Follistatin: a novel therapeutic for the improvement of muscle regeneration


Department of Biology, School of Science, Center for Regenerative Biology and Medicine, Indiana University-Purdue University Indianapolis-(BCY, JMW, GD)
Musculoskeletal Research, Lilly Research Laboratories-(BY, YW, PS, AM, HUB, VK, JA)
Departments of Drug Disposition Development/Commercialization, and Biotechnology Discovery Research. Lilly Research Laboratories-(ADM, JEC)
Translational Sciences-Molecular Pathology. Lilly Research Laboratories-(JMW)
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Corresponding Authors: Venkatesh Krishnan PhD
Lilly Research Laboratories
Eli Lilly & Company
Lilly Corporate Center
Indianapolis, IN 46285, USA
Email: krishnan_gary@lilly.com
Phone: (317) 276-0603

And

Guoli Dai, DVM, PhD
Department of Biology, School of Science
Center for Regenerative Biology and Medicine
Indiana University-Purdue University Indianapolis, Indiana 46202
Email: gdai@iupui.edu
Phone: (317) 278-3895

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List of abbreviations: IgGs, immunoglobulins; IV, intravenous; SC, subcutaneous; IM, intramuscular; LLOQ, lower limit of quantitation; CTX, Cardiotoxin; FBS, fetal bovine serum; GDF, growth differentiation factor; BMP, bone morphogenic factor; TGFβ, tissue growth factor beta; SBE, SMAD binding element; βA, βB, βC, and βE, beta subunits; gdf8, growth differentiation factor 8; ActRIIA/B-Fc, a fusion protein of ectodomain of ActRIIA or B and Fc-region of immunoglobulin; PK, pharmacokinetic; PD, pharmacodynamic; FST288, human follistatin 288 isoform; FST315, human follistatin 315 isoform; FST-Fc, follistatin 315 murine IgG1 Fc fusion; FST-ΔHBS-Fc, follistatin 315 heparan sulfate binding deficient mutant murine IgG1 Fc fusion.
ABSTRACT

Follistatin (FST) is a member of the TGFβ family and is a secreted glycoprotein that antagonizes many members of the family including Activin A, GDF11 and myostatin. The objective of this study was to explore the use of an engineered Follistatin therapeutic created by fusing FST315 lacking heparin binding activity to the N-terminus of a murine IgG1 Fc (FST315-ΔHBS-Fc) as a systemic therapeutic agent in models of muscle injury. Systemic administration of this molecule was found to increase body weight and lean muscle mass after weekly administration in normal mice. Subsequently, we tested this agent in several models of muscle injury which were chosen based on their severity of damage and their ability to reflect clinical settings. FST315-ΔHBS-Fc treatment proved to be a potent inducer of muscle remodeling and regeneration. FST315-ΔHBS-Fc induced improvements in muscle repair after injury/atrophy by modulating the early inflammatory phase allowing for increased macrophage density, and Pax7-positive cells leading to an accelerated restoration of myofibers and muscle function. Collectively, these data demonstrate the benefits of a therapeutically viable form of FST that can be leveraged as an alternate means of ameliorating muscle regeneration.
INTRODUCTION

There are a multitude of diseases that affect skeletal muscle that can range from direct injury to the soft tissue or nerve supply, chronic inflammation, aging and cancer. Irrespective of the mode, it results in the same outcome, limited mobility with the contemporaneous loss of muscle function and mass. Skeletal muscle injuries are one of the most common types of work and sports related injuries that present a major challenge to primary care physicians, physical therapists and sports medicine professionals. Individuals can sustain muscle injuries through a myriad of different mechanisms, including improper handling/biomechanics of heavy materials (e.g. muscle strain, tears, neuronal impingement and pulls), trauma (falls, surgeries and sports collisions), and genetic disorders (muscular dystrophy). Patients who suffer from these types of occurrences have very few options in regards to treatment, which limits their ability to return to work or resume their current lifestyle. In order to mitigate the long term issues with muscle injury/trauma, the current conservative treatment incorporates the minimizing of inflammation with limb elevation and local cooling, in conjunction with a regimented physical therapy plan and an adjuvant treatment of non-steroidal anti-inflammatory drugs (NSAIDs). Irrespective of complying with aforementioned treatment protocol, it can be difficult to achieve complete recovery from injury due to the concomitant development of fibrosis that is most often seen due to co-morbidities associated with metabolic and structural aging.

Myostatin or Gdf8 is a well-studied member of the TGFβ family that regulates muscle mass through development and postnatally. Gdf8 has been the center of focus in developing therapeutics which inhibit and neutralize its activity. A variety of methods to accomplish Gdf8 inhibition include the following; specific antibodies which govern the ability of the ligand to interact with its receptor, dominant negative-mimetic proteins, adenoviruses to deliver a
propeptide inhibitor, and a receptor decoy comprised of the ectodomain of the Activin receptor IIB conjugated to the Fc domain of the IgG molecule (Teo et al, 2012).

Another TGFβ superfamily member, FST, has also been found to play a significant role in the management of skeletal muscle size and mass (Amithor et al, 2004; Lee, 2007). FST is comprised of a 63-residue amino (N)-terminal domain, followed by three successive follistatin domains of 73–75 amino acids termed FSD1, FSD2 and FSD3 (Cash et al, 2009; Wang et al, 2000). There are three reported isoforms that exist; FST288, FST315 and FST300 (or FST303), which are thought to be products of posttranslational modification events (Hashimoto et al, 2000; Sidis et al, 2006). FST288 and 315 proteins differ only in their possession of exon 6, which has been shown to influence their interactions with heparin, while FST303 is produced by proteolytic processing of the C-terminal tail domain (Sugino et al, 1993). Full-length exon 6 reduces the overall binding to cell surface heparin sulfated proteoglycans which is observed with FST315 isoform, whereas FST288 lacks exon 6 and therefore displays greater cell surface affinity (Sidis et al, 2006). Affinity to cell surface receptors helps dictate the general biological actions of FST with FST288 conducting itself more in an autocrine fashion and FST315 (primarily found in circulation) behaving more as an endocrine factor (Bilezikjian et al, 2004; Kumar, 2005; Yuen & Ge, 2004)

FST proteins were found to associate with Gdf8 in the serum and later demonstrated to antagonize Gdf8-mediated inhibition of myogenesis (Hill et al, 2002; Thomas et al, 2000). The FST-transgenic mice produced a robust skeletal muscle hypertrophy phenotype and when additionally crossed in a Gdf8 null mouse resulted in a quadrupling effect on muscle (Lee, 2007). This finding indicated the existence of other muscle regulators besides Gdf8 that Follistatin can modulate. Activin A has been the logical option for the second player in regulating muscle mass
and recent data from several laboratories would confer this as the best choice (Gilson et al, 2009; Lee et al, 2012).

