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**Mechanisms of Fibrosis in Regeneration of Aged Skeletal Muscle**

By

**Fasih A. Rahman**

A Thesis

Submitted to the Faculty of Graduate Studies  
through the Department of Kinesiology  
in Partial Fulfillment of the Requirements for  
the Degree of Master of Human Kinetics at the  
University of Windsor

Windsor, Ontario, Canada

2019

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# **Mechanisms of Fibrosis in Regeneration of Aged Skeletal Muscle**

By

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May 2nd, 2019

## **DECLARATION OF ORIGINALITY**

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## ABSTRACT

Skeletal muscle is a highly adaptive tissue that possesses the ability to regenerate following damage. Regenerative capacity of skeletal muscle declines as age advances, leading to restricted mobility and poor quality of life. The impairment of the regeneration process in aged muscle is partly due to the accumulation of structural proteins in the extracellular matrix (ECM), termed fibrosis. In this study, two important proteins regulating ECM remodeling, plasminogen activator inhibitor-1 (PAI-1) and matrix metalloproteinase-9 (MMP-9), were investigated to determine their role in modulating changes in the ECM during aged muscle regeneration. The regeneration process was studied in young (3 month old) and aged (18 month old) C56BL/6J mice at 3, 5, and 7 days following cardiotoxin-induced muscle damage. The regeneration process was significantly impaired in aged muscle as indicated by decreased muscle mass, cross-sectional fibre area, number of newly regenerated myofibres, and eMHC expression. Greater PAI-1 expression was found in aged regenerating myofibres 5 and 7 days following damage. Aged muscle displayed significantly greater extramyocellular PAI-1 acutely following damage, accumulation of collagen I, and delayed macrophage infiltration. Active MMP-9 was lower in aged muscle 3 and 5 days following damage compared to young muscle. However, MMP-9 primarily colocalized with F4/80+ macrophages in young muscle, but this colocalization was reduced in aged muscle. Taken together, this study provides a foundation for the mechanisms underlying the impairment of muscle regeneration in aged muscle.

## **DEDICATION**

This is dedicated to mother, sister and father who have provided advice, love, and support to pursue my ambitions and succeed. This work would not have been done with your encouragement.

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at 3 days following damage ( $p < 0.05$ ). Active MMP-9 expression declined significantly from 3 to 5 days following damage ( $p < 0.05$ ) to match the expression of aged muscle. (C) No significant differences of active MMP-9 positive area were observed in the regenerating regions of young and aged muscle ( $p > 0.05$ ). (D) A significant interaction between age and recovery time point on MMP-9+ F4/80+ cells was detected following damage ( $p < 0.05$ ). A simple main effects analysis showed significantly greater macrophage-specific (F4/80+) MMP-9 expression in the necrotic regions of young muscle 5 days following damage ( $p < 0.05$ ). Additionally, macrophage-specific MMP-9 expression increased significantly between 3 and 5 days in young, however, the opposite was observed in aged muscle (both  $p < 0.05$ ). (E) A significant main effect of age was found in the regenerating regions, with young muscle displaying a greater percentage of macrophage-specific MMP-9 compared to aged muscle ( $p < 0.05$ ). \* in (B) and (D) indicates significance from the simple main effects analyses ( $p < 0.05$ ). \* in (E) indicates a main effect of age. Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 100  $\mu\text{m}$ .....38

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## LIST OF ABBREVIATIONS

bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CSA	Cross-sectional Area
CTX	Cardiotoxin
ECM	Extracellular Matrix
eMHC	Embryonic Myosin Heavy Chain
FDP	Fibrin Degradation Products
HGF	Hepatocyte Growth Factor
IGF-1	Insulin-like Growth Factor-1
MAPK	Mitogen-activated Protein Kinase
MMP	Matrix Metalloproteinase
MRF	Myogenic Regulatory Factor
NGS	Normal Goat Serum
OCT	Optimum Cutting Temperature
PAI-1	Plasminogen Activator Inhibitor-1
PFA	Paraformaldehyde
ROS	Reactive Oxygen Species
TA	Tibialis Anterior
TGF- $\beta$	Transforming Growth Factor- $\beta$
TIMP	Tissue Inhibitor of Metalloproteinase
tPA	Tissue-type Plasminogen Activator



uPA            Urokinase-type Plasminogen Activator  
uPAR           Urokinase-type Plasminogen Activator Receptor

## INTRODUCTION

Skeletal muscle constitutes approximately 40% of the total mass of the human body, and plays a central role in health and wellbeing (1). Skeletal muscle is able to maintain its structure and function following damage due to its inherent regenerative properties, enabling it to completely restore structure and function within weeks after severe damage (2). The regeneration process can be categorized into three sequential and overlapping stages: 1) inflammation and necrosis of damaged fibres with the aid of immune cells, 2) activation, proliferation, differentiation, and fusion of muscle stem cells, called satellite cells, and 3) maturation and remodeling of the extracellular matrix (ECM) (3–6). Each stage of regeneration must occur for muscle to resume its function within the body. With aging, the regenerative capacity of skeletal muscle declines as a result of a plethora of aberrant changes within the body, and is accompanied by the loss of muscle mass, eventually leading to physical impairments (7, 8).

The combination of neurodegeneration, hormonal alterations, and changes in nutritional habits has been shown to cause numerous pathophysiological modifications in skeletal muscle. These include decreased motor units and muscle fibre number, accumulation of fat within the muscle, fibrosis, and overall weakness (7, 8). Additionally, the regenerative capacity of skeletal muscle declines with age resulting from several intrinsic and extrinsic factors, many of which affect satellite cell homeostasis. Although these negative physiological changes can be combatted via diet, exercise, and medication (9–13), the impact of such strategies on age-related fibrosis has been overlooked.

Several mechanisms underlying the development of fibrosis have been identified in various tissue types. Transforming growth factor- $\beta$  (TGF- $\beta$ ) has been shown to be the master regulator of fibrosis in several tissues, including skeletal muscle (14–17). TGF- $\beta$  has been shown to be

chronically elevated in aged muscle, leading to a pro-fibrotic response within the tissue (14, 18). One of the downstream effects of TGF- $\beta$  is its ability to enhance transcription of plasminogen activator inhibitor-1 (PAI-1), a widely circulated protein that is responsible for the inhibition of the plasminogen system (14, 19). Notably, PAI-1 slows the regeneration of damaged skeletal muscle and allows for greater ECM accumulation (20–22). Further investigation is needed to better understand the role of PAI-1 and the plasminogen system in skeletal muscle regeneration.

In general, the plasminogen system is responsible for the remodeling of the ECM through the degradation of fibrous proteins (23–25). The active substrate of the plasminogen system, plasmin, has been shown to directly degrade fibrin, a protein found in the ECM, while also activating a class of proteins called matrix metalloproteinases (MMPs). Activated MMPs are responsible for the direct degradation of proteins within the ECM. Function of the plasminogen system is tightly regulated by three protein inhibitors: PAI-1,  $\alpha$ 2-antiplasmin, and tissue inhibitor of metalloproteinases (TIMPs). Nonetheless, the role of PAI-1 and its downstream proteins has yet to be investigated in skeletal muscle during regeneration. This is particularly important in aged muscle due to the higher degree of fibrotic tissue.

