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SDF-1/IGF-1 Conjugated to a PEGylated Fibrin Matrix as a Treatment for an Ischemia Reperfusion Injury in Skeletal Muscle Repair

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SDF-1/IGF-1 Conjugated to a PEGylated Fibrin Matrix as a Treatment for an Ischemia Reperfusion Injury in Skeletal Muscle Repair

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Abstract

SDF-1/IGF-1 Conjugated to a PEGylated Fibrin Matrix as a Treatment for an Ischemia Reperfusion Injury in Skeletal Muscle Repair

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Ischemia/reperfusion (I/R) injury causes extensive damage to skeletal muscle, often resulting in prolonged functional deficits. This current study determines the efficacy of controlled release of SDF-1α and IGF-1 by conjugation to biodegradable, polyethylene glycol, (PEG)ylated fibrin gel matrix in skeletal muscle repair of an I/R injury. Male Sprague-Dawley rats underwent a 2-hour tourniquet induced I/R injury on their hind limbs. Twenty-four hours post injury the following treatments were administered: PEGylated fibrin gel (PEG-Fib), SDF-1 conjugated PEGylated fibrin gel (PEG-Fib/SDF-1), or dual protein IGF-1 and SDF-1 conjugated PEGylated fibrin gel (PEG-Fibrin/SDF-1/IGF-1. Following 14 days after injury, functional and histological evaluations were performed. There was no significant difference in maximum tetanic force production recovery between PEG-Fib and

PEG-Fib/SDF-1 groups. However, PEG-Fib/SDF-1/IGF-1 group resulted in significant improvement of force production relative to the other treatment groups. The same results were found for specific tension. Histological analysis revealed a greater distribution of small myofibers in the PEG-Fib/SDF-1 group than the PEG-Fib group, while the PEG-Fib/SDF-1/IGF-1 group had the smallest distribution of small fibers and similar to controls (uninjured). There were also a greater number of centrally located nuclei in the PEG-Fib/SDF-1 group than the PEG-Fib group, while the PEG-Fib/SDF-1/IGF-1 group had similar values to controls. Although these results confirm the protective role of exogenous IGF-1, SDF-1 did not have an effect on skeletal muscle repair.

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Introduction

Ischemia reperfusion (I/R) occurs following reperfusion of ischemic tissue and results from local inflammation causing tissue damage. In severe cases, limb loss results or a systemic response ensues causing multiple organ failure and death (Blaisdell 2002). The first phase of I/R is the ischemic phase characterized by depletion of ATP, accumulation of metabolites and microvascular damage. The next phase, reperfusion syndrome, is the most damaging due to reactive oxidative species causing damage to cell membranes and subsequent cell apoptosis and necrosis (Honda, Korge, and Weiss 2005). I/R causes a prolonged inflammatory response that results in impairment of skeletal muscle function and creates a clinical problem especially from tourniquet use, hence an effective therapy needs to be investigated.

Stem cell therapy has been widely implicated as a potential therapy for I/R skeletal muscle repair, which include embryonic, adult and induced pluripotent stem cells (Jones, Lebkowski, and McNiece 2010). Although the different types have shown potential, adult stems have received the most attention. Specifically bone marrow derived stem cells (BMSCs) have been highly investigated due to their high abundance in adults and myogenic potential (Ferrari et al. 1998). Extensive literature has shown their effects to be highly advantageous in tissue regeneration, however the exact effects of how BMSCs aid in regeneration following injury has been highly debated. BMSCs have been shown to migrate to injured tissue, engraft,

and transdifferentiate into a different tissue's lineage (LaBarge and Blau 2002; Deans and Moseley 2000; Long, Corbel, and Rossi 2005; Ferrari et al. 1998). Conversely, others have shown little engraftment and instead demonstrated BMSCs secrete factors that are anti-apoptotic, anti-inflammatory, pro-angiogenic and activate other stem cells to proliferate and differentiate (Aggarwal and Pittenger 2005; Wagers 2002; Jones, Lebkowski, and McNiece 2010; Deans and Moseley 2000). None the less, the effects of BMSCs have enhanced tissue regeneration.

However, stem cell therapy does not come without risk and problems. Patient morbidity at the donor site can result and there is often a lag between harvesting and treatment from autologous cell transplantation due to the requirement of expansion of the cell population (Jones, Lebkowski, and McNiece 2010). During cell expansion, the BMSCs are living so they require specific growth conditions to survive such as temperature and air composition. They also have to been grown as a single layer and must be expanded to a sufficient number for clinical use which is time consuming. Another problem that may occur during expansion is phenotype switching, in which the original desired cell type is changed and could produce unexpected outcomes.

Stromal derived factor 1 (SDF-1) was pursued as an alternative to alleviate the problems associated with stem cell therapy. Following injury, injured tissue releases a gradient of SDF-1 and in turn SDF-1 causes stem cells to migrate to the injured area to aid in tissue regeneration exhibiting similar effects to stem cell therapy. The cells induced into the area express the CXCR4 receptor which include

circulating BMSCs (monocytes, lymphocytes, hematopoietic stem cells, mesenchymal stem cells), endothelial cells, and other progenitor cells derived from heart, muscle, and liver (Kucia et al. 2004). Several pathways are activated upon SDF-1 and CXCR4 binding stimulating migration, proliferation and differentiation of cells (Kucia et al. 2004). The MAPK and PI3K/Akt are responsible for some of those actions and as well as cell survival by stimulation of anti-apoptotic pathways (Kryczek et al. 2007). In skeletal muscle repair activation of muscle progenitor cells, satellite cells (SCs) are required for effective repair (Seale et al. 2000). SDF-1 is thought to cause activation of SCs, proliferation and differentiation into myoblasts then myofibers .

Insulin-like growth factor (IGF-1) is a pro-regenerative growth factor. In I/R, IGF-1 reduces the inflammatory response by causing activation of PI3K/Akt pathway promoting cell survival (Hammers et al. 2011). Furthermore, proliferation and differentiation of SCs are stimulated by IGF-1 via the MAPK and PI3K/Akt pathways (Bodine et al. 2001; Stitt et al. 2004; Pallafacchina et al. 2002). This is similar to the proposed effects of SDF-1, however IGF-1 has been studied more extensively than SDF-1.

A biodegradeable polyethylene glycol (PEG)ylated fibrin matrix that binds proteins is used to release growth factors, cytokines, cells, and other proteins. The PEGylated fibrin matrix is composed of fibrinogen, PEG and thrombin and holds the proteins in by entrapment, covalent binding to PEG and physical affinity to fibrin (Drinnan et al. 2010). The matrix is injected as a gel and quickly polymerizes

forming a clot. As the matrix degrades, the proteins will be released in a controlled manner and protects the proteins allowing their effects to be exerted over a prolonged period reducing the requirement for multiple treatments.

SDF-1 conjugated to PEGylated fibrin will be used as a therapy in treating I/R injury. Additionally, IGF-1 and SDF-1 as a dual delivery to I/R will also be used to further assess repair of skeletal muscle following I/R. We hypothesize that SDF-1 will enhance skeletal muscle repair and the addition of IGF-1 will further enhance the repair process after I/R injury by functional and histological analysis.

Literature Review

Skeletal Muscle Injury and Repair

Skeletal muscle is the most abundant tissue in the human body making up 40% to 50% of total body and has the incredible ability to regenerate itself after damage. Skeletal muscle injuries result from a variety of mechanism, such as contusions, strains, lacerations and disease but the general damage and repair processes are similar (Huard, Li, and Fu 2002). The process is broken down into three phases: the destruction phase, the repair phase and the remodeling phase.

The Destruction Phase

Immediately after muscle injury, the myofiber plasma membrane is damaged and ruptures along the length of the myofiber. Extracellular calcium flows into the extracellular space and cause neighboring fibers to undergo hypercontraction and tear apart activating a protein kinase cascade. The cascade initiates local inflammation and increases muscle degeneration (Li, Cummins, and Huard 2001). However, the contraction band forms a protective barrier to contain the necrosis and halts necrosis from extending further along the entire myofibers (Järvinen et al. 2005a).

Skeletal muscle vasculature is also damaged due to skeletal muscle being highly vascularized. This induces hemorrhaging and allows blood cells easy access to the injury site. A hematoma fills in the gap between the ruptured fibers and will serve as a scaffold for the fibroblasts in the repair phase. The complement cascade is activated and recruits neutrophils in the injured area to clear cellular debris by

phagocytosis and release of proteases (Chargé and Rudnicki 2004). They also secrete cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8) and interleukin-1 (IL-1) to amplify the inflammatory response and recruit other leukocytes to the injured area (Tidball 2008). TNF-α has been shown to decrease the protein expression of MyoD and Myosin Heavy Chain that play a role in the regenerative phase (Li, Cummins, and Huard 2001). Activated neutrophils also release enzymes that activate reactive oxygen species (ROS) formation. ROS cause lysis of skeletal muscle cell membranes and may play a role in activating nuclear factor kappaB (NF-κB). Decreased MyoD mRNA expression has been associated with the expression of NF-κB. These combined effects serve to hamper or prolong skeletal muscle repair (Chargé and Rudnicki 2004).

Monocytes are later recruited into the area and transform into macrophages. The macrophages become the dominant cell population at the injury site. Two populations of macrophages that have different roles in skeletal muscle repair have been identified and appear at different time points. The M1 population is first to arrive at the injury site and serves as pro-inflammatory cells. They function to debride the damaged tissue and activate satellite cells which are the progenitors to skeletal muscle that reside under the basal lamina. The M2 population appears later on and serves as anti-inflammatory cells. They release growth factors and anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β (Ruffell et al. 2009; Chazaud et al. 2009). The M2 population causes the satellite cells to differentiate forming myoblasts, then myotubes.

