THE ROLE OF INFLAMMATION IN SKELETAL MUSCLE HEALING

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Skeletal muscle injury is a common type of injury in sports medicine. After the injury, the traumatized muscle undergoes sequential and overlapped phases of healing, including degeneration, inflammation, regeneration, and fibrosis. Inflammation is an important phase in the natural healing process of many injured tissues. During this phase, various cytokines and cells participate and form a complex environment. Since uncomfortable symptoms are associated with inflammation, current treatments for skeletal muscle injury focus on inhibiting the inflammation phase by using non-steroidal anti-inflammation drugs (NSAIDs). Given that inflammation is shown to be beneficial to the healing process of many other tissues, it is necessary to study its role in skeletal muscle healing in order to improve the healthcare of sports-related injuries. Here, we investigated the role of the inflammation phase in skeletal muscle injury. We initially compared the healing of injured skeletal muscle and the growth of muscle cells with and without the treatment of NS-398, a cyclooxygenase-2 (COX-2) specific NSAID. We observed that NS-398 impaired muscle healing by delaying muscle regeneration and increasing scar tissue formation. NS-398 inhibited the proliferation and differentiation of muscle cells, and the expression of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Next, we investigated the important components in the inflammation phase, including the COX-Prostaglandins pathway, transforming growth factor-β1 (TGF-β1), and macrophages. We found that $PGF_{2\alpha}$ and PGE_2 may promote muscle cell maturation. The expression of TGF-β1, a fibrotic growth factor, may be suppressed by PGE_2 but increased by NS-398. Macrophages may promote muscle healing by

increasing the release of various cytokines and growth factors, including TGF-β1 and PGE_2. These results suggest that various cytokine, growth factor, and cellular components participate in the inflammation phase and cooperate with each other to modulate the healing process. Simply using NSAIDs to block inflammation may not be the optimal treatment in the effort of achieving complete recovery after muscle injury. In conclusion, our results suggest that the inflammation phase is important in skeletal muscle healing and further research to investigate the role of its components is necessary.

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NOMENCLATURE:

WT, Wild type

COX, Cyclooxygenase

NSAIDs, Non-steroidal anti-inflammatory drugs

PGE_2, Prostaglandins E_2

$PGF_{2\alpha}$, Prostaglandins $F_{2\alpha}$

TGF-β1, Transforming growth factor β1

LP cells, Late-plated cells

MPC, Myogenic precursor cells

MHC, Myosin heavy chain

GM, Gastrocnemius muscle

TA, Tibialis anterior muscle

PM, Proliferation medium

DM, Differentiation medium

siRNA, Small interference RNA

CTX, Cardiotoxin

PREFACE

I would like to thank Dr. Johnny Huard for being my dissertation advisor and guiding me through my graduate study. Especially, when I was looking for a lab, he picked me and allowed me to do what interested me most.

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1.0 INTRODUCTION

1.1 SIGNIFICANCE

Skeletal muscle injury is commonly related to sports injures and accidents in daily life. The outcomes of such injuries range in severity from common and relatively minor muscle injures like strains and contusions, to more unusual and devastating injures like lacerations. Their incidence varies from 10 to 55% of all injuries sustained in sport events. [1] [2] The healing of injured skeletal muscle is composed of degeneration, inflammation, regeneration, and fibrosis. These healing phases generally happen in such an order, although they do overlap with each other. While minor injuries mostly heal without complications, moderate to severe skeletal muscle injury may heal with scar tissue and impaired athletic capability.

The inflammation phase, marked by the symptoms of “Rubor”, “Tumor”, “Calor”, and “Dolor”, is an important phase of the natural healing process in many injured tissues. During the inflammation phase, injured tissue and the coagulation cascade release various types of cytokines and growth factors to increase the permeability of blood vessels and the chemotaxis of inflammatory cells. Neutrophils rapidly invade into the injury site, followed by macrophages. These inflammatory cells can phagocytose necrotic tissue debris and release more cytokines and growth factors to initiate regeneration, or cause more damage. [3] However, the role of many inflammation phase components, for example the COX-2-Prostaglandins pathway, TGF-β1, and macrophages, are not well understood for muscle healing. Clinically, the medical treatment of

skeletal muscle injuries is simply NSAIDs, regardless of the importance of the inflammation phase. However, we believe that in order to improve the outcome of clinical treatment of skeletal muscle injury and healthcare of sports medicine, the research of skeletal muscle injury and healing mechanisms is warranted.

1.2 THE INFLAMMATION OF SKELETAL MUSCLE AFTER INJURY

1.2.1 The Healing of Skeletal Muscle after Injury

Skeletal muscle injuries occur through a variety of mechanisms, including direct trauma (e.g., lacerations, contusions, and strains) and indirect causes (e.g., ischemia and neurological dysfunction). Various types of injuries to skeletal muscle result in the initiation of a similar healing response that undergoes sequential but overlapped phases of degeneration, inflammation, and regeneration. Active muscle degeneration and inflammation occur in the first few days post-injury, whereas muscle regeneration usually occurs five to ten days after injury. The regeneration process usually peaks at two weeks and then decreases at three to four weeks post-injury. Additionally, when injuries are severe, the healing muscle may undergo fibrosis (scar tissue formation). Fibrosis begins between the second and third weeks post-injury. (**Fig. 1-1**) As long as fibrosis occurs, complete regeneration of muscle tissue cannot occur.[4, 5]

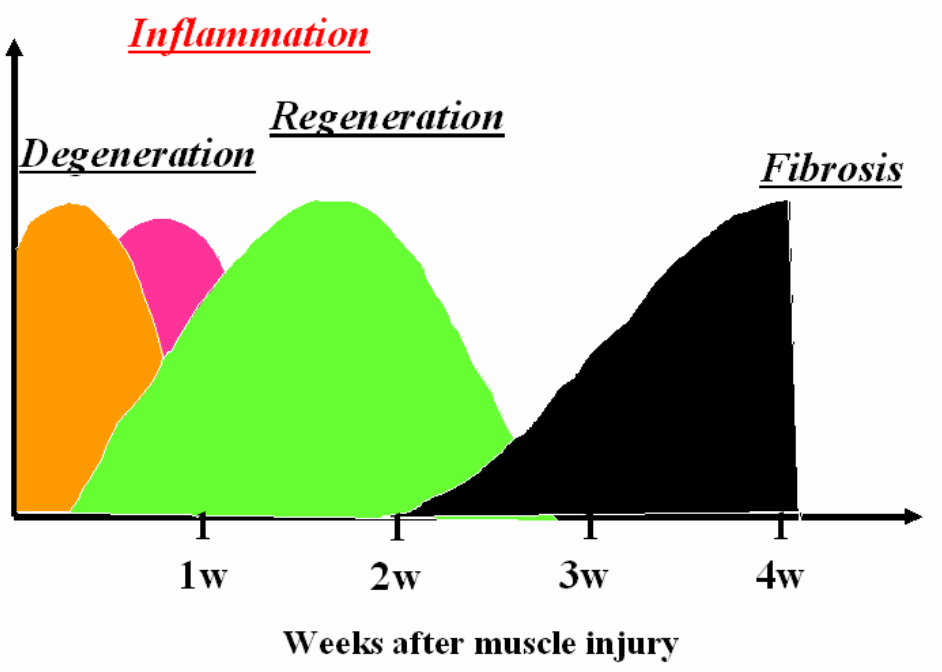


Figure 1-1. Natural Healing Process after Skeletal Muscle Injury

The natural healing process of skeletal muscle injury is composed of degeneration, inflammation, regeneration, and fibrosis. Degeneration and inflammation predominate within the first a few days after injury, followed by regeneration. If the injury is severe, instead of being complete recovered by regenerating muscle tissue, it goes on to form fibrotic tissue around 2 weeks after injury. These healing phases occur sequentially and overlap with each other.

1.2.2 NSAIDs and COX Pathway

Most studies report a favorable effect of NSAIDs in reducing muscle weakness and functional loss in the short term after muscle injury.[6-9] However, beyond the first week after injury, some studies report either no effect or a detrimental effect on long term muscle strength recovery after prolonged NSAIDs treatment.[9, 10]

Why do NSAIDs display such contradictory effects on muscle strength during healing? This may possibly be explained by the complex effects of inflammation in muscle healing. Demonstrated by animal models and in human beings, NSAIDs are capable of decreasing

prostaglandin concentrations, limiting edema, and reducing the inflammatory cell infiltration in traumatized skeletal muscle.[10, 11] By these mechanisms, early NSAIDs treatment reduces uncomfortable acute inflammatory symptoms and improves muscle function in the short-term. However, it is not clear whether the inflammatory phase is essential to subsequent muscle healing, which may, in that case, explain why the extended use of NSAIDs leads to deterioration of muscle injury.[9]

The release of inflammatory metabolites, including prostaglandins, leukotrienes, and thromboxanes, produces inflammatory symptoms such as redness, swelling, pain, and temperature elevation. The prostaglandins, potent modulators of inflammation, appear to be involved in multiple aspects of muscle regeneration after injury.[12] The prostaglandins are signaling molecules synthesized from arachidonic acid released from membrane phospholipids by phospholipase. The arachidonic acid is converted to the prostaglandin PGH_2 by the enzyme cyclooxygenase (COX). The COX product PGH_2 may then be converted to various other prostaglandins through specific synthases. (**Fig. 1-2**) In injured skeletal muscle, PGE_2 and $PGF_{2\alpha}$ have receptor-mediated functions in nociception, inflammation, and regeneration.[12-14] They have been shown to be important in regulating the control of muscle protein synthesis and degradation.[15, 16] $PGF_{2\alpha}$ in particular, has been revealed to promote the growth of skeletal muscle cells by stimulating the secondary fusion of nascent myotubes.[17, 18] PGE_2 has been suggested to reduce fibrosis formation by inhibiting fibroblast proliferation and collagen production.[19] However, it is not clear how COX enzyme is involved in muscle healing, including regeneration and fibrosis formation, and whether the effect of COX enzyme is mediated by its downstream products, the prostaglandins.

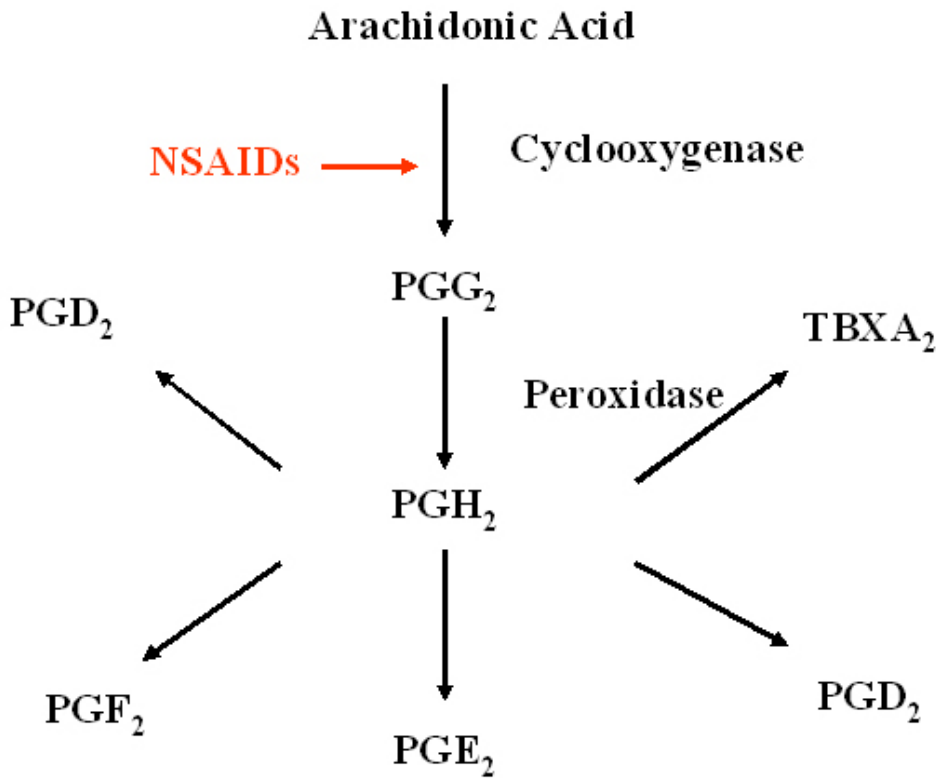


Figure 1-2. The COX Pathway

Cyclooxygenase (COX) converts arachidonic acid into prostaglandin H_2 (PGH_2). PGH_2 is transformed into other prostaglandins, including PGE_2 and $PGF_{2\alpha}$, by synthases specific to each molecule.

1.2.3 Primary and Secondary Fusion of Muscle Cells

There are two stages in the formation of mature myotubes by myoblast fusion. (**Fig. 1-3**) In the primary phase, a subset of differentiated myoblasts fuses together to form a nascent myotube, containing a limited number of nuclei. The secondary phase of myoblast fusion, which finally leads to the formation of mature myotubes, occurs between nascent myotubes and more single myoblasts.[20]

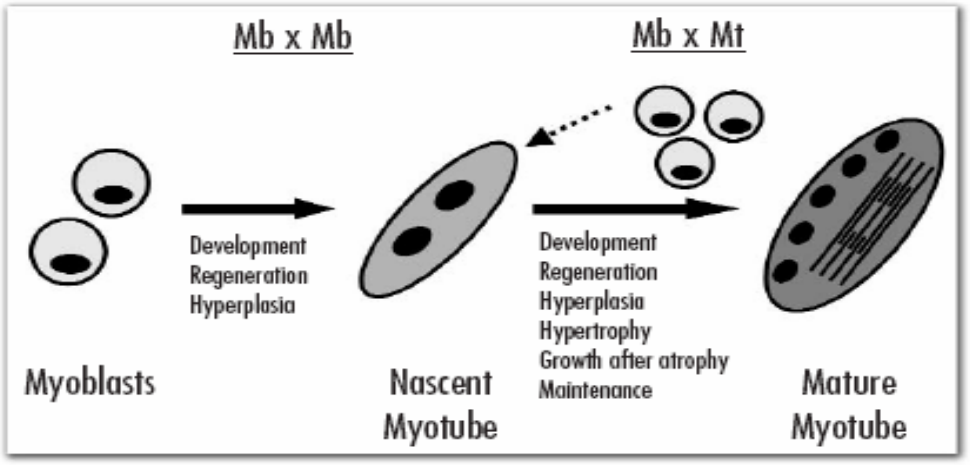


Figure 1-3. The Fusion of myoblasts

The fusion of myoblasts is divided into two stages: Primary fusion, in which two single myoblasts fused into a nascent myotube; and Secondary fusion, which is between nascent myotube and more single myoblasts. (Reproduced with the permission from the journal of *Cell Cycle*)

1.2.4 Inflammatory Cells and Skeletal Muscle Healing

Muscle injury can produce an inflammatory response during which neutrophils rapidly invade, followed by macrophages. Studies have depicted a complex picture in which inflammatory cells promote both injury and repair, through the combined actions of free radicals, growth factors, and chemokines.[3] New findings clearly show a role of neutrophils in promoting muscle damage soon after muscle injury or modified use. No direct evidence is yet available to show that neutrophils play a beneficial role in muscle repair or regeneration. [3]

Macrophages have also been shown to be capable of promoting muscle damage in vivo and in vitro through the release of free radicals, although many other findings indicate that they rather play a role in muscle repair and regeneration. By phagocytosing damaged tissue, macrophages help to remove the debris that could impede muscle regeneration. These cells can stimulate the proliferation of activated satellite cells by releasing growth factors and cytokines

and by direct contact with them.[21-24] Recent study results suggest that macrophages may also fuse with myofibers directly to promote regeneration.[25] To further prove the importance of the macrophage in skeletal muscle healing, a "Loss of macrophage" study is necessary. In addition, it is not clear whether the macrophage affects the expression of growth factors in different muscle-isolated cells, including myoblasts, satellite cells, and fibroblasts.

1.2.5 TGF-β1 and Fibrosis formation

TGF-β1 is a cytokine produced by both immune and non-immune cells, and it exhibits a broad range of functions. TGF-β1 controls the differentiation, proliferation, and state of activation of all immune cells, and is implicated in immune abnormalities linked to cancer, autoimmunity, opportunistic infections, and fibrotic complications.[26] TGF-β1–triggered signals are transduced by Smads, a family of proteins that serve as substrates for TGF-β receptor type I and type II.[27] Interestingly, both IFN-γ and TNF-α inhibit the TGF-β/Smad signaling pathways,[28, 29] suggesting that these proinflammatory cytokines act, at least in part, by blocking the effects of immunosuppressive cytokines like TGF-β1. Work in a variety of murine models provides irrefutable evidence that eliminating TGF-β1 or disrupting its downstream signaling cascade leads to inflammatory disease.[30-33] Thus, TGF-β1 may be a negative regulator of inflammation. However, except for the fact that the Smads pathway that is at the downstream of TGF-β1 and regulated by TGF-β1, we still don't know if TGF-β1 can affect the inflammation by other means, for example the number and functional status of inflammatory cells.

Fibrosis is a complex biologic process involving the acute inflammatory response. Transient activation of fibroblasts to proliferate and produce an elevated quantity of extracellular matrix (ECM) is essential to fibrosis. One of the most potent fibrotic stimuli to fibroblasts is

TGF-β1. Besides its multiple cellular functions including inhibition and stimulation of cell growth, cell death or apoptosis, and cellular differentiation, TGF-β1 is also an inducer of ECM protein synthesis and has been implicated as the key mediator of fibrogenesis in various tissues.[34]

Study has shown that the intersection of the TGF-β1 signal pathways can not only exaggerate inflammation, but also protect tissue from fibrosis. [35] It seems that TGF-β1 is the pivot of the balance between inflammation and fibrosis. A diminished production of TGF-β1 may tip the balance in favor of inflammation. In contrast, increased TGF-β1 secretion may over-ride an anti-inflammatory effect and tip the balance in favor of fibrosis.[36] It has been suggested that the fibrotic effect of TGF-β1 is opposed by other molecules. PGE_2 is a potent inhibitor of fibroblast proliferation[37] and collagen synthesis.[38, 39] This suggests that PGE_2 may play an important role in maintaining normal extracellular matrix homeostasis. It has been reported in lung fibrosis disease models that the production of PGE_2 can be up-regulated by TGF-β1 via the COX-2 pathway.[19, 37] Thus, the more TGF-β1 produced, the stronger opposition to its fibrotic effect will be posed by PGE_2. However, this phenomenon needs to be addressed in skeletal muscle tissue. In addition, it needs to be addressed whether PGE_2 has an inhibitory effect on the expression of TGF-β1 directly to form a negative feedback loop.

1.3 PROJECT OBJECTIVES

1.3.1 Objective #1: Identify the Role of COX-2 Pathway in Skeletal Muscle Inflammation

Previous studies in the literature have suggested that PGE_2 and $PGF_{2\alpha}$ are important cytokines for the growth of muscle cells.[15, 16, 18] Our *in vitro* preliminary data showed that LP cells isolated from COX-$2^{-/-}$ mice had a very low level of PGE_2 and $PGF_{2\alpha}$ expression, and the growth of these COX-$2^{-/-}$ cells was limited. NS-398, a COX-2 specific inhibitor, also decreased the expression of PGE_2 and $PGF_{2\alpha}$ and the synthesis of Myosin Heavy Chain (MHC) in LP cells. *In vivo* preliminary data showed that the administration of NS-398 decreased the number and size of regenerating myofibers in a mouse laceration injury model. Based on these information, we hypothesize that COX-2 pathway is required in muscle regeneration, and its effects are mediated by the end products of the pathway, PGE_2 and $PGF_{2\alpha}$. In this objective, we will assess the effect of NS-398 on muscle regeneration and fibrosis formation, and the role of PGE_2 and $PGF_{2\alpha}$ in skeletal muscle healing. Furthermore, the importance of COX-2 pathway was evaluated by functional testing of injured skeletal muscle.

1.3.2 Objective #2: Identify the Role of Macrophage in Skeletal Muscle Inflammation

It has been postulated that macrophages play an important role in the muscle healing process. By phagocytosing damaged tissue, macrophages help to remove the tissue debris that can impede muscle regeneration. These cells also stimulate the proliferation of activated satellite cells by releasing growth factors and cytokines and by direct contact.[21-24] Recent study results suggest that macrophages may also fuse with myofibers directly to promote regeneration.[25] Our *in vivo* preliminary data showed that the infiltration of macrophages was reduced in NSAIDs-treated

mice, which had delayed muscle healing compared to the non-treated control mice. *In vitro* preliminary data showed that the co-culture of macrophages with LP cells can promote the myogenic differentiation of LP cells. Based on this information, we hypothesize that macrophages are required in muscle healing, and macrophages may regulate the expression of growth factors and cytokines in muscle cells. Because few studies have been done to show the direct impact of "Loss of Function" of macrophages, our approach is to deplete macrophages by injecting clodronate liposome. This agent can deplete macrophages in experimental animals and allows us to assess the importance of macrophages in skeletal muscle healing. In addition, we will investigate the relationship between macrophages and the expression of TGF-β1 and PGE_2 in injured skeletal muscle tissue and muscle cells, as this may be one of the mechanisms that macrophages use to modulate muscle healing process.

1.3.3 Objective #3: Identify the Role of TGF-β1 in muscle inflammation

TGF-β1 has been shown to have an anti-inflammatory effects in many tissues.[40-43] Recently, it has been shown that TGF-β1 is a negative regulator of NF-κB, a transcription factor that can be activated by many proinflammatory cytokines. [33] However, in our preliminary experiments, we found that TGF-β1 can increase the infiltration of macrophages after the intramuscular injection of thioglycolate broth, a chemotaxis agent. Thus, it seems that TGF-β1 may interfere with the inflammation phase in various ways. Increasing the infiltration of macrophages may be one of the mechanisms by which TGF-β1 affects the inflammatory response, and consequently the whole healing process in injured skeletal muscle. We hypothesize that TGF-β1 interferes with the inflammatory response after skeletal muscle injury by increasing the infiltration of

macrophages. We will assess the infiltration of macrophage by using a flow cytometry technique.

It has been reported that the production of PGE_2 can be up-regulated by TGF-β1 via the COX-2 pathway in lung fibroblasts.[19, 37] Our preliminary data suggested that this finding may also be true in myogenic precursor cells. PGE_2 was found to oppose the fibrotic effect of TGF-β1 by inhibiting fibroblast proliferation[37] and collagen synthesis.[38, 39] Thus, it was postulated that the homeostasis of the extracellular matrix may be maintained by this mechanism.[19] However, it is not clear whether PGE_2 has an inhibitory effect on the expression of TGF-β1 itself. Our *in vivo* preliminary data showed that by blocking COX-2, and thus the expression of PGE_2 and $PGF_{2\alpha}$, the expression of TGF-β1 in connective tissue was significantly increased. Thus, a negative feedback loop composed of TGF-β1, COX-2 and PGE_2 may exist to keep the TGF-β1 level from surging and the overproduction of fibrotic tissue. Based on this information, we hypothesize that TGF-β1 may increase the expression of PGE_2, and PGE_2 may decrease the expression of TGF-β1. A negative feedback loop is therefore formed to oppose the production of TGF-β1 and its fibrotic effects.

2.0 THE ROLE OF COX-2 PATHWAY IN SKELETAL MUSCLE INFLAMMATION

2.1 INTRODUCTION

Skeletal muscle injury produces pain, vasodilation, and a series of inflammation-related symptoms. To relieve pain, doctors for years have prescribed non-steroid anti-inflammatory drugs (NSAIDs), a large and chemically diverse group of drugs that inhibit the cyclooxygenase (COX) enzyme and thereby block the conversion of arachidonic acid into prostaglandins and thromboxanes. By so doing, these drugs relieve the pain associated with inflammation. Although some studies have shown that the administration of NSAIDs promotes muscle healing by reducing degeneration and inflammation,[44, 45] other research has demonstrated that NSAIDs are detrimental to the entire healing process.[8, 9, 46] In response to the increasing debate regarding both the beneficial and detrimental effects of the COX-2–specific inhibitors, we performed this study to investigate the influence of these drugs on inflammatory signals, muscle regeneration, fibrosis, and, consequently, overall muscle healing after injury. Here we examined the effects of NS-398, a COX-2–specific inhibitor, both on myogenic precursor cells *in vitro* and in a mouse skeletal muscle laceration model *in vivo*.

