Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
15 NOV 2014	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Garg K., Corona B. T., Walters T. J.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
US Army Institute of Surgical Research, JBSA Fort Sam Houston, Texas	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**13**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

J Appl Physiol 117: 1120–1131, 2014.
First published September 25, 2014; doi:10.1152/japplphysiol.00689.2014.

Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury

Koyal Garg, Benjamin T. Corona, and Thomas J. Walters

US Army Institute of Surgical Research, Extremity Trauma and Regenerative Medicine, Fort Sam Houston, Texas

Submitted 1 August 2014; accepted in final form 23 September 2014

Garg K, Corona BT, Walters TJ. Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury. *J Appl Physiol* 117: 1120–1131, 2014. First published September 25, 2014; doi:10.1152/japplphysiol.00689.2014.—Losartan is a Food and Drug Administration approved antihypertensive medication that is recently emerging as an antifibrotic therapy. Previously, losartan has been successfully used to reduce fibrosis and improve both muscle regeneration and function in several models of recoverable skeletal muscle injuries, such as contusion and laceration. In this study, the efficacy of losartan treatment in reducing fibrosis and improving regeneration was determined in a Lewis rat model of volumetric muscle loss (VML) injury. VML has been defined as the traumatic or surgical loss of skeletal muscle with resultant functional impairment. It is among the top 10 causes for wounded service members to be medically retired from the military. This study shows that, after several weeks of recovery, VML injury results in little to no muscle regeneration, but is marked by persistent inflammation, chronic upregulation of profibrotic markers and extracellular matrix (i.e., collagen type I), and fat deposition at the defect site, which manifest irrecoverable deficits in force production. Losartan administration at 10 $mg \cdot kg^{-1} \cdot day^{-1}$ was able to modulate the gene expression of fibrotic markers and was also effective at reducing fibrosis (i.e., the deposition of collagen type I) in the injured muscle. However, there were no improvements in muscle regeneration, and deleterious effects on muscle function were observed instead. We propose that, in the absence of regeneration, reduction in fibrosis worsens the ability of the VML injured muscle to transmit forces, which ultimately results in decreased muscle function.

losartan; fibrosis; volumetric muscle loss; regeneration

SKELETAL MUSCLE INJURIES ARE among the most common and frequently disabling injuries sustained by civilians and soldiers (16, 20, 35). Volumetric muscle loss (VML), defined as the traumatic or surgical loss of skeletal muscle with resultant functional impairment, often results from battlefield injuries and has limited treatment options and high morbidity (14, 26, 55). Although skeletal muscle has a remarkable regenerative capacity in cases of physical trauma involving minimal loss of tissue, a traumatic muscle injury (e.g., VML resulting in 20% or higher muscle loss) is well beyond the inherent capacity for self-repair and regeneration. Such injuries heal with extensive fibrosis, a chronic overgrowth of the extracellular matrix (ECM) (13, 14, 42, 55). Lieber and Ward (38) defined skeletal muscle fibrosis as "an abnormal and unresolvable, chronic increase in extracellular connective tissue that interferes with function." Besides loss of function, fibrosis also alters the tissue environment and increases susceptibility to reinjury (9, 30, 42). This has led researchers to investigate agents aimed at reducing fibrosis following injury.

Although several growth factors, such as epidermal growth factor or fibroblast growth factor, released from neutrophils, macrophages, fibroblasts, and myogenic precursors, can promote fibrosis, the most profibrogenic growth factor identified in the literature is transforming growth factor-β1 (TGF-β1) (6, 40, 46–48). It is the most dominant regulator of the quantity and composition of ECM deposition after injury in multiple tissues and organs. TGF-β1 is known to promote fibrosis by several mechanisms, such as altering the balance between ECM synthesis and degradation, inducing the expression of other profibrogenic factors, such as connective tissue growth factor (CTGF) and vimentin, and increasing its own overexpression through autocrine signals. In addition, it also has broad-ranging effects on cell growth, differentiation, and immune modulation (53).

Several agents that inhibit TGF-β1 have emerged as antifibrotic therapies (11, 18, 23, 25, 31). Losartan is a Food and Drug Administration (FDA)-approved antihypertensive medication that inhibits angiotensin II type 1 receptor (AT1) activation and thereby blocks TGF-β1 (8, 10, 12, 24) and CTGF (7, 24) activity. In injured skeletal muscle, angiotensin II can be produced by myofibroblasts and macrophages at the site of injury (3) and is known to have profound effects on TGF-β1 secretion, activation, and signaling (58). The activity of the AT1 receptor is also reportedly high at the injury site and colocalizes with areas of fibrous scar deposition (2). Activation of the AT1 receptor by angiotensin II activates TGF-β1 signaling molecules (such as SMAD proteins) and also produces thrombospondin-1, which is a key regulator of latent TGF-β1 activation. Losartan inhibits AT1 activation and thereby blocks TGF-β1 activation and signaling (10). Losartan has been successfully used to reduce fibrosis and improve regeneration and function in several models of endogenously healing skeletal muscle injuries, such as contusion (33) and laceration (2). The antifibrotic effect of losartan has also been effectively combined with other regenerative therapies, such adipose-derived stem cells and platelet-rich plasma, to improve skeletal muscle healing (43, 49).

The efficacy of losartan has not yet been tested in a VML injury model. VML injury involves a substantial loss of muscle tissue that does not regenerate by endogenous mechanisms and results in extensive fibrosis (14, 55). TGF-β1 gene expression in nonrepaired VML injured muscle is upregulated by 2 wk postinjury and remains elevated for several months thereafter, the chronic activity of which is believed to be primarily responsible for the development of fibrosis and impairment of myogenesis (13). Hence, preventing fibrosis development and enhancing regeneration in a VML model presents a significant opportunity to improve healing outcomes. Therefore, the major

Address for reprint requests and other correspondence: T. J. Walters, United States Army Institute of Surgical Research, Extremity Trauma and Regenerative Medicine, 3698 Chambers Pass, Fort Sam Houston, TX 78234 (e-mail: thomas.j.walters22.civ@mail.mil).

objective of this study was to determine the effect of losartan treatment on muscle regeneration and fibrosis development after VML injury in the rat tibialis anterior (TA) muscle.

METHODS

Experimental Design

Male Lewis rats with VML were provided access to drinking water with or without losartan potassium powder (Santa Cruz Biotechnology) at 2.5, 10, or 30 $mg \cdot kg^{-1} \cdot day^{-1}$ from 3 days postinjury for 7 days to determine the dose of losartan that was most effective at reducing fibrotic marker expression. Thereafter, the chosen dose of losartan (10 $mg \cdot kg^{-1} \cdot day^{-1}$) was administered from 3 days postinjury and continued to 14 days [losartan short-term (Los-ST)] or 28 days postinjury [losartan long-term (Los-LT)] (Fig. 1). Losartan treatment was started on *day 3* postinjury based on the results reported by Kobayashi and coworkers (33). This study showed that administration of losartan immediately after injury does not result in significant improvements in muscle regeneration or function. The authors proposed that administration of losartan immediately after injury would significantly interfere with the initial and crucial stages of wound healing (preinflammation). In addition, losartan administration immediately after injury could also impact the initial provisional ECM that is essential for cellular activity at the defect site. Since TGF-β1 activity is reported to be the greatest during the acute phases of muscle injury (27, 37), we hypothesized that blocking TGF-β1 during the early phases of muscle repair by Los-ST administration would slow or delay the progression of fibrosis without interfering with the late phases of muscle remodeling. Losartan was also administered long term to block TGF-β1 during both the early and the late phases of muscle repair after injury, to prevent any relapsing effects of TGF-β1 due to its chronic upregulation in VML injury (13). Rats from each treatment group were recovered out to 7, 14, 28, or 56 days postinjury. At these times, TA muscles were collected for histological and molecular analyses. At 28 and 56 days only, rats underwent in situ mechanical functional testing of the TA muscle before tissue harvest.

Animals

This work has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the United States Army Institute of Surgical Research Institutional Animal Care and Use Committee. Adult male Lewis rats (Harlan Laboratories, Indianapolis, IN) were housed in a vivarium accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and provided with food and water ad libitum. The body weights of animals are listed in Table 2.

Surgical Creation and Treatment of VML Injury

A lateral incision was made through the skin to reveal the TA muscle. The skin was separated from the fascia by blunt dissection. The fascia was dissected away from the muscle. The skin and fascia were reflected from the anterior surface of the TA muscle. The TA muscle and underlying extensor digitorum longus muscle was separated using blunt dissection. A metal plate was inserted between the muscles, and a 6-mm punch biopsy was performed through the TA muscle. The biopsy was removed, weighed, and measured (see Table 2). Bleeding was controlled with light pressure. The wound was closed by suturing the fascia with simple interrupted sutures (6-0 Vicryl), and the skin incision was closed with simple interrupted sutures using Prolene or skin staples.

