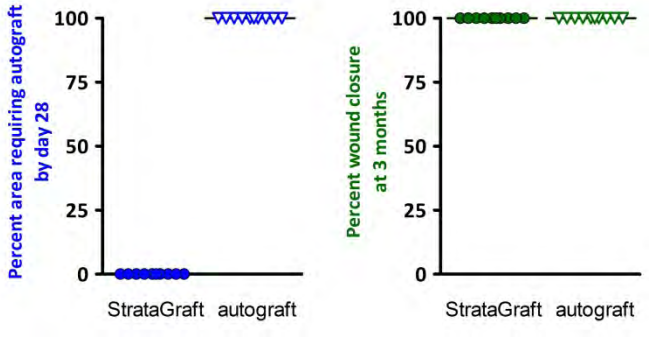


Figure 1. Autografting and wound closure assessments

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**).

There were no adverse events deemed likely product-related in any of the initial subjects. In subjects evaluated to date, 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 three months. Unexpectedly, evaluation of scarring and cosmesis after three months revealed that, in many cases, the sites treated with StrataGraft were smoother, more supple, and less raised than the autograft control sites (**Figure 3**). Although these exciting observations need to be confirmed by continued patient enrollment and long-term follow-up, the results to date have exceeded expectations and suggest that a single application of StrataGraft tissue may be able to promote the healing of deep partial-thickness burn without the need for autografting.

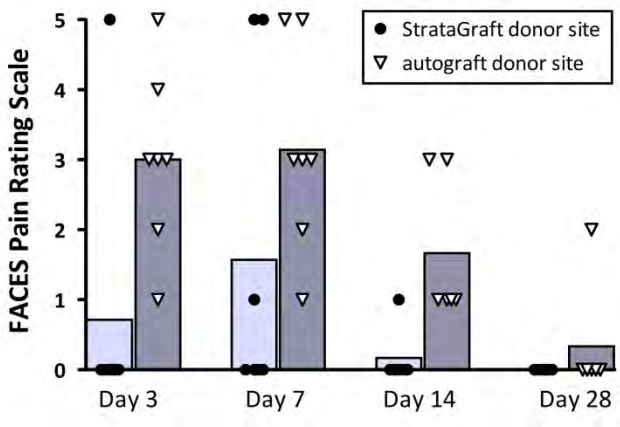
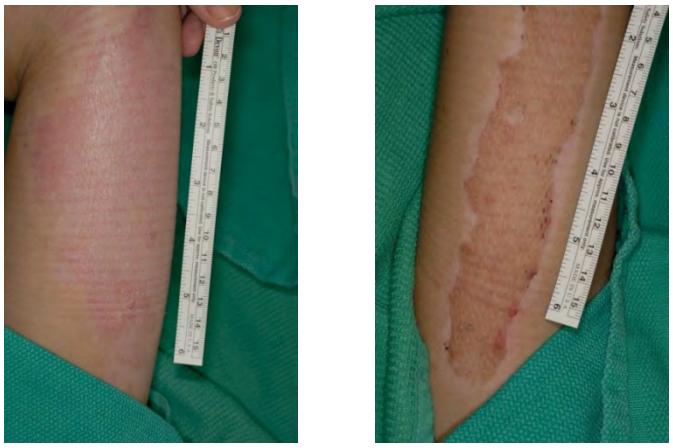


Figure 2. Donor site pain assessments

Following an interim analysis of data from the first subject cohort, the WFSM I-DSMB unequivocally recommended progression to the next patient cohort. Enrollment in the second dose cohort is ongoing and will be completed during year 5. Subjects in this cohort will be treated with up to 440 cm^2 of StrataGraft tissue.



StrataGraft Tissue Autograft

Figure 3. Wound site appearance after 28 days

Key Research Accomplishments:

- Finalized clinical protocol and obtained all regulatory approvals for study initiation
- Identified six clinical sites for this multi-center clinical trial
- Transitioned the StrataGraft tissue production process to a cGMP-compliant biomanufacturing facility and maintained continuous tissue production stream

- Obtained IRB and HRPO approval and conducted site initiation visits at five sites
- Worked with Research Point to provide clinical trial monitoring and data management activities for the study
- Initiated patient enrollment at four of the five sites
- Completed enrollment of all 10 patients in first dose cohort
- Completed interim safety analysis and report
- Received recommendation to proceed to next dose cohort from the WFSM I-DSMB
- Initiated enrollment in second dose cohort

Conclusions

In summary, progress made during the previous project year has enabled initiation of a clinical trial to evaluate the safety and efficacy of StrataGraft tissue as a universal skin replacement for treatment of deep partial-thickness burns. To date, the StrataGraft clinical trial results have exceeded expectations because no StrataGraft treated wound has required subsequent autografting. 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 deep partial thickness burns.

Research Plan for the Following Years

Research plans for the coming years include completion of subject enrollment, follow-up assessments of safety and efficacy outcomes, and preparation of study report.

Planned Clinical Translations

Work done during the previous project year has set the stage for completion of subject enrollment in year 5. Patient follow-up and monitoring will continue beyond year 5.

Corrections/changes planned for next year and rationale for changes

NA

Conflict of interest disclosure

NA

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Reportable Outcomes

N/A for clinical trials

Project BS-10: Multi-functional Bioscaffolds for Promoting Scarless Wound Healing

Team Leader(s)	Newell Washburn, PhD (Carnegie Mellon University)
Project Team Members	Allison Elder, Emily Friedrich, & Mohamed Ramadan (Carnegie Mellon University)
Collaborator(s)	Robert Christy, PhD (Institute for Surgical Research)
Therapy	Burn treatment
Deliverable(s)	Baseline Objective: Scaffolds that control inflammation and promote healing Revised Objective: Gels that control inflammation and promote burn healing
TRL Progress	Start of Program: TRL #3 End Year 3: TRL #5 End Year 1: TRL #3 End Year 4: TRL #5 End Year 2: TRL #4
Key Accomplishments:	(1) Analyzed data from test in rat burn model. (2) Applied for funding for clinical trials. (3) Developed new formulation that does not require antibody conjugation.
Keywords	burns, cytokines, inflammation, antibodies, gels

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 (1). The complications from partial- or full-thickness burns are broad ranging, including compromised protection by the epiderm is and loss of resident leukocytes and lymphocytes, edema, reduced host defenses to bacterial colonization, multiple organ failure, and loss of connective tissue cells that would normally contribute to the repair response. Burned tissue has been modeled as having three concentric zones: (1) irreversibly damaged tissue in the zone of coagulation; (2) hypoperfused tissue in a zone of stasis; (3) edematous tissue in a zone of hyperemia (3). 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. The goal of this research is to determine whether it is possible to use

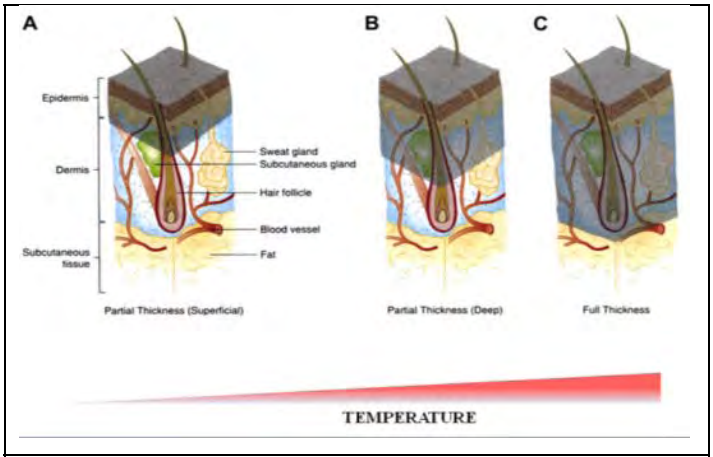

Figure 1. The effects of time and temperature on the extent of tissue necrosis at the site of thermal injury (1).

antibodies against the pro-inflammatory cytokine tumor necrosis factor- (TNF) conjugated to hyaluronic acid (HA) to inhibit burn progression.

The Washburn lab has made excellent progress this year. In collaboration with Dr. Robert Christy at ISR, the team has demonstrated that application of (anti-TNF)-HA conjugates to partial-thickness burns reduces burn progression, rescuing 70% of viable tissue from inflammation-induced necrosis in a rat burn model, shown in Figure 2. The Washburn group has performed in-depth analysis of tissue responses to understand better the mechanism of effect of these conjugates. Researchers recently developed a non-conjugated analogue that could display similar levels of efficacy as the conjugated version. The non-conjugated version will be tested in the rat burn model in Year 5 in preparations for a potential clinical trial, along with developing strategies for long-term storage of these materials. This version, in which all components have established records of safety and efficacy in treating inflammatory conditions, would have much lower regulatory barrier for getting into early phase trials.

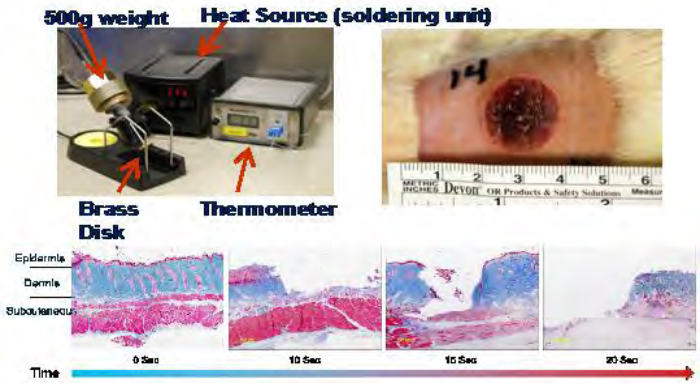

Figure 2. The rat partial-thickness burn model developed in this study.

Research Progress

Details of the rat burn model are shown in Figure 2. A 1" brass disk was heated to 85 ℃ and pressed against the skin of an anesthetized rat for 10 sec to create a deep partial-thickness burn. One day following burn injury, the eschar is removed and the treatments are applied to the site. The goal of the experiments is to test whether the remaining viable tissue beneath the primary injury can be rescued from inflammation-induced necrosis.

The following treatments were tested in this study (all treatments and time points were performed at N=4): (1) Saline; (2) 1% HA solution; (3) 100 µg/mL anti-TNF solution; (4) 1% (anti-TNF)-HA solution (100 µg/mL anti-TNF)

Tissue samples were removed 1 day from the first group following application of the first treatment (Day 1), and the rest of the rats received a second treatment on Day 2. Half these rats were sacrificed on Day 4 to recover tissue, and the other half of the group received a third treatment. This last cohort was sacrificed on Day 7, the final time point in the study.

Trichrome staining of tissue sections was used to provide gross histological assessment of tissue responses. The tissue sections look similar at Day 1, indicating the burn procedure is reproducible. Most sites are forming granulation tissue by Day 4, but by Day 7 sites many sites have formed a thick layer of tissue at the outer layer of the site that stains intensely. This layer appeared to be thinnest in the sites treated with (anti-TNF)-HA solutions.

Vimentin immunostaining was used to identify necrotic tissue. Vimentin is a component of the cytoskeleton, and only viable tissue will stain positively (2). The thickness of the non-stained layer on blinded samples was quantified by volunteers. These results are shown in Figure 5. Sites treated with saline lost 700 μm of viable tissue by Day 7, but this amount was reduced by 70% under treatment with (anti-TNF)-HA conjugates.

The Washburn group has expanded the program in immunostaining tissue sections to determine how the conjugates affect the inflammatory microenvironment at the burn site. These fundamental studies will be important in order to facilitate regulatory approval and adoption by leaders in the burn-treatment community. Quantification of macrophages in the treated and control sites using CD68 immunostaining was completed, with representative images and results shown in Figure 3. It is interesting to note that while there was a 40% reduction in macrophage levels at sites treated with (anti-TNF)-HA conjugates, the lowest macrophage levels were observed in sites treated with anti-TNF alone. It is hypothesized that non-conjugated antibody may have diffused from the burn site into the blood stream and inhibited recruitment of circulating monocytes. Work has also been done in measuring healing responses, such as the formation of granulation tissue at the site. This will continue in Year 5.

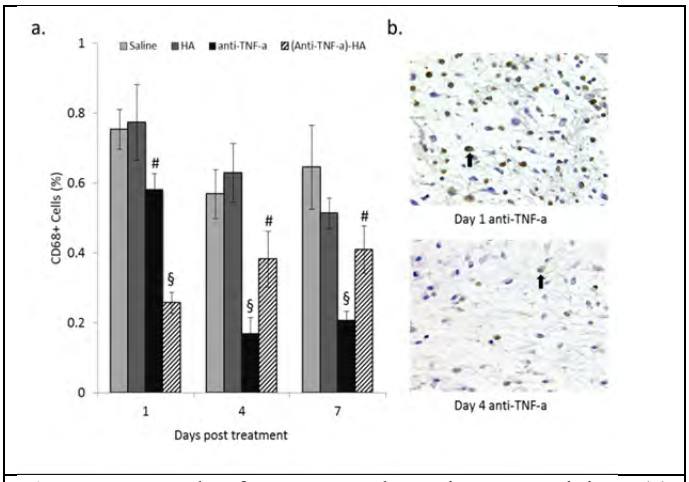

Figure 3. Results from macrophage immunostaining. (a) Quantification of results. (b) Sample data.

To facilitate translating this technology into the clinic, an analogue has been developed that does not require covalent conjugation of the antibody. The Washburn lab used a biolayer interferometer from ForteBio to investigate physical properties of the new formulation. In Figure 4 are shown representative plots. The abscissa shows the time during adsorption or desorption and the ordinate shows the computed change in thickness of the sensor surface. The bottom family of curves is TNF antibody alone, with

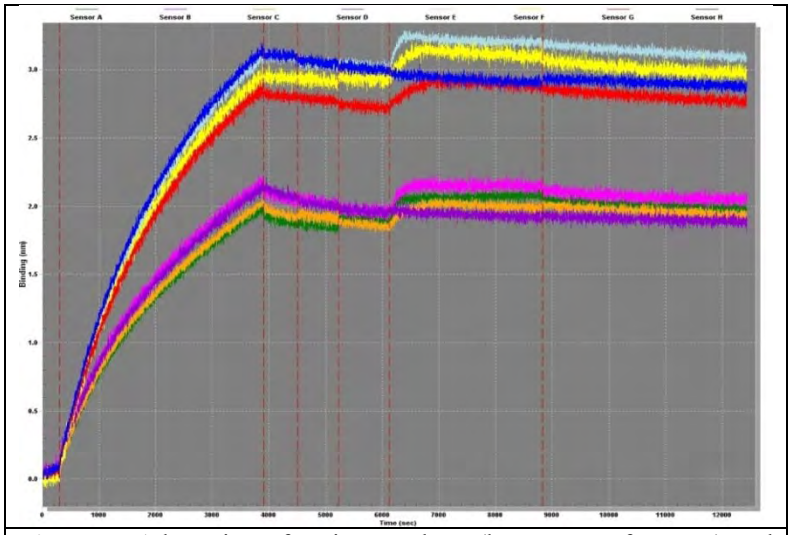

Figure 4. Adsorption of anti-TNF alone (bottom set of curves) and new formulation of anti-TNF (top set of curves) followed by binding of TNF.

a characteristic maximum thickness change of approximately 1 nm. The top family of curves is the new formulation including TNF antibody at the same concentration. There is a significant increase in thickness, indicating that the antibody interacts strongly with the carrier. These stronger interactions could serve a similar function in vivo, increasing antibody residence time at the site of inflammation. CMU is applying for a provisional patent on this approach, and it will be validated in Year 5 of this grant in the rat burn model.

Key Research Accomplishments

- Investigated effects of (anti-TNF)-HA gels in rat burn model and developed mechanistic understanding.
- Developed analogue that does not require covalent conjugation, which will significantly facilitate clinical translation.

Conclusions

The results indicate that (anti-TNF)-HA conjugates are highly effective at inhibiting burn progression in a rat model. Due to fundamental similarities in early inflammatory responses across species, the Washburn lab expects a similar response in humans.

The research plan for the following year will focus on performing more fundamental studies to understand the mechanism of effect of the conjugates. The Washburn lab will also test the non-conjugated analogue and validate its activities in a rat burn model.

The planned clinical transition will involve the CMU spin-off company, Washburn Therapeutics. The company has an exclusive license for patents filed by Carnegie Mellon University and has formed a partnership with a manufacturer of anti-TNF that can be used in humans. The company will begin clinical trials of the new formulation late 2012/early 2013.

The spin-off company, Washburn Therapeutics, could constitute a conflict of interest for Dr. Washburn. Dr. Washburn has up a management plan with Carnegie Mellon University (CMU) to ensure that no real conflicts arise. Research at CMU will focus on establishing a fundamental understanding of the function of these conjugates while research at the company will focus on the ultimate translation to the clinic, so a clear separation should make any real conflicts in how funding is used unlikely.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	1	0
# Post docs	0	1
# grad students	1	2
# undergrad students	1	3
# staff members working for AFIRM	0	0

Other Project Statistics	
# Honors given to AFIRM faculty	1
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	1
# Inventions disclosed	0
# Patents awarded	1
# Peer reviewed publications	1
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Honors and Awards

Coulter Translational Research Award (year 2 was awarded)

Patents and Inventions

Inflammation-regulating compositions and methods (nationalized)

Peer-Reviewed Publications

1. Reduction of burn progression with topical delivery of (antitumor necrosis factor-a)-hyaluronic acid conjugates. Sun LT, Friedrich E, Heuslein JL, Pferdehirt RE, Dangelo NM, Natesan S, Christy RJ, Washburn NR. Wound Repair Regen (in press).

Proposals

"Inflammation-regulating gels for improving burn outcomes" (U.S. Army; pending)

Grants

09/01/10 – 08/31/12 Wallace H. Coulter Foundation "Improving Burn Outcomes with Cytokine-Neutralizing Gels"

Overview of the Scarless Wound Healing Program

Program Leaders: Geoffrey C. Gurtner, MD, Michael T. Longaker, MD

Scar formation following injury is a major biomedical burden for the US health care system. Both soldiers and civilians suffer from the consequences of dysregulated wound repair. The costs associated with treatment of tissue fibrosis in the US are estimated to be over $4 billion per year. Current treatment regimens involving surgery, silicone sheeting, anti-inflammatory medications and laser/radiation have been disappointing. This is largely due to a lack of understanding of the fibrotic process. The pathophysiology of scar formation suggests the need to regulate numerous aspects of the wound environment, including cells, extracellular matrix, mechanics and bio-chemical signaling.

Wound healing proceeds through overlapping and well-defined phases of repair. This process continues for months and often results in irreversible scar formation with resultant contractures and disfigurement. For any therapeutic approach to be truly successful, it must be comprehensive and encompass the myriad in-puts regulating wound healing. Studies of tissue regeneration have implicated the inflam-matory environment, matrix components, me-chanical context and cellular players in pro-ducing a "scarless" wound profile. The WFPC approach encompasses a broad continuum of technologies aimed at modulating the tissue response to injury. Collectively, these projects represent a collaborative effort to address every aspect and stage of wound repair in a single research program, with the overarching aim of developing a more effective wound management paradigm.

Effective strategies to promote wound regeneration and prevent scar formation are needed, especially given the increasing survival of injured soldiers returning from the battlefield. The burden of scarring that follows the 230 million surgical procedures performed world-wide each year is enormous. Although the exact incidence of pathologic scarring is unknown, soldiers and patients continue to suffer from functional disabilities caused by wound contracture and severe disfigurement from hyperproliferative scarring.

Although anti-fibrotic biomolecules have demonstrated effectiveness in vitro, a major hurdle for clinical translation is the ability to maintain drug release and bioactivity in a complex wound environment. There is also a lack of effective animal models to study scar formation. Therefore, development of more appropriate and clinically-relevant animal models of hypertrophic scarring remains another area of unmet need.

This and other strategies undertaken to reduce care are described in the following section.

Project SW-1: Mechanical Manipulation of the Wound Environment to Reduce Manifestation of Scar

Team Leader(s)	Geoffrey C. Gurtner MD (Stanford University)
Project Team Members	Michael T. Longaker MD, MBA (Stanford University) Reinhold Dauskardt PhD (Stanford University)
Collaborator(s)	Neodyne Biosciences Biomaterials and Advanced Drug Delivery (BioADD) Center at Stanford University
Therapy Deliverable(s)	Control of wound environment to minimize scarring *Baseline:* Battlefield-ready, region-specific devices capable of stress-shielding mechanical forces to minimize scar formation. *Revised:* Identify novel molecular targets in scar mechanotransduction and develop drug-eluting mechanomodulatory scaffolds capable of mitigating fibrosis
TRL Progress	Start of Program: TRL #4 End Year 3: TRL #7 End Year 1: TRL #4 End Year 4: TRL #7 End Year 2: TRL #6
Key Accomplishments:	The Stanford group in conjunction with Neodyne Biosciences has successfully completed a phase I/II randomized within-patient control prospective clinical trial and published their results in a top tier surgical journal. A phase III trial has subsequently been initiated with DoD support. The Stanford group has further demonstrated that mechanical forces play a major role in scar formation in human subjects. They have also identified a key role for the molecular target focal adhesion kinase (FAK) in the regulation of inflammatory pathways contributing to skin fibrosis and published on this in Nature Medicine
Keywords	hypertrophic scarring, wound device, mechanotransduction, fibrosis

Introduction

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. Multiple factors are known to influence wound repair (such as inflammation, oxygen tension, and ischemia) but therapeutic modalities aimed at these targets have been largely unsuccessful. Mechanical force has long been recognized to influence cellular behavior *in vitro* and clinical observations based on Langer's lines and hypertrophic scarring corroborate this phenomenon *in vivo* (1,2). Recently, the Gurtner laboratory published the first murine model of hypertrophic scarring based on increasing the skin stress of healing wounds (3). We found that intrinsic skin mechanics correlated with scarring phenotype following wounding, as low mechanical stress fetal wounds exhibit minimal fibrosis and stiffer human skin displays robust scarring (3). 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, we 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.

To our knowledge, there are no commercially available wound care products that specifically address the mechanical stress state of healing wounds to reduce scarring. Elastic bandages and pressure dressings provide a widely variable range of compressive forces and are generally used for hemostatic purposes, not directly for scar attenuation. Negative pressure wound sponge devices (WoundVac) are used on large open exudative wounds but require elaborate components and an electrical energy source. In addition, their mechanism of action is in part based on *increasing* mechanical stimulation. In contrast to existing wound care options, our technology enables precision stress-shielding of area-specific wound forces through a portable, ready-to-use, simple pressure adhesive dressing that can be readily employed on the battlefield immediately following injury. This technology has not been achieved elsewhere and we continue to make significant progress in this application.

Current mechanotransduction literature implicates a central role for cell-matrix interactions in scar mechanotransduction (4). Specifically, focal adhesion kinase (FAK) has been identified as a potential target in the mechanical activation of inflammation and fibrosis (5). This molecular target may be a driving force in the formation of human hypertrophic scarring.

Research Progress

The Stanford group had previously initiated preclinical animal studies suggesting a central role for fibroblast FAK in hypertrophic scarring. A fibroblast-specific FAK knockout (KO) mouse strain was subsequently developed and studies were undertaken to compare hypertrophic scarring in wildtype mice subjected to mechanical loading compared to FAK KO mice. Work for year 3 ended with the finding that FAK KO scars had significantly reduced gross scar area and cross sectional scar area compared to wildtype mice. In year 4, we verified that FAK KO mice had decreased collagen deposition and scar tissue compared to wildtype mice (Figure 1). Further transcriptional analyses of FAK KO wounds demonstrated a decrease in expression of the inflammatory cytokine MCP-1, which was verified by Western blot. Immunolocalization of histologic sections confirmed a reduction in MCP1 in FAK KO wounds as well as a reduction in macrophages and activated fibroblasts. MCP-1 injection into wild type wounds exacerbated scarring and resulted in increased macrophage recruitment into wounds (Figure 2), further demonstrating the relevance of a FAK-MCP1 pathway to hypertrophic scar formation. Topical treatment of mechanically loaded murine wounds with a FAK inhibitor reduced gross scar area and histologic evidence of scarring (Figure 3). These studies suggest the importance of fibroblast FAK in mechanosensation and inflammatory signaling through MCP1 in effecting hypertrophic scarring. Preclinical studies demonstrated that pharmacologic inhibition of FAK is therefore a promising therapeutic strategy for reducing wound inflammation and scarring (Figure 4).

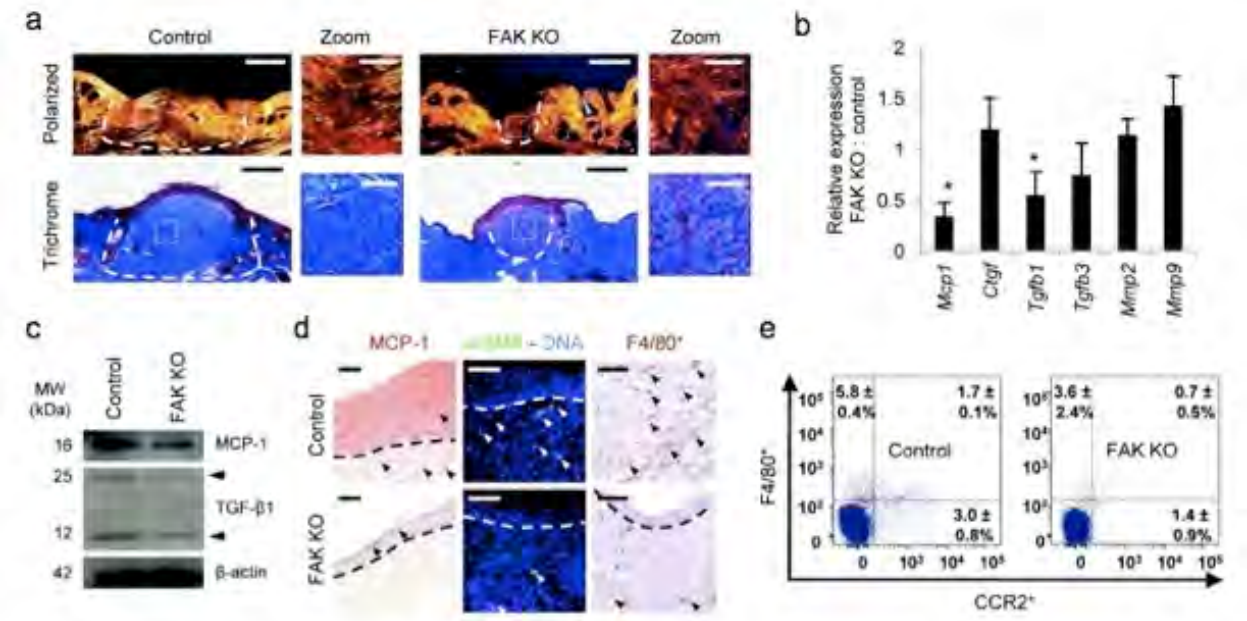

Figure 1: (a) Polarized light and trichrome stained tissue sections demonstrate a reduction in collagen deposition in FAK KO scars. (b) Real time PCR reveals a quantitative decrease in MCP1 andTgfb1 transcription in FAK KO wounds, (c) with validation by immunblot. (d) Decreased immunolocalization of MCP1, a-SMA+ cells, and F4/80+ cells (e) is followed by flow cytometry demonstrating a reduction in F4/80+ and CCR2+ cells in FAK KO tissue.

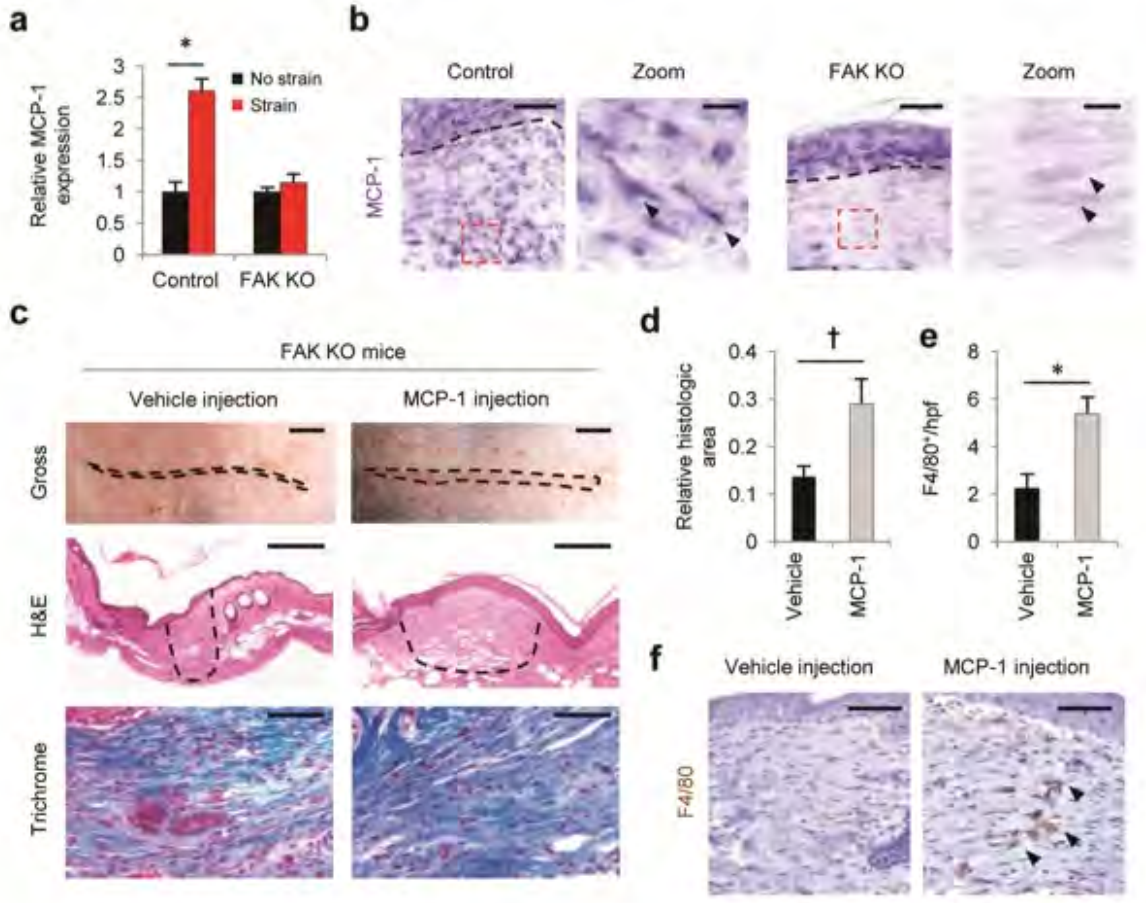

Figure 2: (a) MCP1 transcription in strained FAK KO fibroblasts is reduced. (b) In situ hybridization reveals decreased dermal MCP1 in FAK KO mice at day 10 following injury. (c) Injection of MCP1 into mechanically loaded FAK KO wounds restores scar hypertrophy, with a significant increase in (d) scar area, (e-f) and macrophage infiltration into the wound bed.

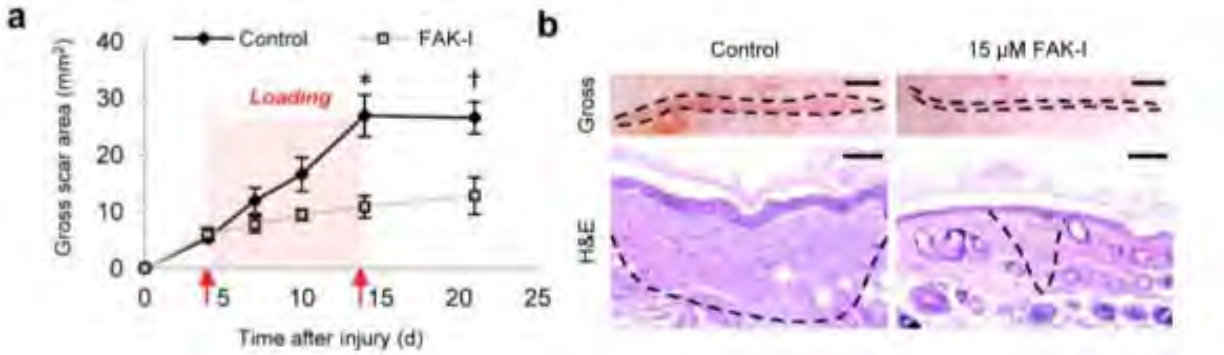

Figure 3: Wounded mice subjected to mechanical loading undergo treatment with a small molecule FAK inhibitor, PF573228 (Tocris), resulting in (a) a significant reduction in gross scar area and (b) a decrease in cross sectional scar area as demonstrated by H&E staining. *Not shown*: Reduction in dermal MCP1 immunlocalization to wounds treated with PF573228.

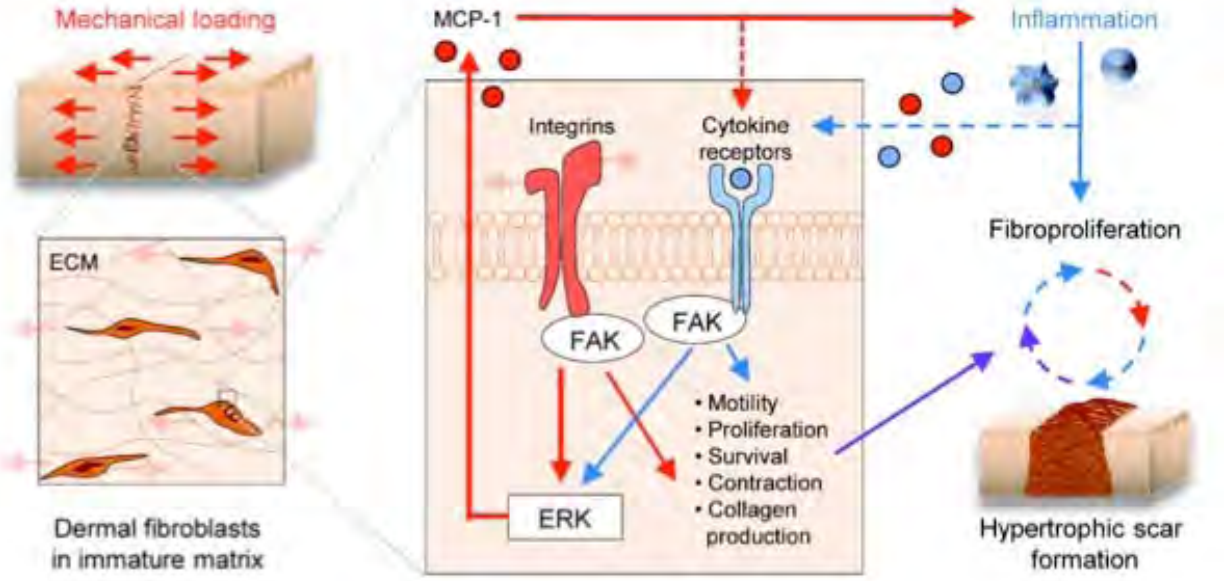

Figure 4: Schematic demonstrating the activation of fibroblast FAK by local and systemic mechanical and inflammatory stimuli, resulting in the activation of ERK and upregulation of MCP1, which further incites fibroproliferative pathways and hypertrophic scar deposition.

Key Research Accomplishments

- Demonstrated that fibroblast FAK plays a critical mechanosensory role in hypertrophic scar formation
- Identified the mitogen activated protein kinase ERK as a critical downstream effector of FAK signaling
- Found that FAK knockdown results in a decrease in transcriptional levels of inflammatory cytokines including MCP1, both in vitro and in vivo
- After demonstrating a signaling relationship between FAK in mechanically stretched wounds and MCP1 secretion, our group demonstrated that exogenous administration of MCP1 to incisional wounds could recapitulate features of hypertrophic scars
- Demonstrated molecular evidence of inflammation in wildtype scars, including activated fibroblasts, macrophage infiltrates and inflammatory cytokines, all of which were reduced in FAK KO scars
- Demonstrated that topical inhibition of mechanically loaded wounds with a small molecule FAK inhibitor reduced hypertrophic scarring
- Published findings in Nature Medicine, a top tier biomedical journal

Conclusions

The Stanford group has successfully demonstrated that fibroblast focal adhesion kinase plays a critical mechanosensory role in the skin. Activation of fibroblast FAK in high tension wound environments precipitates an inflammatory cascade of events, including MCP1 secretion, that results in collagen deposition and scarring. Pharmacologic inhibition of FAK reduces wound inflammation and scarring and holds great potential for treatment and prevention of scarring.

Research Plan for the Following Years

The Stanford group is planning to further investigate the role of focal adhesion kinase in cutaneous wound healing by exploring the function of keratinocyte FAK in wound healing and scarring. A keratinocyte specific FAK KO mouse is currently in development and will be used to investigate the effects of this keratinocyte protein on scarring and wound remodeling. A more rigorous understanding of how keratinocyte FAK signaling contributes to wound healing will help frame our previous findings on fibroblast FAK and ultimately enable us to develop targeted, cell specific strategies towards improving tissue regeneration and reducing scarring.

Planned Clinical Translations

Neodyne Biosciences Inc. is in the process of conducting phase III trials, which will recruit a larger patient population. In conjunction with the Materials Science and Engineering department at Stanford University, they will further refine the polymeric device to custom-design treatments

for various size wounds and tensio n states. Th is will allo w for body-specific reg ional stress-shielding to address a wide variety of surgical wounds.

Corrections/Changes Planned and Rationale:

None

Conflict of Interest Disclosure:

GCG, MTL, and RHD are co-founders of and hold an equity position in Neodyne Biosciences, Inc.

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2. Edlich, R.F. & Carl, B. A. Predicting scar formation: from ritual practice (Langer's lines) to scientific discip line (static and dynam ic skin tensions). *The Journal of emergency medicine* **16**, 759-760 (1998).
3. Aarabi, S. *et al.* Mechanical load initiates hypertrophic scar f ormation through decreased cellular apoptosis. *Faseb J* **21**, 3250-3261 (2007).
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5. Parsons, J.T. Focal adhesion kinase: the first ten years. *J Cell Sci* **116**, 1409-16 (2003).
6. Wong VW, *et al*. Focal adhesion kinase links m echanical force to skin fibrosis via inflammatory signaling. *Nature Medicine* 2011; 18(1):148-52.

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	0
# Post docs	1	2
# grad students	0	2
# undergrad students	0	1
# staff members working for AFIRM	1	1

Other Project Statistics	
# Honors given to AFIRM faculty	7
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	1
# Patents awarded	5
# Peer reviewed publications	2
# Non-peer reviewed publications	2

*Directly supported in whole or part –time by AFIRM

Honors and awards – list them by awardee name

1. Dr. Gurtner: James Barrett Brown Award 2008 (Best plastic surgery paper) from the American Association of Plastic Surgeons
2. Dr. Gurtner: James Barrett Brown Award 2009 (Best plastic surgery paper) from the American Association of Plastic Surgeons
3. Dr. Gurtner: Associate Chairmanship position from Stanford University Department of Surgery

4. Dr. Longaker: Basic Science/Translational Researcher of the Year Award 2010 from the American Association of Plastic Surgeons
5. Dr. Longaker: Frederick Birnberg Award for Excellence in Dental Research from Columbia University 2011
6. Dr. Longaker: Honorary Fellow of the Society of Black Academic Surgeons 2011
7. Dr. Longaker: Flanc-Karl Award from the American Surgical Association 2011

Patents and Inventions – Patents awarded, filed, invention disclosures
Patents Awarded:

1. US Patent #20080033334
Devices and bandages for the treatment or prevention of scars and/or keloids and methods and kits thereof
Filed: February 7, 2008
Inventor: Geoffrey C. Gurtner, MD, et al.

2. US Patent #20060037091
Method for producing hypertrophic scarring animal model for identification of agents for prevention and treatment of human hypertrophic scarring
Filed: February 16, 2006
Inventor: Geoffrey C. Gurtner, MD, et al.

3. US Patent #8168850
Methods for the treatment or prevention of scars and/or keloids
Filed: January 21, 2009
Inventor: Geoffrey C. Gurtner, MD, et al.

4. US Patent #8063263
Methods for the treatment or prevention of scars and/or keloids
Filed: January 22, 2009
Inventor: Geoffrey C. Gurtner, MD, et al.

5. US Patent #7683234
Devices and bandages for the treatment or prevention of scars and/or keloids and methods and kits therefor
Filed: August 3, 2007
Inventor: Geoffrey C. Gurtner, MD, et al.

Patent Applications Filed:

1. Application #: 2012/0046586 A1
Skin Treatment Devices and Methods with Pre-Stressed Configurations
Published: 2/23/12
Inventor: Geoffrey C. Gurtner, MD, et al.

Peer-Reviewed publications

1. Gurtner GC, Dauskardt RH, Wong VW, Bhatt KA, Wu K, Vial IN, Padois K, Korman JM, Longaker MT. Improving cutaneous scar by controlling the mechanical environment: large animal and phase I studies. Ann Surg. 2011; 254(2):217-25.

2. Wong VW, *et al*. Focal adhesion kinase links mechanical force to skin fibrosis via inflammatory signaling. *Nature Medicine* 2011; 18(1):148-52.

Other Publications

1. Wong VW, Rustad KC, Akaishi S, Sorkin M, Glotzbach JP, Januszyk M, Nelson ER, Levi K, Paterno J, Vial IN, K uang AA, Longaker MT , Gurtner GC. Focal adhesion kinase links mechanical force to skin fibrosis via inflammatory signaling. Nat Med. 2011 Dec 11;18(1):148-52.

2. Wong VW, Bhatt KA, Vial IN, Daus kardt RH, Longaker MT, Gurtner GC. Mechanomodulation of the wound environm ent to decrease scar formation in a porcine model. Armed Forces Institu te of Regenerati ve Medicine All Hands Meeting, St. Pete, FL. Abstract/podium presentation.

3. Wong VW, Bhatt KA, Vial IN, Wu K, Pado is K, Dauskardt RH, Longaker MT, Gurtner GC. Beyond Langer's lines: m anipulating wound mechanical forces to control hypertrophic scar formation in the red duroc pig. Am erican College of Surgeons Annual Meeting 2010, Washington DC. Abstract/podium presentation.

4. Beasley B, Longaker MT, Gurtner GC, Da uskardt GC, Dauskardt RH, Yock P. Stress-shielding device dem onstrates dramatic decrease in scar form ation in first-in-m an study. ATACCC meeting August 2010. Abstract.

Project SW-2: Regenerative Bandage for Battlefield Wounds

Team Leader(s)	Geoffrey C. Gurtner MD (Stanford University) Michael T. Longaker MD, MBA (Stanford University)
Project Team Members	Anthony Oro MD, PhD (Stanford University)
Collaborator(s)	None
Therapy	Improved wound healing and reduced scarring
Deliverable(s)	*Baseline:* Regenerative bandage that promotes fetal-like wound healing instead of scarring *Revised:* None
TRL Progress	Start of Program: TRL #2 End Year 3: TRL #4 End Year 1: TRL #3 End Year 4: TRL #5 End Year 2: TRL #3
Key Accomplishments:	The Stanford group has developed a novel biomaterial scaffold with modifiable open porosity and matrix components. This composite matrix is highly biocompatible with numerous cell types important for wound repair. Their group has initiated characterization of the dermal architecture of fetal murine skin and unwounded murine skin using advanced microscopic techniques. They have also demonstrated predictable degradation properties in vivo and the biomaterial scaffold seeded with bone marrow derived, mesenchymal stem cells improved early wound healing in a humanized excisional wound model in mice
Keywords	dermal matrix, wound healing, fetal skin

Introduction

Wounded soldiers returning from the present conflicts have sustained significant trauma to the head, neck, face, and limbs. Timing is critical to optimize salvage of traumatic wounds; once wounds are surgically debrided, coverage is important to reduce a prolonged inflammatory state, infection with subsequent contraction, and disability. A novel approach is needed to minimize this inflammatory and fibrotic cascade in the initial days following injury while promoting tissue regeneration. The research team's technical approach begins immediately post-injury with a regenerative bandage consisting of a fetal biomimetic matrix and human progenitor cells to maintain an acute wound in a pro-regenerative state of "suspended animation" and prevent the onset of scarring, fibrosis, and infection. Utilizing their knowledge of fetal skin development, scarless repair, and burn therapy, the researchers hope to preserve wounds in a "fresh state" by recreating a fetal-like wound-healing milieu to promote regeneration and optimize the results of definitive therapy provided back in the United States.

There are several commercial products used for skin engineering based on human or pig skin. These decellularized matrices are effectively used in a variety of surgical and wound settings and clinical results are improved in many cases compared to no treatment at all (1). However, natural skin sources are limited by availability, cost, and risk of disease transmission. Further, clinical results using skin substitutes remain suboptimal due to poor cosmetic and functional outcomes

(2). Synthetic skin substitutes offer the promise of a widely available, disease-free, cheaply produced replacement skin which can potentially improve current clinical outcomes.

The Stanford group has developed hygroscopic dressings mimicking unwounded dermal micropatterning (3). This engineered construct significantly improved cutaneous wound healing in a mouse model and demonstrated potent immunomodulatory properties that enhanced wound vascularization. They are now focused on using this regenerative template to maintain progenitor cells in suspended animation for delivery into wounds; and to develop a battlefield-ready, rapidly expanding hydrogel that can be used as a regenerative bandage and vehicle for autologous stem cell delivery.

Research Progress

The Stanford group has made significant progress in further developing their carbohydrate-based collagen hydrogel for wound healing by performing both in vitro and in vivo experiments demonstrating the potential therapeutic efficacy of this technology. A new, efficient method of seeding hydrogels with stem cells was developed using capillary force (Figure 4). Mesenchymal stem cells (MSCs) that were plated or seeded in hydrogels were evaluated for expression of transcription factors associated with self renewal and pluripotency including Oct4, Sox2, and Klf4. Transcriptional levels of these stemness markers were increased in the hydrogel group and this was confirmed by immunoblot (Figure 1). We subsequently performed in vivo experiments using a stented excisional wound model and found that wounds treated with the MSC seeded hydrogel were significantly more vascular than wounds that were untreated or injected with MSCs (Figures 2-3). Histologic wound analyses revealed evidence of MSC differentiation into pericytes, endothelial cells, and fibroblasts (Figure 5). Cross sectional H&E staining demonstrated evidence of dermal architecture regeneration in wounds treated with MSC seeded hydrogels (Figure 6).

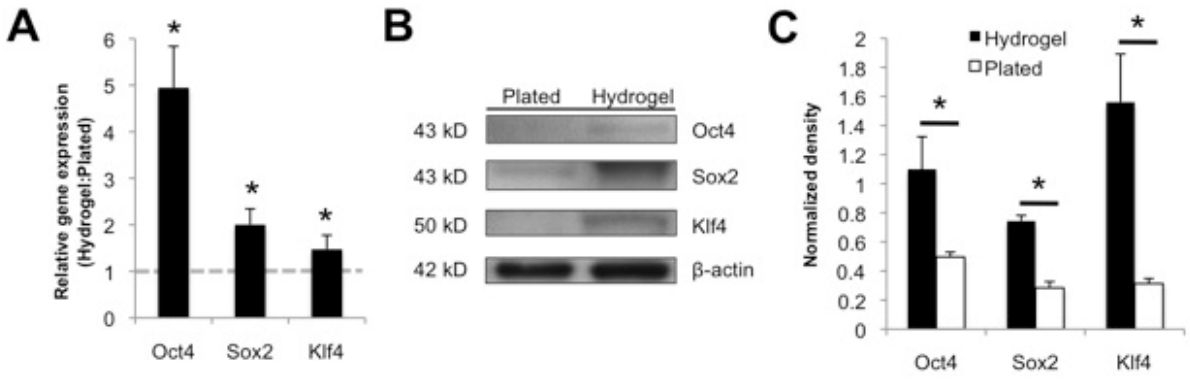

Fig. 1. MSC expression of stemness genes. (A): qRT-PCR analysis of *Oct4, Sox2* and *Klf4* gene expression in hydrogel culture versus standard plating (dotted grey line, n=7). (B): Western blotting of stemness genes. (C) Quantification of western blotting (n=4).