The Repair Phase

Myofibers are postmitotic and are regenerated by a pool of undifferentiated cells called satellite cells (SCs). The repair phase is characterized by the activity of the satellite cells. The SCs migrate from the basal lamina of muscle fibers and into the injured area, where they will become myoblasts and further proliferate and differentiate into myotubes or fuse with existing myofibers (Tedesco et al. 2010). Quiescent and activated SCs express Pax7 and knockouts of Pax7 in SCs failed to generate myoblasts, demonstrating the absolute requirement of Pax7 for SC development (Seale et al. 2000; Kawiak et al. 2006). Pax7 positive cells were shown to increase during regeneration and were expressed in centrally located nuclei in newly regenerated fibers further reiterating the importance of Pax7 on SCs in muscle regeneration. Upon exposure to signals in the environment, SCs are activated and upregulate different MRFs at specific time points. Early MRFs that are expressed include Myf5 and MyoD. Myf5 promotes SC renewal, while MyoD promotes satellite cell differentiation. Late MRFs regulate the terminal differentiation of myoblasts, such as myogenin and MRF leading to fusion with existing fiber or formation of new fibers (Chargé and Rudnicki 2004).

Other populations of cells are also a source of myonuclei in muscle repair such as nonmuscle resident stem cells and muscle resident stems cells. Both populations are multipotent stem cells that have the potential to contribute to the regeneration of skeletal muscle that are derived from the bone marrow, neuronal

compartment and mesenchymal tissue. The cells can differentiate into a myogenic lineage or release factors to promote muscle repair (Chargé and Rudnicki 2004).

The hematoma that was formed from rupture of the vasculature serves as a temporary ECM scaffold for incoming fibroblasts and creates a connective tissue scar. This allows the wound to withstand the contraction force applied to it. More ECM proteins are synthesized to provide further strength and elasticity to the scar. In some cases the scar can become too dense and becomes a mechanical barrier delaying or inhibiting muscle regeneration (Y. Li, Cummins, and Huard 2001; Järvinen et al. 2005a). Other critical parts of muscle regeneration are restoration of vasculature and intramuscular nerves to the injured area. The vasculature will support the growth of regenerating fibers, and the nerves will promote the survival of the myofibers (Järvinen et al. 2005a).

The Remodeling Phase

After the progenitor cells have fused with myofibers or have formed new myofibers, centrally nucleated myofibers are observed. The fibers continue to mature and express proteins such as myosin heavy chain. The newly regenerated myofibers at the ends of the damaged area adhere to the basal lamina and extend through the connective tissue scar that was formed between the damaged ends of the myofibers. The myofibers branch as they extend and overtime the scar diminishes as the myofibers infiltrate through the connective tissue scar. The fusion of the two ends of the transected myofibers may or may not close depending on the extent of the injury and the individual (Järvinen et al. 2005b).

The skeletal muscle repair process described above is a general scheme for minor and mild skeletal muscle injuries. In more severe injuries, the general scheme is not sufficient for full recovery and interventions must be applied or loss of function may result. The following study will focus on ischemia/reperfusion (I/R) injuries that do not heal well on their own. Following (I/R) injury, skeletal muscle has been shown to have functional and histological impairments.

ISCHEMIA REPERFUSION INJURY

Tourniquets are applied worldwide in the clinical operating room and on the battlefield to limit blood loss and clear the field of view for surgeons (Honda, Korge, and Weiss 2005). However, tourniquet applications induce an ischemia/reperfusion (I/R) injury that can exacerbate tissue damage, causing loss of function and hamper regeneration. The first phase of I/R is caused by ischemia resulting in depletion of ATP, microvascular damage, necrosis and local inflammation. The second phase, reperfusion syndrome, restores blood flow to the previous ischemic areas and causes an influx of a large concentration of reactive oxidative species (ROS) (Honda, Korge, and Weiss 2005; Blaisdell 2002). The additional recruitment of leukocytes increases the ROS concentration and inflammation in the area (Honda, Korge, and Weiss 2005). ROS are responsible for further destruction of skeletal muscle cells and mitochondria. In severe cases, a systemic response may result leading to multiple organ failure and death (Blaisdell 2002).

The reperfusion phase of I/R injury accounts for most of the local and systemic damage due to ROS. ROS are activated oxygen derived radicals that are strong oxidizing and reducing agents such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), hypochlorous acid (HOCl) and nitric oxide-derived peroxynitrite. A common source of these ions is xanthine oxidase (XO), which is normally found in microvascular endothelial cells of skeletal muscle as an oxidized nicotinamide-adenine dinucleotide (NAD^+)-dependent dehydrogenase (XDH). During ischemia, XDH is converted to XO and when molecular oxygen encounters XO during reperfusion ROS are formed. Another common source of ROS is activated neutrophils that damage cell membranes by lipid peroxidation. The initial tissue injury causes an increase in lipid peroxidation by activation of cyclooxygenase and lipooxygenase pathways along with transition metal ions responsible for neutrophil activation. The activated neutrophil release granules that enzymes that play a role in the formation of ROS (Gillani et al. 2012).

Ischemia reperfusion injury is currently a problem in the clinical and combat settings due the exacerbation of muscle damage, affecting the regeneration phase. I/R creates an unfavorable environment for regeneration as the result of prolonged inflammation and oxidative stress. Inflammation and oxidative must cease for regeneration to occur. The clinical upper limit for tourniquet application is 2 hours, however in cases such as in combat or prolonged surgeries, tourniquet application often last longer than 2 hours (Walters et al. 2008). The increase in duration of the tourniquet application enhances the severity of the ischemia reperfusion injury

(Honda, Korge, and Weiss 2005) and current I/R clinical therapies have not been effective in attenuating the I/R injury such as hyperthermic, hyperbaric , pharmacological, and pre-conditioning treatments (Y. Li, Cummins, and Huard 2001). Studies have shown the long term detrimental effects of I/R injuries. Fish et al. (1993) demonstrated that at 42 days after 2 hours of I/R the maximal force production was still reduced in the I/R muscles compared to control muscles in rats. Vignaud et al. (2010) also found at 56 days following an I/R injury muscle force and muscle weight did not fully recover in contrast to myotoxic-induced injury, which made a full recovery, and control muscle. However, there have been advances in research that have the potential to be applied in the clinical setting.

STEM CELLS

Stem cell therapy has received a lot of attention in the last decade due to stem cells' unique ability to self-renew and repair damaged tissue. During development, stem cells participate in organogenesis and in the adult stem cells are used to repair and maintain tissues. In research, specific focus has been on bone marrow derived stem cells (BMSCs) due to its abundance in the body and its potential to transdifferentiate into cells such as muscle, liver, heart and brain and engraft into the injury site (Li et al. 2012). However, others have shown that stem cells create an anti-inflammatory environment by decreasing tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) secretions, while increasing VEG-F, interleukin-10 (IL-10) and IL-4 (Aggarwal and Pittenger 2005). The activities of these factors and many others promote tissue regeneration and enhance angiogenesis and

neurogenesis. In Dr. Roger Farrar's lab, Matthew Tierney (unpublished) showed the potential of BMSCs as a therapy for volumetric skeletal muscle mass loss. Implantation of BMSC seeded on a decellularized extracellular matrix (ECM) improved functional recovery and restored mass and vasculature to the defected area compared to implantation with only ECM.

However, there are obstacles in stem cell therapy which include patient morbidity at the donor site and a time lag between harvesting and treatment from autologous cell transplantation (Jones, Lebkowski, and McNiece 2010). During cell expansion, the BMSCs are living so they require specific growth conditions to survive such as temperature and air composition. They also have to been grown as a single layer and must be expanded to a sufficient number for clinical use which requires a lot of space and time. Another problem arising during cell culture is a change in genotype and phenotype altering the initial characteristics of the cell, which will affect the outcomes of the therapy after implantation. Due to these obstacles, cytokines have been investigated as an alternative to aid in tissue repair. Cytokines can be produced easily in large quantities and do not require as stringent conditions for storage. Also, the mechanisms of actions for these commercially available cytokines have been studied and their effects are clearer than stem cells.

STROMAL DERIVED FACTOR-1 (SDF-1)

Stromal derived factor-1 (SDF-1) is a small chemokine (8kDa) that functions in a variety of biological processes including development, infection, inflammation and tissue repair (Kucia et al. 2004). SDF-1 homes cells expressing the CXCR4

receptor present on lymphocytes, monocytes, endothelial cells, neuronal cells, and other progenitor cells which include hematopoietic cells and muscle satellite cells to sites expressing SDF-1(Horuk 2001; Bleul et al. 1997; Gupta et al. 1998; Hesselgesser et al. 1997). Another receptor to SDF-1 has recently been identified as CXCR7 although there has not been as extensive research (Horuk 2001). The importance of SDF-1 in development is supported in mice knockouts of SDF-1 who died in utero and of those survived died one hour after birth from severe heart defects (Nagasawa et al. 1996). In adults, SDF-1 is widely expressed by many organs such as bone, heart, liver, brain, and skeletal muscle upon injury (Kryczek et al. 2007). As stated previously, the homing of progenitor cells by SDF-1 aids in creating a pro-regenerative environment by secreting factors such as vascular endothelial growth factor (VEGF) and IGF-1 to reduce inflammation and induce anti-apototic factors and angiogenesis. Furthermore, the migrated progenitor cells may engraft and differentiate into the organ's cell lineage and activate residential stem cells. Although there has been extensive research in a variety of organ systems such as the immune system, bone and heart there has been limited research on the role of SDF-1 in skeletal muscle regeneration.

CXCR4 is expressed on skeletal muscle satellite cells which are essential to muscle regeneration. Upon injury, such as an I/R injury, gene expression of CXCR4 and SDF-1 are upregulated by hypoxia-inducible factor-1 alpha (HIF-1a) in cooperation with nuclear factor-kb (NF-kb). Other factors associated with inflammation such as C3a complement and hyaluronic acid also stimulate CXCR4

and SDF-1 upregulation (Kucia et al. 2005). After SDF-1 binding to the G protein coupled CXCR4 receptor several pathways are activated: JAK/Stat, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3-K), phospholipase C- (PKC-), adhesions and phosphatases. These pathways facilitate migration, survival and adhesion of the CXCR4 cells to surfaces which include fibrinogen, fibronectin, stroma and endothelial cells (Ratajczak et al. 2006). SDF-1 has also been proposed to activate adhesion molecules on the cell surface such as integrins and in turn trigger further expression of CXCR4 on the cell's membrane to increase its activity (Kucia et al. 2004).