In Situ TA Muscle Functional Testing

Rats were anesthetized, and the TA muscle, its tendon, and its nerve supply were surgically isolated in situ, as previously described (54, 56). The rectal temperature was monitored throughout the procedure, and body temperature was maintained with a water-heated surgical bed. The transected common peroneal nerve was stimulated via a nerve cuff electrode using a pulse stimulator. The distal tendon of the TA was isolated and cut. The distal one-third of the TA was dissected free from the surrounding musculature, leaving the origin and neurovascular pedicle intact. The distal tendon was threaded through a hole in the lever arm of a dual-mode servo muscle lever system (Aurora Scientific, Mod. 309b) and secured with silk suture (3-0 or 4-0). The lower leg was secured and stabilized with pins at the knee and ankle joints. The temperature of the peroneus longus muscle was monitored with a needle thermistor and acted as a surrogate for the TA and maintained at 35 ± 1°C. Isometric forces were determined at 150 Hz.

Histological and Immunohistochemistry Analysis

TA muscles were embedded in a talcum-based gel and frozen in 2-methylbutane (Fisher Scientific) super-cooled in liquid nitrogen using standard methodology reported previously (15). Frozen cross sections (8 μm) were cut from the middle one-third of the TA muscle in the area where the original surgical defect was made. Immunofluorescence stained tissue sections were probed for collagen I (1:500, Millipore AB755P), sarcomeric myosin (MF20: 1:10, Hybridoma Bank), and nuclei (DAPI; 1:100, Invitrogen). Corresponding Alexaflour 488 and 596 labeled secondary antibodies (1:200 to 1:500, Invitrogen) were incubated at room temperature for 1 h. Muscle sections were also stained with hematoxylin and eosin and Oil Red O. Qualitative assessments of immunostained sections were made by observing three sections from $n = 3–4$ muscles per time point per group.

Quantitative RT-PCR

RNA was isolated from snap-frozen cross sections of TA muscle that was composed of the defect area and the remaining muscle mass (50–100 mg). RNA was extracted using Trizol LS reagent (Invitrogen) and purified using RNeasy mini kit (Qiagen) and purified with DNase I (Qiagen) digestion. The yield of RNA was quantified using a NanoDrop spectrometer (NanoDrop Technologies) and optical den-

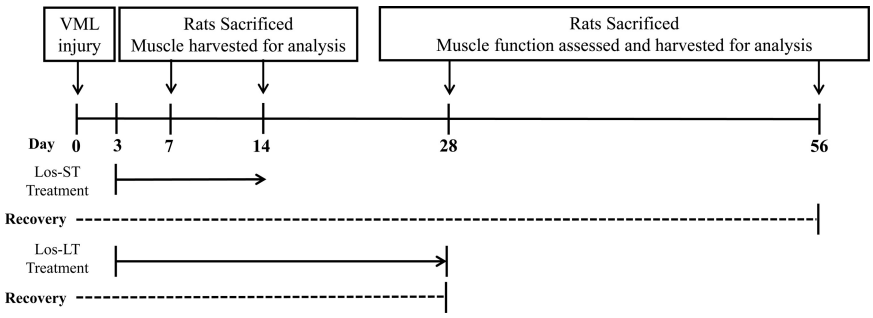


Fig. 1. Schematic representation of the in vivo experimental design. Losartan treatment was started at *day 3* post-volumetric muscle loss (VML) injury. The losartan short-term (Los-ST) and long-term (Los-LT) treatment was continued for up to 14 and 28 days postinjury, respectively. The rats on the Los-ST treatment were allowed to recover for 56 days postinjury. The rats on the Los-LT treatment were allowed to recover for 28 days postinjury. Rats were euthanized at *days 7*, *14*, *28*, or *56* postinjury, and muscle was harvested for histological, gene, or protein expression analysis. Muscle function was assessed at *days 28* and *56* postinjury.

sity 260-to-280-nm ratios were determined. RNA (500 ng) was reverse transcribed into cDNA using the Super-Script III first-strand synthesis kit (Invitrogen). The primer sets used in the study are listed in Table 1. All primer sets have been synthesized by Sigma-Aldrich DNA Oligo design tool. The technical data for each primer set from the Sigma-Aldrich Oligo design tool showed no primer-dimer formation for any of the primer sets used in this study. Aliquots (2 μl) of cDNA were amplified with 200 nM forward/reverse primers, SYBR GreenER (Invitrogen) in triplicate using a Bio-Rad CFX96 thermal cycler system (Bio-Rad). Nontemplate control and no reverse transcriptase controls were run for each reaction. Gene expression was normalized to 18S (housekeeping gene) to determine the ΔCT value. Expression levels for each mRNA transcript were determined by the $2^{-\Delta\Delta CT}$ method by normalizing each group to the contralateral control group (n = 3–6 per group). Melt-curve analysis was used to detect and eliminate any nonspecific amplification.

Western Blot

The soluble protein fraction of cross sections that comprised the defect area and remaining muscle mass of TA muscles was extracted as described previously (n = 4–6 per group) (13). Protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Scientific). Proteins were resolved by SDS-PAGE using total protein from tissue homogenates (20 μg, collagen I and III) on 4–20% Tris-glycine gels (Bio-Rad). Transfer was made onto nitrocellulose membranes subsequently blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% (vol/vol) Tween 20 (TBST) and 5% (wt/vol) nonfat dried milk. Membranes were then incubated overnight at 4°C in TBST containing 5% (wt/vol) bovine serum albumin and primary antibody for collagen type I or III (Abcam) diluted 1:1,000. Membranes were rinsed six times in TBST and then incubated at room temperature for 1 h in TBST and 5% milk containing peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:2,000. Membranes were rinsed six times in TBST before exposure to ECL Reagents (Invitrogen). The membranes were then imaged using the Odyssey Fc system (LI-COR Biosciences).

Statistical Analysis

Dependent variables were analyzed using *t*-tests, one- and two-way ANOVAs. A post hoc means comparison testing (e.g., Dunnett's, Tukey's, Newman-Keuls', or Bonferroni's) was performed when a significant ANOVA was observed. Alpha was set at 0.05. Values are listed as means ± SE. Statistical testing was performed with Prism 5 for Windows (Graphpad, La Jolla, CA).

RESULTS

Losartan Dose Response

To determine the dose of losartan that was most effective at reducing fibrotic marker expression, we tested a range of losartan dosages (2.5, 10, and 30 $mg \cdot kg^{-1} \cdot day^{-1}$), administered 3 days postinjury in drinking water. The rats were euthanized, and TA muscles were harvested on *day 7* postinjury. The water consumption of the rats was monitored daily. No differences between the consumption of drinking water (vehicle) or losartan was noted (Fig. 2*A*). The gene expression analysis revealed that losartan at 10 $mg \cdot kg^{-1} \cdot day^{-1}$ was most effective at reducing fibrotic markers such as TGF-β1 and CTGF (Fig. 2, *B* and *E*). All tested dosages led to a significant reduction in collagen I and III expression (Fig. 2, *C* and *D*). No statistical differences were noted in vimentin expression (Fig. 2*F*). Therefore, 10 $mg \cdot kg^{-1} \cdot day^{-1}$ dosage of losartan was chosen as the most effective at reducing profibrotic gene expression and was used for all of the subsequent experiments in the study.

Early Response After VML Injury (7 and 14 Days Post)

Losartan (10 $mg \cdot kg^{-1} \cdot day^{-1}$) was administered from 3 days postinjury and continued to 14 days (Los-ST). At *days 7* and *14*, TA muscle was harvested for histological and molecular analyses.

Histological analysis. At *days 7* and *14* postinjury, qualitative immunohistological analyses were performed at the site of the defect (Figs. 3 and 4). Little to no evidence of muscle fiber regeneration marked by the absence of myosin$^+$ fibers was seen in either vehicle or losartan-treated groups. Connective tissue (collagen 1$^+$ and laminin$^+$) and inflammatory cells (CD68$^+$ macrophages) were observed in the defect site.

Gene expression. Expression of genes involved in the early response after VML injury was characterized from the cross sections of muscle composed of both the defect area and the remaining muscle mass at 7 and 14 days postinjury. Losartan treatment led to a significant reduction in fibrotic markers, such as collagen I and III at 7 days postinjury (Fig. 2, *C* and *D*), but failed to maintain this effect by *day 14* postinjury (Fig. 3, *F* and *G*). The gene expression of TGF-β1 (Fig. 3*E*), CTGF (Fig. 3*H*), and vimentin (Fig. 3*I*) was not reduced by losartan treatment at *day 14*. The expression of myogenic markers, such as Pax7 (Fig. 4*E*) and myogenin (Fig. 4*F*), was higher in the losartan-treated group at *day 14*. However, little to no myogenesis was observed histologically, indicating that the myogenic events are likely occurring in the remaining muscle mass around the defect. The expression of inflammatory markers, such as CCR7 (an M1-like marker) and CD163 (an M2-like marker), was upregulated at both *days 7* and *14* postinjury. However, no significant differences between the groups were

Table 1. *Nucleotide sequence for primers used for quantitative RT-PCR*

	Forward Sequence	Reverse Sequence	Amplicon Length, bp
CCR7	5′-GCTCTCCTGGTCATTTTCCA-3′	5′-AAGCACACCGACTCATACAGG-3′	107
CD163	5′-TCATTTCGAAGAAGCCCAAG-3′	5′-CTCCGTGTTTCACTTCCACA-3′	101
Collagen I	5′-GACCAATGGGACCAGTCAGA-3′	5′-CTGGTGAACGTGGTGCAG-3′	123
Collagen III	5′-GGGCTTAGAGGTGGAGCTG-3′	5′-ATCCCTTGCAGACCAGGAG-3′	104
CTGF	5′-CAAGCAGCTGGGAGAACTG-3′	5′-ACAGGGTGCACCATCTTTG-3′	127
Myogenin	5′-CTACAGGCCTTGCTCAGCTC-3′	5′-GTTGGGACCAAACTCCAGTG-3′	153
Pax7	5′-GCAGTCGGACCACATTCAC-3′	5′-CGCACGACGGTTACTGAAC-3′	155
TGF-β1	5′-GTCAGACATTCGGGAAGCA-3′	5′-CCAAGGTAACGCCAGGAAT-3′	138
Vimentin	5′-TCAAACGAATACCGGAGACA-3′	5′-CGAGCCATCTCTTCCTTCAT-3′	188
18S	5′-GGCCCGAAGCGTTTACTT-3′	5′-ACCTCTAGCGGCGCAATAC-3′	173

CTGF, connective tissue growth factor; TGF-β1, transforming growth factor-β1.

