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# Recombinant Myostatin (GDF-8) Propeptide Enhances the Repair and Regeneration of both Muscle and Bone in a Model of Deep Penetrant Musculoskeletal Injury

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## Abstract

**Background—**Myostatin (GDF-8) is known as a potent inhibitor of muscle growth and development, and myostatin is also expressed early in the fracture healing process. The purpose of this study was to test the hypothesis that a new myostatin inhibitor, a recombinant myostatin propeptide, can enhance the repair and regeneration of both muscle and bone in cases of deep penetrant injury.

**Methods—**We used a fibula osteotomy model with associated damage to lateral compartment muscles (fibularis longus and brevis) in mice to test the hypothesis that blocking active myostatin with systemic injections of a recombinant myostatin propeptide would improve muscle and bone repair. Mice were assigned to two treatment groups after undergoing a fibula osteotomy: those receiving either vehicle (saline) or recombinant myostatin propeptide (20 mg/kg). Mice received one treatment on the day of surgery, another injection five days following surgery, and a third injection 10 days following surgery. Mice were euthanized 15 days following the osteotomy procedure. Bone repair was assessed using microCT and histological evaluation of the fracture callus. Muscle healing was assessed using Masson trichrome staining of the injury site, and image analysis used to quantify the degree of fibrosis and muscle regeneration.

**Results—**Three propeptide injections over a period of 15 days increased body mass by 7% and increased muscle mass by almost 20% (P<.001). MicroCT analysis of the osteotomy site shows that by 15 days post-osteotomy, bony callus tissue was observed bridging the osteotomy gap in 80% of the propeptide- treated mice, but only 40% of the control (vehicle)-treated mice (P<.01). MicroCT quantification shows that bone volume of the fracture callus was increased by approximately 30% (P<.05) with propeptide treatment, and the increase in bone volume was accompanied by a significant increase in cartilage area (P=.01). Propeptide treatment significantly decreased the fraction of fibrous tissue in the wound site, and increased the fraction of muscle relative to fibrous tissue by 20% (P<.01).

**Conclusions—**Blocking myostatin signaling in the injured limb improves fracture healing and enhances muscle regeneration. These data suggest that myostatin inhibitors may be effective for improving wound repair in cases of orthopaedic trauma and extremity injury.

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## INTRODUCTION

Approximately 2 million cases of delayed and nonunion fractures occur annually in the United States, and the treatment and care of these patients requires considerable time and cost. Bony nonunions are common in cases where fractures are associated with extensive muscle damage or poor muscle coverage, revealing that muscle is a primary factor driving the rate of bone healing with traumatic musculoskeletal injury.[1] For example, size of the fracture callus is increased in regions alongside muscle,[2] and open fractures in sites lacking muscle coverage, such as the tibia, heal much more slowly than fractures where muscle coverage is available.[3–4] Healing of open bone defects is accelerated when a muscle flap is used to cover the wound, and intact muscle is more effective at promoting bone repair than injured muscle.[5–6] It has also been observed that new bone growth can be observed when minced muscle tissue is implanted alongside bone, but minced liver tissue does not have the same osteogenic effect.[7] As noted by Stein et al.[2, p. 1382] "Muscle is perhaps the most crucial factor in the physiological process of fracture healing". It is therefore clear that improving muscle regeneration and muscle coverage in cases of orthopedic trauma has significant potential to accelerate bone repair.

Myostatin (GDF-8), a member of the TGF-beta superfamily of growth and differentiation factors, is most well-known as a potent suppressor of muscle growth, development, and regeneration. Mice lacking myostatin show a significant increase in muscle mass,[8] and congenital absence of myostatin is associated with increased muscle mass in both humans and dogs.[9–11] It has also been shown that factors which inhibit myostatin, such as follistatin, can improve muscle regeneration and decrease fibrosis in injured muscle.[12,13] Recently we have demonstrated that the receptor for myostatin, ActRIIB, is expressed in bone marrow derived stem cells,[14] and mice lacking myostatin show increased bone density and strength.[15,16] These data are further supported by genetic studies showing that myostatin gene polymorphisms are associated with variation in peak bone mineral density,[17] and that inhibition of normal myostatin signaling by transgenic overexpression of myostatin propeptide increases bone mineral density in mice.[18] Although the mechanism(s) by which myostatin regulates bone formation and bone density is not yet well understood, there is also evidence that myostatin plays an important role in fracture healing and bone repair. Myostatin is expressed in the fracture callus early (<48 hrs) in the healing process,[19] and the receptor for myostatin is highly expressed in proliferating chondrocytes of the fracture callus.[20] A role for myostatin in the regulation of fracture repair is also supported by our recent data showing that fracture callus size, strength, and bone volume is increased in mice lacking myostatin.[21]

Myostatin is normally bound to a propeptide from which it must be cleaved to form an active ligand.[22,23] A recombinant myostatin propeptide effectively inhibits active myostatin in vitro and in vivo, and overexpression of the propeptide increases muscle mass.[24,25] Here we evaluate the potential of this myostatin inhibitor to improve muscle and bone repair in a model of deep penetrant injury.

