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14. ABSTRACT This project is a collaborative effort between the United States Army Institute of Surgical Research and Wake Forest Institute of Regenerative Medicine designed to develop engineered muscle constructs that can ultimately be used to replace the physical loss of craniofacial muscle. During this first year, we have optimized the pre-conditioning protocol required to produced aligned constructs with multinucleated myotubes; differentiate adipose derived stem cells (ADCs) in to myotubes on 3-D scaffold (porcine bladder extracellular matrix; BAM) and shown electrophysiological activity similar to muscle progenitor cells (MPCs). We were also successful in developing and validating a rat lattissimus dorsi (LD) muscle defect and method of in situ assessment of muscle function. These accomplishments constitute successful completion of the stated year one milestones.					
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Table of Contents

	Page
Introduction	4
Body	4
Key Research Accomplishments	11
Abstracts	11
Publications	11
References	12

INTRODUCTION:

Trauma to the head and face represents 30% of all battlefield injuries (4) and represents nearly 8,000 soldiers from the current wars (2). These injuries often require extensive reconstructive surgery to repair tissue defects. Current surgical options are limited and often unsatisfactory, leaving **no available clinical treatment. Engineering skeletal muscle tissues *de novo* with the patient's own cells would be a major advance towards a satisfactory treatment option.** It would accelerated wound healing with functional and cosmetic augmentation of the tissue defect, and thus, enhance restoration of tissue function, self-esteem, and quality of life. The overall aim of this project is to probe the feasibility and applicability of creating contractile skeletal muscle tissues through use of a bioreactor system in conjunction with stem cells and novel biomaterials/scaffolds. We will test the feasibility of using this technology beginning with in vitro studies followed by generating viable tissues in an established mouse excision model. The size and complexity of the excised tissue defects will be progressively increased until we are able to repair an injury area that is clinically relevant to those incurred in battlefield facial trauma, which will test the applicability of this novel technology. The overall goal is to utilize this technology in injured armed forces personnel to assist with rehabilitation and restoration soft tissue function. This will be accomplished through a collaborative effort between the United States Army Institute of Surgical Research (USAISR) and Wake Forest Institute of Regenerative Medicine (WFIRM).

BODY:

1. **Demonstrate that MCs¹ seeded with MPCs can restore form and function to small P-MEx in a mouse LD**
 - 1.1. **Optimize bioreactor duration for engineering MCs using MPCs.** This work has been completed. The results were reported in the 2010 annual report.
 - 1.2. **Optimize protocols for driving ADSC's to myoblasts in culture.** While we did observe partial myogenic differentiation of ADSCs on BAM scaffolds using 5-Aza preconditioning (*as reported in the 2010 annual report*), the degree of differentiation was not adequate to support implantation of these scaffolds into a volumetric muscle loss injury (VML). Instead, we developed a seeding strategy to deliver a mixture of bioreactor preconditioned MPCs + ADSCs for subsequent implantation. With this co-delivery *in vivo*, there exists the potential for the ADSCs to become myogenic, as was envisioned in the original protocol.
 - 1.3. **Test scaffold and bioreactor protocol for engineering MCs using ASC.** *See text above from SubAim 1.2.*
 - 1.4. **Implant, retrieve and characterize in vitro MCs seeded with MPCs in vivo using LD P-MEx mouse model.** In the current reporting period, we have completed SubAim 1.4. Although not a specific milestone or requirement of the originally funded aims, in doing this work we uncovered an important and informative relationship between defect size

¹ In the original proposal we referred to our engineered construct as a muscle construct (MC), we have since changed our terminology to Tissue Engineered Muscle Construct (TEMR), they will be used interchangeably in this document.

and the extent of functional recovery, as gauged by the corresponding P_o (maximal tetanic response observed on the retrieved LD muscle) on the same animal. In short, as shown in **Figure 1** there was a significant negative correlation observed between the magnitude of the surgically created defect and the extent of functional recovery. As corresponds to intuition, the larger the initial surgical defect, the lower the extent of functional recovery observed for both the MPC (Panel A) and ADSC-seeded (Panel B) TEMR constructs 2 months post-implantation. In addition, this relationship held generally true for all treatment groups/animals in this study. This observation may have important implications not only for the design of future experiments and generations of this technology, but also, it may have prognostic value with respect to functional recovery with any given technology. As such, we will continue to evaluate the utility of this metric.