Due to the importance of the plasminogen system and its downstream effectors in regulating fibrosis, it is imperative to further explore the underlying mechanisms by which this system works in skeletal muscle, especially in aged muscle. Therefore, this study examined the role of these proteins in modulating changes in the ECM acutely following damage in young and aged muscle.

## REVIEW OF THE LITERATURE

### **Skeletal Muscle Repair and Regeneration: An Overview**

Skeletal muscle plays an important role in overall health, longevity, and quality of life (26, 27). This organ has the unique ability to self-heal following damage and undergoes continuous repair on a day-to-day basis. The coordinated self-healing process is highly dependent upon the severity of damage, and is subtly tailored to cope appropriately (28). Muscle damage can range from minor plasma membrane rupture, myofibrillar disruption and Z-disk streaming to severe injury affecting whole muscle bundles, including blood vessels, nerves, and pericellular tissues (29). Minor damage is caused by exercise, particularly during eccentric (lengthening) contractions, causing the influx of plasma proteins into the damaged fibre, and the efflux of muscle proteins into the extracellular fluid and blood (30). In the case of minor damage, the muscle may only require the closure of the plasma membrane and repair of myofibrils (31).

On the other end of the spectrum, severe damage requires the necrosis of the muscle fibre as part of the regeneration process. The process of skeletal muscle regeneration after damage involves several important molecular pathways across different cell types (3–5, 32) and is coordinated in three overlapping stages. The stages are as follows: 1) inflammation and necrosis of damaged muscle fibres, 2) activation, migration, proliferation, differentiation, and fusion of resident stem cells called satellite cells, and 3) maturation of newly formed muscle fibres and remodeling of the regenerated fibre (3–6). The remainder of this section will outline each stage of skeletal muscle regeneration.

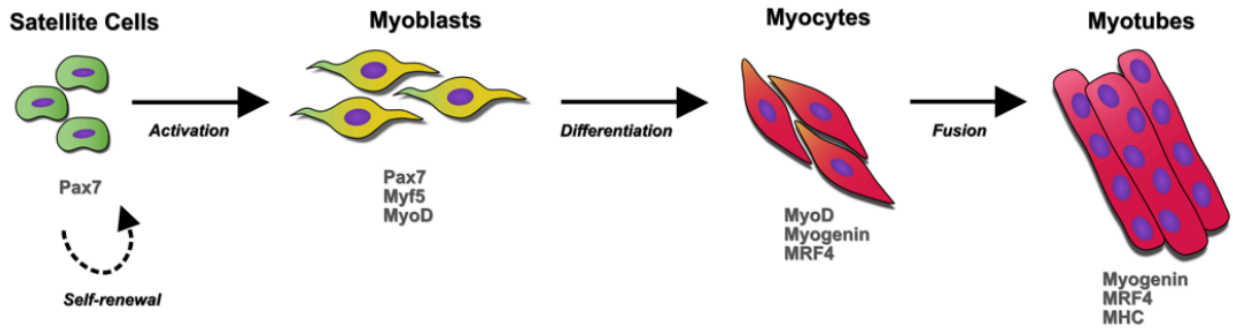
Immediately following muscle damage, disruption occurs at the plasma membrane of muscle fibres resulting in the accumulation of intracellular calcium ions. The presence of high levels of calcium within the muscle fibre triggers the activation of calcium-dependent proteases,

leading to the breakdown of intracellular components (33). Additionally, calcium ions inhibit normal cellular respiration of the mitochondria and overwhelms the calcium uptake capabilities of the sarcoplasmic reticulum (33). Together, these events cause cell death and subsequent leakage of intracellular proteins into the extracellular space. The accumulation of debris in the extracellular space serves as a chemotactic signal for immune cells, enabling the infiltration and initiating the inflammation stage of muscle regeneration (33).

Neutrophils are the first of the immune cells to arrive at the damaged muscle, with numbers increasing rapidly within the first hour following damage and remaining elevated for up to 5 days post-damage (34, 35). Neutrophils function to promote muscle damage and necrosis, through the secretion of reactive oxygen species (ROS), however the role of these cells in muscle regeneration is not essential (36). Rather the role of neutrophils in skeletal muscle regeneration is primarily through the attraction of M1 macrophages (36). M1 macrophages are pro-inflammatory cells that infiltrate the tissue one day after damage and with numbers peaking approximately two days post-damage. While these macrophages remove debris through phagocytosis, perhaps more importantly they express and secrete high levels of cytokines to signal for the amplification of the inflammatory response (37, 38). M2 macrophages are nonphagocytic anti-inflammatory cells that arrive after the pro-inflammatory M1 macrophage activity at the tissue. Concentrations of M2 macrophages peak at day 4 and remain elevated for the remainder of the regeneration process (39). M2 macrophages play an important part in alleviating the inflammation in the tissue to allow for the rest of the regeneration process to continue (39). M2 macrophages also release growth factors, cytokines, and extracellular proteases that promote the activation of satellite cells and remodeling of the interstitial space, enabling the regeneration process to proceed (15, 19).

The active regeneration of muscle fibres occurs with the help of resident muscle stem cells, called satellite cells. Satellite cells are mononucleated cells located between the basement membrane and the plasma membrane. In resting muscle, satellite cells are in a quiescent state but act as a reserve precursor to skeletal muscle cells (3, 4, 6). After muscle damage, satellite cells are stimulated by various signals arising from the environment, including signals secreted from macrophages during the latter portion of the inflammatory phase (3, 4, 41). Satellite cells then migrate to the site of damage and re-enter the cell cycle to proliferate (3). At this stage, satellite cells undergo either symmetric division to expand their reserve or asymmetric division to give rise to committed satellite cells, called myoblasts (Figure 1). A portion of the satellite cells pool will return to their quiescent state while the committed myoblasts will further differentiate into myocytes to aid in the regeneration of damaged fibres (3, 4). The newly formed myocytes can fuse together to form myotubes or fuse directly to the damaged muscle, donating their nuclei and replacing the necrotized tissue (4, 42, 43).

Satellite cells can be identified in unperturbed skeletal muscle and during the different stages of regeneration via immunohistochemical staining of various myogenic regulatory factors (MRFs). Quiescent satellite cells express the transcription factor Pax7 (3, 4) but once activated, the satellite cells (myoblasts) can express a combination of Pax7, Myf5, and/or MyoD (3). During this time, the myoblasts exit the cell cycle and differentiate into mature myocytes. The differentiation process is instigated by reduced expression of Pax7 and Myf5 and increased expression of myogenin and MRF4 (3). At the final step of the active regeneration process, the myocytes can fuse to form myotubes or fuse directly to the damaged muscle fibre. During this step, MyoD expression is greatly reduced while myosin heavy chain (MHC) and other contractile protein expressions increase (3).



**Figure 1.** Myogenic lineage progression during skeletal muscle regeneration. Following muscle damage, quiescent satellite cells ( $Pax7^+$ ) activate into myoblasts ( $Pax7^+$ ,  $Myf5^+$ , and  $MyoD^+$ ). Satellite cells can also self-renew to allow for the maintenance of the muscle stem cell pool. Following several rounds of proliferation, the active myoblasts differentiate into muscle cells called myocytes ( $MyoD^+$ ,  $myogenin^+$ , and  $MRF4^+$ ). Finally, myocytes can undergo fusion to form multinucleated myotubes ( $myogenin^+$ ,  $MRF4^+$ , and  $MHC^+$ ) that eventually mature into muscle fibres and express adult myosin heavy chain (MHC).