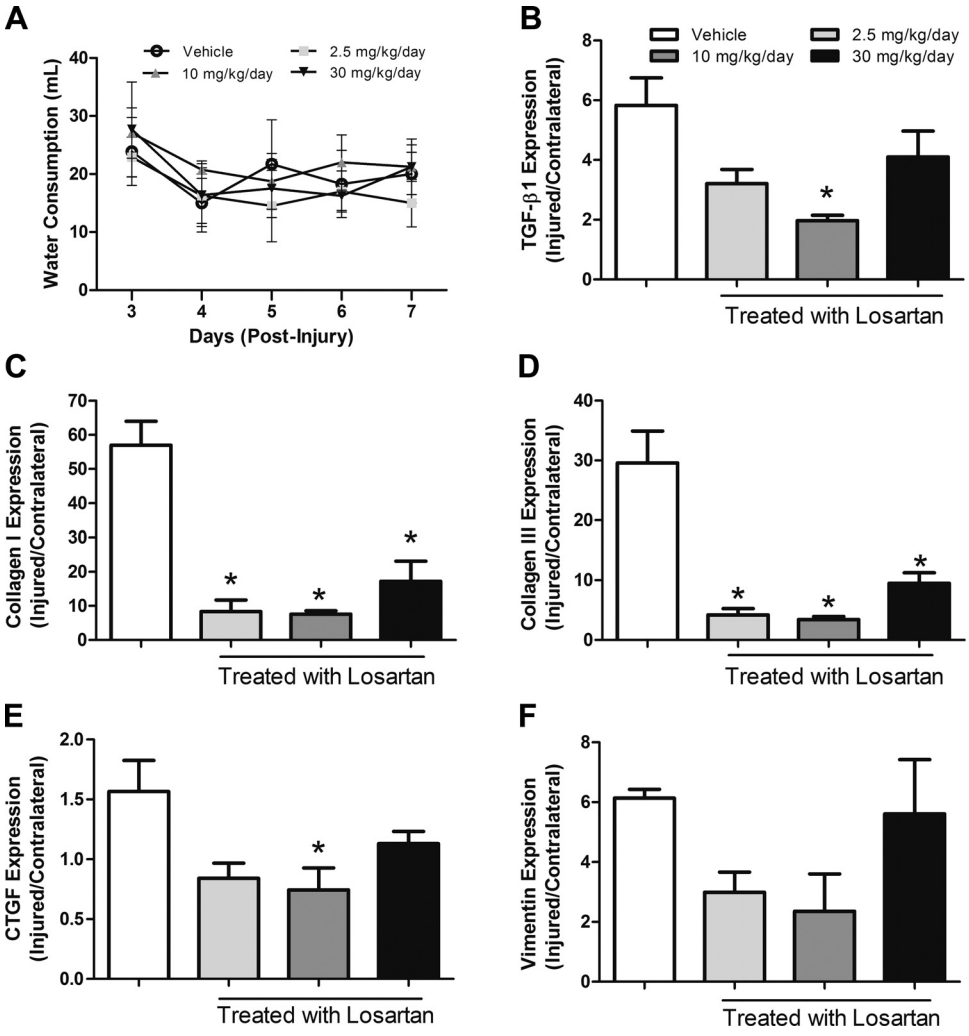


Fig. 2. Dose response of losartan to VML injured muscle. Losartan was administered in drinking water on *day 3* postinjury. *A*: vehicle (drinking water) and losartan consumption over 7 days following VML injury. The effect of losartan dosage on the gene expression of fibrotic markers, such as transforming growth factor (TGF)-β1 (*B*), collagen I (*C*), collagen III (*D*), connective tissue growth factor (CTGF; *E*), and vimentin (*F*) after VML injury, are shown. The 10 $mg \cdot kg^{-1} \cdot day^{-1}$ dose of losartan proved to be the most effective at reducing fibrotic marker expression at *day 7* postinjury. Values are means ± SE (n = 3–4). *Statistical significance with the vehicle group using Dunnett's multiple-comparison test for a significant one-way ANOVA, $P < 0.05$.

noted (Fig. 4, *K* and *L*), indicating that losartan is unable to modulate the innate immune response at *day 14* postinjury. In addition, the expression of CCR7 was approximately twofold higher than the expression of CD163 in all groups at *day 14*, indicating a greater presence of proinflammatory markers in the injured muscle.

Prolonged Response After VML Injury (28 Days Post)

Losartan (10 $mg \cdot kg^{-1} \cdot day^{-1}$) was administered from 3 days postinjury and continued to 14 (Los-ST) or 28 (Los-LT) days postinjury. At *day 28*, muscle function testing was performed before tissue harvest for histological and molecular analyses.

Histological analysis. Representative TA muscle cross sections from vehicle-, Los-ST-, or Los-LT-treated groups are presented in Fig. 5, *A–I*. Immunohistological observations of the defect area at 28 days postinjury indicated on-going remodeling events. Collagen I was still present in the defect area and was observed radiating into the remaining muscle mass (Fig. 5, *A–C*). Centrally located nuclei were observed in the majority of myofibers in all groups. The defect area also showed the presence of inflammatory cell infiltrates (Fig. 5, *D–F*). Some adipocytes (Fig. 5, *G–I*) were also detected in the defect area by Oil Red O staining, but little to no muscle fiber regeneration was observed.

Gene expression. The effect of Los-ST and Los-LT treatment on fibrosis after VML injury was analyzed using the gene and protein expression of fibrotic markers. The gene expression of all fibrotic markers was elevated at *day 28* postinjury in the vehicle-treated muscles, indicating that VML results in chronic injury with continued fibrosis (Fig. 5, *J–N*). The gene expression of TGF-β1 and CTGF was significantly higher in the Los-ST-treated muscles compared with the vehicle and Los-LT-treated muscles. The gene expression of collagen I and III and vimentin was not found to be statistically different between the groups.

Muscle weight and functional analysis. There were no differences in uninjured TA muscle weights among the groups. VML-injured TA muscles were significantly (~20%) lower in weight compared with the contralateral controls 28 days postinjury (Table 2), indicating that the mass of TA excised at the time of injury was not recovered 28 days postinjury. The peak isometric force of the VML injured muscles is shown in Fig. 6, *C* and *D*. All VML-injured muscles, regardless of group, showed significant deficits in force production compared with the uninjured contralateral muscles. The vehicle and Los-ST-treated muscles exhibited ~30% deficit in force compared with the uninjured muscles. The Los-LT-treated muscles showed ~47% deficit (Fig. 6*C*), which was a significantly greater deficit in force