As illustrated in **Figure 2**, these current data confirm and extend previous observations (3), to demonstrate that over a similar range of surgically created defects, cell-seeded bladder acellular matrix (i.e., BAM) scaffolds provide robust improvements in functional recovery within 2 months of implantation. Specifically, we observed a degree of functional recovery with the MPC-seeded, bioreactor-derived TEMR construct in these studies that is virtually identical to our previous report (3).

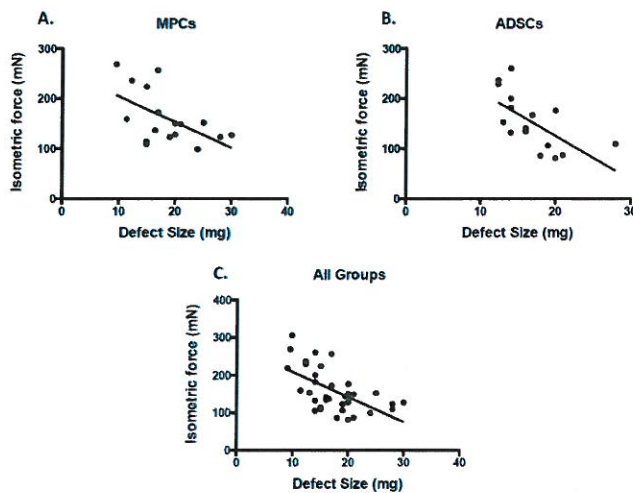


Figure 1 A-C. Graphical depiction of the relationship between the size of the surgically-created VML defect and the extent of functional recovery observed two months post-surgery for the MPC-seeded BAM-based (bladder acellular matrix; Panel A) or ADSC-seeded (adipose-derived stem cells) BAM-based TEMR scaffolds. Of note, the MPC-seeded scaffolds were preconditioned in a bioreactor as previously described (3). Panel C illustrates that this relationship holds generally true for all 3 treatment groups used in these studies (i.e., regression analysis was not statistically significant for the MPC + ADSC and NR groups as these animal cohorts were smaller).

2. Demonstrate that MCs seeded with ASCs can restore form and function to small P-MEx in a mouse LD

Seeding BAM scaffolds with ADSCs (in the absence of bioreactor preconditioning) also lead to statistically significant functional recovery. Furthermore, the addition of ADSCs to the MPC-seeded BAM scaffolds during the course of bioreactor preconditioning tends to further increase the magnitude of functional recovery, although for the present cohort size, this apparent effect is not yet statistically significant. The functional recovery observed with the ADSC-seeded BAM scaffolds was somewhat surprising. However, as illustrated in **Figures 3 and 4**, the functional recovery was associated with significant remodeling,

cellularity and myofiber formation in the TEMR construct, at sites within the scaffold that far removed from the interface of the remaining native muscle tissue with the implanted TEMR-ADSC construct. This type of remodeling and neo-tissue formation is very reminiscent of our prior observations with the MPC-seeded and bioreactor preconditioned TEMR constructs (3).