As the muscle completes regeneration (i.e. establishment of *de novo* myofibres), remodeling of the extracellular matrix (ECM) also takes place. The remodeling of the ECM is largely accomplished by fibroblasts, which become active and migrate to the site of damage (6). Fibroblasts temporarily stabilize the ECM by releasing proteins such as collagen, elastin, fibronectin, laminin, and proteoglycans that acts as a scaffold for new muscle fibres (6). During the different stages of skeletal muscle regeneration, the balance between ECM degradation and accumulation is critical for this process to occur normally. Earlier in the regeneration process, there is a need for the ECM degradation to enable the infiltration of immune cells. This is largely accomplished by a subfamily of matrixin proteins called matrix metalloproteinases (MMPs) that are secreted from immune cells, fibroblasts, and myoblasts (40, 44, 45). The interplay between MMPs and their inhibitors, called tissue inhibitors of MMPs (TIMPs), plays a crucial role in the remodeling of the ECM throughout the regeneration process, as will be discussed later.

The dysregulation of any of these events can severely hinder the regenerative potential of skeletal muscle that ultimately leads to impaired muscle health. Numerous studies have shown the

knockout of genes or pharmacological inhibition of a single factor regulating muscle regeneration to delay the process as a whole. For instance, the inability of immune cells to infiltrate the damaged tissue has functional consequences for skeletal muscle health, specifically the overstimulation of the immune cells can impair normal muscle regeneration (36, 46). The inability of satellite cells to activate, migrate, proliferate, differentiate or fuse can have a significant negative impact on the regeneration of damaged muscle fibres (20, 41, 47, 48). Finally, the inhibition or failed activation of MMPs can lead to the aberrant accumulation of fibrous tissue, which further impairs regeneration and skeletal muscle function (6, 38, 44, 49). In summary, each stage of muscle regeneration needs to occur in a timely fashion for the complete restoration of muscle structure and function.

### **Effects of Aging on Skeletal Muscle Regeneration**

Several physiological changes occur to skeletal muscle during aging leading to a gradual deterioration in skeletal muscle mass, termed sarcopenia (7). Sarcopenia is characterized by a decreased number of muscle fibres (particularly fast twitch fibres), decreased motor unit number, accumulation of fat within the muscle, fibrosis, and overall weakness (8). Several factors have been identified to cause sarcopenia, including neurodegeneration, endocrine factors, and nutritional status (7, 8). Anabolic hormones such as testosterone, growth hormone, and insulin-like growth factor-1 (IGF-1) tend to decline with age (7, 9, 50, 51). These hormones are responsible for increasing muscle protein synthesis, leading to increased muscle mass, while also reducing muscle protein breakdown (52, 53). The supplementation of exogenous hormones coupled with appropriate caloric and protein intake have been shown to slow and prevent sarcopenia (9–13).

The loss of muscle mass is associated with a sharp decline in the regenerative capacity of skeletal muscle with age. A link between a reduced satellite cell pool and poor regeneration in



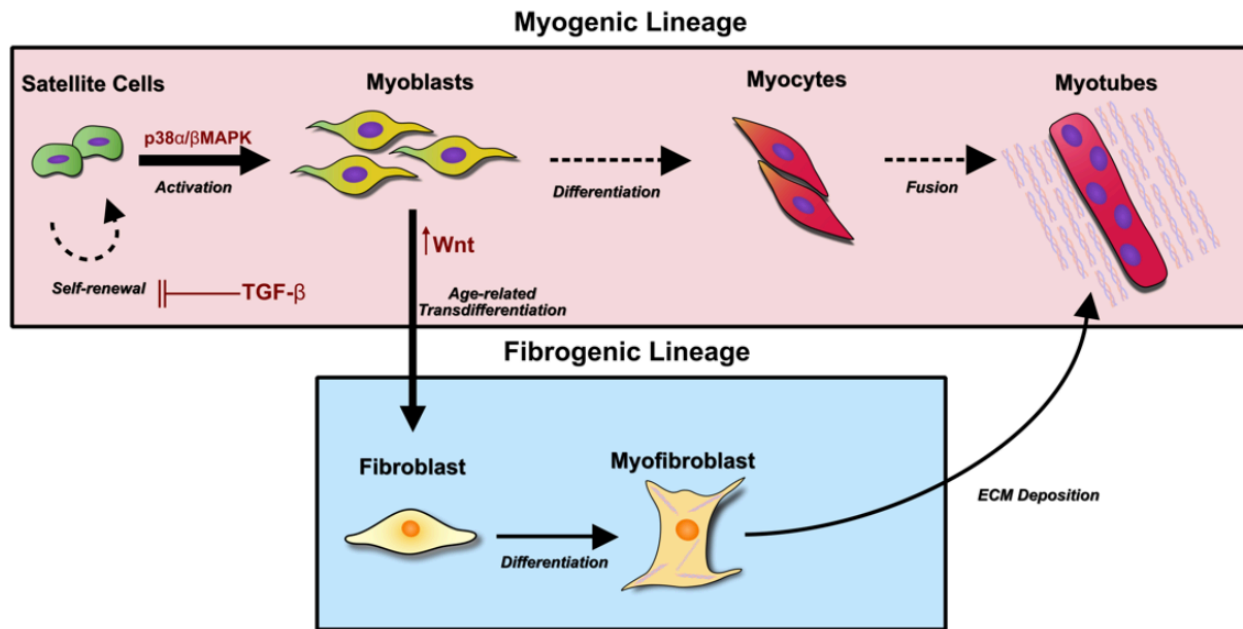
aged muscle has been indicated (54–56). The capacity of the satellite cells to support the maintenance of aged muscle tissue is thought to play an important role during the regeneration process. As a result, the reduced number of satellite cells observed in aged muscle may partially explain its poor regenerative potential (54). Asymmetric cell division and limited proliferation of satellite cells have been proposed as potential mechanism leading to a reduction of satellite cell number (57). However, the contribution of the reduced satellite cell pool and its implications in aged muscle has been debated (58). Although the absolute number of satellite cells is reduced with age, the density (number per total area) of these cells in the muscle tissue remains constant throughout the lifespan of murine models (41). The underlying cause of impaired satellite cell function displayed by aged muscle has been under considerable investigation, however it still remains unclear whether this is due to changes in the intrinsic cell program or a result of extrinsic factors (59).

An important intrinsic factor involved in the poor regeneration observed in aged muscle is the inability of aged satellite cells to return to quiescence. Evidence suggests that the p38 $\alpha$ / $\beta$ MAPK pathway is aberrantly active, causing a disruption in satellite cell proliferation, and asymmetric division, leading to the increased number of committed satellite cells and a loss of the satellite cell reserve for subsequent muscle regeneration (60, 61). Transplantation of old satellite cells into young muscle did not reduce p38 $\alpha$ / $\beta$ MAPK activity, suggesting that intrinsic signaling behaviour was no longer responsive to the extrinsic factors composing a youthful microenvironment (61, 62). Interestingly, pharmacological inhibition of p38 $\alpha$ / $\beta$ MAPK signaling restored satellite cell self-renewal through symmetric division and enhanced engraftment potential (61, 62). Overall, this indicates that there is likely some intrinsic dysfunction in satellite cells as a result of aging.