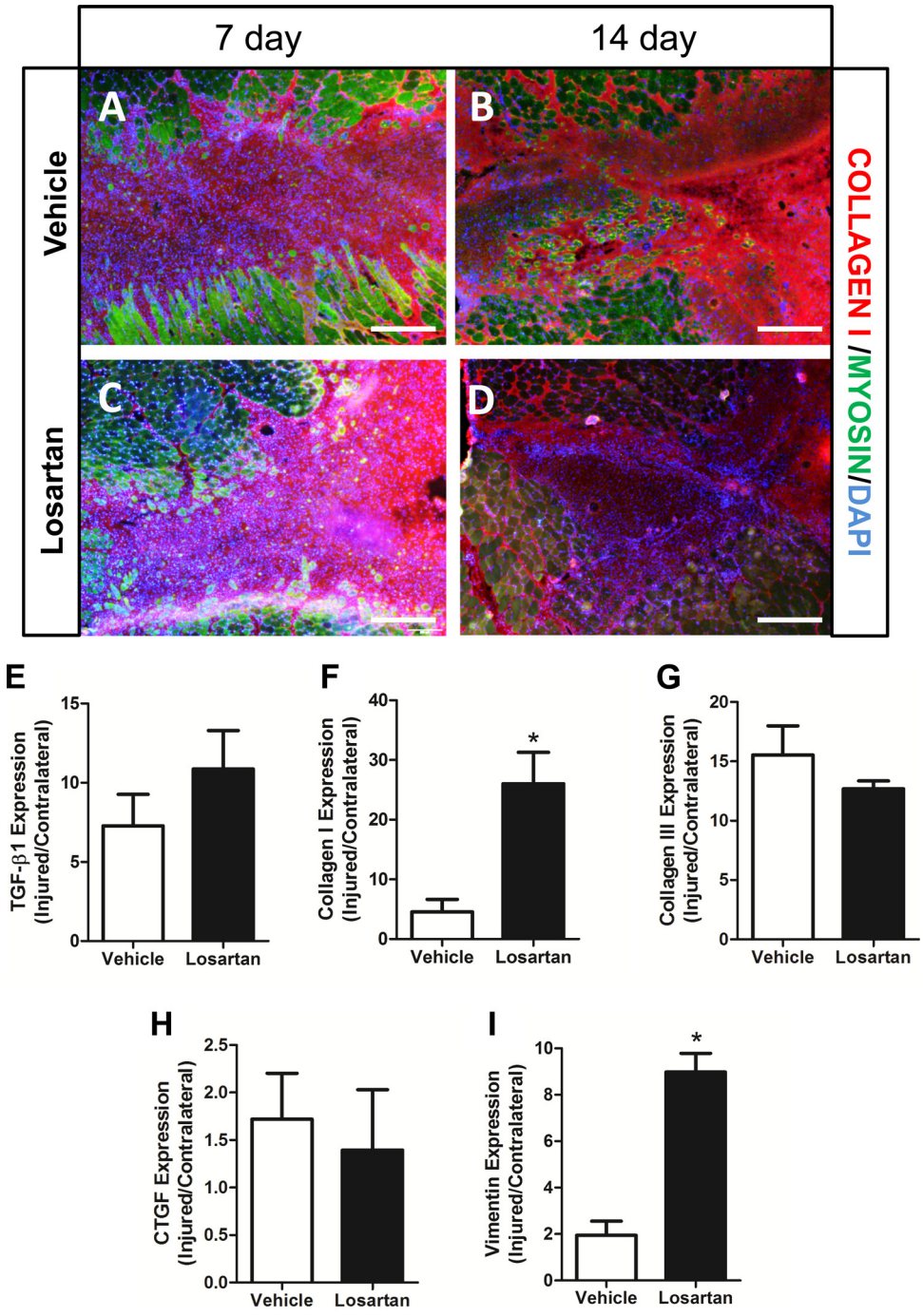
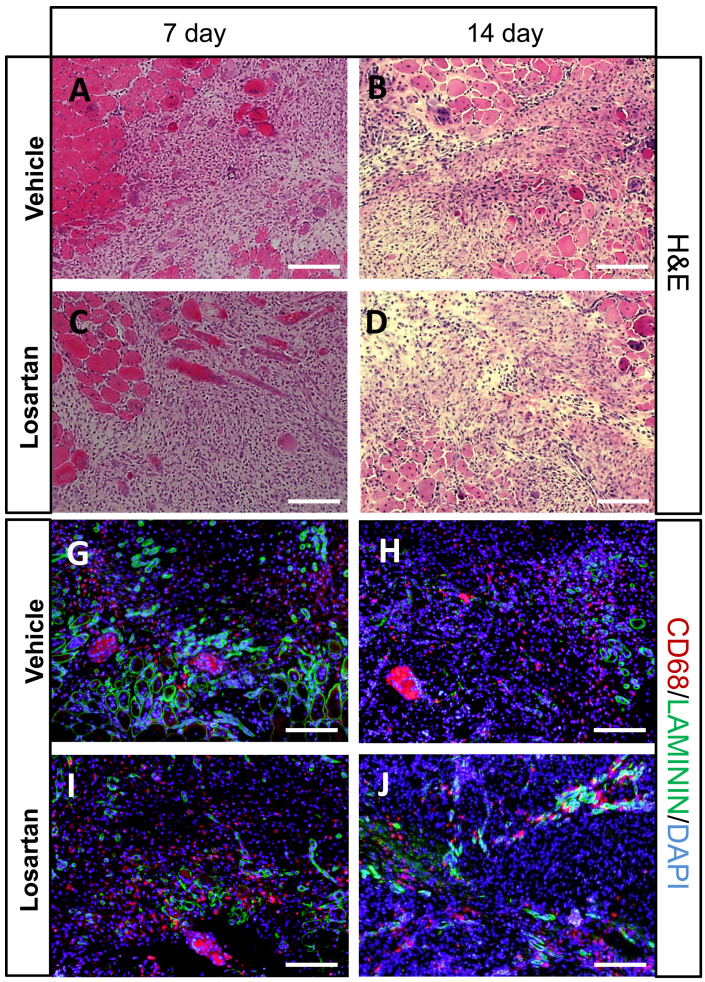


Fig. 3. Early fibrotic response of VML-injured tibialis anterior (TA) muscle to losartan treatment. VML-injured TA muscles of the vehicle-treated and losartan-treated (10 mg·kg^{-1}·day^{-1}) animals were harvested 7 and 14 days postinjury. Losartan treatment was continued for 14 days. The cross-sectional area comprising the defect and the remaining muscle mass is shown. *A–D*: the tissue was stained for collagen and myosin (scale bar = 40 μm) [vehicle treated (*A* and *B*) and losartan treated (*C* and *D*) for 7 days (*A* and *C*) and 14 days (*B* and *D*)]. Each panel is representative of the defect area examined in 3 sections from n = 3–4 animals. The harvested tissue was also assayed for gene expression of fibrotic markers on *day 14*, such as TGF-β1 (*E*), collagen I (*F*), collagen III (*G*), CTGF (*H*), and vimentin (*I*). Values are means ± SE (n = 4–6). *Statistical significance with the vehicle group using t-test, $P < 0.05$.

compared with both the vehicle and the Los-ST treatment. These results indicate that the Los-LT treatment has exacerbated the functional deficit in VML injured muscles.

Protein content. The protein levels of collagen I and III are shown in Fig. 6, *A* and *B*. The protein level of collagen type I was significantly elevated in the vehicle and Los-ST-treated groups compared with the contralateral control muscles. The Los-LT treatment significantly reduced the content of native collagen type I compared with the vehicle-treated TA muscles. The protein expression of collagen type III was significantly elevated in vehicle-, Los-ST-, and the Los-LT-treated groups compared with the contralateral control TA muscles. The collagen III protein levels were not modulated by Los-LT treatment. Losartan has been known to stimulate collagenase activity (52) and inhibit the posttranscriptional synthesis of collagen type I (51), which could potentially explain the differences observed between the gene and protein expression of collagen type I and III.

The correlation between the peak isometric force and the collagen type I content was investigated (Fig. 6*E*). A positive significant correlation (Pearson $r = 0.5706$, $r^2 = 0.3256$, $P = 0.03$) was found between the two experimental measurements, suggesting that up to 33% of the effect on muscle function is partly explained by the collagen type I content of the muscle.



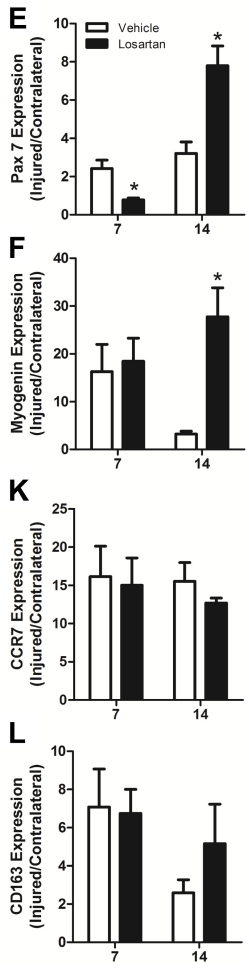


Fig. 4. The early response of VML-injured TA muscle to losartan treatment. Tissue samples comprised the defect area and the remaining muscle mass harvested at *days 7* (*A*, *C*, *G*, *I*) and *14* (*B*, *D*, *H*, *J*) were stained for hematoxylin and eosin (H&E; scale bar = 100 μm; *A–D*) and CD68 and laminin (scale bar = 100 μm; *G–J*) in vehicle-treated (*A*, *B*, *G*, *H*) and losartan-treated animals (*C*, *D*, *I*, *J*). Each panel is representative of the defect area examined in 3 sections from $n = 3–4$ animals. The harvested tissue was also assayed for gene expression of myogenic markers such as Pax7 (*E*) and myogenin (*F*) and inflammatory markers of M1-like macrophage phenotype CCR7 (*K*) and M2-like macrophage phenotype CD163 (*L*) by RT-PCR. Values are means ± SE ($n = 4–6$). *Statistical significance with the vehicle group using a two-way ANOVA and Bonferroni posttests, $P < 0.05$.

A significant correlation was not observed between peak isometric force and collagen type III protein content (data not shown).

Prolonged Response After VML Injury (56 Days Post)

Losartan (10 mg·kg^{-1}·day^{-1}) was administered from 3 days postinjury and continued to 14 days (Los-ST). The Los-LT treatment was not used for the 56-day time point since it resulted in significant deficits in force at 28 days postinjury compared with the vehicle-treated muscles, indicating that the Los-LT treatment regimen is adversely affecting the muscles by hindering the functional recovery after VML injury. The animals in this 56-day postinjury group only received the Los-ST treatment regimen for 14 days. At *day 56*, muscle function testing was performed before tissue harvest for histological and molecular analyses.