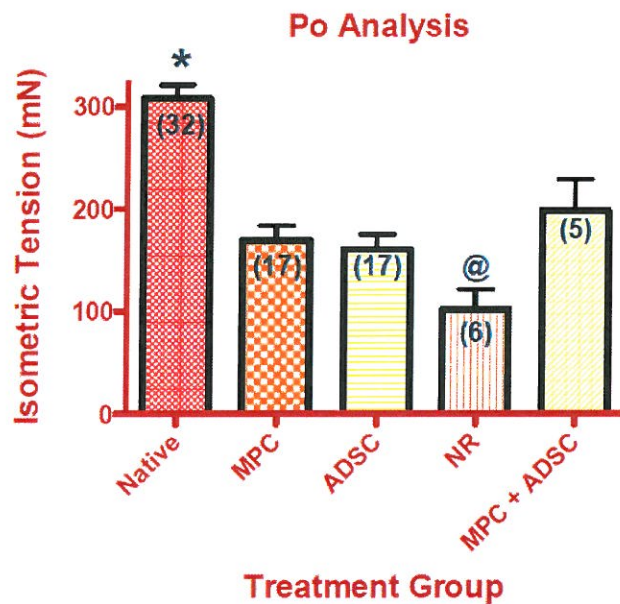


Figure 2. Illustration of the degree of functional recovery observed 2 months post-surgery for the various treatment groups relative to the contractile responses observed on native LD muscle. Shown are the mean and SEM for the maximal tetanic response (i.e., Po) for all retrieved LD tissues in each treatment group. The number of observations is shown in parentheses. As illustrated, all cell-seeded TEMR constructs produced significant functional recovery relative to non-repaired (NR) group.

Peak isometric tension following repair of VML in mouse lat VML. Native = uninjured control, i.e., positive control; MPC=TEMR with MPCs; ADSC = TEMR with ADSCs; NR = No Repair, i.e., negative control; MPC + ADSC, co-cultured TEMR.

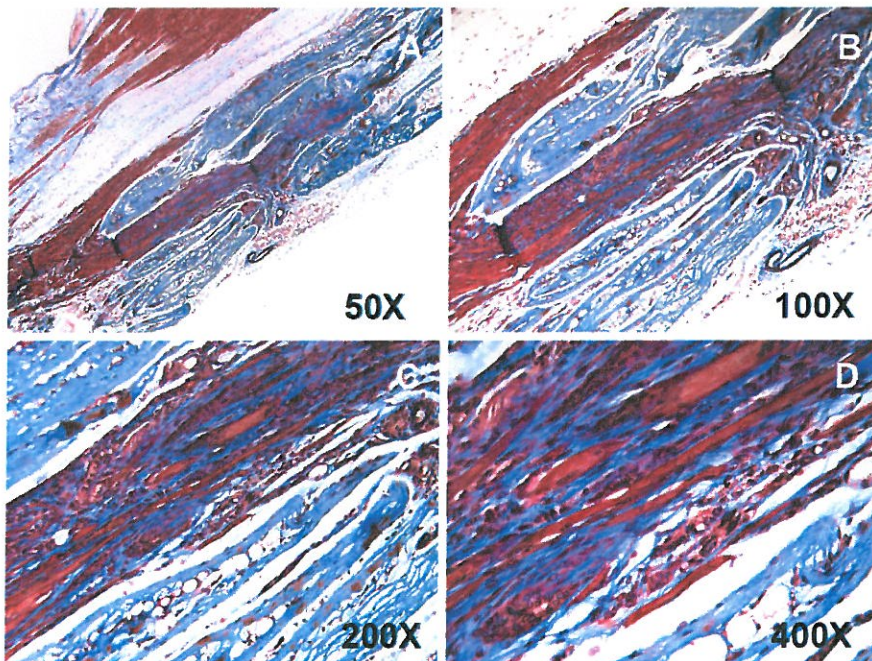


Figure 3. Histological analysis of remodeled TEMR-ADSC constructs retrieved 2 months after implantation. Masson's Trichrome staining of TEMR-ADSC constructs demonstrates tissue remodeling occurring 2 months post implantation