Contrary to the previous findings (61, 62), there is a strong body of evidence to suggest that the aberrant changes in satellite cell function with aging could be a result of changes in the microenvironment of the satellite cell (63–65). One of the changes noted in aged muscle is the chronic elevation of canonical Wnt ligands. The Wnt signaling pathway plays a crucial role in satellite cell differentiation, without which normal regeneration cannot occur (66). In aged muscle, the chronic elevation in Wnt ligands, such as Wnt3A, have been shown to alter the satellite cell lineage from myogenic-to-fibrogenic (67). The addition of Wnt3A to young serum, derived from young mice blood, induced a myogenic-to-fibrogenic conversion of young satellite cells, *in vitro* (67). Conversely, the myogenic-to-fibrogenic conversion by aged serum was prevented with the addition of Wnt inhibitors (67). In regenerating muscle, the direct injection of Wnt3A one day following damage resulted in increased fibrous tissue disposition, similar to that of aged regenerating muscle, while the injection of a Wnt inhibitor resulted in enhanced myogenic cell proliferation (67).

Further investigation into the differences in the microenvironment show the expression of several cytokines and growth factors to be modified in aged muscle. Members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily are one of many important growth factors that are overexpressed in aged animals, and contributes to the dysregulation of the microenvironment (68). TGF- $\beta$  works through the phosphorylation of Smad3 on fibroblasts, which upregulates several cyclin-dependent kinase inhibitors, eventually leading to the deposition of fibrous tissue (69). Activation of the TGF- $\beta$ /Smad pathway has also been shown to cause the differentiation of fibroblasts to myofibroblasts (17). In satellite cells, TGF- $\beta$ -mediated activation of Smad3 opposes the Notch signaling pathway, and as a result, leads to impairments in self-renewal and quiescence

(69). Both the pharmacological inhibition of TGF- $\beta$ /Smad activity and the forced activation of Notch signaling in aged muscle was found to restore its regenerative potential (65,69).



**Figure 2.** Age-related myogenic-to-fibrogenic lineage conversion. Chronic elevation in TGF- $\beta$  in aged muscle has been shown to impair self-renewal of satellite cells, and when coupled with overactivation of p38 $\alpha$ / $\beta$ MAPK signaling leads to the over commitment of satellite cells and a depletion of the satellite cell pool. The elevation in Wnt signaling causes an age-related transdifferentiation or conversion of myoblasts to fibroblasts, which then differentiate to myofibroblasts, contributing to ECM deposition.

Gene expression analyses demonstrate that aged myoblasts display a major alteration in the expression of many genes directly or indirectly involved in the TGF- $\beta$  signaling pathway (70). The effects of TGF- $\beta$ /Smad signaling include the upregulation of an important protein regulator of fibrosis called plasminogen activator inhibitor-1 (PAI-1) (71, 72). PAI-1 is the primary inhibitor of the plasminogen system, and consequently inhibits degradation of the ECM. Therefore, chronic elevation of PAI-1 has been shown to cause the accumulation of connective tissue (23). Inhibition of TGF- $\beta$ /Smad was shown to significantly reduce the expression of profibrogenic genes, including PAI-1, and thus demonstrating a potential mechanism in reducing fibrosis (72).

However, the role of PAI-1 in aged muscle, specifically during regeneration requires further research.

### **Role of the ECM and the Plasminogen System in Muscle Regeneration**

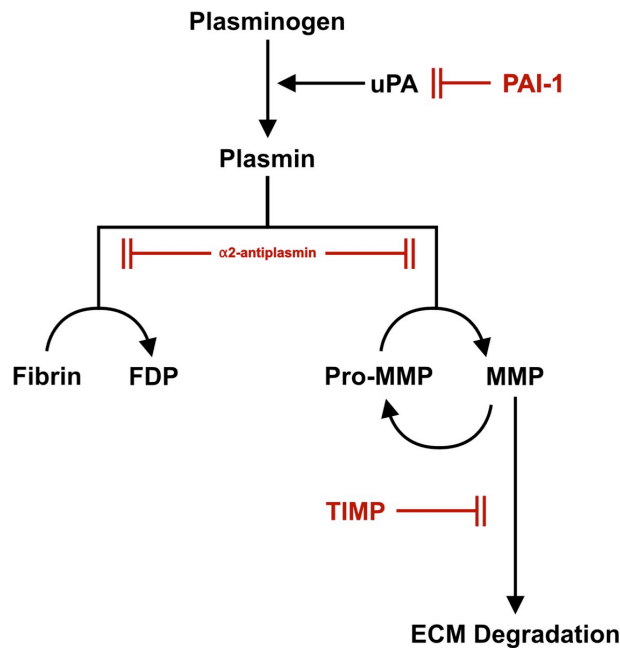
The extracellular matrix (ECM) plays a central role in maintaining structural integrity of skeletal muscle. The breakdown and synthesis of several ECM protein components have been observed following damage to muscle fibres (73). As previously mentioned, skeletal muscle regeneration requires the activation, migration, proliferation, differentiation, and fusion of satellite cells at the site of damage (4). Recently, there has been strong evidence that points to the ECM playing an integral role in normal satellite cell function during regeneration (74). The ECM surrounding the muscle is composed of fibrous and linking proteins such as collagen, laminin, fibronectin, and integrins (75). These proteins form complexes that not only anchor cells, but also allow cells to sense their surrounding and coordinate behavior among themselves.

The remodeling of the ECM during regeneration is important in restoring strength and morphology of the muscle following damage, and has been reviewed in detail (5, 6, 38). The remodeling process follows a similar pattern to the overall regeneration process. After damage, degeneration of the ECM occurs by actions of proteases secreted by muscle fibres, immune cells and activated satellite cells (36, 40, 44). ECM protein fragments and growth factors are released during ECM degeneration, allowing the migration of immune cell and satellite cells (6, 38). Furthermore, the reconstruction of the ECM during the latter portion of muscle regeneration allows for the entrapment of growth factors, terminating the signals needed for migration, and allowing differentiation of satellite cells to occur (6, 38, 74). Transcriptional profiles of cardiotoxin-induced damage shows a temporal pattern of gene expression of matrix degrading enzymes (MMPs) followed by the upregulation of ECM protein components of the basement membrane (76).

One of the contributors to the remodeling of the ECM is the plasminogen system. The plasminogen system is a proteolytic system responsible for the breakdown of fibrous tissue and has received considerable attention in cancer, thrombosis, pulmonary and kidney fibrosis, along with other inflammatory conditions (14, 23, 77–85). Over the last two decades, the effects of this system on ECM remodeling in skeletal muscle, particularly during regeneration, has been a topic of interest (20, 22, 84, 86, 87). The primary agonist of this system is plasmin, which is the active form of plasminogen. Two activators have been identified to catalyze the conversion of plasminogen to plasmin: tissue-specific plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (23, 88). uPA appears to be the primary activator in skeletal muscle and is secreted by macrophages during regeneration (86, 88). uPA activity is not limited to the activation of plasminogen, but it also binds to a well-characterized uPA receptor (uPAR), however, the interaction between uPA/uPAR is not required for normal muscle regeneration as observed in uPAR null mouse models (84).

The active substrate of the plasminogen system, plasmin, has been identified to have two general functions. Firstly, plasmin plays a central role in proteolytic cleavage of ECM proteins, primarily fibrin (23, 24). Secondly, plasmin activates a class of zinc-containing endopeptidases called matrix metalloproteinases (MMPs) to aid in the degradation of the ECM (23, 24). The combined activity of the plasmin and MMPs have been shown to activate several growth factors entrapped in the ECM, including TGF- $\beta$ , hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF). All of these growth factors are important in cell migration and tissue remodeling (89–92), and due to their modulation by plasmin/MMP, it is clear that the role of the plasminogen system can impact the progression of remodeling, including that of skeletal muscle (Figure 3).