Finally, the main objective of this study was to test a newly engineered FST biotherapeutic that fuses a heparin-deficient binding form of Follistatin 315 to the N-terminus of a murine IgG1 Fc (FST315-ΔHBS-Fc) (Datta-Mannan et al, 2012; Sidis et al, 2005a) in multiple models of muscle injury. Our results clearly demonstrate the viability of a systemically administered form of FST that can increase muscle mass, facilitate muscle repair and restore muscle function in multiple models of muscle injury. Combining FST’s effects on inflammation and pro-regenerative capabilities makes it a practical option for the treatment of muscle diseases where injury is associated.
MATERIALS and METHODS

Animal care and use

All mouse experiments were performed with the approval of Eli Lilly and Company’s Institutional Animal Care and Use Committee and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

For all studies described here, ten-week-old C57BL/6 female and male mice were used (Harlan, Indianapolis, IN). Animals were housed in a room with controlled temperature (22±2°C), and a 12:12 hour light-dark cycle (lights on at 06:00 am) with ad lib access to food (TD 5001 with 0.95% calcium and 0.67% phosphorus, Teklad, Madison, WI) and water.

Construction, Expression, and Purification of Recombinant Follistatin Variant Proteins

The Follistatin variants used in these studies (FST288, FST288-ΔHBS, FST315, and FST315-ΔHBS-Fc) were initially described by Datta et al., 2012 (Datta-Mannan et al, 2013). Proteins were expressed in stably transfected Chinese hamster ovary cells at Eli Lilly (Indianapolis, IN). Isolation of non-Fc containing FST variants containing C-terminal hexahistidine tags from concentrated cell culture supernatants was facilitated by a two-step purification method. In a first step, crude conditioned cell culture media containing either FST288 or FST315 was captured using Nickel-charged chelating sepharose (GE Healthcare), Bound proteins were eluted using a step gradient of Imidazole and pooled fractions were concentrated using an Amicon Ultra-15 concentrator (Millipore) and further purified using a Superdex G200 preparative gel filtration step (GE Healthcare). Finally protein purity was assessed using both simply-blue stained (Invitrogen) SDS-PAGE gel analysis and analytical gel filtration on a TSKG3000SWXL column (Tosho Bioscience) and was generally found to be >95%.
Isolation of FST315-ΔHBS-Fc chimeric protein from concentrated cell culture supernatants was performed using a two-step purification method. In the first step, crude conditioned cell culture media containing the specific variant was captured onto Mab select sepharose (GE Healthcare) under high salt conditions (1M sodium chloride) and eluted using a step-gradient of 10 mM sodium citrate, pH 3.0. Pooled protein was concentrated using an Amicon Ultra-15 concentrator (Millipore) and further purified using a Superdex G200 preparative gel filtration step (GE Healthcare). These steps generally resulted in protein purity of >95%, as assessed by simply-blue stained (Invitrogen) SDS-PAGE and analytical gel filtration on a TSKG3000SWXL column (Tosho Bioscience).

**In vivo electroporation**

FST plasmids were constructed by inserting the cDNAs into the CAG expression vector (InvivoGen, San Diego, CA). DNA for all in vivo experiments was prepared using the Qiagen ENDO-Free Maxi prep kit (Valencia, CA). DNA was eluted into physiological saline at a concentration of 0.5 µg/µL. Mice legs were shaved and then percutaneously injected with 25 µg (0.05 mL) of cDNA into the gastrocnemius muscle using an insulin syringe. Shortly following the injection of the cDNA (~20-30 seconds) four pulses of 160 volts/cm for 0.1 ms duration at 100 ms interval was delivered transcutaneously to the DNA injected limb at a rate of one pulse/second with a BTX 830M electroporator (Harvard Apparatus model # 8300, Holliston, MA) utilizing gene calipers.

**In vivo injury/immobilization models**

**Cardiotoxin Injury:** similar to what was described by Garry et al., with slight modifications (Garry et al, 2000). Muscle injury was induced by a 100 µL injection of a 10 µM Cardiotoxin
(CTX) (Cardiotoxin 1 from *naja naja atra*; Sigma-Aldrich, St. Louis, MO; part #C3987) solution into the right gastrocnemius muscle with a three point injection technique to fully cover the lateral and medial gastrocnemius. The injected muscles were harvested at various timepoints following CTX injury. Experiments were performed with each study incorporating an uninjured (no CTX or treatment) control group as a reference.

*Cardiotoxin Injury/hindlimb immobilization*: lower limb muscles were injured using the aforementioned protocol. Following the CTX injections, mice were subjected to hindlimb immobilization with the employment of Vet-Lite casting (Jorgensen Labs, Loveland, Colorado). Animals were placed in a plantar-flexed state to allow for maximal atrophy of the gastrocnemius muscle. Animals were maintained in this flexed state for a period of 7 days.

*Ovariectomy*: female mice at eight weeks of age were anesthetized and placed in ventral recumbency. The dorsal mid-lumbar area was shaved and swabbed and a 2-3 cm dorsal midline skin incision was made halfway between the caudal edges of the ribcage where a single incision was made into the muscle wall. The ovary and the oviduct are exteriorized through the muscle wall. A hemostat was used to clamp the uterine vasculature between the oviduct and uterus. Each ovary and part of the oviduct was removed with single cuts through the oviducts near the ovary. The ovary on the other side is removed in a similar manner.

*Body composition*

Lean body mass and fat mass were measured using nuclear magnetic resonance (NMR; Echo Medical Systems, Houston, TX).

*ELISA for Follistatin*
The quantitative determination of human FST concentration was determined using a Human Follistatin Quantikine Elisa kit (Quantikine; R&D Systems; Cat # DFN00). Briefly, standards and sample lysates were added to a flat-bottomed 96-well ELISA plates that have been pre-coated with a monoclonal antibody specific for follistatin. The plate was washed 4 times and 400 µL of the enzyme-linked monoclonal antibody specific to follistatin was added to each well. The plate was washed an additional 4 times and then one additional time to remove any unbound antibody-enzyme reagent. Substrate is added and incubated for thirty minutes and then plates are read at 450 nm on a microplate reader.

**Histology and Immunohistochemistry**

Muscle tissue was evaluated using hematoxylin and eosin (H&E) and immunolabeled using anti-mouse F4/80 clone BM8 (eBioscences, San Diego, CA) at 0.5 µg/mL or anti-Pax 7 (0.1 µg/mL) (Sigma Aldrich, St. Louis, MO). Fiber diameter was analyzed in H&E-stained gastrocnemius muscle sections using Aperio ImageScope software (Vista, CA). For each muscle, the distribution of the fiber diameter was calculated by analyzing 100 to 150 myofibers. Images were acquired using digital slide scanning (ScanScope XT, Aperio, Vista, CA).