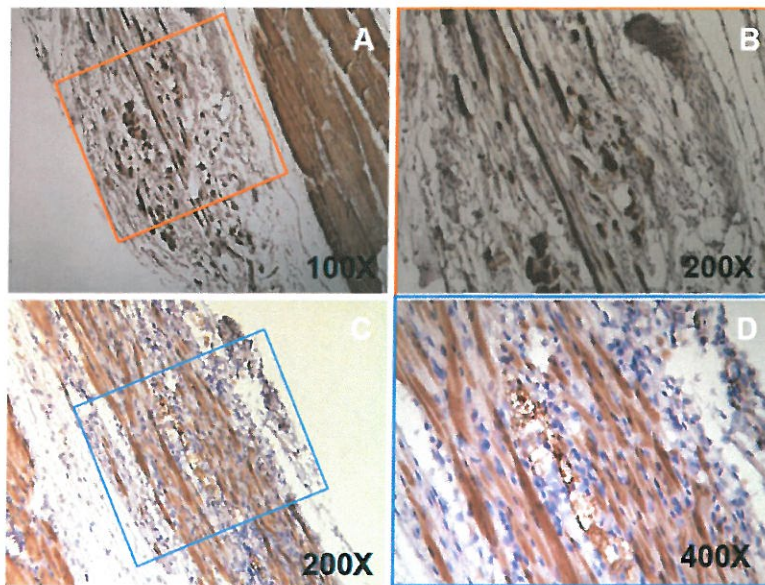


Figure 4. Tissue formation in R-TE-MR-ADSC constructs retrieved 2 months after implantation. Immunohistological staining with MHC antibody (MF 20) (A: 100X and B: 200X) provides evidence for the formation of myofibers on the scaffold side of the engineered tissue. MF 20 positive myoblasts were also observed on the scaffold undergoing remodeling. Immunohistological staining of titin (T12), a structural protein, revealed localization to sarcomeric structures on regenerating muscle fibers (C: 200X and D: 400X) on the scaffold. Figure xxA-D. A & B MHC F20

3. Demonstrate that MCs seeded with MPCs can restore form and function to P-MEx in a rat LD

3.1. Develop a rat LD-PMex Model. Model development and characterization was completed during this reporting period, including a formal study characterizing and validating the model (Figure 5)(1).

3.2. Implant, retrieve, characterize and compare MCs seeded with ASCs in vivo using LD. Because of issues related to the procurement of the bioreactors we were unable to test MCs with MPCs or ADSCs. Rather than halt work, we chose to test rat acellular muscle matrix (RAMM) as a method of repair. Repair of the injury with RAMM significantly improved function (Figure 6). Microscopic examination of the RAMM repair demonstrated widespread vascularization of the formally acellular scaffold (Figure yy). Interestingly, microscopic examination of the RAMM showed no sign of muscle in any form; suggesting the improvements in muscle function may have been due to bridging between existing muscle, resulting in an improved transmission of force across the remaining intact muscle. These results show that RAMM alone is a promising scaffolding material that integrates very well with surrounding musculature. These results have been submitted for publication(1). The fact that we were able to restore function, without restoring muscle parses highlights a major limitation of this model. Additionally, the model is time consuming and technically demanding. Further, we have determined that our TA P-MEx rat model is better suited to testing repair solutions. A modification of the grant was therefore requested and approved for the elimination of this model from future study.