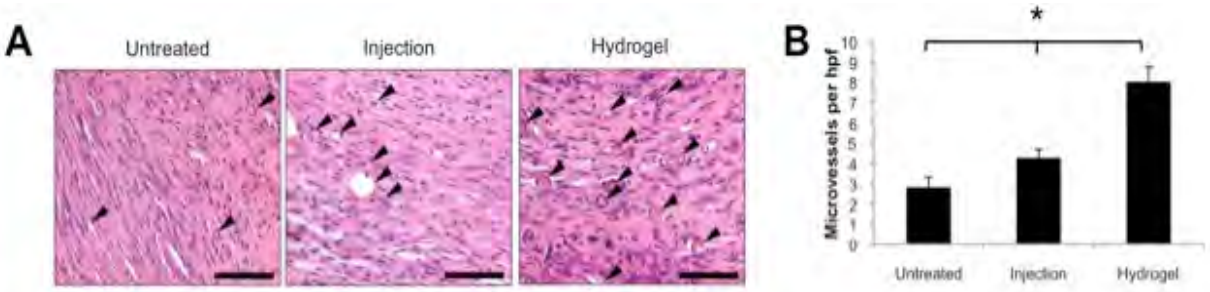

Fig. 2. Effect of MSC delivery method on wound vascularization. (A): H&E-stained sections of day 14 wounds. Arrowheads indicate microvessels. Scale bar 20μm. (B): Microvessel counts.

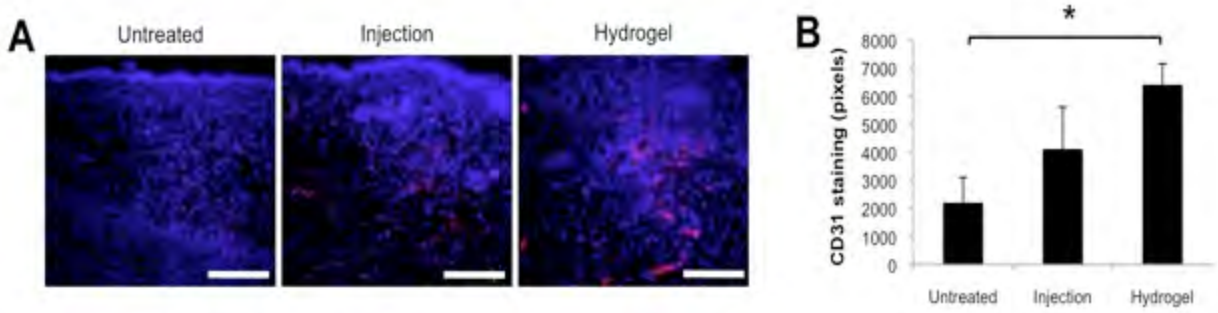

Fig. 3. CD31 analysis. (A): Day 14 wounds stained for CD31 (red). Scale bar 100μm (B): Quantification of CD31 staining intensity.

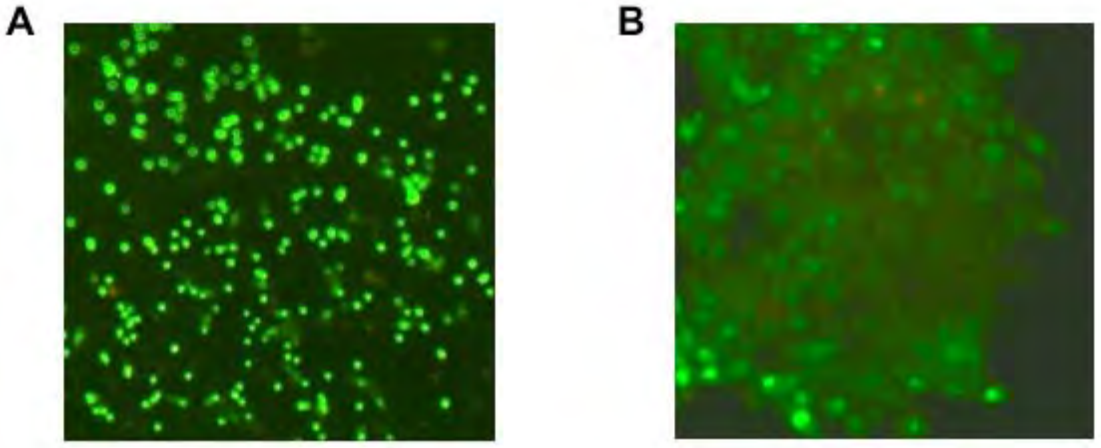

Fig. 4. Cell viability using new seeding method of capillary force. (A): Live/dead assay results at day 3. (B): Cluster of cells using live/dead assay at day 14.

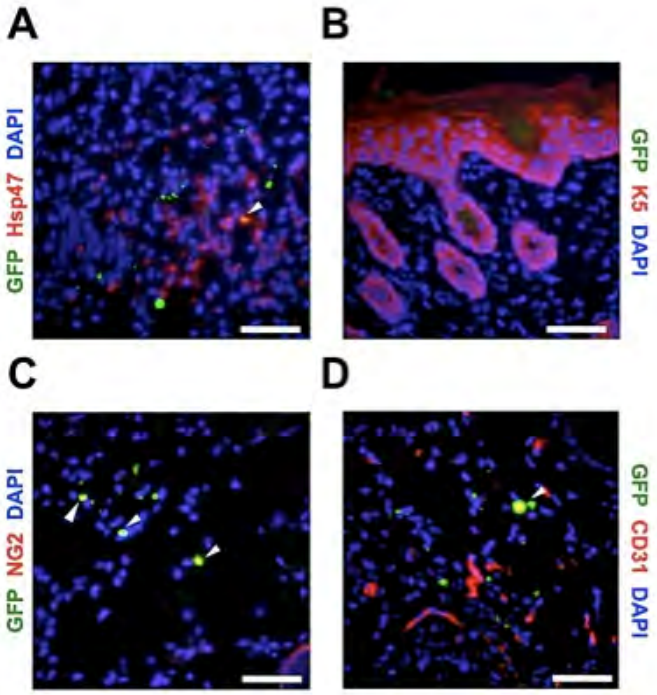

Fig. 5. Co-localization of GFP+ MSCs with cell-specific markers to determine MSC fate within wounds. MSCs co-localized (yellow, arrowheads) with Hsp47 (A), NG2 (C), and CD31 (D). (B): No GFP+/K5+ cells were observed. Scale bar 20μm.

Fig. 6. Effects of MSC delivery method on wound closure. (A): H&E staining of wounds at day 14. Scale bar 100μm. *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.

Key Research Accomplishments

- Developed and tested capillary seeding method for rapid and efficient distribution of stem cells throughout the hydrogel substrate
- Evaluated stemness factor expression among stem cells seeded in hydrogels compared to plated cells and found an increase in expression among hydrogel seeded cells
- Applied MSC seeded hydrogels to stented excisional wounds and identified a more rapid rate of wound closure compared to untreated and injected groups
- Demonstrated increased vascularity of MSC seeded hydrogel treated wounds by both H&E staining and CD31 staining
- Used immune co-localization techniques to demonstrate MSC differentiation into pericytes, fibroblasts and endothelial cells in vivo

Conclusions

The Stanford group has successfully demonstrated the therapeutic potential of a regenerative bandage composed of stem cells seeded on a biocompatible hydrogel scaffold. In vitro studies revealed that the hydrogel creates an environment for MSCs that increases stemness factor expression and in vivo studies revealed an improvement in wound closure and vascularity with evidence of dermal architecture restoration and cell differentiation into stromal subtypes.

Research Plan for the Following Years

The Stanford group will continue to optimize the regenerative bandage by investigating the therapeutic potential of adipose-derived stem cell (ASC) implantation into the hydrogel. ASCs are a promising group of mesenchymal stem cells that are easy to isolate from human patients

and therefore a practical cell to investigate for clinical use. In vitro studies will initially be performed on ASC seeded hydrogels to determine cell viability, stemness factor transcription and characterize the transcriptional profile of wound healing cytokines. In vivo studies will be performed using an excisional wound model that resembles human wound healing in order to determine whether ASC seeded hydrogels can augment wound closure, improve wound growth factor expression and vascularity, and promote dermal architecture restoration. Initial studies using murine cells will be performed prior to using human cells, which would help to move this preclinical work towards clinical trials.

Planned Clinical Translations

The Stanford group plans to continue in vivo small animal studies using murine and human ASCs to further characterize the observed improvement in early wound healing. They plan to file a 510(K) for a cell-free matrix if continued positive results are obtained. This could set the path for clinical trials using this dressing on open wounds to enhance granulation tissue formation.

Corrections/Changes Planned and Rationale

The Stanford group is expanding its use of progenitor cells to include adipose- and bone marrow-derived stem cells to seed into these biomatrices. The use of these cells has greater clinical applicability compared to embryonic stem cells (as initially proposed) and their use bypasses ethical concerns regarding the use of embryonic tissues. Autologous cells can potentially be harvested from injured patients and used to seed biomatrices in vitro for subsequent use as a regenerative wound bandage.

Conflict of Interest Disclosure

None

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Honors and awards – list them by awardee name

1. Dr. Gurtner: Jam es Barrett Brown Award 2008 (Best plastic surgery paper) from the American Association of Plastic Surgeons
2. Dr. Gurtner: Jam es Barrett Brown Award 2009 (Best plastic surgery paper) from the American Association of Plastic Surgeons
3. Dr. Gurtner: Associate Chairm anship position from Stanford University Department of Surgery (2010)
4. Dr. Gurtner: Basic Science Researcher of the Year Awa rd 2011, Association of Plastic Surgeons

1. Dr. Longaker: Basic Science/Translational Re searcher of the Year Award 2010 from the American Association of Plastic Surgeons
2. Dr. Longaker: Frederick Birnberg Award for Ex cellence in Dental Research from Columbia University 2011
3. Dr. Longaker: Honorary Fellow of the Society of Black Academic Surgeons 2011
4. Dr. Longaker: Flanc-Karl Award from the American Surgical Association 2011

Patents and Inventions – Patents awarded, filed, invention disclosures

1. Application #: 2011/0263724 A1
Threads of hyaluronic acid and/or derivatives thereof, methods of m aking thereof and uses thereof. Published: Oct 27, 2011 Inventors: Geoffrey C. Gurtner, MD, et al.
2. Application #: 2011/0305745 A1
Pollulan Regenerative Matrix Published: Dec 1 5, 2011 Inventors: Geoffrey C. Gurtner, MD, et al.

Peer-Reviewed publications

1. Wong VW, Rustad KC, Galvez MG, Neofyotou E, Glotzbach JP, Januszyk M, Major MR, Sorkin M, Longaker MT, Rajadas J, Gurtner GC. Engineered pullulan-collagen composite hydrogel scaffolds improve early cutaneous wound healing. Tissue Eng Part A. 2011 Mar;17(5-6):631-44.

Other Publications

1. Galvez MG, W ong VW, Chang EI, Major M, Carre L, Kandimalla R, Bhatt KA, Rajadas J, Longaker MT, Gurtner GC. Pullulan-co llagen hydrogel scaffold as a derm al substitute. American College of Surgeons 95 th Annual Meeting, Chicago, IL. Abstract/podium presentation.

2. Rustad KC, Wong VW , Galvez MG, Major MR , Nehama D, Sorkin M, Januszyk M, Rajadas J, Longaker MT, Gurtner GC. Pullula n-collagen hydrogel scaffold based on fetal dermal microarchitecture for regenerative wound healing. Ar med Forces Institu te of Regenerative Medicine All Hands Meeti ng, St. Pete, FL. January 13, 2010. Poster presentation.

3. Januszyk M, W ong VW, Rustad KC, Glotzbach JP, Major MR, Longaker MT , Gurtner GC. An Automated Method to Identify and Compare Wound Area in an Excisional Model of Murine Wound Healing. W ound Healing Society 21st Annual Meeting, Dallas, TX. April 15, 2011. Podium presentation.

4. Januszyk M, Rustad KC, Major MR, W ong VW, Glotzbach JP, Gurtner GC. Autom ated Detection of Wound Area in a Stented Excisi onal Model of Murine W ound Healing. Plastic Surgery Research Council 56th Annual Meeting, Louisville, KY. April 30, 2011. Podium presentation.

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	0
# Post docs	1	2
# grad students	0	2
# undergrad students	0	1
# staff members working for AFIRM	1	2

Other Project Statistics	
# Honors given to AFIRM faculty	8
# Doctorates awarded under A FIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	2
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	4

*Directly supported in whole or part –time by AFIRM

Project SW-4: Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration

Team Leader(s)	Patricia A. Hebda, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)
Project Team Members	Joseph E. Dohar, MD and Tianbing Yang, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)
Collaborator(s)	None
Therapy	Attenuate local inflammatory responses to reduce scarring and promote healing
Deliverable(s)	*Baseline:* Combinatorial anti-inflammatory topical therapy to reduce scar formation *Revised:* None
TRL Progress	Start of Program: TRL #3 End Year 3: TRL #3 End Year 1: TRL #3 End Year 4: TRL #4 End Year 2: TRL #3
Key Accomplishments:	Precise characterization of donor cell strains of different phenotypes, including cells strains with regenerative healing capability (adipose stem cells (ASC), fetal skin fibroblasts (embryonic day 15, E15)), for continuing work with cell therapy. Primarily exploration of regenerative healing mechanism guided by ASC and E15 cells in adult wound healing environment.Establishment of preliminary therapeutic strategy for combinatory topical treatment with isogenic ASC cells, E15 cells and anti inflammatory agent (Nimesulide) plus anti-fibrotic agent (PGE2), demonstrating rapid, regenerative healing.
Keywords	scarless healing, inflammation, fibrosis, cell therapy

Introduction

The Hebda laboratory is focusing on two related processes highly relevant to scar formation: inflammation and fibroblast activity. The overriding hypothesis is that the development of fibrosis can be prevented by blunting early wound healing processes leading to fibroblast recruitment and activation of synthetic properties. To achieve regeneration, it is first essential to regulate the inflammatory response and the influx of host fibroblasts. Control of these two fibrogenic processes will serve to establish an optimal foundation for therapies and interventions leading to regenerative healing. The early inflammatory phase of tissue repair has been shown to be important for the long-term outcome of wound healing.

This project has three Specific Aims:

1. To determine the potential of combinatorial anti-inflammatory therapy in decreasing subsequent fibroblast activity in the wound bed.
2. To precisely characterize the contribution of the fibroblast phenotype to the overall degree of tissue fibrosis.

3. To design interventions, based on the results of the first 2 aims, that provide a wound environment for rapid, regenerative healing.

In the previous 2 years, the researchers completed their achievements on the Aim 1, demonstrated that early, short-term treatment with anti-inflammatory agents can attenuate the wound inflammatory response with downstream effects on healing; and on the Aim 2, demonstrated that early, one time topical treatment with isogenic (same strain) adipose stem cells (ASC), fetal skin fibroblasts (embryonic day 15, E15), and even adult skin fibroblasts, leads to reduced scarring and increased healing.

The third goal of this study is to design interventions, based on the results of the first 2 aims, that provide a wound environment for rapid, regenerative healing.

Dr. Hebda's research team proposes to use a novel method, transplantation of fetal fibroblasts into an adult dermal wound bed, to precisely characterize the impact of inflammatory and other soluble mediators on the fibroblast phenotype. This approach will allow them to determine if the fibroblast phenotype is a dynamic one, largely influenced by the wound environment. Should this be the case, then prevention of fibrosis/scarring could be primarily a matter of reducing pro-fibrotic signals in the wound bed. Alternatively, if donor fibroblast phenotype persists within the wound after transplantation, therapeutic efforts will be directed toward enhancing the wound healing contribution of fibroblasts with a regenerative phenotype.

Competing Technologies: While there are a number of studies that have tested the effect of cell-based therapy for improved wound healing, the research team is aware of no studies investigating the combinatorial approach they are taking.

Research Progress

Aim 2: ***To precisely characterize the contribution of the fibroblast phenotype to the overall degree of tissue fibrosis.***

The researchers have generated GFP-expressing cell strains of different phenotypes: 1) embryonic day 15 fetal skin fibroblasts (E15, scarless phenotype), 2) embryonic day 15 fetal skin fibroblasts (E18, reparative phenotype), 3) adult skin fibroblasts (reparative phenotype), and 4) adult adipose stem cells (ASCs) (multipotent progenitor cells). These cells have been used for Aim2, and will be used together with the optimized therapies from Aim 1 to determine effects on the viability and responses of the transplanted cells and the quality of healing.

The researchers adapted protocols from the literature for cell isolation and preparation.[1] To avoid possibly stem cells differentiation and fibroblast instability, the optimal passage of all the cell strains was selected for therapy as 5^{th}, i.e. the stem cells no more than 5^{th} passage, the E15, E18, and adult skin fibroblasts no less than 5^{th} passage. For instance, the researchers observed that the purity of fibroblasts was satisfied after passage 5^{th}[2], and the ASC were full of differentiation potential before passage 5^{th} (Figure1).

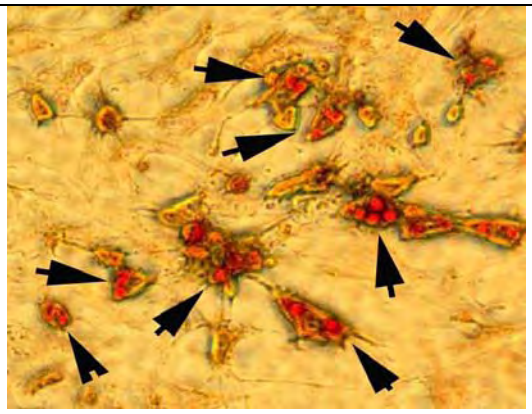

Figure 1a. Adipogenesis induced differentiation of ASC using Stem Cell Kits (R&D, MN, US). Rat adipose stem cells (ASC, passage 4) were induced under adipogenic conditions for 14days. Fixed, and stained with Oil Red O, a marker for lipid-rich vesicles (200× magnification)

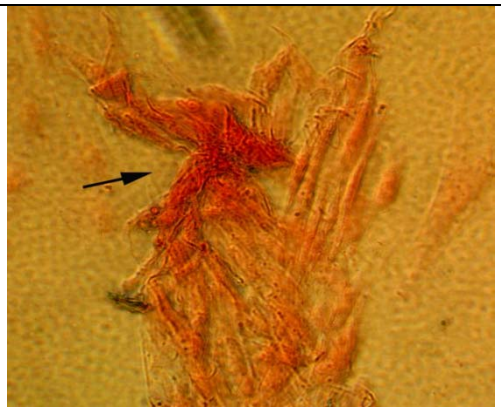

Figure 1b. Osteogenesis induced differentiation of ASC using Stem Cell Kits Rat ASC, passage 4, were induced under osteogenic conditions for 14days, stained with Alizarin Red S, mineralized matrix as indicated (200× magnification)

Since isogenic fibroblast transplant had been traced successfully at the wound beds of day 14 on a histological basis [3], an animal experiment was performed to detect the population balance between host (endogenous) fibroblasts and the isogenic transplants. GFP positive isogenic cells were applied at 5×10^5 cells per incisional wound and 2×10^5 cells per excisional wound. On day 6 post wounding and cell treatment, wound beds were harvested and cells were processed for flow cytometry. After intracellular staining with Vimentin antibody (PE labeled), endogenous fibroblasts were identified as Vimentin single positive, while donor cells as Vimentin & GFP double positive. The results indicated that donor cells consist of 7% to 11% (Figure2) of total fibroblast population at the wound beds, neither different between incisional and excisional wounds, nor among the four cell types.

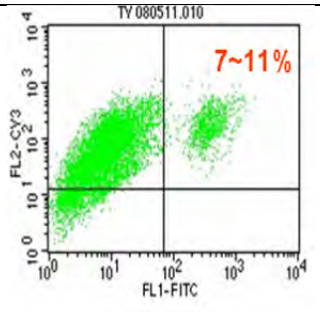

Figure 2. Two parameter histogram Dot Plot displayed the distribution of endogenous (left) and exogenous (right) cells in Day 6 wounds. Sample size=10^4 cells.

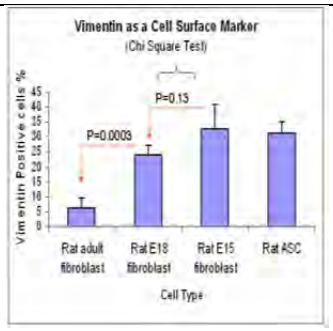

Figure 3. Surface Vimentin positive cell portion in the four cell types (10^4 total cells), Mean Fluorescence Intensity (MFI) showing no differences.

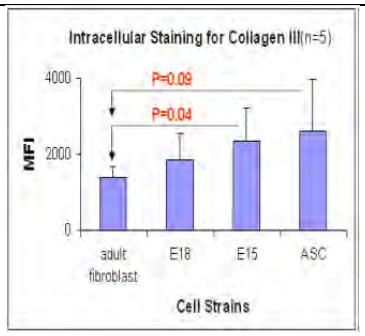

Figure 4. MFI shows differences in intracellular collagen type III (fetal type) level among the four cell types (10^4 total cells).

Based on their previous observations that ASC and E15 fetal skin fibroblasts treatment significantly increased the regain of tensile strength ($p<0.001$, $p=0.007$, respectively)[4], the researchers have been exploring the molecular mechanisms under these phenomena.

Cell surface Vimentin positive population was confirmed to be 6% for adult fibroblast, 24% for E18 fibroblast, 32% for E15 fibroblast, and 31% for adipose stem cells, significantly lower in adult fibroblasts by comparison with other three cell types (Figure 3). The researches believe the cell surface Vimentin serves as a favorite factor for wound healing, since cell surface Vimentin is a receptor for soluble CD44, a hyaluronan binding domain [5], while recruitment of hyaluronan is beneficial at wound beds [6][7].

Cell intermediate collagen type I, collagen type III, and fibronectin were detected by flow cytometry after intracellular staining. No difference was found in intracellular collagen type I among the four cell types, but collagen type III and fibronectin shared a similar pattern, with a higher intracellular expression in adipose stem cells and E15 fibroblasts compared to adult fibroblasts (Data shown for collagen type III, Figure 4). This pattern shows a positive correlation between tissue tensile strength and intracellular collagen type III level and fibronectin level. Secretory collagen level was detected in fetal cell supernatant but not in adult cell supernatant by Western Blotting, indicating both collagen type I and collagen type III play an important role in fetal wound healing comparing to adult wound healing (Figure 5). It has been well documented that fetal cells produce more collagen than adult cells, especially collagen type III [8], and a higher percentage of type III to type I collagen reduces scarring [9]. The researchers believe their E15 fetal cells and ASCs are beneficial for wound healing, due at least partially to their collagen production.

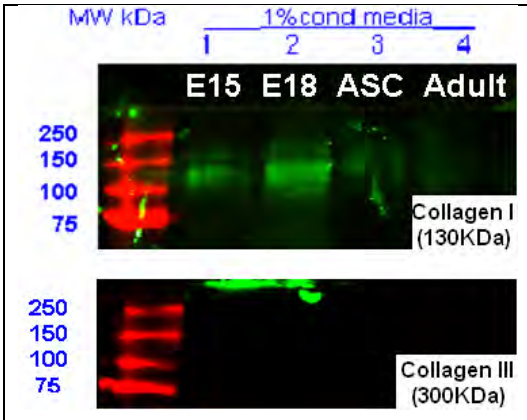

Figure 5b Western Blot Detection of Collagen type I and III in cell culture supernatant: 5×10^5 cells were cultured in 5 ml low serum medium (1% FBS) in 6-well plates, and the supernatant were collected after 24 hours

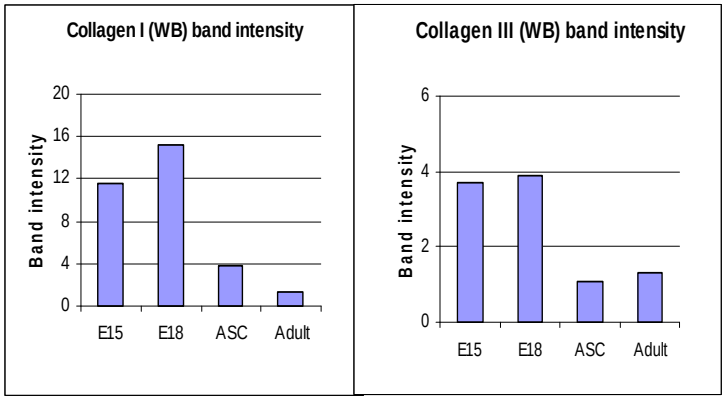

Figure 5b. Quantitative Analysis of Odyssey Western Blots: Band intensity measured by Odyssey Imagine System indicated a much higher collagen secretion in fetal cells than adult cells. The collagen type III / type I ratio was 0.32 in E15 cells, while 0.26 in E18 cells.

The researchers also detected NFkBp65 & and its inhibitor IkB α level in whole cell lysate upon IL-1β stimulation by Western Blotting, and found an interesting lower NF-κB/ IkBα ratio in E18 cells, implying immunological change occurring at the developmental switch point of E18, a critical timing from scarless regeneration to reparative (adult-type) wound healing (Figure 6).

Since NFkBp65 has been shown to be a positive factor for liver regeneration [10] and a negative factor for muscle regeneration [11], it is certainly an exciting start to explore the role of NFkB pathway in skin wound healing.

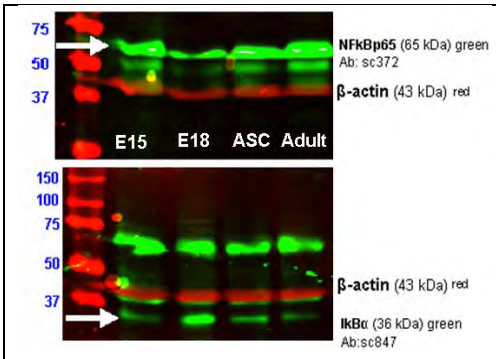

Figure 6a. Western Blot Detection of NFkBp65 & Ik Bα: SDS-PAGE (50µg protein per well) was followed by blotting analysis with two independent channels (green for target protein and red for β-actin) scanning using Odyssey Imagine System.

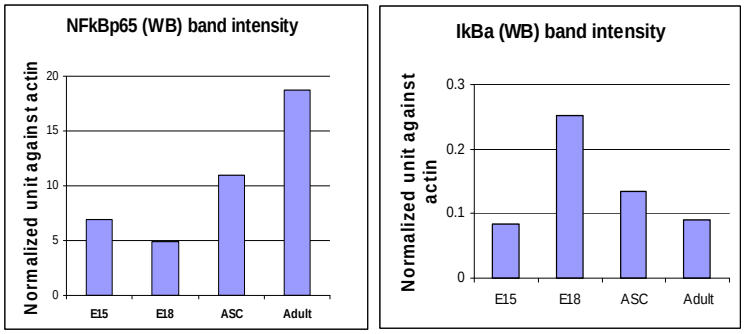

Figure 6b. Quantitative Analysis of Odyssey Western Blots: Band intensity was measured and normalized against β-actin, indicating NFkBp65 expression is higher in adult cells, lower in fetal cells. NFkBp65 / IkBa ratio is 207 in adult fibroblasts, 19 in E18 fibroblasts, 82 in E15 fibroblasts, and 80 in ASC.

Key Research Accomplishments

- Precise characterization of donor cell strains of different phenotypes, including cell strains with regenerative healing capability (adipose stem cells (ASC), fetal skin fibroblasts (embryonic day 15, E15)), for continuing work with cell therapy.
- Characterization of regenerative healing mechanism through differential inflammatory signaling guided by ASC and E15 cells in adult wound healing environment.
- Establishment of preliminary therapeutic strategy for combinatory topical treatment with isogenic ASCs, E15 cells and anti-inflammatory agent (Nimesulide) plus anti-fibrotic agent (PGE2), leading to rapid, regenerative healing.

Conclusions

The current results demonstrate that early, one time topical treatment with isogenic (same strain) adipose stem cells (ASC) and fetal skin fibroblasts (embryonic day 15, E15), leads to reduced scarring and increased healing. ASC and E15 cells share some key similarities, such as better survival in the wound environment, faster regain of wound tissue tensile strength, expression of higher level of cell surface vimentin, production of higher levels of intracellular collagen type III and fibronectin, comparable NFkBp65 / IkBα ratios; and also shared differences in terms of their differentiation nature, collagen secretion ability, and their contribution to collagen fiber organization. Based on their benefits in the healing process, both ASCs and E15 cells are ideal candidates for combination with anti-inflammation agent (Nimesulide) and anti-fibrotic agent (PGE2) for either novel therapeutic strategy development, or molecular mechanism exploration. These results are very encouraging that the experimental plan is feasible and the milestones achievable. Meanwhile the project has progressed to the third aim, designing interventions, based

on the results of the first 2 aims, that provide a wound environment for rapid, regenerative healing.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	2	0
# Post docs	1	3
# grad students	0	1
# undergrad students	0	0
# staff members	1	0

Other Project Statistics	
# Honors given to AFIRM faculty	3
# Doctorates awarded under AFIRM	0
# Masters degrees awarded under AFIRM	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	2
# Non-peer reviewed publications	4

*Directly supported in whole or part –time by AFIRM

Honors and awards – list them by awardee name

1. Patricia Hebda: Keynote Speaker, 2nd TERMI S (Tissue Engineering and Regenerative Medicine) World Congress, in conjunction with the 2009 Seoul Stem Cell Symposium, Seoul, Republic of Korea August 31 to September 3, 2009
2. Patricia Hebda, 2011 Distinguished Service Award from the Wound Healing Society
3. International Advisory Board Member for the 4th Congress of the World Union of Wound Healing Societies, Yokohama Japan, September 2-6, 2012

Peer-Reviewed publications

1. Yang TB, Guo Y, Cetin S, Rivera-Serrano C, Tano Z, Dohar JE, Hebda PA. Synergistic effects of a COX-2 inhibitor and prostagl andin E2 to improve healing and reduce scarring. *Wound Repair Regen* 2012 (manuscript in review).
2. Yang TB, Cetin S, Barsic M, Dohar JE, Hebda PA. Cell-based therapy to reduce scarring and improve healing in the wound bed. (manuscript in preparation).

Other Publications

1. Rivera-Serra C, Guo Y, Cetin-Fe rra S, Barsic M, Yang T, Dohar JE, Hebda PA. Effect of Nimesulide and PGE2 therapy, alone and in co mbination, in a cutane ous excisional wound model in rats. Com bined Otolaryngology Spring Meeting/American Society for P ediatric Otolaryngology. Phoenix, AZ, May 28-31, 2009.
2. Yang TB, Dohar JE, Hebda PA. Com binatorial anti-inflammatory therapy in d ecreasing subsequent fibrotic fibroblast activity in the wound bed. (selected for podium presentation)

Symposium for Advanced Wound Care- Wound Healing Society Joint Meeting. Orlando, FL. April 17-20, 2010. *Wound Repair Regen* 2010; 18:A16.

3. Yang TB, Cetin-Ferra S, Dohar JE, Hebda PA. Cell-based therapy to reduce scarring and improve healing in the wound bed. Symposium for Advanced Wound Care- Wound Healing Society Joint Meeting. Dallas, TX. April 14-17, 2011. *Wound Repair Regen* 2011; 19:A61.
4. Yang TB, Cetin-Ferra S, Dohar JE, Hebda PA. Differential inflammatory signaling and synthesis of collagen and fibronectin by adipose stem cells and fibroblasts of different phenotypes relevant to outcome of cell-based wound therapies. Symposium for Advanced Wound Care- Wound Healing Society/European Tissue Society Joint Meeting. Atlanta, GA, April 19-22, 2012. *Wound Repair Regen* 2012; 20:A47.

PROJECT SW-5 TERMINATED

Project SW-6: Isolation and Expansion of Native Vascular Networks for Organ Level Tissue Engineering

Team Leader(s)	Geoffrey C. Gurtner, MD (Stanford University)
Project Team Members	Michael T. Longaker, MD, MB A (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 *Revised:* None
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #3 End Year 1: TRL #1 : End Year 2: TRL #2
Key Accomplishments:	The Stanford group further developed and improved their technique to isolate and maintain the explantable microvascular beds (EMBs) based on the rat superficial inferior epigastric vessels on an ex vivo bioreactor system. They first improved the decellularization process of the native vascular network that preserved a structural matrix, but removed the majority of the adipose cells. They have established a proof-of-concept that stem cells can be seeded onto intact flaps after a decellularization process and remain viable even long term after reanastomosis into the native circulation
Keywords	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. However, two major obstacles have hindered the development of techniques to fabricate replacement organs: 1.) the inability to adequately vascularize tissue constructs *in vitro* and 2.) the inability to re-integrate these tissues into the systemic circulation. Tissue engineering approaches based on the implantation of cells onto resorbable matrices have had success in replicating simple, relatively avascular structures such as cartilage or bone, but have been unable to create more complex parenchymal organs, such as liver. Despite the obvious promise that stem cell technology holds to "regenerate" partially damaged organs *in vivo*, it is difficult to envision the creation of new organs *in vitro* using existing methodologies. To address this problem, the Stanford group has developed a novel approach to engineer constructs of organ-level complexity by using pre-existing, explanted microcirculatory beds (EMBs) to fabricate autologous, vascularized neo-organs *in vitro* (1-4). 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.

They have successfully constructed a novel perfusion bioreactor system which permits the cultivation of EMBs for extended periods *ex vivo*. During cultivation, they have demonstrated extremely efficient seeding of EMBs with primitive progenitor and stem cells after a decellularization process. The technology has advanced beyond "proof of principle" toward a flexible regenerative environment based on a bioreactor system. This innovative approach has allowed utilization of the pre-existing vascular system as a scaffold that can be manipulated *ex vivo* and subsequently reconnected to the circulatory system *in vivo* using standard microsurgical techniques.

Research Progress

The Stanford group has continued to perform detailed studies to optimize the perfusion rates and perfusate composition to maximize EMB survival and vascular patency. They further developed and improved the technique to isolate and maintain explantable microvascular beds (EMBs) based on the rat superficial inferior epigastric vessels on an ex vivo bioreactor system. They have continued to refine the surgical protocol to maximize preservation of the microvascular structures. Previous studies by the Hagey laboratory have established proof-of-concept that stem cells can be seeded onto intact flaps. However, the persistence of pre-existing mature cells appears to limit the ability to guide stem cell differentiation for neo-organ fabrication. As such, a major goal of this study was to remove the pre-existing parenchymal cells while maintaining the vasculature and matrix scaffolding for progenitor cell seeding. The Stanford group first improved the decellularization process of the native vascular network with external administration of Trypsin (enzymatic digestion) that preserved a structural matrix, but removed the majority of the adipose cells (**Figure 1**). Parallel to this external enzymatic digestion, the vascular architecture was perfused internally with the culture medium DMEM to limit vascular decellularization and thereby preserve these critical structures. A wide variety of available detergents were examined, but after multiple iterations the Trypsin-DMEM protocol was found to be the most effective in removing parenchymal cells while preserving the microcirculation. They were able to successfully decellularize the tissue while preserving the matrix architecture and nearly all vascular structures, most importantly, the microcirculation. Because of this, they were able to perfuse the EMBs for longer than 48 hours on the bioreactor and maintain the viability of the matrix. This was never possible with fully cellular explantable microvascular beds even with exogenous oxygen carrier perfusion (i.e., perflurocarbon, synthetic hb, etc.) which created a host of secondary problems. Finally, as illustrated in **Figure 2**, they futher optimized the mechanical protection of the EMB with the application of a thermoreversible gel, which is a solid mass at 37ºC during the bioreactor period, while transforming into liquid condition at room temperature. This provides preservation of the microvascular pedicle allowing re-anastomosis to the host vasculature for in vivo experiments and minimizes mechanical damage of these delicate structures during manipulation.

The Stanford group has continued efforts to determine the optimal seeding and differentiation conditions for different infused stem cells. Since the EMB is encased in a gel during the ex vivo period, they 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

developed by the Hagey lab to create a functional biomaterial-based stem cell niche for the delivery of mesenchymal stem cells (MSCs) in vivo. Bioluminescence imaging and FACS analysis of luciferase+/GFP+ MSCs indicated that stem cells delivered within the hydrogel remained viable longer (up to two weeks) and demonstrated enhanced engraftment efficiency suggesting that this would be a viable way to increase cell delivery around the EMB and may provide a way to create laminated structures composed of two or more different cell types as would be required for organ replacement. For example, one population of cells could be delivered perivasculary via perfusion while *simultaneously* a second distinct population could be delivered to the EMB via delivery in the encasing gel. Their data suggest that the pullulan-collagen hydrogels provide a functional niche capable of long term cell viability while also upregulating a number of pluripotency genes (please see **Publications**). Thus the hydrogel provides a second route to seed cells on the EMB, and to do so in a spatially oriented fashion. They are not aware of another technology that provides this degree of control which is necessary for large volume tissue engineering.

In a parallel effort, the Stanford group had demonstrated the ability for the first time to seed *human* Luciferase+ adipose derived stem cells (hASCs) on the EMB and confirmed the survival of the cells during and after perfusion on the bioreactor (**Figure 3**). Furthermore, they successfully re-attached the EMB into the original femoral vessels of an immune-compromised rat (**Figure 4**). In vivo, they demonstrated that hASCs can engraft and remain functional for extended periods following seeding onto processed EMBs and reintegration into the host (**Figure 5, 6**). They have continued to refine the protocol for ASC administration on the decellularized EMB-matrix. Bioluminesence imaging with IVIS detection systems allows the non-invasive monitoring of cell survival and has demonstrated successful engraftment of the cells in to the scaffold and cell viability for greater than 14 days after replantation of the EMB back into the native vascular circulation. Thus they have demonstrated that extrinsic seeding of human ASCs onto EMBs is possible, providing a new and readily available cell source for eventual translation.

Much of the work of the first several months of the grant has gone into optimizing a cell seeding protocol that is scalable and potentially translatable for human applications. While these efforts have been underway, they continued to brainstorm and develop alternative approaches to replace physiologic function in vivo that hold the potential to be translated into human trials in a reasonable time frame. Broadly, these approaches fall into two categories: either replace a single protein using gene therapy approaches or replace cells to provide for more diverse functionality. At present, it is unclear which of these approaches is more feasible and for this reason preliminary efforts are underway exploring both approaches.

The easiest goal to accomplish in the short term is to replace a single protein. The translational target for this approach is human factor IX deficiency, which is a single gene defect but has proved difficult to reverse using standard gene therapy techniques. In this effort, EMBs without decellularization were used along with a novel DNA Minicircle delivery technique. They achieved significant progress using the vascularized adipose tissue flaps (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. Adipose tissue flaps (such as human omentum) are ideal candidates for Minicircle transfection

due to their excellent vascularization, peripheral location and expendability. They 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.

More difficult is the delivery of cells in a predictable and oriented fashion. For that reason, they have focused on decellularization strategies which are working extremely well and are much simpler and potentially scaleable. They improved the decellularization process of the native vascular network with external administration of Trypsin (enzymatic digestion) that preserved a structural matrix, but removed the majority of the adipose cells (**Figure 1**). In preliminary studies to simulate the extravascular environment of neo-liver constructs, they have seeded MSCs into the pullulan collagen hydrogel, which is a matrix capable of encasing the EMB microvasculature. Pluripotency gene expression and expression of hepatic growth factor (HGF) and insulin-like growth factor (IGF) were increased, suggesting that this may be a viable strategy to increase hepatocyte cell mass. This preliminary work is ongoing and focuses on describing the differences between mature adult hepatocytes and differentiating hepatocyte like progenitor cells. If adult hepatocytes are the preferred cell type, then the hydrogel delivery mechanism will be required for seeding as prior work has shown that these cells cannot be seeded via perfusion (1).

Figure 1: Processed autologues scaffold (explantable microcirculatory bed) after multiple decellularization steps with external administration of Trypsin. The vasculature architecture remains intact.

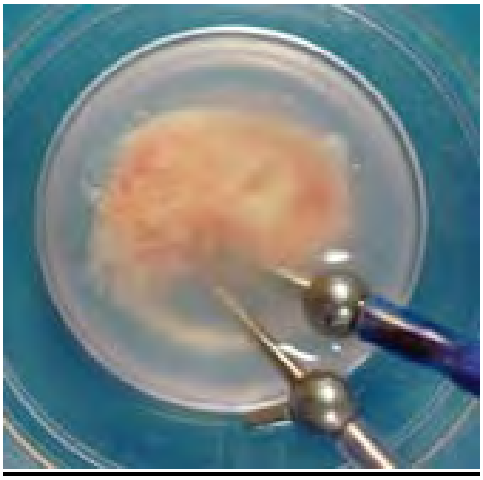

Figure 2: The explanted microvascular bed is covered and secured with a thermoreversible gel (Thermogel) for overnight protection during ex vivo perfusion on the bioreactor.

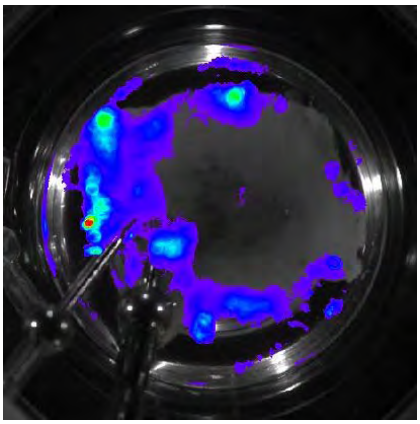

Figure 3: Bioluminescence imaging of the EMB 24 h after perfusion on the bioreactor. The EMB was seeded with luciferase+ human ASCs. The image shows that the luciferase+ human ASCs are viable and persistent after perfusion on the bioreactor.

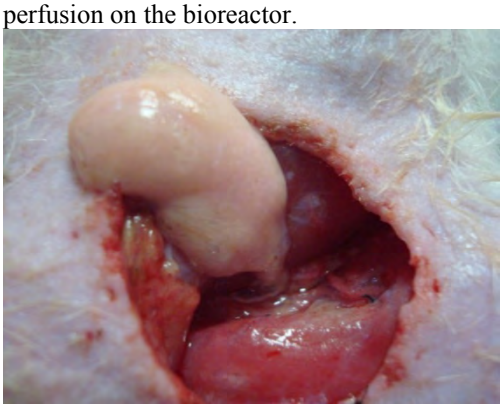

Figure 4: Sucessful re-anastomosis ("replant") of the EMB back into the native femoral vessels of the nude rat after 24 h with the seeded Luciferase+ human adipose derived stem cells (ASC).

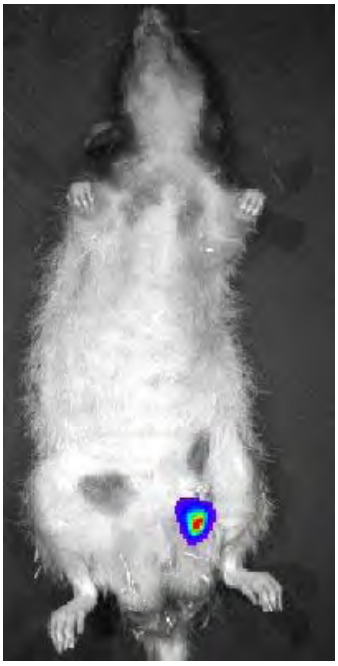

Figure 5: Bioluminescence imaging 14 days after replantation of the EMB back into the native rat circulation. The EMB was seeded with luciferase^{+} human adipose-derived stem cells (hASC). These data demonstrate that hASCs can engraft and remain functional for extended periods following seeding onto processed EMBs and reintegration into the vascular circulation.

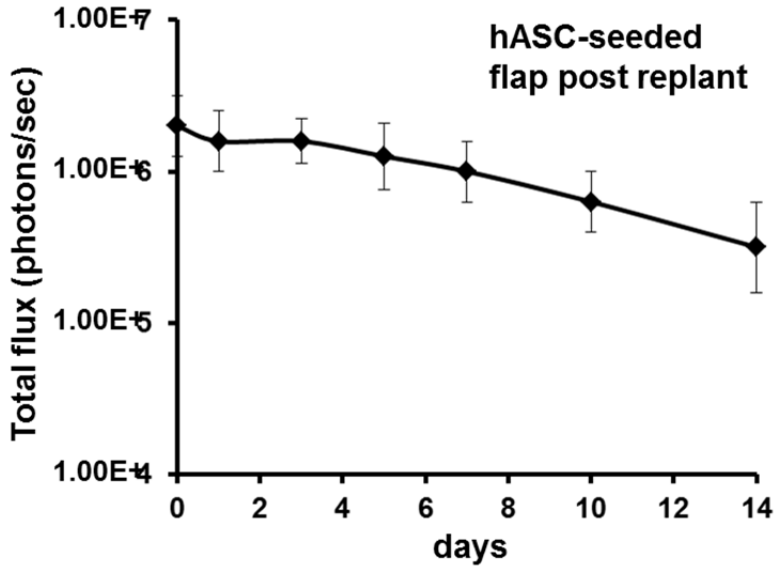

Figure 6: Bioluminescence intensity of human ASC-seeded flap after replant into the native rat circulation over 2 weeks. These data demonstrate that hASC remain functional for extended periods.

Key Research Accomplishments

- Further surgical refinements of explantable microvascular beds (EMB)
- Establishment/Refinement of a decellularization protocol that preserves the matrix and vasculature while removing native parenchymal cells
- Improvement in EMB viability during bioreactor period with thermoreversible gel for tissue protection
- Seeding of processed rat tissue flaps with adipose-derived stem cells (ASC), with engraftment and persistence of cells for extended time periods
- Successful re-anastomosis of EMB into native vascular circulation
- Survival of ASC long term after re-anastomosis

Conclusions

The Stanford group has successfully validated and optimized their protocol for the isolation and maintenance of explantable microvascular beds (EMBs) based on the rat superficial inferior epigastric vessels on an ex vivo bioreactor system. They have developed a decellularization protocol that achieves complete decellularization of tissue while preserving the matrix architecture and the macroscopic vascular structure. They have successfully seeded decellularized rat tissue flaps with adipose-derived stem cells (ASCs), and studies indicate that engrafted cells persist in a functional capacity for extended time periods. Also the group successfully re-anastomosed the EMB into the native vascular circulation and showed a long term survival of the engrafted ASC's after the re-anastomosis. Together, these successful studies suggest that progenitor cell-seeded vascularized scaffolds are a promising approach to fabricate complex organ-level constructs. The potential of this technology is tremendous and they continue to make significant strides toward refining this novel paradigm for organ-level engineering and regenerative medicine.