**Gene expression analysis**

RNA was extracted from isolated injured and uninjured skeletal muscles using TRIzol reagent. Total RNAs were reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). All cDNAs were assayed for genes of interest using TaqMan Gene Expression Analysis (Applied Biosystems, Carlsbad, CA) and the Assay-On-Demand primer/probe set. Gene of interest mRNA levels were quantitated by determining the cycle number at which amplification detection threshold was achieved. Real time-PCR reactions were
performed in 20 µL reactions according to the manufacturer's guidelines. Samples were subjected to quantitative PCR using ABI 7900HT real-time PCR system. After normalization to the housekeeping gene (GAPDH) or ribosomal protein, large, P0 (36B4) for the injury/immobilization, the expression of the gene of interest was examined in the affected leg (gastrocnemius) as compared to the vehicle control or non-injured legs for the subsequent analysis.

**In situ muscle function**

All *in situ* muscle function experiments were performed using the 807B *in situ* small animal apparatus from Aurora Scientific (Aurora, ON, Canada). The knee was clamped using either a buttressing pad or blunted set screw. The foot was then secured in the foot pedal and two platinum electrodes were then inserted to stimulate the tibial nerve. Contractions of the gastrocnemius were induced by direct stimulation via the tibial nerve and single twitches (rectangular pulse, 1 ms) were applied at different muscle lengths to determine the optimal length (resting length \([L_0]\)), measured with calipers as the distance between the medial condyle of the femur and the myotendinous junction near the calcaneus. Experimental tetanic/twitch measurements are then performed upon the optimal placement of the electrodes and the ensuing muscle measurements of force were recorded. Finally, the muscle was subjected to strength and fatigue measurements with external stimulation applied. The stimulation protocol was evaluated and optimized by stimulating the plantar-flexor muscle groups with trains of 0.1 ms square pulses at different pulse frequencies (5 Hz, 10 Hz, 20 Hz, 40 Hz, 60 Hz, 80 Hz, 100 Hz and 150 Hz), train intervals, and current amplitudes. There were 60 seconds in between stimulations. For mice, a 50 ms train of 2.5 milliamp pulses at 100 Hz (five pulses/train) produced intense and forceful muscle contractions. Specific force was calculated by dividing the force generated at
each frequency by cross sectional area as determined by Burkholder et al (Burkholder et al, 1994). The formula to calculate the gastrocnemius bundle CSA was calculated from muscle weight (MW) and muscle length (ML) CSA=MW*cos(25*π/180)/1.056/0.45/ML/1000 (30).

Data analysis and presentation

All results were expressed as mean and (+/-) standard errors of the mean (S.E.M.). Significance (p-value ≤ 0.05) of the effects of Follistatin treatment on various parameters were analyzed by one way ANOVA and student’s t-test. For several experiments the analysis comparison was performed in terms of the log transformed concentrations.
RESULTS

Native FST288 promotes slightly greater muscle anabolic effects than FST315

The first goal was to examine the consequence of local overexpression of the two native FST isoforms (FST288 and 315) and one engineered isoform that has been relieved of its heparin binding capability (FST288-ΔHBS). Removal of heparin binding activity in FST288 was facilitated by replacing the native HBS found in its FSD1 domain with that of the homologous sequence in follistatin-like 3 (FSTL3) as initially described by Sidis et al. (Sidis et al, 2005a). Female C57BL/6 mice were injected into the gastrocnemius with 25 µg of the abovementioned FST constructs and then subjected to electroporation similar to the methods produced by Kawai et al., and Yin et al., but with slight modifications (Kawai et al, 2006; Yin & Tang, 2001). Seven days following electroporation, muscles were harvested to verify FST gene expression was being achieved. Figure 1A and 1B shows day 7 quantification (both RNA and protein) of FST315 from the electroporated muscles. Gene expression was approximately 13-fold higher whereas protein expression obtained by ELISA was approximately 4-fold higher than empty vector.

Once overexpression was successfully achieved, the ensuing experiments were designed to compare and contrast the anabolic effects of the two FST isoforms in adult mice. Female C57BL/6 mice were electroporated with two rounds (day 0 and day 10) of each construct to sustain expression over a longer duration to elicit a more robust phenotype. On day 24 muscles were excised to evaluate changes in muscle mass. Similar to the studies carried out by Gilson et al., all of the FST-transduced muscles produced substantial increases in muscle wet weights. Gross observation of the excised gastrocnemius in FST groups clearly demonstrated a robust increase in mass relative to the empty vector control treated animals (Figure 1C). FST288...
increased muscle 37.5% whereas the FST315 23.7% (Figure 1D). Altering the HBS region of FST288 (FST288-ΔHBS) significantly reduced the local activity of the protein resulting in a 12.3% increase in muscle mass. To better understand the consequence of local FST overexpression histopathological analysis was performed on muscles harvested on day 24 to assess changes and distribution in myofiber size (Figure 1E). This analysis revealed a significant increase in myofiber diameter with FST overexpression reflecting overall hypertrophy (Figure 1F). These data also demonstrate that FST288 overexpression significantly increases myofiber size even when compared to FST315 inferring that the high cell surface affinity of FST288 may lead to a more pronounced local effect in muscle anabolism.

Local delivery of FST288 and 315 rapidly improves muscle repair in CTX muscle injury while systemic delivery does not.

To investigate a potential benefit of FST administration in muscle regeneration, gastrocnemius muscles were subjected to CTX injury similar to what was described by Garry et al., with slight modifications (Garry et al, 2000). CTX snake venom was chosen as a model due to its ability to recapitulate the specific pathologies observed in muscle injury such as trauma and surgery, namely inflammation, tissue necrosis, macrophage infiltration and satellite cell activation. One hour prior to muscle injury, mice were administered a local 0.5 mg/kg/day (IM) or subcutaneous 5 mg/kg/day (SC) injection of FST288 or 315. The treatments continued for a period of seven days to assess the regenerative effects of both local and systemically delivered FST. On the seventh day of treatment, muscles were excised and mass were measured. Local administration of the two FST isoforms produced a significant increase in gastrocnemius wet weight (19.2% FST288 and a 9.2% FST315 or 99.5% and 103% of the contralateral control respectively) and was ineffective when systemically delivered (Figure 2). This was not surprising given the
relative small size of FST and its capability of binding heparin leading to a rapid clearance of the molecule. Removal of the heparin binding sequence also has no effect when systemically delivered, demonstrating that FST’s size is the main factor in clearance of the molecule (data not shown). We predicted based on existing data that FST would indeed diminish the degeneration that is coupled with injury, however, what was unanticipated was FST’s ability to promote rapid repair in the injured muscle in such a short time frame (Zhu et al, 2011).