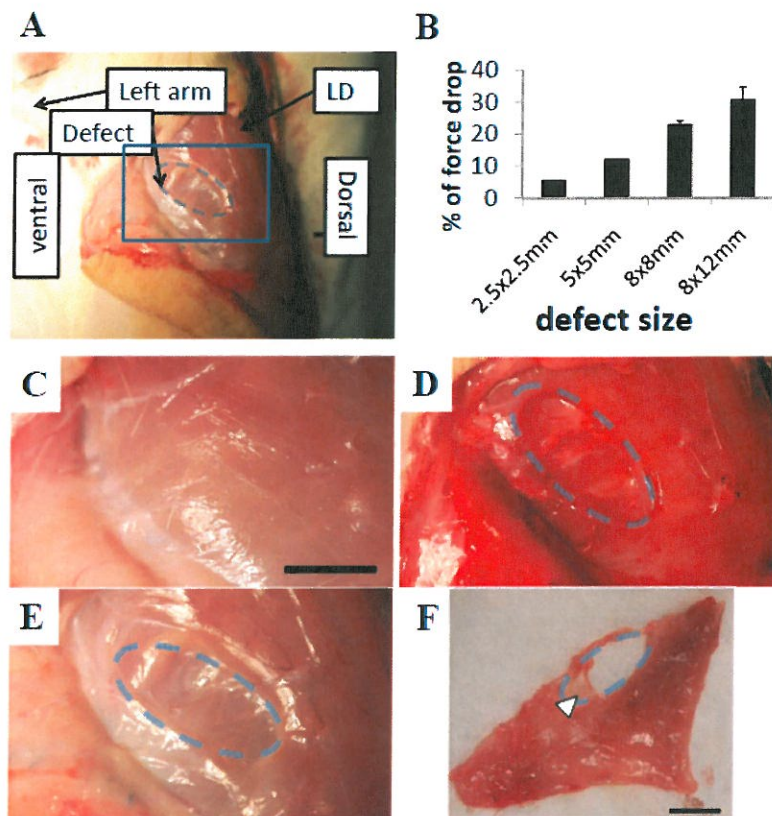


Figure 5. Characterization of LD model based on size of defect. Defect size at 0 and 4 weeks. A: an image illustrating LD defect location in animal; B: the relationship between force drop and defect size; C-E: representative images of LD without defect (C), with freshly created defect (D), and with defect at 4 weeks (E); F: an isolated LD shows a layer of transparent tissue formed at defect area at 4 weeks (arrow head). The blue dotted lines demarcate the defected area in A, D, E and F. Scale bar = 1cm

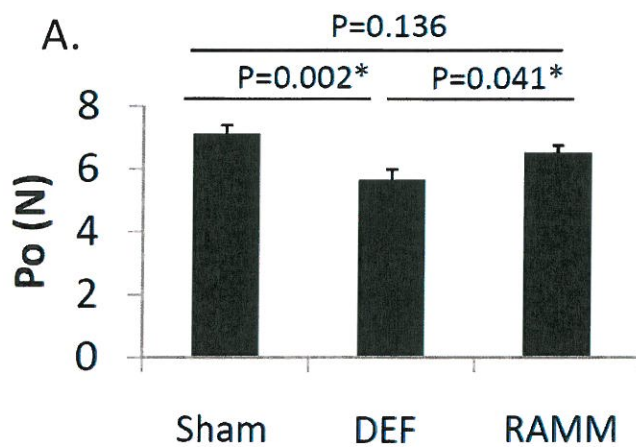
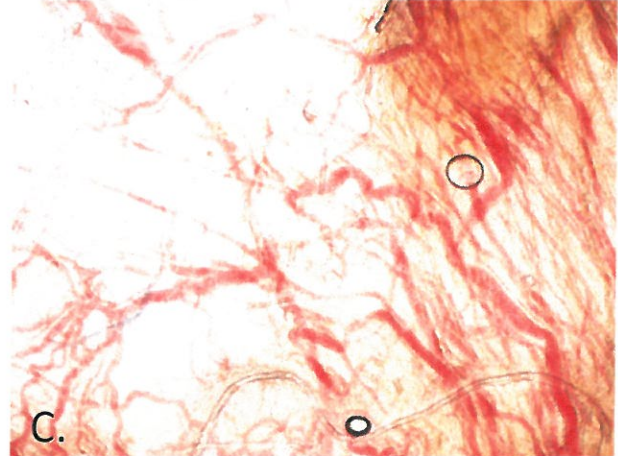
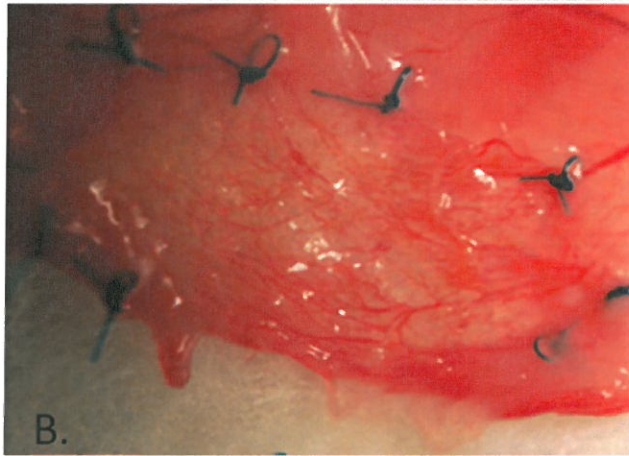


Figure 6. A: Peak tetanic tension in Sham (uninjured), DEF-unrepaired defect, and RAMM-defect repaired with RAMM at 8 weeks post-injury. The defect injury resulted in a significant reduction in force. However repair with RAMM returned function to a level that was not statistically different from the uninjured Sham group. *Statistically significant ($p > 0.05$). B: A close-up image of RAMM repaired area at 8 weeks (outlined by suture), showing the RAMM is well integrated into host tissue and is highly vascularized; C: microscopic images showing vascularization within M-ECM at 8 weeks. DEF (not shown) showed no signs of healing.