Histological analysis. Representative TA muscle cross sections from vehicle-treated and Los-ST-treated groups are presented in Fig. 7, *A–D*. The qualitative histological analysis showed ongoing remodeling events at *day 56* postinjury, marked by the presence of myofibers with centrally located nuclei. The defect area appeared smaller, and the inflammatory reaction seemed subsided compared with 28 days postinjury. The presence of collagen type I was still observed in the defect area. Little to no muscle fiber regeneration was observed.

Muscle weights and functional analysis. There were no differences in TA muscle weights among the groups. VML-injured TA muscles were significantly (~17%) lower in weight at 56 days postinjury compared with the contralateral controls (Table 2), indicating that the mass of TA excised at the time of injury was only partially recovered 56 days postinjury. Muscle function testing revealed that the injured muscles, regardless of treatment (vehicle or Los-ST), still exhibited a significant deficit in peak isometric force compared with the uninjured muscles (Fig. 7, *E* and *F*). Vehicle and Los-ST treatment resulted in ~23 and ~27% deficit in force production, respectively. However, no differences between the vehicle and the Los-ST treatment were noted, indicating that Los-ST treatment is ineffective at improving muscle regeneration or function.

DISCUSSION

It is believed that the development of fibrosis hinders muscle regeneration, and slowing the progression of fibrosis is the key to enhancing muscle regeneration and function (35, 36, 45). The administration of antifibrotic therapies in various models of skeletal muscle injuries has resulted in improvement in muscle regeneration with functional recovery (5, 11, 19, 45). Notably, losartan, an FDA-approved angiotensin II inhibitor, was originally discovered as a therapeutic for cardiac fibrosis in hypertensive diseases (28, 39), but has recently been found

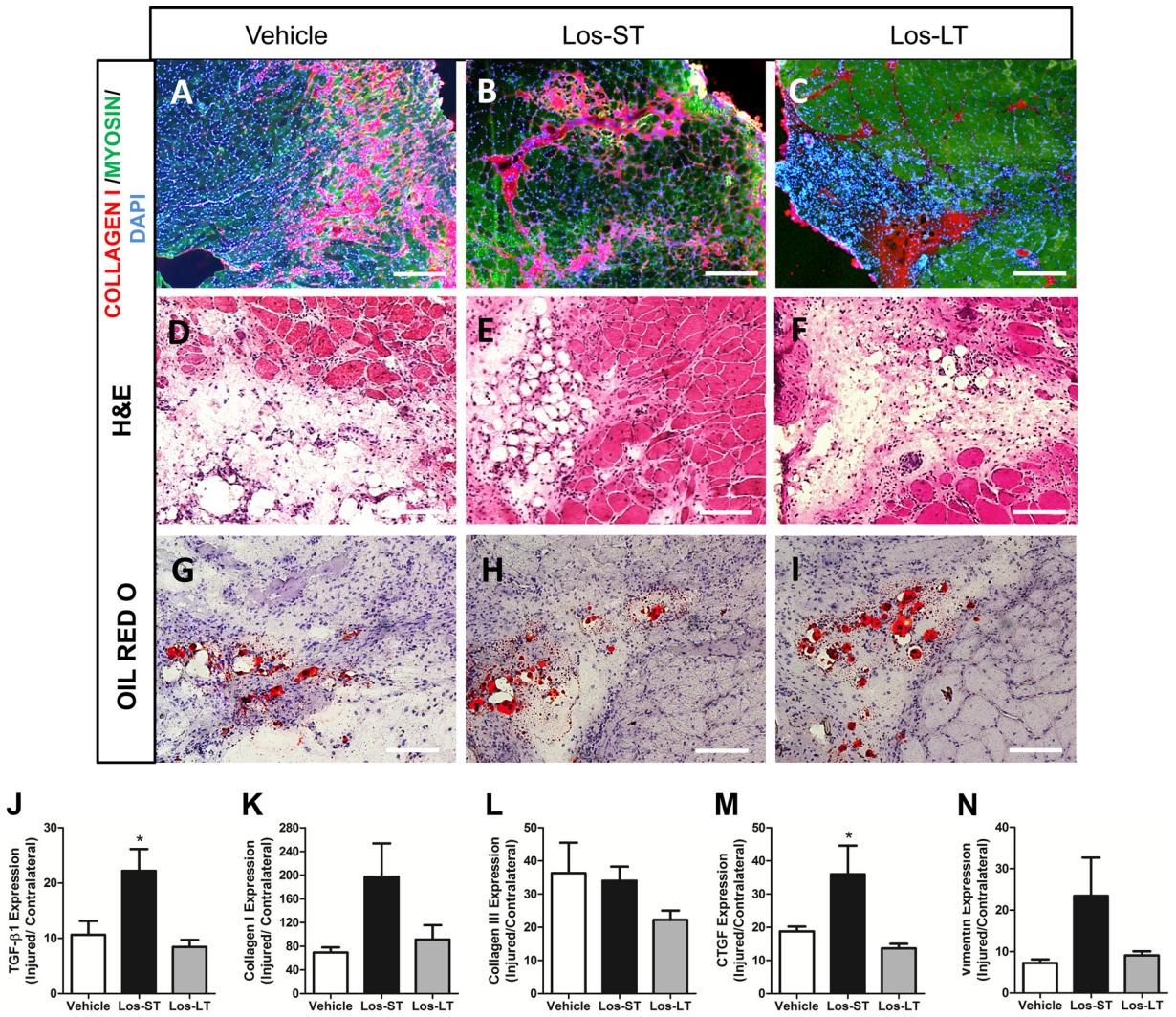


Fig. 5. Prolonged response of VML-injured TA muscles to losartan treatment. VML-injured TA muscles of the vehicle-treated and losartan-treated (10 $mg \cdot kg^{-1} \cdot day^{-1}$) muscles were harvested 28 days postinjury. Losartan treatment was continued for 14 days in the Los-ST group and for 28 days in the Los-LT group. The cross-sectional area comprising the defect and the remaining muscle mass is shown. The tissue was stained for collagen and myosin (scale bar = 40 μm; *A–C*), H&E (scale bar = 100 μm; *D–F*), and Oil Red O (scale bar = 100 μm; *G–I*) in vehicle-treated (*A*, *D*, *G*) and Los-ST- (*B*, *E*, *H*) and Los-LT-treated animals (*C*, *F*, *I*). Each panel is representative of the defect area examined in 3 sections from $n = 3–4$ animals. The gene expression of fibrotic markers such as TGF-β1 (*J*), collagen I (*K*), collagen III (*L*), CTGF (*M*), and vimentin (*N*) was determined by RT-PCR. Values are means ± SE ($n = 4–6$). *Statistical significance using one-way ANOVA and Tukeys multiple-comparison tests, $P < 0.05$.

to prevent fibrosis in recoverable skeletal muscle injuries as well (e.g., laceration or contusion) (2, 5, 33). Unlike recoverable skeletal muscle injuries in rodents and humans, VML injury is beyond the endogenous myogenic repair and regenerative capacity of mammalian skeletal muscle and presents intramuscular fibrosis and chronic muscle weakness (13, 14, 55). In addition, TGF-β1 activity is reported to be transient in recoverable muscle injuries due to cardiotoxin or laceration

Table 2. *Lewis rat body and muscle weight data*

	28 Days Postinjury			56 Days Postinjury	
	Vehicle	Los-ST	Los-LT	Vehicle	Los-ST
Sample size, *n*	8	8	9	8	8
TA excised, %	18.96 ± 0.52	20.11 ± 0.69	19.41 ± 0.50	18.91 ± 0.68	18.34 ± 0.57
Body weight at surgery, g	353.60 ± 5.38	363.38 ± 4.11	359.89 ± 1.05	359.13 ± 4.67	364.50 ± 5.22
Body weight at sacrifice, g	377.90 ± 6.53	383.40 ± 5.50	390.00 ± 2.00	426.50 ± 7.37	425.00 ± 8.96
TA-CON muscle weight, g	0.65 ± 0.02*	0.64 ± 0.01*	0.64 ± 0.01*	0.69 ± 0.01*	0.68 ± 0.01*
TA-VML muscle weight, g	0.52 ± 0.02	0.52 ± 0.01	0.49 ± 0.02	0.57 ± 0.03	0.56 ± 0.01

Values are means ± SE; *n*, no. of animals. Los-ST, losartan short-term; Los-LT, losartan long-term; TA, tibialis anterior; CON, control; VML, volumetric muscle loss. *Statistically different from injured, $P < 0.05$.