Several pathological conditions can arise with the unrestricted activation of proteolytic enzymes, and this is usually the case in cancer cell migration (77–80, 85). As a result, a protein inhibitor tightly regulates each protein of the plasminogen system. PAI-1 is one of the main inhibitors of the plasminogen system. PAI-1 inhibits the activity of uPA, preventing the activation of plasminogen and thus reducing proteolytic breakdown of the ECM (20, 22). At the plasmin level,  $\alpha$ 2-antiplasmin inhibits the activation of MMP and degradation of fibrin. Finally, at the MMP level, tissue inhibitors of metalloproteinases (TIMPs) inhibit MMP function while simultaneously preventing the self-activation of MMPs (23, 25). The inhibition of the plasminogen activator protein, uPA, by PAI-1 is thought to be the most important inhibitor of the system due to its upstream location within the system. In other words, the inhibitory role of PAI-1 is likely enough to reduce the proteolytic activity of all the downstream proteins, including MMPs.



**Figure 3.** The plasminogen system. Plasminogen is the inactive substrate of the plasminogen system that is activated into plasmin primarily by uPA in skeletal muscle. Plasmin can directly degrade fibrin into fibrin degradation products (FDPs) and activate MMPs. The MMPs work to degrade connective tissue in the ECM and also activate additional MMPs in a positive feedback loop. PAI-1,  $\alpha$ 2-antiplasmin, and TIMPs work to inhibit the activation and function of the proteins uPA, plasmin, and MMP, respectively.

## **Role of MMP/TIMP Balance in Muscle Regeneration**

Various types of protease are involved in ECM remodeling, but the main enzyme class are the MMPs (93). MMPs have been shown to play an important role in the remodeling of the ECM in many tissues of the body (20, 88, 94–96). MMPs are divided into eight different categories based on their function. MMP-2 and MMP-9 are members of the gelatinase group and are most predominant during skeletal muscle regeneration (44, 97–99). Both of these proteins directly degrade denatured type I collagen, II and III collagens, and native type IV and V collagens (44, 100). MMP-2 is also able to degrade elastin and has low activity with fibronectin (101). The temporal expression of these MMPs changes throughout the regeneration process to control ECM turnover.

MMP-9 expression is upregulated during the first three days after cardiotoxin injection, as indicated by Western and Northern blot analyses (44). MMP-9 activity significantly dropped after three days and was absent within a week (44). The expression of MMP-9 correlated with the inflammatory stage of regeneration and during the active proliferation of satellite cells (44). Further investigation showed MMP-2 to be significantly upregulated between the third and tenth day after cardiotoxin injection, with peak activity on day seven. The temporal expression of MMP-2 correlated with the migration, proliferation, and fusion of satellite cells (44). Ten days post-injection, when myoblast fusion is completed, MMP-2 levels return to baseline. This suggests that MMP-2 likely plays a role in myoblast fusion, particularly in the degeneration of type IV collagen found in the basement membrane (44).

Tissue inhibitors of MMPs (TIMPs) aid in the regulation of ECM degradation by inhibiting the proteolytic function of MMPs. Chronic high levels of TIMPs cause the accumulation of connective tissue (fibrosis), whereas the loss of TIMPs lead to the enhanced degradation of the

ECM (102). Although there is limited information on the specific role of TIMPs in muscle regeneration, Lewis et al. (2000) observed a partial role of TIMP-1 in myoblast fusion. Similar to the temporal expression of MMP-2 and MMP-9 during regeneration, TIMP-1 was also found to follow a similar expression pattern (103). *In vivo*, TIMP-1 mRNA expression was undetectable during the earlier stages of regeneration, specifically when myoblasts were in their single cell stage (103). However, as regeneration continued, TIMP-1 expression increased until it reached a peak during myotube formation (103). The overall expression of TIMP-1 seemed to be during the latter portion of regeneration, allowing for the accumulation of ECM proteins to anchor the newly formed muscle fibre (103).

### **Implications of MMP/TIMP Balance in Aged Muscle**

The remodeling of the ECM is partially regulated by the plasminogen system, with PAI-1 being the primary inhibitor of this system (20, 23, 44, 104). The effect of plasmin in ECM remodeling has been discussed previously, with its downstream effectors, MMP, playing an important role. The balance between MMPs and TIMPs has been shown to contribute to the efficient regeneration of skeletal muscle following damage (95, 99, 103), however, these factors have been shown to change during aging (6, 63, 67, 68). With age, the regenerative potential of skeletal muscle declines and is accompanied by fibrosis (67). Elevations in pro-fibrotic growth factors and proteins have been investigated in aged skeletal muscle, specifically TGF- $\beta$  and PAI-1 (18, 21, 68). As a result, there is likely a shift in the MMP/TIMP ratio favouring TIMPs and thus contributing to fibrosis in aged muscle. Presently, the intricate relationship between the plasminogen system and MMP/TIMP balance during skeletal muscle regeneration remains unclear, particularly in aged muscle. Furthermore, the function of PAI-1 and its downstream proteases has yet to be elucidated in damaged skeletal muscle.



## **Clinical Significance of the Plasminogen System in Aged Muscle**

The aging population has seen a significant increase over the last decade in Canada. The seniors population is projected to soon become larger than the child population, and this trend continues to increase as technology and health care improves (105). An important, yet often overlooked component of health, longevity, and quality of life is the effects of maintaining healthy muscles (26, 27). Aged muscle has been shown to display several aberrant changes. Fibrosis in skeletal muscle has historically been understudied and often overlooked compared to other age-related changes to skeletal muscle, particularly following damage. However, due to the importance of skeletal muscle health in maintaining overall health, there is a need for a more comprehensive understanding of the modifications in skeletal muscle tissue with age.

## PURPOSE AND OBJECTIVES

Due to the importance of the plasminogen system and its downstream effectors in regulating fibrosis, it is imperative to further explore the underlying mechanisms by which this system works in skeletal muscle, specifically in aged muscle. Therefore, this project will have two primary objectives:

1. to better understand and characterize the physical changes that occur in the extracellular environment throughout the regeneration process in young and aged muscle, and
2. identify the expression pattern and localization of important proteins of the plasminogen system in young and aged muscle.

To test these objectives, five research questions were developed. The research questions and hypotheses are as follows:

Research Question #1: To what extent is the regenerative capacity impaired in aged muscle as indicated by embryonic myosin heavy chain (eMHC) expression?

Hypothesis #1: It was hypothesized that aged muscle would display fewer, and smaller regenerating myofibres compared to young muscle at 5 and 7 days following damage.

Research Question #2: How does the ECM change in young and aged muscle throughout the regeneration process.

Hypothesis #2: It was hypothesized that aged muscle would become fibrotic during regeneration as demonstrated by greater expression of the major ECM protein constituents collagen I and IV, and fibronectin.

Research Question #3: How does the presence of macrophages change throughout the regeneration process in aged muscle and is it consistent with the changes that occur with ECM remodeling?

Hypothesis #3: It was hypothesized that aged muscle would display greater macrophage content at all time points following damage.