In addition to homing stem cells to the injured area, other pathways may be stimulated for the survival of the CXCR4 postitive cells and cause activation, proliferation, and differentiation of satellite cells. The MAPK and PI3-K/Akt pathways have been extensively researched in muscle injury and regeneration, however SDF-1 effects on the pathways has been limited. Briefly, the PI3K/Akt pathway inhibits apoptosis by suppressing release of mitochondrial cytochrome c and caspase 9, activates transcription of anti-apoptotic genes via NF-κB, and increases protein synthesis via activation of mTOR and inhibition of glycogen synthase kinase 3β (GSK-3β) (Harada et al.2004; Honda, Korge, and Weiss 2005; Stitt et al.2004; Jefferson, Fabian, and Kimball 1999; Sacheck et al.2004). PI3-K pathway has also been proposed in signaling of IL-1 and TNF-α which participate in inflammation. MAPK activation affecting the downstream signals ERK1/2 and is responsible for the proliferation of satellite cells, while the PI3K subsequently

causes their differentiation (Roux and Blenis 2004; Coolican et al.1997). These pathways together are possibly responsible for the survival, proliferation and differentiation of cells into the injured area.

The highlights of SDF-1 have made it a potential therapy in I/R injuries by reducing the prolonged inflammatory phase of I/R injuries, inducing angiogenesis and activating the proliferation and differentiation of satellite cells. Additionally, SDF-1 serves as an alternative to stem cell transplantation by serving as a cytokine to home a patients' own stem cells to the site of injury. In this study we will use the alpha variant of SDF-1 as a therapy for I/R injury. One gene encodes both SDF-1α and SDF-β. The alpha variant is more abundant than the beta variant.

INSULIN-LIKE GROWTH FACTOR-1 (IGF-1)

IGF-1 is an extensively studied endocrine growth factor that is mediated in an autocrine and paracrine fashion (Adams 2002; Stitt et al. 2004). In response to skeletal muscle injury, IGF-1 stimulates myoblasts proliferation and differentiation and promotes cell survival (Adams 2002b; Coolican et al. 1997). IGF-1 has also been shown to promote survival of nerves following tissue injury (Pallafacchina et al. 2002). In 2008, Hammers et al. showed IGF-1 upregulation was responsible for survival of muscle fibers and aided in repair after injury through functional and histological analysis.

The mechanisms underlying these results involve the MAPK and PI3-K pathways as discussed previously as possible mechanisms in which SDF-1 functions. However, those mechanisms in IGF-1 have been widely studied. Proliferation is

mediated by the Ras/Raf-1/MAP kinase, while differentiation is activated by the PI3-kinase pathway (Coolican et al. 1997). The PI3-K pathway also involves increased protein synthesis, increased cell survival, and decreased apoptosis further enhancing regeneration. Although the pathways are antagonistic to one another, both are highly regulated and are needed in muscle regeneration.

The key regulatory pathway in which IGF-1 induces its effects is through the PI3-K/Akt pathway. Once activated, glycogen synthase kinase 3β (GSK-3β) is phosphorylated causing inhibition of its normal activity, which decreases protein synthesis (Jefferson, Fabian, and Kimball 1999). Furthermore, FOXO is phosphorylated to block its translocation into the nucleus, where it induces E3 ubiquitin ligases through the MuRF1 and MAFbx genes for protein degradation (Stitt et al. 2004; Sacheck et al. 2004). Meanwhile, mTOR is activated triggering its downstream targets, p70S6K and PHAS-1/4E-BP to increase protein synthesis by increasing translation (Bodine et al. 2001). Inactivation of Bad also occurs which suppresses release of mitochondrial cytochrome c and caspase 9 thereby inhibiting apoptosis. Additionally, IKK is activated and degrades the inhibitor of nuclear factor- κB (NF-κB) allowing NF-κB to translocate to the nucleus and activate transcription of anti-apoptotic genes (Harada et al. 2004).

Due to the potential beneficial effects and the extensive research done in our lab of IGF-1 in I/R injury, this protein was chosen in conjugation with SDF-1 as a therapeutic model for I/R injury in this study.

PEGylated Fibrin Gel as a Mode of Delivery

The use of biomaterials in tissue engineering is desirable as a delivery vehicle for materials such as cells and proteins. Biomaterials are designed to mimic the structure and function of natural tissues to induce, inhibit or enhance biological processes (Hubbell 1995). The retention of the bioactivity, protection from degradation, controlled release, and localization of the proteins or cells added to the biomaterial are key characteristics when selecting a delivery vehicle (Gombotz and Pettit 1995). Therefore, PEGylated fibrin was chosen to deliver SDF-1/IGF-1 to an I/R injury in the lateral gastrocnemius.

Fibrin is the primary component of the PEGylated fibrin biomaterial and naturally participates in clotting, cellular and matrix interactions, the inflammatory response and wound healing in the human body (Zhang et al.2008; Mosesson 2005). It is a biodegradable polymer constructed of fibrinogen monomers, which are composed of two sets of three polypeptide chains (Aα, Bβ, and γ) joined together by disulfide bridges. The addition of thrombin cleaves fibrinopeptide A from the Aα chains and fibrinopeptide B from the Bβ chains causing conformational changes and exposure of polymerization sites. This creates fibrin monomers that self-associate and form insoluble fibrin. The blood coagulation factor XIIIa, a transglutaminase, rapidly covalently cross-links γ chains in the fibrin polymer creating a stable fibrin network (Ahmed, Dare, and Hincke 2008).

The other component of PEGylated fibrin is the bi-functional succinimidylglutarate derivative of PEG (SG-PEG-SG) that creates secondary

crosslinking and mediates covalent binding to proteins (Hammers et al. 2011; Zhang et al. 2008). This protects proteins from recognition and degradation by proteolytic enzymes and the immune system in vivo to allow prolonged release (Roberts, Bentley, and Harris 2002). Most cytokines are released in limited amounts following tissue injury and have short half-lives in circulation usually lasting less than 30 minutes. PEGylated fibrin will release SDF-1 and IGf-1 in a controlled manner and prolong the presence and effects of SDf-1 and IGF-1 on the tissue injury and repair processes. Zhang et al. demonstrated the controlled release of SDF-1 over a 10 day period. SDF-1 retained its bioactivity upon release resulting in increased stem cell homing and heart function following treatment with PEGylated fibrin-SDF-1 on an induced acute myocardial infarction model.

Once the components of PEGylated fibrin are combined it is first present as a gel that rapidly polymerizes after 1 to 2 minutes. Therefore, the biomaterial has the ability to be molded to the site of injury and localize to the area. SDF-1 and IGF-1 are contained in the PEGylated fibrin gel via entrapment, conjugation amine reactive PEG linker, and physical affinity with the fibrin matrix (Drinnan et al. 2010). The proteins will be released in a controlled manner thereby allowing one sufficient administration of the treatment rather than several bolus injections of SDF-1 and IGF-1. The individual components of PEGylated fibrin are used as part of FDA approved devices, yet PEGylated fibrin as a whole has not been approved for clinical use. This study will investigate the potential of PEGylated fibrin as a therapy to I/R. In 2005, Hammers et al. showed the controlled release of IGF-1 with PEGylated

fibrin lasting several days. This resulted in enhanced muscle recovery due to hyper-activation of the PI3K/Akt pathway, reducing cell necrosis and apoptosis.

SIGNIFICANCE OF STUDY

Over 20,000 tourniquets (TK) are applied per day in the clinical setting to reduce blood loss and create a field of view for surgeons. Tourniquet applications are also used in the military combat setting to reduce blood loss from a traumatic injury. Despite these critical benefits, tourniquet applications cause an ischemia/reperfusion (I/R) injury which causes extensive damage exacerbating the damage of an injury or possibly cause loss of a limb. Often I/R causes prolonged or permanent functional deficits in the area of TK applied that affect daily activities, and current methods are not effective in preventing or treating these I/R induced injuries.

Research is needed to create effective treatments that can be used in the clinical setting. Current research has implicated stem cell therapy as a possible treatment. However, the procurement of these cells is difficult and time consuming for practical clinical use. Therefore, attention has been shifted to cytokines, and this study will evaluate a novel chemokine, stromal derived factor-1 (SDF-1), and a highly studied growth factor, insulin like-growth factor-1 (IGF-1). The purpose of this study is to utilize SDF-1 to enhance recovery of an I/R injury and supplement this treatment with IGF-1. This study will also assess biodegradable poly(ethylene) glycol (PEG)ylated fibrin matrix as a delivery vehicle of proteins, cells, or pharmaceuticals to specific areas via injection. The matrix will release the components in a controlled manner and prolong bioactivity, decreasing the need for multiple injections. These results may help in finding effective therapies in other

tissue loss models such as muscular dystrophy and further knowledge of the skeletal muscle repair process.

Methods

ANIMALS

Male Sprague–Dawley rats (6–9 months; Charles River) were used for this study. Rats maintained on a 12-hour light/dark cycle and were housed individually. They were allowed ad libitum access to food and water. The analgesic, carprofen, was administered prior to tourniquet application, 12 hours post, 24 hours post and whenever necessary. All experimental procedures were approved and conducted in accordance with guidelines set by the University of Texas at Austin and the Institutional Animal Care and Use Committee.

TOURNIQUET APPLICATION

The 2-hour tourniquet-induced I/R model of skeletal muscle injury was performed on a hind limb of the rat. The leg was elevated to draw blood out of the leg and a pneumatic tourniquet cuff (D.E. Hokanson, Inc.; Bellevue, WA) was placed proximal to the knee. The cuff was inflated to 250 mm Hg using the Portable Tourniquet System (Delfi Medical Innovations Inc.; Vancouver, BC, Canada) for 2 hours. During the course of this procedure, rats were anesthetized with 2% to 2.5% isoflurane and body heat was maintained with the use of a heat lamp.

PEGYLATED FIBRIN PREPARATION

Human fibrinogen was reconstituted in tris buffered saline (40 mg/mL, pH 7.8) in a 37°C water bath for 1 hour. Bifunctional SG-PEG-SG (NOF America Corp, Irvine, CA) was added to rat SFD-1α (PeproTech Inc.; Rocky Hill, NJ) and reconstituted human fibrinogen (Sigma-Aldrich Co.; St. Louis, MO; 5:1 PEG:fibrinogen molar ratio). The mixture was placed in a 37°C water bath for 1 hour to react. Polymerization was induced by the addition of 25 U/mL of human

thrombin (in 40mMcalcium chloride, Sigma). The final concentrations of fibrinogen and SDF-1 were 10 mg/mL and 10 μg/mL respectively. Twenty-four hours post injury, 0.25mL of PEGylated fibrin (PEG-Fib), PEGylated fibrin conjugated to SDF-1α, or PEGylated fibrin conjugated to SDF-1 and IGF-1 was injected3 into the lateral gastrocnemius (LGAS) muscle of the tourniquet injured limb. PEG-Fib-containing treatments were injected as a fluid and polymerized in situ.