2.2 RESULTS

2.2.1 NS-398 Inhibited the Proliferation and Differentiation of Myogenic Cells in Vitro

LP cells (preplate 5) isolated via the preplate technique[47, 48] were immunostained for 2 well-known markers of early myogenesis: desmin and Pax7.[49-51] More than 90% of the cells were desmin positive and 85% were Pax7 positive (Data not shown). This finding confirms that these cells are myogenic precursor cells. NS-398, at a concentration of 100 μM, significantly inhibited the proliferation of LP cells at day 3 and day 4 (**Figure 2-1, A**). The addition of NS-398 (10 μM or 100 μM) to cells cultured in DM for 2 days significantly decreased their expression of the late myogenic differentiation markers myogenin and MHC-d but not of the early marker MyoD ($P < 0.05$, **Figure 2-1, B and C**). The addition of NS-398 at any of the tested concentrations significantly inhibited the expression of PGE_2 and $PGF_{2\alpha}$ in culture supernatant ($P < 0.05$, **Figure 2-1, D**)

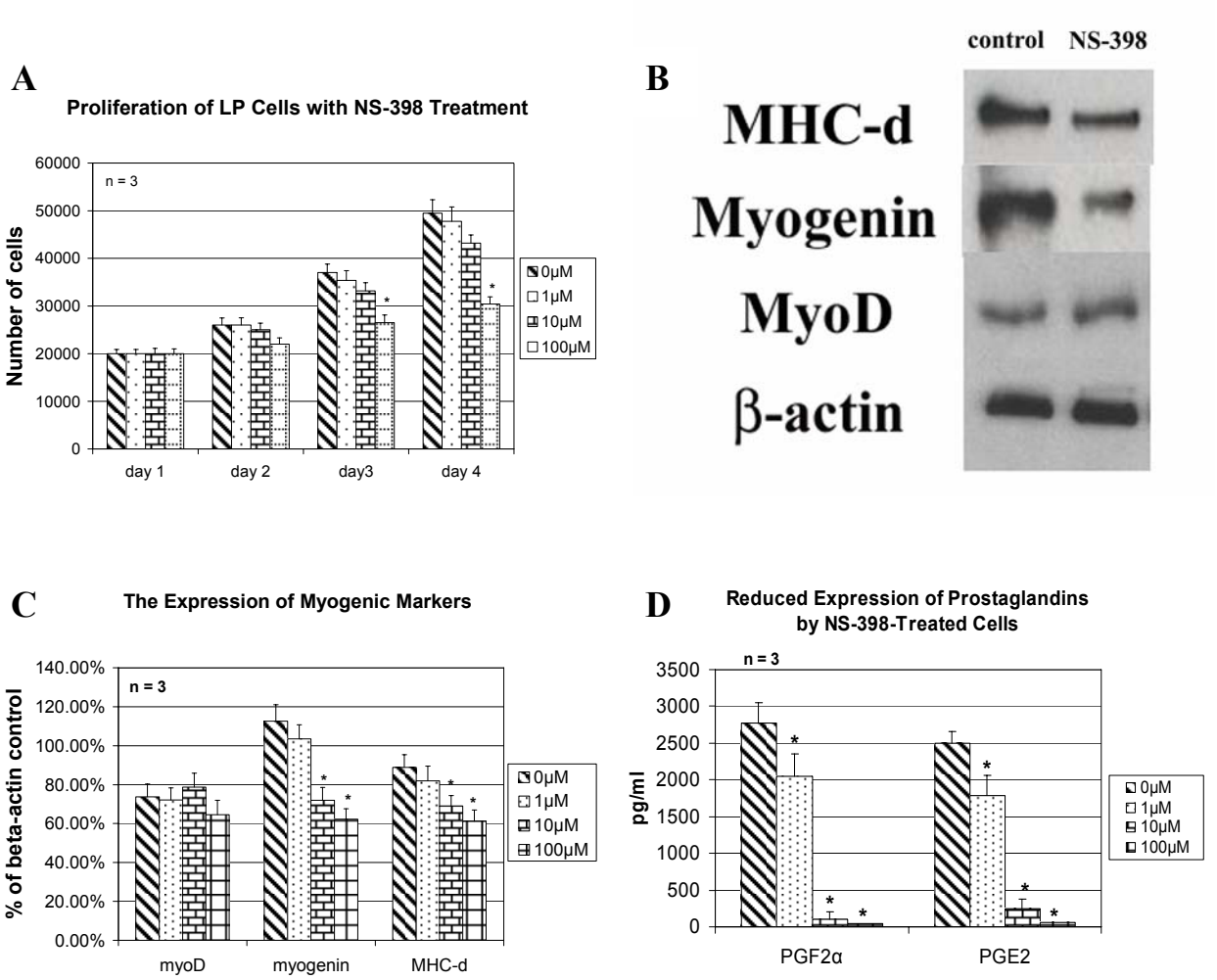


Figure 2-1. The Effect of NS-398 on the Proliferation and Differentiation of Myogenic Cells

NS-398 (100 μM) significantly inhibited the proliferation of myogenic precursor cells at day 3 and day 4 (**A**). Western blot results show that NS-398 (10 μM and 100 μM) decreased the expression of MHC-d and myogenin but not of MyoD (**B** and **C**). The addition of all tested concentrations of NS-398 *in vitro* severely reduced the expression of prostaglandins by LP cells (**D**). The asterisks indicate a significant difference ($P < 0.05$) between the marked groups and the 0μM group. (Reproduced with the permission from *American Journal of Pathology*)

2.2.2 The Effect of NS-398 on Skeletal Muscle Regeneration

H & E staining revealed the presence of centronucleated regenerating myofibers in both the NS-398–treated groups and the non-treated (control) group as early as 3 days after injury (Results not shown). Seven days after injury, the injury site primarily contained regenerating myofibers. Seven and 14 days after injury, significantly more regenerating myofibers were present in the non-treated control group than in each of the NS-398–treated groups ($P < 0.05$). Furthermore, the centronucleated myofibers in the non-treated control group were significantly larger than those in each of the NS-398–treated groups ($P < 0.05$), which indicates more advanced muscle regeneration in the control group. The dosage of NS-398 and the duration of NS-398 administration also contributed to these effects. Twenty-eight days after injury, the control group and the NS-398–treated groups exhibited similar degrees of muscle recovery. There were no significant differences between the groups in terms of the number or size of regenerating myofibers at this time point (**Figure 2-2**).

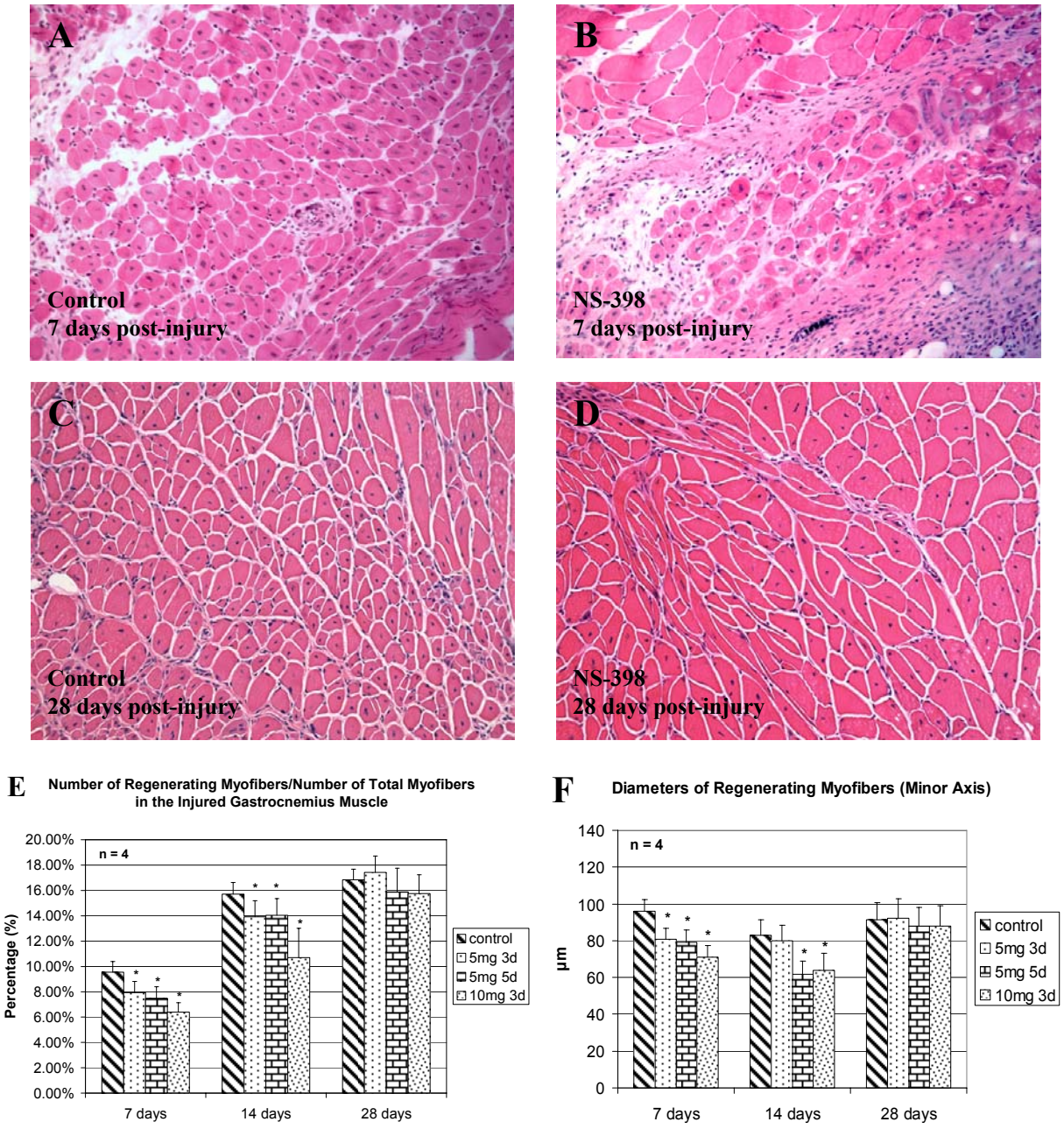


Figure 2-2. NS-398 Decreased the Number and Size of Regenerating Myofibers

Seven and 14 days (**A, B**) after injury, muscle sections in the control group contained a significantly higher percentage of centronucleated myofibers than did muscle sections in the NS-398–treated groups. Furthermore, the minor axis diameters of the centronucleated myofibers in the control group were significantly larger than those of the centronucleated myofibers in the NS-398–treated groups. However, follow-up analysis of these parameters conducted 28 days (**C, D**) after injury revealed no significant difference between the control and treated groups. The asterisks in **E** and **F** indicate a significant difference ($P < 0.05$) between the marked groups and the control group. (Reproduced with the permission from *American Journal of Pathology*)

2.2.3 NS-398 Increased Fibrosis Formation in Injured Skeletal Muscle

We used trichrome staining to observe fibrous tissue formation after injury in different groups. The results are comparable with those observed after H & E staining. At early time points (14 days after injury), the control group exhibited better recovery and contained less fibrous tissue than the NS-398–treated groups ($P < 0.05$). Muscles from the low-dose, short-duration group (5mg-3day) contained less fibrous scar tissue ($P < 0.05$) than muscles in the high-dose group (10mg-3day) or muscles in the longer NS-398 treatment group (5mg-5day). Although it did not reach statistical significance, the percentage of fibrous tissue in the NS-398–treated groups appeared to be higher than that recorded for the control group at the 28-day time point. (**Figure 2-3**)

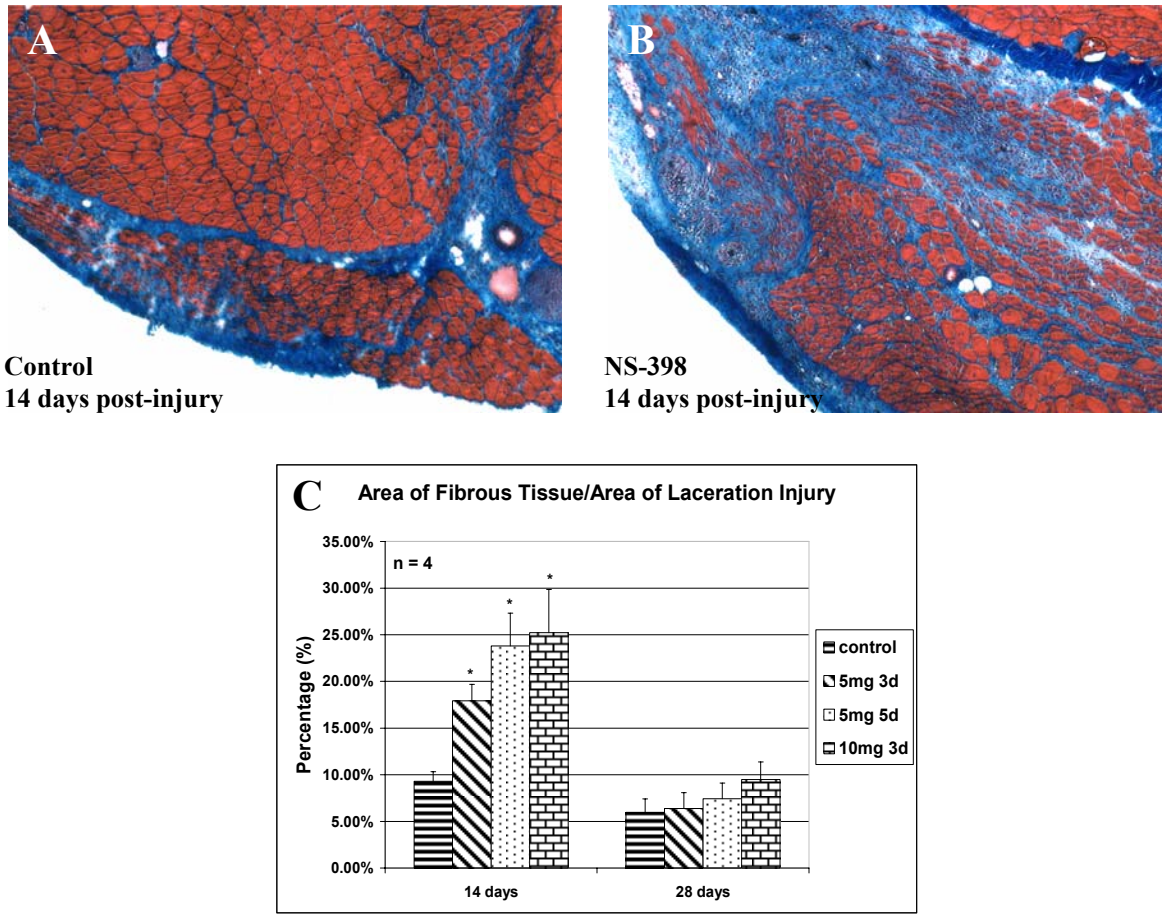


Figure 2-3. NS-398 Increases the Formation of Fibrosis Tissue

Trichrome staining was used to observe fibrosis after injury (100X magnification). Fibrous tissue was stained blue. At one of the earlier time points (14 days after injury), the control group (**A**) contained less fibrous tissue (less stained area) than did either of the NS-398–treated groups ($P < 0.05$, **B**). The low-dose, short duration group (5mg-3d) contained less fibrous tissue than the high-dose (10mg-3d) and longer duration (5mg-5d) groups ($P < 0.05$). However, at the last time point (28 days after injury), no significant differences were observed. The asterisks indicate a significant difference ($P < 0.05$) between the marked groups and the control group. (Reproduced with the permission from *American Journal of Pathology*)

2.2.4 The Effect of NS-398 on the Expression of TGF-β1

We used immunostaining to investigate the expression of TGF-β1 during skeletal muscle healing. We observed the expression of TGF-β1 in the extracellular matrix of all groups on 7, 10, and 14 days after injury. The control muscles expressed significantly less TGF-β1 than the NS-

398–treated muscles ($P < 0.05$). This finding supports the possible involvement of TGF-β1 in the promotion of fibrosis observed in the NS-398–treated muscles. (**Figure 2-4**)

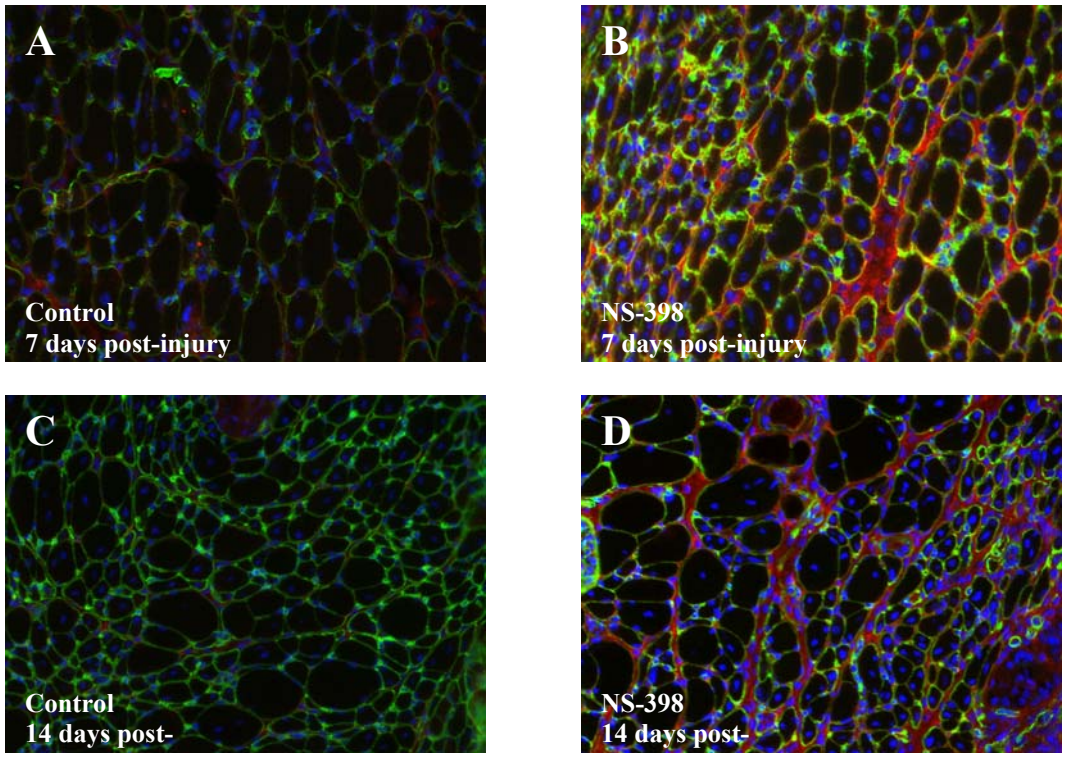


Figure 2-4. The Effect of NS-398 on TGF-β1 Expression

TGF-β1, collagen IV, and cell nuclei are red, green, and blue, respectively. Both the control and the NS-398–treated groups (5mg-3day) exhibited relatively low TGF-β1 expression 7 days after injury (**A** and **B**). However, 10 days and 14 days (**C**) after injury the control group showed hardly any expression of TGF-β1, whereas, relative to the control group, the NS-398–treated groups exhibited significantly higher TGF-β1 expression (**D**). Asterisks in the graph indicate that the TGF-β1–expressing area was significantly less in the control group than in the various NS-398–treated groups ($P < 0.05$). (Reproduced with the permission from *American Journal of Pathology*)

2.2.5 The effect of PGE_2 and $PGF_{2\alpha}$ on COX-$2^{-/-}$ cells

More than 90% of the LP cells isolated from wild-type mice or COX-$2^{-/-}$ mice were positive for desmin, which suggests that these LP populations both contained primarily myogenic precursor cells and were comparable populations. We performed immunostaining to identify MHC-d expression and cell nuclei in the cell populations cultured for 18 days in differentiation medium. Only 5% of the myotubes in the COX-$2^{-/-}$ LP cell culture contained more than 3 nuclei, compared with 60% of the myotubes in the wild-type LP cell culture (**Fig. 2-5 A–C**). However, the total numbers of nuclei were similar in the 2 groups. At 100x magnification, averages of 112 and 120 nuclei were visible in the wild-type cell population and the COX-$2^{-/-}$ cell population, respectively. The expression of PGE_2 and $PGF_{2\alpha}$ was significantly lower in the COX-$2^{-/-}$ LP population than in the wild-type LP population at all time points. The expression levels observed on day 4 are shown as an example (**Fig. 2-5 D**).

Both PGE_2 and $PGF_{2\alpha}$ restored the secondary fusion of COX-$2^{-/-}$ cells. Treatment of the COX-$2^{-/-}$ cell cultures with the 3 different concentrations of $PGF_{2\alpha}$ (10000 ng/ml, 1000 ng/ml, and 100 ng/ml) increased the percentages of myotubes containing more than 3 nuclei to 28%, 40%, and 62%. Treatment of the COX-$2^{-/-}$ cell cultures with PGE_2 at a concentration of 100 ng/ml increased the percentage of myotubes containing more than 3 nuclei to 45% (**Fig. 2-6 A**). At concentrations of 1000 ng/ml and 10000 ng/ml, PGE_2 had a severe toxic effect on COX-$2^{-/-}$ LP cells over the 18-day period. Most cells gradually detached from the culture surface, and very few cells remained attached at the end of the culture period (**Fig. 2-6 B).** Analysis of the floating cells with a trypan blue assay demonstrated that the cells were dead.

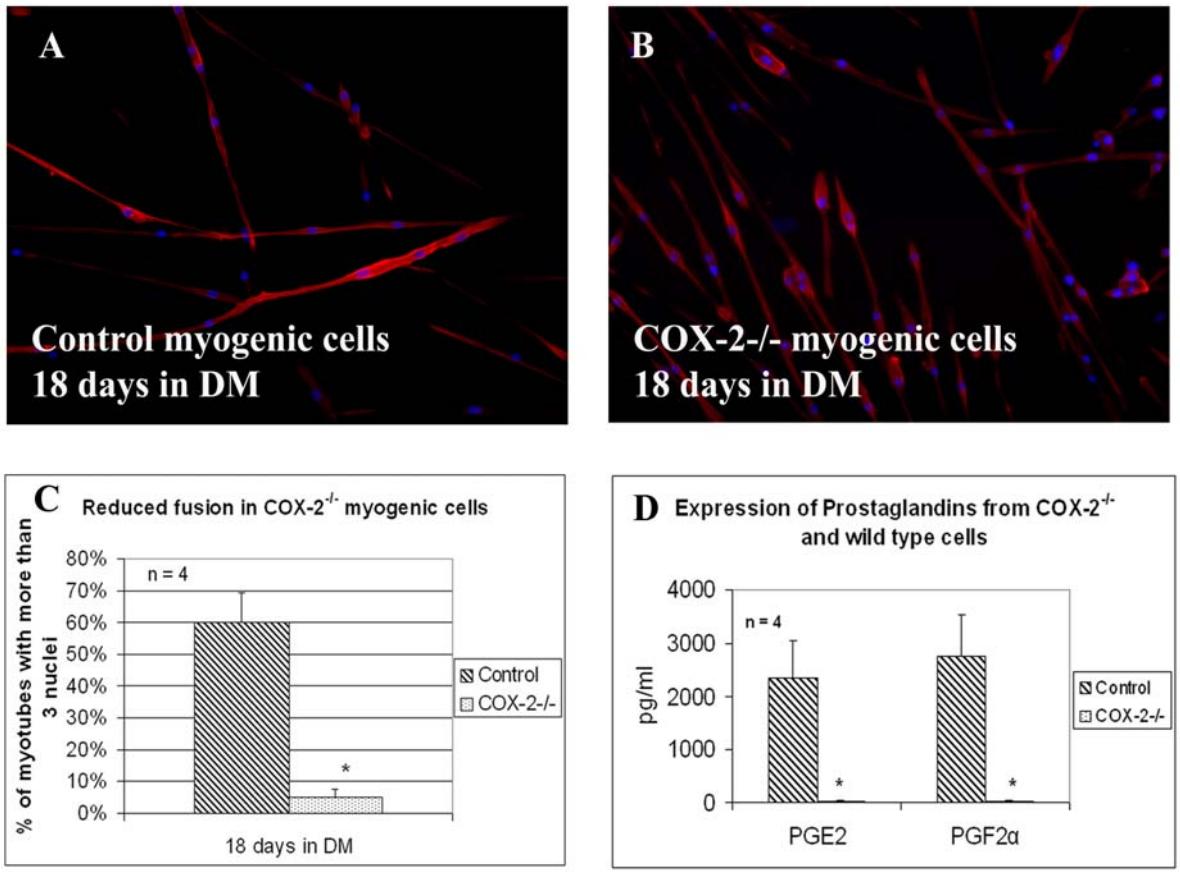


Figure 2-5. The Reduced Expression of Prostaglandins and Fusion in COX-2$^{-/-}$ Cells.