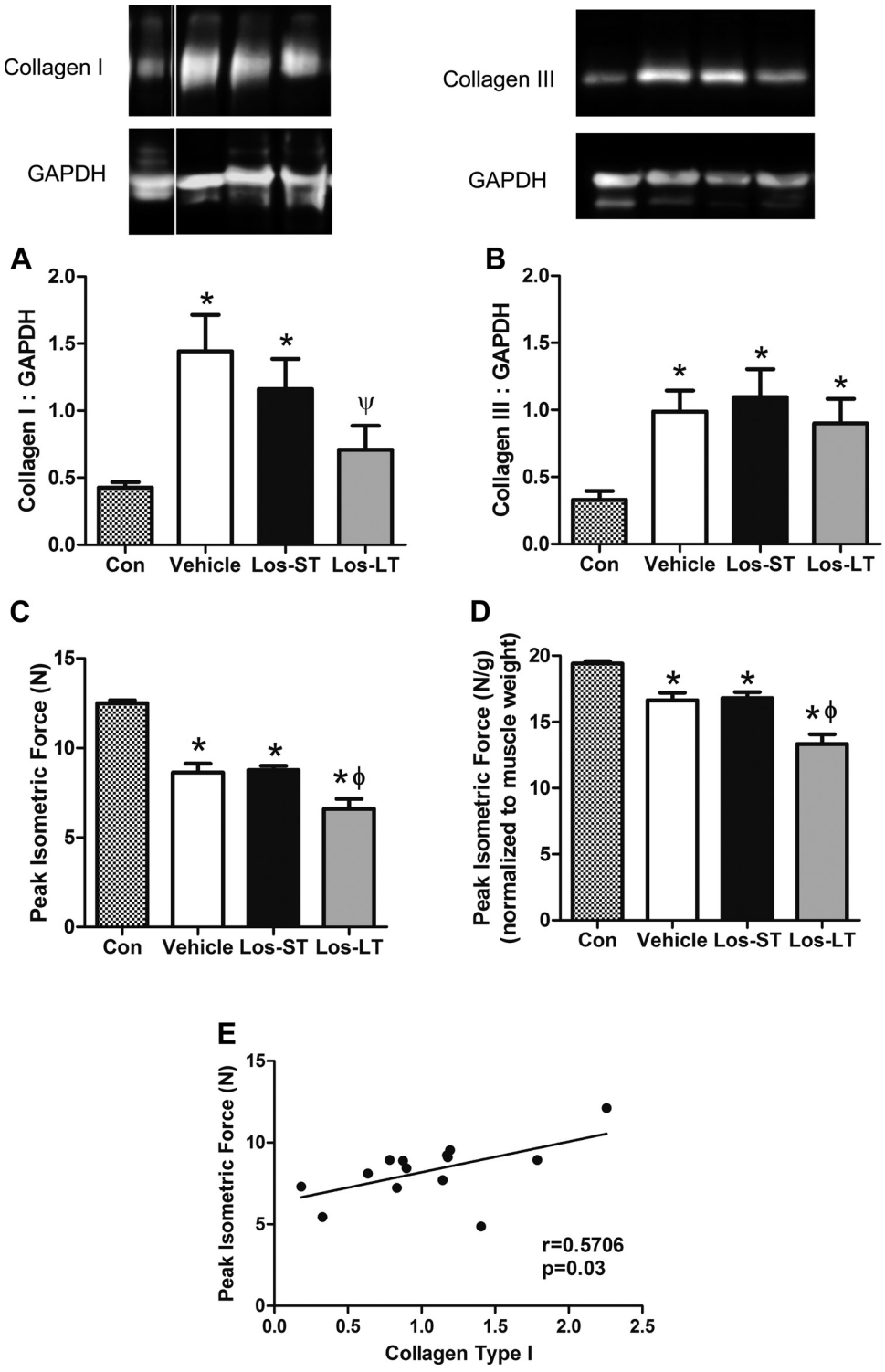


Fig. 6. Prolonged impact of losartan treatment on VML-injured muscles. VML-injured TA muscles of the vehicle-treated and losartan-treated (10 mg·kg^{-1}·day^{-1}) animals were harvested 28 days postinjury. Losartan treatment was continued for 14 days in the Los-ST group and for 28 days in the Los-LT group. The protein levels of the collagen I (*A*) and collagen III (*B*) were assayed by Western blot ($n = 4–6$). Statistical significance from *contralateral controls (Con) and from $^{\Psi}$vehicle using one-way ANOVA, $P < 0.05$. The absolute peak isometric force (*C*) and the peak isometric force normalized to muscle weight (*D*) are shown ($n = 7–9$). *All injured muscles showed a significant deficit in force compared with the uninjured contralateral Con, and $^{\Phi}$the Los-LT treatment also resulted in a significant reduction in force compared with vehicle and Los-ST treatment, $P < 0.05$, using one-way ANOVA and Newman-Keuls multiple-comparison test. Values are means ± SE. *E*: Pearson correlation between collagen type I and peak isometric force was found to be positive and significant.

(12, 37), whereas VML involves chronic upregulation of TGF-β1 several months after injury (13). Therefore, we tested the efficacy of losartan treatment in a rat VML injury model. Our study shows that, even after several weeks of recovery, VML injury results in little to no muscle regeneration and is marked by persistent inflammation, chronic upregulation of fibrotic markers, ECM and fat deposition at the defect site, and irrecoverable deficits in function. A Los-ST treatment regimen was ineffective at modulating any of the above-mentioned observations. Most importantly, a Los-LT treatment regimen was effective at reducing the deposition of collagen type I, but with deleterious effects on the recovery of muscle function.

The impact of losartan treatment on muscle function and fibrosis was assessed by measuring the peak isometric force and the protein content of collagen type I, respectively. Interestingly, the Los-LT treatment regimen resulted in a significant

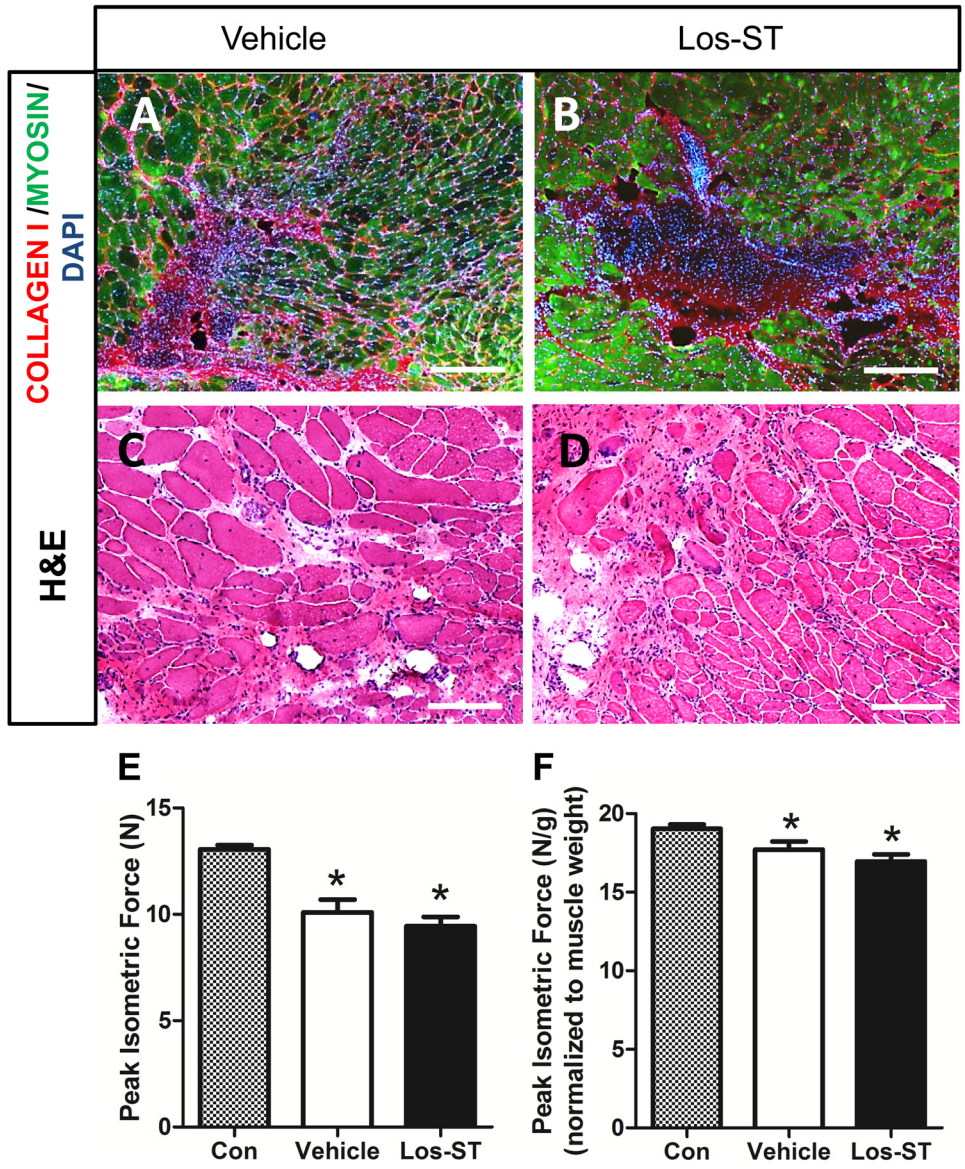


Fig. 7. Chronic injury in VML-injured muscles with or without losartan treatment. TA muscles from the vehicle-treated and losartan-treated (10 $mg \cdot kg^{-1} \cdot day^{-1}$) animals were harvested 56 days postinjury. Losartan treatment was continued for 14 days in the Los-ST group. The cross-sectional area comprising the defect and the remaining muscle mass is shown. The tissue was stained for collagen and myosin (scale bar = 40 μm; *A* and *B*) and H&E (scale bar = 100 μm; *C* and *D*) in vehicle- (*A* and *C*) and Los-ST-treated (*B* and *D*) animals. Each panel is representative of the defect area examined in 3 sections from $n = 3–4$ animals. The absolute peak isometric force (*E*) and the peak isometric force normalized to muscle weight (*F*) are shown ($n = 8$). All injured muscles showed a significant deficit in force compared with the uninjured contralateral Con. No differences between the groups were noted. Values are means ± SE. *Statistical significance, $P < 0.05$, using one-way ANOVA and Newman-Keuls multiple-comparison test.