In a subsequent experiment aimed at investigating additional potential treatments, 0.25 mL of PEGylated fibrin conjugated with two proteins SDF-1α and IGF-1 were injected in LGAS and allowed to recovery for the same period. The final concentrations of SDF-1 and IGF-1 were 10μg/mL and 25μg/mL respectively.

FUNCTIONAL ANALYSIS

Following 14 days of tourniquet induced I/R injury, *in* situ evaluations of lateral gastrocnemius (LGAS) force production were performed on the tourniquet and contralateral leg (uninjured). Rats were anesthetized with 2% to 2.5% isoflurane, and the skin of the hindlimb was removed to expose the hamstring. The hamstring was removed to isolate the LGAS and the medial gastrocnemius was deinnervated. The calcaneus was detached and the LGAS was separated from the plantaris and soleus. The Achilles tendon was attached to the lever arm of a dual mode servomotor (Aurora Scientific Model 310B Inc.; Aurora, ON, Canada). The muscle was stimulated using a stimulator (A-M Systems, Carlsborg, WA, Model 2100) with electrodes applied to the tibial nerve. Optimal length (Lo) was determined by finding the length producing the maximal

twitch force at 0.5 Hz and 5V and maximal peak tetanic tension (Po) was measured at 150 Hz and the minimal voltage required to elicit a maximal Po response. Each tetanic contraction was followed by 2 minutes of rest. Muscle temperature was

maintained with a heat lamp and mineral oil. After the completion of the contractile measurements, the muscles were harvested, weighed, embedded in OCT compound, and frozen in liquid nitrogen-cooled isopentane. The muscles were stored in a -80°C freezer until histological analysis.

HISTOLOGICAL ANALYSIS

Frozen, OCT-embedded muscle samples following 14 days of recovery after an I/R injury were sectioned on a cryostat (Leica CM1900; Leica Microsystems Inc.; Buffalo Grove, IL) and placed on a warm slide. Hematoxylin & eosin (H&E) staining was performed and slides were observed with a light microscope (Nikon Diaphot, Nikon Corp.; Tokyo, Japan) with the 20X objective lens. Images were taken using a mounted digital camera (Optronix Microfire; Optronix; Goleta, CA). Myofiber cross-sectional area (CSA) was measured using ImageJ software).

WESTERN BLOTTING

The following samples were prepared for western blotting: Saline, PEG, Fib, PEG-Fib, PEG-SDF-1, Fib-SDF-1 and PEG-Fib-SDF-1. Samples were boiled in 2X Laemmli's sample buffer at a ratio of 1:1 for 5 minutes, and equal amounts of total protein were loaded into each well of a 5% stacking-15% separating polyacrylamide gel. Following SDSPAGE, proteins were transferred to a PVDF membrane (Millipore) and blocked with 5% milk in 0.1% Tween-20 in TBS (TBST) for 1 h. Membranes were incubated in a 1:5000 dilution of anti-SDF-1α antibody in 5% BSA-TBST overnight at 4°C, then in 1:1000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce) in 5% milk-TBST for 2 h. Blots were imaged with the Chemidoc XRS system (Bio-Rad).

STATISTICAL ANALYSIS

Functional values were analyzed using one-way ANOVA to compare groups, and the Tukey post-hoc test was used to compare between data sets ($p<0.05$). The values are represented as the mean ± SEM, unless noted otherwise.

RESULTS

WESTERN BLOT ANALYSIS

Western blotting was used to indicate SDF-1α binding to PEGylated fibrinogen. Samples prepared and placed in the wells in the following order: Peg-Fib-SDF-1, PEG-Fib, Fib-SDF-1, Fib, PEG-SDF-1, PEG, SDF-1, and Saline. SDF-1α antibody was used as the primary antibody to detect the presence of SDF-1α in the samples. Positive staining was present in all samples containing SDF-1α and was also in the fibrinogen alone, which was most likely due to SDF-1 present in the fibrinogen (Figure 2). Fibrinogen is taken from fresh human plasma and sold for commercial use.

FUNCTIONAL ANALYSIS

Following 14 days of recovery after I/R injury, maximal isometric tetanic force (P_0) was measured and compared to the contralateral limb (no injury) in the LGAS. There was a significant difference between the groups. There was no significant difference seen in P_0 with the treatment of PEG-Fib/SDF-1 with 52.05 ± 6.00 compared to the PEG-Fib group with 49.48 ± 9.87% . However there was a significant difference in the PEG-Fib/SDF-1/IGF-1 group with 66.50 ± 13.37 compared to the PEG-fib group as seen in Figure 3.

Specific tension (SP_0) was also evaluated which normalized the P_0 to the LGAS cross sectional area (Figure 4). A significant difference was seen between the groups, but there was no significant difference between the PEG-Fib with 10.59 ± 2.41 N/cm^2 and PEG-fib/SDF-1 groups with 10.13 ± 2.21 N/cm^2 . There was a

significant increase in specific tension of PEG-Fib/SDF-1/IGF-1 group with 14.22 ± 1.79 N/cm^2 compared to the other groups.

LGAS weight and cross-sectional area were assessed, but there was no significant difference between the groups.

HISTOLOGICAL ANALYSIS

H&E staining was performed for histological evaluation 14 days after I/R injury. PEG-fib/SDF-1 had a greater distribution of smaller fibers than PEG-Fib group, while the PEG-Fib/SDF-1/IGF-1 group had fiber size distribution closer to the size distribution of the uninjured (Figure 5).

Smaller fiber size indicates degenerative/regenerative cycling of injured myofibers therefore fibers < 2000μm^2 were quantified for each group (Figure 6). There was a significantly greater distribution of smaller fibers in the PEG-Fib/SDF-1 group than the PEG-Fib group. The PEG-Fib/SDf-1/IGF-1 group had no significant difference between the uninjured.

Centrally located nuclei were analyzed (Figure 7), which indicate newly regenerating fibers. There were significantly greater centrally located nuclei in the PEG-Fib/SDF-1 group compared to the others, while the PEG-Fib/SDF-1/IGF-1 group had no significant difference from the uninjured group. These results correlated with the small fiber size results.

DISCUSSION

I/R injury induces a prolonged inflammatory phase that negatively impacts the regeneration process causing morbidity and permanent functional deficits. I/R injury cannot be healed using the normal tissue injury and repair processes, therefore a therapy was needed to enhance the processes. This study used SDF-1 conjugated to PEGylated fibrin and subsequently added IGF-1 to study the combined effects of both proteins.

Much literature has investigated the effects of IGF-1 as a therapy for I/R injury inducing intracellular signaling affecting the Ras/Raf-1/ERK MAPK and PI3K/Akt pathways. The effects include increased protein synthesis, cell survival, and activation of myoblasts to proliferate and differentiate. In 2011, Hammers et al. demonstrated an increase in muscle recovery following treatment of an I/R injury with IGF-1 conjugated to PEGylated fibrin in rats. The results were due to prolonged IGF-1 delivery by the PEGyated fibrin matrix and subsequent hyper-activation of the PI3K/Akt pathway stimulating increased cell survival to enhance tissue repair.

There is also ample literature on the effects of SDF-1α, however most are based on heart, liver, blood, or tumor models. Zhang et al. (2007) showed an increase in heart function after treatment of an acute myocardial infarction with PEGylated fibrin conjugated to SDF-1α. The study also showcased PEGylated fibrin as a mode of delivery to increase the presence of SDF-1, enhancing the regenerative effects by the chemokine. There is limited amount of literature on skeletal muscle regeneration and of the ones published they have demonstrated the chemotactic

ability of SDF-1 but have not measured functional recovery, which is important clinically.

The study is in agreement with previous studies on the effects of IGF-1, but not SDF-1. SDF-1α was proposed to induce migration of stem cells to injured tissue and activate similar pathways to IGF-1 though these molecular pathways are implicated on progenitor cell migration. The cells would promote a pro-regenerative environment to stimulate proliferation and differentiation of satellite cells and some of the cells may engraft and transdifferentiate into skeletal muscle. However, there was no significant difference in treatment with PEGylated fibrin and SDF-1α conjugated to PEGylated fibrin in LGAS force recovery. There was also a greater distribution of smaller fibers and centrally located nuclei in the SDF-1α conjugated PEGylated fibrin group than the PEGylated fibrin group. This indicates SDF-1 treated groups were still in the degeneration/regeneration phases. The unexpected results were not due to non-binding of SDF-1 to PEG as demonstrated in the western blot showing large complexes of the PEG-Fib-SDF-1 matrix. Nor were the results due to the biological inactivity of SDF-1, which retention of the bioactivity was shown to be maintained in the matrix (Zhang et al. 2007). The results may be due to an overwhelming migration of cells to the area hampering the regenerative process.

Skeletal muscle injury and repair is a complex process with many cytokines and cells infiltrating the area from specific cues and at certain time points. The first phase of tissue repair is the inflammatory phase, which is necessary for clearing

debris allowing for new fibers to generate in the area. The additional cells in the area may have inhibited clearing of debris thereby disrupting the inflammatory which is crucial for successful tissue repair. The right signals were also not present to induce the beneficial effects as a result of SFD-1 treatment.

In conclusion, the study demonstrates that SDF-1 does not enhance tissue repair following injury, but may actually hamper tissue regeneration. Furthermore, IGF-1 and SDF-1α conjugated to PEGylated fibrin revealed improvements of force relative to injured but Hammers (2011) demonstrated a greater improvement of force using only IGF-1 conjugated to PEGylated fibrin. This indicates SDF-1α hampered the complete beneficial effects of IGF-1. In 2010 Grefte et al. implanted a collagen matrix loaded with SDF-1 into a skeletal muscle defect injury. There was increased $Pax7^+$ satellite cells and $MyoD^+$ myoblasts and myofibers into the injured area, however the cells did not migrate within the scaffold and regenerate myofibers and instead caused fibrosis in the scaffold.