Research Question #4: What is the expression pattern and localization of the upstream inhibitor of the plasminogen system, PAI-1, in young and aged muscle?

Hypothesis #4: It was hypothesized that aged muscle would display a greater overall expression of PAI-1 (extracellular and intracellular).

Research Question #5: What is the expression pattern and localization of the fibrinolytic enzyme MMP-9 in young and aged muscle?

Hypothesis #5: It was hypothesized that young muscle would display greater overall expression of MMP-9 acutely following damage – contributing to an efficient remodeling of the ECM.

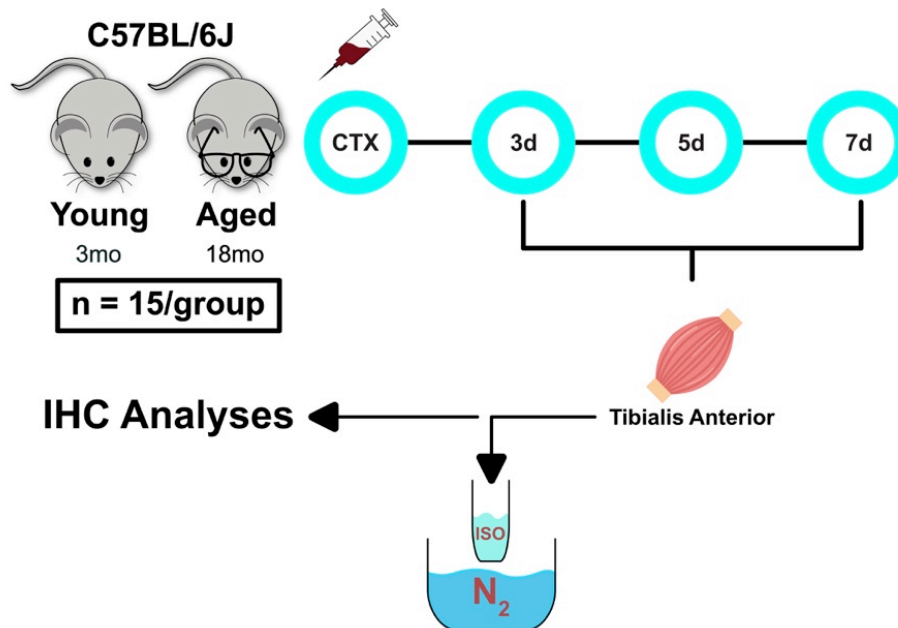
## METHODS

### Animal Care

Male C57BL/6J mice were purchased at 8 weeks of age from Jackson Laboratory (Bar Harbor, ME). Mice were divided into two groups (n = 15 per age group): young (3 months) and aged (18 months). These groups were then subdivided into three groups to investigate muscle regeneration (n = 5 per subgroup): 3, 5, and 7 days post-damage. Male mice were used to avoid the confounding variable of the estrous cycle which impacts the expression of many hormones and/or growth factors (106).

The animal room was maintained at 22°C, 50% humidity and a 12-h/12-h light-dark cycle. All mice had access to standard mouse chow and water ad libitum.

The University of Windsor Animal Care Committee approved all experimental protocols.



**Figure 4.** Skeletal muscle damage and tissue collection protocol. Fifteen C57BL/6J mice received a CTX injection to the left tibialis anterior muscle. Mice were euthanized and muscles were collected at 3, 5, and 7 days following damage. A series of histochemical and immunohistochemical stains were performed.

## **Skeletal Muscle Damage and Tissue Collection**

Skeletal muscle damage was induced with a 50  $\mu$ L intramuscular injection of 10uM cardiotoxin (CTX; L8102, Latoxan, France) to the left tibialis anterior (TA) muscles of the mice. The right TA was uninjured to serve as a control. Following the specified regeneration period, animals were euthanized using carbon dioxide inhalation followed by cervical dislocation. Injured and uninjured TA muscles were extracted, weighed, and mounted with optimum cutting temperature (OCT) embedding compound on a flat piece of cork. Tissue samples were snap frozen in isopentane cooled by liquid nitrogen and stored in a -80°C freezer until further analyses.

## **Histochemical and Immunohistochemical Analyses**

Ten-micron skeletal muscle cross-sections were cut using a Leica CM1860 UV cryostat (Leica Microsystems Inc., Concord, ON, Canada) and were mounted on glass slides. The subsequent stains are outlined below.

*Hematoxylin and eosin:* hematoxylin and eosin (H&E) stains were used to identify the basic morphology of the muscle following CTX-induced muscle damage (complete protocol found in Appendix A). This stain was used to identify regions of necrotic and regenerating muscle and provided information on the severity of muscle damage during at the specific time points.

*Immunofluorescence:* Immunostaining was performed to determine the expression pattern and localization of select proteins (see Appendix B for antibody list and working concentrations). Muscle sections were fixed using ice-cold 2% paraformaldehyde (PFA) for 5 minutes at 4°C and blocked with 5% normal goat serum (NGS), and 0.1% Triton-X100 in neutral PBS for 1 hour at room temperature. In the case of mouse-on-mouse reactivity, mouse IgG block (BMK2202, Vector Laboratories Inc., Burlingame, CA, USA) was applied according to manufacturer's instructions for 1 hour at room temperature. This was followed by a blocking solution of 10% NGS, 1.5%

bovine serum albumin (BSA), and 0.2% Triton-X100 in neutral PBS for 1 hour at room temperature. Primary antibodies were applied and stored at 4°C overnight in a humidified chamber. Primary antibodies were discarded and appropriate secondary antibodies were applied along with 4,6-diamidino-2-phenylindole (DAPI) to identify nuclei. A dilution of TrueVIEW (SP-8400, Vector Laboratories Inc., Burlingame, CA, USA) was used to reduce autofluorescence prior to applying aqueous mounting medium and a cover slip. Specifics of the protocol can be found in Appendices C-F.

### **Slide Scan Analysis**

Whole muscle sections were scanned using a Zeiss Axio Scan.Z1 slide scanner (Carl Zeiss Canada Ltd., Toronto, ON, Canada). Necrotic and regenerating areas were manually measured using the H&E stained sections. Regenerating areas were easily identified due to the myofibres' smaller size and centrally located nuclei. Necrotic regions were identified by the presence of degenerating (i.e. necrotic) myofibres (fragmented borders, lighter H&E stain intensity, and >2 nuclei within the myofibre). Undamaged regions were omitted from the analyses to control for uneven penetration of CTX throughout the muscle.

### **Image Analysis**

Images of stained sections were obtained using a Nikon 90i eclipse microscope (Mississauga, ON, Canada) and analyzed using Nikon Elements software. Five to twenty evenly spaced images were taken within the necrotic and regenerating areas of the muscle. Exposure time remained consistent between slides during the imaging process to allow for comparison of brightness values, and thus, provided data to for the comparison of apparent protein content (particularly useful for myocellular PAI-1 content). Alternatively, some analyses used signal thresholding as a detection method; these analyses included the determination of collagen,

fibronectin, PAI-1, and MMP-9 positive area within the muscle sections. In all staining procedures, a negative control section (no primary antibody applied) was performed and used to set the signal threshold settings. In all cases, the negative control sections displayed no signal. To determine macrophage density, cells were identified using F4/80 cell surface marker and DAPI. Cells were then manually counted within the necrotic and regenerating areas.