decrease in the protein content of collagen type I (Fig. 6*A*), but with deleterious effects on force production (Fig. 6*C*), compared with the vehicle-treated muscles. In contrast, both the vehicle and Los-ST-treated muscles did not present any differences in either force production or collagen content. Collagen type I is the main fibrillar collagen in the muscle ECM. Deposition of ECM or collagen type I after skeletal muscle injury serves as a scaffold for the invading cells and the ruptured myofibers (22, 32). In addition, it also provides a physical means for lateral force transmission between the myofibers in the muscle. Thus the mechanism for the greater reduction in force in the Los-LT treatment may be due to the reduction of collagen type I protein content. This contention is partly supported by a significant positive correlation between the collagen type I content and the peak isometric force (Fig. 6*E*), which further suggests that the deficits in force production are at least ~33% related to the decreased collagen type I content. Recent work has also suggested a link between fibrosis development and force transduction in skeletal muscle. Meyer and Lieber (41) have suggested that the development of skeletal muscle fibrosis in response to desmin deletion is a compensatory mechanism that protects muscle fiber from injury due to excessive strains. In support, our laboratory has previously demonstrated that deposition of a large fibrotic mass in the VML defect area resulted in the recovery of one-third of the original functional deficit by 2 mo postinjury (14). In another study, Gumucio et al. (27) demonstrated that inhibition of TGF-β using a bio-neutralizing antibody initially improved force production following eccentric contraction injury; however, it ultimately led to long-term force deficit due to a disrupted and disorganized ECM. In light of our observations and these recent studies, we conclude that inhibition of ECM deposition (or fibrosis) can be detrimental to muscle function.

To understand the effect of losartan treatment on fibrosis development after VML injury, gene expression of profibrotic markers, including TGF-β1, was quantified from *days 7–28* postinjury. It was observed that the Los-ST treatment significantly reduced the gene expression of fibrotic markers such as

TGF-β1 by *day 7*, but failed to maintain this effect by *day 14* (Fig. 3*E*). This finding could be explained by the redundancy in molecular participants that upregulate TGF-β1, as reported previously (4, 6, 44, 57). When losartan treatment was discontinued in the Los-ST treatment group at *day 14*, the gene expression of the fibrotic markers increased even further, as indicated by the ~10-fold increase in TGF-β1 expression at *day 28* (Fig. 5*J*), suggesting a rebound effect. Continuous administration of losartan for the full 28 days in the Los-LT treatment group was able to significantly reduce the gene expression of TGF-β1 compared with the Los-ST treatment group. Collectively, these data indicate that the Los-LT treatment regimen was more effective than the Los-ST treatment regimen at reducing the profibrotic gene expression in a VML injury model, albeit with potentially deleterious consequences.

Losartan has previously been effective at improving regeneration in several models of skeletal muscle injuries (1, 9, 11, 14). Consistent with these findings, gene expression of myogenic markers such as pax7 and myogenin was higher in the losartan-treated group 14 days after VML injury (Fig. 4, *E* and *F*). However, histologically, little to no myosin-positive fibers were identified in the defect area (Fig. 3, *A–D*), indicating that the myogenic gene expression was localized to regenerating fibers in the remaining muscle mass and not indicative of de novo fiber regeneration in the defect. Beyond 14 days postinjury, little to no evidence of myogenesis in the defect area was observed histologically, and, as a result, functional deficits persisted 28 and 56 days postinjury (Fig. 6, *C* and *D*, and Fig. 7, *E* and *F*). Instead of muscle fiber regeneration, ECM deposition occurred within the defect area. While gene expression of collagens I and III was elevated following VML, regardless of treatment (Fig. 5, *K* and *L*), only protein expression indicated attenuated collagen I content with Los-LT treatment (Fig. 6*A*). Since losartan can inhibit the posttranscriptional synthesis of collagen type I (15) and stimulate collagenase activity (16), the compound may impact both synthesis and degradation of collagen and, therefore, may explain discrepancies between gene and protein expression. Nevertheless, the protein content of collagen type I significantly correlates with the muscle function, as indicated in Fig. 6*E*. We would like to highlight that, in the absence of myogenesis in this VML model, fibrosis development is a positive adaptation of the injured muscle. The fibrotic scar bridges the area of the defect and likely facilitates force transmission to some extent. Losartan treatment inhibits fibrosis and does not improve regeneration and, therefore, has deleterious effects on force transmission and muscle function. This study examined the efficacy of losartan administration in nonrepaired VML-injured muscles because there is no current clinical standard of care for VML injuries. Future studies would include testing antifibrotic agents, such as losartan, in combination with regenerative agents. Combining losartan with a regenerative therapy might prove more effective at decreasing the pace of fibrosis development by simultaneously accelerating muscle regeneration in a VML-injured muscle. In addition, the development of antifibrotic therapies for delayed treatment (approximately months after injury) is essential in a VML model, as it would be more clinically relevant for war-wounded service members. It is also important to consider that TGF-β1 is a multifunctional growth factor with broad spectrum roles in immunomodulation and wound healing (1, 17, 21, 34). TGF-β1 knockout animal models have shown defects in bone development (21), hematopoiesis (17), platelet aggregation (29), and immune modulation (34). Therefore, systemic attenuation of TGF-β1 activity (as a therapy) could impact multiple organs and prove highly detrimental in polytraumatized patients in a clinical setting.

In conclusion, we propose that, in the absence of regeneration, the development of fibrosis in VML-injured muscles is a protective mechanism that lessens prolonged muscle damage by providing mechanical stability and a means for force transduction. Based on the results of this study, it can be presumed that the inhibition of fibrosis by losartan in a more severe VML (with 40–60% muscle mass excision) would have deleterious consequences on the overall muscle structure and function. We reason that development of fibrosis is an adaptive tissue response to injury that is evolutionarily conserved. Natural selection appears to have favored the development of fibrosis around traumatic or infectious injuries to *1*) limit the bleeding and the spread of pathogens or microorganisms to other tissues and organs (50); *2*) partially restore functional use of the limb (14); and *3*) bridge the area of defect to protect the remaining muscle mass from damage and reinjury (14). Hence, while the specific effects of TGF-β1 and ECM deposition may appear detrimental to muscle regeneration, as a whole these are protective mechanisms that attempt to restore muscle function after chronic injuries.

ACKNOWLEDGMENTS

We thank Janet Roe for technical assistance.

GRANTS

These studies were funded by the Combat Casualty Care Research Program, Medical Research and Materiel Command.

DISCLAIMER

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.G. and T.J.W. conception and design of research; K.G. performed experiments; K.G., B.T.C., and T.J.W. analyzed data; K.G., B.T.C., and T.J.W. interpreted results of experiments; K.G. prepared figures; K.G., B.T.C., and T.J.W. drafted manuscript; K.G., B.T.C., and T.J.W. edited and revised manuscript; K.G., B.T.C., and T.J.W. approved final version of manuscript.

REFERENCES

1. **Andreetta F, Bernasconi P, Baggi F, Ferro P, Oliva L, Arnoldi E, Cornelio F, Mantegazza R, Confalonieri P.** Immunomodulation of TGF-beta 1 in mdx mouse inhibits connective tissue proliferation in diaphragm but increases inflammatory response: implications for antifibrotic therapy. *J Neuroimmunol* 175: 77–86, 2006.
2. **Bedair HS, Karthikeyan T, Quintero A, Li Y, Huard J.** Angiotensin II receptor blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. *Am J Sports Med* 36: 1548–1554, 2008.
3. **Berk BC, Fujiwara K, Lehoux S.** ECM remodeling in hypertensive heart disease. *J Clin Invest* 117: 568–575, 2007.
4. **Bish LT, Yarchoan M, Sleeper MM, Gazzara JA, Morine KJ, Acosta P, Barton ER, Sweeney HL.** Chronic losartan administration reduces mortality and preserves cardiac but not skeletal muscle function in dystrophic mice. *PLos One* 6: e20856, 2011.