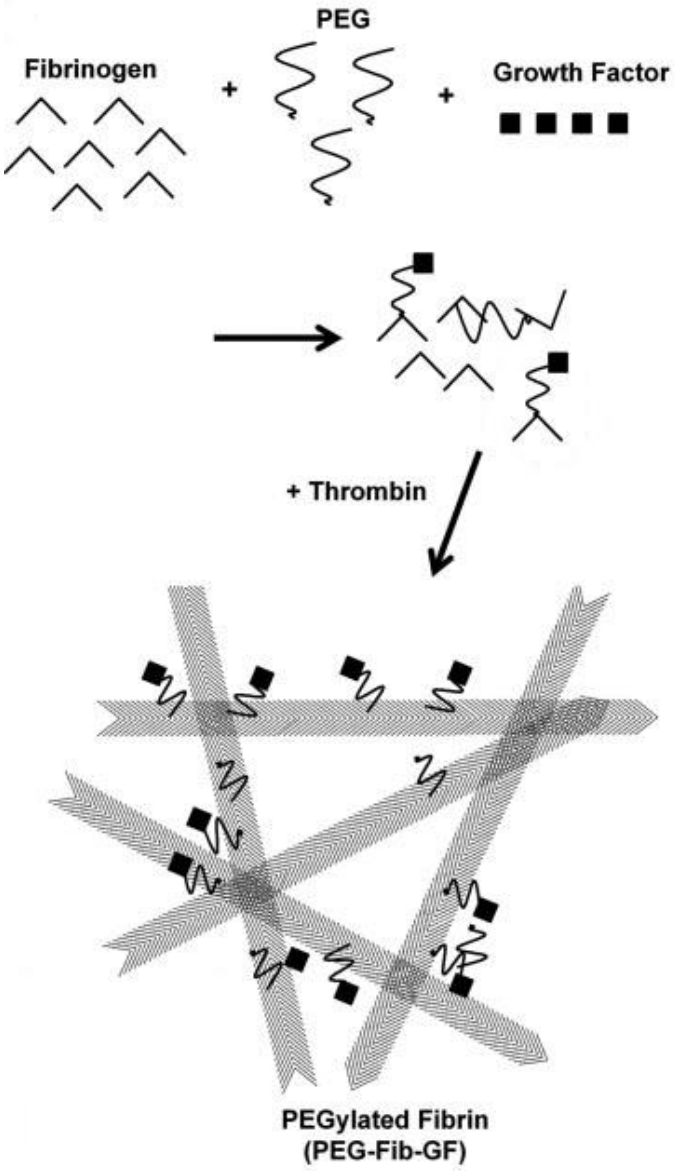


Figure 1: Taken from Hammers et al. (2011). The PEGylated fibrin gel is made by the coincubation of fibrinogen, bifunctional PEG and protein that covalently bind. The protein is sequestered in the complex by entrapment, covalent binding to PEG, and physical affinity to fibrin.

	1	2	3	4	5	6	7	8
PEG	-	-	+	+	-	-	+	+
Fib	-	-	-	-	+	+	+	+
SDF-1	-	+	-	+	-	+	-	+

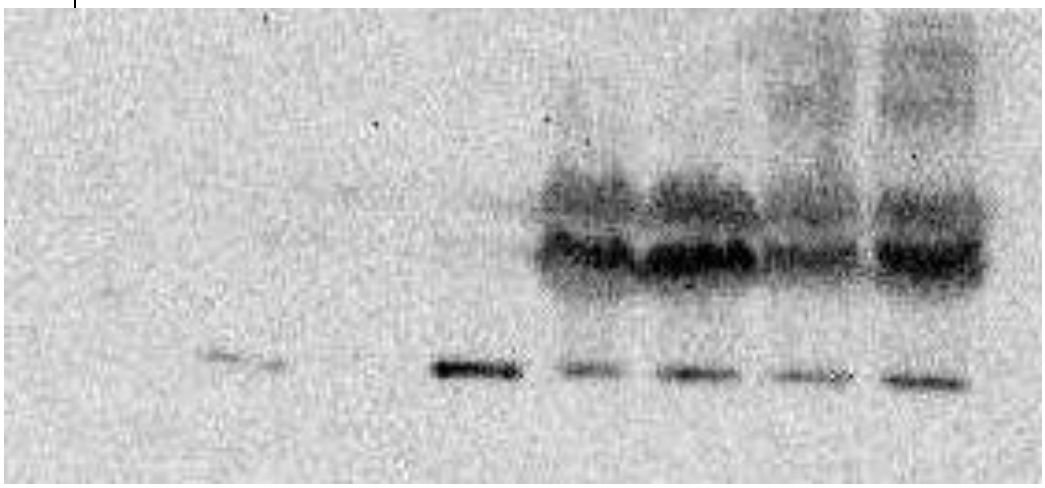


Figure 2: The PEGylated fibrin SDF-1 complex was verified by western blotting using anti-SDF-1α.

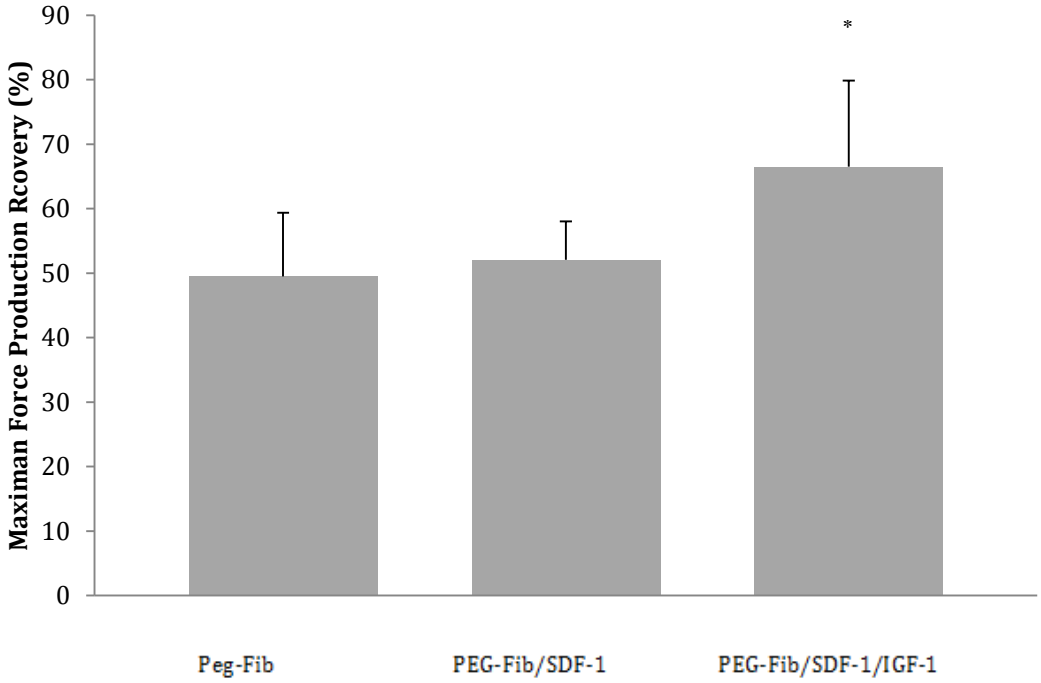


Figure 3: Following 14 days of an I/R injury, maximum tetanic force production (P_0) of the LGAS was measured in situ from the following groups: PEGylated fibrin (PEG-Fib), PEGylated fibrin conjugated with SDF-1α (PEG-Fib/SDF-1), and PEGylated fibrin conjugated to SDF-1α and IGF-I (PEG-Fib/SDF-1/IGF-I). The P_0 were compared to the contralateral leg that received no injury giving percent recovery. Values are represented in mean ± SEM, *P<0.05 versus PEG-Fib, n=6.

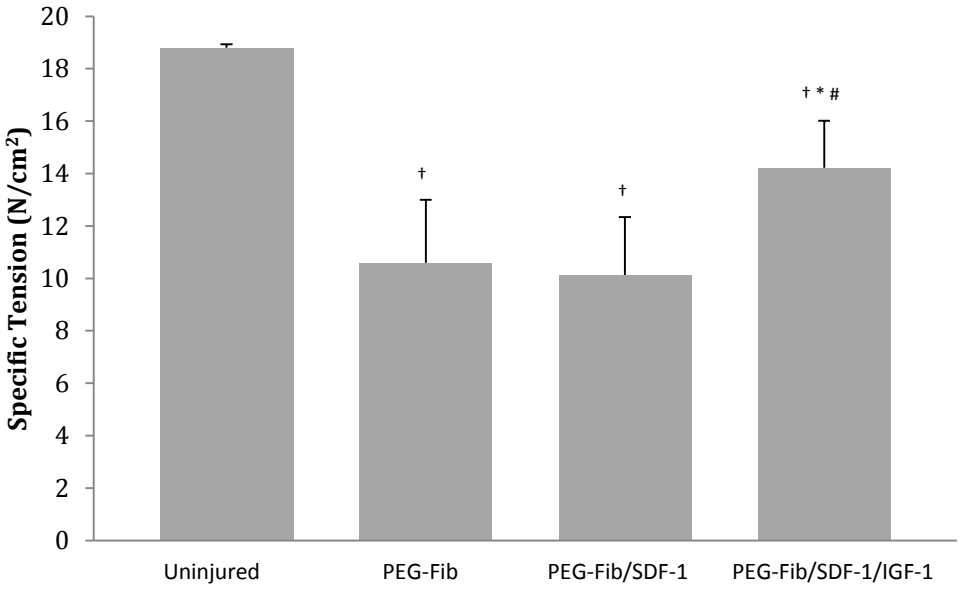


Figure 4: Following 14 days of an I/R injury, specific tension (SP_0) of the LGAS was measured in situ from the following groups: PEGylated fibrin (PEG-Fib), PEGylated fibrin conjugated with SDF-1α (PEG-Fib/SDF-1), and PEGylated fibrin conjugated to SDF-1α and IGF-I (PEG-Fib/SDF-1/IGF-I). Values are represented in mean ± SEM, † $P<0.05$ versus uninjured, * $P<0.05$ versus PEG-Fib, # $P<0.05$ versus PEG-Fib/SDF-1, n=6.