### **Data and Statistical Analysis**

A two-way ANOVA was used to analyze for potential differences in dependent variables between young and aged mice at different time points following induction of muscle damage. The independent variables were age (young or old) and time following muscle damage (3, 5, or 7 days post-damage). Main effects and interaction were considered significant at  $p < 0.05$ . If a significant interaction was found, a subsequent simple main effects analysis with Bonferroni adjustment was performed to determine the effect of both independent variables on the dependent variable (107). T-test analyses were used to analyze for potential differences between young and old in the contralateral undamaged TA muscle (where no time post-CTX was not relevant). All data, including those depicted in figures, are provided as mean  $\pm$  standard deviation.

## RESULTS

### Baseline and Post-CTX Characteristics

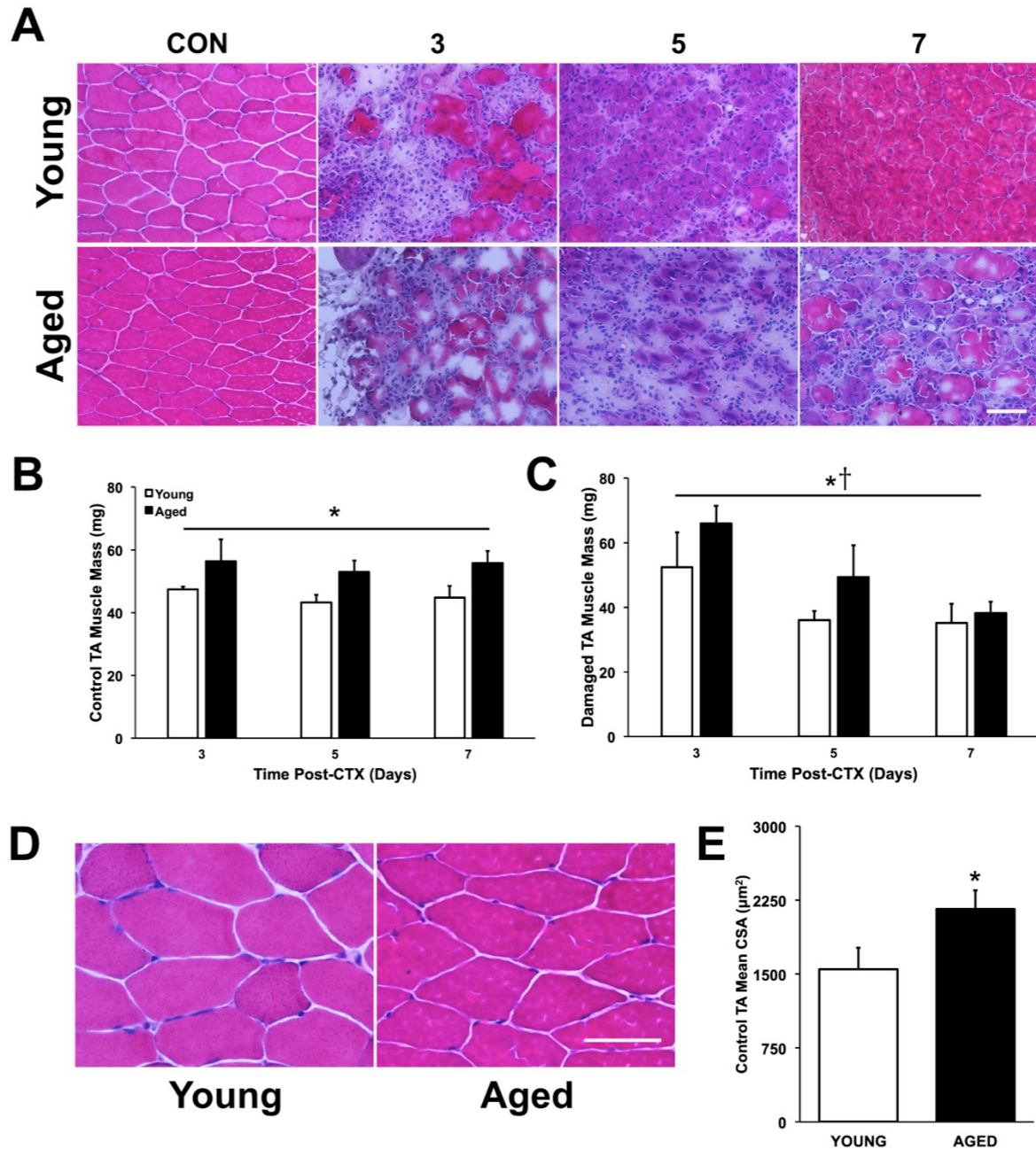
Body masses of mice were measured prior to induction of muscle damage (pre-CTX), and during the time of muscle collection (post-CTX). Aged mice had a greater body mass compared to young mice regardless of time point (main effect of age:  $p < 0.05$ ). Body mass of mice post-CTX was significantly lower in both groups compared to their baseline measures. Body mass of aged mice declined from  $32.7 \pm 0.43\text{g}$  to  $32.0 \pm 0.29\text{g}$  ( $p < 0.05$ ), while young mice declined from  $25.7 \pm 0.49\text{g}$  to  $25.1 \pm 0.49\text{g}$  ( $p < 0.05$ ). Assessment of the uninjured contralateral leg revealed a significantly larger TA muscle mass in aged mice ( $p < 0.05$ ) with no notable change between recovery time points ( $p > 0.05$ ; Figure 5A, B). Mean cross-sectional area (CSA) of the uninjured TA muscle was found to be larger in aged mice as well (Figure 5D, E).

Three days following CTX-induced damage, muscle mass increased in both groups. Aged TA muscle mass increased from  $56.4 \pm 6.9\text{mg}$  in the undamaged leg to  $66.0 \pm 5.4\text{mg}$  in the damaged leg, while young TA muscle mass increased from  $47.4 \pm 0.9\text{mg}$  to  $52.4 \pm 6.7\text{mg}$  (Figure 5B, C). Between 5 and 7 days following damage, TA muscle mass in both groups declined below their contralateral control measures. At the 7-day time point, aged muscle mass declined to  $38.2 \pm 3.5\text{mg}$  while young muscle mass was reduced to  $35.2 \pm 5.9\text{mg}$  (Figure 5C).