Research Plan for the Following Years

In the next two years, the Stanford group will continue to modify the perfusion protocols in order to optimize EMB tissue survival *ex vivo*, in particular further optimizing the degree of decellularization. Here they will focus on protocol refinements regarding duration and amount of the external Trypsin exposure as well as the perfusion rate during the internal perfusion with the culture medium DMEM in order to further maximize the decellularization. Another goal is to preserve the microvascular pedicle of the adipose tissue flap during the decellularization process completely, which is critical for the microsurgical re-anastomosis of the artery and vein back into the systemic circulation of the rat. Here they will concentrate on the refinement of the thermoreversible gel as a protection agent of the vascular pedicle. Also they will continue to improve the stem cell seeding efficiency and post-implantation survival by examining both intravascular and extravascular approaches. They will further investigate the "ideal" amount of administered stem cells as well as refine the protocol regarding several repeated applications of the cells. Repeated applications of the stem cells with intravascular and extravascular approaches seem to be a key for further optimization of the engraftment rate. Modification of the parenchymal tissues may allow for improved stem cell survival and this will be pursued in the

upcoming year. Further, they are planning to apply luciferase+ adipose-derived stem cells from the rat. Due to the immune suppression in a nude rat model (human cells), they will transition this work to employ ASC's from a luciferase expressing rat species. Also they will continue to pursue two different strategies to replace essential hepatic function. As progress continues, this will enable a gradual transition from replacing single proteins to replace cells and fabricate a true vascularized neo-liver construct. Initially, these efforts will focus on albumin production in their seeded flap in a *hypoalbuminemia rat model as a surrogate for global liver function although this is an oversimplification. Eventually, in later years of the grant these efforts will examine using other metabolic and synthetic agents to assess performance.* The critical path over the next year will be to determine the ideal cell type (progenitor vs. differentiated) to use in these studies.

Planned Clinical Translations:

N/A

Corrections/Changes Planned and Rationale:

None

Conflict of Interest Disclosure:

None

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	0
# Post docs	1	2
# grad students	0	2
# undergrad students	0	1
# staff members working for AFIRM	1	0

Other Project Statistics	
# Honors given to AFIRM faculty	8
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	4
# Non-peer reviewed publications	2

*Directly supported in whole or part –time by AFIRM

Honors and awards – list them by awardee name

1. Dr. Gurtner: James Barrett Brown Award 2008 (Best plastic surgery paper) from the American Association of Plastic Surgeons
2. Dr. Gurtner: James Barrett Brown Award 2009 (Best plastic surgery paper) from the American Association of Plastic Surgeons
3. Dr. Gurtner: Associate Chairmanship position from Stanford University Department of Surgery (2010)
4. Dr. Gurtner: Basic Science Researcher of the Year Award 2011, Association of Plastic Surgeons
5. Dr. Longaker: Basic Science/Translational Researcher of the Year Award 2010 from the American Association of Plastic Surgeons
6. Dr. Longaker: Frederick Birnberg Award for Excellence in Dental Research from Columbia University 2011
7. Dr. Longaker: Honorary Fellow of the Society of Black Academic Surgeons 2011
8. Dr. Longaker: Flanc-Karl Award from the American Surgical Association 2011

Peer-Reviewed Publications

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Other Publications

1. Evers LH, Sorkin M, Sim ons D, Longaker MT , Gurtner GC. Isolation and expansion of native vascular networks for or gan level tissue engineering. 97 th Annual Meeting of the American College of Surgeons, San Francisco, CA. October 25, 2011. Abstract supplement.

2. Evers LH, Tran PS, Simons D, Sorkin M, Longaker MT, Gurtner GC. Innovative approach of organ tissue engineering using autologous d ecellularized microcirculatory beds as vascularized bioscaffolds. 7th Annual Academic Surgical Congress, Las Vegas, NV. February 16, 2012. Abstract supplement.

Project SW-7: Neodyne's Device to Actively Control the Mechanobiology during Wound Healing and Prevent Scar Formation

Team Leader(s)	Bill Beasley (Neodyne Biosciences), Geoffrey C. Gurtner, MD (Stanford University)
Project Team Members	John Zepeda (Neodyne), Jasper Jackson (Neodyne), Rich Caligaris (Neodyne), Peggy McLaughlin (Neodyne), Christy Cowley (Neodyne)
Collaborator(s)	Michael T. Longaker, MD, MBA (Stanford University), Reinhold Dauskardt, PhD (Stanford University), Paul Yock, MD (Stanford University)
Therapy Deliverable(s)	Control of wound environment to minimize scarring *Baseline:* Commercially available devices capable of stress-shielding mechanical forces to minimize scar formation *Revised:* None
TRL Progress	Start of Program: End Year 1: TRL #5 End Year 2: TRL #6 End Year 3: TRL #7 End Year 4:: TRL #8
Key Accomplishments:	Neodyne has enrolled and treated over 60 patients in a clinical trial designed to test its commercial-ready device capable of stress-shielding wounds and off-loading pathologic mechanical forces to prevent fibrosis. Neodyne has received FDA clearance for its class-1 product and has released the product for commercial use through its Quality System
Keywords	hypertrophic scarring, mechanobiology, wound device

Introduction

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. Multiple factors are known to influence wound repair but therapeutic modalities aimed at these targets have been largely unsuccessful. Mechanical force has long been recognized to influence cellular behavior *in vitro* and clinical observations based on Langer's lines and hypertrophic scarring corroborate this phenomenon *in vivo* (1,2). Recently, the Gurtner laboratory published the first murine model of hypertrophic scarring based on increasing the skin stress of healing wounds (3). Dr. Gurtner's laboratory found that intrinsic skin mechanics correlated with scarring phenotype following wounding, as low mechanical stress fetal wounds exhibit minimal fibrosis and stiffer human skin displays robust scarring (3). These findings prompted the initial studies to examine the role of mechanical stress in scar formation and to develop a novel device to actively control wound environment mechanics to mitigate fibrosis.

Today, there are no commercially available products that specifically address the mechanical stress state of healing wounds to reduce scarring. In contrast to existing wound care options, Neodyne's technology enables precision stress-shielding of area-specific wound forces through a portable, ready-to-use, pressure sensitive adhesive dressing that can be readily applied after surgery.

The Neodyne technology consists of a load bearing biopolymer that is stretched by means of an applicator and then applied to the skin with a goal of optimizing a regenerative wound

environment for minimal scar formation. The objective of the project is to complete clinical trial(s) utilizing a market-ready device to provide expanded data for de novo surgical incisions as well as explore the potential to improve scar appearance after a scar revision procedure. Ultimately, this data will be used to support the commercial launch of the product, and to make the technology available to both military and civilian patients.

In previous years a Pilot study with a second generation device was conducted on 61 patients after surgical procedures. The device was designed to deliver precise stress shielding at several strain levels in order to determine efficacy and patient tolerance. Trial results indicated a clear correlation between delivery of higher strains and skin irritation. Data collected on strain measurement indicated that the device delivered precise strain that was maintained for the full wearing period. Figure 1 shows treated and control incisions from the Pilot trial.

Research Progress

Neodyne has completed work in the past year in two primary areas: 1) Clinical trial execution, and 2) Manufacturing readiness to prepare for larger scale production and commercialization.

Gen 3 Clinical Trial Execution

Based on results of the previous trials, the biomechanical skin strain research, finalization of the Gen 3 product design, and feedback collected in market research studies, the Gen 3 trial to test market ready product design and build additional clinical evidence has been enrolling and treating patients following de novo abdominoplasty procedures since the summer of 2011.

This trial is a prospective, open label, randomized study of up to 100 subjects to measure scar formation as a primary endpoint in surgical abdominoplasty procedures where patients will serve as their own control. Up to one-half of the incision is treated by the Neodyne device and the contralateral half treated with physician preferred standard of care. Ease of use, pain amelioration, comfort, and scar smoothness are among the outcome measurements. Follow up will occur at six months and one year post-surgery.

In addition to 15 private plastic surgery sites, Neodyne has enrolled patients at the Institute of Surgical Research in San Antonio and has received approval at David Grant Hospital at Travis Air Force Base. The company has future plans to treat military injuries and/or reconstructive surgeries at these and other military hospitals.

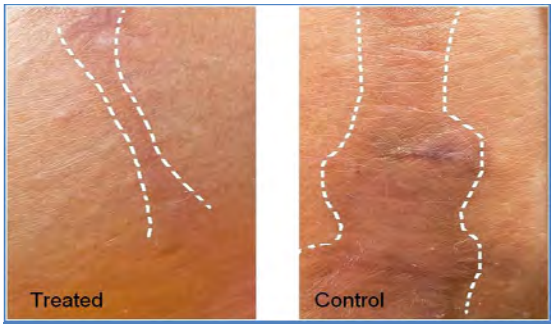

Figure 1. Pilot study treated and non-treated incisions (same patient) after 6 months.

In total, over 60 patients have been enrolled in the clinical trial, with approximately 15-20 who will have reached the six-month post-surgery endpoint of the trial by June 1. It is expected that trial enrollment will be completed by the

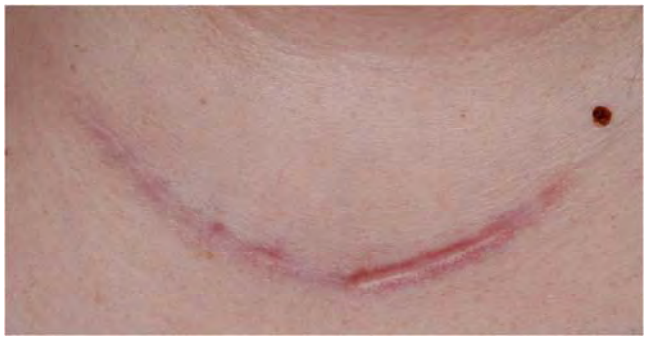

Figure 2. Scar revision – six month post-procedure (treated side on left)

end of June or early July 2012.

In addition to the AFIRM sponsored trial, Neodyne is conducting a scar revision trial with a similar design to the abdominoplasty trial, enrolling 10 patients since October 2011. Results of this trial show dramatic improvements in scar reduction on the treated side of scar revision incisions (Figure 2).

Manufacturing Readiness

In the summer of 2011 Neodyne completed the evaluation of current and potential vendors and suppliers to provide the raw materials, sub-assemblies, and infrastructure to support the manufacturing required to deliver clinical product for the current clinical trial. In support of its 2011-12 clinical trials, the Neodyne manufacturing team has built over 1000 clinical devices.

Subsequently, Neodyne has focused on developing the final specification for the Neodyne device in preparation to outsource commercial manufacturing in the United States. Several contract manufacturers have been evaluated and the selection of the manufacturers for the adhesive, dressing, and final product assembly has been completed. The manufacturing process flow has been finalized for the commercial product (Figure 3). Neodyne will be responsible for the final packaging and delivery of devices to the customer.

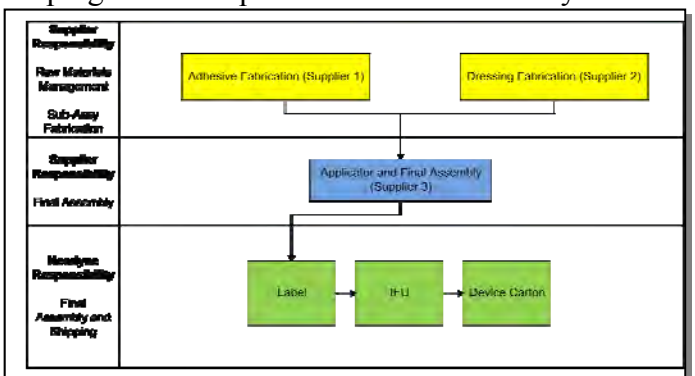

Figure 3. Manufacturing flow for commercial product.

Link to 2011 Annual Report
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Key Research Accomplishments

- Completed process with ISR for military participation in clinical trial
- Began process with Travis AFB for participation in trial
  - Initiated and completed design improvements for improved manufacturing efficiency
  - Conducted ongoing skin strain testing
  - Developed and released second size dressing for smaller incision
  - Completed evaluation of contract manufactures for commercial product manufacturing
  - Conducted clinical product manufacturing to support clinical trial

- Clinical site enrollment and training – 17 sites in California, Texas, Arizona, and Nevada

- Treated over 60 patients in clinical trial
- Received 510(k) clearance of first family of product

Conclusions

In summary, Neodyne has utilized a novel stress-shielding device to safely and effectively modulate the mechanical wound environment in post-surgical human subjects to markedly reduce cutaneous scarring. This innovative device demonstrates the capability to precisely regulate skin fibrosis post-injury and is a promising translational approach to minimize the biomedical burden of hypertrophic scar formation.

The first-in-man study set a strong foundation for the promise of the Neodyne technology. The pilot trial with a second generation device further supported the need for precise control of skin strain and an intuitive user design, and suggests that there is a narrow range of strain levels that provide optimal scar reduction. With these understandings, Neodyne has nearly completed testing the latest design in clinical trials and will soon launch the product commercially.

The current Neodyne devices are able to create an optimal mechanical environment for linear incisions. With the use of digital imaging correlation technology, the company has the capability to accurately measure the compressive and tensile forces in the skin within the treated area and surrounding tissue. This information will enable Neodyne to further improve the precision and efficacy of its incisional wound treatment.

The most recent product design has dramatically improved the usability and intuitiveness of the treatment, and has potential uses beyond the application of Neodyne's stress-shielding device. We are extremely interested in partnering with AFIRM and USAISR to conduct additional clinical trials to test the expanded capabilities of the technology to maximize the benefit for the wounded warrior.

Research Plan for the Following Years

In the next few years, Neodyne has several goals related to the commercialization of its technology using advanced versions of the polymeric device. Neodyne plans to continue with clinical studies in both military and civilian populations to validate the full range of potential uses for the product and to test hypotheses for use on incisional wounds as well as scar revision procedures. Neodyne will continue to collaborate with Stanford University to conduct human trials with advanced versions of the technology that are further developed in the Gurtner laboratory.

Planned Clinical Translations:

Neodyne is in the late stages of enrolling its third device trial, which is recruiting a larger and ethnically broader patient population to test the efficacy and market readiness of its latest product design. In addition to the AFIRM funded trial, Neodyne plans to continue to enroll patients in a

scar revision study to evaluate the efficacy of its product on lower tension incisions. In conjunction with the Materials Science and Engineering departm ent at Stanford Univers ity, Neodyne will further refine the polym eric device to custom-design treatments for various size wounds and tension states. This will allow for b ody-specific regional stress-shielding to address a wide variety of surgical wounds.

Corrections/Changes Planned and Rationale

None

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty	0	2
# Post docs	0	1
# grad students	0	0
# undergrad students	0	0
# staff members	0	0

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded	0
# Masters degrees awarded	0
# Inventions disclosed	0
# Patents awarded	4
# Peer reviewed publications	1
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Honors and awards – list them by awardee name

None

Patents and Inventions – Patents awarded, filed, invention disclosures

Patents

- Neodyne Biosciences is licensed under U. S. Patent No. 7,683,234 and related patents U.S. 8,063,263, 8,168,850, 8,183,428 and U.S. application SN's 13/089,104, 13/089,105 which are not a subject inventions as the inve ntions were first conceived and reduced to practice prior to the date of the AFIRM Agreement with Neodyne Biosciences.

Inventions Disclosed

- Neodyne Biosciences filed Utility Application Serial No. 12/854,859 on Aug. 11, 2011; a related Divisional Application Serial N o. 13/089,129 on April 18, 2011; and related continuation application Serial No. 13/315,214 on December 8, 2011. These applications claims priority of: Provisional A pplication No 61/233,122 filed on August 11, 2009 which relates to an applicator; Provisi onal Application No. 61/243,020 filed on Sept. 16, 2009 which relates to testing com pleted prior to the AF IRM Agreement date; and Provisional Application No.61/264,205 filed on Nov. 24, 2009 which relates to an applicator design. The inventions are not s ubject inventions as they were either: conceived and reduced to pr actice prior to the AFIRM Agreem ent, and/or were not developed in any relationship to the AFIRM funded activities.

- Neodyne Biosciences filed Application Se rial No. 13/345,525 on 1/6/2012 which claim s priority of Provisional applications No. 61/430,908 on Ja n 7, 2011 and No. 61/443,647 on Feb 16, 2011 related to applicators. These inventions are not subject inventions as they were conceived and reduced to practice prior to the AFIRM Agreement and/or were not related to AFIRM funded activities.

- Neodyne Biosciences filed Utility A pplication Serial No. 13/029,023 on Feb. 16, 2011 relating to skin straining devices and m ethods. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM funded activities.

- Neodyne Biosciences filed Utility Applic ation Serial No. 13/411,443 on March 2, 2012 relating to skin tightening devices and m ethods. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM f unded activities.

- Neodyne Biosciences filed Utility Applic ation Serial No. 13/411,394 on March 2, 2012 claiming priority of Provisional Applic ation SN. 61/605,717filed March 1, 2012, relating to alternative controlle d strain skin treatm ent devices and methods. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM funded activities.

- Neodyne Biosciences filed Provisional Application Serial No. 61/512,340 on July 27, 2011 relating to prestrained skin treatment devices and methods. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM funded activities.

- Neodyne Biosciences filed Provisional Application Serial No. 61/566,590 on December 2, 2011 relating to skin treatment devices, methods systems or kits. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM funded activities.

- Neodyne Biosciences filed Provisional Application Serial No. 61/594,931 on Feb. 3, 2012 relating to alteration of biomarkers. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM funded activities.

- Neodyne Biosciences filed Provisional Application Serial No. 61/596,708 on Feb. 8, 2012 relating to chronic wound treatment devices and methods. The inventions are not subject inventions as they were not developed in any relationship to the AFIRM funded activities.

Peer-Reviewed Publications

1. Gurtner GC, Dauskardt RH, Wong VW, Bhatt KA, Wu K, Vial IN, Padois K, Korman JM, Longaker MT. Improving cutaneous scar by controlling the mechanical environment: large animal and phase I studies. Ann Surg. 2011.
2. Wong VW, Akaishi S, Longaker MT, Gurtner GC. Pushing Back: Wound Mechano transduction in Repair and Regeneration. Journal of Investigative Derm. July, 2011

Other Publications

1. Bhatt KA, Vial IN, Wu K, Kelantan M, Dauskardt RH, Longaker MT, Gurtner GC (2009) Stimulating the Regenerative Potential of Adult Wounds Through Mechano-Modulation of Skin Stresses. Stanford University 2nd Annual Resident Research Day, May 12, 2009. Abstract/podium presentation.
2. Wong VW, Bhatt KA, Vial IN, Dauskardt RH, Longaker MT, Gurtner GC. Mechanomodulation of the wound environment to decrease scar formation in a porcine model. Armed Forces Institute of Regenerative Medicine All Hands Meeting, St. Pete, FL. Abstract/podium presentation.

Overview of Extremity Injuries – Compartment Syndrome and Limb & Digit Salvage Programs

Program Leaders: Kenton Gregory, MD and W.P. Andrew Lee, MD

Tissue wounds to the extremities are among the most common battlefield injuries sustained by troops during Operations Iraqi Freedom and Enduring Freedom. Particularly common traumatic injuries caused by improvised explosive devices are blast and projectile injuries. Thus there is a need to develop technologies which address both limb and digit salvage and the consequences of amputated parts. While some times the damage is obvious other times injuries are complicated by Compartment Syndrome (CS). In CS, traumatic related tissue swelling creates increased compartment pressures and this leads to ischemia and infarction of tissues. CS dramatically amplifies the battlefield injury and quickly leads to permanent muscle, nerve and vascular cell death. Soldiers that develop CS have prolonged recovery times and rarely recover complete muscle function, and they usually do not return to active duty at the same level of performance. Most CS injuries of the extremities result in permanent disability.

This program aims to develop regenerative medicine technologies using a number of approaches from autologous stem and progenitor cells, that offer a safe and potentially effective new therapeutic avenue to amplify the body's endogenous regenerative response to injury, to hand transplants, to biomaterials approaches—all with the goal to improve the functional recovery of the injured soldier. The regenerative medicine technologies described herein have been used by AFIRM investigators and others for civilian tissue injuries safely and effectively and thus substantiate the rationale for using this approach to solve an important unmet need in the treatment of battlefield injuries.

Over the past year, it became apparent that while the mission is straightforward, research and development of innovative technologies is not. In an effort to balance mission expectations with the methodical pace of research, a reorganization of the research efforts within AFIRM was performed. Since the limb and digit program and the compartment syndrome program had many similarities, These two programs (limb & digit program and compartment syndrome program) became administratively merged the into the extremities injuries program. Both programs are thematically focused on injuries to the extremities and thus will synergistically benefit from being combined. In addition, the restructured program also parallels that of the RCC C consortium. Certain projects remained under the direct oversight of Drs. Gregory and Lee while others which had more broader impacts on AFIRM where reassigned to the enabling technologies core where they could receive more exposure within AFIRM.

This section of the report details the progress within the Extremity Injuries area has made.

Project EI-1: Hand Transplantation For Reconstruction Of Disabling Upper Limb Battlefield Trauma – Translational And Clinical Trials

Team Leader(s)	W.P. Andrew Lee, MD (Johns Hopkins University School of Medicine)
Project-Team Members	Gerald Brandacher, MD, Damon S. Cooney, MD, PhD, Justin M. Sacks, MD, Stefan Schneeberger, MD, Eric Wimmers, MD, Zuhaib Ibrahim, MD (Johns Hopkins University School of Medicine); Vijay S. Gorantla, MD, PhD, Joseph E. Losee, MD (University of Pittsburgh)
Collaborator(s)	None
Therapy	Reconstructive transplantation of upper extremity under a novel bone marrow/stem cell-based immunomodulatory protocol
Deliverable(s)	*Baseline:* (Phase 1 – Translational/Preclinical Trials) Novel immunosuppressive protocol that combines systemic stem cell-based therapy with local immunomodulation in a swine heterotopic hindlimb model of composite tissue allotransplantation and (Phase 2 – Clinical Trial) Reconstructive transplantation as treatment for hand or forearm loss under a novel cell based immunomodulatory protocol *Revised:* None
TRL Progress	Start of Program: TRL #4 End Year 3: TRL #5 End Year 1: TRL #4 End Year 4: TRL #5 End Year 2: TRL #5
Key Accomplishments:	In Phase 1, the team determined that 60 x 10^6 BM cells result in significantly higher levels of micro-chimerism. During Year 3-4, the BM infusion protocol was combined with costimulation blockade (CTLA4Ig). This novel immunomodulatory protocol resulted in indefinite graft survival in 4 out of 6 animals to date. In Phase 2, no new transplants have been performed since September 2010. Four out of five 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, which correlates with their level of amputation, time after transplant and participation in hand therapy.
Keywords	hand transplantation, immunosuppression, immunomodulation, swine

Introduction

Extremity Trauma account for the majority of battlefield injuries sustained by troops during Operations Iraqi Freedom and Enduring Freedom[1-3]. Composite tissue allotransplantation (CTA) (e.g., hand/face transplants) is an innovative reconstructive modality for such complex injuries. Despite excellent and highly encouraging functional results, CTA has not reached widespread clinical use because recipients require lifelong high-dose multidrug immunosuppression to prevent graft rejection[4]. Our research is aimed at minimizing and possibly eliminating the requirement for maintenance immunosuppression through targeted immunomodulation.

This project has two phases: *Phase 1 (Translational Trials):* A preclinical model of targeted immunomodulation in heterotopic hindlimb transplantation utilizing complete MHC mismatched miniature swine. Research trials are parallel and complementary to clinical trial, and work in each arm will be detailed separately. *Phase 2 (Human/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 bone marrow/stem-cell based protocol.

Research Progress

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

The optimal dose of BM cell infusion (60 million cells/kg) was applied to subsequent experiments evaluating the addition of CTLA4Ig fusion protein (20mg/kg IV on postoperative day 0, 2, 4, and 6) to replace the need for induction treatment with whole body and thymic irradiation (Table 1).

Table 1: Rejection Free Survival of Skin Component of CTA (*Group 6 is currently in progress; BM = bone marrow cell infusion @ 60 million cells/kg IV on postoperative day 0, CTLA4IgIg given at 20mg/kg IV on postoperative day 0, 2, 4, and 6)

Groups	Immunosuppressive Protocol	Rejection free survival of Skin component (Days)
1	No treatment	5, 6, 8
2	FK only (30 days)	30, 31,32
3	XRT + FK (30 days)	35,37
4	XRT + BM + FK (30	50, 52, 53
5	CTLA4Ig + FK (30 days)	100, 127, > 150, >150, >150
6	CTLA4Ig + BM + FK (30	>150, >45*

Group 5 (CTLA4Ig and tacrolimus) animals demonstrated significantly prolonged muscle survival beyond 150 days post-transplant; the skin component survived past 150 days in 3 out of 5 animals (Figure 1). Skin and muscle histology in all long-term surviving animals were rejection-free (Figure 2). Two animals in Group 5 had diarrhea and acute weight loss due to parasitic infestation and were euthanized at post-operative day 45. There was no clinical or histologic evidence of rejection at the time of euthanasia. Group 6 (CTLA4Ig, BM and tacrolimus) is currently in progress.

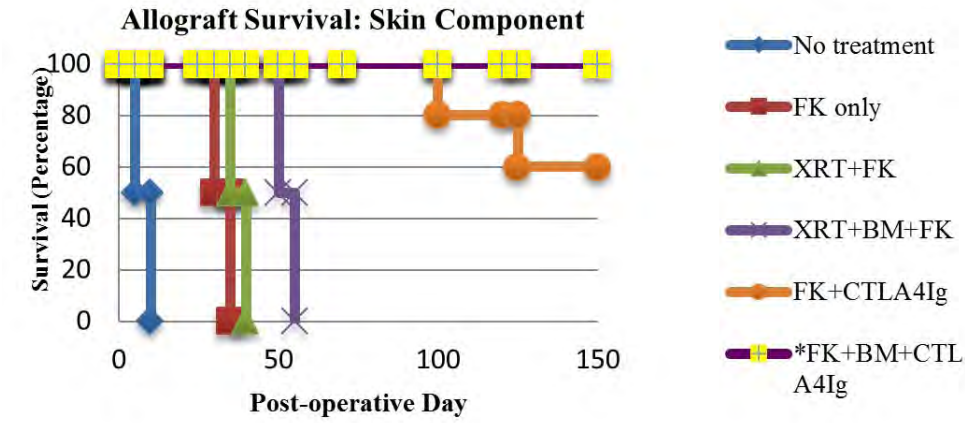

Figure 1: Allograft survival (skin component); FK (tacrolimus); XRT (Total body and thymic irradiation); BM (Bone Marrow Infusion)

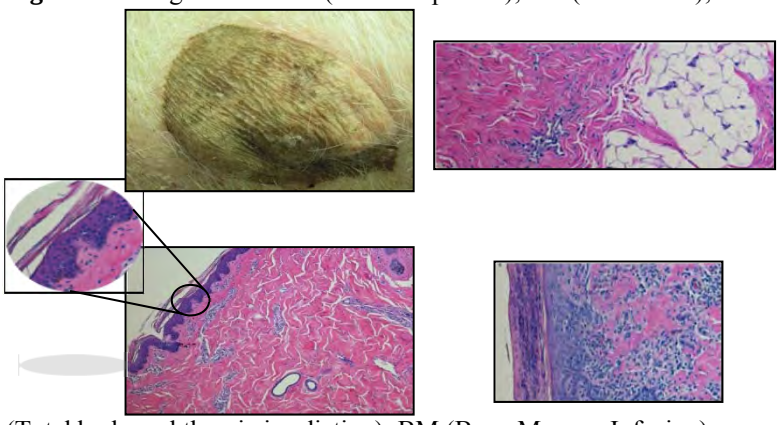

Figure 2: (A) No clinical evidence of rejection at post-op day 173 in a long term survivor (B) Allograft skin biopsy at post-op day 173 showing intact epithelium (H&E stain) (C) Allograft subcutaneous tissue and muscle biopsy at post-op day 173 with no evidence of rejection (D) Skin biopsy from control group showing dermal and intra-epithelial lymphocytic infiltrates with epithelial necrosis at early post-operative days.

The combination of CTLA4Ig and donor BM infusion has provided promising results to date. FK spacing was not necessary since the majority of animals receiving co-stimulatory blockade had indefinite graft survival with short-term tacrolimus treatment alone. However, there are some animals that still rejected the skin component of the allograft after prolonged rejection free survival. In the remaining experiments, local immunomodulatory strategy (as described in objectives) will be evaluated in combination with BM infusion and co-stimulatory blockade to achieve indefinite survival without systemic immunosuppression.

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

Candidates for hand transplantation continue to be screened and accrued via an approved IRB protocol; however no new transplants have been performed since September 2010. Four out of the five 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. Patients demonstrated sustained improvements in motor function (ROM, intrinsic return, grip and pinch strength) and sensory return correlating with the time after transplantation, level of amputation and participation in hand therapy. Four out of five patients have regained function, allowing resumption of independent living and are highly satisfied with their results. Side effects were few and included transient increase in serum creatinine, hyperglycemia managed with oral hypoglycemics, minor wound infection, an episode of hyperuricemia and bony non-union in two cases. No systemic infectious (bacterial or viral) complications occurred.

**In addition to the Armed Forces Institute for Regenerative Medicine (AFIRM), support for these transplants was provided by the Orthopedic Extremity Trauma Research Program (OETRP) W81XWH-08-1-0421 and the University of Pittsburgh Medical Center.*

Key Research Accomplishments

- The novel immunomodulatory protocol was optimized using BM infusion in combination with costimulatory blockade (CTLA4Ig) in a complete MHC mismatched MGH miniature swine model.
- Indefinite graft survival off immunosuppression using this protocol has been achieved in 4 out of 6 animals to date.
- Eight successful hand/forearm transplants were performed in five patients including the first bilateral and first above elbow arm transplant in the US. Four out of five patients are maintained on a single immunosuppressive drug at low levels and continue to have increased motor and sensory function of their transplanted hands correlating with the level of amputation, time after transplantation and participation in hand therapy.

Conclusions

This project features the development of a preclinical heterotopic hindlimb transplant model for CTA using a novel immunomodulatory protocol. Stable levels of microchimerism were achieved in all groups after bone marrow cell infusion. The addition of co-stimulatory blockade (CTLA4Ig) enabled us to optimize induction therapy, further reduce maintenance immunosuppression, and indefinitely prolong graft survival. Such targeted immunomodulatory protocols that combine BM cell-based strategies and biologics might facilitate immune tolerance and eliminate the need for multi-drug immunosuppression to maintain graft survival after CTA. Such immunomodulatory concepts have been applied in parallel in performing human hand transplantation using a novel BM cell based strategy that aims to reduce maintenance immunosuppression necessary for successful CTA. Five patients have been transplanted with a follow up ranging between 19-39 months. The success of this experimental protocol will allow for greater clinical application of hand transplantation for the reconstruction of upper extremity amputations.

Research Plans for the Following Year

Based on the striking data obtained in Years 1-4, CTLA/Ig represents a potential paradigm shift in the immunosuppression protocol in CTA. Additional experiments will be performed using combination of donor BM infusion with costimulation blockade plus topical treatment.

Planned Clinical Transitions

The project goal is to promote long-term hand transplant acceptance while minimizing the need for immunosuppressive drug therapy. The successful large animal protocol will be further optimized by using topical treatment of the skin component. Once successfully implemented in the translational large animal model we will obtain FDA approval to utilize this regime in our ongoing clinical trial of human upper extremity transplantation. This might enable tolerance induction and widespread clinical application of hand transplantation for the reconstruction of upper extremity amputations.

Corrections/Changes Planned for Year 5

Transition of the team and laboratory from the University of Pittsburgh to Johns Hopkins University School of Medicine delayed the Translational Trials for a total of 14 months (see detailed explanation outlined in Year 3 annual report). Therefore, remaining large animal experiments involving topical treatment of the skin component will have to be completed during year 5 prior to the adaption of this regimen to our human clinical trial for hand transplantation.

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Conflict of Interest Disclosure: NONE.

REPORTABLE OUTCOMES

Personnel Statistics	Paid*	Unpaid
# Faculty	5	3
# Post docs	1	0
# grad students	0	0
# undergrad students	0	0
# staff members working for AFIRM	2	0

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	7
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Peer-Reviewed Publications

1. Wachtman GS, Wimmers EG, Gorantla VS, Lin CH, Schneeberger S, Unadkat JV, Zheng XX, Brandacher G, Lee WP. Biologics and donor bone marrow cells for targeted immunomodulation in vascularized composite allotransplantation: a translational trial in swine. Transplant Proc. 2011 Nov; 43(9): 3541-4.
2. Brandacher G, Lee WP. Hand transplantation. Hand Clin. 2011 Nov;27(4):xiii-xiv.
3. Gorantla VS, Brandacher G, Schneeberger S, Zheng XX, Donnenberg AD, Losee JE, Lee WP. Favoring the risk-benefit balance for upper extremity transplantation--the Pittsburgh Protocol. Hand Clin 2011; Nov; 27(4): 511-20, ix-x.
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7. Ibrahim Z, Lin C, Zhang W, Ng TW, Schneeberger S, Zheng XX, Lee WPA, Brandacher G. The vascularized bone marrow component of composite tissue allografts (CTA) combined with costimulatory blockade promotes donor-derived hematopoietic stem cell engraftment, chimerism and tolerance. Transplant Int. 2011; Vol.24, Suppl.2, 93.

Project EI-3: Cellular Therapy for Treatment and Consequences of Compartment Syndrome

Team Leader(s)	Johnny Huard, PhD, McGowan Institute for Regenerative Medicine, University of Pittsburgh, and Shay Soker, PhD, Wake Institute for Regenerative Medicine
Project Team Members	Burhan Gharaibeh, PhD; Nick Oyster, B.S.; Minakshi Poddar, M.S.; Johannes Schneppendahl, M.D. MIRM -- Tracy Criswell, PhD, Zhan Wang, PhD, WFIP
Collaborator(s)	Dr. William Wagner and Dr. Stephen Badylak from WFIP
Therapy	Cellular Therapies for Treatment of Compartment Syndrome
Deliverable(s)	*Baseline:* Muscle tissue regeneration by delivering muscle stem and progenitor cells together with angiogenic and anti-fibrosis factors *Revised:*

TRL Progress

Start of Program:	TRL #1	End Year 3:	Projenitor cells TRL #2Lorsartan TRL #4
End Year 1:	Compartment Syndrome Model TRL# 3 Progenitor cells TRL# 2	End Year 4:	Projenitor cells TRL #2Lorsartan TRL #5
End Year 2:	Projenitor cells TRL #2 Lorsaten TRL #4		

Key Accomplishments:	Dr. Huard's group developed a novel model of a traumatic lower limb injury which combines aspects of compartment syndrome, ischemia-reperfusion and crush injuries to cause significant damage to the underlying skeletal muscle, nerve, and vascular structures. 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. Similarly, a two-person case study conducted at the University of Pittsburgh Medical Center showed that Losartan is an effective treatment for grade II hamstring injuries (1) . Finally, mouse muscle derived stem cells over-expressing vascular endothelial growth factor (VEGF) or soluble fms-like tyrosine kinase 1 (sFlt-1) transplanted into injured skeletal muscle indicate the timing of VEGF regulation is critical in treating an injury as complex as compartment syndrome
Keywords	Compartment syndrome, skeletal muscle, stem cells, fibrosis, Losartan

Introduction

Compartment syndrome (CS) is a serious injury characterized by an increase in pressure in an enclosed space leading to compromised circulation, severe pain, and damage to muscle, nerves and vasculature. CS can be caused by a multitude of injuries that include trauma, contusion, fractures and blast injuries that affect the musculoskeletal system and if left untreated can lead to amputation, Volkmann's contracture(2) or death. On a cellular level ischemia can lead to necrosis (3) allowing for minimal or no regeneration of musculoskeletal tissues. CS mechanisms can be divided into two categories; those that increase the content of the compartment and those that decrease the size of compartment(4-6). The clinical standard of care for CS is surgical fasciotomy allowing the compartment volume to increase. Fasciotomy is used with varying degrees of success in civilian and combat trauma centers and is often done prophylactically as alternative (pharmacologic) treatments for regulating compartment pressure are not available. With fasciotomy rates increasing among US armed forces casualties (7) and the understanding that fasciotomies are often too late or insufficient to prevent compartment syndrome, there is a need for an increased understanding of the damage to the underlying tissues. This project aimed to develop a small animal model of CS to allow for future testing of therapies treating CS sequelae based on our expertise in contusion and laceration injury models in rodents and the use of murine skeletal muscle derived stem cell therapies (8-12). Additionally, 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 immunosuppresants. Furthermore, histopathological and physiological data obtained allowed for a more thorough assessment of the heterogeneity of the CS injury than has been done in other studies.

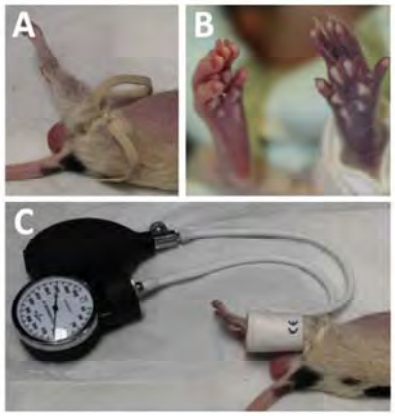

Figure 1: The CS injury model. A) The tourniquet was placed just above the trochanter major and secured by double-knotting. B) Venous blood flow occlusion was confirmed by a lack of pink color in the sole of the injured foot (right). C) The neonatal cuff was secured using over the lower hindlimb that included the anterior compartment and inflated to a pressure above 300 mmHg. The tourniquet and cuff remained in place for 3 hours during which time the animal remained anesthetized with 2% isoflurane.

Research Progress (Year 4)

Johnny Huard's Team:

Dr. Huard's lab completed development of the injury model (Figure 1) and a manuscript describing the injury is being submitted. As part of the study, the group used a novel method to measure the intracompartmental pressure of the affected muscle (Figure 2). An implantable blood pressure monitor/transmitter (Figure 2B) (Data Systems International) was partially implanted (Figure 2C) into a rat prior to injury. For this experiment, only the probe was implanted into the muscle body while the transmitter remained outside the subject.

Following implantation into two athymic rats, the tourniquet and cuff (TKA + compression) or tourniquet alone (TKA alone) were secured over the hind limb with the monitor implanted and the intracompartmental pressure was recorded every 10 seconds for 3 hours. The results shown in Figure 2D show external compression is necessary to elevate the anterior compartment pressure to the level necessary for a diagnosis of compartment syndrome.

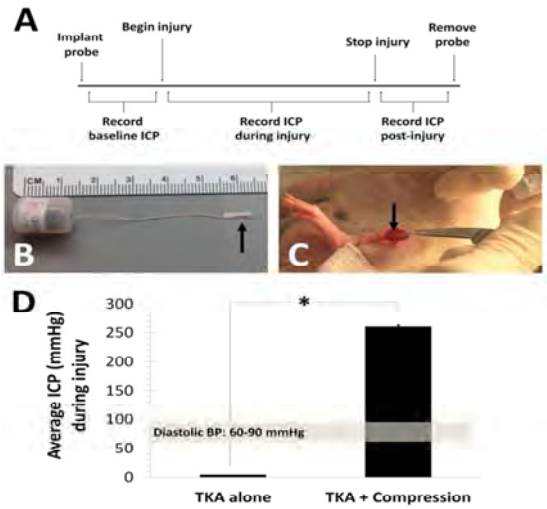

Figure 2: Intracompartmental pressure measurements during the 3 hour injury. A) The implantable transmitter used to measure and transmit anterior compartment pressures. It consists of a transmitter and a 5 cm catheter, the tip of which (arrow) is implanted into the muscle B) The tip of the transmitter being inserted into the centroid of the TA (arrow). For these measurements only the tip was implanted while the rest of the catheter and the transmitting component were left extracorporeal. C) The average compartment pressures recorded inside the anterior compartment when injured with a tourniquet alone or a tourniquet + external compression for 3 hours. The range of diastolic pressure for rats is represented by the shaded brown bar. The average pressure when the tourniquet only is applied is 4.41±0.02 mmHg. The average pressure when the cuff is applied in addition to the tourniquet is 260.70±2.70 mmHg. D) Experimental design for the measurement of compartment pressures. After implanting the tip of the catheter baseline pressure measurements were recorded for 5-10 minutes. The injury was started by applying the tourniquet or the tourniquet + cuff. Once the materials were secured pressures were recorded for 3 hours. After 3 hours the tourniquet and/or cuff were removed and pressures were recorded for another 20 minutes after which the transmitter was removed. *(* $p<0.05$).*

Using the Huard injury model, 35 athymic rats were injured and randomly assigned to 1 of 4 treatment groups; PBS, LacZ, VEGF or sFlt-1. The latter three treatment groups consisted of mouse muscle derived stem cells isolated via the Huard group's preplate technique. The mouse MDSCs were transduced with LacZ retrovirus and then separated in to 3 groups. Two groups were additionally transduced with either VEGF or sFlt-1 retrovirus. Secreted levels of VEGF and sFlt-1 in the transduced MDSCs were analyzed with enzyme-linked immunosorbent assays (ELISA) (Figure 3A-B). Detectable levels of VEGF and sFlt-1 are present only in the cell lines transduced to overexpress the particular protein (either VEGF or sFlt-1). The negative values are due to the comparison to standard curve as a linear relationship since the curve deviated from a linear relationship at low concentrations. This group has previously shown VEGF can have a negative effect on muscle regeneration at high concentrations (13). To reduce the secreted amount of VEGF to beneficial levels, the final number of injected cells consisted of 50% LacZ only and 50% VEGF overexpressing cells. Seventy-two hours post injury, PBS (20uL) or $1x10^6$ MDSCs (suspended in 20uL) were injected into the injured tibialis anterior muscles. Animals were sacrificed 7 and 14 days after injury and the tissues collected, frozen, section and stained to look for implanted cells (Figure 3C). The function of injured anterior muscle groups was assessed using an *in situ* test apparatus (Aurora Scientific) just before sacrificing the animals. The data in Figure 3D show no significant differences

in function between the treatment groups. Fibrosis (Figure 3E) and necrosis (Figure 3F) levels indicate timing the regulation of VEGF levels following compartment syndrome is important in expediting skeletal muscle regeneration.

Key Research Accomplishments

- Completed development of a small animal model of traumatic lower limb injury that features aspects of crush, ischemia-reperfusion and compartment syndrome injuries.
- Established a novel method of measuring intracompartmental pressure in the anterior compartment of rats.
- Created 3 murine MDSC lines which stably express LacZ, LacZ/VEGF or LacZ/sFlt-1. The cells were characterized for levels of VEGF and sFlt-1 secretion and transplanted into the tibialis anterior muscle of injured athymic rats.

Conclusions

Research Plans for the Following Years

Therapies combing the benefits of MDSCs and the anti-fibrotic effect of Losartan (*AFIRM 2011 annual report, pg. 443-444*) are currently being tested. Along with the previously mentioned murine cell lines, this group has created 3 human muscle derived cell lines that stably express green fluorescent protein (GFP), GFP and VEGF, or GFP and sFlt-1. Dr. Huard's team previously reported on the successful transplantation of human muscle derived cells into CS injured athymic rats. Tests using these newly developed and characterized muscle cells will build upon year 4's successful transplantation of murine MDSCs. The Huard group will optimize the timing and dosing of MDSC transplantation and Losartan to better regulate VEGF and mimic the clinical setting. The

data from this previous year indicate VEGF regulation is important in treating the complex tissue damage compartment syndrome creates; however the current treatment protocol may not be ideal. Currently, both MDSC and Losartan treatment is started 72 hours after CS injury but clinically treatments will be started immediately following fasciotomy. The injury models developed by both the Huard and Soker groups mimic fasciotomy (removal of external compression) allowing for testing of a clinically relevant treatment protocol.

Planned Clinical Transitions

Drs. Huard and Soker are submitting an AFIRM II proposal that outlines a research plan to determine the effect of losartan on tissue fibrosis and time to return to prior level of function in a limited case study of acute grade II or III hamstring strain patients. Briefly, skeletal muscle fibrosis will be quantified in the injured hamstring of the subject with a 3 Tesla MRI during the initial visit and compared to a follow up MRI 6-months post-injury. Research subjects will be followed to determine the time to return to the prior level of sports activity and a correlation between scarring and time to return will be determined.

Corrections/changes planned for the next year and rationale for changes:

No deviations from the previous plan

Conflict of Interest Disclosure

Johnny Huard received remuneration as a consultant and royalties from Cook MyoSite, Inc. during the period of time the studies for this manuscript were performed.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	6	0
# Post docs	2	1
# grad students	1	3
# undergrad students	0	2
# staff members working for AFIRM	4	0

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	6
# Non-peer reviewed publications	11

*Directly supported in whole or part –time by AFIRM

 # Honors given to AFIRM faculty

2010 Best Oral Presentation in the Compartment Syndrome Category.

Uehara K, Kobayashi T, Ota S, Sun B, Tobita K, Ambrosio F, **Gharaibeh B**, Fu FH, **Huard J**. Angiotensin Receptor Blocker Improves Skeletal Muscle Function Recovery in a Dose Dependent Manner. AFIRM All Hands Meeting; St. Pete, FL; January 11-14, 2010.

AFIRM Career Enrichment and Travel Support – February 2012

Gao X, Usas A, Lu A, Tang Y, Poddar M, Tebbets JC, Huard M, Cummins JH, **Huard J**. Role of donor and host cyclooxygenase-2 in muscle derived stem cell mediated bone healing in critical sized calvarial defect model.

Armed Forces Institute of Regenerative Medicine (AFIRM) WFPC Career Enrichment & Travel Support - December 2011

Mu XD, Takayama K, Tang Y, Wang B, Weiss K, **Huard J**. Role of Notch signaling in stem cell behavior in dystrophic muscle. 51th Annual meeting of American Society for Cell Biology (ASCB), December 3-7 2011; Denver CO.

Peer Reviewed Publications

1. Burhan Gharaibeh, Yuri Chun, Tanya Hage n, Sheila Jean McNeill Ingham , Vonda Wright, Freddie Fu and Johnny Hu ard. Biological approaches to improve skeletal muscle healing after injury and disease. Birth Defects Res C Embryo Today. 96(1):82-94.
2. Masahiro Nozaki, Shusuke Ota, Satoshi Terada, Yong Li, Kenji Uehara, Burhan Gharaibeh, Freddie H. Fu, Johnny Huard. Ti ming of the adm inistration of Suram in treatment after muscle injury. Muscle and Nerve. Proof corrected.
3. Gharaibeh, B, Deasy, B, Lava sani, M, Li, Y and Huard, J. 2012. Muscle derived stem cells for musculoskeletal disease: Role of cell survival, neo vascularization and microenvironment in tissue repair. Vol. 2, Part 3, Pa ges 891-908. In Muscle: Fundamental Biology and Mechanisms of Disease. Elsevier. (Joseph A. Hill and Eric N. Olson, Eds.).
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Non Peer Reviewed Publications

1. . Gharaibeh B, Oyster N, Boyer A, Poddar M, Witt M, Huard J. Patterns of muscle injury in a new model of com partment syndrome in athymic rat. ORS Annual Meeting; February 4-7, 2012; San Francisco, CA.
2. Oyster N, Gharaibeh B, Boye r A, W right A, W itt M, Mu X, Huard J . Muscle-derived Cells with High Levels of Aldehyde Dehydrog enase Activity Increase Force and R educe Fibrosis in Com partment Syndrome Injure d Skeletal Muscle. ORS Annual Meeting; February 4-7, 2012; San Francisco, CA.