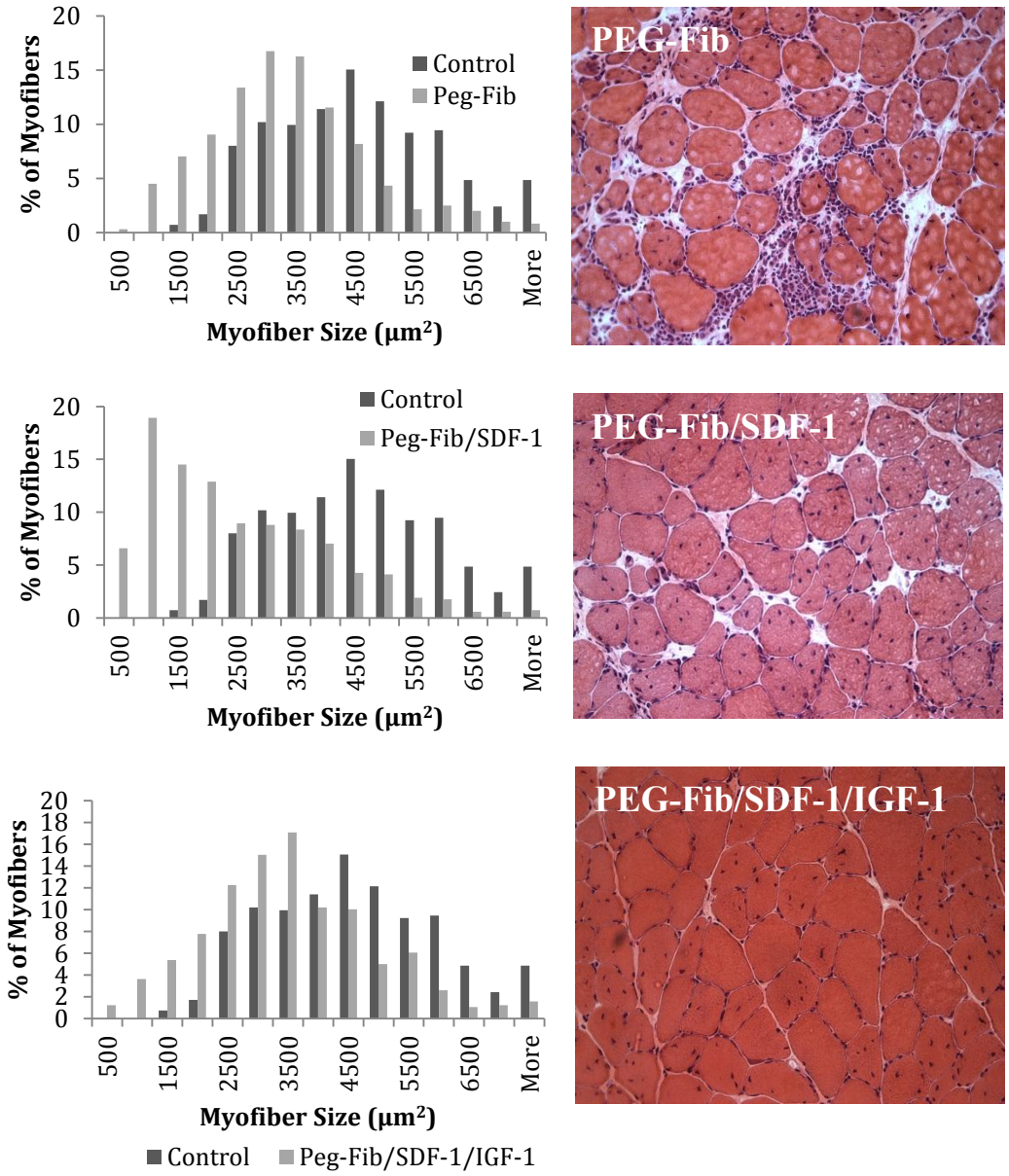


Figure 5: H&E stained sections were examined 14 days post-injury from each group for fiber size composition at 200X magnification. Smaller fibers were indicative of degenerative/regenerative cycling of injured myofibers, while very large rounded myofibers were due to focal edema and/or hypercontraction.

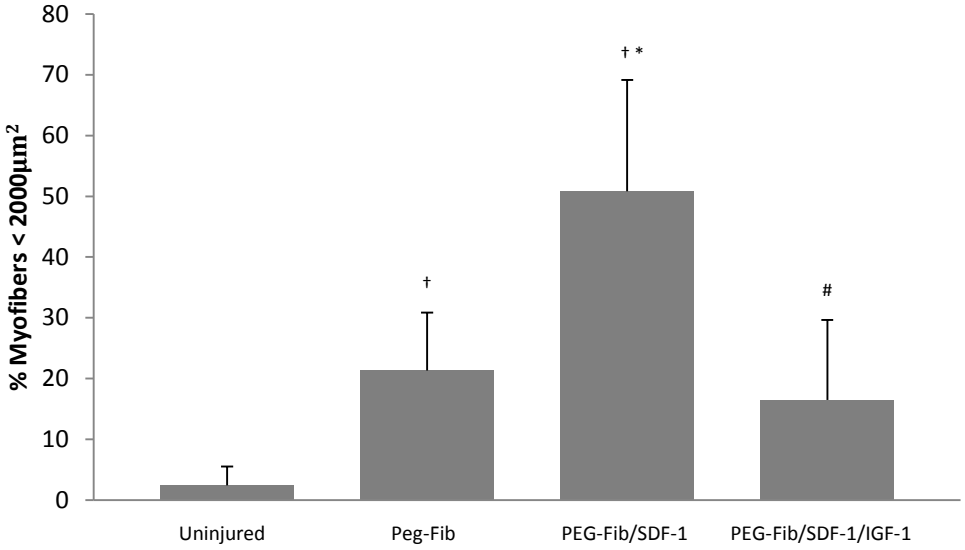


Figure 6: Small myofibers (< 2000 µm^2) were compared among the different groups, which indicate degenerative/regenerative cycling of injured myofibers. Values are represented in mean ± SEM, † P<0.05 versus uninjured, * P<0.05 versus PEG-Fib, # P<0.05 versus PEG-Fib/SDF-1, n=6, N=9.

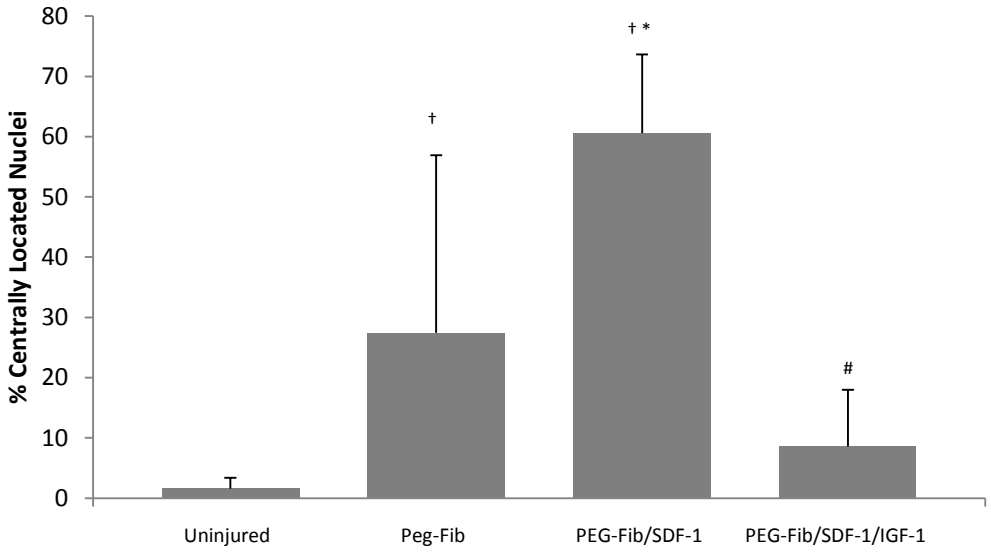


Figure 7: Centrally located nuclei were compared among the different groups, signifying regenerating myofibers. Values are represented in mean ± SEM, † P<0.05 versus uninjured, * P<0.05 versus PEG-Fib, # P<0.05 versus PEG-Fib/SDF-1, n=6, N=9.

Appendices

APPENDIX A: INSTRUMENTATION (TAKEN FROM MATTHEW TIERNEY)

A. Dual-Mode Muscle Lever System: Series 310B-LR, Aurora Scientific, Inc.

   Measurement and control of the dynamic physical properties, including length and force, of all types of muscle and connective tissue; can operate isotonically, isometrically or auxotonically.

B. Isolated Pulse Stimulator: Model 2100, A-M Systems.

   Physiological stimulation of neurological structures, used in conjunction with the dual-mode muscle lever system.

C. Rapid Sectioning Cryostat: Leica CM1900, Meyer Instruments, Inc.

   Reproducible sectioning of frozen skeletal muscle tissue for use in histological and immunohistological analysis.

D. Inverted Tissue Culture Microscope: Nikon Daiphot, Nikon Instruments, Inc.

   Magnification and visualization of histologically identifiable structures contained by frozen tissue sections obtained from the cryostat.

E. Digital Microscope Camera: Microfire, Optronics.

   Acquisition and collection of images visualized by the Nikon Diaphot inverted tissue culture microscope.

APPENDIX B: TOURNIQUET PROCEDURE

1. Connect the tubing to the chamber.
2. Turn on the oxygen tank and adjust it to 40%.
3. Turn on the isoflurane to 5% for 5 minutes and decrease to 2.5% for 5 mins.
4. Weigh the rat before you put it into the chamber.
5. Prepare the tourniquet bed and supplies while you wait for the rat to be completely anesthetized.
6. Transfer the rat to the cone and lay the rat face up on the tourniquet bed. Inject the rat with analgesic, carprofen. The stock is 50mg/mL and 5mg/kg is required for the rat.
7. The randomly selected tourniquet leg was shaved and suspended for 5 minutes.
8. The pneumatic tourniquet cuff was placed proximal to the knee and the pressure was set to 250mm Hg for 2 hours. As you inflate the cuff, hold it down.
9. Monitor breathing rate, temperature, isoflurane %m and response to toe and/or tail pinch.
10. After the procedure, monitor the rat for recovery.