Compared with the differentiation of wild-type (control) LP cells (**A**), the differentiation of LP cells derived from COX-2$^{-/-}$ mice (**B**) was severely compromised. After COX-2$^{-/-}$ cells and wild-type cells were cultured for 18 days in differentiation medium, the percentage of myotubes containing more than 3 nuclei was significantly lower in the COX-2$^{-/-}$ cell culture than in the wild-type cell culture (P<0.05; **C**). ELISA results indicate that the expression of both PGE_2 and $PGF_{2\alpha}$ was significantly lower in COX-2$^{-/-}$ cell cultures than in wild-type cell cultures (P<0.05; **D**). The asterisks indicate a significant difference (P < 0.05) between the marked groups and the control group.

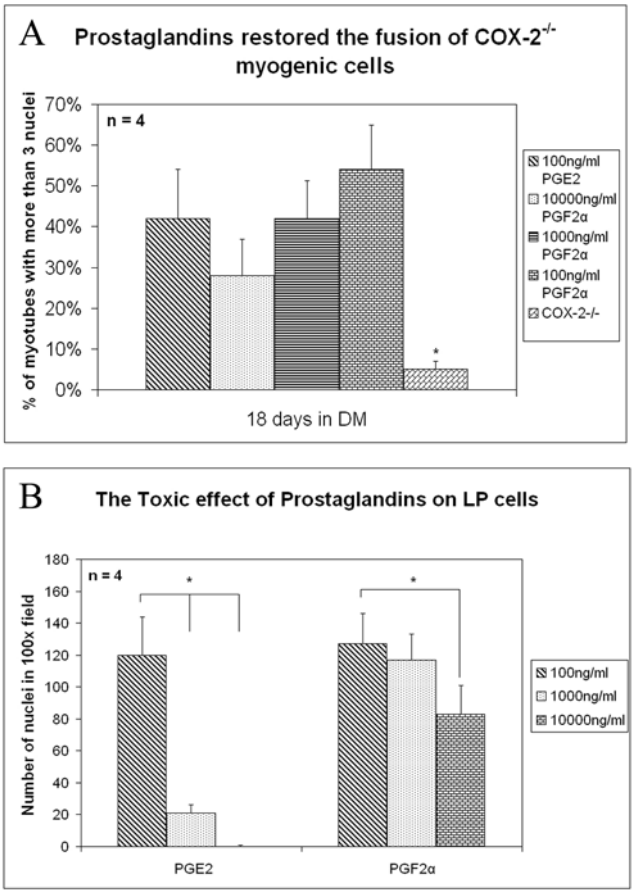


Figure 2-6. Prostaglandins Restores the Fusion of COX-2$^{-/-}$ Cells.

The addition of PGE_2 (100 ng/ml) or $PGF_{2\alpha}$ (100, 1000, or 10000ng/ml) significantly improved the fusion ability of COX-2$^{-/-}$ cells to varying degrees. (P<0.05; **A**) The graph in panel **B** shows the average number of nuclei in 100x magnification fields at the end of the culture period (18 days). High concentrations of PGE_2 (1000 or 10000 ng/ml) or $PGF_{2\alpha}$ (10000 ng/ml) were toxic to LP cells and caused cell detachment from the culture surface. The asterisks indicate a significant difference (P < 0.05) between the marked groups and the control group.

2.2.6 Skeletal Muscle Recovery after Injury in Wild Type and COX-2$^{-/-}$ Mice

Our evaluation of TA muscle sections obtained 5 days after laceration revealed fewer regenerating myofibers in the COX-2$^{-/-}$ muscle sections than in the wild-type sections (P<0.05). At 14 days after injury, muscle sections from the COX-2$^{-/-}$ mice again contained significantly fewer regenerating myofibers than observed in the wild-type sections, and the average diameter of the minor axes of the myofibers in the COX-2$^{-/-}$ sections was significantly lower than those

measured in the wild-type sections (P<0.05; **Fig. 2-7 A, B**). We did not compare the minor axis diameters in the 2 groups 5 days after injury because the sections obtained from the COX-2$^{-/-}$ mice contained too few regenerating myofibers.

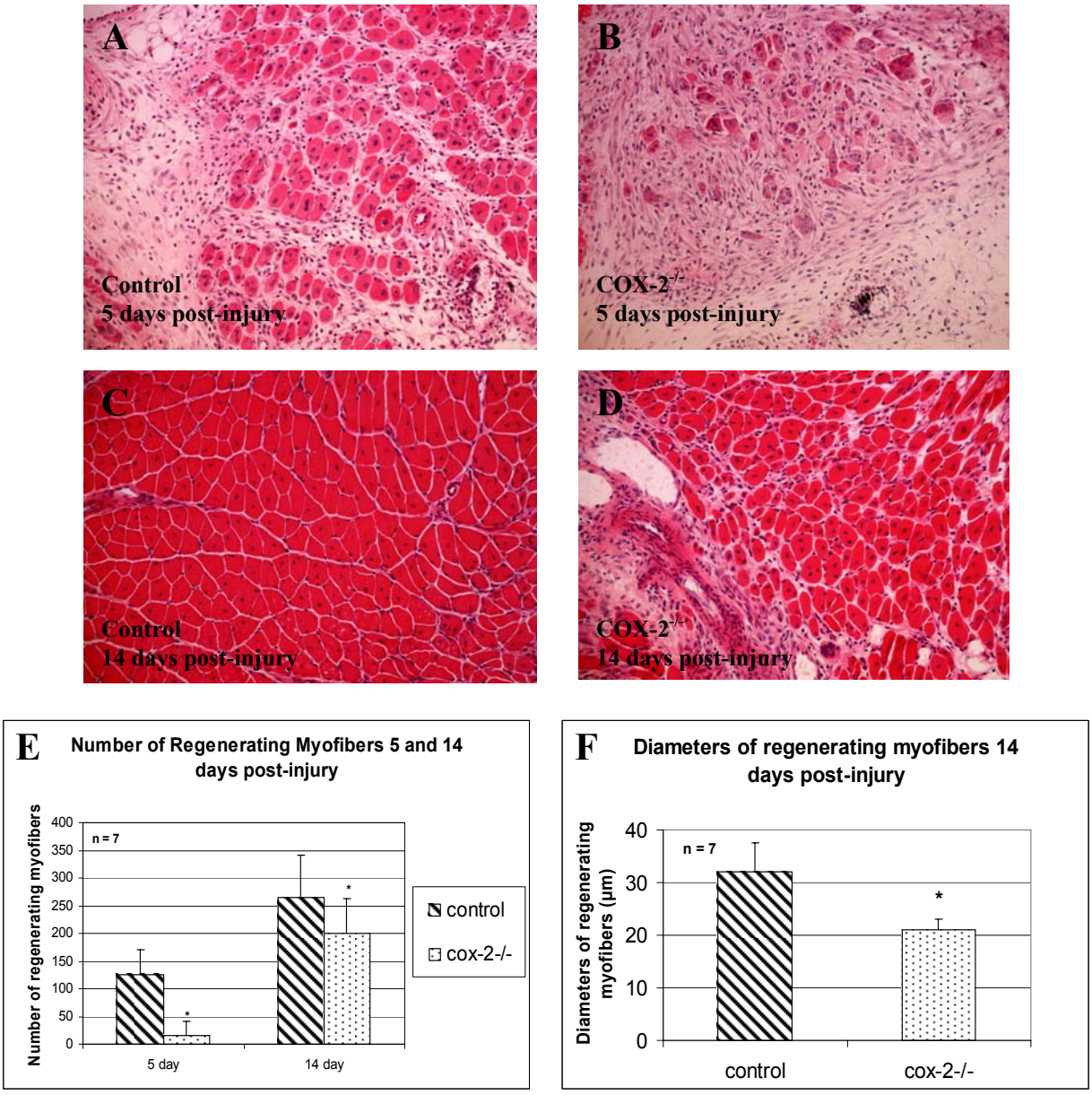


Figure 2-7. Skeletal Muscle Healing in Wild Type and COX-2$^{-/-}$ Mice

The recovery of wild-type mice (**A**, **C**) and COX-2$^{-/-}$ mice (**B**, **D**) 5 and 14 days after injury is shown by hematoxylin and eosin staining. The number of centronucleated regenerating myofibers was significantly lower in COX-2$^{-/-}$ mice 5 and 14 days after injury than in wild-type (control) mice (P<0.05; **E**). The minor axis diameters of regenerating myofibers 14 days after injury were significantly smaller in COX-2$^{-/-}$ mice than in wild-type (control) mice (P<0.05; **F**). The asterisks indicate a significant difference (P < 0.05) between the marked groups and the control group.

2.2.7 The Effect of NS-398 on Immune Response after Skeletal Muscle Injury

Using flow cytometry, we identified a population of CD-11b (FITC)-positive cells and a population of CD-11b/F4/80 (FITC/APC) double-positive cells in both the NS-398–treated groups and the non-treated (control) groups as early as 12 hours after injury; these 2 populations represent neutrophils and macrophages, respectively. The percentages of these cells (relative to the entire cell population) continued to increase until they peaked 48 hours after injury; these percentages dropped dramatically thereafter. At all time points, the percentages of neutrophils and macrophages in the NS-398–treated group were lower than those in the control group. However, statistically significant differences between the control group and the treated groups occurred only at 48 hours after injury for neutrophils and 24 hours after injury for macrophages ($P < 0.05$, **Fig. 2-8**).

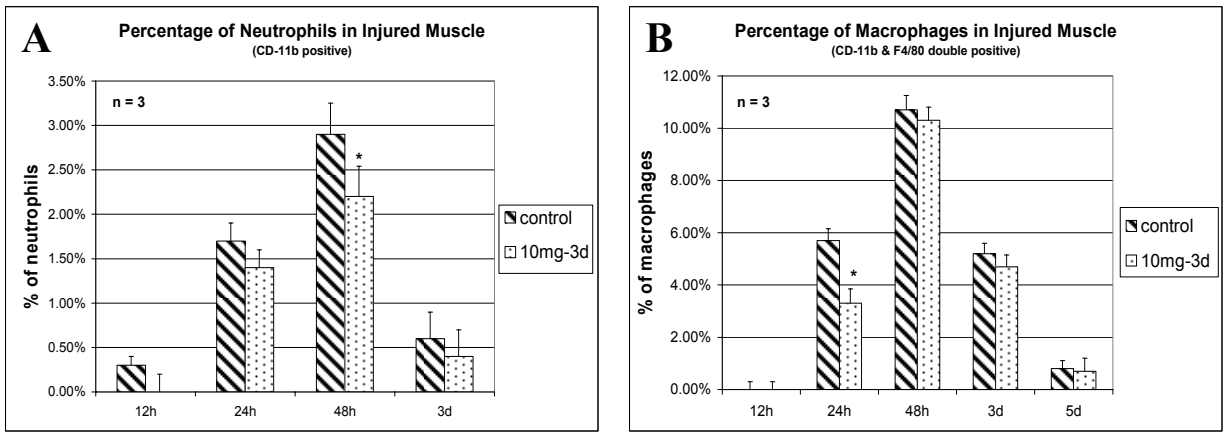


Figure 2-8. Immune Response after NS-398 Treatment

Neutrophils (**A**) and macrophages (**B**) infiltrated the injury site as early as 12 hours after injury. The percentages of these cells (in terms of the entire muscle cell population) peaked 48 hours after injury. At all time points, the NS-398–treated groups contained lower percentages of inflammatory cells than did the control group, but we observed significant differences ($P < 0.05$) only at 48 hours after injury for neutrophils and at 24 hours after injury for macrophages. The asterisks indicate a significant difference ($P < 0.05$) between the marked groups and the control group. (Reproduced with the permission from *American Journal of Pathology*)

2.2.8 Immune Response after Skeletal Muscle Injury in COX-2$^{-/-}$ Mice

We performed CD-11b immunohistochemical staining to assess the infiltration of inflammatory cells, including macrophages and neutrophils, in the lacerated TA muscles. Muscle sections from the COX-2$^{-/-}$ mice contained fewer CD-11b–positive cells than did those from wild-type mice (P<0.05; **Fig. 2-9**). This finding suggests that the COX-2$^{-/-}$ mice exhibited a lower inflammatory response than did the wild-type mice.

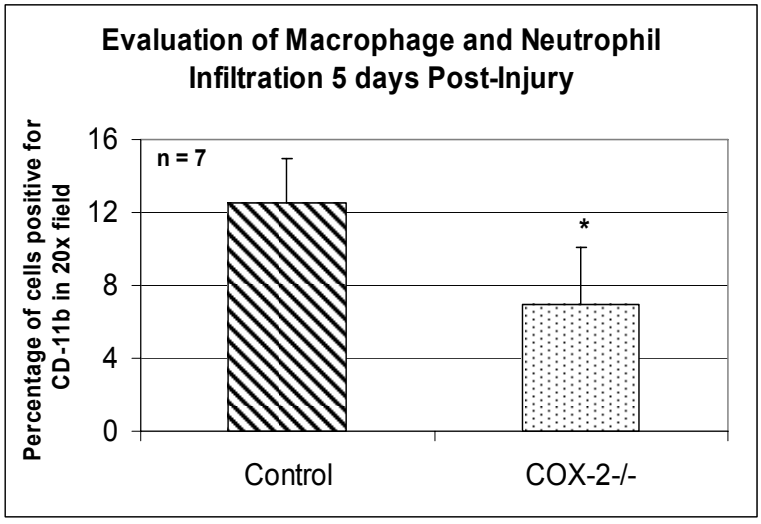


Figure 2-9. Infiltration of Inflammatory Cells after Muscle Injury

CD-11b immunostaining revealed significantly reduced infiltration of macrophages and neutrophils in COX-2$^{-/-}$ mice compared to wild-type control mice (P<0.05). The asterisks indicate a significant difference (P < 0.05) between the marked groups and the control group.

2.2.9 The Importance of COX-2 Pathway Evaluated by Physiologic Evaluation

We tested the specific peak force (N/cm^2) and the specific tetanic force (N/cm^2) of TA muscles isolated from wild-type and COX-2$^{-/-}$ mice 14 days after laceration. We calculated the functional recovery percentage and used it as an indicator of functional recovery of the injured muscles. The wild-type (control) group muscles exhibited better functional recovery than the COX-2$^{-/-}$

muscles, as indicated by a higher functional recovery percentage in the former group of muscles (P<0.05; **Fig. 2-10**).

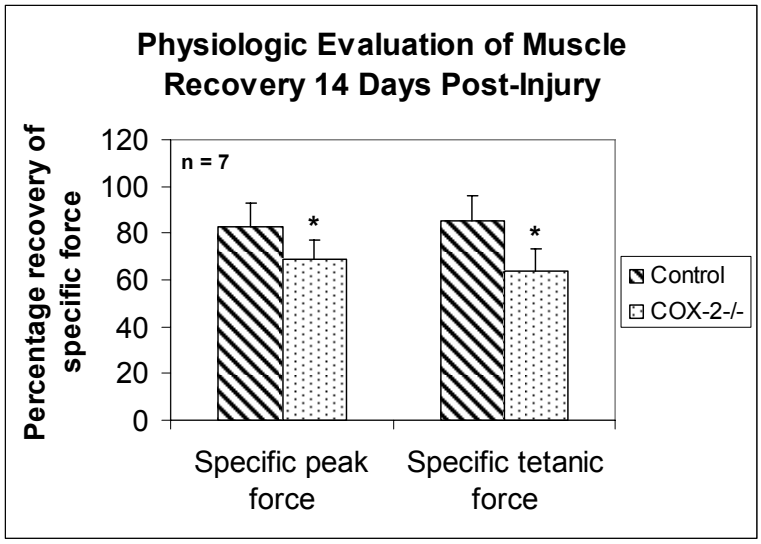


Figure 2-10. Physiologic Evaluation of Muscle Recovery

Physiologic testing measured specific peak force and specific titanic force of injured skeletal muscle on 14 days after injury. It revealed significantly reduced functional recovery in COX-$2^{-/-}$ mice compared to wild-type control mice (P<0.05). The asterisks indicate a significant difference (P < 0.05) between the marked groups and the control group.

2.3 DISCUSSION

2.3.1 NS-398 Inhibited the Proliferation and Differentiation of Myogenic Cells

Satellite cells are the cells primarily responsible for the regeneration of muscle tissue. When a muscle is injured, quiescent satellite cells begin to proliferate and generate myogenic cells, [52] [53] which are identifiable by their expression of the early myogenic markers MyoD and Myf5. [54] [55] Before they terminally differentiate to form new myofibers or fuse with previously existing myofibers, satellite cells begin to express myogenin and MRF4. [51, 56] (**Figure 2-11**) The study reported here demonstrates that NS-398 decreased the expression of myogenin and MHC-d by myogenic precursor cells but did not affect MyoD expression. This finding suggests that NS-398 interferes with the maturation of myogenic cells (i.e., with late-stage differentiation) but not with their early activation or differentiation.[49, 51, 56, 57] NS-398 administration also inhibited the proliferation of myogenic precursor cells, although a significant difference was only noted at the highest dose (100μM). Since the expression of prostaglandins (PGE_2 & $PGF_{2\alpha}$) was severely impaired at low doses of NS-398 (1μM and 10μM), this proliferation inhibition may be at least partially due to a COX-2-independent pathway. The results of trypan blue assay indicated good cell viability (data not shown) and cell numbers continued to increase in all NS-398–treated groups during the 4-day period of the experiment. Therefore the proliferation inhibition does not appear to be due to a toxic effect of NS-398. In a recently published study, Mendias et al.[58] showed that NS-398 can inhibit the proliferation and differentiation of rat satellite cells, a finding that our results strongly support. In their study, however, NS-398 concentrations ranging from 0.01 μM to 100 μM showed similar inhibitory effects on the proliferation of the rat satellite cells. This discrepancy between their results and ours may be related to species-related differences.

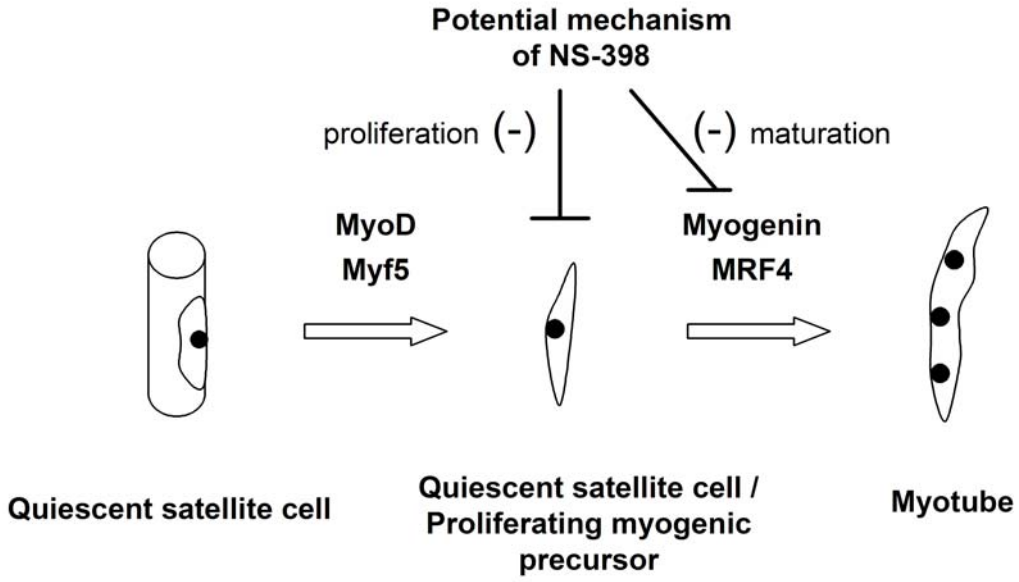


Figure 2-11. The Proposed Mechanism of Myogenic Cell Differentiation

Different markers distinguish various stages of myogenic differentiation. MyoD and myogenin denote the early and late stages of muscle differentiation, respectively.[51, 56, 59-61] The detrimental effects of NS-398 on myogenic cells likely occur via inhibition of both cell proliferation and maturation of differentiated myogenic cells. (Reproduced with the permission from *American Journal of Pathology*)

2.3.2 NS-398 Inhibited the Regeneration of Injured Skeletal Muscle

We used the number of centronucleated regenerating myofibers and their minor axis diameters to quantify histological recovery of the injured muscles. Results from 7 days and 14 days after injury demonstrated better recovery in the control group than in the NS-398–treated groups. The low-dose group (5mg-3day) showed better recovery than the high-dose and the longer NS-398 treatment groups (5mg-5day and 10mg-3day, respectively). However, 28 days after injury, all groups exhibited similar final outcomes. We observed that NS-398 delayed muscle healing and that this effect was dependent on the dose of NS-398 and the duration of its administration. We hypothesize that, after cessation of NS-398 treatment and subsequent clearance of the compound from the body, the normal healing process resumes. Using a muscle freeze injury model, Bondesen et al.[62] found that SC-236, another Cox-2–specific inhibitor, decreased the size of

regenerating myofibers, even regenerating myofibers analyzed 5 weeks after injury. That study, however, involved the ongoing administration of SC-236 for the duration of the 5-week-long experiment, unlike our study which administered for a maximum of 5 days. The prolonged inhibition of muscle healing observed in that study may be due to the extended administration of the COX-2-specific inhibitor and gives more credence to the argument in favor of using these drugs only with caution—especially over the long term.

2.3.3 NS-398 Increased the Expression of TGF-β1 and Fibrosis Formation

Fibrosis frequently occurs after injury in many organs and tissues. When fibrous tissue forms in an injured area, the recovery of normal tissue architecture and functionality is compromised. After muscle injuries, fibrosis causes decreased muscle contractility and range of movement.[63] More importantly, the resultant fibrosis tissue makes the repaired muscle more susceptible to recurrent injuries. [64] The results of trichrome staining in our study showed that fibrosis began as early as 10 days after injury and that fibrous tissue (rather than regenerating myofibers) filled the injury sites. The NS-398–treated muscles contained fewer regenerating myofibers and more fibrous tissue (in terms of area) than did control muscles. However, as demonstrated by the results of H & E staining of the muscles 28 days after injury, the final outcomes in all groups were similar.