3. Terada S, Ota S, Kobayashi T, Mifune Y, Ta kayama K, Lee S, Gharaibeh B, Otsuka T, Fu F, Huar d J. Com bination Treatment of Platelet-Rich Plasma and Angiotensin II Receptor Blocker for Contusion Sk eletal Muscle Injury in Mice. ORS Annual Meeting; February 4-7, 2012; San Francisco, CA.
4. Oyster N, Gharaibeh B, Boye r A, W right A, W itt M, Mu X, Huard J . Muscle-derived Cells with High Levels of Aldehyde Dehydrog enase Activity Increase Force and R educe Fibrosis in Com partment Syndrome Injured S keletal Muscle. AFIRM; St. Pete, FL; February 13-15, 2012.
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6. Mifune Y, Matsumoto T, Ota S, M eszaros L, Usas A, Gharaibeh B, Fu F, Johnny Huard. Platelet-rich plasma combined with blocking VEGF enhances the efficacy of MDSCs for cartilage repair in OA. 2011 ORS Annual Me eting; January 13-16, 2011; Long Beach, CA.
7. Gharaibeh B, Oyster N, Boyer A, Blackwell T, Poddar M, Distefanno G, Kobayashi T, Al Hallak A, Viswanathan V, Ahani B, Lava sani M, W itt M, Huard J. Histolo gical analysis of muscles and nerve injury in a new model of compartm ent syndrome. 2011 ORS Annual Meeting; January 13-16, 2011; Long Beach, CA.
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Project EI-4: Epimorphic, Non-Blastemal Approach to Digit Reconstruction

Team Leader(s)	Stephen F. Badylak, DVM, PhD, MD (University of Pittsburgh)
Project Team Members	Lisa E. Carey (Institute for Regenerative Medicine, University of Pittsburgh), Vineet Agrawal (McGowan Institute for Regenerative Medicine, University of Pittsburgh),Scott A. Johnson (McGowan Institute for Regenerative Medicine, University of Pittsburgh), Neill Turner (McGowan Institute for Regenerative Medicine, University of Pittsburgh) Li Zhang (McGowan Institute for Regenerative Medicine, University of Pittsburgh), Janet Reing (McGowan Institute for Regenerative Medicine, University of Pittsburgh)
Collaborator(s)	Ron Stewart (University of Wisconsin) Jamie Thomson (University of Wisconsin) Susan Braunhut (University of Massachusetts, Lowell) David Kaplan (Tufts University) Eileen Moss (The University of Texas at Arlington) Muthu Wijesundara (The University of Texas at Arlington)
Therapy Deliverable(s)	Treat digit loss with epimorphic regeneration strategies 1. *Baseline:* A biologic scaffold based strategy for inducing epimorphic regeneration in limb and digit soft tissues 2. A biomaterial that can facilitate epimorphic regeneration in soft tissues (multiple forms, solid sheet, gel, powder, etc.). *Revised:*
TRL Progress	Start of Program: TRL #3 End Year 3: TRL #5 End Year 1: TRL #4 End Year 4: TRL #5 End Year 2: TRL #5
Key Accomplishments:	The Badylak lab has shown in a mouse model of mid second phalanx digit amputation that treatment with bioactive molecules derived from extracellular matrix (ECM) can recruit endogenous multipotential stem cells to the site of injury [1]. The lab has identified and characterized specific potent fractions of bioactive peptides [2, 3]. The lab has also characterized the source of at least a portion of the recruited multipotent stem cells. Additionally, the concurrent innate immune response has been analyzed, with results showing a more positive, alternatively activate macrophage (M2) environment in response to ECM treatment [4-6]. Finally, we have shown that treatment with bioactive homing molecules from ECM causes bone growth in an amputated digit [3]
Keywords	limb regeneration, extracellular matrix (ECM), epimorphosis, multipotential cell cluster (MCC), innate immune response, M2 Macrophage

Introduction

Background

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 (1). Conventional treatment methods are inadequate to restore functional tissue in most patients and it is therefore attractive to investigate unconventional treatment methods. To address this problem, the present work investigates regeneration of lost limbs/digits following acute trauma via a non-blastemal epimorphic regeneration approach. Certain non-mammalian species, such as the amphibian urodele, are capable of full regeneration of limbs through accumulation of preprogrammed stem cells at the site of amputation, forming 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, 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 does not have this capacity as a component of the default mechanism for wound healing. The present work, in large part, is focused on inducing this non-blastemal regenerative capacity in alternative tissues. The signals to facilitate this resurrection of non-blastemal regeneration reside within the extracellular matrix (ECM). Developing therapeutic strategies that can take advantage of this matrix based approach is the fundamental objective of the present work.

Specific Aims

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

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

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

Specific Aim #4: To evaluate digit reconstruction in a human pilot study in years 7 – 10 of this project. (Progress dependent upon success in Specific Aims 1 – 3.)

Previous Year's Achievements

In the past year, the Badylak group and collaborators have characterized the genetic signature of cells recruited in both blastemal (in urodeles) and non-blastemal (in mice) formation in response to limb injury. Additionally, the Badylak lab has begun to characterize the innate immune

response to bioactive ECM to a site of amputation. Finally, we have shown that treatment with bioactive ECM results in a positive tissue-remodeling macrophage (M2) microenvironment that may improve functional tissue formation, in comparison to without treatment *in vitro*. This work has resulted in the following manuscripts:

1. Agrawal, V., et al., Epimorphic regeneration approach to tissue replacement in adult mammals. Proc Natl Acad Sci U S A, 2009. 107(8): p. 3351-5.
2. Agrawal V, Tottey S, Johnson SA, Freund JM, Siu BF, Badylak SF. Recruitment of Progenitor Cells by an Extracellular Matrix Cryptic Peptide in a Mouse Model of Digit Amputation. *Tissue Eng Part A*. Vol. 17, No. 19-20, October 2011: 2435-2443.
3. Agrawal V, Kelly J, Tottey S, Daly KA, Johnson SA, Siu BF, Reing J, Badylak SF. An Isolated Cryptic Peptide Influences Osteogenesis and Bone Remodeling in an Adult Mammalian Model of Digit Amputation. *Tissue Eng Part A*. 2011 Aug 29.
4. Badylak, S. F., Valentin, J., Ravindra, A., McCabe, G., Stewart-Akers, A. Macrophage Phenotype as a Determinant of Biologic Scaffold Remodeling. *Tissue Engineering* 2008 Nov;14(11):1835-1842.
5. Brown BN, Valentin JE., Stewart-Akers AM, McCabe GP, Badylak SF. Macrophage Phenotype and Remodeling Outcomes in Response to Biologic Scaffolds With and Without a Cellular Component. *Biomaterials.* 2009. Mar:30(8):1482-1491.
6. Brown BN, Ratner BD, Goodman SB, Amar S, Badylak SF. Macrophage polarization: An opportunity for improved outcomes in biomaterials and regenerative medicine. Biomaterials, 2012 May; 33(15): 3792-3802

Analysis of Competitive Technologies

There is currently no viable treatment for functional regeneration of limbs and digits following traumatic injury. At this time, there are no competing technologies. Composite tissue transplantation is possible in a very small number of select patients.

Leveraged Funding

We have continued to collaborate with Drs. Susan Braunhut and David Kaplan through leveraged funding ("Control of the microenvironmental niche to promote epimorphic regeneration in amputated digits", Department of Defense and Pittsburgh Tissue Engineering Initiative, W81XWH-07-1-0415). This funding has also supported work with Drs. Ron Stewart and Jamie Thomson at the University of Wisconsin. The work with these co-investigators involves the full spectrum of basic science to translational medicine.

We also have a partial collaboration with Dr. Eileen Moss, Ph.D. at the Automation & Robotics Research Institute, The University of Texas at Arlington. As will be seen later in this document, we propose to expand that collaboration as a follow-up phase to our first 3 years of work. This proposal involves the development of a BIODOME.

Research Progress

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

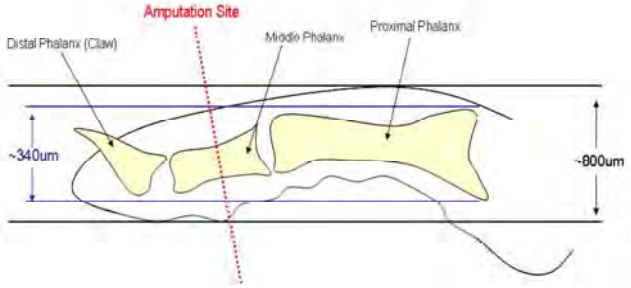

Figure 1. Site of P2 amputation in mouse model.

Using an established mouse model (C57/BL6) of mid-second phalanx (P2) digit amputation (Figure 1) [1], we have definitively shown that bioactive homing signals from ECM results in a dense cell accumulation at the site of amputation. These cells express multiple markers characteristic of primitive, multipotent stem cells.

Work from the Thomson laboratory (publication pending) has identified specific transcription factors, oncogenes, and blastemal genes that peak throughout the healing and regeneration of the salamander limb through deep RNA 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. There is a manuscript in preparation that compares the genetic profile of regenerating newts and non regenerating mice as part of this collaboration.

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

In previous years, the lab has demonstrated the capability of bioactive matrix peptides to recruit multipotential cells to the site of injury after acute trauma [1-3]. An additional aspect of evaluating the effect of these therapeutic molecules is to characterize the innate immune response. Recent studies from the Badylak lab and other labs have indicated the importance of the innate immune response in tissue remodeling [5-7]. To investigate the effect that ECM has on the immune system, macrophage polarization was characterized *in vitro*. Work in the Badylak lab has analyzed this effect both in mouse and human macrophages. In one study, monocytes harvested from human donors were cultured with different concentrations of decellularized urinary

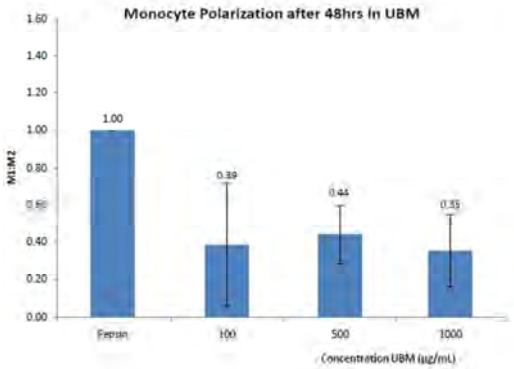

Figure 2. Human Monocyte Polarization Response. M1:M2 ratio- greater than 1 is predominantly an M1 response, lower than 1 is a predominantly M2 response

bladder matrix (UBM) digested in pepsin. Th en they were labeled with imm unofluorescent antibodies for markers of pro-inflammatory macrophages (M1) and regulatory macrophages (M2). The m arkers used were CCR7 and CD206, respectively. F low cytometry results demonstrated that at concentrations from 100 to 1,000 µg/ml monocytes are predom inantly polarized to an M2 phenotype compared to a pe psin control (Figure 2). In a m ouse model, macrophages were harvested from the bone m arrow and cultured with thre e different types of ECM then stained with F4/80 (a m ouse specific macrophage marker), FIZZ1 (a m ouse-specific M2 marker), and iNOS (an M1 marker). Pre liminary results show that the EC M cultured macrophages take on a predom inantly M2 phenotype when cultured in the presence of ECM degradation products.

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

The potential of bioactive m olecules to constr uctively stimulate tissue rem odeling in a m ouse digit amputation model has been shown in previous work [1-3]. As a consequence of this evaluation, the B adylak lab is now developing strategies to ch aracterize changes in the innate immune response to ECM degradation product treatment. Using immunofluorescent staining, results indicated that m ice treated with UBM in a digit amputation model showed an increase in M2 phenotype m acrophages over tim e, compared to digit amputation without treatment [Figure 3].

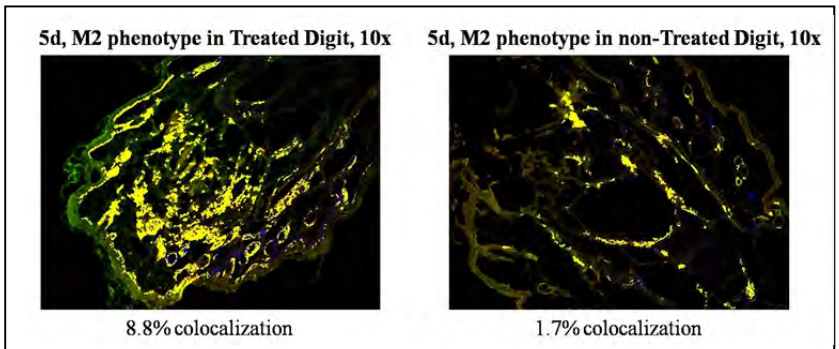

Figure 3. Frozen mouse digit sections immunofluorescently labeled with markers for macrophages (F4/80 in green), M2 phenotype (FIZZ1 in red), and nuclei (DAPI in blue) show a greater M2 macrophage presence at the digit amputation injury site 5 days after treatment (A) compared to 5 days after no treatment (B). C olocalization of F4/80+, FIZZ1+, and DAPI+ cells is indicated in yellow and expressed as a percentage of total pixels.

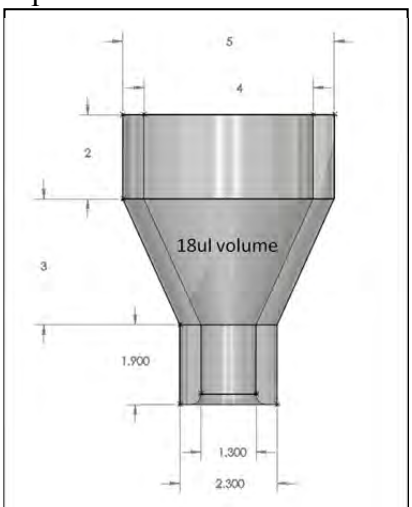

Figure 4. BIODOME prototype schematic, measurements in millimeters

Specific Aim #4: To evaluate digit reconstruction in a human pilot study in years 7 – 10 of this project. (Progress dependent upon success in Specific Aims 1 – 3.)

In collaboration with the Automation & Robotics Research Institute (ARRI) at the University of Texas Arlington, work in the past year resulted in a novel prototype device designated

as the BIODOME (Biomechanical Interface for Optimized Delivery of MEMS Orchestrated Mammalian Epimorphosis) that was developed for an adult mouse model of digit amputation. The device has received a number of design improvements from the previous models, including better flexibility to allow for toe swelling, smaller profile for greater mouse mobility, and easier to remove for reuse and better preservation of the healing digit (Figure 4). This new prototype is currently in testing in the mouse model. The ARRI group is working on concurrent development of a device for a large animal study in canines. Optimization of both mouse and large animal designs is ongoing and will inform future designs for a BIODOME device to control digit reconstruction in a human pilot study. The proposed work will focus on utilization of the findings of aims 1-3 and the mouse and canine BIODOMEs to refine a prototype for a human BIODOME.

Key Research Accomplishments

- Established consistent model of digit amputation in the C57Bl/6 mouse (mid P2 amputation).
- Established a method for recruiting multipotential stem cells to the site of amputation by regional injection.
- Isolated, sequenced, and synthesized a potent ECM fraction with chemotactic properties for multiple progenitor cells *in vitro*
- Characterized the innate immune response to ECM treatment, *in vitro* and *in vivo*
- Refined a prototype BIODOME device for controlling the microenvironment of the site of amputation.
- Designed a new prototype BIODOME for controlling the microenvironment of a soft tissue injury site in a large animal model.

Conclusions

We have shown the ability to recruit endogenous multipotential cells to the site of injury in a non-regenerating mammalian system (i.e., a step toward non-blastemal epimorphic regeneration). Work continues 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. We also are continuing to further define the population of cells involved in the formation of the MCC and to examine 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 we face. We believe that control of the "microenvironmental niche" will be required, and to that end we have developed a conceptual prototype for a BIODOME 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 Following Years

Research in the following years will focus on the testing and utilization of the BIODOME functional delivery device, with an objective of eventual use in human trials in specific aim #4. 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, we will seek industry partners for the mass production, further development, and potential clinical testing of the bioactive peptides for treatment of limb/digit loss.

Planned Clinical Transitions

The Badylak lab is in a continuing collaboration with Dr. Peter Rubin at the University of Pittsburgh to evaluate a powder form of extracellular matrix for the treatment of distal digit amputations. The product being used is a commercially available FDA approved form of powdered ECM. The patients who present 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.

In collaboration with ARRI, the lab is developing the prototype for a canine BIODOME treatment delivery device to be used in a preclinical trial for tissue regeneration using control of the injury microenvironmental niche.

Corrections/changes planned for next year and rationale for changes

None

Conflicts of Interest Disclosure

None

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1. Agrawal, V., et al., Epimorphic regeneration approach to tissue replacement in adult mammals. Proc Natl Acad Sci U S A, 2009. 107(8): p. 3351-5.
2. Agrawal V, Tottey S, Johnson SA, Freund JM, Siu BF, Badylak SF. Recruitment of Progenitor Cells by an Extracellular Matrix Cryptic Peptide in a Mouse Model of Digit Amputation. *Tissue Eng Part A.* Vol. 17, No. 19-20, October 2011: 2435-2443.
3. Agrawal V, Kelly J, Tottey S, Daly KA, Johnson SA, Siu BF, Reing J, Badylak SF. An Isolated Cryptic Peptide Influences Osteogenesis and Bone Remodeling in an Adult Mammalian Model of Digit Amputation. *Tissue Eng Part A.* 2011 Aug 29.

4. Badylak, S. F., Valentin, J., Ravindra, A., McCabe, G., Stewart-Akers, A. Macrophage Phenotype as a Determinant of Biologic Scaffold Remodeling. *Tissue Engineering* 2008 Nov;14(11):1835-1842.
5. Brown BN, Valentin JE., Stewart-Akers AM, McCabe GP, Badylak SF. Macrophage Phenotype and Remodeling Outcomes in Response to Biologic Scaffolds With and Without a Cellular Component. *Biomaterials.* 2009. Mar:30(8):1482-1491.
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7. Tidball JG, Villalta SA. Regulatory interactions between muscle and the immune system during muscle regeneration. American Journal of Physiology. 2009 Nov; 298(5): 1173-1187

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty w orking on AFIRM projects	2	0
# Post docs	2	0
# grad students	5	0
# undergrad students	4	0
# staff members working for AFIRM	4	1

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	1
# Masters degrees awarded under A FIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	6
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Peer-Reviewed publications

1. Agrawal, V., et al., Epim orphic regeneration approach to tissue replacem ent in adult mammals. Proc Natl Acad Sci U S A, 2009. 107(8): p. 3351-5.
2. Agrawal V, Tottey S, Johnson SA, Freund JM, Siu BF, Badylak SF. Recruitment of Progenitor Cells by an Extracellular Matrix Cryptic Peptide in a Mouse Model of Digit Amputation. *Tissue Eng Part A.* Vol. 17, No. 19-20, October 2011: 2435-2443.
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Proposals: Over the past year, the following proposals were submitted:

1. NIH F31 proposal, "A Novel Delivery System to Modulate Tissue Remodeling Response in a Mouse Digit Amputation Model" (April 13, 2012) – Pending Review

Project EI-5: Use of Bone Marrow Derived Cells for Compartment Syndrome

Team Leader(s)	Kenton Gregory, MD, Oregon Health and Sciences University (OHSU);Consortium: Wake Forest University-University of Pittsburgh's McGowan Institute for Regenerative Medicine Consortium
Project Team Members	Bo Zheng, MD; Cynthia Gregory, PhD; Michael Rutten, PhD; Hua Xie, MD, PhD; Rose Merten, BS; Brya n Laraway, BS; Annabe th Rose, BS; James Hunt, BS; Carrie Charlton, BS ; Amy Jay, BS; Cher Hawkey, BS; Teresa Malarkey, CVT, LAT
Collaborator(s)	OHSU Center for Regenerati ve Medicine; U.S. Ar my Institute of Surgical Research; Special Operations Medical Command-Fort Bragg; Biosafe-AmericaBiologics Consulting Group; Torston Tonn, M.D., Johann Wolfgang University, Frankfurt, Germany
Therapy	Autologous Bone Marrow Stem Cell Treatm ent for Compartment Syndrome (CS)
Deliverable(s)	*Baseline:* Develop a large-anim al CS model to evaluate efficacy of multiple stem cell trea tment to reg enerate muscle and n erve damage in extremity wounds complicated by CS *Revised:* None
TRL Progress	Start of Program: TRL #2 End Year 3: TRL #3-4 End Year 1: TRL #2 End Year 4: TRL #4-5 End Year 2: TRL #3
Key Accomplishments:	The Gregory group completed the pivot al proof of main concept pre-clinical study in Sinclair Mini-S wine during year f our to evalu ate the deployment strategy of Autologous Bone Marrow Mononuclear cells (BM-MNC) to treat extremity injuries complicated by CS. They first completed a prospective, random ized, blinded, sham-controlled dose ranging study (W81XWH-08-2-0032) to determ ine a therapeutic concentration of Autologous BM-MNCs for treatment one-week post CS injury (n=30). The selected concentration was used in the m ulti-dose study to determ ine the deployment strategy of single randomized against multiple cell treatments (1, 2 and 3 cell-treatm ents post inju ry; n=16). No adverse events or complications were associated with any cell treatm ents. Both studies showed statistically significant improvement in muscle strength and gait. Robust engraftment of transplanted cells was observed at 3 months. This study demonstrates the potential of a safe, new treatment for severe extremity injury that offers injured troops an improved functional recovery
Keywords	Extremity compartment syndrome, autologous bone marrow derived stem cells, porcine model, automated bone marrow stem cell separator, gait analysis, cell engraftment

Introduction

Extremity injuries are the most common battlefield injuries sustained by troops in current military conflicts. These injuries are often complicated by compartment syndrome (CS), where secondary edema and swelling increases compartment pressure that stops blood flow, resulting in ischemia and infarction of muscle and nerve tissue. Fasciotomy is required to relieve the pressure. Troops developing CS have prolonged recovery times and permanent disability is common. The goal of this AFIRM program is to improve the endogenous cellular regenerative response by local treatment with autologous bone marrow stem and progenitor cells in order to produce an improved functional recovery.

The Gregory group completed their pivotal pre-clinical study in Sinclair Mini-Swine during year four. The researchers first completed a single dose study (W81XWH-08-2-0032) to determine a therapeutic dose of autologous bone-marrow mononuclear cells (BM-MNC) for treatment one-week post CS injury. The study evaluated total cell dosages of 50 million or 100 million BM-MNCs randomized against injections of media alone as control in a prospective, blinded study with 10 swine per group (total n=30). Robust stem cell engraftment was observed at 3 months with statistically significant improvement in motor strength and gait. The high cell dose of 100 million BM-MNCs was selected as the cell concentration for the AFIRM pre-clinical study to optimize the deployment strategy of single vs. multiple cell treatments (1, 2 and 3 cell-treatments post injury). Under AFIRM, swine received Autologous BM-MNC treatments post-injury at weeks 1 & 2 (n=8) and at weeks 1, 2 & 4 (n=8).

Research Progress

Pre-clinical Study

A prospective, randomized, blinded, sham-controlled study comparing treatment of CS with autologous bone marrow cells versus control (media only) was performed in a chronic (3 month) swine model. Severe extremity injury with CS was created by infusing autologous plasma into the left-hind anterior tibialis muscle compartment resulting in elevated compartment pressures (>120 mmHg) for six hours, producing severe ischemia and infarction of the muscle and nerve with resultant motor and nerve deficit.

One-week post CS injury, approximately 50 cc of bone marrow was aspirated for mononuclear cell isolation using a Sepax (Biosafe Inc) automated cell-processing device. The cells were sampled for phenotype characterization, labeled with CM-DiI florescent cell tracker, and injected according to a standardized three-dimensional grid pattern throughout the injured muscle. BM-MNCs were harvested, isolated and administered again at week 2 post-injury (n=16) and at week 4 post injury (n=8). In this study, BM-MNC flow cytometry analysis was performed for each therapeutic BM-MNC preparation, totaling either two or three Autologous BM-MNC preparation per subject. In addition to assessing each preparation independently, consecutive cell preparations from the same subject were assessed for any BM-MNC morphological changes that occur between bone marrow draws.

Prior to administration to autologous recipients, aliquots of the BM-MNCs were reserved for cell function analysis. The functional capacity of the BM-MNCs was assessed by their ability to invade and migrate through a Matrigel coated membrane filter in response to a stromal-derived factor-1α (SDF-1α) gradient. The assays were performed using 8.0 micron Matrigel-coated transwell filter plates.

Muscle and nerve function data were recorded at five time points: pre-injury, post-injury, 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).

After sacrifice, the injured skeletal muscle was harvested and investigated for evidence of previously injected CM-DiI cells. Cryosections of the harvested muscle were fixed and the tissue was characterized using primary antibodies and antigens against skeletal muscle myofibers, vascular-like cells neural phenotypes, proliferation nuclear proteins, and macrophage cells. To assess the differentiation fate of the CM-DiI labeled cells that were injected into the injured muscle, a 1-cm^3 portion was taken from the injured muscle and enzymatically (Roche, Liberase) digested. The digested cells were then sorted using a BD-FACS Aria II into CM-DiI positive and negative CM-DiI stained cells.

Results

Muscle Function Analysis

Muscle function was determined by dorsiflexion force measurements of the injured limb. The absolute torque (**Figure 1)** suggests that the control, cell media only, animals did not clinically improve between weeks 6 and 12 while the cell treatment groups continued to improve. The

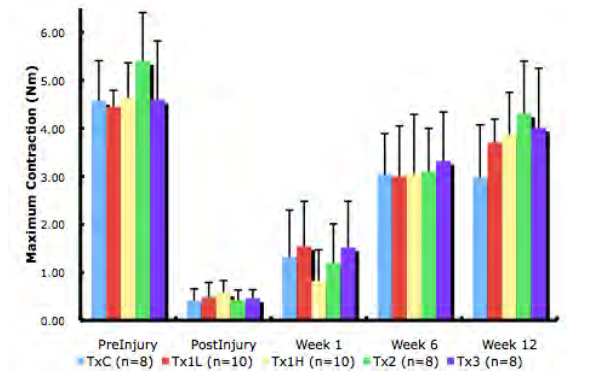


Figure 1. Absolute Torque (Nm). TxC-Control animals, cell-media only injections at week 1. Tx1L-Low cell dose ($50x10^6$ BM-MNCs) single treatment at week 1. Tx1H-High cell dose ($100x10^6$ BM-MNCs) single treatment at week 2. Tx2-High cell dose ($100x10^6$ BM-MNCs) two cell treatments, week 1 & 2. Tx3 -High cell dose ($100x10^6$ BM-MNCs) three cell treatments, week 1, 2 & 4.

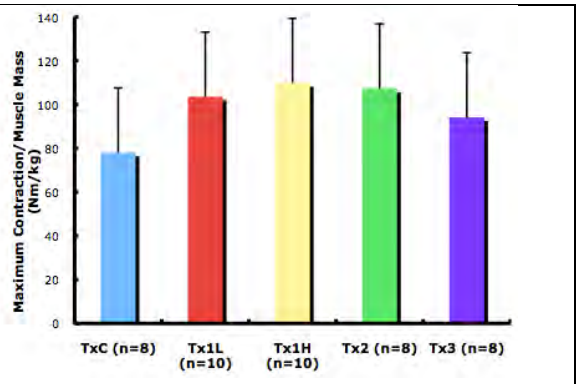


Figure 2. Normalized Torque (Nm). TxC-Control animals, cell-media only injections at week 1. Tx1L-Low cell dose ($50x10^6$ BM-MNCs) single treatment at week 1. Tx1H-High cell dose ($100x10^6$ BM-MNCs) single treatment at week 2. Tx2-High cell dose ($100x10^6$ BM-MNCs) two cell treatments, week 1 & 2. Tx3-High cell dose ($100x10^6$ BM-MNCs) three cell treatments, week 1, 2 & 4.

normalized torque represents the quality of muscle function at the final time point week 12 by normalizing the maximum contraction to the muscle size. In **Figure 2**, single treatment high cell dose (109.9 ± 28.6 Nm/kg) appears to be the optimum cell-therapy regime for improved muscle function.

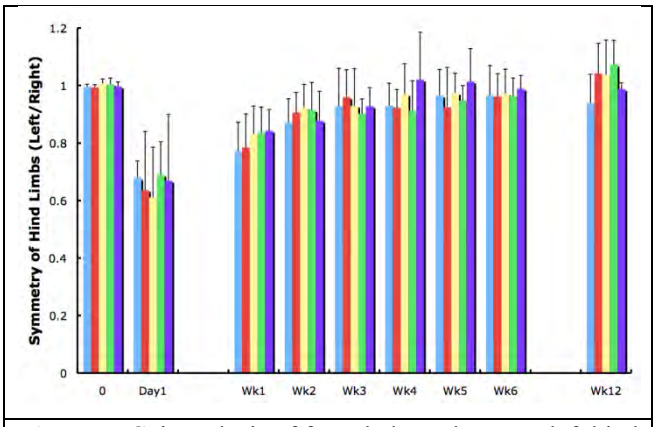


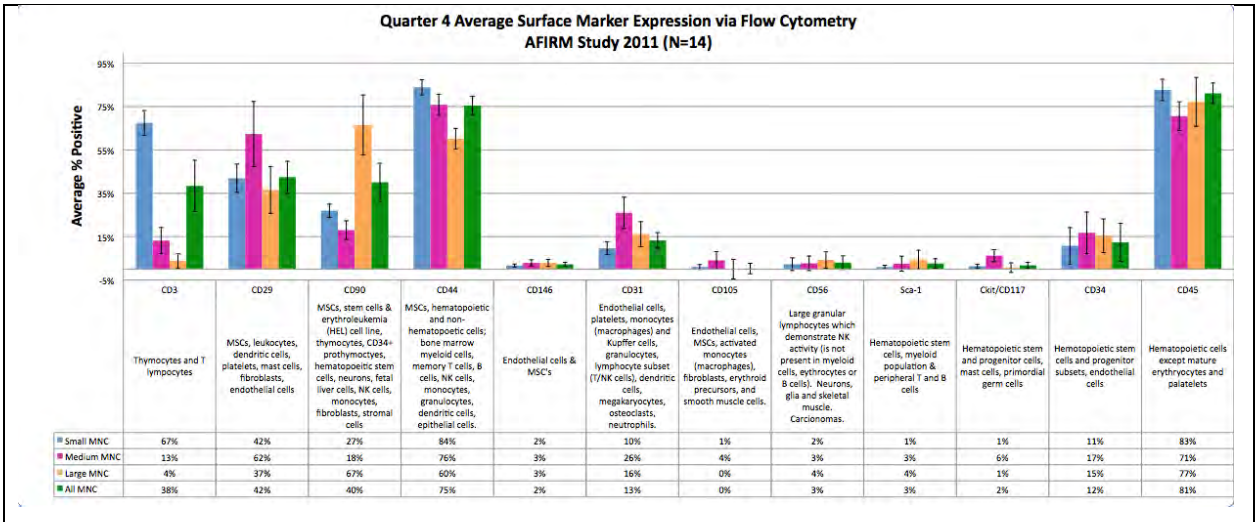
Figure 3. Gait analysis of force balance between left hind injured limb and the right hind uninjured limb. TxC-Control animals, cell-media only injections at week 1. Tx1L-Low cell dose ($50x10^6$ cells) single treatment at week 1. Tx1H-High cell dose ($100x10^6$ cells) single treatment at week 2. Tx2-High cell dose ($100x10^6$ cells) two cell treatments, week 1 & 2. Tx3-High cell dose ($100x10^6$ cells) three cell treatments, week 1, 2 & 4.

Gait Analysis

Gait analysis is represented by the hind-limb force symmetry. Immediately following injury the animals bare most of their weight on their un-injured right side. As they begin to improve the same pattern of healing is observed, as the muscle function data, where treatment groups continue to improve clinically between weeks 6 and 12 while the control group does not. (**Figure 3)**

Bone Marrow Mononuclear Cell Characterization

Subsets of BM-MNCs were detected by their staining with antibodies specific for several distinct cell populations (**Figure 4**). The antibody panel was chosen to stain cell-surface determinants found on cells including mesenchymal stromal cells and endothelial/vasculature related cells, primitive stem cells, and hematopoietic lineage cells. Ongoing analyses demonstrate the



	CD3	CD29	CD90	CD44	CD146	CD31	CD105	CD56	Sca-1	Ckit/CD117	CD34	CD45
	Thymocytes and T lympocytes	MSCs, leukocytes, dendritic cells, platelets, mast cells, fibroblasts, endothelial cells	MSCs, stem cells & erythroleukemia (HEL) cell line, thymocytes, CD34+ prothymocytes, hematopoeitic stem cells, neurons, fetal liver cells, NK cells, monocytes, fibroblasts, stromal cells	MSCs, hematopoietic and non-hematopoetic cells; bone marrow myeloid cells, memory T cells, B cells, NK cells, monocytes, granulocytes, dendritic cells, epithelial cells.	Endothelial cells & MSC's	Endothelial cells, platelets, monocytes (macrophages) and Kupffer cells, granulocytes, lymphocyte subset (T/NK cells), dendritic cells, megakaryocytes, osteoclasts, neutrophils.	Endothelial cells, MSCs, activated monocytes (macrophages), fibroblasts, erythroid precursors, and smooth muscle cells.	Large granular lymphocytes which demonstrate NK activity (is not present in myeloid cells, erythrocytes or B cells). Neurons, glia and skeletal muscle. Carcionomas.	Hematopoietic stem cells, myeloid population & peripheral T and B cells	Hematopoietic stem and progenitor cells, mast cells, primordial germ cells	Hematopoietic stem cells and progenitor subsets, endothelial cells	Hematopoietic cells except mature erythrocytes and palatelets
Small MNC	67%	42%	27%	84%	2%	10%	1%	2%	1%	1%	11%	83%
Medium MNC	13%	62%	18%	76%	3%	26%	4%	3%	3%	6%	17%	71%
Large MNC	4%	37%	67%	60%	3%	16%	0%	4%	4%	1%	15%	77%
All MNC	38%	42%	40%	75%	2%	13%	0%	3%	3%	2%	12%	81%

Figure 4. Analysis of surface marker expression via Flow Cytometry suggest that our treatment BM-MNCs are a multi-potent cell source for our compartment syndrome treatment.

presence of these BM-MNC subsets, which is consistent with the adult Sinclair Mini-Swine bone marrow being a multi-potent cell source for injured tissue repair (**Figure 4**).

Flow cytometry assessments of the BM-MNCs were monitored for viability pre- and post-CM-DiI labeling through observation of 7AAD positive cells (dead); Annexin V positive cells (apoptotic). Additional analyses revealed that within the isolated BM-MNC population, there are three distinct populations of cells based on their size and internal granularity (i.e., their forward and side light-scattering properties, respectively). This data was observed to assess whether the cell subset populations varied pre and post CM DiI Labeling (**Figure 5**).

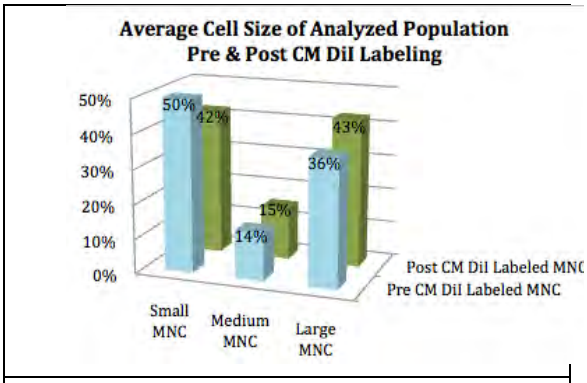


Figure 5. Analysis of cell size subpopulations show that cell distribution is consistent pre and post CM-DiI labeling of the BM-MNCs.

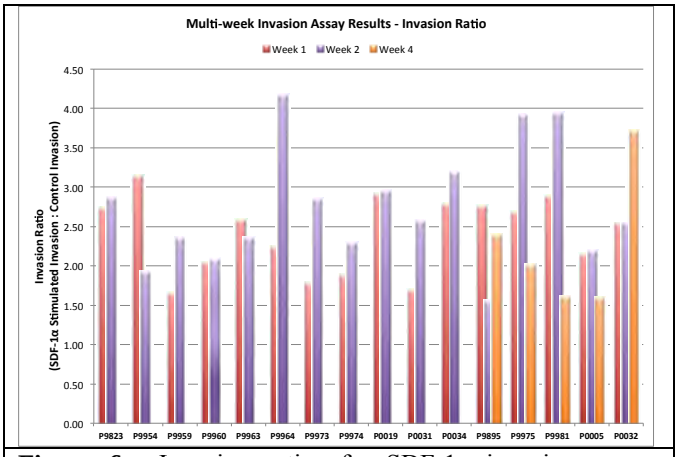


Figure 6. Invasion ratios for SDF-1α invasion assays performed to determine cell function at the time of injection. (The invasion ratio is defined as the ratio of SDF-1α-stimulated invasion to non-stimulated invasion.) All samples resulted in a greater than 1.5 fold increase in invasion in response to SDF-1α as compared to control levels of invasion.

Cell Function Assay

In all cases the BM-MNCs were functional as demonstrated by their increased invasion in response to SDF-1α vs. no chemokine. CyQUANT-based SDF-1α invasion assays demonstrated that all BM-MNC doses contained functional cells at the time of injection (**Figure 6**). Post-study analysis will attempt to correlate cell functionality with improvements in muscle function.

Bone Marrow Mononuclear Cell CM-DiI-Labeling and in vivo Tracking

Evidence of successful engraftment of injected BM-MNCs, as shown by CM-DiI-labeled cells (red) seen on confocal microscopy, was found 11 weeks post-injection (Figure 7). Skeletal muscle myofiber markers **(dystrophin, desmin and Phalloidin) showed that CM-DiI labeled cells appear to preferentially distribute within regions of skeletal muscle fibers or reside near new regenerated myofibers (Figures 7 A, B, E).** CM-DiI cells were located close to or colocalized with myogenitor antigen (MyoD and Pax7) **(Figures 7 C-D)**, or **also found located close to neuronal type markers (β-III tublin, GFAP, CNPase, O4 and** α-Bungarotoxin) **(Figures 7 F-J**); and to **vascular structures where they expressed vascular endothelial markers (CD31, vWF and α-SMA) (Figures 7 K-M**). Some CM-DiI-labeled cells were found to be positive for the proliferation nuclear protein (Ki-67) (**Figures 7**

N), and negative for the m acrophage antigen expression within the skeletal muscle tissu e (**Figures 7 O**).

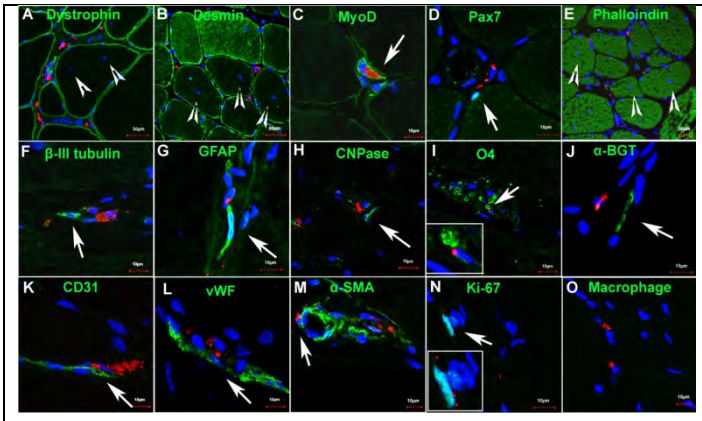


Figure 7. Representative confocal microsc ope images of fluorescent immunohistochemical staining of DiI-labeled cells in injured skeletal muscle at 11 week s post injection. Data sh owed CM-DiI labeled BM-MNCs (red cel ls) distributed between myofibers (green) ((**A)**. dystrophin; **(B)** desmin and **(E)** Phalloidin) (Note, newly regenerated skeletal myofibers are central nuclei arrow hea ds). CM-DiI cells (re d, arrow) located close to or colo calized with myogenitor antigen (green, arrow) (**(C)** MyoD and **(D)** Pax7), or nervous system cell type markers (green, arrow) (**(F)** β-II tubulin, (**G)** GFAP; **(H)** CNPase; **(I)** O4 and **(J)** α-Bungarotoxin), or with vascular relative antigen (green, arrow) (**(K)** CD31; **(L)** vWF; and **(M)** α-SMA). Some CM-DiI-labeled cells sh owed positive for the proliferation nuclear protein (green, arrow) (**(N)** ki-67) and negative for macrophage antigen **(O)**. Nuclei (blue) were stained with DAPI. Th e image (left corner) was magnified form the arrowed antigens.

Recovery of CM-DiI-labeled Cells from Digested Skeletal Muscle Tissue by Cell Sorting

The sorted CM-DiI-pos itive cells were cytocentrifuged onto glass slides (**Figure 8A**) for phenotypic characterization using confocal immunofluorescence microscopy (**Figure 8B**). The enzym atic digestion of two grams of anterior tibialis skeletal m uscle yielded o n average 3.24 x 10^6 total isolated cells and 1.23 x 10^5 sorted CM-DiI-positive cells (n = 5). The purity of the sorted CM-DiI-pos itive cells was found to be 97.25% positive for CM-DiI labeling. In prelim inary single-antibody staining experim ents, CM-DiI-labeled cells exp ressed the neural markers S-100, GFAP, O4, and beta-III tubulin, and they expressed v on Willebrand factor and α-smooth muscle actin (**Figure 8C**). The muscle digest was com pleted for all animals moving forward in the pivotal study.

Key Research Accomplishments

- Muscle function recovery continued to im prove significantly in an imals treated with Autologous BM-MNCs compared to media only control animals 3 months post compartment syndrome injury. Control animal strength and gait did not improve after 6 weeks.
- CM-DiI dye can be used to track the persis tence and distribution of CM-DiI labeled BM-MNCs over an 11-week period following cell in jection. The data dem onstrated that the transplantation of BM-MNCs into injured muscle might contribute to the synchronized reconstitution of blood vesse ls, muscle fibers, and peri pheral nerves and prom ote myogenesis, angiogenesis and neurogenesis.
- Enzymatic digestion of intact skeletal muscle can produce a single-cell population that can be used for cell sorting to generate a pure popul ation of CM-DiI-label ed cells for phenotypic

identification. In preliminary data, it appear s these injected CM-DiI BM-MNCs are capable of multi-lineage differentiation desirable for muscle, nerve, and vascular regeneration.

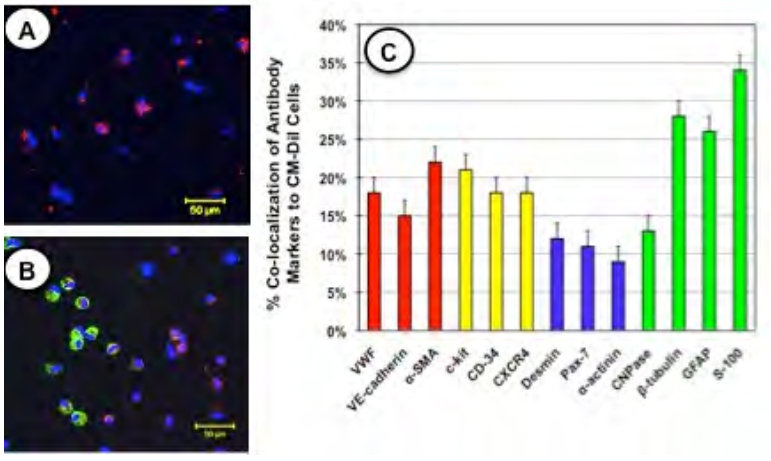


Figure 8. **(A)** Confocal image s howing sorted CM-DiI-labeled cells; nuclei (blue) are stained with DAPI. **(B)** Example of CM-DiI sorted cells co-stained with a phenotypic antibody, *i.e.*, neuronal **G**lial **F**ibrillary Associated Protein (gree n); nuclei (blue) are stained with DAPI. **(C)** Graph showing the co-localization of different phenotypic antibodies with CM-DiI sorted cells. Bars represent mean +/- SE; n = 5.

Conclusions

Treatment of severe anterior tibialis CS muscle in jury with autologous bone marrow stem and progenitor cells in a large animal model resulted in significantly improved muscle function as measured by tetanic force contraction 3 months after injury. W hile control animals treated with m edia alone stopped clinical improvement 6 weeks after injury, tre atment animals continued clin ical improvement through to the 12-week study endpoint. Significant gait im provement and reduction of foot drop were observed at 3 m onths in treated animals compared to control anim als. No adv erse events or complications were associated with any cell treatments. This study demonstrates the potential of a safe, new treatm ent for severe extrem ity injury that offers injured troops an i mproved functional recovery. Important to the clinical translation of this protocol, the Sepax device is FDA approved for hum an cord blood cell s eparation, which will allo w for a shorter p athway through the FDA resulting in a cost effective product.

Research Plans for the Following Years

The Gregory group will finish their 6- month safety and effi cacy study with 10 control an imals and 10 treatment animals before the end of year 5 and AFIRM I. TRL 4

Planned Clinical Transitions

The Gregory group will begin a m ulti-center Phase I human clinical trial. The Sepax device has been 510(k) approval through the FDA for hum an use to separate bone m arrow stem cells. Working closely with the commercial partner Biosafe Group SA, they plan on treating more than 20 patients with Autologous BM -MNCs following a com partment syndrome extremity injury in year 5 if funding is obtained.

Corrections/changes planned for next year and rationale for changes

The Gregory group has been preparing for the 6-month safety and efficacy study in year 4. All IACUCs and other approvals had to be transferred from Providence Health and Services to Oregon Health and Science's University as the department transferred institutions in January 2012. The 6-month animal surgeries are scheduled to begin July 9, 2012 and will conclude in April 2013.

Conflict of Interest Disclosure

None to report.

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2. Owens, B.D., et al., Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. J Trauma, 2008. 64(2): p. 295-9.
3. Ritenour, A.E., et al., Complications after fasciotomy revision and delayed compartment release in combat patients. J Trauma, 2008. 64(2 Suppl): p. S153-61; discussion S161-2.

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty	4	0
# Post docs	9	0
# grad students	4	0
# undergrad students	0	0
# staff members	0	1

Other Project Statistics

# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under A FIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	2

*Directly supported in whole or part –time by AFIRM

Peer-Reviewed publications

1. Rutten M, Janes MA, Laraway B, Gregory C, Gregory K. Comparison of Quantum dot and CM-DiI for Labeling and Function of Adult Po rcine Bone Marrow Derived Progenitor Cells. *The Open Stem Cell Journal,* 2:25-36, 2010.
2. Rutten MJ, Janes MA, Chang I, Gregory C, Gregory K. Development of a Functional Schwann Cell Phenotype from Autologous Po rcine Bone Marrow Mononuclear Cells for Nerve Repair. *Stem Cells International*, (2012; *in press*).