4. **Demonstrate that MCs seeded with ASCs can restore form and function to P-MEx in a rat LD** **Demonstrate that MCs seeded with MPCs can restore form and function to P-MEx in a rat TA.** This objective has been eliminated (see 3.2)
5. **Demonstrate that MCs seeded with MPCs can restore form and function to P-MEx in a rat TA.** VML injury in the rat TA muscle was created via by surgically excising ~20% of the muscle's mass. This created an injury that was irrecoverable under assisted endogenous regenerative mechanisms. In response to the loss of the muscle tissue, the functional capacity of the TA muscle remained reduced by ~30% three months post-injury (See **Figure 7** for more detail). As a first attempt to repair the TA muscle VML injury, BAM scaffolding without cells and MPC seeded muscle constructs were implanted at the site the injury immediately after the surgical defect was created. Muscle constructs were generated in a manner similar to that reported previously (3). At this time, neither implant significantly improved *in vivo* TA muscle isometric torque. However, as a first attempt at repair of a larger defect (compared to our previous studies with mouse LD

muscle) the surgical procedures and arrangement of muscle implant may not have been optimized for cell delivery and construct integration. Specifically, a 3 x 1 cm TEMR construct was folded twice to make a 1 x 1 cm construct with three layers. This folding process may have induced significantly cellular damage that impeded its efficacy. In future studies, we will alter the geometry of the VML injury to accommodate the sheet-like characteristics of the TEMR technology, and to permit the greatest surface area for integration with native tissue, and therefore, optimize the opportunity for tissue repair.

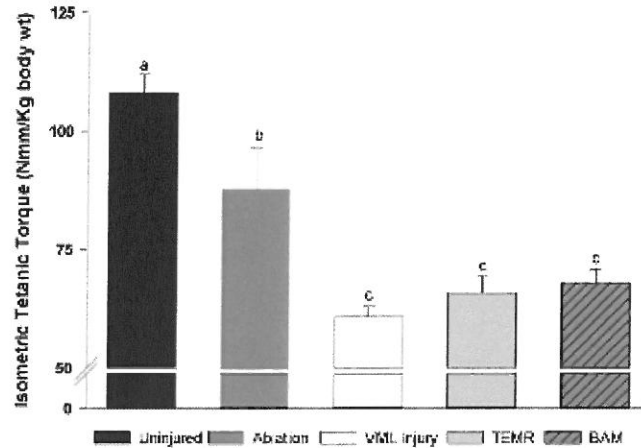


Figure 7. Rat anterior crural muscle *in vivo* peak isometric torque 12 weeks post-VML injury. VML injury was created by surgically excising ~20% of the TA muscle mass. Additionally, the TA muscle's synergists in the anterior crural muscle unit (i.e., EDL and EHL muscles) were ablated at the time of VML injury. Removal of the synergists without (Ablation Group) and with VML injury resulted in a loss of ~30 and ~50% of peak isometric torque, respectively. The VML injury was repaired at the time the injury was created with either a bladder acellular collagen matrix (BAM) or a tissue engineered muscle repair construct (TEMR). TEMR was generated via seeding muscle derived cells on BAM and then preconditioning them in a bioreactor. Neither TEMR nor BAM significantly improved the functional capacity of VML injured muscle. Values are presented as means $\pm$ SEM. Group means denoted with different letters are significantly different ($p < 0.05$).

6. **Demonstrate that MCs seeded with ASCs can restore form and function to large P-MEx in a rat TA.** Based on our current observations and upcoming milestones, we plan to focus this last year largely on the utility of the MPC and MPC + ADSC combination on functional recovery from a surgically created VML injury in the TA model. The rationale for selection of these two groups is to focus resources on the most relevant TEMR technologies. Unpublished observations, as well as the data included herein, clearly indicate that the use of ADSCs alone will yield little, if any, functional improvement over that currently observed with the existing TEMR technology. In an effort to further advance this technology during the last year of funding and gain additional mechanistic insight, we

will combine the advantages of the MPC TEMR construct (control over cell phenotype and composition through bioreactor preconditioning) with the apparently synergistic capabilities of the ADSCs (namely, increased angiogenesis).