To investigate how the administration of NS-398 promotes fibrosis, we used immunohistochemistry to examine the expression of TGF-β1 in the area filled with fibrous tissue. TGF-β1 plays a key role in initiating the fibrosis cascade.[65, 66] Its expression level correlates with the proliferation of fibroblasts and their production of collagen type I, the major component of fibrous tissue.[67] The increased TGF-β1 expression observed in the NS-398–

treated groups relative to that observed in the control group suggests that NS-398 may slow down muscle regeneration by increasing TGF-β1 production, which in turn increases fibrosis. In addition, TGF-β1 may stimulate muscle-derived stem cells to differentiate into fibroblast-like cells.[68, 69] The differentiation of such muscle-derived cells may also explain the fibrosis that occurred in our experiments and those seen in clinical cases of muscle injury.

2.3.4 Prostaglandins Mediate the Fusion of Myogenic Cells

The results of the in vitro experiments demonstrated the importance of COX-2 and its downstream prostaglandin products in myogenesis. COX-$2^{-/-}$ LP cells had a reduced ability to grow into larger myotubes (that is, myotubes containing more than 3 nuclei) from nascent myotubes, and this inhibition of fusion correlated with the cells' significantly reduced expression of PGE_2 and $PGF_{2\alpha}$. The addition of $PGF_{2\alpha}$ and, to a lesser extent, PGE_2 to the COX-$2^{-/-}$ LP cell culture, restored the secondary fusion ability of these cells. Earlier studies have demonstrated the involvement of $PGF_{2\alpha}$ in protein synthesis within skeletal muscle and in muscle cell growth. [16, 70] It was postulated that $PGF_{2\alpha}$ is more important for the secondary fusion of myofibers into larger myofibers than for the initial fusion of 2 single myoblasts. [17] Horsley et al. [18] lead this area of research by showing that $PGF_{2\alpha}$ enhances the fusion of skeletal muscle cells not by stimulating the cells to form myotubes but by recruiting additional cells to fuse with the preexisting multinucleated nascent myotubes. In accordance with this theory, our observations revealed a higher percentage of large myotubes (that is, myotubes containing more than 3 nuclei) in the wild-type LP cell cultures than in the COX-$2^{-/-}$ LP cell cultures (in which the vast majority of myotubes contained fewer than 3 nuclei). However, the total numbers of nuclei within these 2 types of cell cultures at the end of the culture period were similar, which suggests that the higher

percentage of large myotubes in the wild-type cell culture was due to increased fusion rather than heightened cell proliferation. Many scientists view PGE_2 as an important inflammatory mediator; however, research also has shown that PGE_2 can influence the rate of protein turnover in skeletal muscle. [16] In addition, PGE_2 appears to induce the fusion of other cell types, such as osteoclasts. [71] Here we found that PGE_2 administered at a concentration of 100ng/ml restored the secondary fusion ability of COX-$2^{-/-}$ LP cells. At higher concentrations (1000 and 10000 ng/ml), however, PGE_2 had a toxic effect on the growing LP cells. Our results showed that $PGF_{2\alpha}$ was less toxic than PGE_2, but high concentrations of $PGF_{2\alpha}$ (at 10000 ng/ml) also significantly inhibited cell growth. A comparison of $PGF_{2\alpha}$ and PGE_2 delivered at the same concentration (100 ng/ml) revealed that $PGF_{2\alpha}$ administration resulted in better restoration of the fusion ability of COX-$2^{-/-}$ LP cells. $PGF_{2\alpha}$ may be more potent than PGE_2 in stimulating muscle cell fusion.

2.3.5 Inflammation and Regeneration of Injured Skeletal Muscle in COX-$2^{-/-}$ Mice

The results of our in vivo studies revealed that COX-2 gene deficiency had a significant effect on both inflammation and muscle regeneration. Our observations of CD-11b expression[72, 73] at the site of injury revealed significantly reduced infiltration of macrophages and neutrophils 5 days after injury in COX-$2^{-/-}$ mice compared with wild-type mice. We also noted significantly fewer regenerating myofibers in the COX-$2^{-/-}$ mice 5 and 14 days after muscle laceration injury, and those regenerating myofibers had smaller diameters than did myofibers in the wild-type mice. The COX-$2^{-/-}$ mice also showed significantly reduced recovery of skeletal muscle force 14 days after injury.

PGE_2 appears to play multiple roles in the muscle inflammatory process, including induction of pro-inflammatory cytokine synthesis and nitric oxide synthase expression,

vasodilation with increased vascular permeability, and chemotaxis of inflammatory cells. [14, 74, 75] The reduced PGE_2 synthesis in COX-$2^{-/-}$ mice appears to markedly limit the inflammatory process, as evidenced by the reduced infiltration of CD-11b–positive inflammatory cells noted in this study. CD-11b is a marker of both macrophages and neutrophils. [72, 73] These inflammatory cells, especially macrophages, are important mediators of the regenerative process. Macrophages secrete growth factors and cytokines that act in a paracrine fashion to stimulate myoblast regenerative events. [21, 23, 76] Furthermore, direct contact with macrophages can rescue myogenic precursor cells from apoptosis after muscle injury. The rescued cells can act synergistically with macrophages to amplify chemotaxis and enhance muscle growth. [22] In the case of COX-$2^{-/-}$ mice, the clearing of necrotic debris and growth factor–stimulated satellite cell and myoblast proliferation by infiltrating macrophages may be delayed. COX-$2^{-/-}$ mice may also exhibit limited synthesis of $PGF_{2\alpha}$, a product of the COX-2 pathway that appears to be important for the secondary fusion of myoblasts. [18] All of these factors could explain the limited muscle regeneration observed in COX-$2^{-/-}$ mice. The study reported here provided evidence of this delayed muscle regeneration and subsequent reduction in functional recovery of muscle, as evidenced both histologically (by reduced numbers and size of regenerating myofibers) and physiologically (by limited recovery of muscle force).

2.4 CONCLUSION

Our study provided evidence that NS-398 has a negative effect on the proliferation and maturation of differentiated myogenic precursor cells *in vitro*. We also found that the *in vivo* administration of NS-398 delays muscle healing by interfering with the normal inflammatory response and the maturation of regenerating myofibers, and by increasing fibrosis, possibly by up-regulating TGF-β1. By using COX-$2^{-/-}$ mice as an injury model, COX-2 pathway appears to play an important role in the healing of lacerated muscles. Prostaglandins were shown to be the key mediators that regulate the secondary fusion of myogenic precursor cells. Our results indicate that athletes or patients who are taking NSAIDs to treat acute or chronic pain due to muscle injuries might have an increased risk of reduced functional healing or prolonged rehabilitation. In light of the results from our study and other reports,[58, 62] the use of COX-2–specific inhibitors to treat skeletal muscle injuries should be used with caution. When confronted with cases that require the clinical use of such inhibitors to treat skeletal muscle injuries, clinicians probably should consider a reduced dosage or a shorter duration of administration.

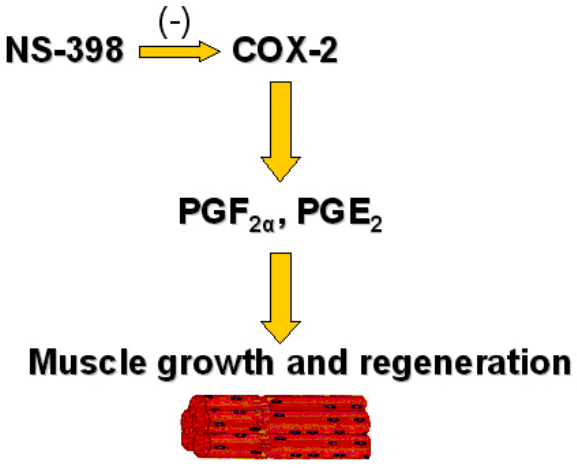


Figure 2-12. COX-2 Pathway and Muscle Regeneration

COX-2 pathway is an integral component of inflammation phase and can be blocked by the use of NSAIDs like NS-398. The products of COX-2 pathway, PGE_2 and $PGF_{2\alpha}$, are important for the regeneration of myofibers after injury.

2.5 MATERIAL AND METHODS

2.5.1 Cell Isolation and Culturing

Myogenic precursor cells were isolated via a previously described preplate technique.[47, 48] Muscles were removed from 4-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and COX-$2^{-/-}$ mice (Taconic Farm, Germantown, NY). The muscles were minced with a surgical blade and were enzymatically digested by sequential exposure to collagenase type XI, dispase, and trypsin. The muscle cell extracts then were plated on collagen-coated flasks. Different populations were isolated by re-plating the extracts after different time intervals. The late preplate (LP) population contains cells with higher myogenic potential than cells in the earlier preplate populations.[48] LP cells were used for our *in vitro* experiments and were maintained in proliferation medium (PM: Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS], 10% horse serum [HS], and 0.5% chicken embryo extract).

The myogenic precursor cells used for the cell proliferation experiments were plated at the same cell density per well in 12-well plates. On day 0, cells were grown overnight in serum-free medium to synchronize the cells by starvation. On day 1, the serum-free medium was replaced with PM. Different concentrations of NS-398 (0, 1, 10, or 100 μM; Cayman Chemical, Ann Arbor, MI), selected on the basis of previous studies involving NS-398,[58, 77, 78] were dissolved in culture media (with dimethyl sulfoxice [DMSO; 10mg/ml] as the stock solution) to examine the effect of NS-398 on cell proliferation. The PM and NS-398 were replenished on day 3. After cells were trypsinized and collected on days 1 to 4, a hemocytometer was used to count the cells manually. Cells in 3 wells/NS-398 concentration were counted each day.

The myogenic precursor cells used for the cell differentiation experiments were plated at the same cell density per well in 6-well plates. On day 0, cells were grown overnight in serum-free medium to synchronize the cells by starvation. The following day, the serum-free medium was removed and replaced with differentiation medium (DM: DMEM supplemented with 1% FBS and 1% HS) supplemented with different concentrations of NS-398. As described above, the cells were permitted to grow for an additional 2 days. At that time, the cells and supernatants were collected for western blot analysis and ELISA.

To observe the differentiation of LP cells isolated from COX-$2^{-/-}$ mice and wild-type control mice, LP cells from each source were plated at the same cell density (20 000 cells per well) in 12-well plates. On day 0, cells were grown overnight in serum-free medium to synchronize the cells by starvation. On day 1, the serum-free medium was replaced with differentiation medium (Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum and 1% horse serum). The LP cells then were cultured until day 18, with replacement of differentiation medium every 3 days. The culture medium was collected for analysis by enzyme-linked immunosorbent assay (ELISA). To observe the effect of PGE_2 and $PGF_{2\alpha}$ on cell fusion, LP cells from COX-$2^{-/-}$ mice were starved on day 0 for synchronization (as detailed above). On day 1, the serum-free medium was replaced with differentiation medium containing different concentrations of PGE_2 and $PGF_{2\alpha}$ (100 ng/ml, 1000 ng/ml, 10 000 ng/ml; Sigma, St. Louis, MO). The differentiation medium containing PGE_2 and $PGF_{2\alpha}$ was replaced every 3 days during the 18-day culture period.

2.5.2 ELISA Assay

Supernatants collected from the differentiation experiments were analyzed by ELISA. Analysis of PGE_2 and $PGF_{2\alpha}$ was performed as suggested in the instructions provided by the manufacturer (DE0100 PGE_2 ELISA kit, DE1150 $PGF_{2\alpha}$ ELISA kit, R & D Systems, Minneapolis, MN). Briefly, antibodies against PGE_2 or $PGF_{2\alpha}$ and PGE_2 or $PGF_{2\alpha}$ conjugates were sequentially applied and incubated for certain length of time. After adding the substrate and stop solution sequentially, the optical density of each well was determined immediately using a microplate reader.

2.5.3 Animal Model

In the NS-398 experiments, the gastrocnemius muscles (GMs) of 68 mice (C57BL/6J, female, 5 weeks of age, Jackson Laboratories) were lacerated in accordance with the injury model detailed below. The Animal Research and Care Committee at the authors' institution approved all experimental protocols (Protocol 5/01). The muscle injury model, developed in mice and used in prior studies,[79, 80] entailed laceration of the GMs in both legs. The mice were anesthetized by intramuscular injection of 0.03 ml ketamine (100 mg/ml) and 0.02 ml xylazine (20 mg/ml). A surgical blade (#11 SteriSharps, Mansfield, MA) was used to lacerate each GM at 60% of its length from its distal insertion through the lateral 50% of muscle width and 100% of muscle thickness. After laceration, the skin was closed with black silk 4-0 suture (Ethicon, Somerville, NJ).

NS-398 (Cayman Chemical) was dissolved in DMSO and injected intraperitoneally into the mice, which received 1 of 2 doses (5 mg/kg of body weight or 10 mg/kg of body weight) for

1 of 2 durations (3 days or 5 days continuously) immediately after surgery. The same amount of DMSO (no NS-398) was injected intraperitoneally into other mice that served as the negative control. Thus, the mice used in the *in vivo* study received 1 of 4 treatment regimens: 5mg NS-398 per day for 3 days (5mg-3day group), 5mg NS-398 per day for 5 days (5mg-5day group), 10mg NS-398 per day for 3 days (10mg-3day group), or only DMSO (control group). The mice were sacrificed at different time points after injury (3, 5, 7, 10, 14, or 28 days), and the GMs were harvested from both legs, flash-frozen in 2-methylbutane pre-cooled in liquid nitrogen, and stored at –80°C pending histological analysis.

In the experiments using COX-$2^{-/-}$ mice, fourteen COX-$2^{-/-}$ mice and 14 wild-type mice (10–12 weeks of age, Taconic Farms, Germantown, NY) were used. The skeletal muscle laceration model used in this study entailed laceration of the TA muscle on both legs, as described previously. [79, 80]

2.5.4 Hematoxylin and Eosin (H & E) Staining

Cryosections were fixed in 1% glutaraldehyde for 1 minute, and then were dipped in hematoxylin for 30 seconds. After being washed with alcohol acid and ammonia water, they were immersed in eosin for 15 seconds. After each step, sections were rinsed with distilled water. The sections then were dehydrated by treatment with alcohols of increasing concentrations (70%, 80%, 95%, and 100%). Finally, the sections were treated with xylene and covered with glass slips.

Slides were analyzed manually via bright field microscopy (NIKON Eclipse E800, Nikon, Tokyo, Japan) and by using Northern Eclipse software. (Empix Imaging, Cheektowaga, NY). Sections containing the largest injury area were analyzed. The centronucleated regenerating

myofibers in those sections were counted under 100X magnification (4 animals/group), and results were recorded as the number of centronucleated myofibers/total number of myofibers in each section. In addition, an image of the central injury area was taken at 200X magnification, and the minor axis diameters (i.e., the smallest diameter) of 200 centronucleated myofibers in each of the images were measured.

2.5.5 Immunohistochemistry

Standard techniques were used to prepare serial 8-μm cryostat sections and cell cultures. For immunohistochemistry, the following primary antibodies were used at the indicated dilutions: monoclonal mouse anti-myosin heavy chain-developmental (MHC-d) (Novocastra Laboratories, Ltd., Newcastle, UK; 1:100), rabbit anti-mouse collagen IV antibody (Biodesign, Saco, ME; 1:200), rat anti-mouse TGF-β1 (BD Biosciences Pharmingen, San Diego, CA; 1:100), polyclonal rabbit anti-desmin (Sigma, D8281; 1:200), monoclonal anti-mouse Pax7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; 1:200), monoclonal rat anti-F4/80 (Abcam, Cambridge, MA; 1:200), rat anti-CD-11b (Chemicon, Temecula, CA; 1:150), and monoclonal anti-α-smooth muscle actin (FITC-conjugated, Sigma, St. Louis, MO; 1:150). The sections and cell cultures then were exposed to the following secondary antibodies for 50 minutes at room temperature: anti-mouse-conjugated Cy3 (Sigma; 1:250) and anti-rabbit-conjugated fluorescein (Sigma; 1:100). Collagen type IV was colocalized with TGF-β1. Negative controls (stainings without the primary antibody) were performed concurrently with all immunohistochemical staining. The nuclei of the sections were revealed via 4,6-diamidino-2-phenylindole staining (DAPI, Sigma). Fluorescent microscopy was used to visualize all immunofluorescence results (Nikon E800). For the in vivo staining, the area of TGF-β1 expression (red after

immunostaining) was measured with Northern Eclipse software (200X magnification; slides from 4 animals/group; Empix Imaging). The percentage of CD-11b–positive cells within the TA muscle sections was determined. For the cell culture staining, the number of nuclei within each myotube and the total number of nuclei in each field were counted under 200x magnification. The percentage of myotubes containing more than 3 nuclei was determined.

2.5.6 Western Blot Analysis

Laemmli sample buffer (BioRad, 161-0737) was used to collect proteins from live cells. After being prepared via standard procedures, protein samples were separated on 10% SDS-polyacrylamide electrophoresis gel and were transferred to nitrocellulose membranes that then were used to perform immunoblotting. Mouse anti-MyoD (Pharmingen 554130; 1:250), anti-myogenin (Pharmingen 556358; 1:250), and anti-MHC-d (Novocastra; 1:500) were applied as primary antibodies, and mouse anti-β-actin (Sigma; 1:8000) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) were diluted to 1:5000 and applied. Blots were developed by using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on X-ray film. All results were analyzed with Northern Eclipse software (Empix Imaging).

2.5.7 Trichrome Staining

Trichrome staining was performed to determine the collagen content of the muscle tissue. Slides were processed as detailed in the manufacturer's protocol (Masson Trichrome stain kit, K7228;

IMEB, Inc., Chicago, IL). Northern Eclipse software (Empix Imaging) was used to measure the area of fibrous tissue (slides from 3 samples/group; 100X magnification).

2.5.8 Flow Cytometry Analysis

The GMs from non-treated (control) and NS-398–treated groups (10mg-3day group) were surgically removed before injury or 12 hours, 24 hours, 48 hours, 3 days, or 5 days after injury. Collagenase, dispase, and trypsin were used to digest the tissue matrix and isolate the cells. Debris was removed via filtration with 100-μm filters.

Isolated cells first were treated with 10% mouse serum (Sigma) to block nonspecific binding sites. Primary rat anti-CD-11b (conjugated with FITC, R & D Systems, Minneapolis, MN) and rat anti-F4/80 (conjugated with APC, Serotec, Raleigh, NC) antibodies were used in combination to distinguish the neutrophil and macrophage populations: F4/80 is specific to macrophages[81] and CD-11b is expressed by macrophages and neutrophils.[72, 82] 7-amino-actinomycin D (7-AAD; Pharmingen) was added to all tubes to exclude nonviable cells from the analysis. Marked cell samples then were analyzed with a FACS Caliber flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

2.5.9 Physiologic Testing

The physiologic testing was conducted as described previously. [83] Briefly, the mice were euthanized and the TA muscles were isolated. Each TA muscle was mounted in a vertical tissue chamber that was constantly perfused with mammalian Ringer's solution aerated with 95% O_2–5% CO_2 and maintained at 25 °C. The TA muscle was secured at both ends and was connected

by fine wire to a force transducer and length servo system (model 305B, dual mode, Aurora Scientific, Aurora, ON, Canada). The muscle was then stimulated (Grass model S-88 stimulator and current amplifier) by monophasic rectangular pulses of cathodal current to obtain the specific peak force (N/cm^2) and specific tetanic force (N/cm^2). Fourteen days after laceration injury, TA muscles from the wild-type mice and the COX-$2^{-/-}$ mice were tested in this manner. The non-injured wild-type mice and COX-$2^{-/-}$ mice also were used to generate baseline values. The values of injured muscles were divided by the baseline values to calculate the percentages of functional recovery.

2.5.10 Statistics

Comparisons between 2 groups were made by unpaired Student's *t* test. A *chi*-square test was used to analyze the percentage differences in the numbers of neutrophils and macrophages identified via flow cytometry. All other data were analyzed by one-way ANOVA statistical analysis. Post hoc multiple comparison tests were performed to determine which means differ. Error bars on figures represent the standard deviation. $P < 0.05$ was considered statistically significant.

3.0 THE ROLE OF MACROPHAGE IN SKELETAL MUSCLE INFLAMMATION

3.1 INTRODUCTION

It has been indicated that macrophages play an important role in the healing process of skeletal muscle injury. By phagocytosing damaged tissue, macrophages may help to remove the debris that can impede muscle regeneration. [84] Besides phagocytosis, macrophage may promote muscle healing by other mechanisms, including stimulating the proliferation of activated satellite cells by releasing growth factors and cytokines [21-24] and rescuing satellite cells from apoptosis by direct contacts. [22] A recent study suggested that macrophages may also fuse with myofibers directly to promote regeneration of injured muscle.[25] Our in vivo results in Objective #1 showed that the infiltration of macrophage into injured muscle was reduced in NSAIDs-treated (NS-398) mice, and these mice had inferior muscle healing than the non-treated control mice. These results all suggested that macrophage is important and probably beneficial to muscle healing. However, some results from irradiation experiments in mice are contradictory in terms of the necessity of macrophages in muscle regeneration *in vivo*. [85] Therefore in this study, we decided to determine the role of macrophages in skeletal muscle healing by using clodronate liposome, an agent that has been proved to be able to deplete macrophage populations in experimental animals. [86] [87] [88] In addition, we examined the effect of macrophages on TGF-β1 and PGE_2 expression both in vivo and in vitro.

3.2 RESULTS

3.2.1 Macrophages Can Be Depleted by Clodronate Liposome Injection

In Objective #1, we have observed that the infiltration of macrophage into muscle tissue could be triggered by an injury and macrophages dominated the following inflammation phase. We used flow cytometry to quantify the number of infiltrating macrophages on 1d, 2d, 3d, and 5d after injury. The infiltration peaked on 2 days post-injury, and decreased quickly on 5 days post-injury. In this objective, liposome clodronate was injected intraperitoneally 2 days prior to the muscle injury to deplete the macrophage populations in the animal. It significantly reduced the number of infiltrating macrophages after muscle injury at all the time points observed. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2% respectively ($P<0.05$, **Figs. 3-1 A**). On the flow cytometry read-out graph, the macrophage population, which is shown in the S2 quadrant in non-treated mice, was depleted in the clodronate liposome-treated mice. (**Figs. 3-1 B**)

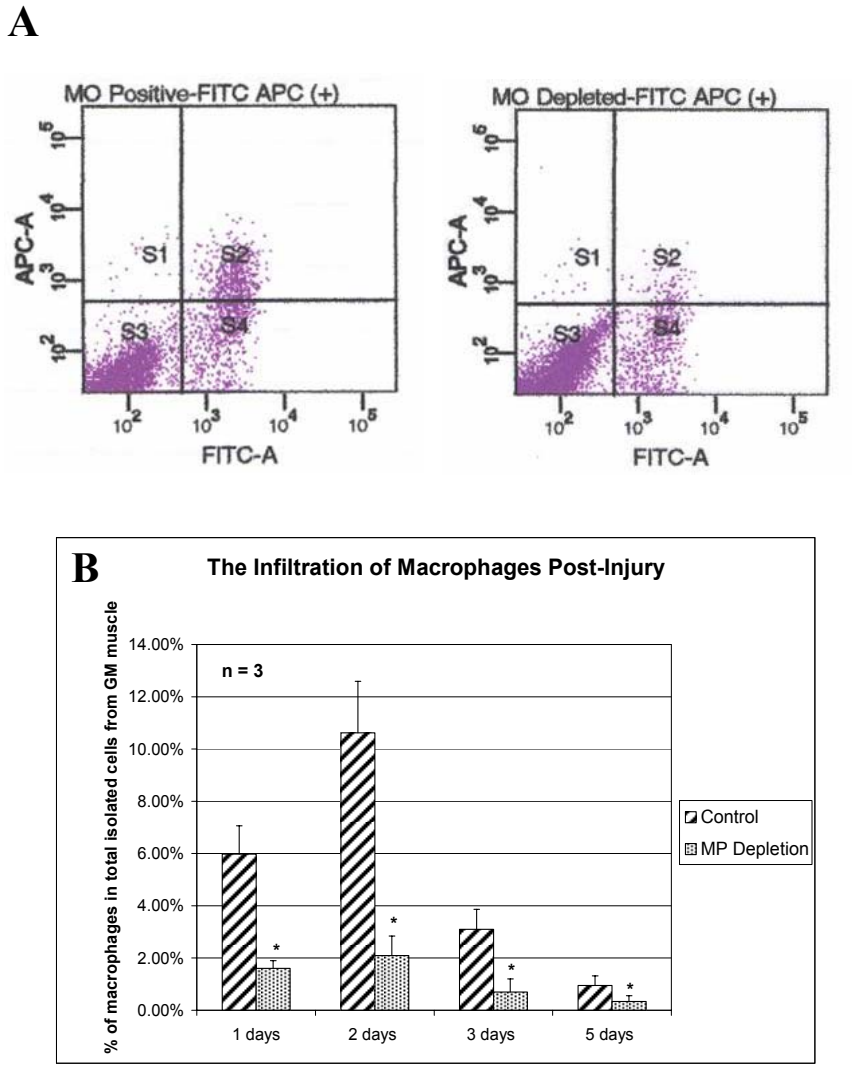


Figure 3-1. Clodronate Liposome Depleted Macrophages in Injured Muscle

Infiltration of macrophages was observed after the injury to gastrocnemius muscle (GM). Injection of clodronate liposome significantly reduced macrophages in injured GM at all time points observed. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2% respectively ($P<0.05$, **B**) The asterisks indicate a significant difference ($P < 0.05$) between the marked groups and the control group. A sample flow cytometry results showed that the macrophage population, which was shown in S2 quadrant in non-treated mice at 48h after injury (**A left**), was depleted in clodronate liposome-treated mice (**A right**). X axis represents the CD-11b cell surface marker; Y axis represents the F4/80 cell surface marker.