Other Publications

1. Wang, Lian, et al: Polychrom atic flow cytometry analysis of bone m arrow cells response to compartment syndrome injury in swine model; 2010, Manuscript in preparation.

Proposals

1. BISC Grant Proposal "Accelerated and Improved Healing of Severe Muscle Inju ries Using the Angiotensin Receptor Blocker Losartan"

Grants

1. W81XWH-09-1-0688 Tissue Replacement and Regeneration for Battlefield Injuries

Project EI-6: Biodegradable elastomeric scaffolds microintegrated with muscle-derived stem cells for fascial reconstruction following fasciotomy

Team Leader(s)	William R. Wagner McGowan Institute for Regenerative Medicine, Dept. of Surgery,450 Technology Dr., Suite 300University of Pittsburgh, Pittsburgh, PA, 15219
Project Team Members	Keisuke Takanari MD, PhD, Ryotaro Hashizume MD, PhD, Yi Hong PhD, Nicholas J. Amoroso BSE, Tomo Yoshizumi MD, Hongbin Jian MD, Antonio D'Amore PhD, Christopher L. Dearth PhD (University of Pittsburgh)
Collaborator(s)	Stephen F. Badylak, DVM, MD, PhD, Johnny Huard, PhD (University of Pittsburgh)
Therapy	Treatment of abdominal compartment syndrome; development of fascial repair technology
Deliverable(s)	*Baseline:* Biodegradable elastomeric scaffolds for fascial reconstruction *Revised:* None
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #3 End Year 1: TRL #1 End Year 4: TRL #3 End Year 2: TRL #2-3
Key Accomplishments:	The in vivo evaluation of sandwich biohybrid scaffolds comprised of dermal extracellular matrix (dECM) and poly(ester urethane)urea (PEUU) was completed. Applying a new "sandwich" fabrication technique provided mechanical properties which mimicked native abdominal muscle tissue, and resulted in a higher deposition of collagen and a better remodeling response compared to the control biohybrid construct. In addition, an assessment of a tissue construct comprised of green fluorescent protein (GFP) transgenic muscle-derived stem cells (MDSCs) and PEUU using a microintegration technique has proceeded. The presence of GFP positive cells was confirmed at both 4 and 8 wks after implantation. MDSC integration into an elastic scaffold facilitated improved microvascular regeneration relative to controls in this animal model of abdominal wall repair. Bi-axial mechanical properties of explanted tissue constructs were similar to native tissue
Keywords	abdominal compartment syndrome, abdominal defect, biodegradable elastomer, extracellular matrix, muscle derived stem cells, mechanical property

Introduction

A severe abdominal injury often requires decompressive laparotomy to avoid pathological levels of intra-abdominal pressure and subsequent abdominal compartment syndrome. The large

abdominal wall defect created due to these lifesaving laparotomies remains a challenging problem for surgeons[1]. Common techniques used to reconstruct full thickness abdominal wall defects utilize synthetic meshes[2-6]. The disadvantages for using prosthetic materials are the risks of intestinal fistula formation, prosthetic infection, adhesions and recurrent hernias which are related to the foreign body response and mechanical property mismatch[7].

A biohybrid composite material that offers both strength and bioactivity for optimal healing towards native tissue behavior may overcome these disadvantages and will be applicable in a variety of fascial tissue repair and replacement procedures. Dermal extracellular matrix (dECM) gel possesses attractive biocompatibility and bioactivity with weak mechanical properties and rapid degradation[8-17], while conventionally electrospun biodegradable, elastomeric poly(ester urethane)urea (PEUU) has strong mechanical properties with limited cellular infiltration and tissue integration[18-24]. The Wagner group hypothesized that these two different materials could be combined in a manner that would possess both advantages. In the past year, the Wagner group completed the evaluation of a "sandwich" design for a biohybrid scaffold in a rat full thickness abdominal wall defect model.

Research Progress

a. PEUU and dECM processed by "sandwich" technique

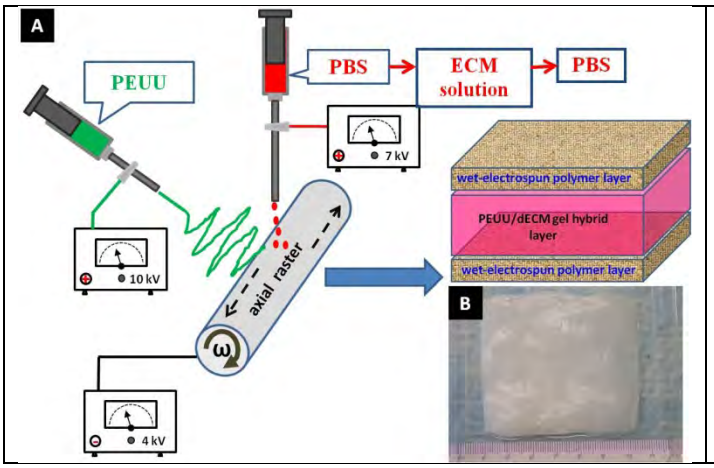


Figure 1. Schematic of the "sandwich" fabrication technique. The green stream represents polymer (PEUU) electrospinning and the red stream is electrosprayed aqueous solutions of: saline, followed by ECM gel, and then saline. The saline spraying results in formation of the denser upper and lower layers of the sandwich scaffold.

The outer layers of the scaffold sandwich were generated by electrospraying saline concurrently with PEUU electrospinning at the beginning and end of the processing period. For the PEUU fiber/dECM gel hybrid layer, porcine dECM gel solution was electrosprayed instead of saline while PEUU was electrospun (**Figure 1**). 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 down at the later time point (**Figure 2**).

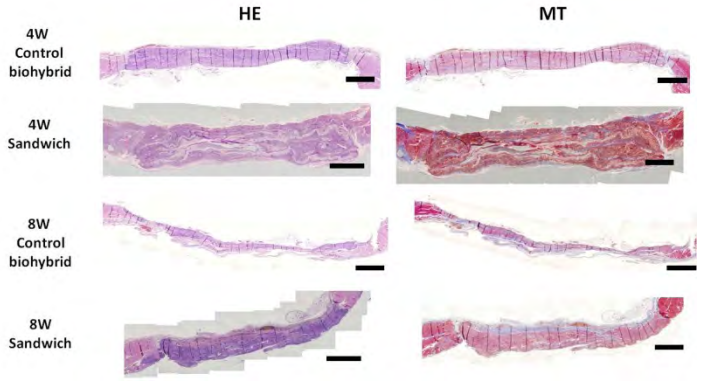


Figure 2. Representative cross-sections of implanted dECM /PEUU (control) and dECM/PEUU (sandwich process) staining with hematoxylin and eo sin, and Masson's trichrome (Scale bar = 1 mm).

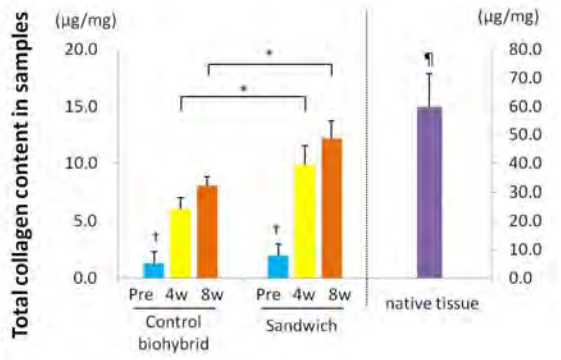
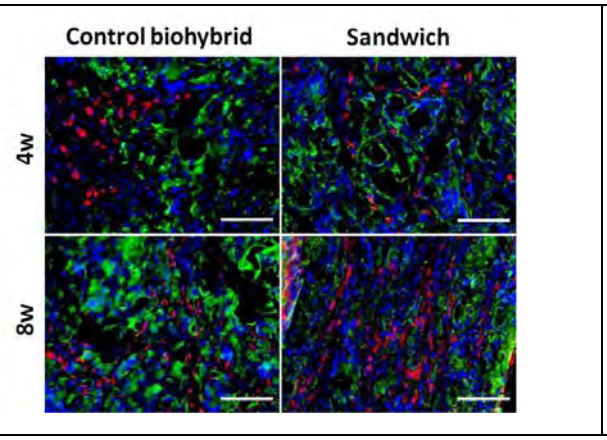


Figure 3. Collagen content in samples. *p< 0.05. † p< 0.01 compared to 4 and 8 wk samples. ¶ p<0.05 compared to 4 and 8 wk s amples in experimental groups and p<0.01 compared to preimplant samples.

Collagen assays showed a marked increase in total collagen content for both patch types from the time of i mplant to 4 and 8 wk (p<0.01, **Figure 3**). The amount of collagen was higher in the sandwich versus control scaffold for both time points (p<0.05). Assessment of scaffold site remodeling with imm unostaining for m acrophage phenotype showed that the ratio of CD163 (M2 macrophages) to CCR7 (M1 macrophages) in creased in the sandw ich group fro m 4 to 8 weeks, and at 8 weeks was greater than that observed in control biohybrid. (**Figure 4**).



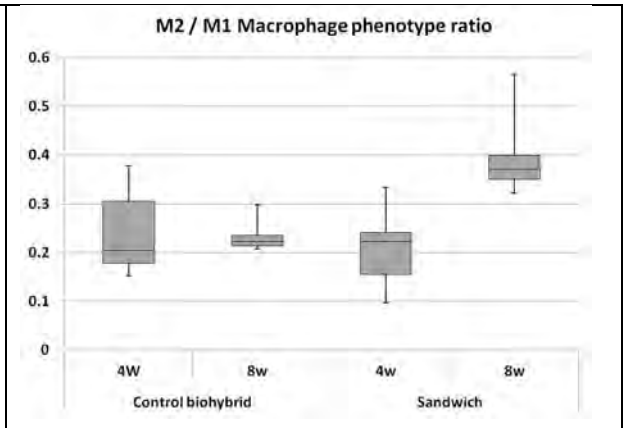


Figure 4. Immunostaining against CD163 (M2 macrophages, red) and CCR7 (M1 macrophages, green). Nuclei are stained with Hoechst (blue). Scale bar = 200 µm. Graph shows the ratio of M2 vs M1 macrophages.

MDSC integrated wet electrospun PEUU

The Wagner group is currently investigating concurrent electrospinning/electrospraying to microintegrate GFP transgenic MDSCs into the scaffolds. Allogenic transplantation was performed with this MDSC-integrated material (**Figure 5**). Control scaffolds were created utilizing the same method without cells. After 4 and 8 wk, GFP positive cells were seen in the scaffold (5.9 and 5.1% of cells respectively). Representative cross sections showed high cell infiltration in both groups (**Figure 6A**) and the thickness of the abdominal wall markedly increased in both scaffolds (**Figure 6B**), whereas the cell integrated scaffold had higher vascularity at both time points compared to the control scaffold (**Figure 6C**).

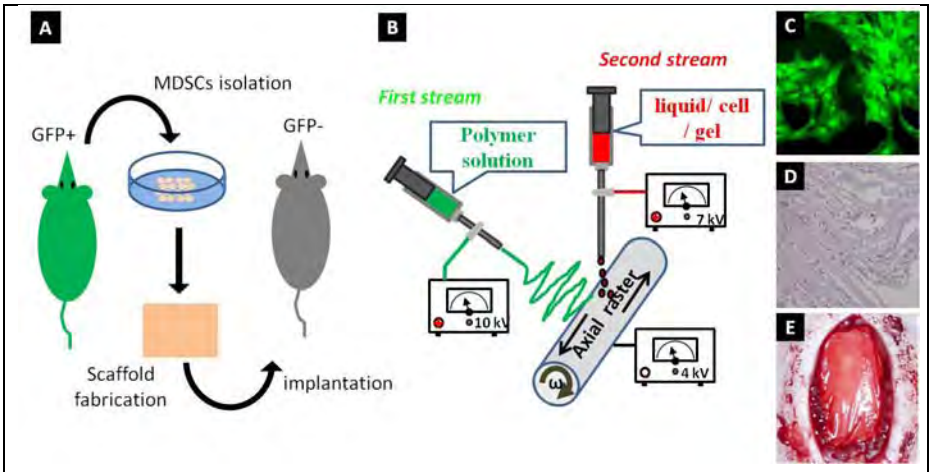


Figure 5. A: Schematic of the study design. GFP positive MDSCs were harvested, seeded in the scaffold and implanted into an allogenic rat of the same strain. **B:** scaffold fabrication method **C:** MDSCs after 1 day culture. **D:** H & E staining image of the MDSC microintegrated scaffold. **E:** Macroscopic image of the scaffold just after implantation.

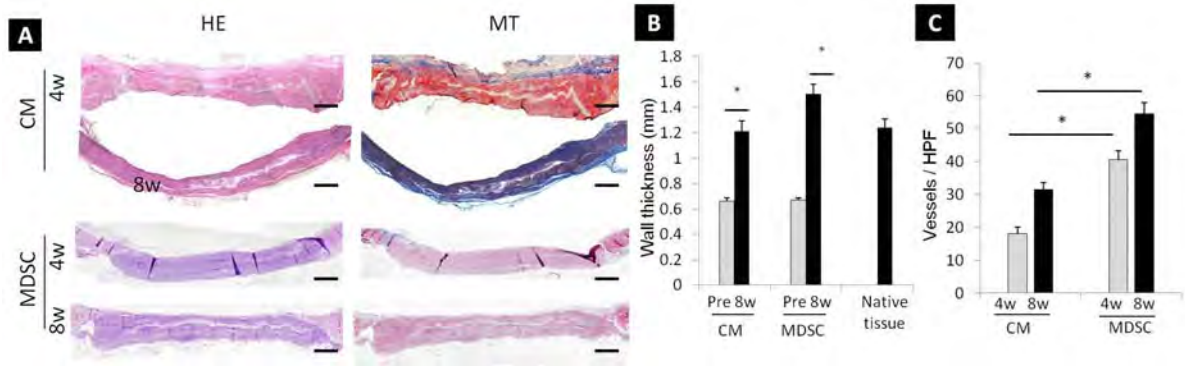


Figure 6. A: Representative cross-sections of implanted cell-integrated scaffolds at 4 and 8 wk after implantation. Scale bar = 1 mm. **B:** Wall thickness of preimplant sample, 8 wk after implantation, and native tissue. Both scaffolds showed increased thickness after 8 wk implantation which was equivalent to the native tissue. **C:** Vessel number in the explanted scaffold expressed as # per high power field (hpf). MDSC integrated scaffolds had signifi- cantly greater vessel numbers compared to scaffolds utilizing culture medium alone. * $p<0.05$

Key Research Accomplishments

- After seeing encouraging preliminary results last year, this year the sandwich scaffold technique was examined extensively. The benefit of this approach was demonstrated in terms of maintaining the scaffold thickness, collagen elaboration, biaxial mechanical mimicry of native tissue behavior, the infiltrating macrophage phenotype response, and collagen orientation.
- Implanting tissue constructs with integrated GFP muscle derived stem cells showed cell survival at 8 weeks and a significant benefit of cell presence in terms of improved vascularity. Biaxial mechanical behavior was also closer to native tissue with these cells.

Conclusions

The sandwich scaffold approach overcomes previous limitations encountered with biohybrid elastomeric scaffolds, maintaining wall thickness and exhibiting higher levels of collagen production with structural and mechanical properties similar to native tissue. This technique is ready to progress to the porcine model. The results with the MDSC microintegrated scaffolds are progressing well, with further analysis ongoing. Cell survival at 8 wk has been demonstrated along with improved vascularity.

Research Plans for the Following Years

In the coming year, the Wagner group will finish its study of MDSC microintegrated scaffolds in the rat model and potentially combine this approach with the dECM sandwich scaffold approach. Porcine trials will begin now that final animal approvals have been obtained. The developed materials will be considered for other applications. Potential applications include skin, craniofacial, and soft tissue reconstruction. When the large animal model is completed, it is anticipated that this technology may potentially be evaluated in concert with other approaches developed in the AFIRM projects.

Planned Clinical Transitions

The Wagner group is close to an agreement with a major manufacturer of abdominal wall and pelvic floor repair materials to license some of this technology. Upon successful completion of the first set of porcine experiments, the FDA will be engaged in discussions to determine the preclinical data that would be required to justify filing for an investigational device exemption. This work would occur in Years 6 and 7. Once this milestone is met, clinical trials can commence, potentially in Year 8.

Corrections/changes planned for next year and rationale for changes

No substantial changes are anticipated for next year.

Conflict of Interest Disclosure:

The investigators have no conflicts of interest.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty	2	0
# Post docs	0	0
# grad students	0	1
# undergrad students	0	0
# staff members	1	0

Other Project Statistics	
# Honors given to AFIRM faculty	6
# Doctorates awarded	0
# Masters degrees awarded	0
# Inventions disclosed	1
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	2

*Directly supported in whole or part –time by AFIRM

Honors and awards:

2011 Keynote Presentation at the 27th Southern Biomedical Engineering Conference

2011 Plenary Presentation at Opening Session, 38th Congress of the European Society for Artificial Organs and 4th Bie nnial Congress of the Internat ional Federation on Artificial Organs

2011 Clemson Award for Applied Research, Society for Biomaterials

2011 Keynote Presentation at th e Materials and Processes fo r Medical Devices (MPMD) Conference (ASM International)

2012 Tissue Engineering and Regenerative Medici ne International Soci ety (TERMIS) Chair-Elect of the Americans Region

2012 Chancellor's Distinguished Research Award, University of Pittsburgh

Inventions disclosed

U.S./International Patent Application (#PCT/US2011/048071) "Biohybrid elastomeric scaffold", through the University of Pittsburgh, filed 8/2011.

Publications:

1. Hong Y, Takanari K, Amoroso NJ, Hashizume R, Brennan-Pierce EP, Freund JM, Badylak SF, Wagner WR. An elastomeric patch electrospun from a blended solution of dermal extracellular matrix and biodegradable polyurethane for rat abdominal wall repair. Tissue Eng Part C Methods. 18(2):122-32. (2012)

Abstracts (non-Peer reviewed publications):

1. Takanari K, Hashizume R, Hong Y, Huber A, Amoroso NJ, D'Amore A, Badylak SF, Wagner WR. Creating a biohybrid material for fascial repair by regionally-controlled processing of an extracellular matrix digest with a biodegradable elastomer. Tissue Engineering and Regenerative Medicine International Society North America Meeting (2011)
2. Takanari K, Hashizume R, Hong Y, Amoroso NJ, Jian H, Gharaibeh B, Huard J, Wagner WR. Muscle-derived stem cell (MDSC) microintegrated elastic scaffolds for the reconstruction of abdominal wall defects. Armed Forced Institute for Regenerative Medicine (AFIRM) All-Hands Annual Meeting (2012).

Project EI-7: Spatial and Temporal Control of Vascularization and Innervation of Composite Tissue Grafts

Team Leader(s)	Robert Guldberg, PhD (Georgia Institute of Technology)
Project Team Members	Barbara Boyan, PhD (Georgia Institute of Technology), Ravi Bellamkonda, PhD (Georgia Institute of Technology), Robert Taylor, MD, PhD (Emory University), Yash Kolambkar, PhD (Georgia Institute of Technology), Natalia Landazura, PhD (Emory University), Nick Willett, PhD (Georgia Institute of Technology), Brent Uhrig (Georgia Institute of Technology), Isaac Clements (Georgia Institute of Technology), Angela Lin (Georgia Institute of Technology)
Collaborator(s)	Dietmar Hutmacher (Queensland University of Technology), Andres Garcia (Georgia Institute of Technology), David Kaplan (Tufts University), Benjamin Harrison (Wake Forest Institute for Regenerative Medicine), Shawn Gilbert (University of Alabama-Birmingham), Thomas Clemens (Johns Hopkins University), George Muschler (Cleveland Clinic), Josh Wenke (US Army Institute for Surgical Research)
Therapy	Functional limb regeneration following severe combined bone, nerve, and vascular injuries
Deliverable(s)	*Baseline:* Develop composite injury animal models that simulate complex military wounds Establish and test spatiotemporal delivery strategies for regeneration of bone, nerve, and vascularity *Revised:* None
TRL Progress	Begin Year 1: TRL #1 End Year 4: TRL #3 End Year 2: TRL #2 End Year 3: TRL #3
Key Accomplishments:	The Georgia Tech team has successfully established 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 five papers, including one in PNAS, were published in 2011, establishing critical dose response relationships and direct clinical comparisons. Large animal studies were initiated in Year 4 with leveraged funding, which will allow progression to TRL #4. The small animal models and imaging methods at Georgia Tech are also being used to assess strategies developed at other AFIRM laboratories.
Keywords	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 in the rat with peripheral nerve resection and/or femoral artery ligation. Importantly, quantitative evaluation methods such as 3D micro-CT imaging, electrophysiology, biomechanics, and gait analysis have been integrated into these models to provide comparison of competing regenerative strategies. The models are being used to quantitatively evaluate spatial and temporal delivery of biological cues that direct nerve, vascular, and bone growth in a synchronized manner. In addition to regenerative technologies developed at Georgia Tech, these quantitative evaluation models are being used to test technologies developed in several other AFIRM laboratories.

The specific aims of this project are:

I. To develop composite injury rodent models of severe limb trauma

II. To quantitatively evaluate strategies for delivering spatial and temporal information to direct segmental bone regeneration, peripheral nerve repair and vascular regrowth

Research Progress

Over the past year, Dr. Guldberg and his colleagues have made significant progress on the composite models of limb injury and hybrid construct bone repair technology. A series of five papers were published in 2011 on the development of the hybrid nanofiber mesh/hydrogel construct to restore function to large segmental bone defects (1-5). These studies have defined in vitro and in vivo release kinetics, dose responses to determine the minimum effective dose, degradation characteristics, effectiveness relative to the current clinical standard, and ability to overcome concomitant soft tissue injury and mechanical instability. Over the past year, we have completed additional studies on the effects of varying the hydrogel composition, volume, and degradation characteristics. We previously demonstrated that macro perforations around the periphery of the nanofiber meshes accelerated bone regeneration. Over the past year, we followed up on this by investigating whether the size of the macro-perforations affects vascular ingrowth and bone formation. We found that all three sizes tested accelerated bone healing equally well. In March 2012, Dr. Guldberg travelled to Australia to initiate large animal (sheep) studies. As part of this, Pfizer provided a gift of recombinant BMP-2 and FMC Biopolymer manufactured the alginate hydrogel. Prior to starting the large animal study, we performed rat studies to verify that the commercially produced hydrogel was functionally equivalent to the

laboratory produced hydrogel. The results verified that the FMC hydrogel performed equally well. Finally, we performed a pilot study to assess the potency of the Pfizer recombinant BMP-2. Comparisons were made in the rat segmental defect model and using an in vitro alkaline phosphatase assay (Figure 1). Pfizer uses a different reconstitution buffer than R&D so we tested that as well. The results demonstrated a significant effect of reconstitution buffer and a small but significant reduction in Pfizer BMP potency. Based on these results we decided to use the HCl reconstitution buffer and a 25% increase in Pfizer BMP-2 dose for the large animal studies.

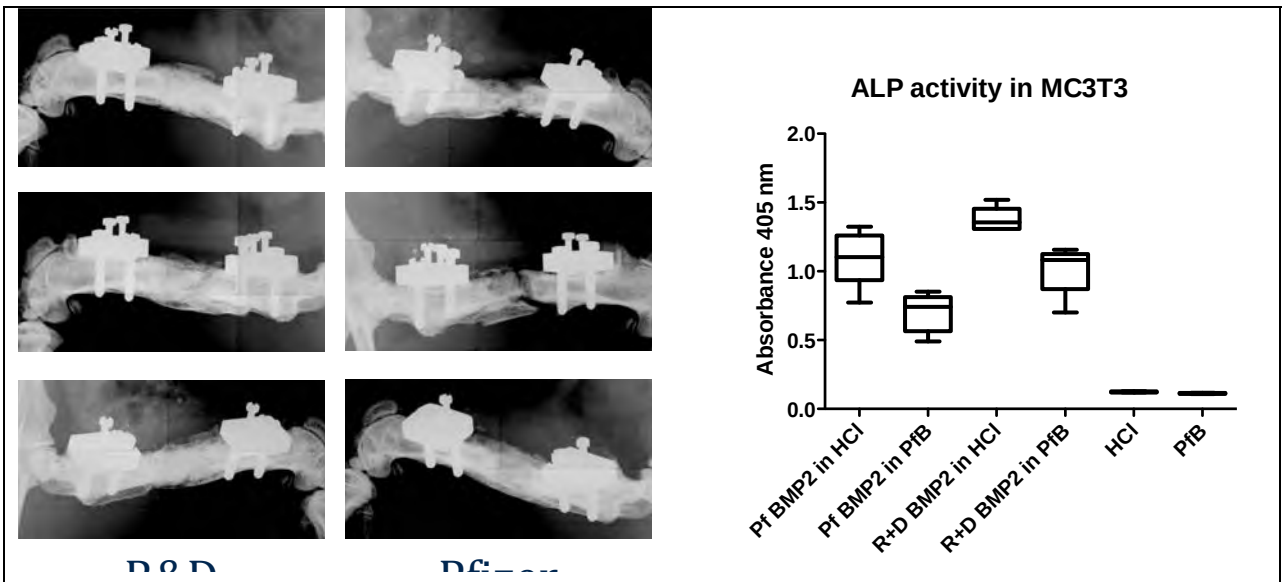

Figure 1. In vivo comparison of recombinant human BMP-2 source delivered using the hybrid nanofiber mesh/alginate BMP delivery system (left). Alkaline phosphatase in vitro assay showing supporting results.

Three composite injury models have now been fully established: bone defect/ischemia, bone defect/nerve defect, and bone defect/muscle defect. The bone/vascular composite injury studies revealed an unexpected outcome in that transient limb ischemia was found to accelerate BMP-2 mediated large bone defect regeneration. We therefore repeated this study over the past 12 months and verified this exciting and interesting result. We also initiated shorter-term endpoint studies to assess the mechanism of this stimulatory effect. Updates on the other two composite models were provided in the previous report. The bone/peripheral nerve composite injury study has now been accepted for publication, while two manuscripts on bone/muscle composite injury and muscle graft treatment of volumetric muscle defects are in preparation.

Key Research Accomplishments

- Composite bone/nerve injury model has been established and study completed showing increased functional deficit associated with the composite injury. Paper accepted.
- Composite bone/vascular injury model has been established and study completed showing that transient ischemia promotes BMP-mediated repair of bone defects. As part of this work,

a method was developed to assess the spatial distribution of vascular ingrowth into muscle and bone and used to demonstrate a gradient reduction in vascular ingrowth from the proximal to the distal end of limb injuries. Study repeated this year.

- Composite bone/muscle injury model has been established and study completed with leveraged funds showing that massive muscle loss significantly impairs BMP-mediated bone repair. Two manuscripts in preparation.
- Contact nerve guidance scaffold has been developed and patent filed.
- Nanofiber mesh/hydrogel spatiotemporal growth factor delivery system has been developed and patent filed.
- Partner and leveraged funds for large animal study have been procured, commercial sources for BMP-2 protein (Pfizer) and alginate hydrogel (FMC Biopolymer) have been validated, large animal study has been initiated
- Collaborative study published with David Kaplan (Tufts University) to test his silk-based hydrogels for delivery of BMP in the standardized 8 mm bone defect model

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-CT assessment of vascularization and bone formation). Variations of the composite injury model include bone/nerve injuries, bone/vascular injuries, and bone/muscle injuries. These models are available for testing regenerative strategies developed by other AFIRM investigators. At Georgia Tech, we have established promising patent-pending regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of clinically approved inductive protein (BMP-2).

Research Plans for the Following Years

We will complete the large animal study at QUT to test the nanofiber mesh/hydrogel BMP delivery technology. Completion of this study will allow transition to TRL #4. Once the patent has issued and the large animal study is completed, companies will be approached to license the technology. Companies with potential interest in this technology include: Pfizer, Medtronic-Sofamor Danek, MiMedx, Zimmer, Smith & Nephew, Stryker, and Synthes. We will also complete ongoing optimization studies of hydrogel composition and degradation characteristics. Having completed development of an improved approach to treating acute segmental bone injuries, we will now turn our attention to overcoming the challenges of chronic non-union with and without adjacent concomitant soft tissue injury or mechanical instability.

Planned Clinical Transitions

Additional funds have been identified for sheep defect studies from the Australian Research Council. Contract details for this study betwee n Georgia Tech and Queensland University of Technology have been com pleted. No AFIRM funds will be used but in kind support of tim e will be prov ided. A Mater ials Transfer Agreement with Pf izer to pro vide BMP for the la rge animal study has been com pleted. The addition of the large an imal study is a revision to our original plan and im proves the ch ances of su ccessful transition to a hum an pilot study. Th e intellectual property will be m arketed to m embers of the Georgia Tech industry partners program. We have previously had success licensi ng patent rights to indus try partners following successful large anim al studies. Once proof of concept has been demonstrated in the large animal model, our goal is to initiate a human clinical trial pilot study.

Corrections/Changes Planned

Large animal studies have been added to the project plan to accelerate prog ression towards clinical translation of the spatiotemporal delivery systems.

Conflict of Interest Disclosure

Dr. Guldberg is an inventor on the biohybrid c onstruct patent application, has consulted for Pfizer who is providing BMP for t he large anim al evaluation of the biohybrid construct. Dr. Bellamkonda is an inventor on the oriented nanofiber mesh patent.

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2. Boerckel JD, Kola mbkar YM, Stevens HY, Lin AS, Dupont KM, Guldberg RE 2011 Effects of in vivo mechanical loading on large bone defect regeneration. J Orthop Res.
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5. Kolambkar YM, Boe rckel JD, Dupont KM, Bajin M, Huebsch N, Mooney DJ, Hutmacher DW, Guldberg RE 2011 Spatiote mporal delivery of bone morphogenetic protein enhances functional repair of segmental bone defects. Bone **49**(3)**:**485-92.

Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	1	3
# Post docs	0	2
# grad students	2	0
# undergrad students	0	2
# staff members working for AFIRM	1	0

Other Project Statistics	
# Honors given to AFIRM faculty	5
# Doctorates awarded under AFIRM support	2
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	2
# Patents awarded	0
# Peer reviewed publications	18
# Non-peer reviewed publications	28

*Directly supported in whole or part –time by AFIRM

Honors and Awards

1. Robert Guldberg was named the Parker H. Petit Director's Chair for Engineering and Medicine.
2. Barbara Boyan received the ASTM Manny Horowitz Award for contributions to standards development on the Committee on Medical and Surgical Materials and Devices
3. Barbara Boyan was elected to National Academy of Engineering
4. Ravi Bellamkonda was named the Carol Ann & David D. Flanagan Professor of Biomedical engineering
5. Robert Guldberg received the 2011 Sigma Xi Best Paper Award for work funded by AFIRM

Patents and Inventions

1. U.S. Patent Application (Pending) –: Systems and Methods to Affect Anatomical Structures. Yash Kolambkar and Robert E. Guldberg

2. U.S. Patent Application (Pending) –: Orie nted Nanoscaffolds for Tissue Repair. Isaac Clements and Ravi Bellamkonda

Peer-Reviewed Publications

1. Kolambkar, Y., Dupont, K.M., Huebsch, N., Mooney, D.J, Hutmacher, D.W., Guldberg, R.E., "An Alginate Based Hybrid System for Growth Factor Delivery in the Functional Repair of Large Bone Defects," Biomaterials, 32(1):65-74, 2011.
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2. Allen, A., Matheny, R.G., Gazit, D., Gazit, Z., Su, S., Stevens, H.Y., Guldberg, R.E., "Synthetic vs. Natural Membranes for Cell- Based Bone Tissue Engineering," The 57th Annual Meeting of the Orthopaedic Research Society, Long Beach, California, January, 2011.
3. Boerckel, J.D., Lin, A., Kolambkar, Y.M., Dupont, K.M, Guldberg, R.E., "Non-Destructive Finite Element Analysis Prediction of Biomechanical Properties in Mechanically Stimulated

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8. Willett, N., Li, M.T.A., Uhrig, B.A., Warren, G., Guldberg, R.E., "Functional and Structural Analysis of Limb Restoration Using a Novel Rat Model of Composite Bone and Muscle Injury," Armed Forces Institute for Regenerative Medicine(AFIRM) Annual Conference, January 19, 2011, Tampa, Florida.
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Grants

1. ARRA - Engineered Delivery of Adult vs. Feta l Stem Cells for Bone Regeneration (R01-AR056694) (PI: R. Guldberg)
2. Engraftment Strategies for F unctional Repair of Com posite Musculoskeletal (Bone and Muscle) Injuries (Dept. of the US Army) (PI: R. Guldberg)
3. Center for Advanced Bioengine ering and Soldier Survivability (Director B. Boyan, Assoc. Director R. Guldberg)
4. Australian Research Council Award (PI: D. Hutmacher, Investigator: R. Guldberg)

Project EI-8: Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome

Team Leader(s)	Stephen F. Badylak, DVM, PhD, MD (McGowan Institute for Regenerative Medicine, University of Pittsburgh)
Project Team Members	Christopher L. Dearth, PhD (McGowan Institute for Regenerative Medicine, University of Pittsburgh) Matthew T. Wolf, BS (Department of Bioengineering, University of Pittsburgh) Scott Johnson, MS (McGowan Institute for Regenerative Medicine, University of Pittsburgh)
Collaborator(s)	Dr. Johnny Huard, PhD (McGowan Institute for Regenerative Medicine, University of Pittsburgh), Dr. Kenton Gregory, MD (Oregon Medical Laser Center, Oregon Center for Regenerative Medicine)
Therapy Deliverable(s)	Treatment for peripheral compartment syndrome (PCS) Baseline: Reconstruction of functional compartmental tissue in animal models utilizing the inductive properties of biologic scaffolds and stem cells. Revised: None
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #5 End Year 1: TRL #2 End Year 4: TRL #5 End Year 2: TRL #3
Key Accomplishments:	Completion of surgical induction and treatment surgeries of PCS in the rabbit model. Analysis of the 3 and 6 month time points. Autologous compartment ECM elicits improved remodeling outcomes compared to control. Successful optimization of strategies to translate to a porcine model of PCS.
Keywords	Peripheral compartment syndrome, extracellular matrix, bone marrow derived mononuclear cells

Introduction

Peripheral compartment syndrome (PCS) represents a serious complication of traumatic extremity injury; especially the type of trauma sustained by soldiers in combat. Severe swelling within a confined space (compartment) is associated with increased intracompartmental pressure, severely compromising blood flow resulting in ischemia, and necrosis of all tissues within the compartment (e.g., muscle, nerves, and associated structures). The loss of functional tissue is frequently severe enough to require amputation of the affected limb. The standard of care for peripheral compartment syndrome is fasciotomy with an attempt to salvage the viability of as much functional tissue as possible, though morbidity of this approach is high. The Badylak Lab's work investigates a method for utilizing the inductive properties of extracellular matrix (ECM) as a scaffold for constructive remodeling into functional tissue. Previous work has shown that manufactured forms of extracellular matrix (e.g., porcine small intestinal submucosa, porcine urinary bladder, porcine and bovine dermis, pericardium, among others) have the potential to promote constructive remodeling of damaged or missing body parts in place of inflammation and scarring [1-5]. The present work extends this concept by investigating methods of deriving

autologous compartment ECM from in-situ decellularization of the necrotic tissue (with the presence of stem cells). Stated differently, the extracellular matrix within the compartment would be isolated from its original cell population (which has now become necrotic) and this matrix would then be used as a template for tissue reconstruction.

In the previous years, a reproducible model of peripheral CS was established in 2 species (rabbit and canine) [1]. This CS induction model has now become the basis for current preclinical animal studies. Previous work also included optimization of compartment tissue decellularization and characterization of its properties considered to be important for biocompatibility *in vitro*. In addition, preclinical studies of the suitability of currently available biologic scaffolds and stem cells for treatment in our animal models were initiated in the previous years.

Research Progress

The primary accomplishments include: 1) Completion of surgical induction and treatment of PCS using autologous compartment ECM. 2) The long term outcome (3 and 6 months) after PCS treatment in a rabbit model using autologous compartment ECM derived *in situ* and autologous bone marrow derived mononuclear cells (BM-MCs) was analyzed. 3) Optimization of methods to induce compartment syndrome in a porcine model.

Reconstruction of functional compartmental tissue in animal models utilizing the inductive properties of biologic scaffolds and bone marrow derived mononuclear cells

In the previous years, decellularization of compartment ECM was optimized using saline (S), 0.1% peracetic acid (PAA), and 2% deoxycholate (DOC). The DOC treatments optimally resulted in a reduction in DNA and cellular protein, but retention of growth factors. The methods for creation of autologous compartment ECM were used for treatment of PCS in a previously validated rabbit model [1]. In addition, autologous BM-MCs were delivered to the affected compartment after decellularization of the compartment and isolation in-situ of autologous ECM. This approach overcomes many of the current concerns about ECM in that both the cells and ECM are autologous, thus avoiding concerns about xenogeneic sources.

PCS induction and autologous ECM treatment were all completed within the past year (**Figure 1A**). It was established that compartment syndrome pressures [1] above 150 mmHg were maintained for all treatment groups, which was greater than the initial pressure prior to induction (**Figure 1B**). There were also no significant differences in the induction procedure between groups.

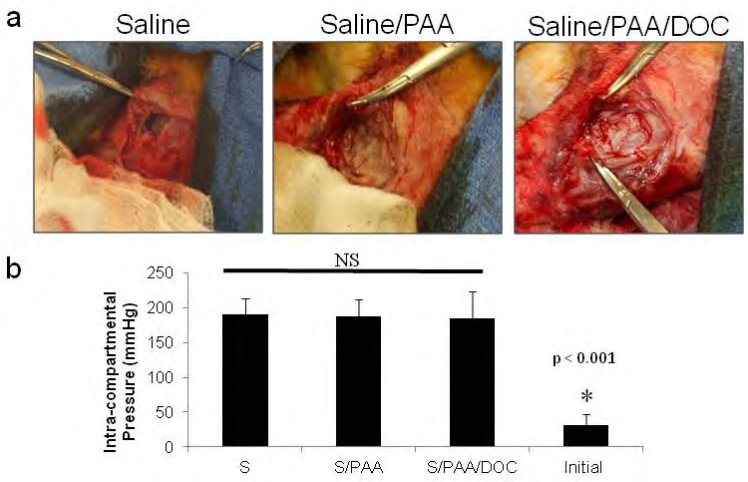

Figure 1. A – Macroscopic images showing the creation of autologous compartment ECM by flushing of the compartment with saline, 0.1% PAA and 2% DOC. B- Intracompartmental pressure measurements for each of the methods showing that all were inductions were similar and significantly higher than prior to infusion.

At 3 months after treatment, fibrous tissue and new muscle cells are found in the defect area in groups treated with saline/PAA and saline/PAA/DOC , while only dense fibrous tissue is present in the saline control group (**Figure 2a**). There were greater am ounts of m yogenesis and less dense fibrous tissue in the saline/P AA/DOC group than in the saline/P AA. Therefore, only the saline control and saline/PAA/DOC groups were c ontinued for the 6 month tim e point. After 6 months, there was adipose tissue and some connective tissue in both groups. However, there were more numerous and larger islands of skeletal muscle present in the saline/PAA/DOC group.

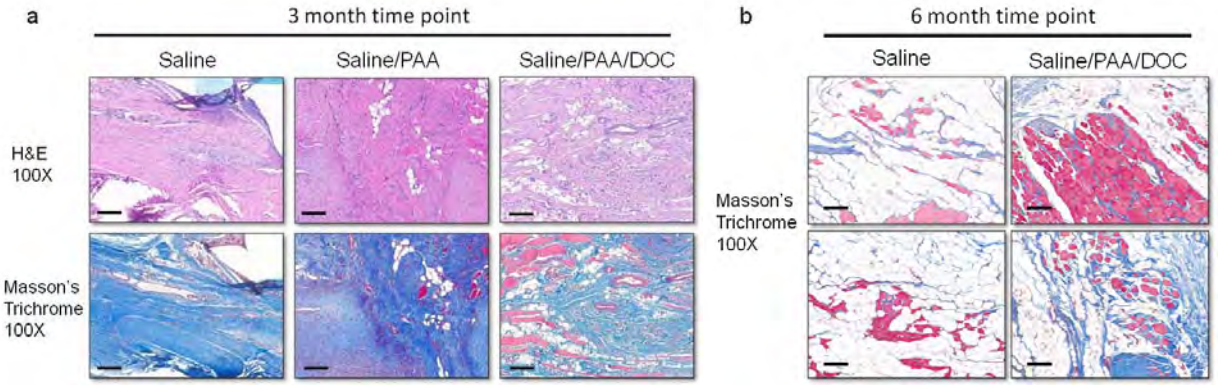

Figure 2. A –After 3 months, the saline only control showed only fibrous tissue while the saline/PAA and saline/PAA/DOC groups showed early evidence of myogenesis. B – After 6 months, there was ad ipose tissue, fibrous tissue, and muscle tissue found in the defect saline/PAA/DOC groups, but less myogenesis in the saline control.

To determine if the potential ro le of the autologous BM-MCs in the myogenesis seen in the saline/PAA/DOC group, BM-MCs were labeled with Qdots (Qtracker-655, Invitrogen). One month following their im plantation in saline/ PAA/DOC treated anim als, (Qdot-labeled) numerous Qdot labeled cells were found within the remodeling tissue (**Figure 3a, arrows**). There were few cells labeled cells after 3 months, but they were present in remodeling tissue and

around muscle fiber bundles (**Figure 3b, arrows**). The survival and integration of Qdot labeled BM-MCs is currently being assessed for the 6 month time point.

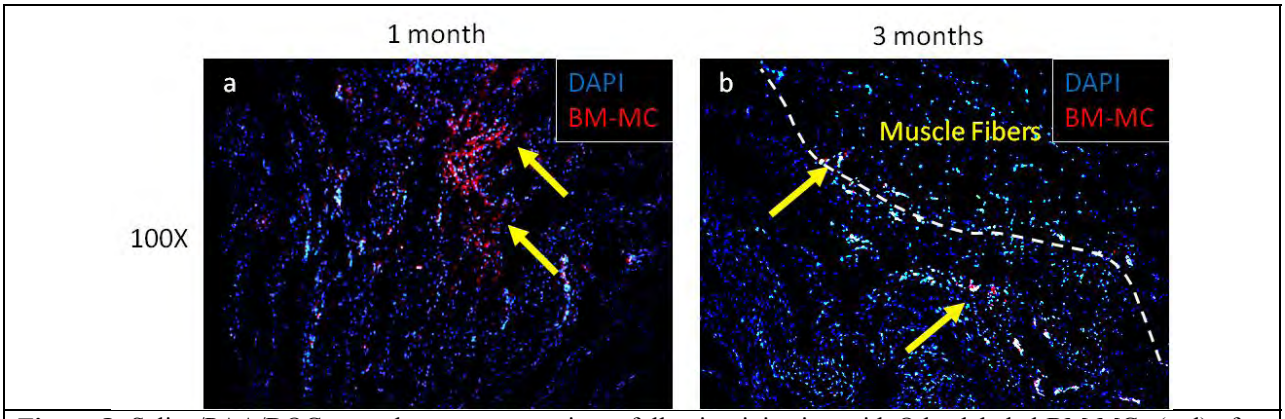

Figure 3. Saline/PAA/DOC treated compartment tissue following injection with Qdot labeled BM-MCs (red) after (A) – one month, where numerous labeled cells are present in the remodeling tissue and (B) – three months post treatment, where there are fewer cells. Sections counterstained with DAPI (blue).

Optimization of PCS induction in a porcine model

PCS induction methods were translated and optimized for use in a porcine model. Cadaveric pigs were exposed to PCS conditions by exposing the anterior tibial compartment, insertion of a saline infusion needle, insertion of a pressure measurement needle, and tying a tourniquet around the proximal portion of the leg (**Figure 4a**). Pressure was initiated by injecting a saline bolus, maintained on a pressure bag drip. Elevated intracompartment pressures exceeding 300 mmHg were maintained for an extended period of time.

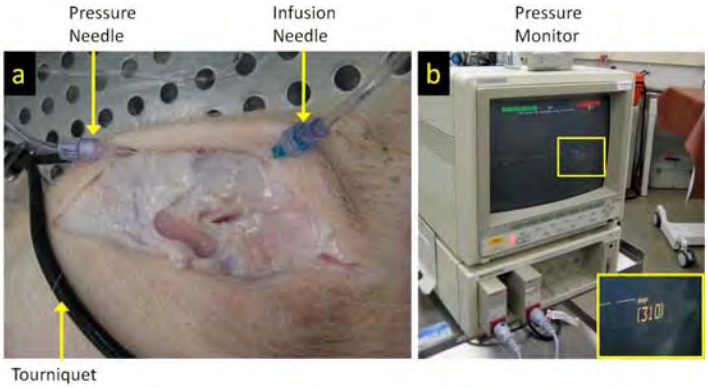

Figure 4. A – Optimization of PCS induction in a cadaveric pig. A tourniquet was tied around the leg and a saline infusion needle and pressure monitor needle inserted into the compartment. B – Pressure was maintained above 300mmHg (inset) for extended periods of time using this configuration.

Key Research Accomplishments

- Induction and treatment surgeries completed for all rabbits.
- Evaluated host response to *in situ* autologous compartment ECM at 3 and 6 month time points.

- Demonstrated clear differences in the host regenerative response after 3 and 6 months of treatment with *in situ* autologous compartment ECM used in conjunction with bone marrow derived mononuclear cells.
- Showed survival of Qdot labeled cells 1 and 3 months post injection.
- Optimized methods inducing PCS in a porcine model.

Conclusions

Research Plans for the Following Years

The research plan for the last 12 months of this 5 year project includes final evaluation of the final 6 month time point in the rabbit, which are due to be collected within the next 4 months. Samples will be collected for assessment via histomorphometric staining and quantification, and immunolabeling for indicators of myogenesis, innervation, and vascularization in the remodeling tissues. Qdot technology has been used to show that injected BM-MCs with autologous extracellular matrix do indeed remain and potentially participate in the constructive remodeling response. The fate of Qdot labeled cells will be determined via immunofluorescent colocalization with skeletal muscle, vasculature, and putative stem cell markers. Concurrent with the final analysis of the rabbit study, PCS will be induced in the porcine model and treated with the saline/PAA/DOC method with autologous BM-MCs and compared to a saline control. The study will utilize the surgical and logistical methods optimized in Year 4.

Planned Clinical Transitions

There are limited options for the treatment of advanced PCS. In most cases, complete loss of functional compartmental musculature results in the need for prosthetic devices. Alternative approaches to reconstructing individual components of the compartment such as muscle, blood vessels, and nerves are in development, but to current knowledge, there is no work currently being done to reconstruct the complex architecture of the innervated, vascularized skeletal muscle architecture of the compartment. Many approaches are investigating the use of stem cells, deposited at the site of damaged tissue, for their ability to reconstitute functional tissue, but this approach would combine such cells with the autologous ECM bioscaffold to optimize the remodeling outcome. Pending similar positive results in the large animal (porcine) preclinical studies, a human clinical trial is currently in the planning stage. Part of this planning involves discussion with our collaborators, Drs. Gregory, Huard, and Guldberg. It is likely that the therapeutic trial will involve the optimal methods derived from each of our projects.