## MATERIALS AND METHODS

### Animals, Treatments, & Surgical Procedures

We performed an initial dose-response study to evaluate efficacy of the myostatin inhibitor in male CD-1 mice four months of age. Mice were treated with the propeptide at 0 mg/kg, 10 mg/kg, 20 mg/kg, or 50 mg/kg at day 0, day 5, and 10 and then sacrificed one week after the last treatment. Results showed that three injections of the propeptide over a 15 day treatment period increased fore- and hindlimb muscle mass by 10% at the 10 mg/kg dose and increase muscle mass by more than 15% at the 20 mg/kg dose. The 50 mg/kg dose did

not increase muscle mass beyond the increase observed in the 20 mg/kg group and so we have used the 20 mg/kg dose here. Adult CD-1 mice were separated into two groups: those receiving the propeptide (PRO) or saline (VEH). Each treatment group included 10–12 male and 10–12 female mice, for a total of 20–24 mice per treatment group. Fibula osteotomy was performed on the left leg under isoflurane anesthesia as described previously,[21] and the lateral compartment muscles fibularis longus and brevis severed in the region overlying the osteotomy site (Fig. 1). The skin incision is closed using VetBond$^{tm}$ skin glue. Treatments were administered immediately following osteotomy, 5 days following surgery, and 10 days following surgery. Animals were weighed at each of these timepoints and euthanized according to IACUC-approved procedures 5 days after the last treatment (15 days after surgery; Fig. 1). Mice were weighed and the left quadriceps femoris and triceps brachii muscles weighed. The left leg was removed and fixed in 10% buffered formalin for 24–48 hrs, washed, and then stored in 70% ETOH.

### MicroCT, Histology, & Histomorphometry

Intact legs with surrounding muscle were first imaged using a FAXITRON small-animal x-ray cabinet at 35kVP, 2.5 mA for 45 seconds to verify that the fibula osteotomy was successful and that the tibia was not damaged. Specimens were sent to the Savannah River Site National Laboratory (Aiken, SC) for micro-computed tomography using a 160 kV micro-focus X-ray machine (Kevex Inc., Model 16010), a four-axis positioning system (New England Affiliated Technologies series 300), and an amorphous silicon imager (Varian Inc, Paxscan 4030) at 12 micron resolution. Measurements of total callus volume and callus bone mineral density were calculated 0.5 mm either side of the callus center. MicroCT images were then scored by a technician blind to the treatments as either having bone crossing the osteotomy site (`bridged') or showing no bone crossing the fracture gap `unbridged'). Specimens were then decalcified using EDTA, embedded in paraffin, and sectioned at 6–8 μm. Paraffin sections were stained with safranin-O and fast green for measurement of cartilage area (Cg.Ar) in the callus. Histomorphometric nomenclature follows recommended standards.[26] Alternate sections were stained using Masson trichrome, which stains fibrous collagen-rich tissue blue and skeletal muscle red. A 0.80 mm$^2$ region of interest was examined lateral to the fibula fracture callus, 90° from an axis running through the center of the tibia and fibula (Fig. 1). The image was captured using a QImaging digital camera at 100X, and the relative fraction of red and blue pixels in each image quantified using SigmaScan software.

### Statistical Analysis

Experiments were performed in two blocks, with osteotomy performed in half the mice (n=20–24) for Block 1 and then a second group of 20–24 mice included for Block 2 approximately six weeks later. Single-factor ANOVA was used to detect significant effects of treatment and block on the outcome measures described above. Chi-square test was used to test for differences between treatment groups in the frequency of bridged or unbridged osteotomy sites.