5. **Burks TN, Andres-Mateos E, Marx R, Mejias R, Van Erp C, Simmers JL, Walston JD, Ward CW, Cohn RD.** Losartan restores skeletal muscle remodeling and protects against disuse atrophy in sarcopenia. *Sci Transl Med* 3: 82ra37, 2011.
6. **Burks TN, Cohn RD.** Role of TGF-beta signaling in inherited and acquired myopathies. *Skelet Muscle* 1: 19, 2011.
7. **Cabello-Verrugio C, Morales MG, Cabrera D, Vio CP, Brandan E.** Angiotensin II receptor type 1 blockade decreases CTGF/CCN2-mediated damage and fibrosis in normal and dystrophic skeletal muscles. *J Cell Mol Med* 16: 752–764, 2012.
8. **Campbell SE, Katwa LC.** Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol* 29: 1947–1958, 1997.
9. **Carlson BM.** Regeneration of entire skeletal muscles. *Fed Proc* 45: 1456–1460, 1986.
10. **Chamberlain JS.** ACE inhibitor bulks up muscle. *Nat Med* 13: 125–126, 2007.
11. **Chan YS, Li Y, Foster W, Horaguchi T, Somogyi G, Fu FH, Huard J.** Antifibrotic effects of suramin in injured skeletal muscle after laceration. *J Appl Physiol* 95: 771–780, 2003.
12. **Cohn RD, van Erp C, Habashi JP, Soleimani AA, Klein EC, Lisi MT, Gamradt M, Rhys CM, Holm TM, Loeys BL, Ramirez F, Judge DP, Ward CW, Dietz HC.** Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. *Nat Med* 13: 204–210, 2007.
13. **Corona BT, Garg K, Ward CL, McDaniel JS, Walters TJ, Rathbone CR.** Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. *Am J Physiol Cell Physiol* 305: C761–C775, 2013.
14. **Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ.** The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials* 34: 3324–3335, 2013.
15. **Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ.** The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials* 34: 3324–3335, 2013.
16. **Cross JD, Ficke JR, Hsu JR, Masini BD, Wenke JC.** Battlefield orthopaedic injuries cause the majority of long-term disabilities. *J Am Acad Orthop Surg* 19, *Suppl* 1: S1–S7, 2011.
17. **Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ.** Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 121: 1845–1854, 1995.
18. **Foster W, Li Y, Usas A, Somogyi G, Huard J.** Gamma interferon as an antifibrosis agent in skeletal muscle. *J Orthop Res* 21: 798–804, 2003.
19. **Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J.** The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med* 29: 394–402, 2001.
20. **Gates C, Huard J.** Management of skeletal muscle injuries in military personnel. *Oper Tech Sports Med* 13: 247–256, 2005.
21. **Geiser AG, Zeng QQ, Sato M, Helvering LM, Hirano T, Turner CH.** Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene. *Bone* 23: 87–93, 1998.
22. **Gillies AR, Lieber RL.** Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve* 44: 318–331, 2011.
23. **Giri SN, Hyde DM, Braun RK, Gaarde W, Harper JR, Pierschbacher MD.** Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. *Biochem Pharmacol* 54: 1205–1216, 1997.
24. **Gomez-Garre D, Martin-Ventura JL, Granados R, Sancho T, Torres R, Ruano M, Garcia-Puig J, Egido J.** Losartan improves resistance artery lesions and prevents CTGF and TGF-beta production in mild hypertensive patients. *Kidney Int* 69: 1237–1244, 2006.
25. **Gosselin LE, Williams JE, Personius K, Farkas GA.** A comparison of factors associated with collagen metabolism in different skeletal muscles from dystrophic (mdx) mice: impact of pirfenidone. *Muscle Nerve* 35: 208–216, 2007.
26. **Grogan BF, Hsu JR.** Volumetric muscle loss. *J Am Acad Orthop Surg* 19, *Suppl* 1: S35–S37, 2011.
27. **Gumucio JP, Flood MD, Phan AC, Brooks SV, Mendias CL.** Targeted inhibition of TGF-beta results in an initial improvement but long-term deficit in force production after contraction-induced skeletal muscle injury. *J Appl Physiol (1985)* 115: 539–545, 2013.
28. **Hao J, Wang B, Jones SC, Jassal DS, Dixon IM.** Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and in vitro. *Am J Physiol Heart Circ Physiol* 279: H3020–H3030, 2000.
29. **Hoying JB, Yin M, Diebold R, Ormsby I, Becker A, Doetschman T.** Transforming growth factor beta1 enhances platelet aggregation through a non-transcriptional effect on the fibrinogen receptor. *J Biol Chem* 274: 31008–31013, 1999.
30. **Huard J, Li Y, Fu FH.** Muscle injuries and repair: current trends in research. *J Bone Joint Surg Am* 84-A: 822–832, 2002.
31. **Huebner KD, Jassal DS, Halevy O, Pines M, Anderson JE.** Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone. *Am J Physiol Heart Circ Physiol* 294: H1550–H1561, 2008.
32. **Jarvinen TA, Jarvinen TL, Kaariainen M, Aarimaa V, Vaittinen S, Kalimo H, Jarvinen M.** Muscle injuries: optimising recovery. *Best Pract Res Clin Rheumatol* 21: 317–331, 2007.
33. **Kobayashi T, Uehara K, Ota S, Tobita K, Ambrosio F, Cummins JH, Terada S, Fu FH, Huard J.** The timing of administration of a clinically relevant dose of losartan influences the healing process after contusion induced muscle injury. *J Appl Physiol* 114: 262–273, 2013.
34. **Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S.** Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 90: 770–774, 1993.
35. **Li Y, Cummins J, Huard J.** Muscle injury and repair. *Curr Opin Orthop* 12: 409–415, 2001.
36. **Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J.** Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *Am J Pathol* 164: 1007–1019, 2004.
37. **Li Y, Huard J.** Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle. *Am J Pathol* 161: 895–907, 2002.
38. **Lieber RL, Ward SR.** Cellular mechanisms of tissue fibrosis. 4. Structural and functional consequences of skeletal muscle fibrosis. *Am J Physiol Cell Physiol* 305: C241–C252, 2013.
39. **Lim DS, Lutucuta S, Bachireddy P, Youker K, Evans A, Entman M, Roberts R, Marian AJ.** Angiotensin II blockade reverses myocardial fibrosis in a transgenic mouse model of human hypertrophic cardiomyopathy. *Circulation* 103: 789–791, 2001.
40. **Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Munoz-Canoves P.** Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle* 1: 21, 2011.
41. **Meyer GA, Lieber RL.** Skeletal muscle fibrosis develops in response to desmin deletion. *Am J Physiol Cell Physiol* 302: C1609–C1620, 2012.
42. **Mu X, Bellayr I, Walters T, Li Y.** Mediators leading to fibrosis–how to measure and control them in tissue engineering. *Oper Tech Orthop* 20: 110–118, 2010.
43. **Park JK, Ki MR, Lee EM, Kim AY, You SY, Han SY, Lee EJ, Hong IH, Kwon SH, Kim SJ, Rando TA, Jeong KS.** Losartan improves adipose tissue-derived stem cell niche by inhibiting transforming growth factor-beta and fibrosis in skeletal muscle injury. *Cell Transplant* 21: 2407–2424, 2012.
44. **Pohlers D, Brenmoehl J, Loffler I, Muller CK, Leipner C, Schultze-Mosgau S, Stallmach A, Kinne RW, Wolf G.** TGF-beta and fibrosis in different organs–molecular pathway imprints. *Biochim Biophys Acta* 1792: 746–756, 2009.
45. **Sato K, Li Y, Foster W, Fukushima K, Badlani N, Adachi N, Usas A, Fu FH, Huard J.** Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. *Muscle Nerve* 28: 365–372, 2003.
46. **Serrano AL, Mann CJ, Vidal B, Ardite E, Perdiguero E, Munoz-Canoves P.** Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. *Curr Top Dev Biol* 96: 167–201, 2011.
47. **Serrano AL, Munoz-Canoves P.** Regulation and dysregulation of fibrosis in skeletal muscle. *Exp Cell Res* 316: 3050–3058, 2010.
48. **Sheppard D.** Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc* 3: 413–417, 2006.
49. **Terada S, Ota S, Kobayashi M, Kobayashi T, Mifune Y, Takayama K, Witt M, Vadala G, Oyster N, Otsuka T, Fu FH, Huard J.** Use of an antifibrotic agent improves the effect of platelet-rich plasma on muscle healing after injury. *J Bone Joint Surg Am* 95: 980–988, 2013.

50. **Thannickal VJ.** Mechanisms of pulmonary fibrosis: role of activated myofibroblasts and NADPH oxidase. *Fibrogenesis Tissue Repair* 5, *Suppl* 1: S23, 2012.
51. **Varo N, Etayo JC, Zalba G, Beaumont J, Iraburu MJ, Montiel C, Gil MJ, Monreal I, Diez J.** Losartan inhibits the posttranscriptional synthesis of collagen type I and reverses left ventricular fibrosis in spontaneously hypertensive rats. *J Hypertens* 17: 107–114, 1999.
52. **Varo N, Iraburu MJ, Varela M, Lopez B, Etayo JC, Diez J.** Chronic AT(1) blockade stimulates extracellular collagen type I degradation and reverses myocardial fibrosis in spontaneously hypertensive rats. *Hypertension* 35: 1197–1202, 2000.
53. **Wells RG.** Fibrogenesis. V. TGF-beta signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 279: G845–G850, 2000.
54. **Wu X, Baer LA, Wolf SE, Wade CE, Walters TJ.** The impact of muscle disuse on muscle atrophy in severely burned rats. *J Surg Res* 164: e243–e251, 2010.
55. **Wu X, Corona BT, Chen X, Walters TJ.** A standardized rat model of volumetric muscle loss injury for the development of tissue engineering therapies. *Biores Open Access* 1: 280–290, 2012.
56. **Wu X, Wolf SE, Walters TJ.** Muscle contractile properties in severely burned rats. *Burns* 36: 905–911, 2010.
57. **Wynn TA.** Cellular and molecular mechanisms of fibrosis. *J Pathol* 214: 199–210, 2008.
58. **Wynn TA.** Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest* 117: 524–529, 2007.