Corrections/Changes Planned

N/A

Conflict of Interest Disclosures

None

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty	2	0
# Post docs	1	0
# grad students	2	0
# undergrad students	1	0
# staff members	1	1

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded	0
# Masters degrees awarded	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Peer reviewed publications

Daly, K.A., et al., *A Rabbit Model of Peripheral Compartment Syndrome with Associated Rhabdomyolysis and a Regenerative Medicine Approach for Treatment.* Tissue Eng Part C Methods, June 2011. **17**(6): p. 631-640.

Project EI-9: Peripheral Nerve Repair for Limb and Digit Salvage

Team Leader(s)	Kacey Marra,PhD, University of Pittsburgh; David Kaplan,PhD, Tufts University; Tom Smith, PhD, Wake Forest University
Project Team Members	YenChih Lin, PhD (University of Pittsburgh),Mostafa Ramadan, MD (University of Pittsburgh),Lauren Kokai, PhD (University of Pittsburgh) Amir Mahan Ghaznavi, MD (University of Pittsburgh),Ryan Nolan (University of Pittsburgh),Samantha Beckowski (University of Pittsburgh) Danielle Minteer, BS (University of Pittsburgh),Marie Tupaj, PhD (Tufts University),James White, PhD(Tufts University),Lee Tien, BS (Tufts University),Jonathan Barnwell, MD (Wake Forest University) Zhongyu John Li, MD, PhD (Wake Forest University),Mark Van Dyke, PhD (Wake Forest University),Lauren Pace (Wake Forest University)
Collaborator(s)	Tirrell Laboratory at University of California, Santa Barbara Harrison Laboratory at Wake Forest Institute for Regenerative Medicine University of Virginia Department of Orthopaedic Surgery (Hand Surgery)
Therapy	Combined strategy for regeneration over long (>3 cm in human) peripheral nerve gaps
Deliverable(s)	*Baseline:* Proactive biodegradable nerve guide system for peripheral nerve regeneration. *Revised:*
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #4 End Year 1: TRL #2 End Year 4: TRL #4 End Year 2: TRL #2
Key Accomplishments:	Study of thermal stability and activity of chondroitinase relased from silk for the reduction of scar tissue proteoglycan formation during nerve regeneration. Quantitative characterization of mechanical properties (Young's modulus and ultimate tensile strength) of silk nerve conduits. Preparation of silk microspheres encapsulating growth factors for controlled release from silk nerve conduits. Improved gradient design and delivery of neurotrophic factors. Study polymer-based tubes containing glial cell-line derived neurotrophic factor embeeed in double wall microsphere, will improve the functional recovery of critical peripheral nerve deficits (5 cm) compared to nerve autografts in non-human primate. Completion of a non-human primate study using keratin gel-filled conduits. Submission of request for designation to the Office of Combination products at the US Food and Drug Administration (FDA). Submission of pre-IND data package to FDA and held pre-IND meeting with CDER/FDA to review preclinical data and clinical trial study design
Keywords	limb regeneration, silk fibroin, nerve guides, drug delivery, keratin, functional electrical stimulation

Introduction

Background

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 (PNR) is a critical issue as 2.8% of trauma patients present with this type of injury. Following trauma, incomplete nerve regeneration and permanent demyelination may result leading to lifelong disability. Several regeneration strategies are currently being employed including biophysical guidance, biochemical applications, and protein modification. The specific aims of this project are to create a biodegradable nerve guidance system that delivers nerve growth factors (NGF, GDNF, NT-3) and biophysical guidance to regenerating peripheral nerves (2) move nerve guidance system *in vivo* (3) Utilize expertise from all three laboratories (biomaterial expertise, small and large animal facilities) Tufts University, University of Pittsburgh, Wake Forest University

Previous Year's achievements

This past year, all three labs have made significant progress. The Tufts group continues to use silk biomaterial protocols and integrate regenerative approaches towards a biodegradable nerve guidance system. Regenerative approaches include incorporating biophysical cues (surface patterning, electrophysiology applications) as well as chemical cues (protein coatings, growth factor incorporation) into silk nerve guides. A new strategy of incorporating chondroitinase enzyme into the silk fibroin nerve guides was investigated as a way of degrading proteoglycans of scar tissue known to inhibit axon outgrowth during PNR. Chondroitinase activity studies released from silk were conducted, with the aim of achieving both controlled release and improving the thermal stability of the enzyme. In addition, the mechanical properties of the silk nerve guides were evaluated using an Instron materials testing system, providing further support that the guides are of sufficient strength for *in vivo* application. Silk fibroin conduits containing controlled gradients of growth factors are being developed. The Marra group was approved to utilize a non-human primate animal model for peripheral nerve repair by the IACUC committee at the University of Pittsburgh, and their group has begun negotiations with both a drug company and a biomaterials company in order to manufacture GMP guides for eventual clinical trials. The group has operated on the first two non-human primates (NHP). These two NHP were treated with two different technologies: a) autograft and b) clinical control (decellularized nerve allograft) in a 5 cm median nerve gap. Analysis of recovery is currently ongoing. The Smith lab completed the initial NHP nerve gap repair studies initiated in 2010. Ten female macaq monkeys underwent a median nerve injury with a 1 cm gap. This gap was repaired using a commercially available nerve guide (Neuragen ®) with either a human hair keratin hydrogel matrix filler or a physiological saline filler (the clinical standard). Monkeys were followed for 1 year post-repair and functional and histological assessments of recovery were obtained. Histologic analyses as well as functional analyses determined that recovery from a critical size

nerve injury was accelerated in the keratin hydrogel treatment group. Final histolog ic and statistical analyses are being completed for manuscript submission. These data were submitted to the FDA in a series of filings with the goa l of obtaining approval for an already funde d (CDMRP) clinical study. The pre-IND data packag e was well r eceived and a cle ar path to the full IND applica tion was outlined at a subsequ ent pre-IND meeting with the FDA. Additiona l preclinical testing will be requi red before the IND can be filed, and funding to support these studies has been requested from multiple sources including AFIRM.

Analysis of Competitive Technologies

There remains a clinical need for an off-the-sh elf nerve guide that can bridge large gaps in human nerve defects (e.g., >3 cm). At this tim e, there are no co mpeting technologies formally approved by the FDA to treat nerve gaps > 3 cm. One guide that has been used in long gaps is AxoGen's AVANCE guide, a decellularized nerve allograft. The team is collab orating with AxoGen and combining their technologies for an optimal guide.

Leveraged Funding

AFIRM funding has been leveraged by a National Science Foundation awarded to the team at the University of Pittsburg h. A CDRMP grant has been awarded to the group at Wake Forest. Additional leveraging is in the for m of donated drugs and conduits from MedGene sis (GDNF), AxoGen (AVANCE), and Integra Lifesciences.

Research Progress

- Target Product Profiles
    - We have focused on three products: Silk Conduit; PCL/GDNF Conduit; PCL/Keratin filler Conduit
    - We envision examining each guide in the NHP median nerve defect model.
- The following table indicates the variety of nerve guides/fillers/drugs that have been or are being tested:

Nerve guide material	Filler	Drug (GDNF) delivery	Animal model
PCL (Pitt)	Keratin gel (WFIRM)	PLGA double-walled microspheres (Pitt)	Lewis rat 1.5 cm sciatic nerve defect(Pitt)
PCL (Pitt)	-	PLGA double-walled microspheres (Pitt)	Non-human primate 5.0 cm median nerve defect (Pitt)
Silk (Tufts)	-	Silk single-walled microspheres (Tufts)	Lewis rat 1.5 cm sciatic nerve defect (Pitt)

PCL (Pitt)	Keratin gel (WFIRM)	PLGA double-walled microspheres (Pitt)	Lewis rat 1.5 cm sciatic nerve defect (Pitt)
PCL (Pitt)	Keratin gel (WFIRM)	PLGA double-walled microspheres (Pitt)	Macaca fasicularis – 1 cm median nerve defect (Wake Forest)
O_2-generating biomaterials (WFIRM)	-	-	Lewis rat 1.5 cm sciatic nerve defect (Pitt)
Peptide Amphiphile (UCSB)	PA gel	PLGA double-walled microspheres (Pitt)	Lewis rat 1.5 cm sciatic nerve defect (Pitt)

- **Integration Synergies**

We have coordinated several meetings within our laboratories:

- Prof. Marra visited the Kaplan laboratory in Boston on April 3-4, 2012 for discussions on *in vivo* experiments with current Tufts laboratory nerve guide designs.
- All investigators met in Feb 2012 in Fort Lauderdale, FL at the AFIRM All-Hands meeting.
- All investigators met in Jan 2011 in Clearwater Beach, FL at the AFIRM All-Hands meeting.
- Prof. Marra visited the Smith laboratory at Wake Forest in November 2010 for discussions regarding the nonhuman primate median nerve defect model.
- All investigators met in Dec 2010 at the AFIRM Interim meeting in Washington, DC.
- Additionally, Dr. Mark Van Dyke has initiated cross-consortium discussions with the nerve repair teams in the Rutgers/Cleveland Clinic AFIRM consortium, and several meetings and teleconferences have been completed.

Key Research Accomplishments

Chontroitinase-Containing Silk Biomaterial Nerve Guides

- Enzyme chondroitinase stabilization and activity assays were completed for the enzyme in silk films, silk lyogels, and for compositions with added stabilizing agents. Chondroitinase activity degrades chondroitin sulfate proteoglycans present in scar tissue, which are inhibitory to axonal outgrowth.
- Stabilization and release of chABC in a silk system at 37°C has been achieved for ~ 4 days with the incorporation of trehalose and lipid microtubules
- The samples released at 4°C indicate that chABC is compatible with silk material processing, that altering silk's crystallinity affects release kinetics, and that incorporating trehalose significantly enhances recovery

Mechanical property testing of silk fibroin conduits

- This study examined the relationship of the silk nerve conduit tensile strength and Young's modulus as a result of processing conditions. The material properties and nerve cell growth

studies were completed on nerve guides with varying stiffness. These in vitro results provided an optimum guide processing condition for neuronal growth.

Concentration gradients of neurotrophic factors in silk fibroin nerve conduits

- Silk microspheres containing NGF were fabricated using self assembly of silk in a silk/poly(vinyl alcohol) polymer blend. ELISA indicated ~ 90% incorporation of NGF into the microspheres, with picogrammed levels of release detected over a 2-week period. A simple dip coating method was used to construct a gradient distribution of the NGF-containing microspheres from high (distal end of the conduit) to low (proximal end of the conduit). Silk films containing gradients of bulk-loaded growth factor were also prepared and characterized.

Keratin gel filled nerve guides

- A keratin hydrogel scaffold system, used as a conduit lumenal filler material, has previously been successfully tested in mice, rats, and rabbits. A definitive large animal preclinical trial was completed in an NHP model at Wake Forest. These data confirmed earlier studies and showed that peripheral nerve regeneration is significantly improved over the use of a conduit alone. Data from group at Wake Forest has been favorably reviewed by CDER/FDA and the path to IND approval has been outlined. One additional preclinical study and limited assay development will be required prior to application of the full IND. Funding has been requested for this work.

This past year, the Tufts Group continued to establish methods for developing biodegradable custom designed silk nerve guides. Their toolbox of strategies for enhancing PNR include the **introduction** of pores, patterned silk films, electrospun silk fibers, growth factors, and electronics to the guides. Recent investigations are targeted at incorporating the enzyme chondroitinase for degrading scar tissue build-up during nerve regeneration, as well as generating controlled concentration gradients of growth factor gradients. A quantitative study of the silk nerve guide mechanical properties was completed and neuron growth was assessed on guides made with varying Young's modulus values. Below are detailed descriptions of each of these developments to the multifunctional nerve guides.

I. Chondroitinase ABC (chABC) Stabilization and Release from Silk

1. chABC-Silk Encapsulation: Films and Lyogels

Chondroitinase has therapeutic potential to improve the outcome of PNR by degrading chondroitin sulfate proteoglycans present in glial scar tissue. However, the activity of chABC drops dramatically under physiologic conditions, and current use requires bolus delivery every other day for 10-14 days in order to achieve clinical efficacy. We first evalulated incorporating chABC into the silk-based nerve guides as means of increasing the stability and providing sustained localized delivery. Cumulative effective release of chABC was monitored over 2 weeks, for the enzyme incorporated into silk fibroin films and lyogels. Nearly linear release at

4°C was observed indicating active enzym e encapsulation. Variation of water annealing tim e tuned the silk film crystallinity, providing cont rol over the rele ase kinetics. The to tal enzyme activity was diminished however, upon heating to 37°C with no active chABC released after day 3. Investigatation of blendi ng the chABC with stabilizing agents f or improving the therm al stability of the enzyme was conducted (Part 2 below).

2. Additives for chABC stabilization

Poly(ethylene glycol) (PEG), tre halose, and lipid microtubes have been blended with the silk fibroin/chABC mixture to improve the therm al stability of chABC. Trehalose a nd lipid m icrotubules have shown effective for thermal stabilization of chABC. We found that dissolving chABC in 1M trehalose before dilution into silk solution s ignificantly improves recovery and overall enzym e activity. However, trehalose treatment does not app ear to stab ilize the enzyme at 37°C beyond day 3. Combinations of trehalose with lipid microtubules led to a small improvement in enzym e efficacy, increas ing the ac tive release of the chABC for up to 4 days (Figure 1). Current experim ents are directed at asses sing the activity of chABC/antibod y complexes released from silk fibroin as well as studying the therm al stability of PEG-chABC conjuagates.

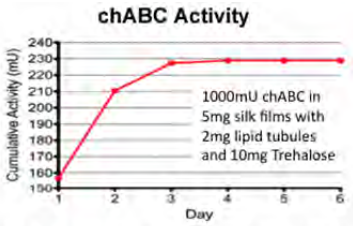

Figure 1. chABC activity for enzyme stabilized in silk fibroin film containing both trehalose and lipid microtubules.

II. Mechanical testing of silk fibroin conduits

1. Nerve Guide Elasticity

The Young's Modulus for the patte rned silk nerve guides was m easured using an Instron materials testing system. Young' s modulus values ranging fro m 0.058 MPa to 0.243 MPa following three days of hydration in PBS, could be prepared by va rying the porosity of the silk fibroin nerve guide during proces sing. The Young's Modulus in the longitudinal direction for peripheral nerves has been reported as approxim ately 8.5-40 MPa. Fro m our measurements, our nerve guides are more elastic than the native peripheral nerve in the longitudinal direction.

2. Nerve Guide Strength

Ultimate tensile strength of the silk fibroin guides was measured to be ~0.4-3.15 MPa f ollowing three days of hydration. Ultimate tensile strength in our nerve guides also vary depending on the porosity. Maximum stress on peripheral nerves has been reported as 11.7 MPa.

3. Neuron growth on guides with varying Young's Modulus

SH-SY5Y derived neurons were grown onto th e guides with varying porosities and Young's Modulus values. Liv e-dead staining was conduc ted to compare cell viability on each of th e

guides. Representative results are shown in Figure 2, and indicate that guides with an approximate Young's Modulus of ~0.06 MPa afford optimal neuron attachment and growth.

III. Growth factor gradient concentration studies

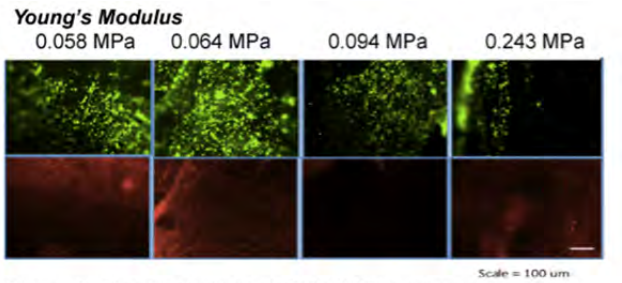

Figure 2. Live-Dead staining of SH-SY5Y derived neurons grown on silk fibroin nerve guides with varying Young's Modulus ranging from 0.06-0.24 MPa. Optimal cell viablility was observed for cells grown on guides of lower stiffness.

Previous research has shown that controlled delivery of GDNF concentrated at the distal end of the nerve conduit leads to enhanced scwann cell migration and nerve fiber density. Gradient delivery of the growth factor was achieved by creating a nerve conduit in which the distal half of the nerve guide contained the GDNF-encapsulated silk microscpheres, while the proximal half of the guide contained no delivery agents. Improved nerve density and Schwann cell recruitment was observed in a rat sciatic nerve defect model at 6 weeks recovery (Lin). We hypothesize that optimization of the growth factor delivery and gradient formation will lead to improved therapeutic outcomes for the silk fibroin conduits.

1. Growth factor gradient formation

Silk microspheres were generated encapsulating nerve growth factor, for growth factor release studies. ELISA showed ~90% incorporation of the positively charged growth factor into the anionic silk biopolymer spheres. Controlled release of the growth factor from sectioned guides indicated ~1% growth factor release (picogram level) over a period of two weeks. A simple dip coating procedure was then used to establish a gradient of growth factor from high to low from distal to proximal end respectively. Following gradient formation, the guides were sectioned, dissolved in hexafluoroisopropanol to release the encapsulated growth factor, and the GDNF was quantified using ELISA. Results show that the dip coating procedure was effective at creating a concentration gradient, but the processing conditions only led to detection of the ~ 25% of the loaded growth factor. As an alternative strategy, silk films containing gradients of growth factors were developed. These films can be rolled into a cylinder, inserted into pre-formed silk fibroin nerve guides, and water annealed to set the shape. Gradient formation on the films was quantified using fluorescently labeled FITC-poly(lysine) as a model growth factor. The films display gradients similar to those obtained for the dip coating procedure. Importantly, the film method does not expose encapsulated growth factors to methanol during guide fabrication, and will allow other biophysical strategies of improving PNR including patterning and electrode incorporation (wireless stimulation, AFIRM II proposal) to be tested in *in vivo* studies with the Prof. Marra's laboratory.

- Polymer-based tubes containing glial cell-line derived neurotrophic factor (GDNF):

- As of January 2012, there is an ongoing non-human primate (NHP) median nerve defect study at University of Pittsburgh:
- The aim of this study is to further investigate the hypothesis that the Polycaprolactone conduit containing glial cell-line derived neurotrophic factor embedded in double walled microspheres, will improve the functional recovery of critical sized peripheral nerve deficits (5 cm) compared to nerve autografts. Functional testing will occur in the cage, and will be performed on the animal every fourteen days following surgery. All functional testing is completely non-invasive, harmless, and does not limit motion for the animal. Prior to surgery, the NHPs were extensively trained by Marra lab members to pick up treats in the right hand from Klüver Board (the arm to be operated on) using only their finger and thumb (which is directly affected by a median nerve injury). We measured the NHPs ability to retrieve food pellets from the Klüver Board at least 50 times (Figure 3).

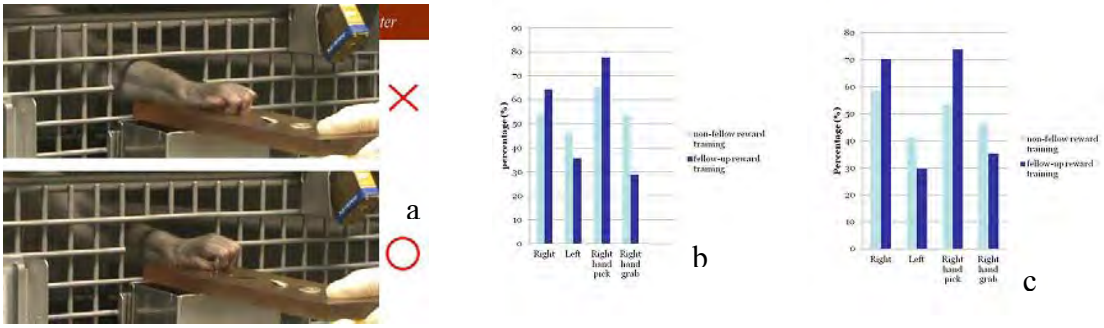

Figure 3. Functional behavior test. That test involved the training of a monkey to pick up 5 mm diameter 50 mg, roughly spherical food pellets from small wells on a modified Klüver Board (a). Baseline measurement of functional behavior test (b and c). NHP performances improved continuously with practice. After 30 days of limited daily practice (30 pellet retrievals/day), NHPs successfully retrieved and carried these small round pellets to the mouth by right hand at an average rate of 70-78% retrieval.

Conclusions

Research Plans

The Pittsburgh-Wake-Tufts labs will begin integrating electrode, drug delivery, and chemical coupling techniques with natural biodegradable polymer nerve guides and test them in the NHP model. Functionalization studies will be carried out with natural biodegradable polymer nerve guides in vitro. In the NHP model, implantations will continue with the next generation biodegradable polymer nerve guides (porosity, multiple growth factors, biophysical stimulation)

Planned Clinical Transitions

All 3 teams have begun planning a pathway to clinical studies. At Pitt, Dr. Marra has met with members of the Clinical Translation Science Institute twice to begin the process of transitioning from pre-clinical to clinical studies, and has had numerous discussions with members of the FDA, as has the Wake Forest group. After completion of the non-human primate model, Dr.

Marra will be able to conduct a Phase I clinical trial. The Wake Forest Group has a funded multi-center clinical trial in place that will begin as soon as the FDA sends certification of an IND to the external IRB that is approving this trial. Funding has been requested to fulfill keratin fate and distribution studies as well as studies to verify the GMP standards of the keratin hydrogel that has been requested by the FDA.

Corrections/changes planned for next year and rational for changes

One important change has been the removal of the rabbit tibial nerve defect model, and the rapid progression into the NHP median nerve defect model. This will permit earlier clinical translation.

Conflict of Interest Disclosure

N/A

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Reportable Outcomes

Patents and Invention: The University of Pittsburgh filed a US patent on the PCL/microsphere nerve guide (inventors Marra and Kokai) in 9/11.

Personnel Statistics	Paid*	Unpaid
# Faculty	4	1
# Post docs	1	3
# grad students	2	2
# undergrad students	1	3
# staff members		1

Other Project Statistics	
# Honors given to AFIRM faculty	1
# Doctorates awarded	1
# Masters degrees awarded	0
# Inventions disclosed	1
# Patents awarded	1
# Peer reviewed publications	9
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Peer-Reviewed publications:

1. Lin, Y-C.; Ramadan M.; Hronik-Tupaj M.; Kaplan, D. L.; Phillips, B. J.; Sivak, W.; Rubin, J. P. ; and Marra, K. G. Spatially Controlled Delivery of Neurotrophic Factors in Silk Fibroin-Based Nerve Conduits for Peripheral Nerve Repair. *Annals of Plastic Surgery*, **2011**, 67(2):147-55.
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Proposals:
Over the past year, the following proposals were submitted: PRORP, Marra PI

Grants

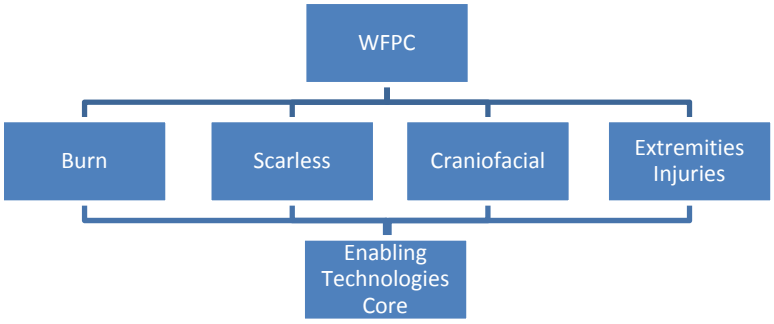
Dr. Marra is a Co-I on a National Sc ience Foundation ERC grant that is related to this project. Dr. Marra is also in discussion with MedGenesis Inc to partner with them as they own the rights to GDNF Dr. Sm ith, along with his collabora tor Dr. Van Dyke, has obtained funding from CDMRP.

Overview of the Enabling Technologies Core

Core Leader: Kacey Marra, PhD

The mission of AFIRM is clear—to accelerate regenerative technologies to the wounded warrior. While the mission is straightforward, research and development of innovative technologies is not. In an effort to balance mission expectations with the methodical pace of research, a reorganization of the research efforts within AFIRM was performed.

To improve synergies in 2010, an enabling technologies (ET) core was created with the objective of developing state-of-the-art tools and/or techniques applicable to the four program areas. The ET core functions to mature basic or platform technologies for insertion into the program areas of best fit and/or serve as a resource in multiple core areas.



This core serves as the gateway for introducing new technologies into AFIRM, and Project Leaders within ET are expected to interact synergistically to develop and apply multi-platform technologies to advance the translational mission of the four clinically based programs. This section of the report details the progress these enabling technologies have made.

Project ET-1: Fluid-Derived and Placenta-Derived Stem Cells for Burn

Team Leader(s)	John D. Jackson, Ph.D., Wake Forest University
Project Team Members	Chad D. Markert, Ph.D., Wake Forest University
Collaborator(s)	David Mack Ph.D., Aleksander Skardal, Ph.D., James Yoo, M.D., Ph.D., Wake Forest University
Therapy Deliverable(s)	Amniotic fluid-derived and placental-derived stem cells for burn *Baseline:* To utilize broadly multipotent stem cells from perinatal sources (amniotic fluid and/or placental tissue) to develop an improved 'off-the-shelf' bioengineered skin product for the treatment of extensive burns *Revised:*
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #2 End Year 1: TRL #1 End Year 4: TRL #2 End Year 2: TRL #2
Key Accomplishments:	Delivered amniotic stem cells with and without mature skin cells (keratinocytes) into wounds in a mouse model and demonstrated more redid wound healing and increased vascularization of the wound bed
Keywords	Stem Cells, Trophic Factors, Regenerative Medicine, Living Skin Equivalents

Introduction

Directed differentiation of amniotic fluid-derived stem (AFS) cells presented many challenges and resulted in lack of consistency in epithelial marker expression. Therefore, the project was redirected to the role of undifferentiated AFS cells in wound healing during this past year. This redirection resulted in faster progress into an in vivo animal model for AFS cells in wound healing. A nude mouse full thickness skin wound model was used to assess the ability of AFS cells to accelerate wound healing (Figure 1). The objectives were to determine whether AFS cells can augment wound healing rates when bioprinted in the wound model and whether increased wound healing rates might be due to integration of the AFS cells in the regenerating tissue or their secreted trophic factors. AFS cells were also bioprinted with mature keratinocytes to examine if the combination of stem cells and mature epithelial cells would enhance wound healing. Bioprinting of the AFS cells into the skin wound increase wound healing rates and induced the neovascularization of the wound area. Interestingly, the number of AFS cells decreased over time suggesting that they did not directly contribute to wound healing by incorporating into the epidermis but influenced wound closure and vascularization potentially via production of trophic factors.

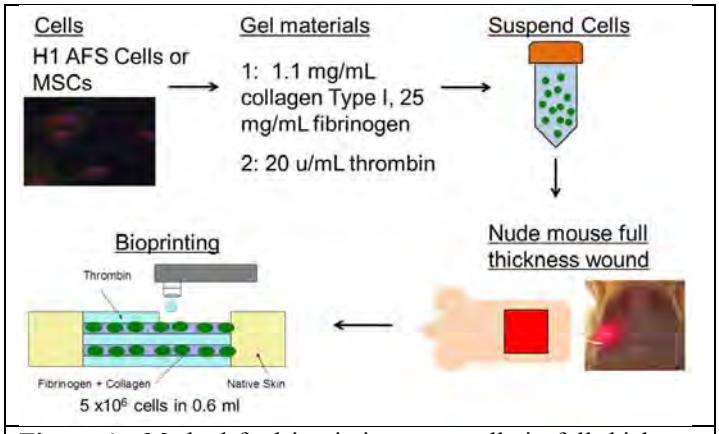


Figure 1. Method for bioprinting stem cells in full thickness wounds

Research Progress

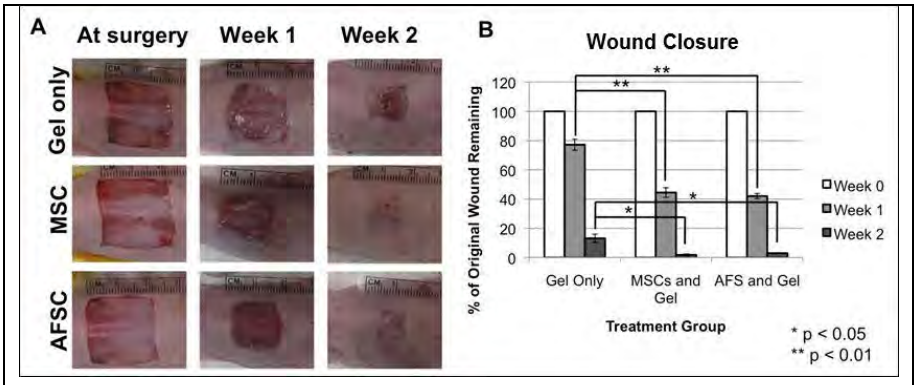


Figure 2. Wound closure after treatment with AFS cells and MSC

Amniotic fluid-derived stem (AFS) cells posses s properties found also in mesenchymal stem cells (MSCs), such as multipotent differentiation, immunomodulatory activity and the lack of significant immunogenicity[1-3]. MSCs have shown therapeutic potential fo r repair and regeneration of tissues dam aged by injury or disease. In particular, MSC treatm ent of acute and chronic wounds result in accelerated wound closure, increased epith elialization, granulation tissue and angiogenesis[4]. Given that AFS cells can be o btained less invasively than MSCs and show greater proliferative capacity in cu lture[2], we investig ated whether they could augment wound healing in a similar fashion as MSCs.

Wound size, contraction, and re-e pithelialization were m easured and quantified. At both time points, wound closure was faster in AFSC and MSC treated groups compar ed to gel-only group (Figure 2). Cell treated groups showed greater le vels of contraction at week 1 (Figure 3). However, re-epithelialization levels were greate r in AFSC and MSC treated groups co mpared to the gel-only group (Figure 3). More blood vessel s were seen in the AFS cell and MSC groups compared to the gel-only group. At one week post treatment, micro vessel density and blood vessel diameter were g reater in the AFS cell a nd MSC gro ups when co mpared to the gel-only group; however, at 2 weeks post treatm ent, AFS cell treatment had greater vessel diam eters that both MSC and gel-only groups (F igure 4). Cell tracking study showed the number of AFS cells and MSC decreased in the regenerating wound which demonstrated that the stem cells did not integrate in to the ep ithelium (Figure 5). 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 AFC cells decreased over tim e the increas e in wound healing activity may be due to trophic factors produced by the stem cells early in the healing process.

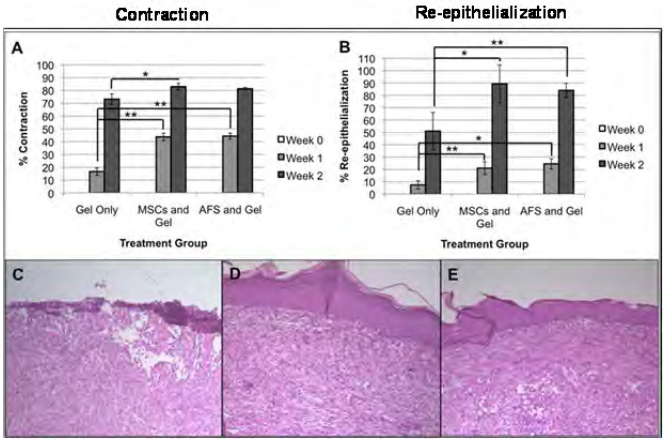


Figure 3. Wound contraction and epithelialization after treatment with AFS cells and MSC.

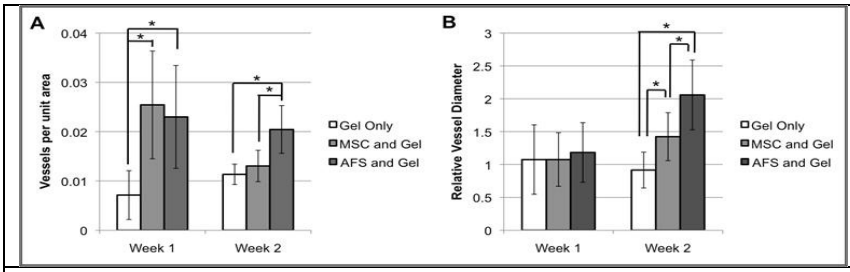


Figure 4. Micro vessel density and vessel diameter after treatment with stem cells.

AFS cells and porcine keratinocytes were bio printed on to full thickness wounds on the back of nude mice. The cells were printed in two layers with the AFS cells on the bottom layer and the keratinocytes on the top layer (Figure 6). In addition, each cell type was bio printed alone as comparative groups. Wound healing was rapid with wound closure occurring by day 14 in all groups (Figure 7). The number of AFS cells began to decrease by day 7 post-bio printing and were undetectable by day 14 post-bio printing. This finding confirms the earlier results of the transient nature of the AFS and MSC cells post-bio printing.

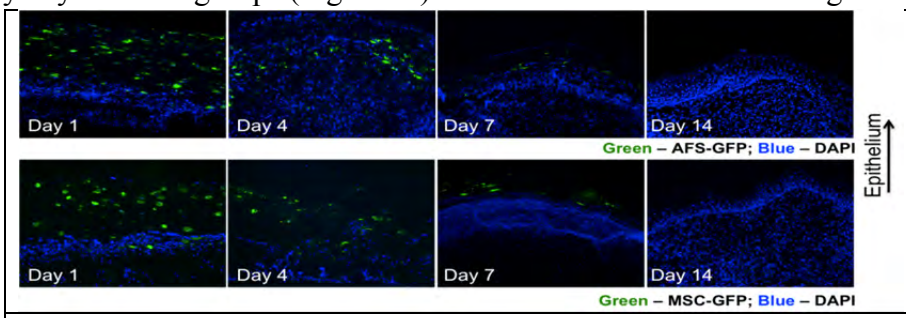


Figure 5. Stem cell tracking after bio printing onto wound.

Key Research Accomplishments

- Established a nude mouse full thickness wound model and bio printing AFS cell into the wound.
- Demonstrated enhanced wound healing as well as increased neovascularization using AFS cells.
- Combined AFC cells with mature keratinocytes to enhance wound healing

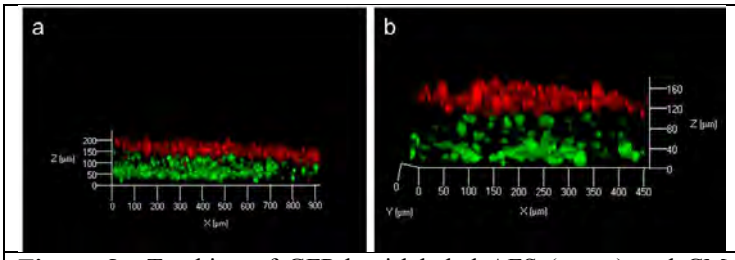


Figure 6. Tracking of GFP-lenti-labeled AFS (green) and CM-DiI-labeled KC (red) in wounds at the 4-day time point. a: Confocal microscopy showing cell distribution along the z-axis (10X objective lens). b: Confocal microscopy showing cell distribution along the z-axis (20X objective lens). AFS: Amniotic fluid-derived stem cells, KC: Keratinocytes.

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 the AFS cells delive red trophic factors im portant in wound healing. These results suggest that AFS cells bio printed as a cell therapy may be a potentially powerful tool for burn and wound healing treatments.

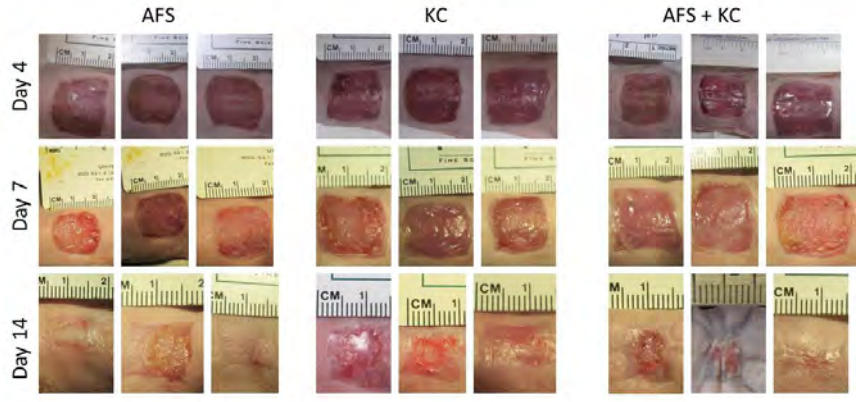


Figure 7. Wound healing over 14 days in a nude m ouse excisional model. AFS: Amniotic fl uid-derived stem cells, KC: Keratinocytes, AFS+KC: Both cell types applied in a s tratified manner, with AFS applied first (derm al layer) and KC applied on top (epidermal layer).

Research Plans for the Following Year

In the next year, a large animal model (porcine) will be use d to bio print AFS cells alone and in combination with autologous keratinocytes in a burn injury. Sim ilar parameters associated with wound healing will be examined including wound epithelialization, wound contracture, and vascularization.

Planned Clinical Transitions

Although no clinical trials are currently planne d under this AFIRM project , the data collected from the porcine burn model will be critical in transitioning to a future clinical trial.

Corrections/Changes Planned

No changes are planned for the refocused project in the next year.

Conflict of Interest Disclosure

The research team has no conflict of interest to disclose.

References

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3. Moorefield, E.C., et al., *Cloned, CD117 Selected Human Amniotic Fluid Stem Cells Are Capable of Modulating the Immune Response.* PLoS ONE, 2011. **6**(10): p. e26535.
4. Maxson, S., et al., *Concise Review: Role of Mesenchymal Stem Cells in Wound Repair.* Stem Cells Translational Medicine, 2012. **1**(2): p. 142-149.

F. Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	2	3
# Post docs	2	2
# grad students	0	2
# undergrad students	0	0
# staff members working for AFIRM	1	6

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	0
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Project ET-2: Peptide-mediated Delivery of Therapeutic Compounds into Injured Tissues During Secondary Intervention

Team Leader(s)	Erkki Ruoslahti, M.D., Ph.D. (Sanford-Burnham Medical Research Institute at University of California, Santa Barbara [UCSB]
Project Team Members	Tero Järvinen, M.D., Ph.D. (Sanford-Burnham Medical Research Institute at UCSB and University of Tampere, Finland); Eunhye Lee, Ph.D., Sajid Hussain, Ph.D., Shweta Sharma, Ph.D., Gary Braun, Ph.D. and Chris Brunquell (Sanford-Burnham Medical Research Institute at UCSB and Institute for Collaborative Biotechnologies, Olivia Yu, Undergraduate student UCSB.
Collaborator(s)	None
Therapy	Systemic drug targeting to injured tissues/preventing scarring/enhancing tissue regeneration.
Deliverable(s)	*Baseline:* Systemic and local wound targeting with peptides that penetrate into wound and scar tissue. *Revised:* None
TRL Progress	Start of Program: TRL #1 End Year 1: TRL #1 End Year 2: TRL #3 End Year 3: TRL #5 End Year 4: TRL #5 (targeted decorin); TRL #3 (CAR peptide as a therapeutic)
Key Accomplishments:	(1) Identified peptides that home to wounds and can deliver a therapeutic payload to wounds and other injured tissues. 2) Developed wound-targeting peptides that penetrate into wound and early scar tissue. 3) Designed a wound-targeted biological anti-scarring agent. (4) Discovered a wound healing promoting activity by one of the homing peptides
Keywords	Wound angiogenesis, homing peptides, anti-scarring, TGF-β

Introduction

We previously published data concerning two wound-homing peptides that recognize wound blood vessels at different stages of healing (Järvinen & Ruoslahti, 2007). One of these peptides (CAR) appears to recognize a wound-specific form of heparin sulfate; the target molecule for the other is not known. We have used the CAR peptide to selectively deliver decorin, a natural inhibitor of TGF-β into skin wounds and obtained significant reduction of several markers of scarring (Järvinen and Ruoslahti, 2010). We have also shown that CAR homes to injured tissue in other conditions (pulmonary fibrosis and pulmonary hypertension; Urakami et al., 2011), and that the peptide can promote tissue-specific accumulation in injuries of compounds that are co-administered with the peptide, not chemically coupled to it. The latest discovery reveals an inherent wound healing promoting activity of the CAR peptide (unpublished results; Year 3 report). Work in Year 4 has focused on optimizing the CAR-decorin conjugate for completion of the preclinical work and introduction of the compound into the clinic. Another focus has been to confirm and extend the results on the healing-promoting activity of the CAR peptide.

Research Progress

Specific Aim 1

As reported last year, we have generated a truncated form of our wound targeting peptide CAR (tCAR; CARSKNK) by making the second lysine the C-terminal residue and shown that tCAR phage binds more avidly to CHO-K cells than CAR. Like CAR, tCAR depends on heparan sulfate for the cell binding as it tCAR binds much less to the glycosaminoglycan-deficient CHO mutant pgsA-745 cells than the parental CHO cells. tCAR also homed more strongly to skin wounds that CAR and penetrated deeper into the wound tissue.

Our latest results provide a partial explanation for the superior performance of tCAR in wound homing *in vivo*. We deleted the N-terminal cysteine and found that this peptide showed poor homing efficiency (shown for tumor homing in Figure 1). We have also made peptides in which the cysteine is converted into an alanine, serine, or methionine and find them inactive. Therefore, the free sulfhydryl group of the cysteine residue is needed for the homing.

We have preliminary data to the effect that the reason for the sulfhydryl requirement in tCAR is that it links the peptide to the free sulfhydryl in albumin (and likely in other plasma proteins), which prolongs the half-life of the peptide in the circulation, resulting in stronger homing. However, this may not be the only reason for the strong homing activity, as nanoparticles coated with tCAR through the sulfhydryl group also efficient and specific homing (Figure 2), although the nanoparticle coupling also prolongs the blood half-life of the peptide. During the coming year, we will determine whether tCAR is more effective than CAR in the various applications we are working on.

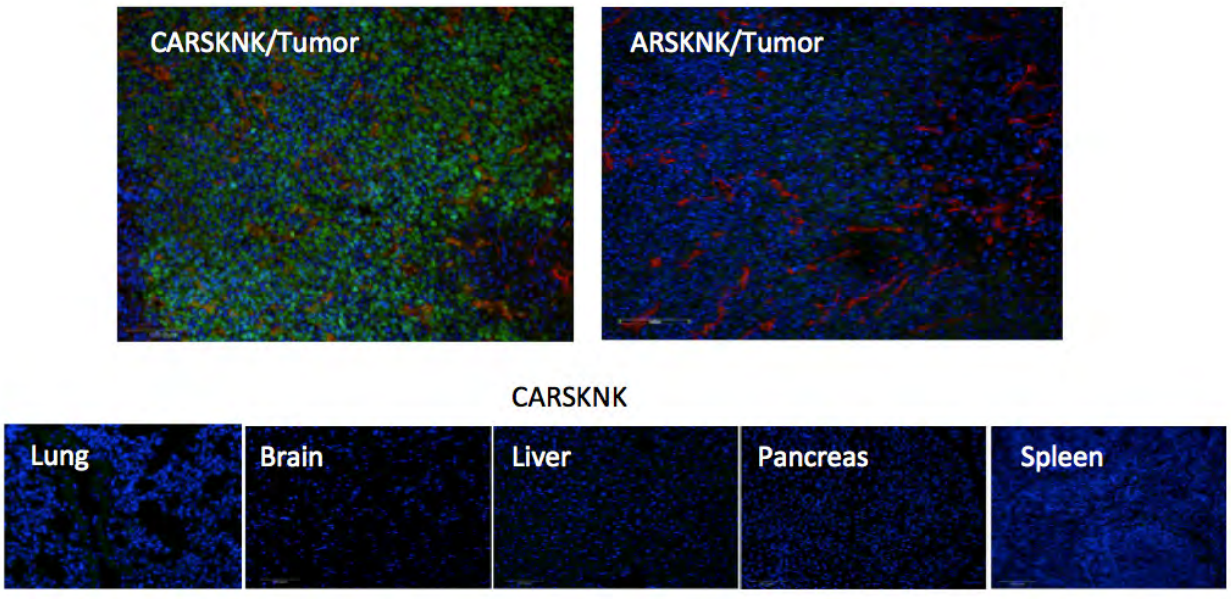

Figure 1. *In vivo homing* of tCAR peptide. Confocal images of sections of a 4T1 tumor and normal organs from mice injected with the 200 µL of 1 mM FAM-tCAR peptide. The circulation time was 2 hours. The tCAR peptide shows extensive homing and spreading within tumor tissue, whereas tCAR that lacks the cysteine residue (ARSKNK) shows no detectable tumor homing. There was not detectable accumulation of either peptide in normal

tissues (shown for tCAR), except in the kidney (peptides are excreted into the urine). Red, CD31; green, peptides; blue, nuclei. Representative fields from multiple sections of tissues from 3 tumor mice are shown. Scale bars, 100 mm.

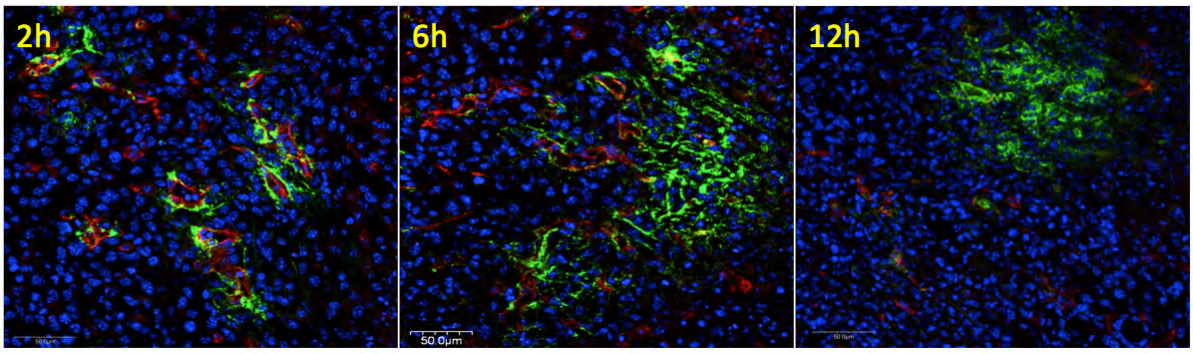

Figure 2. *In vivo* homing of nanoparticles coated with the tCAR peptide. Confocal images of 4T1 tumors from mice injected with tCAR iron oxide nanoworms (Park et al., 2009) in 130 μl were intravenously injected into the tumor mice. The circulation times were as shown. The tCAR nanoworms were initially found in and around the blood vessels, but over time extravasated and spread in the tumor tissue. Red, CD31; green, nanoworms; blue, nuclei. Representative fields from multiple sections of 3 tumors are shown. Scale bars, 50 mm.

A remarkable new discovery also reported last year is that systemic injections of the CAR peptide alone to mice with skin wounds accelerates wound healing. CAR penetrates into cells and tissues in a manner similar to the recently identified CendR peptides (Sugahara et al. 2010). Based on these results, we hypothesize that CAR might enhance wound healing by improving the availability to the regenerating tissue of natural growth factors from the blood and serum, and that because of the wound specificity of CAR, this effect would be specific to wounds. In essence, we would be pharmacologically manipulating a previously described plasma->serum->plasma transition that takes place during normal tissue repair and controls tissue regeneration (Figure 3).