3.2.2 The Effect of Macrophage Depletion on Skeletal Muscle Healing

Using the liposome clodronate technique, we were able to evaluate the importance of macrophages in skeletal muscle healing. With the percentage of infiltrating macrophages significantly reduced, liposome clodronate-treated mice exhibit reduced muscle regeneration at both 14 days and 28 days after injury. The size of regenerating myofibers in liposome clodronate-treated mice was significantly smaller than those observed in the non-treated group. (P<0.05) However, at 7 days post-injury, there was no significant difference between these two groups. (**Fig. 3-2**) These findings suggested that macrophages play at least some beneficial roles in skeletal muscle healing.

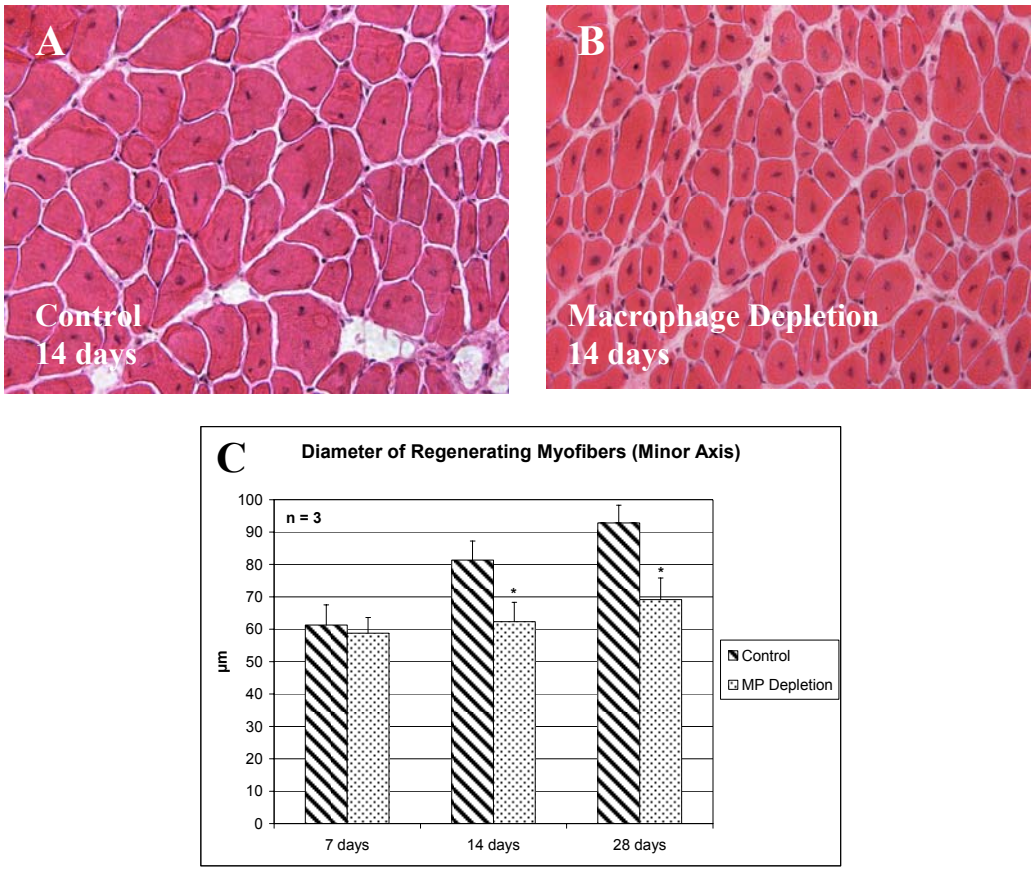


Figure 3-2. Macrophage Depletion Reduced the Size of Regenerating Myofibers

Compared with non-treated mice, liposome clodronate-treated mice exhibit reduced muscle regeneration. At 14 days and 28days post-injury, the size of the regenerating myofibers was significantly smaller in the macrophage-depletion group. (**C,** P<0.05) The asterisks indicate a significant difference (P < 0.05) between the marked groups and the control group. These findings suggest that macrophages might contribute to the growth of regenerating myofibers. Sample pictures from non-treated and macrophage-depletion groups at 14 days post-injury were shown. (**A & B**)

3.2.3 Macrophages Can Increase the Expression of TGF-β1 and PGE_2 of Muscle Cells in Vitro

To examine the interaction between macrophages and muscle cells, macrophages were isolated and co-cultured with different muscle cell types, including satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line). We found that the supernatants collected from the co-cultured cells contained significantly more TGF-β1 and PGE_2 than those from macrophages culture or muscle cells cultures alone. (P<0.05) The expression level was higher than simply the addition of macrophages expression and muscle cells expression. It suggested that macrophages may stimulate and enhance the expression of TGF-β1 and PGE_2 in the major cell types of skeletal muscle tissue, including fibroblasts, myoblasts, and satellite cells (**Figs. 3-3**). In addition, the expression level of TGF-β1 and PGE_2 varied between different muscle cell types, which indicated that different muscle cells have their unique roles in muscle healing at least by releasing different growth factors and cytokines.

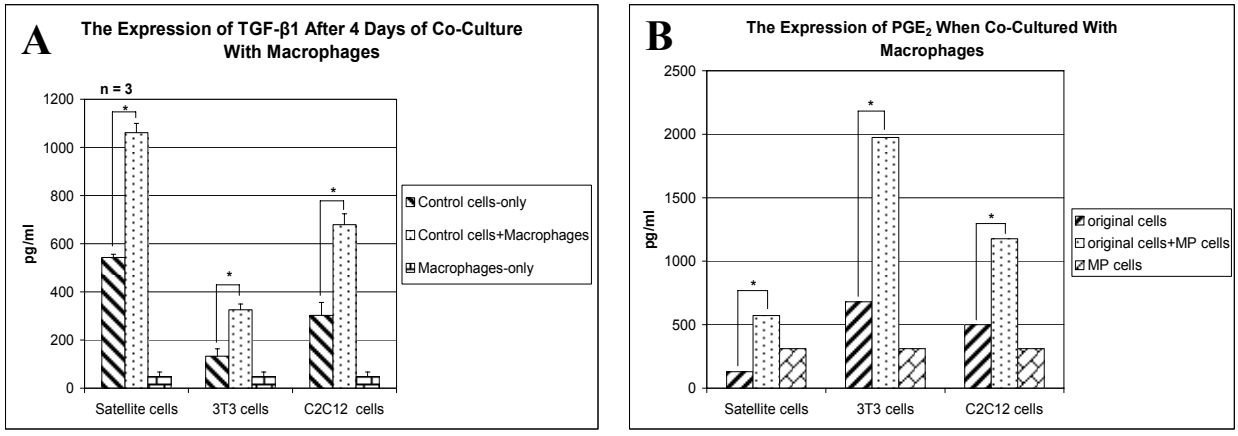
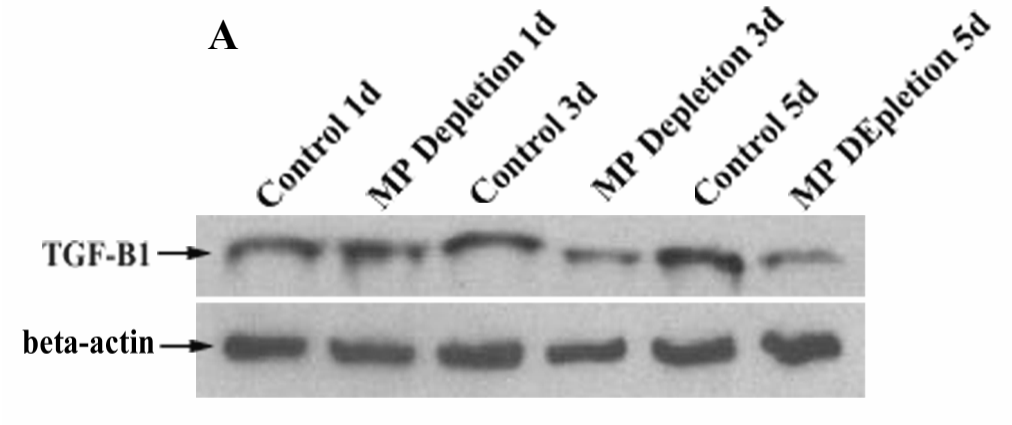


Figure 3-3. Macrophage Increased the Expression of Growth Factors from Muscle Cells

Co-culturing with macrophages significantly increased the expression of TGF-β1 and PGE_2 from major muscle cell types, including fibroblasts, myoblasts, and satellite cells. However, the expression level of TGF-β1 and PGE_2, varied among the different cell types. The asterisks indicate a significant difference (P < 0.05) between the compared groups.

3.2.4 Macrophage Depletion Decreased TGF-β1 Expression in Vivo

To verify the role of macrophage in TGF-β1 expression we found in vitro, injured gastrocnemius muscles were isolated from non-treated and clodronate liposome-treated mice. We examined the TGF-β1 expression level in injured muscle tissue by using western blot. We found that compared to the non-treated group the TGF-β1 expression was significantly decreased 3 days and 5 days after injury in the clodronate liposome-treated group, the group in which macrophage was depleted before the creation of muscle injury. (**Fig. 3-4**) The results from both in vivo and in vitro studies (See **3.2.3**) suggested that macrophage may be an important part in inducing the expression of TGF-β1 during the inflammation phase of skeletal muscle injury.



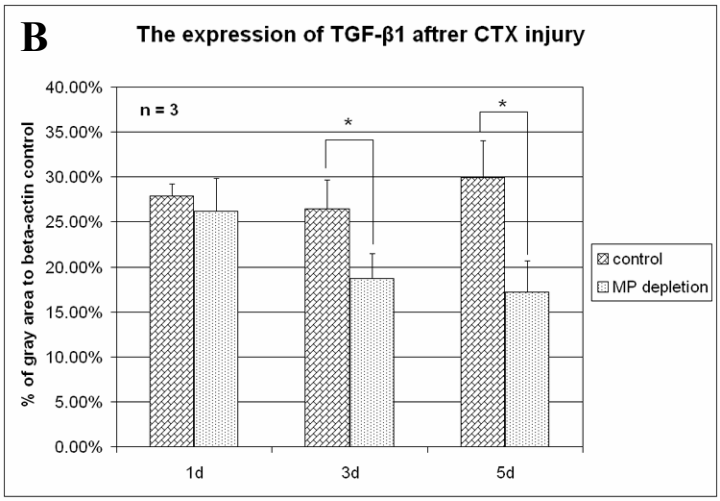


Figure 3-4. The Effect of Macrophage on TGF-β1 Expression in Vivo

The expression level of TGF-β1 was significantly lower in the macrophage depletion group compared to the non-treated group 3 days and 5 days after injury. (**A**, **B**, $P<0.05$) The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. It was assessed by western blot technique, and a sample result of the assessment was shown in panel **A**. β-actin was used as a quantity control for the total amount of proteins.

3.2.5 TGF-β1 Increases the Infiltration of Macrophages

To examine the effect of TGF-β1 on macrophage infiltration after skeletal muscle injury, we compared the number of infiltrating macrophages after the injection of cardiotoxin and the injection of cardiotoxin+TGF-β1 by using flow cytometry. We found that on both 1 day and 3 days after injury, the addition of TGF-β1 significantly increased the infiltration of macrophage. This suggested that TGF-β1 may interfere with the inflammation phase of muscle healing by increasing the number of infiltrating macrophages.

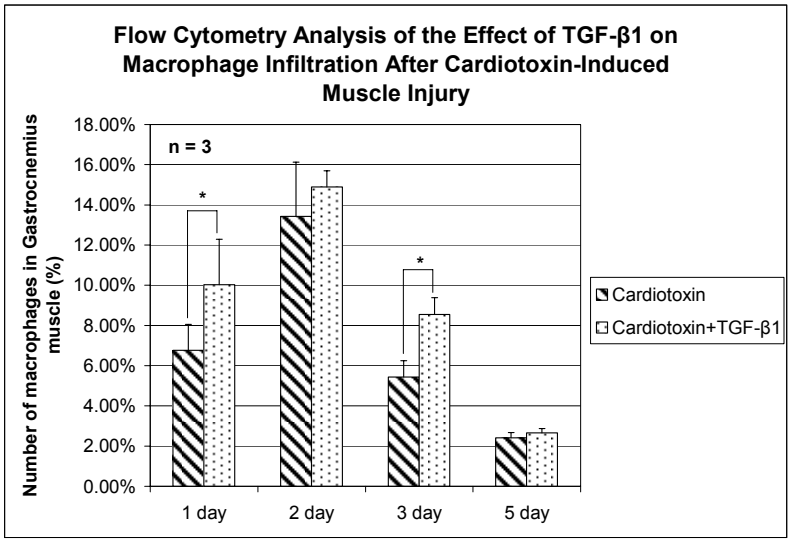


Figure 3-5. TGF-β1 Increased Macrophage Infiltration

Flow cytometry results showed that cardiotoxin injection can create injury in skeletal muscle and induce the infiltration of macrophages. The addition of TGF-β1 significantly increased the number of infiltrating macrophages on both 1 day and 3 days after CTX-induced muscle injury. (P<0.05) The asterisks indicate a significant difference (P < 0.05) between the compared groups.

3.3 DISCUSSION

3.3.1 Macrophages Are Beneficial for Muscle Regeneration

Using whole body and local irradiation to ablate local and emigrate macrophages, a previous study reported that both replication and fusion of muscle precursors could occur in the absence of infiltrating macrophages. [85] However, more evidence suggested that macrophages were important for muscle regeneration. The Cantini group [89] showed that macrophage mediated medium significantly enhanced the regenerative processes in damaged muscle. Chazaud and et.al. [22] demonstrated that macrophages not only stimulate satellite cell proliferation by soluble factors, but rescue satellite cells from apoptosis by direct contacts. They found that macrophages had a much stronger chemotaxis effect after they interplay with satellite cell, and they are responsible for the amplification of monocyte recruitment. Furthermore, macrophages may promote the growth of regenerating myofibers by fusing with myofibers directly.[25] To emphasize the importance of macrophages in skeletal muscle healing, especially the inflammation process, we used the clodronate liposome injection technique to deplete the macrophage population in mice. With the injection of clodronate liposome, most of the macrophages were killed and very few of them infiltrated into muscle tissue during the inflammation phase. We found that although regeneration of injured muscle did occur, the macrophage depletion group had significantly smaller myofibers than the non-treated group. It suggests that macrophages participate in the healing process and play some role in the growth of regenerating myofibers.

3.3.2 Macrophage Increased the Expression of Growth Factors and Cytokines

Macrophages have been shown to contribute to both the regulation of proliferation and differentiation activity of satellite cells by secreting growth factors and cytokines. [21-24] Cantini and his colleagues [21] [90] showed that macrophage conditioned media can increase the number of differentiated myotubes from satellite cells, suggesting that the increased satellite cell proliferation and differentiation is mediated by soluble factors released by macrophages. They also showed [91] that the factors secreted by macrophages exerts its effects not only on specialized satellite cells during muscle regeneration, but also has a broader mitotic activity on all myogenic cells. In this study, we found that when macrophages were co-cultured with other muscle cells, they can significantly increase the expression of TGF-β1 and PGE_2 of these cells. This finding suggested that macrophages may not only express growth factors to help the regeneration of injured muscle, but may also stimulate other muscle cells to enhance their expression of growth factors. This effect may help to amplify the expression of growth factors and lead to an expedited regeneration of injured muscle tissue. It may also explain why the macrophage depleted mice had smaller regenerating myofibers.

In our in vivo experiments, we found that macrophage depleted mice had significantly lower TGF-β1 level in their injured GM muscle compared to the GM muscle from non-treated control mice. This result further supports the notion that macrophages are important for the regeneration of injured muscle, and enhancing the production of growth factors and cytokines may be part of the mechanism.

3.3.3 TGF-β1 Increased the Infiltration of Macrophages

It has been shown that TGF-β1 interferes with the inflammation phase via various ways. [33, 40-43] Mostly, TGF-β1 was thought to inhibit inflammation because TGF-β1 was a negative regulator of NF-κB activation. Smad7 maintains high NF-κB activity in inflammation by blocking TGF-β1 signaling. [33] [40] [41] To find out if TGF-β1 modulates inflammation by other means, for example macrophage infiltration, we injected TGF-β1 with cardiotoxin. The addition of TGF-β1 in cardiotoxin significantly increased the infiltration of macrophages. This suggested that TGF-β1 may interfere with the inflammation phase of muscle healing by increasing the number of macrophages. In addition, based on the previous finding that macrophages increased the expression of TGF-β1, (See **3.2.3**, **3.2.4**) macrophage and TGF-β1 may form a positive feedback to further enhance the number of macrophages in the injured muscle. (**Fig. 3-6**) In the future, it would be interesting to see whether TGF-β1 also affects NF-κB activation in muscle cells and explore what is the dominant role of macrophages in skeletal muscle inflammation.

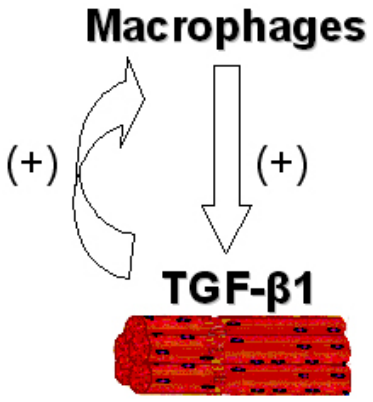


Figure 3-6. The Relation between Macrophage and TGF-β1

Macrophage and TGF-β1 formed a positive feedback in muscle healing. Macrophages increase the expression of TGF-β1 in cultured muscle cells and injured muscle tissue; TGF-β1 increases the infiltration of more macrophages.

3.4 CONCLUSION

We showed that the infiltration of macrophages mainly occurred during the first 5 days after muscle injury. Our study provided evidence that macrophage depletion during this period may be detrimental to the growth of regenerating myofibers. We also found that macrophage may enhance the expression of growth factors both in vitro and in vivo. In addition, the infiltration of macrophage was shown to be increased by TGF-β1. Our results indicate that macrophages may be actively involved in the skeletal muscle healing process. Macrophages play at least some role in muscle healing by increasing the levels of growth factors that are important to muscle healing. On the other hand, these growth factors, like TGF-β1, may modulate the muscle healing process by modulating further infiltration of more macrophage. Based on these results, we postulate that macrophages, as an integral part of inflammation phase, may be beneficial for muscle healing. The use of NSAIDs, which was shown to decrease the infiltration of inflammatory cells in Objective #1, may harm muscle healing through this mechanism. In light of these results, we again postulate that the use of COX-2–specific inhibitors to treat skeletal muscle injuries warrants caution.

3.5 MATERIAL AND METHODS

3.5.1 Cell Isolation and Culturing

Myogenic precursor cells (MPC) were isolated via a previously described preplate technique.[47, 48] Gastrocnemius muscles were removed from 4-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and minced with scissors. The meshes were enzymatically digested by sequential exposure to collagenase, dispase, and trypsin. The muscle cell extracts then were plated on collagen-coated flasks and different populations were isolated by re-plating the extracts after different time intervals. The late plated population (LP cells) is made up of myogenic precursor cells that have high myogenic potential when induced by low serum culture medium.[48] LP cells were used for our *in vitro* experiments along with two other cell lines, NIH 3T3 and C2C12, which are fibroblast cell line and myoblast cell line respectively. These three types of cells were maintained in proliferation medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS], 10% horse serum [HS], and 0.5% chicken embryo extract).

3.5.2 ELISA Assay

A low serum–containing medium (DMEM supplemented with 1% FBS and 1% HS) was used to culture cells in the experiments of growth factor and cytokine expression. The mediums from the macrophage co-culture experiment were collected and kept in -80°C pending ELISA assay. The assay was performed as suggested by the manufacturer's protocols (DE0100 PGE_2 ELISA kit, MB100 TGF-β1 ELISA kit, R & D Systems, Minneapolis, MN) Briefly, antibodies against PGE_2 or TGF-β1 and PGE_2 or TGF-β1 conjugates were sequentially applied and incubated for certain

time. After adding the substrate and stop solution sequentially, the optical density of each well was determined immediately using a microplate reader.

3.5.3 Animal Model

Twenty four C57BL/6J mice (male, 6 weeks of age, Jackson Laboratories, Bar Harbor, Maine), 3 COX-2 knock-out mice (male, 6weeks of age, Taconic farms**,** Hudson, NY) and their wild-type control (male, 6weeks of age, Taconic farms) were used for in vivo experiments. The gastrocnemius muscles (GMs) of the mice were injected with cardiotoxin (c3987, Sigma**,** St. Louis, MO). The Animal Research and Care Committee at the authors' institution approved all experimental protocols (Protocol 29/04). Briefly, the mice were anesthetized by intramuscular injection of 0.03 ml ketamine (100 mg/ml) and 0.02 ml xylazine (20 mg/ml). Ten μl of diluted cardiotoxin (50μg/ml) was injected in the middle mass of each gastrocnemius muscle. The mice were sacrificed at different time points after injection (1, 3, 5, or 14 days).The GMs were harvested from both legs for either flow cytometry experiment or histological analysis. For the later purpose, the GMs were fresh-frozen in 2-methylbutane precooled by liquid nitrogen, and stored at -80°C pending cryosection.

3.5.4 Hematoxylin and Eosin (H & E) Staining

Cryosections were fixed in 1% glutaraldehyde for 1 minute, and then were dipped in hematoxylin for 30 seconds. After being washed with alcohol acid and ammonia water, they were immersed in eosin for 15 seconds. After each step, sections were rinsed with distilled water. The sections then were dehydrated by treatment with alcohols of increasing concentrations (70%,

80%, 95%, and 100%). Finally, the sections were treated with xylene and covered with glass slips.

Slides were analyzed manually via bright field microscopy (NIKON Eclipse E800, Nikon, Tokyo, Japan) and by using Northern Eclipse software. (Empix Imaging, Cheektowaga, NY). An image of the central injury area was taken at 200X magnification, and the minor axis diameters (i.e., the smallest diameter) of 200 centronucleated myofibers in each of the images were measured.