Systemic delivery of a re-engineered FST315 leads to significant increases in lean body mass in normal male mice

The next objective was to explore the therapeutic use of a newly engineered FST molecule. FST288 with its high cell surface interaction capabilities, poor protein expression and purification attributes was determined unsuitable for further development, therefore FST315 was chosen for continued therapeutic development (Datta-Mannan et al, 2012). In order to improve the systemic exposure characteristics of FST315, additional modifications were performed to increase the size and reduce its cell surface interactions by removal of the HBS domain found in follistatin domain 1 (FST315-ΔHBS) (Sidis et al, 2005b). FST315-ΔHBS was then fused to a murine IgG1 Fc which aided in increasing the molecular mass of the protein from 28 kDa to approximately 128 kDa allowing circumvention of the renal clearance mechanism along with increasing the FcRn recycling attributes (Roopenian & Akilesh, 2007). Ultimately, these modifications significantly improved FST315-ΔHBS-Fc exposure (AUC) and half-life (~200-fold and ~30-fold respectively) enabling systemic delivery of the molecule (Datta-Mannan et al, 2012).
To test the capability of the newly engineered biotherapeutic to produce gains in muscle, we administered the molecule in two different regimens (low frequency-Q7D and high frequency-Q2D) at 10 and 30 mg/kg to male C57Bl/6 mice. Studies were conducted for a period of two weeks to inquire changes in overall lean body mass (LBM) and individual muscle mass. Weekly dosing generated only modest increases in lean body mass (Figure 3A) while the more frequent (Q2D) treatment regimen dramatically enhanced LBM and muscle mass endpoints as shown in Figure 3B.

**Systemic deliver of a re-engineered Follistatin (FST315-ΔHBS-Fc) in a CTX model of muscle injury**

A timecourse study was performed employing the newly designed FST315-ΔHBS-Fc to assess its ability to promote muscle repair in the CTX injury model. FST315-ΔHBS-Fc was administered at 10 mg/kg/Q2D for 14 days following muscle injury. FST315-ΔHBS-Fc treatment significantly attenuated the day 1 increase in gastrocnemius bundle muscle wet weight (gastrocnemius, plantaris and soleus) that is observed during the inflammatory phase shortly after injury (Figure 4A). As early as 5 days of FST315-ΔHBS-Fc treatment, significant gains in body weight (8.8%) were already observed when compared to vehicle IgG (supplemental figure 1). Finally, by day 14 the injured gastrocnemius wet weight was 9.4% over the uninjured control (Figure 4A).

Histological analysis (H&E) was performed to assess qualitative changes in myocellular structure at critical timepoints of repair and regeneration. By day 9, histological evidence of accelerated repair was clearly present demonstrating differences between FST315-ΔHBS-Fc and vehicle-IgG. The vehicle IgG group was found to have a greater presence of myocellular degeneration and monocytic infiltration. The FST315-ΔHBS-Fc treated groups exhibited traits of
accelerated myofiber regeneration as evidenced by the increased presence of centronucleated fibers (Figure 4B). Regenerating myofiber size in the FST315-ΔHBS-Fc group tended to be larger when compared to the vehicle IgG group on day 9 and became strikingly evident by day 14 where regenerating myofiber diameter was found to be significantly increased (Figure 4C).

The increased muscle mass observed with 14 days of FST315-ΔHBS-Fc treatment in the context of injury was found to translate into improvements in various aspects of muscle function as determined by in situ contractile assessments (Figure 4D). Maximum tetanic force was significantly improved with FST315-ΔHBS-Fc and was comparable to the uninjured (non-treated) group. Increases in specific force were also observed albeit not as robust; demonstrating the muscles’ ability to efficiently produce stronger contractions per calculated physiological cross sectional area (CSA). FST315-ΔHBS-Fc also resulted in significant improvements in max integration indicating an ability of the muscle to sustain contractions for longer durations (Figure 4E). CTX injection significantly decreased maximum force and twitch tension and FST treatment significantly improved it over the vehicle group (Figure 4E). Maximum force, upon normalization to gastrocnemius bundle wet weight produced only a slight trend in normalized force (~10%), however when normalized to overall body mass a significant 37% increase is found (Supplemental figure 2A and B). Collectively, this improvement in maximum and specific force on day 14 upon FST315-ΔHBS-Fc is an important finding and validates a key outcome that delay in speed of muscle repair may lead to functional deficiency and is typically not measured in animal studies.

Next, we investigated the presence of macrophages in the time course. F4/80 which is a highly glycosylated proteoglycan extracellular antigen found on mature murine macrophages was measured to evaluate the presence of macrophages in muscle sections (Haidl & Jefferies, 1996).
F4/80 staining revealed an early increase in the density of F4/80 positive cells in FST315-ΔHBS-Fc treated samples on day 3, which persisted until day 5 (Figure 5A). These data indicate that the FST315-ΔHBS-Fc alteration of day 1 inflammation may be affecting infiltration or proliferation of macrophages.

Paired box protein Pax 7 belongs to a family of genes that encode for transcription factors and in this case play a distinct role in specification of myogenic satellite cells (Seale et al, 2000). Satellite cells are activated upon injury and are the key drivers in muscle regeneration. IHC was performed on tissue sections from CTX time course samples to measure changes in Pax7 protein expression. Pax7 positive cells were found to be more abundant on day 7 and continued until day 9 (Figure 5B and supplemental figure 3) when compared to vehicle IgG1 treated animals (Figure 5B). These histopathological observations help corroborate the increases in mass changes seen with FST315-ΔHBS-Fc treatment.

To explore the molecular basis of FST315-ΔHBS-Fc’s ability to improve muscle recovery, we explored several genes that are associated with critical aspects of the regeneration process. First off, we measured numerous genes associated with the inflammatory process and the polarized state of macrophages in an attempt to understand the early attenuation in wet weight gain seen with FST treatment. Of these genes explored, three genes in particular were regulated providing a potential mechanism of action. Prostaglandin-endoperoxide synthase 2 (Cox2) and chemokine receptor 7 (CCR7) were both induced following injury and further potentiated with FST treatment (Figure 6A). This is intriguing because both of these genes have been shown to be essential in cell adhesion, proliferation and facilitating repair (Sasaki et al, 2008; Shen et al, 2006). Further investigation revealed that on day 3 resistin like alpha (Retnla) was also
additionally increased in the FST treated group (Figure 6A) demonstrating another alteration in early inflammatory modulators.