We have obtained evidence to support the plasma protein hypothesis. Evans Blue dye injected into the circulation, where it binds to albumin accumulated more strongly in wound tissue when injected together with CAR than when combined with a control peptide (Figure 4)

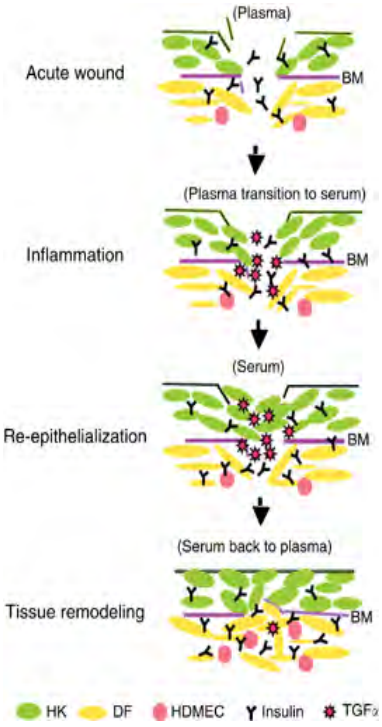

Figure 3. A schematic representation of how serum promotes re-epithelization during wound healing. The three classical and sequential events of wound healing; inflammation, re-epithelialization, and tissue remodeling are schematically depicted. Three major types of cells involved in wound repair, keratinocytes (HKs), dermal fibroblasts (DFs), and human dermal microvascular endothelial cells (HDMECs), are shown. The serum derived TGFα levels are dramatically increased in the wound fluid, following the transition from plasma to serum in the wound bed. After the wound is closed and after transition of serum back to plasma, the levels of TGFα go back to those in unwounded skin (Li et al. 2006).

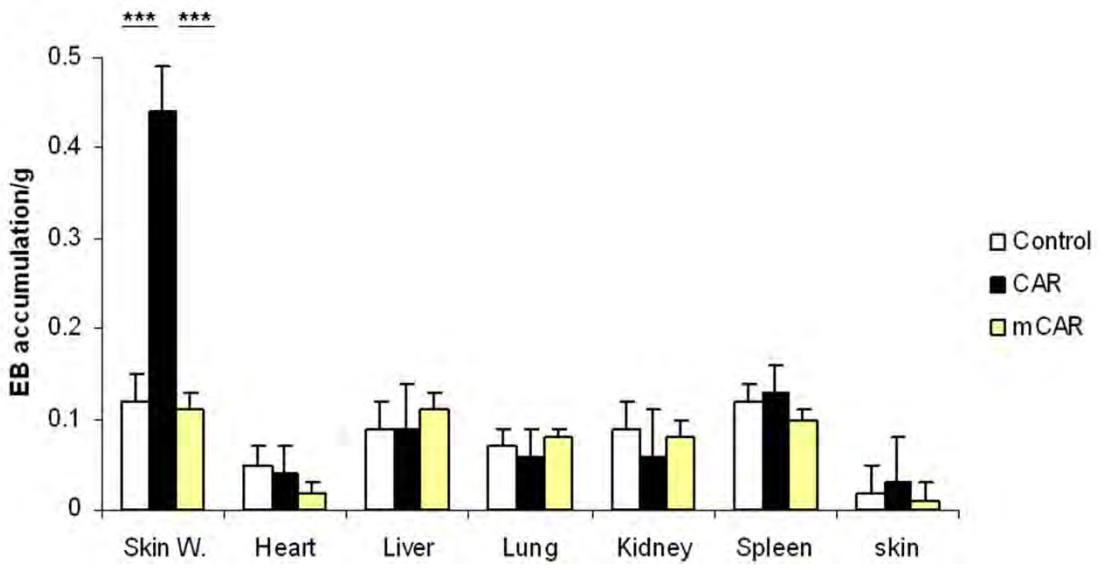

Figure 4. CAR peptide drives accumulation of blood-derived proteins in skin wounds. Mice with full thickness skin wounds received an intravenous injection of CAR or control peptide together with the albumin-binding dye Evans Blue on Day 7 after the wounding. After 30´, the mice were perfused through the heart, the wounds and the control organs harvested and Evans Blue was extracted with formamide. Systemically injected CAR peptide induces "bystander effect" i.e. drives blood derived proteins into the wound bed, whereas the inactive *m*CAR control peptide does not. The "bystander effect" takes place specifically as no accumulation is seen in uninjured organs. N=20, the statistical significance was examined with ANOVA, (***) $P < 0.001$. Mean ± SD.

We next plan to compare wounds from CAR-treated and control-treated mice by using proteomics analyses and mRNA microarrays. The CAR peptide may provide a new way of enhancing wound healing, and perhaps tissue regeneration in general, that is systemic, yet target-specific, and non-toxic. These studies may result in new systemic treatments that can not only accelerate the tissue regeneration in major traumas, but also limit the permanent damage caused by fibrosis in injured, operated and inflamed tissues. In addition, CAR co-injection may enable injury-selective delivery of other compounds with beneficial effects on tissue healing.

Specific Aim 2

Wound-targeted decorin. In the Y1 and Y2 annual reports, we presented data to the effect that wound-targeted version of the anti-fibrotic protein decorin is more effective in suppressing various indicators of subsequent scarring than non-targeted decorin. In Y3, we completed and published the scar suppression study (Järvinen and Ruoslahti 2010). One of the key pieces of data in that publication is the unique selectivity of CAR-decorin against the different TGF-βs. As shown in our article, CAR-decorin is significantly more active than decorin against TGF-β1 and TGF-β2 but, remarkably, had no effect on TGF- β3. The differential inhibitory activity of CAR-decorin against the TGF-β isoforms could have important implications; TGF-β1 is the isoform responsible for scar formation, and TGF- β2 augments the pro-fibrotic activity of TGF-β1. In contrast, TGF-β3 inhibits scarring. We propose that CAR-decorin inhibits induced scar formation induced by TGF- β1- and TGF- β2, while leaving the beneficial TGF-β3 untouched. The *in vitro* assay we have developed for the TGF-β inhibition gives us an important tool in

evaluating the potential of the various modified decorins we will produce to optimize the CAR-decorin compound to be taken into the clinic.

Decorin is a proteoglycan, and the heterogeneity of its single glycosaminoglycan chain makes recombinant decorin produced in mammalian cells heterogeneous (e.g. Järvinen and Ruoslahti 2010), which could cause regulatory problems. Previous data indicate that the protein core is responsible for the binding of decorin to TGF-β, and that the chondroitin sulfate (CS) side chain can actually hinder this interaction (Hildebrand et al. 1994). Thus, the CS chain may not be needed for the inhibition of TGF-β1 activity by decorin, and it may be possible to use homogeneous, GAG-free CAR-decorin that is actually more active than the native decorin as an anti-scarring agent.

In Y3, we reported the preparation of a mammalian cDNA expression vector for decorin in which the serine that serves as the attachment site for the CS chain has been changed into alanine. The alanine mutation produces CAR-decorin with no CS chain. The same result can be achieved by expressing decorin in a baculovirus system, and we reported the cloning of CAR-decorin into a baculovirus vector in Y3. We have now produced CS-free CAR-decorin in the mammalian and baculovirus systems and are in the process of testing these preparations for TGF-β inhibition, circulation half-life, wound homing, and anti-scarring activity. In addition, we have made CAR-mouse decorin for immunogenicity studies in mice.

Key Research Accomplishments

- Shown that a shorter, linear form of the CAR peptide (CARSKNK), which is an effective homing peptide that provides a potential an easier-to-synthesize alternative for the cyclic CAR, requires the free sulfhydryl group for the enhanced activity.
- Shown that prolonged blood half-life is a major reason for the enhanced activity.
- Completed studies of the enhanced wound healing activity of the CAR peptide to a point where a patent application could be filed.
- Provided evidence to the effect that systemically administered CAR enhances the penetration of blood-borne substances into wounds.
- Produced glycosaminoglycan-free decorin.

Conclusions

The development of systemic delivery of therapeutic agents to injured tissues is progressing as planned. The new tissue-penetration technology offers particular promise, particularly the potential of enhancing drug delivery to wound tissue of blood-borne compounds that are not chemically coupled to the tissue-penetrating peptide. The inherent activity of the peptide in wound healing is another potentially important new lead. The activity of the anti-scarring agent, decorin, can be greatly enhanced by fusing this protein with a homing peptide. Ongoing work to improve the properties of the targeted decorin to make the regulatory path easier is in progress in

parallel with efforts to find a corporate partner, which have brought four companies to licensing discussions so far.

Research Plans

The work on the peptide delivery systems will focus on the cell and tissue-penetrating properties of the CAR peptide and its use in enhancing wound and scar penetration of co-administered compounds. Characterization of the CAR variant tCAR, which appears to be more potent in wound homing than the original CAR, will be another focus area. The decorin project will focus on improving the properties of CAR-decorin to facilitate commercial production and regulatory approvals. Comparisons of CAR-decorin and tCAR-decorin in wound homing and scar prevention experiments will also be carried out. The inherent biological activity of CAR in promoting wound healing suggested by initial experiments will be confirmed and its molecular basis explored. Possible synergies of the CAR-decorin and CAR peptide treatments will also be studied. Finally, the molecular basis of the wound-healing promoting activity of the CAR peptide will be studied to facilitate the transition of this treatment into the clinic. Two-year TRL-goals for our products should be CAR-decorin (TLR7), CendR (TLR7), CAR/tCAR (TLR5).

Planned Clinical Transitions

The hope is that the improved efficacy demonstrated for the targeted decorin will encourage clinical trials. The advantages are that less of the recombinant protein needs to be manufactured and that patent coverage will extend into the late 2020s. Our publication at the end of 2010 has already generated substantial interest among academia. Several promising collaborations have been initiated with prominent scientists in the field of scar and fibrosis prevention. The aim is to prove the effectiveness of the targeted decorin in other experimental models of fibrosis in order to generate additional interest from the biotechnology and pharmaceutical industry. Discussions with potential commercial partners and public funding agencies are under way to advance the project.

Corrections/Changes Planned

The main new element that was not foreseen when the original application was submitted is that it is possible to use tissue-penetrating homing peptides to deliver drugs to a target tissue without coupling the drug to the peptide. The peptide appears to activate a transport system in the specific target tissue that sweeps along any compound in the blood. Another exciting, unexpected result was the inherent wound healing-promoting activity of the CAR peptide. These new leads may result in major improvements in the delivery of drugs to injured tissues.

Conflict of Interest Disclosure

Dr. Ruoslahti owns stock in and is a director of a company (VBS Pharma) that is in negotiations with the Sanford-Burnham Institute regarding a license to the CAR homing peptide and CAR-decorin.

References

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Sugahara, K.N., Teesalu, T, Karmali, P.P., Kotamraju V.R., Agemy, L. Greenwald, D.R., and Ruoslahti E. Co-administration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. *Science*, 328: 1031-1035 (2010). PMCID: PMC2881692

Teesalu, T., Sugahara K., Kotamraju, V.R., and Ruoslahti E. C-end rule peptides mediate neuropilin-1-dependent cell, vascular, and tissue penetration. *Proc. Natl. Acad. Sci. USA* 106:16157-16162 (2009). PMCID: PMC2752543

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	1	1
# Post docs	4	0
# grad students	1	0
# undergrad students	1	0
# staff members working for AFIRM	0	1

Other Project Statistics	
# Honors given to AFIRM faculty	2
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	3
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	1

*Directly supported in whole or part –time by AFIRM

Reportable Outcomes

 # Honors given to AFIRM faculty –

Keynote Speaker – Visiting Scientist Training Program, Univ. of Wisconsin - 2012

Visiting Professor, Sass Foundation - 2011

#Patents Awarded

1. None.

2. Submitted patent applications:

Application number	Application Date	Title
PCT/US11/26535	Feb 28, 2011	CAR Peptide for Hom ing, Diagnosis & T argeted Therapy for Pulmonary and Fibrotic Disorders.
13/224,193	Sep 1, 2011	Truncated CAR Peptides and Methods and Compositions Using Truncated CAR peptides
13/406,699	Feb 28, 2012	Methods And Compositions For Enhancing W ound Healing Using Car Peptides
PCT/US12/26863	Feb 28, 2012	Truncated Car Peptid es And Methods And Compositions Using Truncated Car Peptides
61/597,076	Feb 9, 2012	Methods And Compositions For Enhancing W ound Healing Using Car Peptides

 # Peer Reviewed Publications

1. Urakami, T., Järvinen, T., Toba M, Sawada J., Namasivayam, A., Mann D., McMurtry I, Oka, M., Ruoslahti, E., and Kom atsu, M. Peptide-Directed highly selective targeting of pulmonary arterial hypertension. Am . J. Pathol. 178:2489-2995, (2011). PMCID: PMC3123986

 # Non-Peer Reviewed Publications

1. Jarvinen, T., and Ruoslahti, E. Targeted anti-scarring therapy for tissue injuries. In: Advances in Wound Care. Mary Ann Liebert, Inc. (in press).

Project ET-3: Modular, Switchable, Synthetic Extracellular Matrices for Regenerative Medicine

Team Leader(s)	Matthew Tirrell, Ph.D. (UC Berkeley, University of Chicago)
Project Team Members	Katie Megley, B.S. (UC Berkeley), Won H. Suh, M.S./Ph.D. (UC Berkeley), Brian Lin, B.S./M.S. (UCSB), Dan Krogstad, B.S. (UCSB), Nickesh Viswanathan, B.S. (UC Berkeley), Seema Desai (UC Berkeley)
Collaborator(s)	Kacey Marra (UPMC)
Therapy	Injectable synthetic extracellular matrices for regenerative medicine
Deliverable(s)	*Baseline: As they appear in the original proposal* *Revised:*
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #2 End Year 1: TRL #1 End Year 4: TRL #2 End Year 2: TRL #2
Key Accomplishments:	Peptide-based gel system has been developed as injectable extracellular matrices with nanofibrous structures. The hydrogel system can incorporate bioactive peptide sequences and/or growth factors. The three-dimensional matrix system, in addition, allows for mammalian cell growth.
Keywords	Synthetic extracellular matrix, peptide amphiphile, nerve regeneration, tissue engineering, micelles and vesicles

Introduction

The main objective of the ET-3 project is to develop injectable synthetic extracellular matrices for regenerative medicine. The Tirrell lab members, as an Enabling Technologies team of the WFPC AFIRM Consortium, are developing synthetic three-dimensional extracellular matrices that are gel-like and designed to aid in the regenerative processes involved in peripheral nerve regrowth following traumatic injury. Using the Tirrell lab's platform material, peptide amphiphiles, short peptides attached to fatty acid tails, are designed to self-assemble in solution into extended wormlike micelles. These micelles then entangle at high concentration to form a fibrous hydrogel which can be tuned to reflect the stiffness of the native tissue of interest.

Research Progress

At the end of Year 3, Tirrell and co-workers redesigned their PA system with new branched head group peptide architecture. This new PA, termed "GSH", included a histidine arm, glycine spacer, and a serine arm, all attached to a 16 carbon fatty acid tail (Figure 1). With this design the group applied a new mode of peptide head group stabilization by controlling hydrogen bonding between imidazole groups on the histidine residues and primary alcohol side residues on the serines after self-assembly by modulating pH. Below pH 6, histidines are predominately acidic and form weak hydrogen bonds with serines as proton donors. Above pH 6.5, histidines are mostly basic and form strong hydrogen bonds as proton acceptors. As a result, the PAs form weak fibers at low pH and above pH 6.5, strong fibers form. Weak fibers in solution resemble a low viscosity liquid and strong fibers form self-supporting hydrogels. Additionally the biocompatibility of the GSH hydrogel system was studied using a NIH 3T3 model cell line and found to support proliferation and growth (See Year 3 Report Key Accomplishments 5 & 6, Figures 9 & 10.

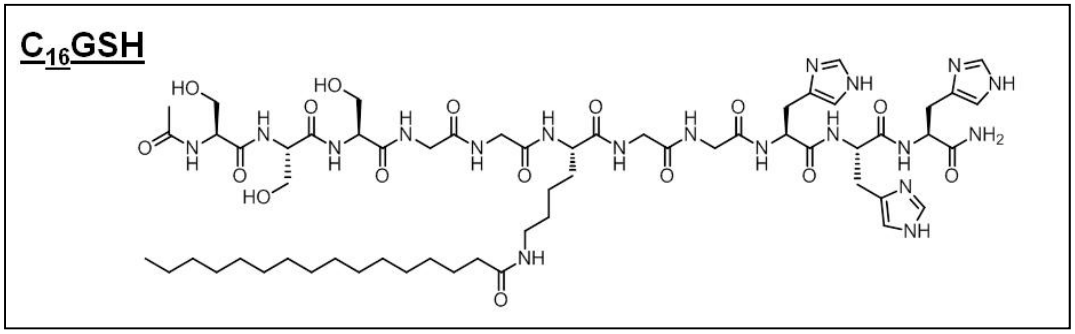

Figure 1. PAs were synthesized in a branched architecture with serine and histidine peptides conjugated to a fatty acid through a lysine residue. "C16GSH" (S3G2KC16G2H3)

Continuing with the GSH system , in Year 4, the T irrell lab used rheology to study the mechanical properties of this system in terms of concentration and pH. Gel solutions were loaded onto the instrument at pH 4 and storage modulus measured (Figure 2A, Open Squares). Next, pH was increased by adding concentrated drops of ba se to the perimeter of the sample and modulus measured again (Figure 2A, Closed Squares). M odulus was found to scale with concentration in the raised pH sample set. A stiff gel of 10 kPa (G') is achieved at 1% by wt (10 mg/mL) of PAs. This corresponds to the stiffne ss of muscle tissue of the body. [1] This in crease in stiffness was found to be caused by increased fiber density w ith increasing concentration as evident by the SEM images (Figure 2B and C).

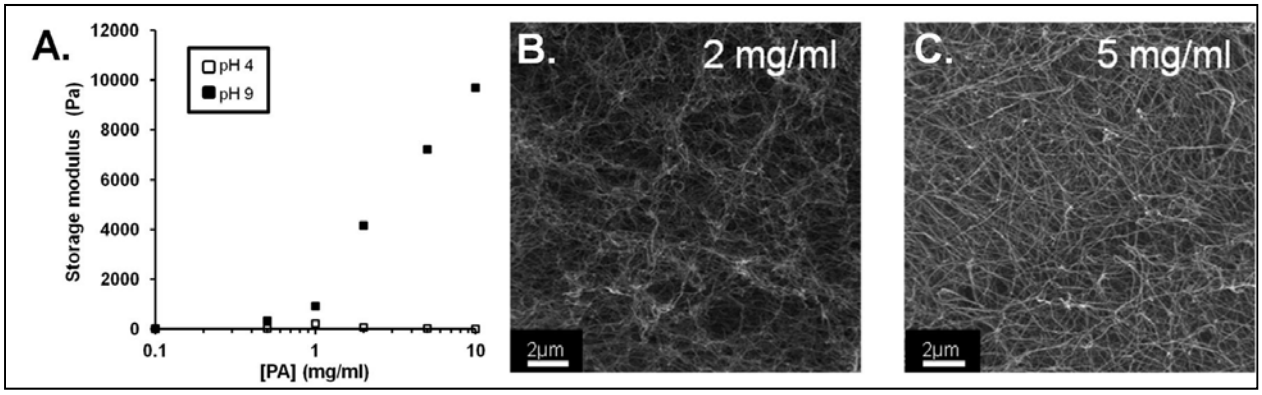

Figure 2. C16GSH hydrogels stiffened when the pH was raised. (A) At pH 4, the samples remained as viscoelastic liquids over the range of concentrations studied. At pH 9, the modulus increased with increasing concentration. (B-C) Representative SEM images of C16GSH depicting the increase in fiber density with increased PA concentration.

Moving towards the application of nerve regenera tion, the Tirrell lab studied the effect of gel modulus on in vitro cell behavior using a Schwann cell. Schw ann cells were sensitive to stiffness of the material and spread most on the softest gel (G'=0.92 kPa) tested (Figure 3). Cell proliferation was m easured after cells were cultured for 48 hours; comm ercially available collagen gel and tissue culture plastic (TCP) were used as controls. After 48 hours cell

proliferation on the 0.92 kPa gel was statistically similar to that of TCP. Stiffer gels showed less proliferation but were statistically similar to the commercially available collagen gel (Figure 4).

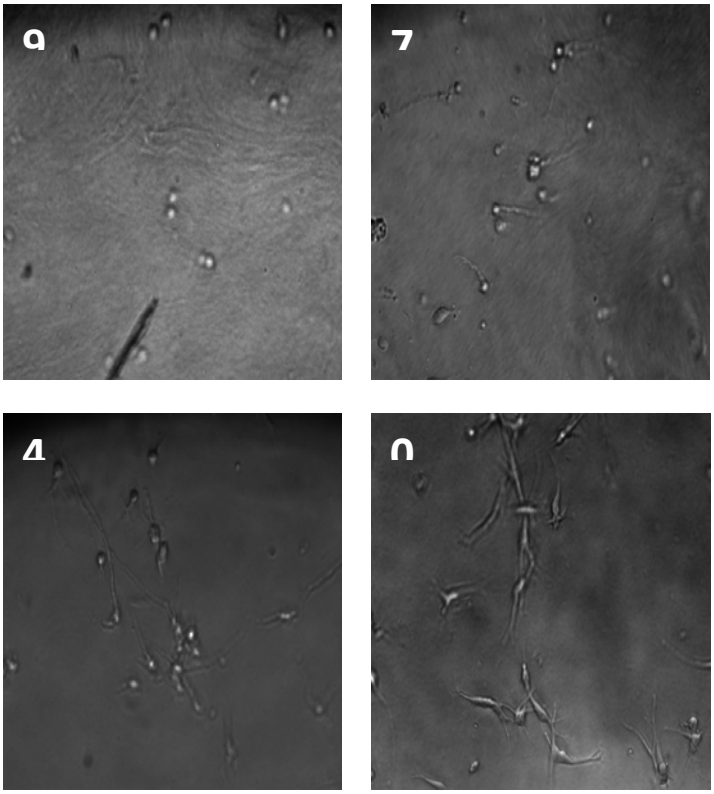

Figure 3: Cell spreading after 18 hours on C_{16}GSH gels, images taken at 20x magnification.

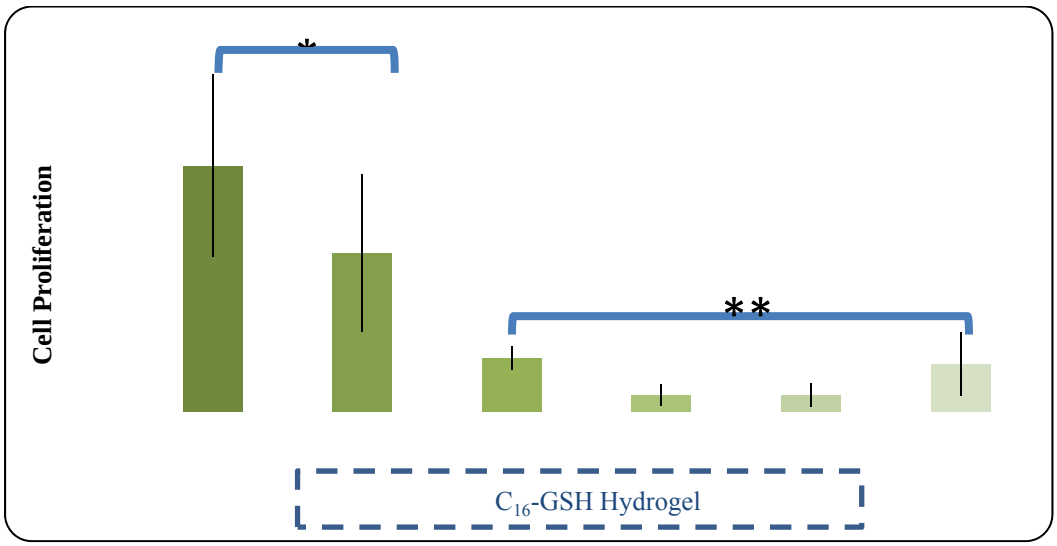

Figure 4. Cell proliferation measured after 48 hours using Alamar Blue viability assay. *0.92kPa and control are not statistically different, Tukey Test ($p<.05$), **no statistical difference, Tukey Test ($p<.05$)

To better mimic the cellular environment during repair of the peripheral nerve the Tirrell lab designed a 3D microchannel experiment to measure migration of Schwann cells across the C_{16}GSH hydrogels. Briefly, passive pumping microchannel arrays developed by Bell Brooks Labs were used. C_{16}GSH solution at low pH was added to the inlet port and allowed to wick across the channel (1mm width x 0.14mm height x 5mm length). Next, basic solution was added to crosslink the matrix as previously described and washed with PBS to bring the pH back down to 7.4. Finally, a suspension of Schwann cells in media was added to the outlet port and allowed to migrate through the 3D GSH hydrogel for a period of 5 days. A representative image is shown from a longitudinal slice where cells have migrated from the inlet port (white arc) along the channel through a 1 mg/mL C_{16}GSH hydrogel (Figure 5). A range of gel concentrations was used, and the number of cells that migrated into the channel for each gel concentration was counted using ImageJ software (Figure 6). A maximum in number of cells migrated occurs at 0.5mg/mL gels.

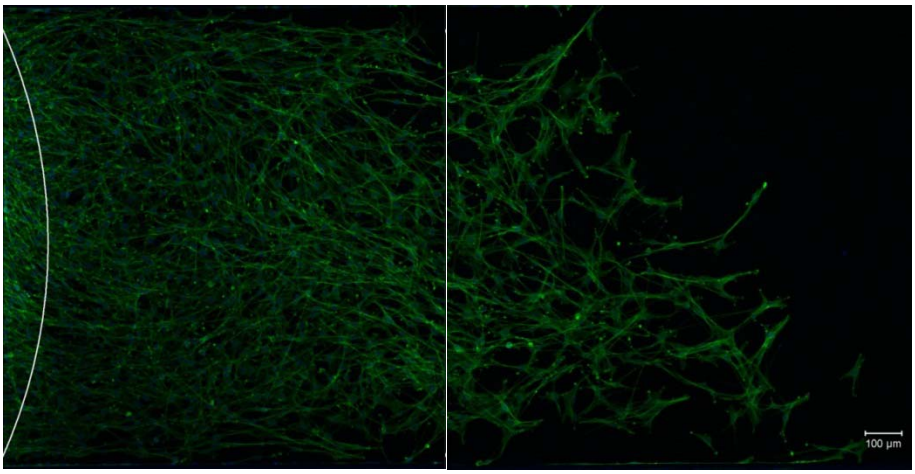

Figure 5. Migration through channels loaded with 1mg/mL C_{16}GSH. White half circles show outline of port where cells were added. Cells were stained with hoescht (blue) and phalloidin (green).

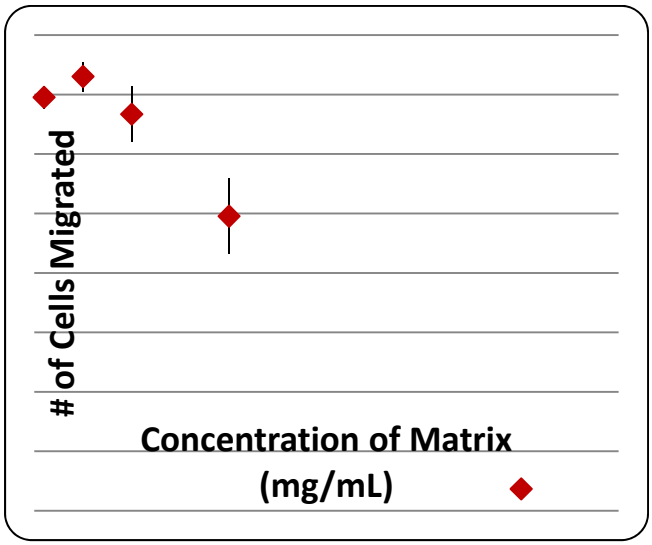

Figure 5. Number of cells migrated into 3D channel per gel concentration after a period of 5 days.

Key Research Accomplishments

- Modulus of gels measured using rheology with varying pH and concentration
- Gel pore structure imaged using scanning electron microscopy
- Cell morphology and proliferation studied on gels of varying modulus
- 3D migration assay developed using commercial microchannel technology
- Migration studied as a function of gel stiffness

Conclusions

1. C_{16}GSH hydrogels span a range of relevant stiffness and can be useful in many regenerative medicine applications to the fibrous, extracellular matrix mimicking, structure.
2. The concentration, (which is directly linked to stiffness) of the C_{16}GSH hydrogels can be tuned to promote the spreading, proliferation, and migration of a model cell type, Schwann cells.
3. Using the relevant in vitro experiments named above a relevant concentration is picked which performs best in the in vitro models, and will be pursued in vivo.

Planned Clinical Transitions
None

Corrections/Changes Planned

Please refer to A above.

Conflict of Interest Disclosure

None

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	0	1
# Post docs	1	0
# grad students	1	2
# undergrad students	0	2
# staff members working for AFIRM	0	0

Other Project Statistics	
# Honors given to AFIRM faculty	1
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	2
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

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1. Engler, Griffin, Sen, Bonnem ann, Sweeney, and Discher. "Myotubes differentiate optimally on substrates with tissue-like stiffness" *Journal of Cell Biology*, 2004: 166,6,877-887.

Reportable Outcomes

Honors and Awards

Matthew Tirrell Awarded 2012 APS Polymer Physics Prize

Peer Reviewed Publications

Brian F. Lin, Katie A. Megley, Nickesh Viswanathan, Daniel V. Krogstad, Laurie B. Drews, Yi C. Qian, and Matthew V. Tirrell. "pH Responsive Branched Peptide Am phiphile Hydrogel Designed for Applications in Regenerative Medicine with Potentia l and Injectab le Tissue Scaffolds" *Journal of Materials Chemistry*, (accepted DOI:10.1039/C2JM31745A)

PROJECT ET-4 TERMINATED

Project ET-5: Material-Induced Host Cell Recruitment for Muscle Regeneration

Team Leader(s)	Sang Jin Lee, PhD (Wake Forest)
Project Team Members	James J. Yoo, MD, PhD, In Kap Ko, PhD, Young Min Ju, PhD, NaJung Kim (Wake Forest)
Collaborator(s)	Shay Soker, PhD (Wake Forest)
Therapy	Treatment of muscle injuries through *in situ* muscle tissue regeneration
Deliverable(s)	*Baseline:* Demonstration of *in situ* muscle tissue regeneration using a target specific scaffolding system *Revised:* None
TRL Progress	Start of Program: TRL #2 End Year 3: TRL #3 End Year 1: TRL #2 End Year 4: TRL #3 End Year 2: TRL #3 End Year 5: TRL #4
Key Accomplishments:	In the past year, we have developed novel injectable and implantable scaffolding systems that effectively released myogenic inducing factors for *in vivo* demonstration of host muscle satellite/progenitor cell differentiation as well as new muscle tissue formation. We have investigated whether the target specific scaffolds can regenerate muscle tissue in a rat muscle defect model.
Keywords	Biomaterials, myogenic-inducing factor, *in situ* tissue regeneration, host stem cell mobilization, volumetric muscle loss, compartment syndrome, muscle regeneration

Introduction

Compartment syndrome is a common traumatic injury which results in muscle, nerve and vessel damage due to increased pressure within a confined space in the body (1). Although compartment syndrome can affect any limb or muscle compartment, including the abdomen, it frequently occurs after trauma to the lower leg such as fracture (2). The standard treatment is fasciotomy, which is considered as the definitive and only treatment for acute compartment syndrome. Although this procedure is able to relieve immediate concerns, muscle weakness and atrophy are continued sequelae (2). 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 the compartment syndrome in wounded soldiers (3-5). In this project we aim to utilize stem or progenitor cells residing in the host to regenerate muscle tissue through the use of a target specific scaffolding system. This approach is based on the demonstration that almost every tissue in the body contains some type of stem or progenitor cells (6-8). The putative healing mechanisms and classic foreign body reaction to implanted biomaterials have also been characterized (9,10). However, these two mechanisms would seem to be in conflict with one another, particularly with respect to functional outcome. While small, localized day-to-day injuries are regenerated by the body's stem and progenitor cell machinery, large traumatic injury overwhelms this system and survival mechanisms take over. This process often creates a deficit

of functional recovery. The specific aim s of this project are to investigate this possibility using an animal model to in itiate cell mobiliza tion, recruitment, and dif ferentiation *in vivo* and to demonstrate *in situ* muscle tissue regeneration using a target specific scaffolding system.
Specific Aims in the Approved Statement of Work:

Aim 1. To develop a biom aterial system that pr omotes stem/progenitor cell m obilization into target specific sites and facilitate differentiation into myogenic lineage *in vivo*

a. To develop and fabricate a reliable biomaterial system (Year 1)
b. To incorporate myogenic-inducing agents with the biomaterial system (Year 1-2)
c. To characterize the biomaterial system (Year 2)

Aim 2. To dem onstrate that m obilized stem/progenitor cells can be dif ferentiated into m uscle cells in a rodent model

a. To evaluate the host response to the biomaterial system in a rodent model (Year 3)
b. To differentiate the recruited cells into the muscle cells within the biomaterial system in a rodent model (Year 3-4)
c. To evaluate the myogenic differentiation within the biomaterial system (Year 3-4)

Aim 3. To demonstrate that functional muscle tissue can be regenerated and repair muscle tissue defects/insufficiency in a rodent model

a. To establish the muscle injured rodent model (compartment syndrome) (Year 3-4)
b. To evaluate the therapeutic potential of the myogenic inducing scaffolds (Year 4-5)
c. To evaluate long-term stability of the scaffolds (Year 4-5)
d.

Research Progress

1. Development of injectable device using heparin-conjugated gelatin microparticles: To immobilized myogenic factors in to the gelatin m icroparticles, heparin was conjugated onto gelatin microparticles (60 – 100 μm). Heparin, a su lfated polysaccharide, has been w idely used as a surface modifier to enhance the stability of biologically im portant proteins such as growth factors and cytokines via binding affinity. This conjugation was pe rformed as described below. The gelatin microparticles were pre-equilibrated in 2-morpholinoethanesulfonic acid (MES, 0.05 M, pH 5.60) for 30 m in. The carboxyl groups of heparin (Sigma–Aldrich) were activated by a reaction with EDC/NHS in MES-buffer (pH 5.60), at a weight ratio of heparin:EDC:NHS of 1:2:1.2 (10 m in, 37°C). After this activation step, the gelatin particles we re immersed into the EDC/NHS activated heparin so lution and incubated for 4 h at 37°C. During this incubation period, heparin became conjugation on the gelatin microparticles as confirmed by Toluidine Blue staining (**Figure 1A**).

For the protein release kinetics, lysozyme was used as a m odel protein. The lysozym e was loaded onto both heparin-conjugated and unmodi fied gelatin microparticles using a so lution dropping method. 60 μL of PBS c ontaining 120 μg of lysozym e was dropped onto the dried gelatin microparticles and the sam ples were allo wed to react at 4°C for 12 h to allow protein loading. The protein-immobilized gelatin microparticles were then suspended in 1 m L PBS (containing 0.1 % bovine serum albumin) at 37°C. At various tim e points, the supernatants were

collected and the amount of released lysozyme was determined by direct absorbance using a spectrophotometer (405 nm) (**Figure 1B**).

To evaluate effects of myogenic factors, we developed heparin-conjugated gelatin microparicles which immobilized IGF-I via electrostatic interactions. For *in vivo* study, a traumatic defect was created by excising approximately 30-40% of TA muscle of SD male rats (age: 10-12 weeks). IGF-1-immobilized biomaterial system were implanted in the TA muscle defect injury of rats and retrieved at 1, 2, and 4 weeks after implantation (**Figure 1C**). The retrieved tissue samples were characterized by histological and immunohistochemical staining.

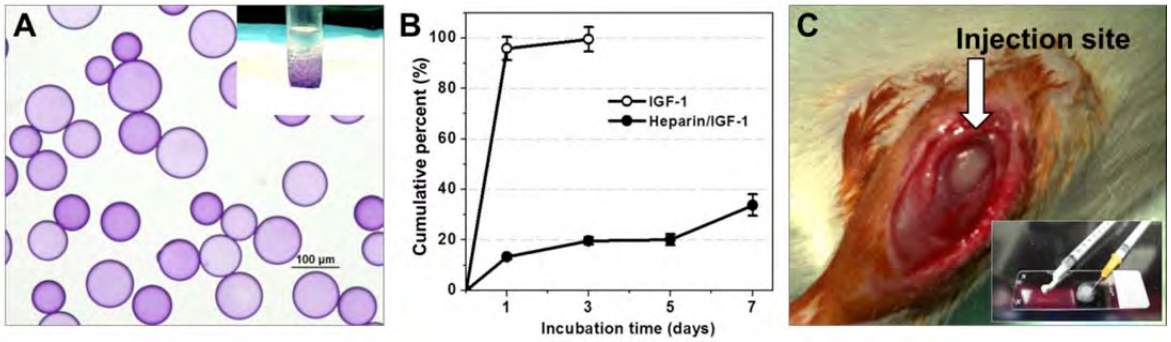

Figure 1. (A) Heparin-conjugated gelatin microparticles as confirmed by Toluidine Blue staining. (B) Release profiles of IGF-1 from heparin-conjugated gelatin microparticles. (C) IGF-1-immobilized gelatin microparticles were injected in the TA muscle defect injury of rats.

The myogenic factor (IGF-1) released from gelatin microparticles has effectively promoted myogenic cell migration and regenerated newly formed muscle tissue in *vivo* (**Figure 2**). By the 1st and 4th weeks, host cells had accumulated within the injury region and abundant host vasculature was found within the region. H&E and immuostaining for myosin heavy chain (MHC) of representative sections after 4 weeks of injection showed the gradual buildup of a neo-muscle fiber bundle structure around the IGF-1-loaded gelatin microparticles **(Figure 2)**.

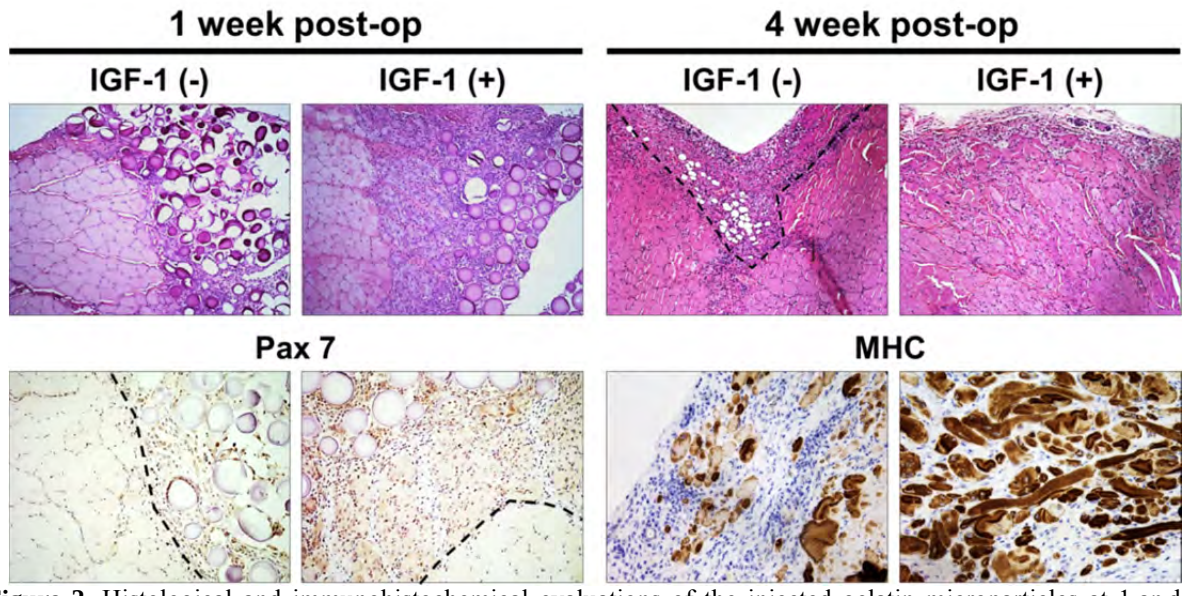

Figure 2. Histological and immunohistochemical evaluations of the injected gelatin microparticles at 1 and 4

weeks post operation (×100).

2. Development of implantable device using heparin-conjugated decellularized muscle matrix: Muscle scaffolds were fabricated by decellularization of tibialis anterior (TA) muscle of Sprague-Dawley (SD) rats using Triton X-100 and ammonium hydroxide (**Figure 3A,B**). For efficient delivery of SDF-1α and IGF-1, heparin molecules were conjugated on the decellularized muscle scaffold using EDC/NHS chemistry (**Figure 3C**).

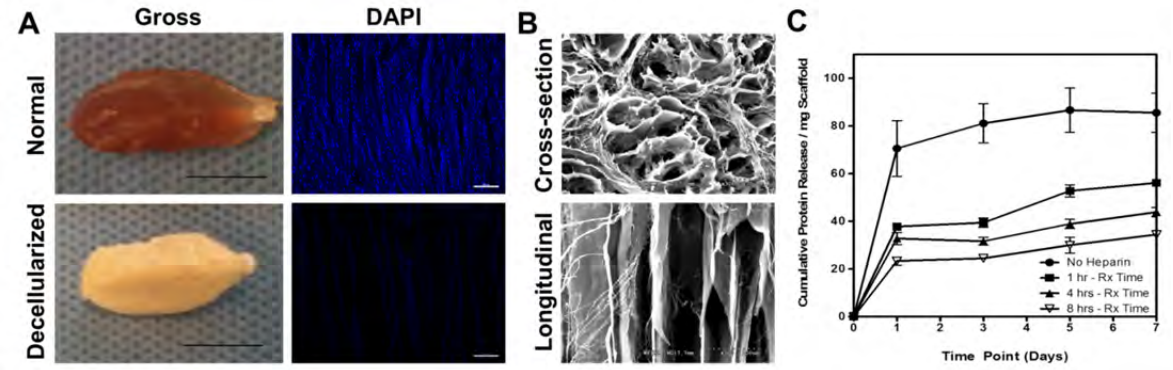

Figure 3. (A) Complete decellularization of tibialis anterior (TA) muscle, confirmed by DAPI staining, (B) while maintaining of structural integrity of TA muscle. (C) Release profiles of protein released from heparin-conjugated decellularizd muscle matrices.

For *in vivo* study, a traumatic defect was created by excising approximately 30-40% of TA muscle of SD male rats (age: 10-12 weeks). Combination delivery with heparin-conjugated decellularized muscle matrices were implanted in the TA muscle defect injury of rats and retrieved at 1, 2, and 4 weeks after implantation.

Our results show that our combination delivery system enhanced the recruitment of satellite cells and pericytes into the muscle-targeting scaffold and also facilitated more well-aligned newly formed myofibers into the interface and interior of the implants than uncrosslinked scaffolds and no delivery system controls. Furthermore, morphological integration of newly formed myofibers with host muscle is confirmed (**Figure 4**). The incorporation of multiple regulatory signals into a scaffolding system and our combined delivery may be a promising approach for more efficient and effective muscle regeneration *in situ*.

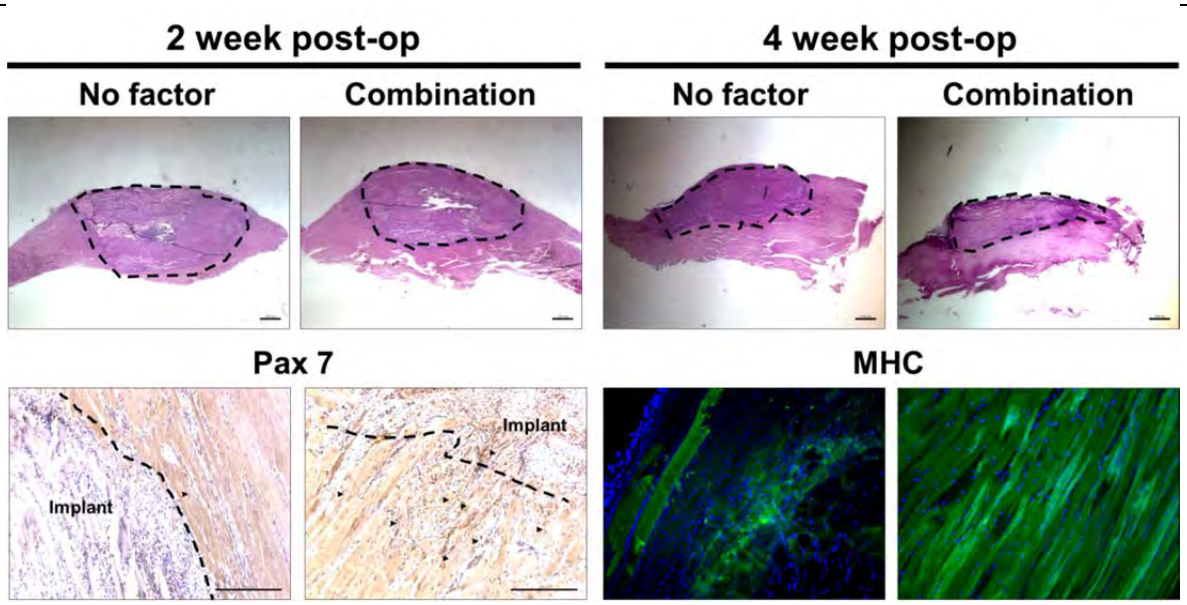

Figure 4. H&E staining of the harvested TA muscle tissue. The implants are identified by circled areas in the low magnified images. Immunohistochemical staining for Pax 7 for host stem cells infiltrated into the implants at 2 weeks. Immunofluorescent staining for MHC to identify mature muscle fibers adjacent to the implants. Combination delivery system induced efficient integration of newly formed myofibers in the interior sites with the host muscle tissue.

Key Research Accomplishments

- Development of injectable device using heparin-conjugated gelatin microparticles
- Development of implantable device using heparin-conjugated decellularized muscle matrix
- *In vivo* demonstration of host muscle satellite/progenitor cell infiltration into myogenic-inducing factor incorporated scaffolding systems
- *In vivo* demonstration of host stem cell mobilization and muscle regeneration using the combination delivery system

Conclusions

We have successfully completed the tasks proposed for Year 4 and met the proposed milestones. We evaluated various myogenic-inducing factors for muscle cell migration, proliferation, and differentiation *in vitro* and investigated the possibility of using an appropriate biomaterial to initiate cell mobilization and recruitment *in vivo*. This study suggests that it may be possible to use the body's biologic and environmental resources for *in situ* muscle tissue regeneration.

In the past year, we have developed novel injectable and implantable devices, which can mobilize host muscle cells and form neo-muscle tissues in a muscle defect region in rats. This study suggests that it may be possible to use the body's biologic and environmental resources for *in situ* muscle tissue regeneration. We 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.

Research Plans for the Following Years

Plans include:
Continue long-term *in vivo* evaluation of the myogenic-inducing factor incorporated biomaterials

Continue development of a smart scaffolding system for application

In vivo studies investigating *in situ* muscle tissue regeneration in a compartment syndrome rat model

Planned Clinical Transitions

This basic research project is not slated for clinical trials during the 1st 5 years of the award.