3.5.5 Macrophage Depletion by Clodronate Liposome

Clodronate liposomes were prepared as described previously [86]. The resultant liposomes act as carriers for clodronate that is toxic to cells. When injected intraperitoneally, the liposome is phagocytosed by macrophages; and the clodronate is released from liposome and kills the host macrophages. Two days before muscle injury, 1 mg of clodronate liposomes (20 mg/ml concentration) was injected intraperitoneally into C57/BL 10J mice to deplete macrophages. Mice injected with liposomes which do not contain clodronate were used as controls. Flow cytometry using macrophage marker antibodies (F4/80 and CD-11b) was used to verify the effectiveness of macrophage depletion.

3.5.6 Flow Cytometry

The GMs from non-TGF-β1-treated (10μl cardiotoxin injection) and TGF-β1–treated groups (9μl cardiotoxin plus 1μl of 5ng/ml TGF-β1 injection) were surgically removed before injury and at 1 day, 3 days, and 5 days after injury for serial evaluation. Collagenase, dispase, and trypsin were

used to digest the tissue matrix and isolate the cells. Debris was removed via filtration with 70-μm filters.

Cells were treated with 10% mouse serum (Sigma) to block nonspecific binding sites. Primary rat anti-CD-11b (conjugated with FITC, R & D Systems, Inc., Minneapolis, MN) and rat anti-F4/80 (conjugated with APC, BD Biosciences, Franklin Lakes, NJ) antibodies were used as a combination to identify neutrophil and macrophage populations. 7-amino-actinomycin D (7-AAD; Pharmingen) was added to exclude nonviable cells from the analysis. Samples then were analyzed with a FACS Caliber flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

3.5.7 Western Blot Analysis

Muscle samples were sliced into 10μm-thick pieces by cryosection and were collected in eppendorf tubes. T-PER tissue protein extraction agent (78510, Pierce) was added to digest the tissue and extract the protein component from the sample. After centrifuging at 10000 rpm for 10 minutes, the supernatant was isolated from the pellet and mixed with equal volume of Laemmli sample buffer (161-0737, BioRad). After being boiled for 5min, samples were centrifuged at 3500rpm for 5min and stored in -20°C pending western blot assay.

Samples were separated on 10% SDS-polyacrylamide electrophoresis gel and were transferred to nitrocellulose membranes that then were used to perform immunoblotting. Rat anti-TGF-β1 (555052, Pharmingen, San Diego, CA) was applied as primary antibody, and mouse anti-β-actin (Sigma; 1:8000) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) were diluted to 1:5000 and applied. Blots were developed by using SuperSignal West Pico Chemiluminescent substrate (Pierce), and

positive bands were visualized on X-ray film. All results were analyzed with Northern Eclipse software (Empix Imaging).

3.5.8 Statistics

A *chi*-square test was used to analyze the percentage differences in the number of macrophages identified via flow cytometry. Student's *t* test was used otherwise in Objective #2. Error bars on figures represent the standard deviation. $P < 0.05$ was considered statistically significant.

4.0 THE ROLE OF TGF-B1 IN SKELETAL MUSCLE INFLAMMATION

4.1 INTRODUCTION

TGF-β1 has been shown to have anti-inflammatory effect in many tissues.[40-43] Recently, it was shown that TGF-β1 is a negative regulator of NF-κB, a transcription factor that can be activated by many proinflammatory cytokines. [33] However, in our Objective # 2, we found that TGF-β1 can increase the infiltration of macrophages with cardiotoxin injury. Thus, it indicated that TGF-β1 may interfere with the inflammation phase via various ways. Increasing the infiltration of macrophages may be one of the mechanisms by which TGF-β1 affects the inflammatory response, and subsequently the whole healing process in injured skeletal muscle.

It has been reported that the production of PGE_2 can be up-regulated by TGF-β1 via the COX-2 pathway in lung fibroblasts.[19, 37] PGE_2 can oppose the profibrotic effect of TGF-β1 by inhibiting fibroblast proliferation[37] and collagen synthesis.[38, 39] It is postulated that the homeostasis of extracellular matrix may be maintained by this mechanism.[19] However, the above mechanism was not testified in muscle cells. It is also unclear whether PGE_2 has a direct inhibitory effect on the expression of TGF-β1. In our Objective # 1, it was shown that by blocking COX-2, and thus the expression of PGE_2 and $PGF_{2\alpha}$, the expression of TGF-β1 is increased in vivo. (See **2.2.4**) Thus, a negative feedback loop composed of TGF-β1, COX-2 and prostaglandins may exist to keep the TGF-β1 level from surging.

4.2 RESULTS

4.2.1 TGF-β1 Increased the Production of COX-2 Enzyme

To examine the effect of TGF-β1 on COX-2 enzyme production in vitro, LP cells were cultured and treated by TGF-β1 for 4 days. We tested the COX-2 enzyme level from the treated LP cells and non-treated control cells. Western blot results showed that the COX-2 production was significantly higher in the treated LP cells compared to the non-treated control cells. ($P<0.05$, **Fig. 4-1**) This result suggested that TGF-β1 may be able to increase the production of COX-2 enzyme and therefore increase the production of downstream products of the COX-2 pathway, which includes PGE_2 and $PGF_{2\alpha}$.

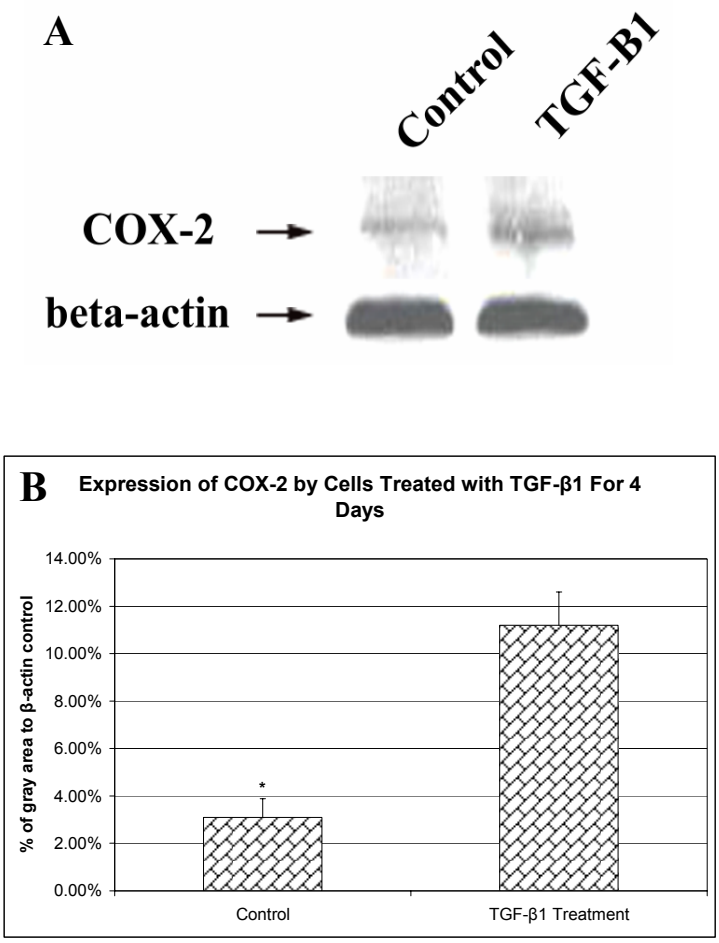


Figure 4-1. TGF-β1 Increased the Production of COX-2 Enzyme

LP cells that were treated by TGF-β1 for 4 days had significantly higher production of COX-2 enzyme than the non-treated control cells. (**A**, **B**, $P<0.05$) The asterisks indicate a significant difference ($P < 0.05$) between the marked groups and the control group. A sample western blot result was shown in panel **A**. β-actin was used as a quantity control for the total amount of proteins.

4.2.2 TGF-β1 Increased the Expression of PGE_2

To examine the effect of TGF-β1 on prostaglandin expression, we tested the expression of PGE_2 from different muscle cells, including satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line), after they were treated by TGF-β1 for 4 days. We found that TGF-β1 treatment increased the expression of PGE_2 significantly in all cell types tested, when

compared to non-treated control cells. (P<0.05, **Fig. 4-2**) This result further supported our hypothesis that TGF-β1 may have a stimulatory effect on the COX-2 pathway, including the production of COX-2 enzyme and the expression/secretion of PGE_2, the end product of COX-2 pathway.

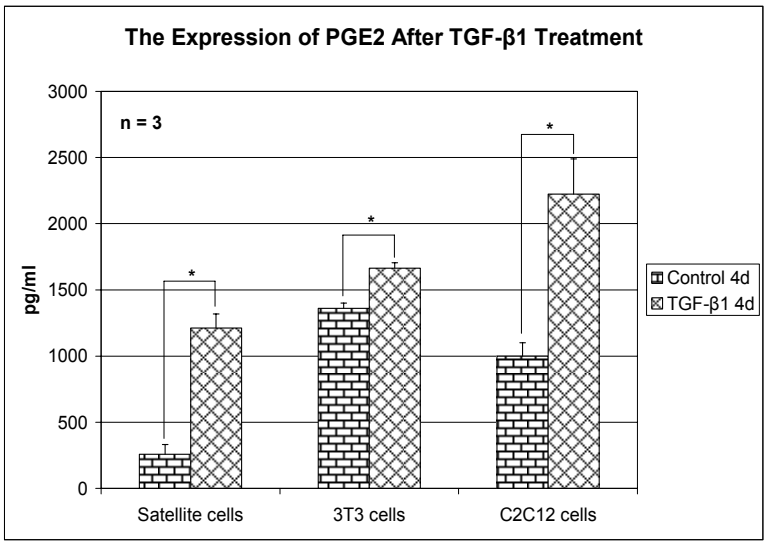


Figure 4-2. TGF-β1 Increased the Expression of PGE_2

Muscle cells, including satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line), were treated by TGF-β1 for 4 days. They all had significantly higher expression of PGE_2 compared to the non-treated control cells. (P<0.05) The asterisks indicate a significant difference (P < 0.05) between the compared groups.

4.2.3 The Relationship between TGF-β1 and the COX-2 Pathway

To further investigate the relationship of TGF-β1 and the COX-2 pathway, we used wild type LP cells as a model system. In this experiment, we first verified that TGF-β1 treatment can significantly increase the expression of PGE_2. (P<0.05) Then, NS-398, the COX-2 specific inhibitor, was added into the cell culture to block the COX-2 enzyme activity. This additional treatment ablated the increased PGE_2 expression that was induced by adding TGF-β1 into the cell culture. (P<0.05) This finding suggested that TGF-β1 may increase the expression of PGE_2 by increasing the production of COX-2 enzyme.

In the second part of the experiment, we used LP cells isolated from COX-$2^{-/-}$ mice as our model system. We were not able to increase the expression of PGE_2 by adding TGF-β1 into the COX-$2^{-/-}$ cell culture. In addition, non-treated COX-$2^{-/-}$ LP cell had a lower level of PGE_2 expression compared to the non-treated wild type LP cells. (**Fig. 4-3**) These results support the notion that TGF-β1 may be able to interfere with the inflammation phase after muscle injury by increasing the expression of PGE_2, an important inflammatory mediator. This effect is probably mediated by the increased production of COX-2 after TGF-β1 treatment.

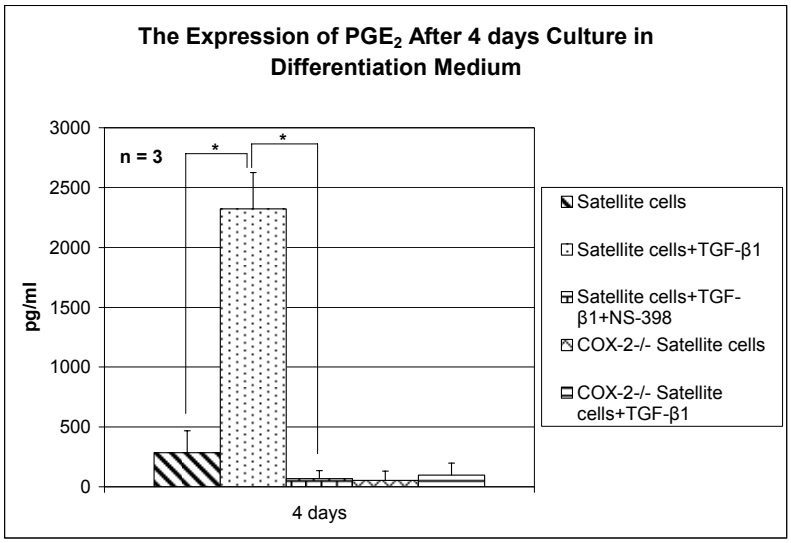


Figure 4-3. The Relationship between TGF-β1 and the COX-2 Pathway

NS-398 can ablate the increased PGE_2 expression that was induced significantly by TGF-β1. ($P<0.05$) Non-treated COX-$2^{-/-}$ LP cells expressed PGE_2 at a relatively lower level than the non-treated wild type LP cells. Even with the treatment of TGF-β1, the expression of PGE_2 can not be increased in the COX-$2^{-/-}$ LP cells. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups.

4.2.4 The Effect of TGF-β1 Small Interference RNA (TGF-β1-siRNA) on PGE_2 Expression

TGF-β1 was known to have an autocrine effect on its continuous expression. [92] [93] [94] Because the addition of TGF-β1 was shown to increase the expression of PGE_2 in our study, it was necessary to elucidate whether the effect was due to a single loading dose of TGF-β1, or it was also due to the autocrine effect and thus, a continuous stimulation of TGF-β1. We used TGF-β1-siRNA to block the production of TGF-β1 in all tested muscle cell types. At first, with the treatment of 100nM TGF-β1-siRNA and an incubation time of 4 days, we significantly reduced the continuous expression of TGF-β1 that was induced by the initial loading of TGF-β1. Then we tested the expression of PGE_2 in the same setting, and found that for satellite cells (LP cells) and fibroblasts (NIH 3T3 cell line) there was no difference in terms of PGE_2 expression with or without TGF-β1-siRNA treatment. However, this treatment reduced the expression of PGE_2 in myoblasts (C2C12 cell line) significantly. These results suggested that the initial loading of TGF-β1 may be enough to stimulate PGE_2 expression in satellite cells and fibroblasts, but myoblasts may need continuous TGF-β1 stimulation to express PGE_2.

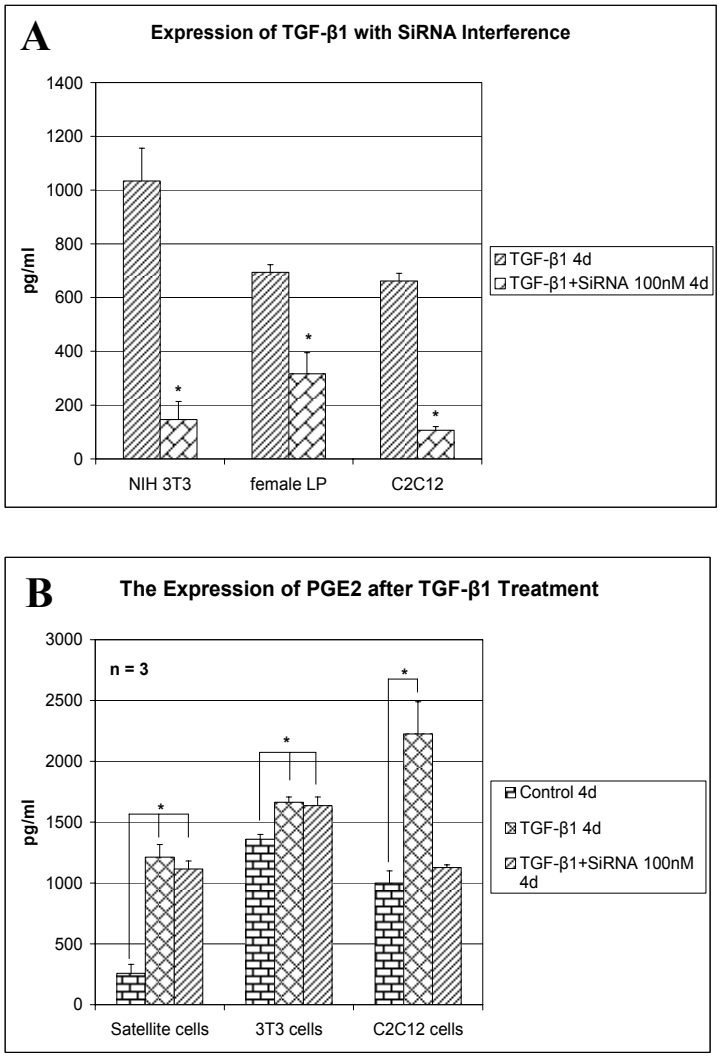


Figure 4-4. The Effect of TGF-β1 siRNA on PGE_2 Expression

The use of TGF-β1-siRNA blocked the autocrine loop of TGF-β1 expression in satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line). The expression of TGF-β1 was significantly reduced after 4 day 100nM TGF-β1-siRNA treatment. (**A**) The TGF-β1-siRNA treatment did not alter the PGE_2 expression induced by the initial loading of TGF-β1 in satellite cells and fibroblasts. However, it reduced PGE_2 expression in myoblasts. (**B**) The asterisks indicate a significant difference ($P < 0.05$) between the marked groups and the control group.

4.2.5 PGE_2 Decreased the Expression of TGF-β1

To examine the involvement of PGE_2 on fibrosis formation, we chose to examine the effect of PGE_2 treatment on the expression of TGF-β1, which was well-known for its fibrotic effect. Three different muscle cell types, including satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line), were tested for their TGF-β1 expression after they were treated with PGE_2 for 4 days. We found that PGE_2 treatment decreased the expression of TGF-β1 significantly in all cell types tested, when compared to non-treated control cells. (**Fig. 4-4**) This result suggested that PGE_2 may be able to decrease fibrosis formation after muscle injury by decreasing the expression of fibrotic growth factor TGF-β1.

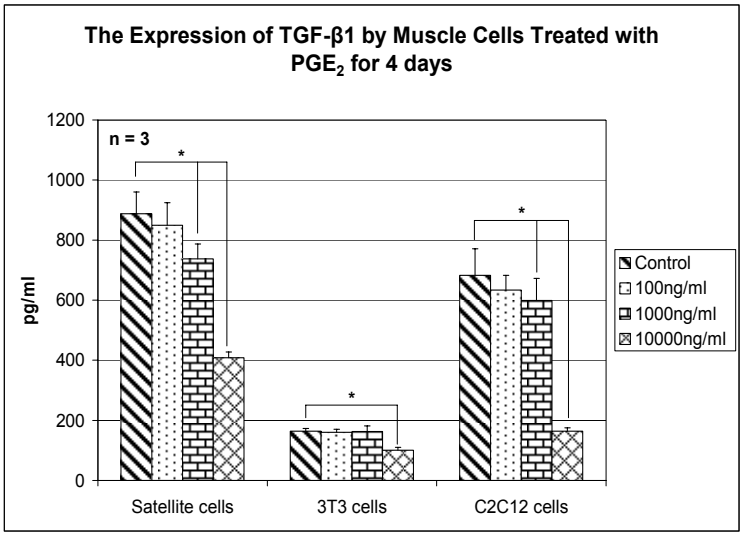


Figure 4-5. TGF-β1 Decreased the Expression of PGE_2

At the concentration of 1000ng/ml and 10000ng/ml, PGE_2 decreased the expression of TGF-β1 significantly in both satellite cells (LP cells) and myoblasts (C2C12 cell line). (P<0.05) However, PGE_2 was able to decrease the expression of TGF-β1 in fibroblasts (NIH 3T3 cell line) only at a concentration of 10000ng/ml. (P<0.05) The asterisks indicate a significant difference ($P < 0.05$) between the compared groups.

4.2.6 PGE_2 and Muscle Cell Proliferation

To examine other effects of PGE_2 on fibrosis formation, we examined the effect of PGE_2 treatment on the proliferation of different muscle cell types, including satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line). We found that a high concentration of PGE_2 (10000ng/ml) was able to inhibit the proliferation of all muscle cell types that were tested. However, at relatively lower concentrations (100ng/ml, 1000ng/ml), PGE_2 was shown to increase the proliferation of both fibroblasts (NIH 3T3 cells) and myoblasts (C2C12) cells, but not satellite cells (LP cells). (**Fig. 4-5**) These results suggested that a high level PGE_2 may decrease cell proliferation in injured skeletal muscle. When the level of PGE_2 was low, its effect may be different depending on cell type and the exact concentrations of PGE_2.

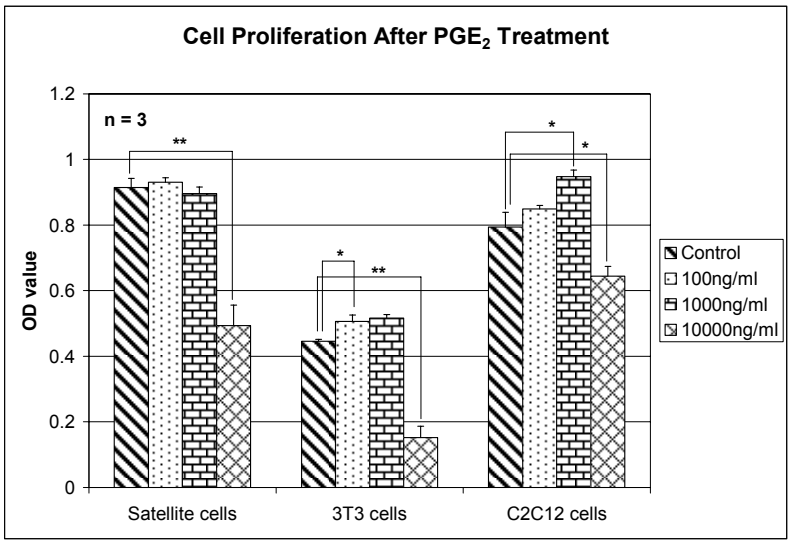


Figure 4-6. PGE_2 Affected Muscle Cell Proliferation

A high concentration of PGE_2 (10000ng/ml) inhibited the proliferation of all muscle cell types. ($P<0.05$, $P<0.01$ for satellite cells) However, at relatively lower concentrations (100ng/ml, 1000ng/ml), PGE_2 increased the proliferation of both fibroblasts (NIH 3T3 cell line) and myoblasts (C2C12 cell line), ($P<0.05$) but not satellite cells (LP cells). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups.

4.3 DISCUSSION

4.3.1 TGF-β1 Affects Inflammation by Interfering with COX-2 Pathway

Fibrosis is a complex biological process that is usually seen in severe muscle injury. Fibroblasts are activated to proliferate and produce an abnormal amount of extracellular matrix (ECM). Damaged skeletal muscle tissue is replaced by the deposition of overproduced ECM, the fibrosis (scar) tissue, instead of by regenerating myofibers. TGF-β1 is one of the most potent fibrotic stimuli. It is an inducer of ECM protein synthesis and fibroblast proliferation, [95] [96] and it has been involved in the fibrogenesis of various tissues.[34] However, the role of TGF-β1 in skeletal muscle healing is not limited to fibrosis. Previous studies provided evidence that TGF-β1 may be an immunosuppressive molecule, because eliminating TGF-β1 or disrupting its downstream Smads signaling cascade leads to severe inflammatory disease.[30-33] On the other hand, TGF-β1 was suggested to be able to increase PGE_2 expression in other tissues. [19, 37, 97, 98] Since we have shown in objective #1 that the COX-2 pathway including PGE_2 and $PGF_{2\alpha}$ are important inflammatory mediators and induce regeneration in skeletal muscle healing, it is necessary to explore their relationship with TGF-β1. In the in vitro studies, we found that the addition of TGF-β1 can significantly increase the production/expression of both COX-2 enzyme and PGE_2. By blocking COX-2 enzyme, the effect of TGF-β1 on PGE_2 expression was ablated. Furthermore, the effect of TGF-β1 can not be seen in COX-$2^{-/-}$ cells. These results clearly indicate that TGF-β1 is not only a fibrotic inducer, but also an inflammatory modulator in muscle injury. TGF-β1 may modulate the inflammatory response by enhancing the COX-2 pathway, especially the production of PGE_2. This was an interesting discovery, because it indicates that TGF-β1 may up-regulate and down-regulate inflammation through different pathways. The next

step would be to testify the negative impact of TGF-β1 on inflammation in muscle tissue/cells. It would be intriguing to find out which pathway is dominant in muscle injury, and why TGF-β1 would have two contradictory effects.