## RESULTS

### Mice assigned to vehicle or propeptide treatment did not differ (<5%) in body weight at the time of surgery (P=.38)

Mice treated with the propeptide were slightly (~7%) but not significantly (P=.12) larger than saline-treated mice at the end of the treatment period (Fig. 1d), and the propeptide significantly (P<.001) increased muscle mass in the mice by almost 20% (Fig. 1e). This increase is significant not only in absolute terms but also when the data are normalized by body mass (P<.001). MicroCT reconstructions of the osteotomy site show that bone is

observed to bridge the osteotomy gap in approximately 40% of cases among the control mice whereas bridging is observed in 80% of the mice treated with the propeptide (P<.01, Fig. 2a,b). MicroCT quantification of bone volume 0.05 mm either side of the osteotomy center shows that propeptide treatment significantly increases the volume of bone in the fibula fracture callus (Fig. 2c). Histological preparations reveal that the increase in callus bone volume with propeptide treatment is accompanied by an increase in callus cartilage volume as well (Fig. 3). Finally, examination of histological sections stained with Masson trichrome indicates that collagen-rich fibrous tissue is abundant in the area of injured muscle lateral to the osteotomy site in control (vehicle) treated mice, whereas fibrous tissue is less prolific and regenerative muscle fibers more common in the area of injury among propeptide treated mice (Fig. 4a). Quantification of blue (fibrous) versus red (muscle) staining using image analysis shows that propeptide treatment significantly increases the fraction of muscle staining and decreases the fraction of fibrous tissue in the injury site (Fig. 4b). In vehicle-treated mice the fraction of red-staining tissue is approximately 15% greater than the regions staining positive for fibrous (blue) tissue, whereas in propeptide treated mice the fraction of red tissue is 35% greater than the blue-positive area (Fig. 4b).

## DISCUSSION

The effects of myostatin inhibitors on muscle mass are now relatively well known, but the effects of these molecules on bone formation and regeneration have only recently been investigated. We have found, for example, that myostatin deficiency directly increases the osteogenic potential of bone-marrow derived stromal (cells),[7] and mice lacking myostatin show increased bone density in the limb and spine.[8,9] We have also recently shown that absence of myostatin increases size and bone volume in the fracture callus,[14] and another group has demonstrated that a soluble decoy myostatin receptor increases bone formation and trabecular bone volume.[27] Previous work has indicated that myostatin is highly expressed in the earliest stages of fracture healing,[12] suggesting that this factor may play a key role in the recruitment and proliferation of progenitor cells in the fracture callus. This hypothesis is supported by the data presented here, showing that cartilage area and bone volume in the fibula fracture callus are both increased with propeptide treatment. These findings point to a role for myostatin in regulating the early sequence of events in endochondral ossification, such that inhibition of myostatin increases the number and/or proliferative capacity of these cells. This increase in the progenitor cell population appears to then have subsequent downstream effects, such that during the chondrogenic and osteogenic phases of fracture healing the soft- and hard-callus remains relatively large. It is also possible that this increase in the progenitor cell population with inhibition of myostatin function enhances the rate of endochondral ossification, so that by two weeks post-fracture the formation of new bone across the fracture gap is accelerated (e.g, Fig. 2a).

To date, the therapeutic potential of myostatin inhibitors has received greatest attention in the area of muscular dystrophy treatment.[28,29] Inhibitors such as a myostatin antibody (MYO-029), decoy soluble myostatin receptor (ActRIIB-Fc), and myostatin propeptide can enhance muscle regeneration, increase myofiber hypetrophy, and decreases fibrosis in healing muscle.[30] Other factors that can inhibit myostatin, such as follistatin and decorin, may also have potential for treating muscle injury and congenital muscular disorders.[6,31] The realization that myostatin inhibitors may also enhance bone healing, together with the substantial evidence for a role of these inhibitors in improving muscle regeneration, suggest that myostatin inhibitors may represent novel therapeutic molecules in the treatment of musculoskeletal injuries in which both muscle and bone are damaged. Perhaps the most immediate application of these inhibitors would be in treating the penetrating extremity injuries frequently encountered in the battlefield setting. Musculoskeletal injuries are the most common wounds encountered in modern warfare, representing 60–70% of all combat-

related injuries.[32] The Joint Theater Trauma Registry indicates that out of 3575 extremity wounds in Iraq, 53% were penetrating soft-tissue wounds and 26% were bone fractures.[33] Most of these injuries are caused by fragments from detonating explosives, which produce extensive soft-tissue damage, bone fractures, and frequently lead to secondary infections.[34] As noted in the introduction, impaired muscle healing and damage to skeletal muscle has a direct effect on bone healing, as muscle serves as a local source of stem cells, growth factors, and vascular supply for bone.[1,35] Myostatin inhibitors may therefore represent novel therapeutic agents for enhancing the repair of battlefield injuries, but also for improving bone and muscle healing in cases of orthopaedic trauma resulting from non-combat injuries such as motor vehicle accidents.