Later timepoints were explored to identify genes affecting the restoration of tissue. In doing so, FST treatment was found to significantly reduce connective tissue growth factor (CTGF). CTGF along with TGFβ is a pro-fibrotic maker that is tightly linked to diseases such as Duchenne muscular dystrophy (DMD) (Sun et al, 2008). Aberrant expression of CTGF has been shown to induce phenotypes of fibrosis. In our studies, when compared to vehicle IgG control, FST significantly down regulated CTGF expression on day 5 following CTX-injury (Figure 6B). NOS1 or nNOS was measured due to its expression and capability in regulating satellite cell differentiation and fusion (Wozniak & Anderson, 2007). Five days after injury, FST treatment increased nNOS expression was approximately 2.4 fold higher than vehicle IgG treated animals (Figure 6B).

In addition to gene changes we also wanted to also explore chemokine and cytokine protein expression two days after injury to better understand the effects of FST315-ΔHBS-Fc on muscle inflammation. We chose 2 days (post-injury) due to the increase in Smad 2/3 protein induced by CTX-injury that was attenuated with FST treatment (Supplemental figure 4). Due to the uncertainty regarding which cytokines may be altered with FST treatment, we utilized the mouse cytokine/chemokine magnetic bead panel to measure a variety of them (Millipore). This panel allowed us to explore 32 proteins that are related to inflammation. We found that FST reduced both Il17a and Interleukin 3 (Il3) in the context of CTX-induced injury (Figure 6C and supplemental data figure 5).

**FST315-ΔHBS-Fc delivered systemically improves muscle mass in a clinically relevant model of muscle disuse and injury (CTX+HI)**
To further test the potential therapeutic utility of FST315-ΔHBS-Fc, a study was performed in a more clinically relevant model which merges both CTX injury and immobilization into one. In this scenario, mice were injured using the standard CTX injury protocol and then immediately subjected to immobilization in a plantar flexed position using a 2 mm vet-lite cast material (Jorgensen laboratories). We have shown that combining these two models extends the inflammatory response, with a concomitant increase in the degenerative phase that further delays muscle repair. In order to maintain clinical relevance, treatment commenced two hours after the mice had recovered from anesthesia to better reflect a muscle trauma/treatment setting. Experiments with FST315-ΔHBS-Fc in this model focused on two dosing regimens, everyday dosing (QD) and every other day (Q2D) at both 10 and 30 mg/kg. At the termination of the study (14 days) bodyweights had increased 21% in the FST Q2D group and 14% in the QD treated group (Figure 7A). The injured and immobilized gastrocnemius (gastrocnemius only) muscles lost approximately 29.7% mass when compared to the unaffected muscle as a result of injury and immobilization. FST treated muscles increased gastrocnemius mass anywhere between 27 to 36% and in most cases above that of the control (untreated) muscles (Figure 7B). It was also noted, that the immobilized quadriceps atrophied 9.6% and was completely protected with FST treatment (Figure 7B). Subsequent to these findings, we wanted to look into the rapidity with which FST treatment can recover muscle mass in the CTX-injured and immobilized model.

A timecourse was performed to investigate the temporal regulation of regeneration with FST treated mice in the context of injury/immobilization. Based on the previous results, a high frequency treatment regimen of 30 mg/kg/Q2D, of FST315-ΔHBS-Fc was chosen. We also included a less frequent once weekly dose (Q7D) to query the lowest amount required for efficacy. Due to the severity of the model we chose the higher 30 mg/kg dose of FST. Mice were
injured and then immobilized and muscles were collected on days 1, 3, 7, 10, and 14 to evaluate changes in muscle mass over the two week period. In this study we found that by day 10 the FST315-ΔHBS-Fc Q2D group had already increased their body weight by 14% where it continued to increase up to 30% by the termination of the study (Figure 8A). The less frequent FST Q7D treated group experienced a 6.7% increase in overall body weight. Total lean body mass acquired from QNMR readings on days 9 and 14, confirmed that the gain in body weight observed was indeed bona fide fat-free lean muscle (Figure 8B). In regards to the effects on injured/immobilized muscle tissue, the FST315-ΔHBS-Fc Q2D dose significantly increased injured immobilized muscle mass as early as day 10 and exhibited signs of hypertrophy by day 14 (Figure 8C).

Gene expression analysis was also performed on the timecourse to correlate the increased mass findings to gene signatures reflective of repair. Alpha-cardiac actin (Actc1) expression has been shown to be regulated during muscle regeneration following trauma with pathological diseases that appears characteristic of activated satellite cells and regenerating myofibers (Dennis et al, 2008). On day 10, Actc1 was significantly increased with both the high and low frequency doses of FST, whereas on day 14 it was only significant with the high frequency dose producing a 3.7 fold increase (Figure 9A). Myosin heavy polypeptide 4 (Myh4), a late or structural marker in myogenesis was also measured and found to be significantly increased with only the high dose group on day 14 (Figure 9A).

Muscle samples were taken from day 14 to examine histological changes with FST315-ΔHBS-Fc treatment. Representative cross and longitudinal sections from FST treated muscles show an overall reduction in the mononuclear cell staining along with significantly larger centronucleated fiber diameter when compared to the vehicle-IgG control (Figure 9B and 9C). In light of the
significant muscle recovery being observed both by mass and histological observations, we wanted to ensure this translated into qualitative improvements in muscle function. *In situ* skeletal muscle contractile properties of the gastrocnemius bundle were assessed on the FST315-ΔHBS-Fc Q7D treatment group. Similar experiments were conducted as before and were extended to 21 days of treatment (1 week of immobilization and two weeks of re-ambulation). Maximum tetanic force and absolute twitch tension were found to be significantly improved even with weekly FST315-ΔHBS-Fc treatment when compared to the vehicle IgG group. Force frequency relationships were also calculated to show a significant increase in the functionality of muscle across multiple frequencies (Figure 9D).