APPENDIX C: PEGYLATED FIBRIN GEL PREPARATION

1. Reconstitute 0.016g of human fibrinogen in a sterile microcentrifuge tube with 200μL of sterile PBS (pH 7.8). Vortex and place in the 37°C sterile water bath for an hour.
2. Deactive bifunctional PEG with nitrogen gas and reconstitute 0.8mg of PEG in a sterile microcentrifuge tube with 200μL of sterile PBS (pH 7.8). Vortex.
3. Filter the PEG and fibrinogen into separate sterile microcentrifuge tubes. Half of the fibrinogen will be lost upon filtering.
4. Combine 41.25μL of PEG, 82.5μL of SDF-1α, then 41.25μL of fibrinogen. Place the mixture in the 37°C sterile water bath for an hour.
5. Place an aliquot of thrombin in the 37°C water bath for 10 minutes.
6. At this time anesthetize a rat with 2% to 2.5% isoflurane and place it faced down for ease of injection into the lateral gastrocnemius (LGAS).
7. Wipe down the tourniquet leg done 24 hours before with 70% alchohol.
8. Draw up thrombin first, then an equal amount of the PEgylated fibrin SDF-1 mixture with a 27 gauge syringe. The total volume in the syringe should be 250μL.
9. Swish the syringe around gently and quickly inject the needle intramuscularly in the middle of the LGAS. When inserting the needle into the LGAS, enter from the back of the knee and move toward the Achilles tendon. Then gently plunge the syringe along the entire length of the LGAS as you remove the needle back up towards the knee.

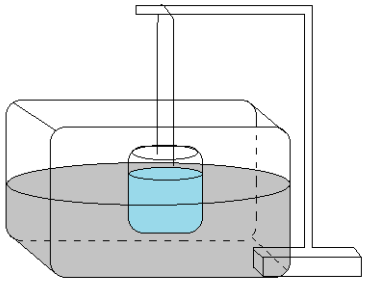
APPENDIX D: *IN SITU* FORCE MEASUREMENT

1. Anesthsize a rat (see Appendix B) and shave both legs.
2. Make an incision on the lateral leg, 5mm below and parallel to the femur towards the hip.
3. Carefully separate the muscles to reveal the sciatic nerve and sever the nerve as proximal to the hip as you can.
4. Make an incision in the skin down the midline of the posterior portion of the lower limb from the popliteal area to the calcaneus.
5. With blunt scissors separate the skin from the biceps femoris which inserts along the distal portion of the tibia in rats. Tie off major veins with polypropylene sutues to minimize blood loss.
6. Cut and separate the biceps femoris from the medial (MGAS) and lateral (LGAS) gastrocnemius.
7. Near the popliteal area cut the tibial nerve branch supplying MGAS.
8. Tweeze away superficial skin and the biceps femoris around the achilles tendon and the sides of the LGAS.
9. Cut the Achilles tendon and a portion of the calcaneus where it inserts near the foot so that the distal end of the muscle is unattached.
10. Using the calcaneus as an anchor, tie the Achilles tendon to the muscle lever arm of the dualmode servomotor with 3-0 silk thread.
11. Stimulate the muscle to contract utilizing a stimulator with leads applied to the deinnervated sciatic nerve.

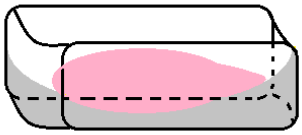
12. Throughout the remainder of the procedure, keep the muscle wet in mineral oil, and maintain the temperature between 35 and 37.5°C with a radiant heat lamp.
13. Adjust the muscle length to optimal length with a micrometer, and determine maximal twitch tension using stimulation of 0.5 Hz and 5V.
14. After determining optimal length, stimulate the muscle at 150 Hz voltage and the minimum voltage necessary to elicit maximal isometric tetanic contraction . After each contraction, allow the muscle to rest for two minutes.
15. Repeat for the contralateral leg.

Appendix E: Tissue Harvesting

1. After functional measurement, the LGAS, MGAS, plantaris and soleus were isolated and cleaned of excess fat and connective tissue.
2. Clean the muscles with 0.9% saline solution and leave them in the solution.
3. Prepare the freezing apparatus by pouring liquid nitrogen in a Styrofoam container. Partially submerge a container into the liquid nitrogen filled with isopentane.



4. In another container filled with ice, place an optimal cutting temperature compound (OTC, Tissue-Tek) filled bottle in the container to make the OTC more viscous.
5. Prepare boats with tape attached to the ends to hold OCT and the muscle (see below). Prepare aluminum foil squares and label each piece with each tissue harvested. When the isopentane is halfway frozen it is ready for tissue freezing.



6. Blot off excess liquid and weigh all the muscles separately on a scale. Measure the length of the LGAS. Record the values.
7. Fill the bottom of the boat with cold OCT and place the muscle on top. Then cover the top of the muscle with more OCT, and quickly place the entire boat into the isopentane for about 10 to 15 seconds.
8. Wrap the entire boat and muscle in labeled aluminum foil and place in the liquid nitrogen until the tissues are reasy to be stored in the -80°C freezer.
9. Repeat steps 7 & 8 for the other muscles.

APPENDIX F: HISTOLOGICAL ANALYSIS

1. Transport frozen tissue from -80°C freezer to the cryostat.
2. Using single-edge razor blades, cut the muscle crossectionally in the middle of the LGAS.
3. Mount tissue onto specimen disks using OCT using cold tweezers.
4. Insert specimen disk into specimen head and orient specimen head if necessary.
5. Initially adjust base of the blade holder to bring blade close to tissue using coarse feed settings and handwheel.
6. Begin sectioning tissue, ensuring sections are sliding under the anti-roll plate. Clean the plate with the cold brushes onto the discard pan.
7. Using a room temperature microscope slide, press the sectioned tissue onto the slide from the blade holder. Two sections are placed on each side.
8. Label the microscope slides with:
    a. Type of muscle/treatment
    b. Date
    c. Stain

A. Hematoxylin and Eosin Staining

Place slides in the coplin jar when staining. The harris hematoxylin and eosin reagents can be reused so put them back in their jars after use.

1. Add Harris Hematoxylin -- 5 min
2. Gently rinse with tap water until clear
3. Add Eosin -- 2 min
4. Gently rinse with tap water until clear
5. Rinse with 70% ethanol -- several seconds
6. Rinse with 100% ethanol -- several seconds
7. Rinse with xylene under the hood -- several seconds
8. Allow the stained slides to dry in the hood and then coverslip with Permount.

B. Masson's Trichrome Staining

The Working Weigert's Iron Hematoxylin is an equal volume mix of bottles A & B. The solution can be reused several times, but over time oxidizes and cannot be reused. Mix 20 ml of A with 20 ml of B to make the solution.

The Working Phosphotungstic/Phosphomolybdic Acid solution is a mix of the 2 acids with DI water. Mix 10 ml of phosphotungstic acid with 10 ml of phosphomolybdic acid and then add 20 ml of water.

Bouin's solution, Beibrich Scarlett Acid Fuchshin and Aniline Blue can also be reused.

1. Bouin's Solution -- 15 min at 56°C or overnight at room temp.
2. Gently rinse with tap water until clear.
3. Working Weigert's Iron Hematoxylin -- 5 or 6 min
4. Gently rinse with tap water until clear.
5. Rinse with DI water
6. Beibrich Scarlett Acid Fuchshin -- 5 min
7. Gently rinse with tap water until clear.
8. Rinse with DI water
9. Working Phosphotungstic/phosphomolybdic Acid -- 5 min
10. Aniline Blue -- 5 min
11. Quick 1% Acetic Acid rinse
12. 1% Acetic Acid -- 2 min
13. Rinse with tap water
14. Rinse with 70% ethanol -- several seconds
15. Rinse with 100% ethanol -- several seconds
16. Rinse with xylene under the hood -- several seconds
17. Allow the stained slides to dry in the hood and then coverslip with Permount.

APPENDIX G: RAW DATA

i. PEG-Fib Group

Tourniquet Leg										
Animal	**Weight (g)**	**MGAS (g)**	**Plantaris (g)**	**Soleus (g)**	**Max Tetanic Contraction (N)**	**Optimal Length (N)**	**LGAS Length (mm)**	**LGAS (g)**	**PCSA (cm^2)**	**Specific Tension (N/cm^2)**
CPF141	700	1.429	0.677	0.436	15.38	0.75	31	1.559	1.239	11.807
CPF142	650	1.238	0.522	0.357	12.87	0.80	30	1.495	1.228	9.830
CPF143	680	1.199	0.665	0.346	13.77	0.80	32	1.465	1.128	11.498
CPF144	660	1.246	0.556	0.376	13.84	0.65	32	1.331	1.025	12.871
CPF145	586	1.188	0.535	0.251	11.54	0.77	32	1.224	0.942	11.428
CPF146	628	1.318	0.499	0.340	7.58	0.75	33	1.499	1.119	6.103
Avg	650.67	1.27	0.58	0.35	12.50	0.75	31.67	1.43	1.11	10.59
Std Dev	40.19	0.09	0.08	0.06	2.72	0.06	1.03	0.13	0.12	2.41
Control Leg										
Animal	**% Recovery**	**MGAS (g)**	**Plantaris (g)**	**Soleus (g)**	**Max Tetanic Contraction (N)**	**Optimal Length (N)**	**LGAS Length (mm)**	**LGAS (g)**	**PCSA**	**Specific Tension**
CPF141	56.28%	1.495	0.722	0.431	27.33	0.80	32	1.734	1.335	19.871
CPF142	52.81%	1.407	0.612	0.329	24.37	0.90	31	1.662	1.321	17.767
CPF143	51.73%	1.565	0.756	0.306	26.62	0.85	34	1.695	1.228	20.980
CPF144	52.09%	1.616	0.728	0.395	26.57	0.65	34	1.577	1.143	22.681
CPF145	54.36%	1.337	0.622	0.244	21.23	0.70	33	1.508	1.126	18.234
CPF146	29.62%	1.508	0.711	0.335	25.59	0.70	33	1.788	1.335	18.645
Avg	49.48%	1.49	0.69	0.34	25.29	0.77	32.83	1.66	1.25	19.70
Std Dev	9.87%	0.10	0.06	0.07	2.24	0.10	1.17	0.10	0.10	1.87