This study has several limitations. First, the surgical model used, while involving muscle damage, does not attempt to simulate the introduction of foreign particles and debris that often occurs with blast trauma. The extensive wound debridement and irrigation that must be performed when treating such wounds is likely to affect the rate of soft tissue healing and regeneration, as is the potential application of antibiotics either locally or systemically. Second, traumatic musculoskeletal injuries frequently occur alongside injuries to other organ systems such as the liver, lungs, or brain,[36] and the damage to multiple organ systems as well as the extensive blood loss that can occur complicates bone healing possibly having both negative and even positive effects.[37] Finally, mice are relatively small mammals and they have they ability to regenerative bone rapidly. We have observed that by two weeks following osteotomy the callus is already more than 50% bone.[21] Future studies will be directed at validating the results presented here in a larger animal model. Nevertheless, our initial findings reported here are promising, and suggest that blocking myostatin function with various recombinant peptides is likely to have significant potential in the area of musculoskeletal tissue regeneration and repair.

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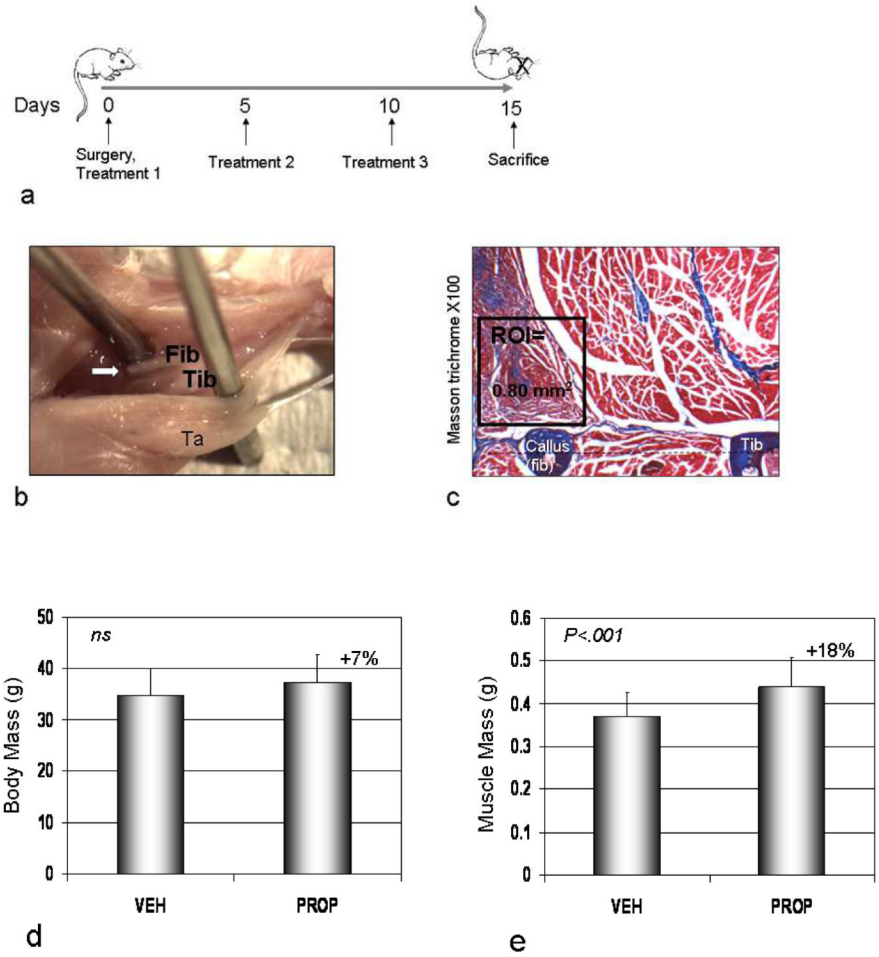


**Fig. 1.**

(a) Mice received treatments on the day of surgery, 5 days post-op, 10 days post-op, and were euthanized 15 days following the initial treatment. (b) A fibula osteotomy procedure was used (arrow) and the lateral compartment muscles were cut. Fib-fibula, Tib-tibia, TA-tibialis anterior. (c) Histological sections at the osteotomy site were stained using Masson trichrome and 0.80 mm2 region of interest lateral to the fracture callus examined for fraction of fibrotic tissue (blue). (d) Body weight and (e) muscle mass (b; triceps brachii + quadriceps femoris) in saline (VEH) and propeptide (PROP; 20 mg/kg) treated mice. Error bars represent S.D.