As a final point, we wanted to extend the amount of damage as well as introduce variables that would better correspond to a potential patient. Elderly patients are typically more susceptible to damage and tend to have a body composition high in fat. Also, since our injury studies have focused on primarily females, we wanted to recapitulate some of the key aspects of menopausal women where increases in FSH are seen to compensate for a loss of estrogen hormones (Rodin et al, 1990). In doing so female mice where ovariectomized for an extended period of time to allow for a body compositional switch and a reduction in insulin sensitivity potentially making it more difficult for mice to repair their injured immobilized muscles (Jansson et al, 2006; Vieira Potter et al, 2012). Experiments were performed in treatment mode (2 hours after injury) using a dose titration and carried out for a period of two weeks. Similar to the previous experiments, FST treatment resulted in a dose dependent increase in injured-immobilized gastrocnemius wet weight with 14 days of treatment (Figure 10). At the two high dose groups of FST, injured-immobilized gastrocnemius wet weights were back to normal uninjured loaded control (indicated by the dotted line). Additionally, serum FSH levels were measured from the vehicle and high
dose group of FST. The re-engineered FST was shown to significantly decrease the elevated FSH levels seen in these mice as a result of ovariectomy (supplemental figure 6).
DISCUSSION

In this report the primary objective was to investigate the beneficial application of a novel FST therapeutic in ameliorating the effects of muscle damage in a variety of clinically relevant injury models. We believe that FST’s unique ability in neutralizing multiple members of the TGFβ family results in an improved ability to augment muscle repair more so than merely inhibiting one member alone. Ultimately, we found that FST possesses attributes beyond that of a simple growth factor increasing myofiber size. We were able to demonstrate that these additional attributes include the ability to modify the inflammatory response potentially altering macrophage density/polarity to bolster satellite cell activation and recruitment. This concurrent alteration of inflammation and macrophage density observed with FST treatment may promote necrotic debris clearance and sustain myogenesis leading to a robust increase in muscle mass and function. Our studies took the next step in determining the therapeutic viability of FST by employing a newly engineered protein that afforded us the means of systemic delivery in a treatment paradigm. These data are the first of its kind reflecting a truer clinical scenario which has not been limited to preventative or prophylactic treatments.

Follistatin, originally known as an Activin-binding protein, interacts with several members of the TGFβ family with varying affinities such as GDF11, BMP2/7 and Activins A and B. It has been debated over the years which members of the TGFβ family are inhibited by FST contributes to its robust increase in skeletal muscle mass. Activin A recently has been established as an inducer of muscle atrophy when exclusively overexpressed in muscle via electroporation and through overexpressing Activin A tumors (Gilson et al, 2009; Zhou et al, 2010). One group re-engineered a FST variant by removing domain II (FSD2) rendering the molecule incapable of binding Activin A (Gilson et al, 2009). Their findings uncovered a diminution in the increase in muscle
mass, producing a similar growth response that is observed with myostatin-inhibition. It has even been shown in humans that serum concentrations of Activin A or the bioavailability increase in an age-related progressive manner between 20 and 50 years of age maxing out in males between 70 and 90 years of age and in females at perimenopause (loss of FST), possibly contributing to the onset or progression of sarcopenia in both sexes (Loria et al, 1998; Reame et al, 2007). This is why we felt it was critical to introduce parameters such as loss of hormonal status, body compositional switch and increased Activin A signaling to better simulate the effect that these co-morbidities would have on muscle regeneration.

It has been empirically verified that Follistatin improves skeletal muscle healing in models of muscle injury including those of laceration and genetically modified animals exhibiting muscle dystrophy (Tsuchida, 2008; Zhu et al, 2011). It has been proposed, however, that the amelioration of fibrosis is achieved by an increase in angiogenesis (Zhu et al, 2011). The authors felt that Follistatin’s unique ability to block negative regulators of muscle such as myostatin, Activin A and TGFβ1 lead to the rapid repair in recovery. TGFβ1, myostatin and Activin A have all been described as potent inducers of scar formation; however we have been unable to show direct interactions between FST and TGFβ. One piece of data that corroborates these anti-fibrotic effects is the reduction of CTGF on day 5 by FST treatment. CTGF has been known play a direct role in contributing to muscle dystrophy and has been shown to be regulated by Activin A in hepatocytes (Gressner et al, 2008; Morales et al, 2011). In future experiments these gene changes could be monitored in other models of fibrosis where FST is tested.

The data described in this article reveal a mechanism that helps elucidate the rapidity in which FST can promote muscle regeneration and recovery. Follistatin is able to accomplish accelerated muscle restoration not only by leveraging the regenerative effects of myostatin inhibition but
potentially through modulating inflammation and pro-reparative aspects via Activin A inhibition (Fumagalli et al, 2007; Jones et al, 2007). Prolonged inflammation can have a deleterious effect on skeletal muscle repair by impeding proper satellite stem cell activation and activating myofibroblast differentiation, both of which result in scar formation (Mann et al, 2011). Nevertheless, it is necessary to maintain some level of inflammation as it is an obligatory cue to initiate repair through satellite cell activation and expansion. This is evidenced by the early regulation of several genes reflective of inflammation and activated macrophages. Potentiated expression of Retnla and CCR7 early in regeneration may help expedite the activation of macrophages as it has been described as playing a key role in tissue regeneration (Munitz et al, 2008). These findings were validated by the heightened density of F4/80 positive cells found in the epimysium on day 3 and 5 post injury. This increase in macrophage infiltration has also been observed with gdf8 inhibition in a similar model of muscle injury, inferring a common mechanism. One hypothesis could be that infiltration of mononuclear cells may be transiently more inflammatory (more M1-CCR7) thus making a quick transition to the M2-pro-myogenic macrophage. Activin A also plays a role in establishing polarity of macrophages supporting a pro-inflammatory phenotype identified by EGLN3 expression and inhibiting the attainment of an anti-inflammatory macrophage (Sierra-Filardi et al, 2011). In our CTX timecourse studies EGLN3 expression was down regulated on day 1, however was unable to produce significance (p value 0.09). The increase in infiltration and rapid switch of macrophages to M2 may be facilitating muscle repair by enhanced activation/proliferation of satellite cells, debris clearance and growth in injured muscles (Arnold et al, 2007). Also, we were able to identify trends in gene expression of CD163 on days 1 and 3; however significance was not achieved. These gene
changes combined with pathology have shown an alteration in the macrophage density indicating a potential M2 anti-inflammatory, pro-myogenic state switch.

A potential switch in macrophage polarity could in turn be activating quiescent satellite cells promoting efficient recruitment to the injured area. Cox2 and nNOS expression may represent gene profiles reflecting the enhancement in satellite cell recruitment and fusion which was substantiated by increased Pax7 positive cells. Early in repair, Cox2 has been shown to be a critical component in the repair process as knockouts are severely compromised (Shen et al, 2006). This may suggest a mechanism in which the muscle may be better prepared for additional insult(s) by fully replenishing their satellite cell reservoir. Finally, we are pursuing the potential involvement of FST-mediated blockade of Activin A in regulating intracellular calcium levels and recycling which could augment cross-bridging of myofilaments that ultimately results in enhanced force generation. Evidence supporting this has been described by Funaba et al., 2003, showing that Activin A is increased by rising levels of cytosolic calcium levels in basophilic mast cells (Funaba et al, 2003). If this mechanism holds true in muscle cells, FST could then reduce the ongoing damaging effects of calcium in injured muscles by neutralizing the production of Activin A.