ii. PEG-Fib/SDF-1 Group

Tourniquet Leg										
Animal	**Weight (g)**	**MGAS (g)**	**Plantaris (g)**	**Soleus (g)**	**Max Tetanic Contraction (N)**	**Optimal Length (N)**	**LGAS Length (mm)**	**LGAS (g)**	**PCSA (cm^2)**	**Specific Tension (N/cm^2)**
SDF142	723	1.495	0.650	0.373	11.16	0.67	28	1.578	1.389	7.555
SDF143	718	1.346	0.635	0.379	9.56	0.68	33	1.624	1.213	7.324
SDF144	787	1.383	0.715	0.373	16.75	0.80	32	1.636	1.260	12.662
SDF145	605	1.277	0.600	0.367	14.59	0.85	31	1.512	1.202	11.433
SDF146	594	1.228	0.484	0.243	12.49	0.80	30	1.245	1.023	11.433
SF147	580	1.128	0.635	0.250	13.90	0.65	29	1.502	1.276	10.383
Avg	667.83	1.31	0.62	0.33	13.08	0.74	30.50	1.52	1.23	10.13
St Dev	85.88	0.13	0.08	0.07	2.56	0.08	1.87	0.14	0.12	2.21
Control Leg										
Animal	**% Recovery**	**MGAS (g)**	**Plantaris (g)**	**Soleus (g)**	**Max Tetanic Contraction (N)**	**Optimal Length (N)**	**LGAS Length (mm)**	**LGAS (g)**	**PCSA**	**Specific Tension**
SDF142	50.70%	1.728	0.7780	0.345	22.01	0.65	31	1.958	1.556	13.726
SDF143	44.12%	1.898	0.7200	0.407	21.67	0.68	32	1.907	1.468	14.295
SDF144	62.04%	1.256	0.5990	0.319	27.00	0.90	32	1.751	1.348	19.359
SDF145	48.86%	1.479	0.7110	0.332	29.86	0.90	31	1.740	1.383	20.941
SDF146	54.30%	1.329	0.5640	0.229	23.00	0.70	31	1.563	1.242	17.951
SDF147	52.26%	1.642	0.6070	0.227	26.60	0.65	29	1.650	1.402	18.511
Avg	52.05%	1.56	0.66	0.31	25.02	0.75	31.00	1.76	1.40	17.46
St Dev	6.00%	0.25	0.08	0.07	3.29	0.12	1.10	0.15	0.11	2.86

iii. PEG-Fib/SDF-1/IGF-1 Group

Tourniquet Leg										
Animal	**Weight (g)**	**MGAS (g)**	**Plantaris (g)**	**Soleus (g)**	**Max Tetanic Contraction (N)**	**Optimal Length (N)**	**LGAS Length (mm)**	**LGAS (g)**	**PCSA (cm^2)**	**Specific Tension (N/cm^2)**
Combo141	609	1.34	0.636	0.376	13.96	0.65	31	1.4	1.112719	11.96169
Combo142	583	1.39	0.323	0.676	18.41	0.7	32	1.658	1.276596	13.87283
Combo143	619	1.387	0.623	0.299	18.69	0.7	32	1.558	1.1996	14.99666
Combo144	465	1.107	0.512	0.2	17.18	0.6	30	1.184	0.97241	17.05042
Combo145	530	1.17	0.586	0.263	17.58	0.65	31	1.455	1.156433	14.63985
Combo146	493	0.961	0.49	0.209	12.57	0.65	30	1.132	0.929703	12.8213
Avg	549.83	1.23	0.53	0.34	16.40	0.66	31.00	1.40	1.11	14.22
St Dev	63.57	0.18	0.12	0.18	2.53	0.04	0.89	0.21	0.13	1.79
Control Leg										
Animal	**% Recovery**	**MGAS (g)**	**Plantaris (g)**	**Soleus (g)**	**Max Tetanic Contraction (N)**	**Optimal Length (N)**	**LGAS Length (mm)**	**LGAS (g)**	**PCSA**	**Specific Tension**
Combo141	48.71%	1.528	0.734	0.351	28.66	0.65	32	1.814	1.39671	20.05427
Combo142	70.81%	1.519	0.687	0.32	26	0.65	32	1.838	1.415189	17.9128
Combo143	69.48%	1.347	0.64	0.293	26.9	0.65	33	1.675	1.250604	20.98985
Combo144	81.93%	1.311	0.56	0.212	20.97	0.65	30	1.468	1.205657	16.85388
Combo145	51.80%	1.382	0.595	0.287	33.94	0.7	31	1.607	1.277242	26.02482
Combo146	76.27%	1.213	0.539	0.248	16.48	0.6	30	1.433	1.176912	13.49294
Avg	66.50%	1.38	0.63	0.29	25.49	0.65	31.33	1.64	1.29	19.22
St Dev	13.37%	0.12	0.08	0.05	6.09	0.03	1.21	0.17	0.10	4.25

iv. Myofiber Distribution

Control		
Fiber size (μm^2)	**Frequency**	**% of Myofibers**
500	0	0.00%
1000	0	0.00%
1500	3	0.73%
2000	7	1.70%
2500	33	8.01%
3000	42	10.19%
3500	41	9.95%
4000	47	11.41%
4500	62	15.05%
5000	50	12.14%
5500	38	9.22%
6000	39	9.47%
6500	20	4.85%
7000	10	2.43%
More	20	4.85%
Total	412	100.00%

Peg-Fib		
Fiber size (μm^2)	**Frequency**	**% of Myofibers**
500	2	0.34%
1000	27	4.52%
1500	42	7.04%
2000	54	9.05%
2500	80	13.40%
3000	100	16.75%
3500	97	16.25%
4000	69	11.56%
4500	49	8.21%
5000	26	4.36%
5500	13	2.18%
6000	15	2.51%
6500	12	2.01%
7000	6	1.01%
More	5	0.84%
Total	597	100.00%

Peg-Fib/SDF-1		
Fiber size (μm^2)	**Frequency**	**% of Myofibers**
500	45	6.60%
1000	129	18.91%
1500	99	14.52%
2000	88	12.90%
2500	61	8.94%
3000	60	8.80%
3500	57	8.36%
4000	48	7.04%
4500	29	4.25%
5000	28	4.11%
5500	13	1.91%
6000	12	1.76%
6500	4	0.59%
7000	4	0.59%
More	5	0.73%
Total	682	100.00%

Peg-Fib/SDF-1/IGF-1		
Fiber size (μm^2)	**Frequency**	**% of Myofibers**
500	7	1.21%
1000	21	3.63%
1500	31	5.35%
2000	45	7.77%
2500	71	12.26%
3000	87	15.03%
3500	99	17.10%
4000	59	10.19%
4500	58	10.02%
5000	29	5.01%
5500	35	6.04%
6000	15	2.59%
6500	6	1.04%
7000	7	1.21%
More	9	1.55%
Total	579	100.00%

v. Small fiber size (< 2000µm²)

Slide	Total Fibers	Total Fibers < 2000µm²	% Fibers < 2000µm²	Total Fibers with Centrally Located Nuclei	% Centrally Located Nuclei
Control 1a	56	1	1.79%	1	1.79%
Control 1b	44	0	0.00%	0	0.00%
Control 1c	60	1	1.67%	0	0.00%
Control 2a	52	1	1.92%	1	1.92%
Control 2b	46	2	4.35%	0	0.00%
Control 2c	42	1	2.38%	1	2.38%
Control 3a	32	0	0.00%	1	3.13%
Control 3b	39	0	0.00%	2	5.13%
Control 3c	41	4	9.76%	0	0.00%
PEG-FIB 1a	71	10	14.08%	3	4.23%
PEG-FIB 1b	71	7	9.86%	2	2.82%
PEG-FIB 1c	60	9	15.00%	13	21.67%
PEG-FIB 2a	61	18	29.51%	34	55.74%
PEG-FIB 2b	71	9	12.68%	3	4.23%
PEG-FIB 2c	62	20	32.26%	50	80.65%
PEG-FIB 3a	70	11	15.71%	2	2.86%
PEG-FIB 3b	70	21	30.00%	12	17.14%
PEG-FIB 3c	61	20	32.79%	35	57.38%
PEG-FIB/SDF-1 1a	88	48	54.55%	35	39.77%
PEG-FIB/SDF-1 1b	67	36	53.73%	50	74.63%
PEG-FIB/SDF-1 1c	76	39	51.32%	54	71.05%
PEG-FIB/SDF-1 2a	82	61	74.39%	46	56.10%
PEG-FIB/SDF-1 2b	73	38	52.05%	33	45.21%
PEG-FIB/SDF-1 2c	99	79	79.80%	78	78.79%
PEG-FIB/SDF-1 3a	63	21	33.33%	38	60.32%
PEG-FIB/SDF-1 3b	66	21	31.82%	42	63.64%
PEG-FIB/SDF-1 3c	68	18	26.47%	38	55.88%
PEG-FIB/SDF-1/IGF-1 1a	59	6	10.17%	3	5.08%

Slide	Total Fibers	Total Fibers < 2000μm²	% Fibers < 2000μm²	Total Fibers with Centrally Located Nuclei	% Centrally Located Nuclei
PEG-FIB/SDF-1/IGF-1 1b	51	11	21.57%	13	25.49%
PEG-FIB/SDF-1/IGF-1 1c	63	6	9.52%	4	6.35%
PEG-FIB/SDF-1/IGF-1 2a	57	1	1.75%	4	7.02%
PEG-FIB/SDF-1/IGF-1 2b	72	17	23.61%	1	1.39%
PEG-FIB/SDF-1/IGF-1 2c	63	5	7.94%	0	0.00%
PEG-FIB/SDF-1/IGF-1 3a	56	2	3.57%	5	8.93%
PEG-FIB/SDF-1/IGF-1 3b	75	22	29.33%	0	0.00%
PEG-FIB/SDF-1/IGF-1 3c	83	34	40.96%	19	22.89%

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