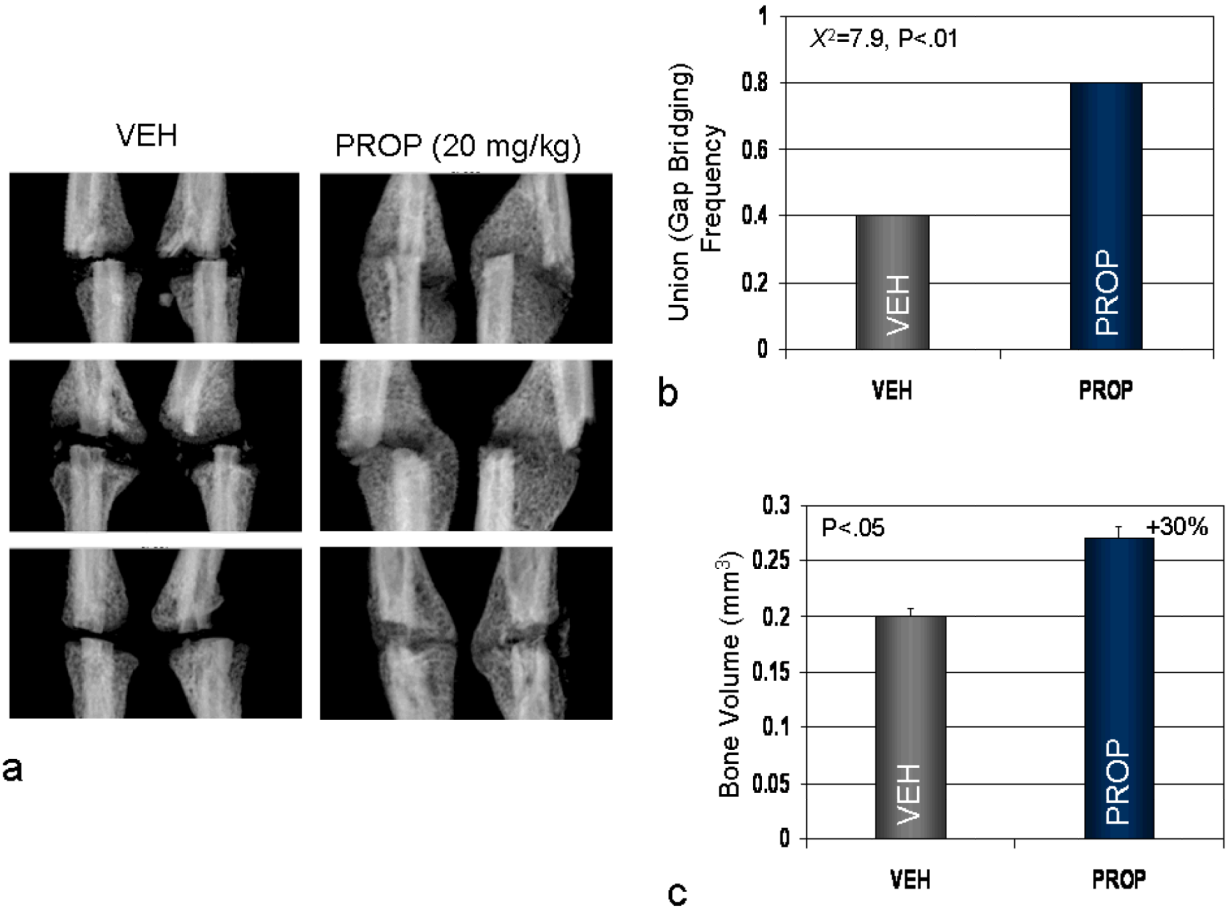


**Fig. 2.**

(a) MicroCT images of the fibula osteotomy site in saline (VEH) and propeptide (PROP; 20 mg/kg) treated mice. Note extensive bridging across the osteotomy gap in the propeptide-treated animals. (b) Bony bridging across the osteotomy gap is increased significantly in the propeptide (PROP) treated mice, and (c) bone volume of the fracture callus is increased significantly in the propeptide (PROP) treated mice.