Treatment with FST was found to inhibit Il17a and Il3 cytokine expression. Il17a is described as a pro-inflammatory cytokine that is produced by subset of T-cells known as helper T (Th) 17 cells (Torchinsky & Blander, 2010). Il17a has been found to be upregulated in Duchenne’s muscular dystrophy and has been demonstrated to negatively affect myoblast migration and terminal differentiation (De Pasquale et al, 2012; Kocic et al, 2013). Furthermore, another group has postulated that Il17 which is induced by Il6 and activated by Il23 might have a significant contribution in promoting activation of neutrophils and ensuing muscle damage following...
prolonged endurance exercise (Sugama et al, 2012). Future studies would include the exploration of additional days and the effect FST may have throughout regeneration on the chemokine/cytokine profile.

Currently the ongoing efforts are focused either on Myostatin inhibitors or decoy receptors (ACVRIIB-Fc). Gdf8 inhibitors maybe faced with limited efficacy in conditions where there are concomitant increases in Activin A or persistent inflammation and or scarring. The Activin receptor IIB decoy therapeutic is a weak Activin A inhibitor and has already faced many issues in the clinic with patient safety as a result of neutralizing many members of the TGF-β family.

In summary these findings support a role for FST in its ability to improve muscle repair and recovery by inhibiting multiple members of the TGFβ superfamily. Our data offer a new alternative for the development of biotherapeutics for indications in which skeletal muscle injury and immobilization are present. Our studies provide the first evidence demonstrating a treatment dosing paradigm of Follistatin that would mimic a real life scenario; an elderly patient (low muscle and high fat mass) suffering traumatic injury to a lower limb (e.g. hip fracture) and within an hour or so arrive at the emergency or critical care unit and have access to regenerative therapeutics. This situation would not be amenable to adenoviral delivery of a molecule due to its long term, uncontrolled expression and lag time in which it takes to reach efficacious levels. In certain unique situations, exploiting Follistatin’s “engineerable” duality could be applied locally for a non-weight bearing injury or even systemically for patients who would be exposed to long-term immobilization resulting in global atrophy. These are just prime examples illustrating the need to have molecule(s) that could be delivered as proximal to the injury as possible. This information should encourage the examination of FST or Follistatin mimetics for the clinical use for muscle injury.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Yaden, Dai, Andrews, and Krishnan


Performed data analysis: Yaden and Wang.

Wrote or contributed to the writing of the manuscript: Yaden, Dai, Datta-Mannan, Croy, Andrews, and Krishnan
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FIGURE LEGENDS

Figure 1: Follistatin increases muscle mass in postnatal mice. Gastrocnemius muscles of female C57BL/6 mice were electroporated with DNA constructs containing one of the following cDNAs; FST315, FST288, FST288-ΔHBS, or empty vector (CAG). (A) Gastrocnemius muscle was collected 7 days after the first injection to assess muscle gene expression (normalized to Gapdh) and (B) protein levels of FST315. (C) Morphology of excised FST315 electroporated gastrocnemius muscles 24 days following electroporation. (D) Mice were electroporated with empty vector, FST315, FST288, or FST288-ΔHBS and gastrocnemius muscle mass (GW) was assessed 24 days after the initial injection. (E) Representative H&E cross-sectional images of myofibers from electroporated gastrocnemius muscle from day 24. (F) Fiber diameter of the electroporated gastrocnemius and the frequency distribution of corresponding fibers (black-empty vector, light gray-FST315, dark gray-FST288). Significance is indicated *P ≤ 0.05, FST group versus empty vector control (Dunnett’s one-way ANOVA).

Figure 2: Local administration of both FST isoforms improves muscle regeneration in the CTX injury model. C57BL6 female mice were subjected to skeletal muscle injury by intramuscular injection of CTX into the gastrocnemius muscle. Mice received FST315 or FST288 daily by either subcutaneous (systemic) or intramuscular (local) injections which started three hours after muscle injury was induced. Muscle mass was assessed at seven days post injection/treatment. Significance is indicated *P ≤ 0.05, drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).

Figure 3: Systemic delivery of FST315-ΔHBS-Fc protein increases muscle mass endpoints. (A) Male C57BL/6 mice were administered either a low frequency (Q7D) or (B) high frequency (Q2D) dose of FST315-ΔHBS-Fc. Gastrocnemius (GW) and quadriceps wet weight (QW) along
with lean body mass (LBM) was measured two weeks following the first administered dose. Significance is indicated \(*P \leq 0.05\), drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).

**Figure 4:** Systemic administration of FST315-ΔHBS-Fc (timecourse) improves muscle repair. (A) Mice received 10 mg/kg of FST315-ΔHBS-Fc every other day for a period of 14 days. A time course was performed to monitor the early and late phase changes in gastrocnemius bundle (including plantaris and soleus) muscle mass (GW). (B) Histology on day 9 and day 14 (black arrows indicating centronucleated fibers) demonstrating the increase in the presence of centronucleated fibers on day 9 and (C) fiber diameter and fiber diameter frequency distribution of Vehicle-IgG and FST treated mice (black bars-Veh IgG and gray bars-FST). (D) \textit{In situ} muscle function on day 14 (black open squares-uninjured, red squares-vehicle CTX and green open triangles FST-CTX). (E) Additional contractility measurements from day 14. Data for the \textit{in situ} measurements are expressed as means ± S.E.M. (n=8 mice/group). Significance is indicated \(*P \leq 0.05\), drug-treated group versus IgG-treated group or IgG-treated group versus uninjured/non-treated group (Dunnett’s one-way ANOVA).

**Figure 5:** FST315-ΔHBS-Fc treatment increases macrophage density and markers of muscle precursor cells. Gastrocnemius muscle samples collected from 3, 5 and 9 days following CTX injury. (A) macrophage-F4/80 positive cell quantifications and their representative sections on days 3 and 5 at 20 and 10X respectively; (B) Pax7 IHC (black arrows indicate Pax7 positive cells on day 9). Significance is indicated \(*P \leq 0.05\), drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).
**Figure 6:** FST315-ΔHBS-Fc treatment alters markers associated with inflammation and repair. Relative mRNA (A) changes from day 1 and 3; and (B) Gene changes from day 5. (C) Day 2 protein expression levels of Il17a in injured gastrocnemius muscle were measured by luminex multiplexing magnetic bead panel (Millipore) in uninjured, vehicle IgG1 and FST treated mice. Data are expressed as means ± S.E.M. of seven mice per group for the day 2 timepoint experiment and five mice per group for the remaining timepoints. Significance is indicated *P ≤ 0.05, drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).