TGF-β1 has been shown to have an autocrine effect on its continuous expression in many cell types. [92] [93] [94] This expression pattern enhances the level of TGF-β1 and allows it to continuously exert its effect. Since we found TGF-β1 could increase PGE_2 expression and may be involved in the inflammation phase of muscle injury, it was important to examine whether the autocrine effect contributed to the enhanced PGE_2 expression. Interestingly, we found that the autocrine effect was critical for myoblasts to express PGE_2, but not for satellite cells and fibroblasts, because blocking the autocrine loop did not change the PGE_2 expression in TGF-β1-treated satellite cells and fibroblasts. This suggested that certain muscle cell types may respond differently to TGF-β1 stimulation. In the inflammation phase, different muscle cell types may play different roles.

4.3.2 PGE_2 Inhibits Fibrosis Formation by Decreasing TGF-β1 Expression

PGE_2 is a potent inhibitor of fibroblast proliferation [38] [99] and collagen synthesis.[38, 39] This suggests that PGE_2 may play an important role in maintaining a low level of extracellular matrix production. It is especially important in an environment favoring the formation of fibrosis tissue, like in the inflammation of damaged liver and skeletal muscle tissue. Previous study on liver fibrosis showed that PGE_2 inhibited TGF-β1-mediated induction of collagen alpha I production in hepatic cells. [100] This finding suggested that PGE_2 and TGF-β1 may be able to regulate the level of each other by forming a negative loop and control the homeostasis of fibrosis formation. To testify this phenomenon in skeletal muscle, we treated different muscle

cell types with PGE_2 and tested for their TGF-β1 expression. We found that PGE_2 treatment significantly decreased the expression of TGF-β1 in all muscle cell types tested, when compared to non-treated control cells. From another aspect, our *in vivo* study (See **2.2.4** result) showed that by using NS-398 to block COX-2, and thus the expression of PGE_2, the expression of TGF-β1 was increased in injured muscle tissue. These results indicated that the TGF-β1 level was probably checked by PGE_2. The use of NS-398 probably inhibited PGE_2 expression and led to a high level of TGF-β1, and therefore increased fibrosis formation in injured muscle. These findings are consistent with the results from the previous liver fibrosis study [100] and further suggest the existence of the negative feedback loop between TGF-β1 and PGE_2. If this is true, the more TGF-β1 produced, the stronger opposition to its fibrotic effect will be posed by PGE_2. (**Fig. 4-7**)

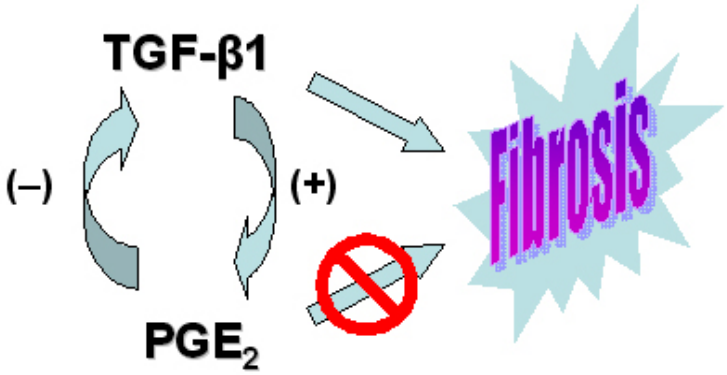


Figure 4-7. The Relation between TGF-β1 and PGE_2

TGF-β1 and PGE_2 may form a negative feedback loop in muscle healing. TGF-β1 increases fibrosis formation and the expression of PGE_2 through the COX-2 enzyme in muscle cells; PGE_2 decreases the expression of TGF-β1 to lower fibrosis formation.

4.3.3 PGE_2 Modifies the Proliferation of Different Muscle Cells

PGE_2 was reported to be capable of inhibiting fibroblast proliferation. [38] [99] This may be one of the mechanisms by which PGE_2 help to reduce fibrosis formation. To examine its effect on muscle cell types, we treated satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line) with PGE_2 for 4 days. We found that the proliferation of all muscle cell types were significantly inhibited when a high concentration of PGE_2 (10000ng/ml) were applied. However, at relatively lower concentrations (100ng/ml, 1000ng/ml), the results differed from those with the high concentration and also between different cell types. The proliferation of both NIH 3T3 cells and C2C12 cells were increased by low concentrations of PGE_2. But it did not seem to have an effect on satellite cell proliferation. These results suggested that the effect of PGE_2 on muscle cell proliferation depended on its concentration. Thus, the next step in our study would be to assess the level of PGE_2 in injured skeletal muscle, which would help us to know the exact effect of PGE_2 on muscle cell proliferation.

4.4 CONCLUSION

Our study showed that TGF-β1 increased the production of the COX-2 enzyme and the expression of PGE_2 *in vitro*. By using NS-398 and COX-$2^{-/-}$ cells, we proved that the effect of TGF-β1 on PGE_2 is through the activity of the COX-2 enzyme. Furthermore, we showed that PGE_2 decreased the expression of TGF-β1 and the proliferation of different muscle cell types. These results suggest that the level of TGF-β1 and PGE_2, the two important molecules in the inflammation phase of muscle healing, are modulated by each other by forming a negative feedback loop. Based on previous studies, it has been indicated that TGF-β1 may be an anti-inflammatory molecule through the Smads signaling pathway. However, our results suggested that TGF-β1 may also enhance inflammation in injured muscle by inducing the COX-2 pathway. Obviously, the role of TGF-β1 in the inflammation phase is complicated and warrants further attention. We postulate that the natural healing mechanism may try to adjust the extent of inflammation through the dual effect of TGF-β1; and try to limit TGF-β1-induced fibrosis formation by increasing the level of PGE_2. To improve the clinical outcome of muscle injury treatment in the future, it is critical to understand the complex mechanism.

4.5 MATERIAL AND METHODS

4.5.1 Cell Isolation and Culturing

Myogenic precursor cells (MPC) were isolated via a previously described preplate technique.[47, 48] Gastrocnemius muscles were removed from 4-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and minced with scissors. The meshes were enzymatically digested by sequential exposure to collagenase, dispase, and trypsin. The muscle cell extracts were then plated on collagen-coated flasks, and different populations were isolated by re-plating the extracts after different time intervals. The late plated population (LP cells) is made up of myogenic precursor cells that have high myogenic potential when induced by low serum culture medium.[48] LP cells were used for our *in vitro* experiments along with two other cell lines, NIH 3T3 and C2C12, which are a fibroblast cell line and a myoblast cell line respectively. These three types of cells were maintained in proliferation medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS], 10% horse serum [HS], and 0.5% chicken embryo extract).

Twenty four hours before the addition of PGE_2 or TGF-β1, we changed from proliferation medium to non-serum medium for overnight to synchronize the cell cycle. For proliferation experiment, proliferation medium was used again and PGE_2 (0, 100, 1000, or 10000 ng/ml) was added to examine their effect on cell proliferation. MTT assay was utilized to test the cell proliferation 4 days later. For the experiments of PGE_2 or TGF-β1 on growth factor and cytokine expression, differentiation medium was used to culture the cells during the 4 days period. Differentiation medium with the addition of PGE_2 (0, 100, 1000, or 10000 ng/ml) and TGF-β1 (5ng/ml) was not replaced during this time. The supernatant was collected at the end and

centrifuged at 3500rpm for 5min to remove the cell debris. They were stored at -80ºC pending ELISA assay.

4.5.2 ELISA Assay

The assay was performed as suggested by the manufacturer's protocols (DE0100 PGE_2 ELISA kit, MB100 TGF-β1 ELISA kit, R & D Systems, Minneapolis, MN) Briefly, antibodies against PGE_2 or TGF-β1 and PGE_2 or TGF-β1 conjugates were sequentially applied and incubated for a length of certain time. After adding the substrate and stop solution sequentially, the optical density of each well was immediately determined using a microplate reader.

4.5.3 Western Blot

After washing with PBS, Laemmli sample buffer (BioRad, 161-0737) was applied to the surface of culture dishes to collect proteins from live cells. After being boiled for 5 minutes, protein samples were separated on 10% SDS-polyacrylamide electrophoresis gel and were transferred to nitrocellulose membranes. Mouse monoclonal anti-COX-2 antibody (160112, Cayman, Ann Arbor, Michigan) was applied as primary antibodies, and mouse anti-β-actin (Sigma; 1:8000) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) were diluted to 1:5000 and applied. Blots were developed by using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL), and positive bands were visualized on X-ray film. All results were analyzed by Northern Eclipse software (Empix Imaging).

4.5.4 Small Interference RNA

siRNA for TGF-β1 gene was pre-designed by and purchased from Ambion Inc. (siRNA ID #187280, Ambion, Austin, TX) They are 21bp long RNA oligonucleotide sequences with a sense chain as CCAAGGAGACGGAAUACAGtt, and an antisense chain as CUGUAUUCCGUCUCCUUGGtt. These sequences were transfected to satellite cells (LP cells), fibroblasts (NIH 3T3 cell line), and myoblasts (C2C12 cell line) following the Pre-Plated Transfection Protocol provided by the producer. Briefly, cells were first plated in 12 well plates at a density of 6.75×10^4 cells/well overnight using proliferation medium. It was changed to differentiation medium before adding the transfection complex. siRNA and SiPort Amine® (4502, Ambion, Austin, TX), the transfection agent, were diluted with Opti-MEM I medium (31985-062, Invitrogen, Carlsbad, CA) and mixed together to form the transfection complex. It was dispensed onto the cells without swirling and incubated for 8h before any treatment.

TGF-β1 (5ng/ml) was added to the cells with or without the treatment of TGF-β1-siRNA 8h later and cultured for another 96h. The supernatant was collected and centrifuged at 3500rpm for 5 min to remove the debris. Samples were kept in -80ºC pending ELISA assay.

4.5.5 Statistics

Comparisons between 2 groups were made by unpaired Student's *t* test. All other data was analyzed by one-way ANOVA statistical analysis. Post hoc multiple comparison tests were performed to determine which means differed. Error bars on figures represent the standard deviation. $P < 0.05$ was considered statistically significant.

5.0 CONCLUSIONS

Based on the background knowledge and preliminary results, we have hypothesized that inflammation is an important phase in skeletal muscle healing. In this study, we showed that COX-2 pathway, macrophages, and TGF-β1 are important components of inflammation phase. Inflammation affects the overall healing of skeletal muscle through these cellular and molecular components. In addition, we found that these components may modulate the production of each other, forming a complex mechanism. (**Figure 5-1**) By investigating the influence of these components of inflammation, the importance of inflammation in muscle healing is emphasized and the mechanism may be used as a guide to improve medical interventions in the future. However, a large part of the skeletal muscle healing mechanism is still unveiled. Although we have started to realize the fact that simply blocking inflammation phase by using NSAIDs may not be an elixir, more studies are warranted in order to tell us exactly how to further improve the quality of healthcare for patients who suffer from skeletal muscle injuries.

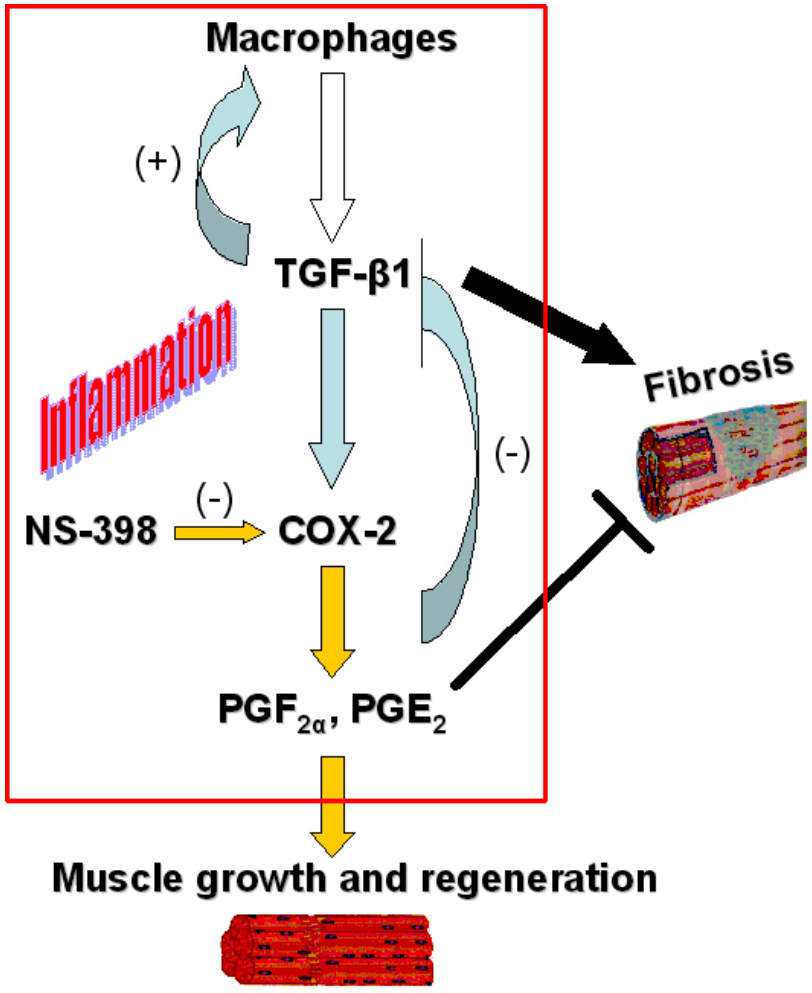


Figure 5-1. The Relationship among Macrophages, TGF-β1, and COX-2 Pathway components in the Inflammation Phase of Skeletal Muscle Healing

5.1 FUTURE DIRECTIONS

5.1.1 The Role of Neutrophils in Skeletal Muscle Healing

Neutrophil is another important inflammatory cell type that is involved in muscle healing. Neutrophils invade into skeletal muscle as early as 2h and last up to 5 day after injury. Studies have depicted a complex picture in which inflammatory cells promote both injury and repair, through the combined actions of free radicals, growth factors, and chemokines. [3] The role of neutrophil, however, is confusing since there is no direct evidence yet available to show how they benefit muscle repair or regeneration, although they are part of the natural healing process. [3] Would nature allow something meaningless or harmful to occur when repairing itself? In the future, we should examine the role of neutrophils, beneficial or detrimental, in skeletal muscle healing.

5.1.2 Fusion between Macrophages and Muscle cells/Myofibers

We found that the depletion of macrophages resulted in reduced regenerating myofiber size. This indicates that macrophages may be involved in the proliferation of myogenic cells and/or the fusion of these cells and regenerating myofibers. Many studies have reported that macrophages can increase the proliferation of myogenic cells. However, new findings just emerged to suggest that macrophages may fuse with myofibers directly to promote regeneration.[25] [101] However, in our effort, the direct fusion between mature macrophages (Marked by F4/80 surface molecule) and myogenic cells was not verified in vitro. (Data not shown) We postulate that the fusion may only occur during a certain differentiation stage of monocytes/macrophages, and certain

molecular environment may be necessary to trigger the fusion between muscle cells and monocytes/macrophages. It will be intriguing to explore the mechanism in the future.

5.1.3 In Vivo Application of TGF-β1-siRNA to Decrease Fibrosis Formation

We have successfully blocked the autocrine loop of TGF-β1 in vitro by using oligonucleotide TGF-β1-siRNA. This leads us to believe that if we could block the autocrine loop in vivo, the expression of TGF-β1 and thus the fibrosis formation would be greatly reduced. This may serve as a powerful weapon against scar tissue formation in skeletal muscle injury. However, there are a few technical problems that need to be solved before we can achieve this goal. Most importantly, the oligonucleotide TGF-β1-siRNA we used in the in vitro study is only active for a short period of time (8h-72h) and makes it unsuitable for the in vivo study. To stably express TGF-β1-siRNA in vivo, we need to construct the siRNA sequence into a viral vector (ex. AAV) and transfect it into muscle cells.

5.1.4 The Role of PGE_2 on TGF-β1 and Cell Proliferation in Vivo

The effect of PGE_2 on TGF-β1 and muscle cell proliferation was analyzed in vitro in this study. It was shown that PGE_2 decreased TGF-β1 expression and muscle cell proliferation at a high concentration (10000ng/ml). However, at lower concentrations (100ng/ml, 1000ng/ml), the effect varied based on concentration and cell types. Thus, it is critical to measure the level of PGE_2 in vivo to determine its actual effect on different muscle cells types in a real injury environment.

APPENDIX A

PREPLATE TECHNIQUE: ISOLATION OF LP CELL POPULATION [102]

Gastrocnemius muscles of mice were obtained under aseptic techniques. A single cell suspension was obtained by digestion and enzymatic dissociation of combined muscles to obtain the appropriate number of cells. Enzymatic dissociation was performed by serial digestion of hand-minced muscles in 0.2% (by weight) collagenase-type XI solution (Sigma) for 1 hour, 0.3% dispase (Gibco-BRL) for 45 minutes, and 0.1% trypsin (Life Technologies) for 30 minutes. The final cell suspension was re-suspended in serum-supplemented Dulbecco's modified Eagle's medium (DMEM, containing 10% fetal bovine serum, 10% horse serum, 0.5% chick embryo extract by volume, and also 100 U/mL penicillin and 100 μg/mL streptomyocin; Gibco-BRL), which was also used for subsequent culturing, and added to a T-75 collagen-coated flask (collagen Type I, Sigma). After 2 hours, floating cells contained within the supernatant were removed and transferred to a second T-75 flask. Fresh medium was added to the first set of adherent cells (termed preplate 1, or PP1), and this procedure was continued for PP2 through PP6 at subsequent 24 hour periods. A smaller surface area flask, T-25, was used for PP6 as the number of remaining non-adherent cells by this point was comparatively lower. This process resulted in six primary cultures of adherent cells with increasing initial adhesion times that are highly fibroblastic in nature[47] [48] [103] [104] that were subsequently used for surface protein

and desmin analysis. Cells in the pp5 and pp6 cell population were termed “late preplate cells” (LP). A flow chart for the isolation of LP cells based on their adhesion characteristics to collagen coated flasks are shown below (**Figure Appendix.1**).

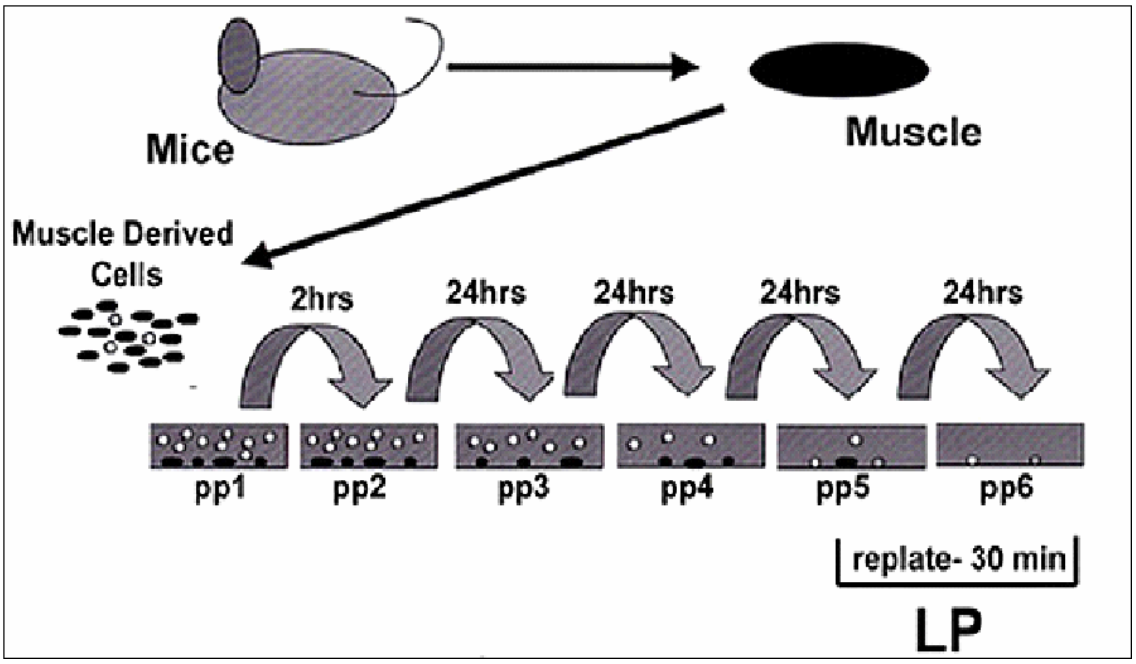


Figure Appendix 1. Schematic Diagram of the Preplating Technique Used for the Isolation of LP Cell Population (Reproduced with the permission from *Journal of Cell Biology*)

APPENDIX B

A DETAILED DESCRIPTION OF THE PHYSIOLOGIC TESTING PROTOCOL [83]

Animals were anesthetized intraperitoneally with pentobarbital sodium (70 mg/kg) and the TA muscle was isolated by removing the overlying biceps femoris and gently opening the fascia of the anterior compartment. The entire TA muscle was removed with its tendon insertion on the foot and tibial origin intact. The TA muscle was mounted in a vertical tissue chamber that was constantly perfused with mammalian Ringer's solution aerated with 95% O2–5% CO2 and maintained at 25°C. The TA muscle origin was secured via a Michel clip (Fine Science Tools, Foster City, CA) placed through the distal head of the tibia and attached to the mounting pin of a glass tissue support rod (Radnoti Glass Technology, Monrovia, CA) connected in series to a micropositioner near the base of the tissue chamber. The tendon insertion on the dorsum of the foot was secured with a Michel clip (Fine Science Tools) that was connected to a force transducer and length servo system (model 305B, dual mode; Aurora Scientific, Aurora, ON, Canada) via fine wire. This wire provides a noncompliant attachment to the force transducer that is necessary for the lengthening activations. In addition to characterizing the force-generating capacity of control and dystrophic muscle in the isometric mode, muscle function was also assessed during repetitive lengthening activations, a paradigm of mechanical stress. The latter measurement is thought to be particularly important, as evidence suggests that a function of the

dystrophin-based membrane skeleton is to protect against stress-induced muscle damage. [105] The muscle was stimulated (Grass model S-88 stimulator and current amplifier) by monophasic rectangular pulses of cathodal current (1.0-msec duration) delivered through platinum plate electrodes placed ~1 cm apart. The TA muscle was positioned midway between the two electrodes. To ensure supramaximal stimulation, current was increased by 50% over the current necessary to obtain peak twitch force (~250–300 mA). Muscle length was adjusted incrementally by using a micropositioner until maximal isometric twitch force responses were obtained (i.e., optimal length [*L*o]). *L*o was measured with a microcaliper accurate to 0.1 mm (Fisher Scientific, Pittsburgh, PA). Maximum tetanic force (*P*0) was assessed with a stimulation frequency of 75 pulses per second (pps) delivered in a 500-msec duration train. In preliminary studies we found that higher frequencies of stimulation, up to 200 pps, were not associated with any further increase in *P*0. After the *in vitro* study each TA muscle was weighed on an analytic balance (model 2100; Fisher Scientific) after tendon and bone attachments were removed and the muscle was blotted dry. Force was expressed in newtons and normalized for muscle cross-sectional area (CSA), the latter estimated on the basis of the following formula: muscle weight (g)/[*L*o (cm) 3 1.056 (g/cm3)] to determine specific titanic (*P*0/CSA) forces. A picture of the device is shown as below. (**Figure Appendix 2**)