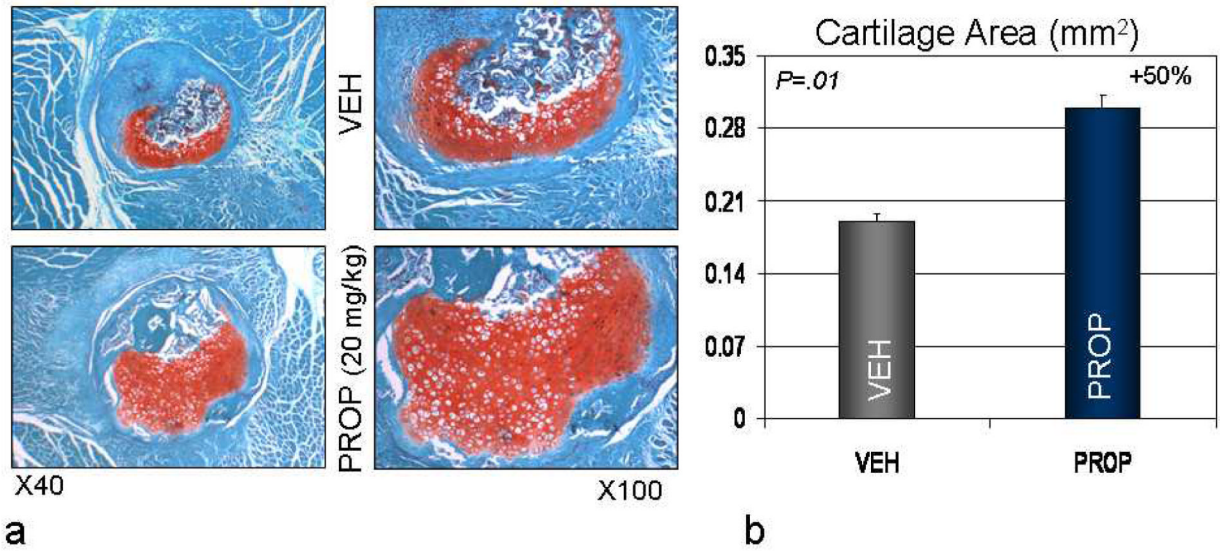


**Fig. 3.**

(a) Cartilage area, as indicated by safranin-O staining, is increased in fracture callus of propeptide (PROP)-treated mice (b).

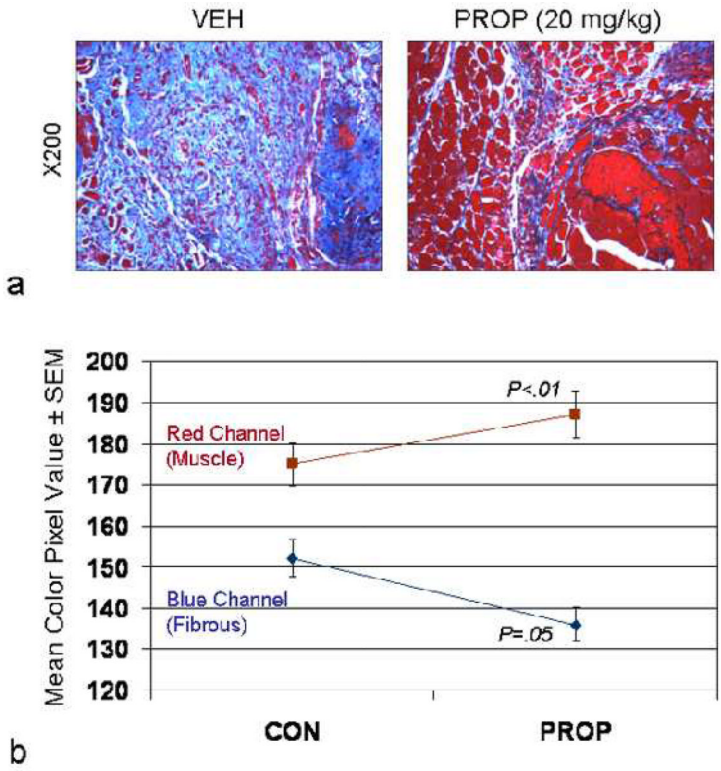


**Fig. 4.**

(a) Masson trichrome staining of the soft-tissue injury site lateral to the fracture callus showing greater fibrotic tissue staining (blue, left panel) in the vehicle (VEH) treated animal compared to greater muscle staining (red, right panel) in the propeptide (PROP) treated animal. (b) Quantification of red and blue pixel fractions, where a value of 250 is either pure red or pure blue), indicates a significant increase in the fraction of red (muscle) pixels and decrease in blue (fibrous tissue) pixels with propeptide (PROP) treatment.