7. **Demonstrate that MCs seeded with selected cells can restore form and function to large PMEx in a rat LD.** This aim has been eliminated (see 3.2)
8. **Demonstrate that MCs seeded with selected cells* can restore form and function to C-MEx in a rat TA.**
- 8.1. **Develop surgical procedure for implanting MC in TA C-MEx rat model.** We are currently engaged in ex vivo studies to develop the surgical repair techniques. The data used from these studies is being used to inform the writing of the animal use protocol that will be submitted for review by the IACUC in January 2012.

Key Accomplishments:

- **We have tested MPCs, ADSCs, and combined MPCs and ADCs in a mouse LD PMex model**
- **We have developed and tested the rat LD PMex model using RAMM**
- **We have tested TEMRs seeded with MPCs in a rat TA PMex model**

Abstracts:

1. Chen X, Christ GJ, and Walters TJ. A Critical Size Defect Model of the Latissimus Dorsi Muscle of the Rat. Armed Forces Institute of Regenerative Medicine, St. Pete's Beach, 2010.
2. Chen X, Roe JL, Rathbone CR, and Walters TJ. Early Assessment of Volumetric Muscle Loss Repaired With Muscle Derived Extracellular Matrix. Advanced Technology Applications for Combat Casualty Care Fort Lauderdale, FL, 2011.
3. Kesireddy V, Stern MM, Nader TP, Christy RJ, Walters TJ, and Christ GJ. Characterization of Adipose Derived Stem Cells Induced to Myotubes on Bladder Acellular Matrix Scaffold. In: Tissue Engineering and Regenerative Medicine Society. Orlando, FL, 2010.
4. Walters TJ. Engineered Skeletal Muscle for the treatment of Volumetric Muscle Loss (VML): Testing Platforms and Functional Assessment Tissue Engineering and Regenerative Medicine Society, Orlando, FL, 2010.
5. Walters TJ. Functional Assessment of Muscle Trauma and Regeneration in Rodent Models. Armed Forces Institute for Regenerative Medicine, St. Pete's Beach, FL, 2010.
6. Walters TJ and Wu X. Rat Model for Traumatic Volumetric Muscle Loss: A Platform for Testing Engineered Skeletal Muscle. Society for Biomaterials, Seattle, 2010.

Publications

1. Machingal MA, Corona BT, Walters TJ, Kesireddy V, Koval CN, Dannahower A, Zhao W, Yoo J, and Christ GJ. A Tissue Engineered Muscle Repair (TE-MR) Construct for Functional Restoration of an Irrecoverable Muscle Injury in a Murine Model. *Tissue engineering*, 2011.
2. Chen X and Walters TJ. Muscle Derived Decellularized Extracellular Matrix Improved Function Recovery in a Critical Size Latissimus Dorsi Muscle Defect A Novel Rat Model. *The Journal of surgical research*, Submitted.

References

1. **Chen X and Walters TJ.** Muscle Derived Decellularized Extracellular Matrix Improved Function Recovery in a Critical Size Latissimus Dorsi Muscle Defect A Novel Rat Model. *The Journal of surgical research*, Submitted.
2. **Hale R, Walker J, Lew T, Wenke JC, and Holcomb JB.** Maxillofacial Battle-Injuries Experienced by U.S. Soldiers in the Current Iraq/Afghanistan Conflict. In: *2008 FDI World Dental Congress*. Stockholm, Sweden, 2008.
3. **Machingal MA, Corona BT, Walters TJ, Kesireddy V, Koval CN, Dannahower A, Zhao W, Yoo J, and Christ GJ.** A Tissue Engineered Muscle Repair (TE-MR) Construct for Functional Restoration of an Irrecoverable Muscle Injury in a Murine Model. *Tissue engineering*, 2011.
4. **Owens BD, Kragh JF, Jr., Wenke JC, Macaitis J, Wade CE, and Holcomb JB.** Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. *J Trauma* 64: 295-299, 2008.