**Figure 7:** Therapeutic administration of FST315-ΔHBS-Fc shows robust efficacy with daily and every other day dosing in a more severe model of injury and immobilization. (A) FST315-ΔHBS-Fc was administered approximately three hours after the animals were injured and immobilized. Animals received either everyday (QD) or every other day (Q2D) treatments and body weight (BW) was collected on day 14. (B) Muscle wet weights were collected on injured immobilized gastrocnemius (GW) and immobilized quadriceps (QW). Significance is indicated *P ≤ 0.05, drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).

**Figure 8:** FST315-ΔHBS-Fc timecourse in injury and immobilization model. (A) Subsequent timecourse was performed in female mice where they received either 30 mg/kg of FST315-ΔHBS-Fc (Q2D or Q7D) or IgG1 control by subcutaneous injections (commenced three hours after muscle injury was induced). Body weight (BW) was assessed on days 1, 3, 7, 10 and 14 days post injection/treatment. (B) Lean body mass (LBM) was measured on days 9 and 14 indicating significant increases with the Q2D dosing. (C) Gastrocnemius wet weight (GW) was assessed on days 1, 3, 7, 10 and 14 days post injection/treatment. Data are expressed as means ± S.E.M. of five to seven mice per group. Significance is indicated *P ≤ 0.05, drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).
**Figure 9:** FST315-ΔHBS-Fc improves muscle regeneration in the injury and immobilization model. A further analysis of several aspects of muscle regeneration in the injury and immobilization study was performed. (A) Relative mRNA expression levels of Actc1 (Day 10 and 14) and Myh4 (Day 14) in injured gastrocnemius muscle were measured by real time PCR in vehicle IgG and FST315-ΔHBS-Fc treated mice on days 10 and 14. (B) Representative H&E cross-and longitudinal sectional images (10X and 20X) of myofibers in the gastrocnemius muscle from days 14. (C) H&E-stained gastrocnemius muscle sections from IgG and FST315-ΔHBS-Fc Q2D treated mice 14 days post-injury/immobilization were used to measure myofiber diameter and fiber diameter frequency. (D) In situ muscle function assessments on day 21. Data are expressed as means ± S.E.M. of five to seven mice per group, except in situ muscle function where eight mice per group were used. Significance is indicated *P ≤ 0.05, drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).

**Figure 10:** FST315-ΔHBS-Fc improves muscle repair and attenuates atrophy/degeneration in a clinically relevant model of injury and immobilization in an OVX background. Mice were treated with a titrated dose of FST315-ΔHBS-Fc starting three hours after muscle injury was induced and repeating every other day. Gastrocnemius muscle mass (GW) was measured at 14 days post injury-immobilization/treatment. A dotted line has been superimposed to indicate the weight of a normal load-bearing muscle. Data are expressed as means ± S.E.M. of seven mice per group. Significance is indicated *P ≤ 0.05, drug-treated group versus IgG-treated group (Dunnett’s one-way ANOVA).
Figure 1

A) Gene expression (fold change)

B) FST (µg/mL)

Empty Vector  |  FST315
---|---

* indicates significant difference.
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Figure 2

Graph showing percent contralateral (%) for different conditions: Veh-IgG, FST 315 SC, FST 315 IM, FST 288 SC, FST 288 IM. The graph includes error bars and asterisks (*) indicating significant differences.
Figure 4 B) 

Day 9

Veh-IgG

FST

Day 14

Veh-IgG

FST

Day 9

Day 14

Centronucleated fiber density

Fibers/mm²

0.00000

0.00005

0.00010

0.00015

0.00020

0.00025

0.00030

*
Figure 4

C) Fiber Diameter (µm)

Day 14

Fiber Diameter Frequency

Fiber Diameter Range (µm)

C)
Figure 4

D)
Figure 4

**E)**

**Tension-time Integral**

- Non-injured
- Veh-IgG
- FST

**Max Tetanic Force**

- Non-injured
- Veh-IgG
- FST

**Twitch Tension**

- Non-injured
- Veh-IgG
- FST

* denotes statistical significance.
Figure 5

A)  Day 3  Day 5

Veh-IgG  F4/80  F4/80  FST

Veh-IgG  F4/80  F4/80  FST

Day 3  Day 5

F4/80+ cells/mm²

N=5

Day 3  Day 5

Veh-IgG  FST  Veh-IgG  FST

*
Figure 5

B) Day 9

Veh-IgG  Pax7  FST  Pax7

20x  20x

Pax7 (+)/mm²

Vehicle-IgG  FST
Figure 6

A) Day 1

- Cox2
  - Non injured
  - Veh-IgG
  - FST

- CCR7
  - Non injured
  - Veh-IgG
  - FST

Day 3

- Retnla
  - Non injured
  - Veh-IgG
  - FST
Figure 6

B) Day 5

- CTGF
- n=5
- Non injured, Veh-IgG, FST

C) Day 2

- Il17a (pg/mL)
- n=5
- Non injured, Veh-IgG, FST

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Figure 7

A) Day 14

![Bar graph showing body weight (grams) for different treatments on Day 14. The treatments are Veh-IgG, 10 QD, 30 QD, 10 Q2D, and 30 Q2D. The graph includes error bars and asterisks indicating statistically significant differences.](image-url)
Figure 8

B)

Day 9

Day 14

Veh-IgG
FST Q2D
FST Q7D

Veh-IgG
FST Q2D
FST Q7D

LBM (grams)

LBM (grams)

*
Figure 8

C)

GW (mgs)

Day 1  Day 3  Day 7  Day 10  Day 14

Veh-IgG-CTX
FST-CTX-Q2D
FST-CTX-Q7D
Figure 9  A)

Day 10

![Graph showing Actc1 levels on Day 10 with Veh-IgG, FST Q2D, and FST Q7D conditions.]

Day 14

![Graph showing Actc1 levels on Day 14 with Veh-IgG, FST Q2D, and FST Q7D conditions.]

Day 14

![Graph showing Myh4 levels on Day 14 with Veh-IgG, FST Q2D, and FST Q7D conditions.]

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Figure 9

C) 

Day 14

Fiber Diameter (µm)

Veh-IgG  FST Q2D

* 

Fiber Diameter Frequency

Frequency (# of Fibers)

Fiber Diameter Range (µm)

[Graph showing fiber diameter distribution and statistical comparison between Veh-IgG and FST Q2D on Day 14]
Figure 9

D)

Max Tetanic Force

Twitch Tension

Hz
Figure 10

Day 14

Injured GW (mgs)

Non-Injured GW (mgs)

Veh-IgG 1 3 10 30

Day 14

Veh-IgG 1 3 10 30

mg/kg

mg/kg

*