Corrections/Changes Planned

None

Conflict of Interest Disclosure

None

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	3	
# Post docs	2	
# grad students	0	
# undergrad students	0	
# staff members working for AFIRM	0	1

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	1
# Non-peer reviewed publications	19

*Directly supported in whole or part –time by AFIRM

Peer Reviewed Publications

1. Ko IK, Ju YM, Chen T, Atala A, Yoo JJ, and Lee SJ, Combined systemic and local delivery of stem cell inducing/recruiting factors for *in situ* tissue regeneration, *FASEB J.*, 26(1), 158-168 (2012)

Non-Peer Reviewed Publications (Abstracts)

1. Ju YM, Hwang CM, At ala A, Yoo JJ, Atala A, and Lee SJ, Biom aterial induces host stem cell recruitment for in situ m uscle regeneration, the Society for Biom aterials 2010 Annua l Meeting and Exposition: Giving LIFE to a wo rld of m aterials, April 21-24, 2010, Seattle, WA, USA
2. Ju YM, Yoo JJ, Atala A , Lee SJ, Biom aterial induced host stem cell recruitment for in situ muscle regeneration, 2010 Advanced Technology Applications for Combat Casualty Care (ATACCC), August 16-19, 2010, St. Pete Beach, FL, USA
3. Ju YM, Yoo JJ, Atala A, Lee SJ, Biom aterial induces host stem cell recruitment for in situ muscle tissue regeneration, 18th Annual Residents' and Fellows' Research Day, Novem ber 12, 2010, Wake Forest University School of Medicine, Winston-Salem, NC, USA
4. Ko IK, Chen T, Ju YM, Yoo JJ, Atala A, Lee SJ , Enhancing stem cell recruitment for in situ tissue regeneration, 18th Annual Residents' and Fellows' Research Day, November 12, 2010, Wake Forest University School of Medicine, Winston-Salem, NC, USA
5. Ju YM, Yoo JJ, Atala A, Lee SJ, Biom aterial induces host stem cell recruitment for in situ muscle tissue regeneration, North Carolina Ti ssue Engineering & Re generative Medicine Conference, November 13, 2010 in NC Bi otechnology Center, Tr iangle Park, North Carolina, USA
6. Ko IK, Chen T, Ju YM, Yoo JJ, Atala A, Lee SJ , Enhancing stem cell recruitment for in situ tissue regeneration, N orth Carolina Tissu e Engineering & Rege nerative Medicine Conference, November 13, 2010 in NC Bi otechnology Center, Tr iangle Park, North Carolina, USA
7. Ju YM, Yoo JJ, Atala A, Lee SJ, Biom aterial induces host stem cell recruitment for in situ muscle tissue regeneration, Tissue Engineerin g and Regenerative Medicine International Society (TERMIS)-North America, December 5-8, 2010, Hilton in the Walt Disney W orld Resort, Orlando, FL, USA
8. Ko IK, Chen T, Ju YM, Yoo JJ, Atala A, Lee SJ , Enhancing stem cell recruitment for in situ tissue regeneration, Tissue Engi neering and Regenerative Medi cine International Society (TERMIS)-North America, December 5-8, 2010, Hilton in the W alt Disney World Resort, Orlando, FL, USA
9. Ju YM, Atala A, Yoo JJ, Lee SJ, Biom aterial-Induced Host Stem Cell Recruitm ent for *In Situ* Muscle Tissue Regeneration, 3rd AFIRM " All Hands" Meeting, January 17-20, 2011 i n Hilton Clearwater Beach Resort, Clearwater, FL, USA
10. Ko IK, Chen T, Ju YM, Andersson K-E, At ala A, Yoo JJ, Lee SJ, Enhancing Stem Cell Recruitment for *In Situ* Tissue Regeneration, 3rd AFIRM "All Hands" Meeting, January 17-20, 2011 in Hilton Clearwater Beach Resort, Clearwater, FL, USA
11. Ju YM, Yoo JJ, Atala A , Lee SJ, Biom aterial induced host stem cell recruitment for in situ muscle tissue regeneration, Society for Bi omaterials 2011 Annual Meeting & Ex position, April 13-16, 2011, Disney's Contemporary Resort, Orlando, FL, USA
12. Ko IK, Ju YM, Chen T, Atala A, Yoo JJ, Le e SJ, Enhanc ing Stem Cell Recruitment for In Situ Tissue Regeneration, Advanced Technolo gy Applications for Combat Casualty Care (ATACCC) 2011 Conference, August 15-18, 2011, Marriott-Harbor Beach Co nference Center, Fort Lauderdale, FL, USA

13. Ju YM, Atala A, Yoo JJ, Lee SJ, Host stem cell recruitment using a target specific scaffold for in situ muscle tissue regeneration, North Carolina Tissue Engineering & Regenerative Medicine Society 13th Annual Conference, November 4, 2011, Old Salem Visitors Center, Winston-Salem, NC, USA
14. Ko IK, De Leon J, Lee B, Atala A, Yoo JJ, Lee SJ, Enhancing host stem cell recruitment for in situ muscle regeneration, North Carolina Tissue Engineering & Regenerative Medicine Society 13th Annual Conference, November 4, 2011, Old Salem Visitors Center, Winston-Salem, NC, USA
15. Ju YM, Yoo JJ, Atala A, Lee SJ, Host stem cell recruitment using a target specific scaffold for in situ muscle tissue regeneration, The TERMIS-NA 2011 Annual Conference & Exposition, December 11-14, 2011, Hilton Americas-Houston, Houston, TX, USA
16. Ko IK, Ju YM, De Leon J, Atala A, Yoo JJ, Lee SJ, Enhancing host stem cell recruitment for in situ muscle regeneration, The TERMIS-NA 2011 Annual Conference & Exposition, December 11-14, 2011, Hilton Americas-Houston, Houston, TX, USA
17. Ju YM, Atala A, Yoo JJ, Lee SJ, Biomaterial Induced Host Stem Cell Recruitment for In Situ Muscle Tissue Regeneration, 4th AFIRM "All Hands" Meeting, February 13-16, 2012 in TradeWinds Resort, St. Pete Beach, FL, USA
18. Ko IK, Ju YM, De Leon J, Atala A, Yoo JJ, Lee SJ, Combined Systemic and Local Delivery of Stem Cell Recruitment for In Situ Muscle Regeneration, 4th AFIRM "All Hands" Meeting, February 13-16, 2012 in TradeWinds Resort, St. Pete Beach, FL, USA
19. Lee SJ, Ju YM, Ko IK, Yoo JJ, Atala A, Biomaterial induce stem cell recruitment for in situ tissue regeneration, The Annual Hilton Head Workshop; Regenerative Medicine, Harnessing Biology for Regeneration, March 14-17, 2012 in the Sea Pines Resort, Hilton Head Island, SC, USA

PROJECT ET-6 TERMINATED

Project ET-7: Scarless Wound Healing through Nanoparticle-mediated Molecular Therapies

Team Leader(s)	Sandeep Kathju, MD, PhD,University of Pittsburgh
Project Team Members	Latha Satish, MSc, MPhil, PhD, University of Pittsburgh
Collaborator(s)	None
Therapy	Formulation containing siRNAs that can be applied to wounds to mitigate scar formation; probiotic therapy to burn wounds to inhibit pathogenic infection and reduce scar
Deliverable(s)	*Baseline:* to arrive at a formulation of molecular agents that can be applied to healing wounds such that they repair with diminished or absent scar formation using nanoparticulate technology. 2. to employ probiotic therapy to reduce infection and scar in a burn wound scenario. *Revised:* None
TRL Progress	Start of Program: TRL #1 End Year 3: TRL #3 End Year 1: TRL #1 End Year 4:: TRL #4 End Year 2: TRL #3
Key Accomplishments:	This project uses siRNA in novel nanoparticulate formulations to mitigate scar formation in healing wounds. siRNA versus CCT-eta applied to incisional wounds was demonstrated to reduce α-smooth muscle actin in healing wounds, suggesting an inhibition of myofibroblast activity. A novel probiotic therapy for infected burn wounds using Lactobacillus was applied to a new mouse model and is capable of rescuing the animal from burn wound induced sepsis and death
Keywords	Scarless wound healing, nanoparticles, siRNA, probiotics, burns

Introduction

The purpose of this project is to arrive at technologies that will enable the reduction of scar formation after injury. Scar, while useful in sealing an injured area, is also the source of significant morbidity, including restriction of movement (eg. in tendons and muscle), narrowing of viscera, entrapment of nerves, etc. (as well as the psychosocial damage associated with severe facial disfigurement). Burn injuries are particularly prone to extensive and crippling hypertrophic scarring.

Mammalian fetal wound healing proceeds without scar and has served as a model for our investigations. We have noted that the chaperonin containing T-complex polypeptide subunit eta (CCT-eta) is specifically reduced in fetal wounds, but increased in scar-forming adult wounds. CCT-eta is permissive for the accumulation of α-smooth muscle actin (α-SMA) and therefore the function of myofibroblasts. We are using siRNA constructs that deplete CCT-eta to attempt to reconstitute a more fetal pattern of wound healing in adult wounds and thereby mitigate scar formation.

A second focus has been on the use of probiotic intervention using Lactobacillus to reduce scar from burn wound infection. We have tested bacteriotherapy with Lactobacillus in a rabbit model of Pseudomonas-infected burn injury as a coun termeasure to the hypertr ophic scarring that can typically ensue.

We have previously rep orted that siRNA versus CCT-eta can reduce d eposited collagen in a healing wound, while actually improving its tensile strength. We have also noted that probiotic therapy of infected bu rn wounds results in attenuation of the infection and res ulting scar deposition (see AFIRM Annual Report 2011, pp. 278-285). We now report further studies along these two lines of enquiry.

Research Progress

siRNA versus CCT-eta as an anti-fibrotic agent

We have continued to analyze various molecular and biochemical parameters in our rabbit model of adult incisional wound healing. We have examined whether in vivo siRNA therapy using our nanoparticulate formulation results not only in reduction of targ et mRNA, but also of target protein species. W e have previously shown that siRNA versus CCT-eta effectiv ely depletes CCT-eta protein *in vitro* (Satish et al., 2010a). Western blot analysis of healing wounds was therefore carried out to determine if the same could be achieved *in vivo* (Figure 1).

Animals were wounded on their dorsum s and wounds were treated with either vehicle only, CCT-eta siRNA, or a scram bled control siRNA. After 4 weeks wounds were re-excised and assayed for both CCT-eta and th e downstream target pro tein α-SMA. An incre ase in CCT-eta protein was seen in wounded specim ens compared to control unwounded skin, consistent with our previous results (S atish et al., 2010b). C CT-eta siRNA significantly decreased CCT-eta protein expression, whereas the nonspecific control siRNA had no such effect.

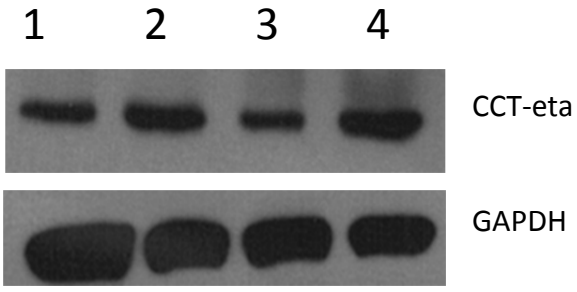

Figure 1. Expression of CCT-eta protein in healing wounds. 1 = control unwounded skin. 2 = co ntrol wound with vehicle only. 3 = wound treated with CCT-eta siRNA. 4 = wound treated with scrambled siRNA. A representative image of five replicate experiments is shown. GAPDH was used as the loading control.

Similar results were noted with α-SMA; treatment of wounds with CCT-eta siRNA significantly diminished α-SMA expression, but control scrambled siRNA had no such effect (Figure 2).

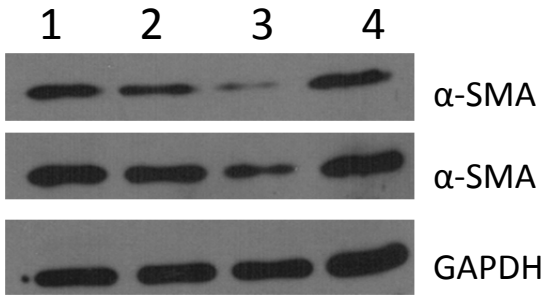

Figure 2. Expression of α-SMA protein in healing wounds. 1 = control unwounded skin. 2 = control wound with vehicle only. 3 = wound treated with CCT-eta siRNA. 4 = wound treated with scrambled siRNA. Two images from three replicate experiments are shown. GAPDH was used as the loading control.

These results reinforce our previous observations that siRNA versus CCT-eta can meaningfully and beneficially modulate the physiology of a healing adult wound.

Suppression of scar in infected burn wounds by probiotics

We have previously observed that a single administration of prophylactic probiotic therapy with Lactobacillus is sufficient to attenuate the length and severity of a Pseudomonal infection of the burn wound, and that this therapy also significantly reduces the amount of deposited collagen in the wound, amounting to a reduction in the scar burden. Over this past year we have processed more samples to finalize data from more animals/specimens, such that conclusive data may be obtained. We now believe that this expanded data set is complete, and it is being readied for publication. Because no new conclusions are at hand, we have not reproduced the data here.

Concomitant with that effort, we have established a new model of probiotic therapy that demonstrates that this approach can rescue an animal from burn wound induced sepsis and death.

Additional Note: On July 1, 2011, the AFIRM investigators on this project (Drs. Sandeep Kathju and Latha Satish) changed institutions, taking positions at the University of Pittsburgh. AFIRM funds were only made available for continuation of this project in February 2012.

Key Research Accomplishments

- We have determined that sustained administration of CCT-eta siRNA does deplete our target proteins of interest.
- We have completed the data set that shows that probiotic therapy with Lactobacillus is effective in mitigating scar deposition in an infected burn wound.
- We have established a new animal model of burn wound infection.
- We have demonstrated that septic translocation leading to death occurs in this animal model.
- We have demonstrated that probiotic therapy can rescue this animal model from burn wound-induced sepsis and death.

Conclusions

We conclude that siRNA versus CCT-eta, delivered as a complexed nanoparticle in an agarose matrix, can effectively deplete its cognate protein and inhibit scar formation without any deleterious effects on wound healing.

We also conclude that probiotic therapy with *Lactobacillus plantarum* can effectively abrogate Pseudomonal (and possibly other) infections, and significantly mitigate the scarring that can ensue after such infected burn injuries. We further conclude that such probiotic therapy can rescue a burn-infected organism from sepsis and death.

Research Plan for the Following Years

With regard to our siRNA-mediated anti-fibrotic therapy, we will plan to re-capitulate our system in a porcine model (which more closely mimics human skin), and examine whether our agents are effective in burn wounds as well as incisional wounds. In addition, we will investigate if an injectable formulation of our siRNA is practical/effective.

With regard to burn injury and probiotics, we will continue to define the utility of probiotics in reducing the local and systemic inflammation elicited by an infected burn wound, and attempt to delineate the mechanisms involved. We will also test the ability of probiotics to treat already infected burn wounds, and determine how to counteract other burn wound pathogens with probiotics.

Planned Clinical Transitions

At this point, both interventional strategies have essentially demonstrated proof-of-concept benefit in animal models, although some minor clarifying work still needs to be finished. The siRNA formulation is ready to proceed to a porcine model (which more closely resembles human skin architecture) as the final step before considering Phase I studies in human to evaluate for safety, toxicity, immunogenicity etc.

The probiotic therapy has several possible routes to clinical use. Probiotics already have a much more extensive history of clinical use in other scenarios (eg. gastrointestinal and genitourinary infections), and direct application of live bacteria onto burn wounds may be one avenue. Another possibility is to construct dressing materials for burn wounds that incorporate dehydrated (and rehydratable) probiotic agents as an off-the-shelf therapy. We will explore with our Burn surgeon colleagues the most optimal pathways forward in this regard.

Corrections/changes planned for next year and rationale for changes

In order to have the strongest data set possible, we will expand the use of our siRNA formulation to a porcine burn injury model. This will be the final animal data to obtain before moving to clinical trial planning. We will also investigate if an injectable form of our agent is practical/effective.

With our probiotic therapy, we will attem pt to unders tand the m echanism by which it is effective, and especially attempt to determine if whole bacteria are required or whether they are secreting some product that may in itself be sufficient.

Conflict of interest disclosure

The Investigators have no conflicts of interest to disclose.

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Reportable Outcomes

Personnel Statistics	Paid* Unpaid
# Faculty working on AFIRM projects	2
# Post docs	2
# grad students	0
# undergrad students	0
# staff m embers working for AFIRM	1

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	0
# Masters degrees aw arded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	1 pending
# Peer reviewed publications	1
# Non-peer reviewed publications	0

*Directly supported in whole or part –tim e by AFIRM

Patents and Inventions:

Patent application pending: "Compositions and Methods for Reduced Scarring in Healing Wounds and for Treatment and Prevention of Fibrosis."

Peer-Reviewed publications:

Satish L, Lo N, Gallo PH, Johnson S, Haberman S, Kathju S. Chaperonin containing T-complex polypeptide (CCT) subunit expression in oral mucosal wounds and fibroblasts. Cell Stress Chaperones. 2011 Nov;16(6):675-80.

Project ET-8: Oxygen-Generating Biomaterials for Large Tissue Salvage

Team Leader(s)	Benjamin Harrison, PhD (Wake Forest University)
Project Team Members	Benjamin Rowe, MS (Wake Forest University), Catherine Ward, Ph.D. (Wake Forest University)
Collaborator(s)	George Christ PhD (Wake Forest University), James Yoo MD PhD (Wake Forest University), Shay Soker (Wake Forest University)
Therapy Deliverable(s)	Supply temporary oxygen to hypoxic tissue Baseline: Injectable oxygen generating materials for tissue salvage Revised: None
TRL Progress	Start of Program: 2 End Year 1: 2 End Year 3: 3 End Year 2: 3
Key Accomplishments:	A controllable, injectable, oxygen-generating biomaterial has been created. The material has been tested for sustained release of oxygen *in vitro* and feasibility of injection *in vivo*.
Keywords	oxygen, tissue engineering, tissue salvage, hypoxia, ischemia

Introduction

Replacement or restoration of tissue loss caused by traumatic injury, congenital defects, tumor removal or severe burns is a challenge. For example, current treatment for reconstruction of volumetric muscle loss is associated with donor site morbidity and limited functional restoration. In addition, following traumatic injury or chronic peripheral vascular disease, vascular integrity is compromised (or absent) and the metabolic needs of downstream organs/tissue will not be met. Both scenarios produce an ischemic environment, which if not corrected, can result in decreased organ/tissue function and, ultimately, tissue necrosis. Restoring blood flow can take time, as natural angiogenesis 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 angiogenesis. While such approaches are able to stimulate host tissue responses associated with neovascularization, the extended time needed to establish the vascular network may be inadequate.

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

Particulate oxygen generators (POGs) are particles that have the ability to release oxygen when placed in aqueous environments such as in culture or in the body.

Previously published work has shown that these materials can provide oxygen in an ischemic skin flap model in mice (3) and in cell culture (4). Our project has shown that POGs can provide a supplemental source of oxygen for cells and tissue in a hypoxic environment and increase their

viability and minimize apoptosis and necrosis. Our ultimate goal is to develop a method to maintain cell and tissue viability during the time it takes for a vascular network to be established or repaired.

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

POGs may overcome one of the major limitations in muscle salvage and tissue engineering by acting as a supplemental oxygen source in several regenerative models.

Therefore, the goal is to provide oxygen at a therapeutic concentration and not necessarily to replicate standard cell culture conditions. The materials used are based on encapsulated solid peroxides that decompose upon contact with water to oxygen, water and other biocompatible byproducts. Examples of the chemical equations governing oxygen generation are shown below:

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

During year 1, efforts were primarily focused on identifying formulations capable of generating oxygen which were non-toxic to cells and could be delivered as an injectable.

During year 2, the laboratory has begun to use the technology developed during year 1 to establish collaborations with other AFIRM investigators and use the POG technology in relevant *in vivo* models.

During year 3, we have demonstrated that our POG technology can be tolerated well and can improve the functional muscle responses over non-treated controls. Optimization of our in vivo models needed to be continued to move our research forward.

Research progress

Development and optimization of hind limb ischemia model to evaluate the utility of POGs for preservation of tissue structure and function.

Skeletal muscle is a highly metabolic tissue at major risk of irrecoverable functional loss following traumatic injury and development of peripheral vascular disease in both military and civilian populations. As such, skeletal muscle provides an excellent model system for evaluating the ability of POGs to preserve tissue structure and function. An extended series of studies were conducted using leveraged funding from the NIH and have established the boundary conditions for POG-mediated preservation of skeletal muscle tissue structure and function *in vitro* (Ward et al., manuscript in preparation). The goal of this past year of investigations was to establish an *in vivo* model for evaluating the applicability of POGs. In this regard, several experimental procedures were reviewed as potential candidates for development of a hind limb ischemia model to test the POG technology. For example, we previously placed a pressure cuff on the hind limb for three hours to create an ischemic injury, and 14 days later force measurements were recorded (see Figure 1; dorsoflexion of the foot to electrical stimulation of the peroneal nerve). These initial studies showed no significant differences in the functional responses between the injured and non-injured animals, and moreover, we determined that using a pressure cuff produces a tourniquet effect resulting in unwanted nerve damage; this neuropathy, in turn,

impedes our ability to study the impact of POGs. To avoid this complication, we redesigned our experimentation.

Our reworked experimental design created ischemia to the rat hind limb via arterial ligation. Through multiple iterations, we concluded that to counter the robust collateral vascularization inherent in rodents, the iliac artery and vein needed to be ligated directly distal to the abdominal bifurcation. Moreover, this approach also allows the animal's contralateral limb to serve as an internal control for future experiments. After a twenty-four hour recovery period, we will deplete the oxygen reserve in the tibialis anterior muscle via electrical stimulation of the peroneal nerve. The experimental setup is shown in Figure 1.

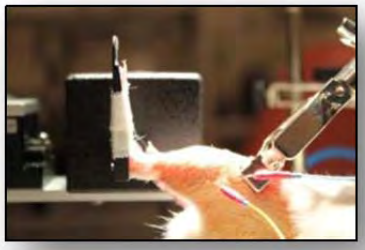

Figure 1: *In vivo* servomotor with foot pedal, illustrating stimulus through electrodes positioned around nerve. Contraction of hindlimb muscles (anterior crural compartment) is measured as torque.

Functional assessments *in vivo* were performed using a customized servomotor (Aurora Scientific) with a foot pedal. Electrodes were placed in the limb, surrounding the common peroneal nerve, to elicit muscle contraction.

Our initial results show the promising potential of the POG technology (Figure 2). In this pilot study we evaluated three different experimental groups: a) a non-injured control, b) an ischemic injury group that did not receive POG treatment, and c) an ischemic injury group that received a single POG injection. Our results are summarized in Figure 2. As illustrated, with POGs on board, the TA muscle exhibited a 25-35% functional contractile reserve for up to 30 minutes of stimulation (i.e., stimulating every 5 minutes as depicted in Figure 2).

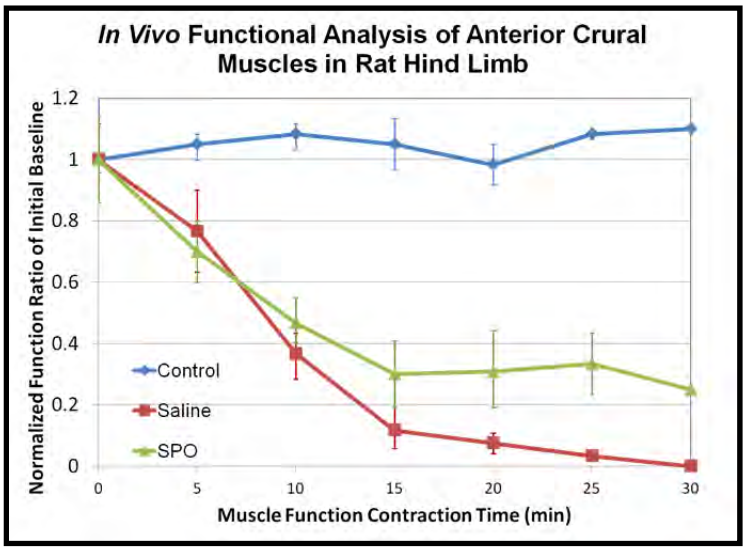

Figure 2: Force Measurement Comparison between Non-Injured, POG-treated, and Saline-treated Ischemic Animals. POG-treated animals showed a 25-35% functional reserve as compared to non-treated injuries.

Parallel morphological differences were also documented between experimental groups. For example, the POG-treated experimental group showed similar fiber morphology to the non-injured control, while the non-treated (saline injection) injury group displayed signs of irregularly-shaped muscle in cross-

section and early signs of necrosis (Figure 3). C onsistent with these findings, our initial studies also documented that the POG-treated group mainta ined similar glycogen levels to non-injured animals, while non-treated groups displayed significant glycogen depletion. This clearly suggests that the POG-treated group can m ore efficiently metabolize glycogen via the aerobic respiration pathway, while the non-treated group is relegated to anaerobic respiration.

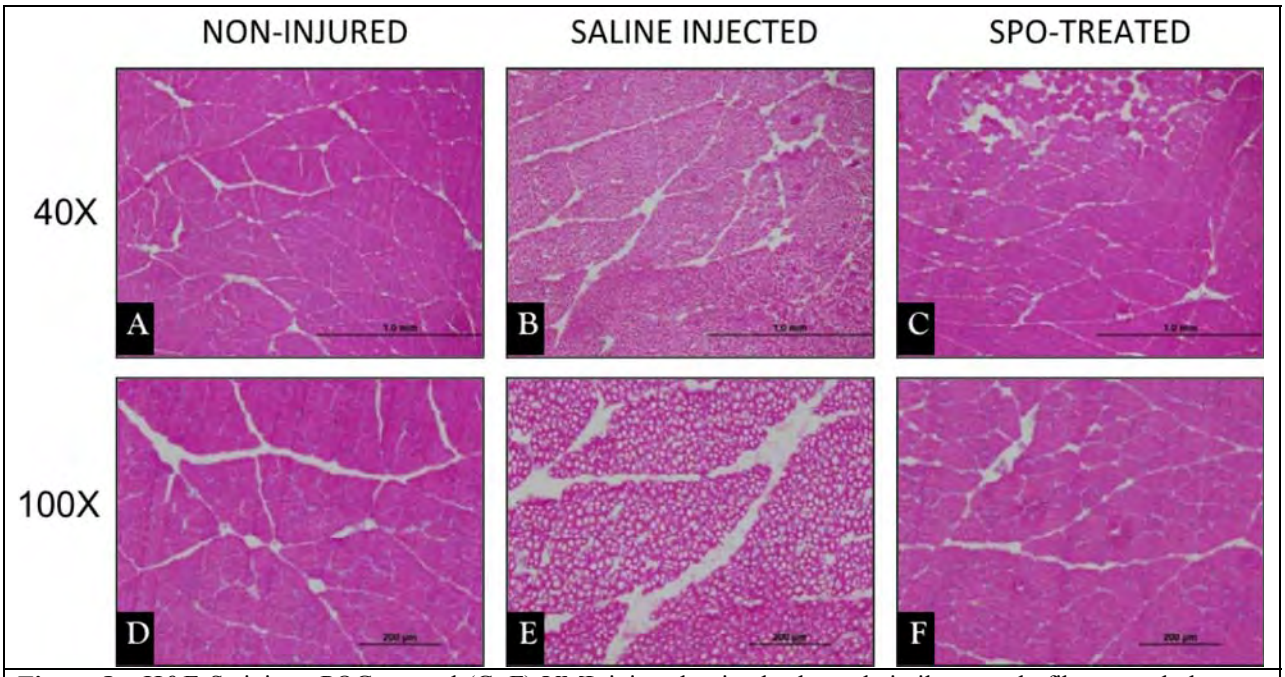

Figure 3: H&E Staining. POG-treated (C, F) VML injured animals showed similar muscle-fiber morphology to Non-Injured (A, D). Non-treated Injuries (B, E) showed irregular-shaped fibers and early signs of necrosis.

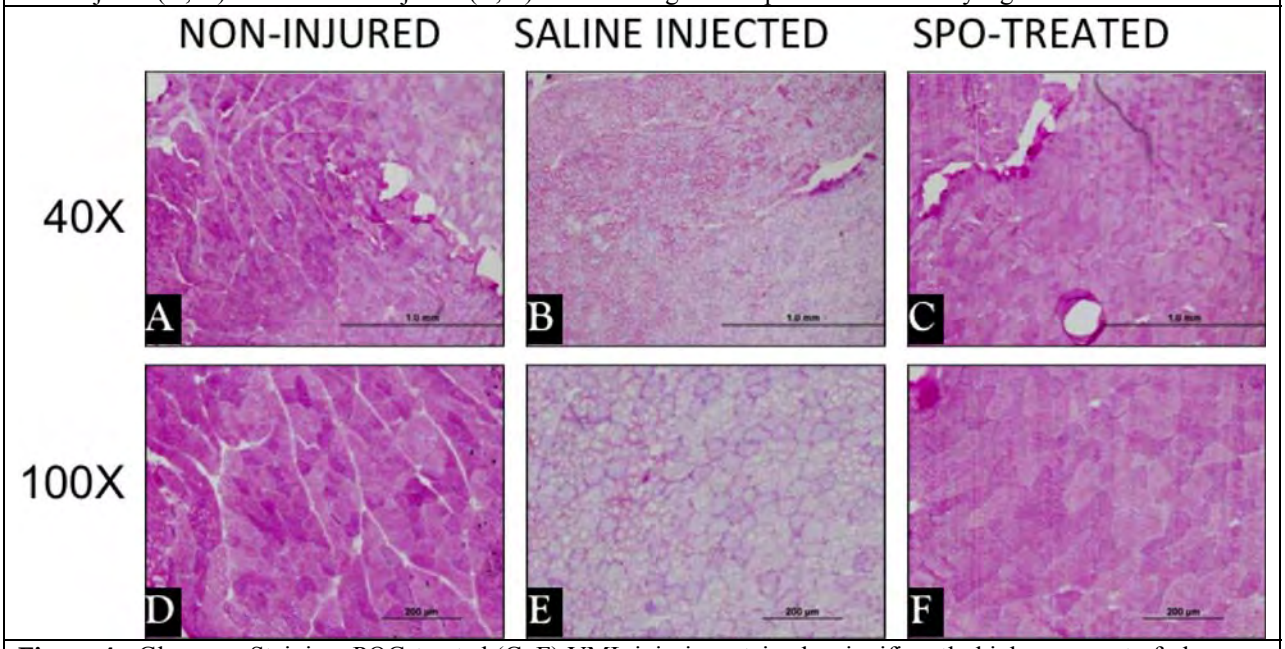

Figure 4: Glycogen Staining. POG-treated (C, F) VML injuries retained a significantly higher amount of glycogen than Non-treated injuries (B, E).

While further studies are clearly required, this pilot study shows that POGs have the potential to be beneficial for the preservation of skeletal muscle structure and function in an acute ischemic environment *in vivo*.

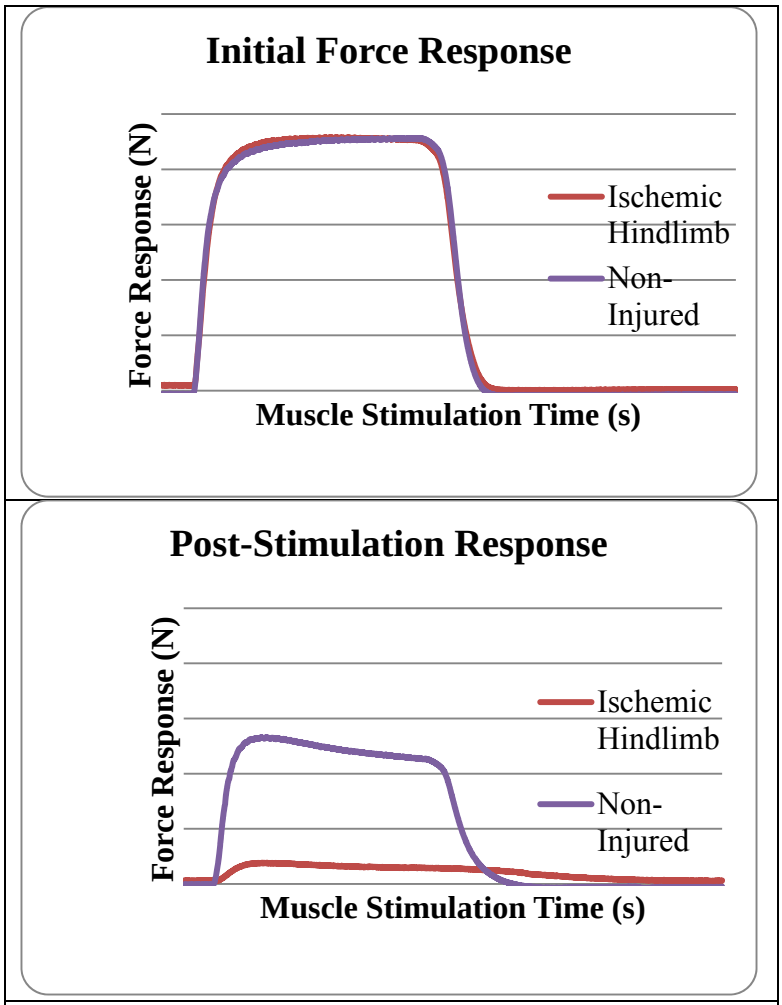

Figure 5: Initial Tetanic Force Measurement Curves of Ischemic versus Non-Injured Hind limb TA Muscles Before and After a Fatigue-Inducing Sub-maximal Stimulation Protocol.

To further expand the utility and "signal-to-noise" ratio in our *in vivo* studies, we wanted to develop an animal model which would better mimic a clinically relevant injury, i.e. compromised or injured vasculature requiring limb salvage from, for example, battlefield casualties, PAD (peripheral arterial disease), or extremity thrombosis. The above pilot study utilized a periodic maximal tetanic contraction, which suboptimally addresses this more realistic scenario. Therefore, our near-term goal is to standardize an experimental protocol which induces ischemia in a two-step process, by 1) removing blood flow to the hind limb via arterial ligation (as described previously) and 2) fatiguing the tibialis anterior muscle through sub-maximal contraction.

Our initial findings have been promising. Following a 24-hour post-ligation surgical recovery time period, we see similar tetanic responses from both non-injured and ligated hind limb animal groups. After fatiguing the hind limb via a continual sub-maximal stimulation protocol, we see a significant decrease in a second tetanic stimulus from our ischemic injury group as compared to our non-injured controls (Figure 5). More work is needed to increase our group size, and to monitor a time-course recovery of our injured versus non-injured experimental groups.

Key Research Accomplishments

- Demonstrated that POGs can preserve skeletal muscle structure and function *in vivo* in an ischemic environment.

- Initial development of an improved *in vivo* model for evaluating the utility of POGs for preservation of skeletal muscle structure and function under clinically relevant ischemic conditions.

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 Following Years

Animal studies will continue to evaluate the efficacy of the material. The technology will be tested in multiple systems where ischemia/hypoxia may cause detrimental effects and POGs may be most beneficial. Because organs are composed of multiple cell types, we will continue to analyze several different tissues 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 in the last year will be to optimize the model and delivery protocol for POGs to demonstrate physiologically relevant improvement in skeletal muscle structure and function under clinically relevant experimental conditions.

Translation Strategy

The ultimate deliverable of this project is to provide an oxygen delivery system to improve the viability of hypoxic tissue. While this project is currently at TRL 3, it is anticipated that a clinical trial will be ready within the next few years. Table 1 details the target product profiles of this project. This project is expected to produce the following product that will potentially enter clinical stages during the first 5 years of AFIRM.

Planned Clinical Transitions

While no immediate human clinical trials are currently slated under this AFIRM project, options are being explored including multiple pathways to incorporate into other AFIRM research as well as to leverage other funds to accelerate the time to clinic.

Corrections/Changes Planned

For the next year, the laboratory will continue to focus on the potential application of POGs for muscle tissue preservation in an ischemic injury.

Conflict of Interest Disclosure

The research team has no conflicts to disclose.

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Reportable Outcomes

Personnel Statistics	Paid*	Unpaid
# Faculty working on AFIRM projects	2	
# Post docs	1	
# grad students	1	
# undergrad students	0	
# staff members working for AFIRM	1	

Other Project Statistics	
# Honors given to AFIRM faculty	0
# Doctorates awarded under AFIRM support	1
# Masters degrees awarded under AFIRM support	0
# Inventions disclosed	0
# Patents awarded	0
# Peer reviewed publications	0
# Non-peer reviewed publications	0

*Directly supported in whole or part –time by AFIRM

Appendix A. Abbreviations

ACS	abdominal compartment syndrome
ACURO	Animal Care and Use Review Office
AFS	amniotic fluid-derived stem
ARRI	Automation & Robotics Research Institute
ASCs	adipose derived stem cells
a-SMA	*a*-smooth muscle actin
ASTM	American Society for Testing and Materials
BAM	bladder acellular matrix
bFGF	basic fibroblast growth factor
BIODOME	Biomechanical Interface for Optim ized Delivery of MEMS Orchestrated Mammalian Epimorphosis
BLA	Biologics License Application
BMP	bone morphogenetic proteins
BSA	bovine serum albumin
CABSS	Center for Advanced Bioengineering and Soldier Survivability
CCT-eta	chaperonin containing T-complex polypeptide
CDER	Center for Drug Evaluation and Research
CDI	carbonlydiimidazole
CDMRP	Congressionally Directed Medical Research Program
CEA	cultured epithelial autograft
cGMP	current good manufacturing practice
cGTP	current good tissue practice
CPH	calcium phosphate
CRO	contract research organization
CS	Compartment Syndrome
CT	Computed (Axial) Tomography
CTA	composite tissue allografts
CT-MACS	Continuous-Trapping Magnetic Activated Cell Sorter
CTP-Os	connective tissue progenitors
DC	direct current
DMEM	Dulbecco's modified eagle medium
DOD	Department of Defense
DPI	dual polarization interferometer
ECD	external compression device
ECM	extracellular matrix
EDC or EDAC	1-ethyl-3-[3-dimethylaminopropy] carbodiimide
EDL	extensor digitorum longus
EGF	epidermal growth factor
EMBs	explanted microvascular beds
ESC	embryonic stem cells
FACS	fluorescence-activated cell sorting

FDA	Food and Drug Administration
FGF-6	fibroblast growth factor-6
GAGs	glycosaminoglycans
GFAP	glial fibrilliar acidic protein
GFP	green fluorescent protein
GLP	good laboratory practice
H&E	hemotoxylin & eosin stain
HBOT	hyperbaric oxygen therapy
HDFs	human dermal fibroblasts
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
hMDCs	human myo-endothelial and pericyte cells
HPF	high power fields
HUVECs	human umbilical vein endothelial cells
IACUC	Institutional Animal Care and Use Committee
IC	inductively coupled
ICP	intracompartmental pressures
ICX	Intercytex
ICX-SKN	permanent dermal skin graft replacement
IDE	investigative device exemption
IEDs	improvised explosive devices
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IND	investigational new drug
IRB	Internal Review Board
iRGD	“internalizing”-RGD
LD	latissimus dorsi
LDE	living dermal equivalents
LSE	living skin equivalents
LTA	left tibialis anterior
MACS	magnetically-activated cell sorting
MCC	multipotential cell cluster
MDSCs	muscle derived stem cells
MEA	microelectrode array
MFS	microfabricated ferromagnetic strips
MHC	myosin heavy chain
MIT	Massachusetts Institute of Technology
MMP1	matrix metalloproteinase 1
MSC	mesenchymal stromal cells
MSMR	Medical Surveillance Monthly Report
NanoCaPs	nano-crystalline calcium phosphate
NDA	new drug application
NGF	nerve growth factor
NIH	National Institutes of Health

NS	none significant
NSAIDs	non-steroidal anti-inflammatory drugs
OETRP	Orthopaedic Extremity Trauma Research Program
ORIF	open reduction internal fixation
OTRP	Orthopaedic Trauma Research Program
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PCL	polycaprolactone
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG2	polyethylenglycol-2
PEUU	poly(ester urethane urea)
PHA	phytohemagglutinin
PI	primary investigator
PLGA	poly lactic-co-glycolic acid
PMMA	poly methyl methacrylate
POG	particulate oxygen generating
pSKN	prior to lyophilisation
PTFE	polytetrafluoroethylene
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RBAC	Rodent Behavior Analysis Core
RBC	red blood cells
RT	room temperature
RTA	right tibialis anterior
SD	standard deviation
SDF-1*a*	stromal cell-derived factor-1*a*
SEM	scanning electron microscopy
siRNA	small interfering ribonucleic acid
SIS	small intestinal submucosal
SOP	standard operating procedures
SRA	sponsored research agreement
STMG	split thickness meshed grafts
TATRC	Telemedicine & Advanced Technology Research Center
TBD	to be determined
TBSA	total burned surface area
TCP	tissue culture plastic
TE-SKM	tissue engineered skeletal muscle
TGF-B1	transforming growth factor-B1
TNF-*a*	tumor necrosis factor-*a*
UBM	urinary bladder matrix
UCSB	University of California, Santa Barbara
UGH-MACS	Ultrahigh-Gradient Magnetic Activated Cell Sorter
UPMC	University of Pittsburgh Medical Center
USAMRMC	US Army Medical Research and Material Command

USMRMC	US Medical Research and Material Command
VEGF	Vascular Endothelial Growth Factor
VML	volumetric muscle loss
W/O/W	water-in-oil-in-water
WFIRM	Wake Forest Institute for Regenerative Medicine
WFPC	Wake Forest Pittsburgh Consortium
WFUSM	Wake Forest University School of Medicine
WIRB	Western Institutional Review Board
XRD	x-ray diffraction

Appendix B. AFIRM-WFPC Revised Project Numbers

This internal change help better identify projects

Craniofacial Regeneration	Project Leader	Previous Prog.	New Project Number	Old Project Number
Engineered Cartilage Covered Ear Implants for Auricular Reconstruction	Yoo (Wake)	Craniofacial	CF-1	4.1.1
SWOR (Space Maintenance, Wound Optimization, Osseous Regeneration and Reconstruction) for Craniomaxillofacial Defects	Mikos (Rice), Wong (UTHSC)	Craniofacial	CF-2	4.1.2
Novel Synthetic Bone for Craniofacial Regeneration	Sfeir (U Pitt)	Craniofacial	CF-3	4.1.3
Injectable and Implantable Engineered Soft Tissue for Trauma Reconstruction	Rubin, Yoo, Marra, Lee, Kaplan	Craniofacial	CF-4	4.1.4 & 4.1.5
Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle	Christ (Wake)	Craniofacial	CF-5	4.1.6
Burn				
Tissue-engineered Skin Substitute for Burns at Intercytex/DFB/HealthPoint	Ronfard (Intercytex/DFB)	Burn	BS-1	4.2.1
Tissue-engineered Skin Substitute for Burns at Organogenesis	Baites (Organogenesis)	Burn	BS-2	4.2.1a
Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device	Gerlach (U Pitt)	Burn	BS-3	4.2.2
Novel Keratin Biomaterials That Support the Survival of Damaged Cells & Tissues	Van Dyke (WFIRM)	Burn	BS-4	4.2.3
Artificial Extracellular Matrix Proteins for Regenerative Medicine	Tirrell (Caltech)	Burn	BS-5	4.2.4
In Situ Bio-printing of Skin for Battlefield Burn Injuries	Yoo (Wake)	Burn	BS-6	4.2.5
A Comparative Study of the ReCell® Device and Autologous Split-thickness Meshed Skin Grafting in the Treatment of Acute Burn Injuries	Holmes (WFUHS)	Burn	BS-7	4.2.7
In vitro expanded living skin for reparative procedures	SJ Lee (Wake)	Burn	BS-8	4.2.8
Stratatech Technology for Burn	Holmes (WFUHS)	Burn	BS-9	4.2.9
Extremities Injuries				
Hand Transplantation for Reconstruction of Upper Limb Trauma	Lee (U Pitt)	LD	EI-1	4.4.2
Biologic Scaffold for Functional Muscle Replacement: Evaluation for 10 Patients	Badylak (U Pitt)	LD	EI-2	4.4.7
Cellular therapy for the treatment and consequences of compartment syndrome	Huard (U Pitt), Soker (Wake)	CS	EI-3	4.3.1
Blastemal Approach to Digit Reconstruction	Badylak (U Pitt)	LD	EI-4	4.4.1
Use of bone marrow derived stem cells for treatment of compartment syndrome	Gregory (OLMC)	CS	EI-5	4.3.2

Biodegradable elastomeric scaffolds microintegrated with muscle-derived stem cells for fascial reconstruction following fasciotomy	Wagner (U Pitt)	CS	EI-6	4.3.3
Spatial & Temporal Control of Vascularization & Innervation of Compos Tis. Grafts	Guldberg (GaTech)	LD	EI-7	4.4.3
Use of autologous inductive biologic scaffold materials for treatment of CS	Badylak (U Pitt)	CS	EI-8	4.3.4
Peripheral Nerve Repair	Marra (U Pitt), Kaplan (Tufts), Smith (Wake)	LD	EI-9	4.4.4
Scarless Wound Healing				
Mechanical Manipulation of the Wound Environment to Reduce Manifestation of Scar	Gurtner (Stanford)	Scarless	SW-1	4.5.1
Regenerative Biomimetic Dressings for Primary Intervention in the Field	Gurtner (Stanford)	Scarless	SW-2	4.5.2
Multi-functional Bioscaffolds for Promoting Scarless Wound Healing	Washburn (CMU)	Scarless	SW-3	4.5.3
Regulation of Inflammation, Fibroblast Recruitment & Activity for Regen. Healing	Hebda (U Pitt)	Scarless	SW-4	4.5.4
Scar Mitigation via Matrix Metalloproteinase-1 Tertiary Therapy	Russell (U Pitt)	Scarless	SW-5	4.5.7
Isolation and Expansion of Native Vascular Networks for OrganLevel Tissue Engineering (Basic Science)	Gurtner (Stanford)	Scarless	SW-6	4.5.8
Neodyne's Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation	Beasley / Gurtner	Scarless	SW-7	4.5.9
Enabling Technologies				
Amniotic Fluid Stem (AFS) Cells for Burn Injuries	Furth (Wake)	Burn	ET-1	4.2.6
Peptide-mediated Delivery of Therapeutic Compounds into Injured Tissues During Secondary Intervention	Ruoslahti (UCSB)	Scarless	ET-2	4.5.6
Modular, Switchable, Synthetic Extracellular Matrices for Regenerative Medicine	Tirrell (Berkley)	LD	ET-3	4.4.5
High Throughput Approaches to Tissue Regeneration	Thompson (U Wis)	LD	ET-4	4.4.8
Material-induced host cell recruitment for muscle regeneration	Lee (Wake)	CS	ET-5	4.3.5
High Purity Magnetophoretic Sorting for Transplant Therapies	Soh (UCSB)	LD	ET-6	4.4.7
Scarless Wound Healing through Nanoparticle-mediated Molecular Therapies	Kathju (ASRI)	Scarless	ET-7	4.5.5
Oxygen Generating Biomaterials for Engineering Large Tissue Mass	Harrison (Wake)	LD	ET-8	4.4.6