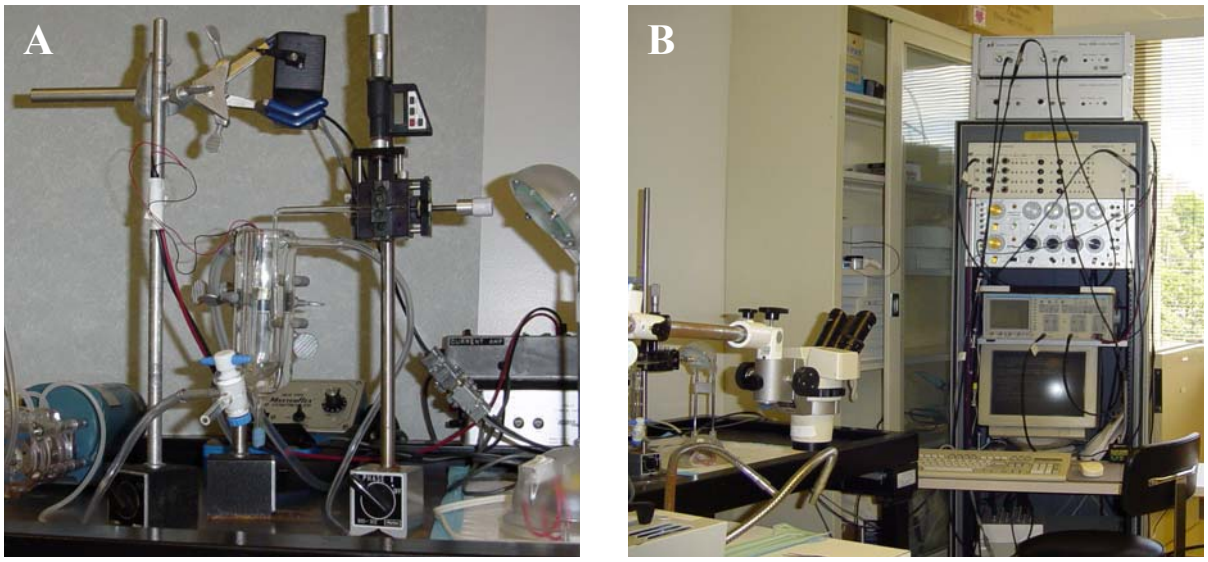


Figure Appendix 2. The Physiologic Testing Device

Picture **A** showed the muscle mounting component. Picture **B** showed the data collecting and processing component. (Courtesy from Mr. Terry O'Day)

APPENDIX C

A BRIEF DESCRIPTION OF HOW TO MAKE LIPOSOME CLODRONATE

The preparation of liposome clodronate was described briefly as below. Soy phosphatidylcholine, DL-α-tocopherol, and cholesterol (all from Sigma-Aldrich, St. Louis, MO; 1:0.01:0.3 molar ratio) were dissolved in chloroform. After evaporation of the solvent, dry lipid film was dispersed in 10 ml of clodronate solution (Ostac, Roche, Switzerland) by careful shaking. Suspension was sonicated three times for 5 min and freeze-thawed in three cycles of liquid nitrogen and water at 37°C. Liposomes were washed twice in PBS (50,000×*g*) to remove free clodronate. [86] The structure of liposome clodronate is shown as below. (**Figure Appendix 3**)

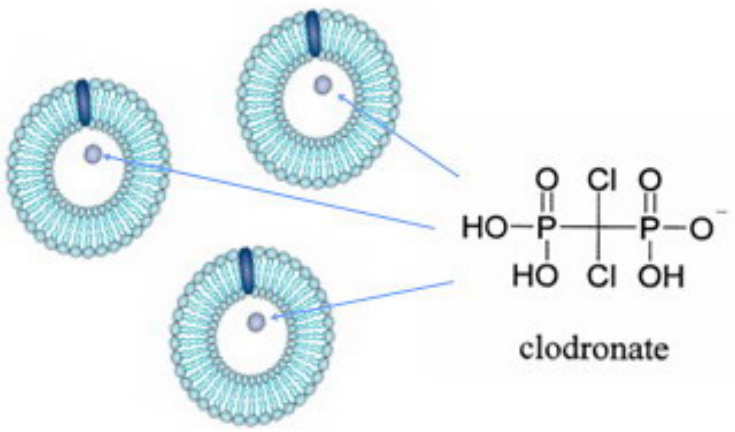


Figure Appendix 3. The Schematic Structure of Liposome Clodronate (Courtesy from Dr. Reto Schwendener)

BIBLIOGRAPHY

1. Garrett, W.E., Jr., *Muscle strain injuries.* Am J Sports Med, 1996. **24**(6 Suppl): p. S2-8.

2. Jarvinen, M.J. and M.U. Lehto, *The effects of early mobilisation and immobilisation on the healing process following muscle injuries.* Sports Med, 1993. **15**(2): p. 78-89.

3. Tidball, J.G., *Inflammatory processes in muscle injury and repair.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(2): p. R345-53.

4. Huard, J., Y. Li, and F.H. Fu, *Muscle injuries and repair: current trends in research.* J Bone Joint Surg Am, 2002. **84-A**(5): p. 822-32.

5. Li, Y., J. Cummins, and J. Huard, *Muscle injury and repair.* Current opinion in orthopaedics, 2001. **12**: p. 409-415.

6. Hasson, S.M., et al., *Effect of ibuprofen use on muscle soreness, damage, and performance: a preliminary investigation.* Med Sci Sports Exerc, 1993. **25**(1): p. 9-17.

7. Dudley, G.A., et al., *Efficacy of naproxen sodium for exercise-induced dysfunction muscle injury and soreness.* Clin J Sport Med, 1997. **7**(1): p. 3-10.

8. Obremsky, W.T., et al., *Biomechanical and histologic assessment of a controlled muscle strain injury treated with piroxicam.* Am J Sports Med, 1994. **22**(4): p. 558-61.

9. Mishra, D.K., et al., *Anti-inflammatory medication after muscle injury. A treatment resulting in short-term improvement but subsequent loss of muscle function.* J Bone Joint Surg Am, 1995. **77**(10): p. 1510-9.

10. Almekinders, L.C., *Anti-inflammatory treatment of muscular injuries in sport. An update of recent studies.* Sports Med, 1999. **28**(6): p. 383-8.

11. Trappe, T.A., et al., *Skeletal muscle PGF(2)(alpha) and PGE(2) in response to eccentric resistance exercise: influence of ibuprofen acetaminophen.* J Clin Endocrinol Metab, 2001. **86**(10): p. 5067-70.

12. Prisk, V. and J. Huard, *Muscle injuries and repair: the role of prostaglandins and inflammation.* Histol Histopathol, 2003. **18**(4): p. 1243-56.

13. Hatae, N., Y. Sugimoto, and A. Ichikawa, *Prostaglandin receptors: advances in the study of EP3 receptor signaling.* J Biochem (Tokyo), 2002. **131**(6): p. 781-4.

14. Sakamoto, A., et al., *A prostaglandin E2 receptor subtype EP4 agonist attenuates cardiovascular depression in endotoxin shock by inhibiting inflammatory cytokines and nitric oxide production.* Shock, 2004. **22**(1): p. 76-81.

15. Palmer, R.M., *Prostaglandins and the control of muscle protein synthesis and degradation.* Prostaglandins Leukot Essent Fatty Acids, 1990. **39**(2): p. 95-104.

16. Rodemann, H.P. and A.L. Goldberg, *Arachidonic acid, prostaglandin E2 and F2 alpha influence rates of protein turnover in skeletal and cardiac muscle.* J Biol Chem, 1982. **257**(4): p. 1632-8.

17. Horsley, V. and G.K. Pavlath, *Forming a multinucleated cell: molecules that regulate myoblast fusion.* Cells Tissues Organs, 2004. **176**(1-3): p. 67-78.

18. Horsley, V. and G.K. Pavlath, *Prostaglandin F2(alpha) stimulates growth of skeletal muscle cells via an NFATC2-dependent pathway.* J Cell Biol, 2003. **161**(1): p. 111-8.

19. Keerthisingam, C.B., et al., *Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-beta in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice.* Am J Pathol, 2001. **158**(4): p. 1411-22.

20. Pavlath, G.K. and V. Horsley, *Cell fusion in skeletal muscle--central role of NFATC2 in regulating muscle cell size.* Cell Cycle, 2003. **2**(5): p. 420-3.

21. Cantini, M., et al., *Macrophages regulate proliferation and differentiation of satellite cells.* Biochem Biophys Res Commun, 1994. **202**(3): p. 1688-96.

22. Chazaud, B., et al., *Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth.* J Cell Biol, 2003. **163**(5): p. 1133-43.

23. Lescaudron, L., et al., *Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant.* Neuromuscul Disord, 1999. **9**(2): p. 72-80.

24. Merly, F., et al., *Macrophages enhance muscle satellite cell proliferation and delay their differentiation.* Muscle Nerve, 1999. **22**(6): p. 724-32.

25. Camargo, F.D., et al., *Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates.* Nat Med, 2003. **9**(12): p. 1520-7.

26. Letterio, J.J. and A.B. Roberts, *Regulation of immune responses by TGF-beta.* Annu Rev Immunol, 1998. **16**: p. 137-61.

27. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system.* Embo J, 2000. **19**(8): p. 1745-54.

28. Ulloa, L., J. Doody, and J. Massague, *Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway.* Nature, 1999. **397**(6721): p. 710-3.

29. Verrecchia, F., et al., *Tumor necrosis factor-alpha inhibits transforming growth factor-beta /Smad signaling in human dermal fibroblasts via AP-1 activation.* J Biol Chem, 2000. **275**(39): p. 30226-31.

30. Kulkarni, A.B. and S. Karlsson, *Inflammation and TGF beta 1: lessons from the TGF beta 1 null mouse.* Res Immunol, 1997. **148**(7): p. 453-6.

31. Yang, X., et al., *Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta.* Embo J, 1999. **18**(5): p. 1280-91.

32. Nakao, A., et al., *Blockade of transforming growth factor beta/Smad signaling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity.* J Exp Med, 2000. **192**(2): p. 151-8.

33. Monteleone, G., et al., *A failure of transforming growth factor-beta1 negative regulation maintains sustained NF-kappaB activation in gut inflammation.* J Biol Chem, 2004. **279**(6): p. 3925-32.

34. Border, W.A. and N.A. Noble, *Transforming growth factor beta in tissue fibrosis.* N Engl J Med, 1994. **331**(19): p. 1286-92.

35. Munger, J.S., et al., *The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis.* Cell, 1999. **96**(3): p. 319-28.

36. Clancy, R.M. and J.P. Buyon, *Clearance of apoptotic cells: TGF-beta in the balance between inflammation and fibrosis.* J Leukoc Biol, 2003. **74**(6): p. 959-60.

37. McAnulty, R.J., et al., *Indomethacin suppresses the anti-proliferative effects of transforming growth factor-beta isoforms on fibroblast cell cultures.* Biochem J, 1997. **321 (Pt 3)**: p. 639-43.

38. Goldstein, R.H. and P. Polgar, *The effect and interaction of bradykinin and prostaglandins on protein and collagen production by lung fibroblasts.* J Biol Chem, 1982. **257**(15): p. 8630-3.

39. Saltzman, L.E., et al., *Modulation of collagen production by fibroblasts. Effects of chronic exposure to agonists that increase intracellular cyclic AMP.* Biochem J, 1982. **204**(1): p. 25-30.

40. Wang, W., et al., *Signaling Mechanism of TGF-{beta}1 in Prevention of Renal Inflammation: Role of Smad7.* J Am Soc Nephrol, 2005. **16**(5): p. 1371-83.

41. Monteleone, G., F. Pallone, and T.T. MacDonald, *Smad7 in TGF-beta-mediated negative regulation of gut inflammation.* Trends Immunol, 2004. **25**(10): p. 513-7.

42. Warshamana, G.S., et al., *Titration of non-replicating adenovirus as a vector for transducing active TGF-beta1 gene expression causing inflammation and fibrogenesis in the lungs of C57BL/6 mice.* Int J Exp Pathol, 2002. **83**(4): p. 183-201.

43. Fiocchi, C., *TGF-beta/Smad signaling defects in inflammatory bowel disease: mechanisms and possible novel therapies for chronic inflammation.* J Clin Invest, 2001. **108**(4): p. 523-6.

44. Abramson, S.B. and G. Weissmann, *The mechanisms of action of nonsteroidal antiinflammatory drugs.* Arthritis Rheum, 1989. **32**(1): p. 1-9.

45. Cheung, E.V. and J.G. Tidball, *Administration of the non-steroidal anti-inflammatory drug ibuprofen increases macrophage concentrations but reduces necrosis during modified muscle use.* Inflamm Res, 2003. **52**(4): p. 170-6.

46. Almekinders, L.C. and J.A. Gilbert, *Healing of experimental muscle strains and the effects of nonsteroidal antiinflammatory medication.* Am J Sports Med, 1986. **14**(4): p. 303-8.

47. Rando, T.A. and H.M. Blau, *Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy.* J Cell Biol, 1994. **125**(6): p. 1275-87.

48. Qu, Z., et al., *Development of approaches to improve cell survival in myoblast transfer therapy.* J Cell Biol, 1998. **142**(5): p. 1257-67.

49. Jankowski, R.J., B.M. Deasy, and J. Huard, *Muscle-derived stem cells.* Gene Ther, 2002. **9**(10): p. 642-7.

50. Lee, J.Y., et al., *Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing.* J Cell Biol, 2000. **150**(5): p. 1085-100.

51. Seale, P., et al., *Pax7 is required for the specification of myogenic satellite cells.* Cell, 2000. **102**(6): p. 777-86.

52. Mauro, A., *Satellite cell of skeletal muscle fibers.* J Biophys Biochem Cytol, 1961. **9**: p. 493-5.

53. Lipton, B.H. and E. Schultz, *Developmental fate of skeletal muscle satellite cells.* Science, 1979. **205**(4412): p. 1292-4.

54. Cooper, R.N., et al., *In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle.* J Cell Sci, 1999. **112 (Pt 17)**: p. 2895-901.

55. Fukada, S., et al., *Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody.* Exp Cell Res, 2004. **296**(2): p. 245-55.

56. Cornelison, D.D. and B.J. Wold, *Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells.* Dev Biol, 1997. **191**(2): p. 270-83.

57. George-Weinstein, M., et al., *In vitro and in vivo expression of alpha 7 integrin and desmin define the primary and secondary myogenic lineages.* Dev Biol, 1993. **156**(1): p. 209-29.

58. Mendias, C.L., R. Tatsumi, and R.E. Allen, *Role of cyclooxygenase-1 and -2 in satellite cell proliferation, differentiation, and fusion.* Muscle Nerve, 2004. **30**(4): p. 497-500.

59. Beauchamp, J.R., et al., *Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells.* J Cell Biol, 2000. **151**(6): p. 1221-34.

60. Seale, P. and M.A. Rudnicki, *A new look at the origin, function, and "stem-cell" status of muscle satellite cells.* Dev Biol, 2000. **218**(2): p. 115-24.

61. Yoshida, N., et al., *Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'.* J Cell Sci, 1998. **111 (Pt 6)**: p. 769-79.

62. Bondesen, B.A., et al., *The COX-2 pathway is essential during early stages of skeletal muscle regeneration.* Am J Physiol Cell Physiol, 2004. **287**(2): p. C475-83.

63. Shanmugasundaram, T.K., *Post-injection fibrosis of skeletal muscle: a clinical problem. A personal series of 169 cases.* Int Orthop, 1980. **4**(1): p. 31-7.

64. Croisier, J.L., *Factors associated with recurrent hamstring injuries.* Sports Med, 2004. **34**(10): p. 681-95.

65. Gaedeke, J., et al., *Angiotensin II, TGF-beta and renal fibrosis.* Contrib Nephrol, 2001(135): p. 153-60.

66. Ihn, H., *Pathogenesis of fibrosis: role of TGF-beta and CTGF.* Curr Opin Rheumatol, 2002. **14**(6): p. 681-5.

67. Ghosh, A.K., *Factors involved in the regulation of type I collagen gene expression: implication in fibrosis.* Exp Biol Med (Maywood), 2002. **227**(5): p. 301-14.

68. Li, Y. and J. Huard, *Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle.* Am J Pathol, 2002. **161**(3): p. 895-907.

69. Li, Y., et al., *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, 2004. **164**(3): p. 1007-19.

70. Vandenburgh, H.H., et al., *Mechanical stimulation of skeletal muscle increases prostaglandin F2 alpha production, cyclooxygenase activity, and cell growth by a pertussis toxin sensitive mechanism.* J Cell Physiol, 1995. **163**(2): p. 285-94.

71. Wani, M.R., et al., *Prostaglandin E2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading, and fusion.* Endocrinology, 1999. **140**(4): p. 1927-35.

72. Kishimoto, T.K., et al., *Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors.* Science, 1989. **245**(4923): p. 1238-41.

73. Landay, A., G.L. Gartland, and L.T. Clement, *Characterization of a phenotypically distinct subpopulation of Leu-2+ cells that suppresses T cell proliferative responses.* J Immunol, 1983. **131**(6): p. 2757-61.

74. Stenson, W.F., K. Chang, and J.R. Williamson, *Tissue differences in vascular permeability induced by leukotriene B4 and prostaglandin E2 in the rat.* Prostaglandins, 1986. **32**(1): p. 5-17.

75. Tetsuka, T., et al., *Cross-talk between cyclooxygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by interleukin 1.* Proc Natl Acad Sci U S A, 1994. **91**(25): p. 12168-72.

76. Massimino, M.L., et al., *ED2+ macrophages increase selectively myoblast proliferation in muscle cultures.* Biochem Biophys Res Commun, 1997. **235**(3): p. 754-9.

77. Adeagbo, A.S., et al., *NS-398, a selective cyclooxygenase-2 blocker, acutely inhibits receptor-mediated contractions of rat aorta: role of endothelium.* Eur J Pharmacol, 2003. **458**(1-2): p. 145-54.

78. Nasrallah, R., et al., *Effect of COX-2 inhibitor NS-398 on expression of PGE2 receptor subtypes in M-1 mouse CCD cells.* Am J Physiol Renal Physiol, 2001. **281**(1): p. F123-32.

79. Fukushima, K., et al., *The use of an antifibrosis agent to improve muscle recovery after laceration.* Am J Sports Med, 2001. **29**(4): p. 394-402.

80. Menetrey, J., et al., *Growth factors improve muscle healing in vivo.* J Bone Joint Surg Br, 2000. **82**(1): p. 131-7.

81. Austyn, J.M. and S. Gordon, *F4/80, a monoclonal antibody directed specifically against the mouse macrophage.* Eur J Immunol, 1981. **11**(10): p. 805-15.

82. van Eeden, S.F., et al., *The use of flow cytometry to measure neutrophil function.* J Immunol Methods, 1999. **232**(1-2): p. 23-43.

83. Watchko, J., et al., *Adeno-associated virus vector-mediated minidystrophin gene therapy improves dystrophic muscle contractile function in mdx mice.* Hum Gene Ther, 2002. **13**(12): p. 1451-60.

84. Grounds, M.D., *Phagocytosis of necrotic muscle in muscle isografts is influenced by the strain, age, and sex of host mice.* J Pathol, 1987. **153**(1): p. 71-82.

85. Robertson, T.A., M.D. Grounds, and J.M. Papadimitriou, *Elucidation of aspects of murine skeletal muscle regeneration using local and whole body irradiation.* J Anat, 1992. **181 (Pt 2)**: p. 265-76.

86. Aichele, P., et al., *Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses.* J Immunol, 2003. **171**(3): p. 1148-55.

87. Tyner, J.W., et al., *CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection.* Nat Med, 2005. **11**(11): p. 1180-7.

88. Beck-Schimmer, B., et al., *Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury.* Respir Res, 2005. **6**: p. 61.

89. Cantini, M., et al., *Macrophage-secreted myogenic factors: a promising tool for greatly enhancing the proliferative capacity of myoblasts in vitro and in vivo.* Neurol Sci, 2002. **23**(4): p. 189-94.

90. Cantini, M., et al., *Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by activated monocytes.* Biochem Biophys Res Commun, 1995. **216**(1): p. 49-53.

91. Cantini, M. and U. Carraro, *Macrophage-released factor stimulates selectively myogenic cells in primary muscle culture.* J Neuropathol Exp Neurol, 1995. **54**(1): p. 121-8.

92. Kubota, T., et al., *Autocrine/paracrine function of transforming growth factor-beta 1 in porcine granulosa cells.* Hum Reprod, 1994. **9**(11): p. 2118-22.

93. Ploemacher, R.E., P.L. van Soest, and A. Boudewijn, *Autocrine transforming growth factor beta 1 blocks colony formation and progenitor cell generation by hemopoietic stem cells stimulated with steel factor.* Stem Cells, 1993. **11**(4): p. 336-47.

94. Huang, F., et al., *Transforming growth factor beta 1 (TGF beta 1) is an autocrine positive regulator of colon carcinoma U9 cells in vivo as shown by transfection of a TGF beta 1 antisense expression plasmid.* Cell Growth Differ, 1995. **6**(12): p. 1635-42.

95. Kahari, V.M., H. Larjava, and J. Uitto, *Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-beta 1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but down-regulates decorin (PGII) mRNA levels in human fibroblasts in culture.* J Biol Chem, 1991. **266**(16): p. 10608-15.

96. Taipale, J., et al., *Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein.* J Cell Biol, 1994. **124**(1-2): p. 171-81.

97. Fawthrop, F.W., et al., *Effects of transforming growth factor beta on the production of prostaglandin E and caseinase activity of unstimulated and interleukin 1-stimulated human articular chondrocytes in culture.* Br J Rheumatol, 1997. **36**(7): p. 729-34.

98. Han, C., et al., *Transforming growth factor-beta (TGF-beta) activates cytosolic phospholipase A2alpha (cPLA2alpha)-mediated prostaglandin E2 (PGE)2/EP1 and peroxisome proliferator-activated receptor-gamma (PPAR-gamma)/Smad signaling pathways in human liver cancer cells. A novel mechanism for subversion of TGF-beta-induced mitoinhibition.* J Biol Chem, 2004. **279**(43): p. 44344-54.

99. Durant, S., D. Duval, and F. Homo-Delarche, *Effect of exogenous prostaglandins and nonsteroidal anti-inflammatory agents on prostaglandin secretion and proliferation of mouse embryo fibroblasts in culture.* Prostaglandins Leukot Essent Fatty Acids, 1989. **38**(1): p. 1-8.

100. Hui, A.Y., et al., *Prostaglandin E2 inhibits transforming growth factor beta 1-mediated induction of collagen alpha 1(I) in hepatic stellate cells.* J Hepatol, 2004. **41**(2): p. 251-8.

101. Ojima, K., et al., *Mac-1(low) early myeloid cells in the bone marrow-derived SP fraction migrate into injured skeletal muscle and participate in muscle regeneration.* Biochem Biophys Res Commun, 2004. **321**(4): p. 1050-61.

102. Qu-Petersen, Z., et al., *Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration.* J Cell Biol, 2002. **157**(5): p. 851-64.

103. Richler, C. and D. Yaffe, *The in vitro cultivation and differentiation capacities of myogenic cell lines.* Dev Biol, 1970. **23**(1): p. 1-22.

104. Qu, Z. and J. Huard, *Matching host muscle and donor myoblasts for myosin heavy chain improves myoblast transfer therapy.* Gene Ther, 2000. **7**(5): p. 428-37.

105. Petrof, B.J., et al., *Dystrophin protects the sarcolemma from stresses developed during muscle contraction.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3710-4.