### Assessment of Regenerative Capacity

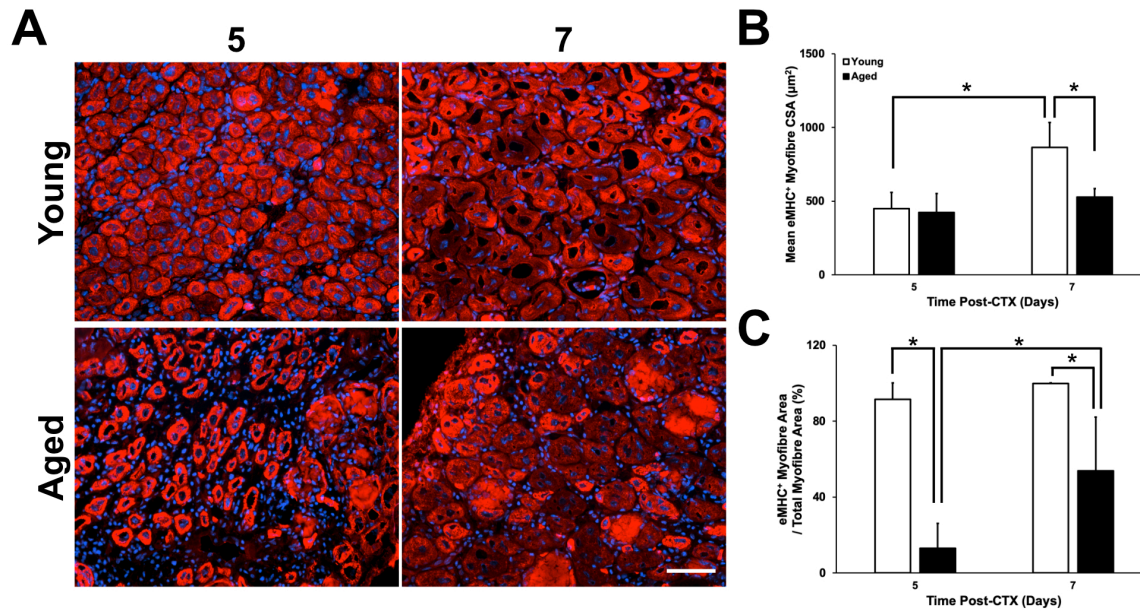
The active regeneration stage involves the *de novo* formation of myofibres from the fusion of myoblasts (Figure 1). The regenerating myofibres express a premature isoform of myosin heavy chain (MHC), embryonic myosin heavy chain (eMHC), before maturing and expressing adult MHC isoforms. The newly formed myofibres contain centrally located nuclei, which can then be used in conjunction with eMHC<sup>+</sup> cells to identify regenerating myofibres.





**Figure 5.** Aged muscle is unable to return to its original morphology following CTX-induced muscle damage. (A) H&E-stained cryosections of the TA muscle throughout the recovery time course demonstrate slow regeneration and persistent myofibre necrosis. (B) Control (uninjured) and (C) damaged TA muscle masses measured and collected at their respective post-CTX time points. (D) Magnified image of young and aged of the contralateral control (undamaged) TA muscle used to assess baseline CSA. (E) Control aged TA muscle were observed to have a greater mean CSA compared to young, similar to TA muscle mass data (B). \* denotes a significant main effect / difference of age (young vs. aged;  $p < 0.05$ ). † denotes a significant main effect of recovery time point following damage ( $p < 0.05$ ). Scale bar represents 50  $\mu\text{m}$ .

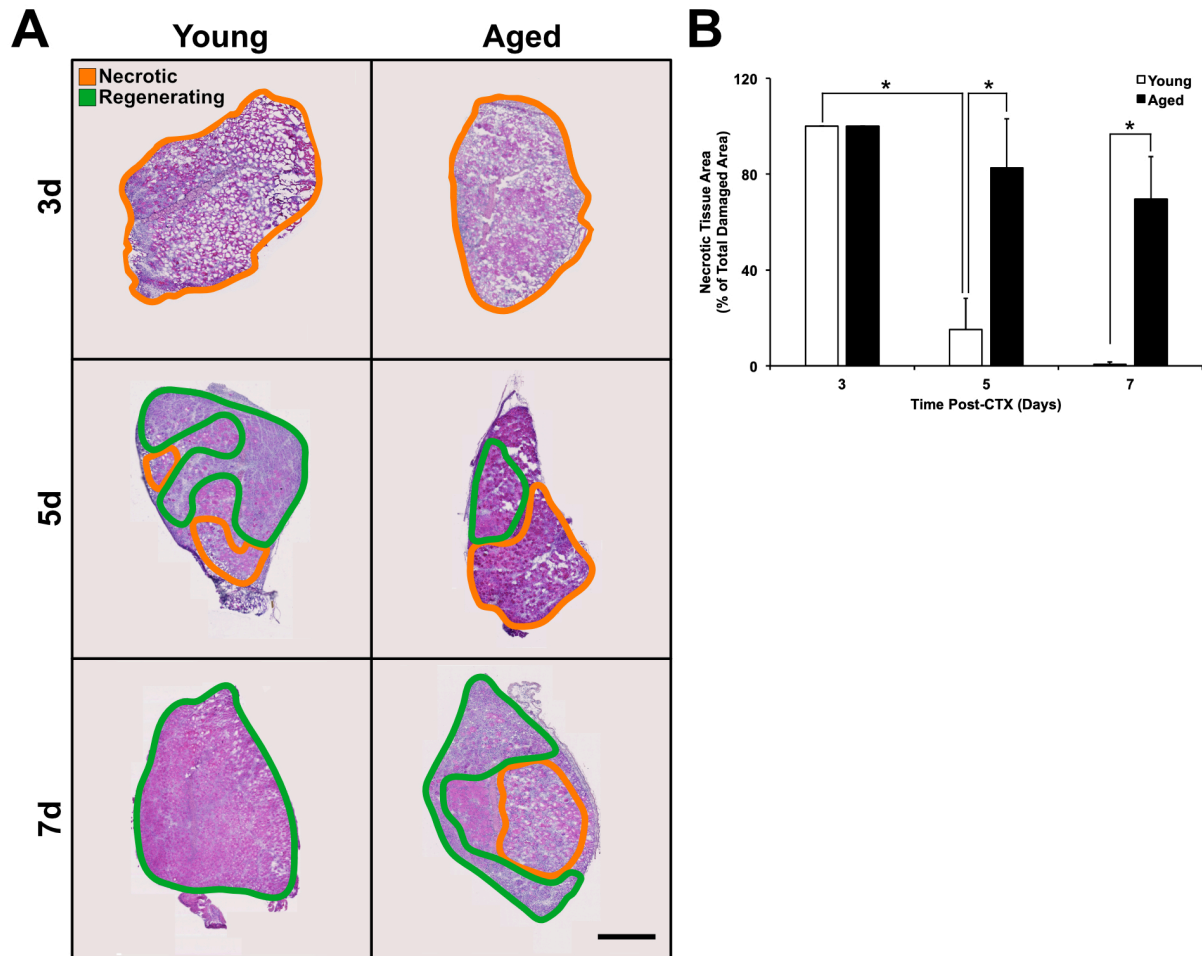
Thus, to assess the regenerative capacity of aged skeletal muscle, CSA of eMHC+ (regenerating) myofibres was analyzed. Regenerating myofibres were not present at 3 days following damage in young and aged muscle, as a result, the analysis was only conducted at the 5 and 7-day time point. Results from the analysis demonstrated no differences in mean myofibre CSA between groups at 5 days following damage ( $p>0.05$ ), however, young regenerating myofibre CSA increased significantly between 5 and 7 days following damage ( $p<0.05$ ; Figure 6A, B). A similar increase in CSA was not observed in aged muscle ( $p>0.05$ ), and at 7 days following damage, the CSA of young regenerating myofibre exceeded that of the aged regenerating myofibre ( $p<0.05$ ). Further investigation into the proportion of area occupied by regenerating myofibres relative to total myofibre area was used to determine the regenerative response of the muscles following damage (Figure 6C). Total myofibre area was defined as the summated area occupied by eMHC+ regenerating myofibres plus the summated area occupied by necrotic myofibres. The results from this analysis showed a significantly greater regenerative response in young muscle, as indicated by roughly 92% of the total myofibre area occupied by regenerating myofibres compared to only 13% in aged muscle ( $p<0.05$ ; Figure 6C). Nearly all the myofibre area present 7 days following damage was regenerating in young muscle, while 54% were regenerating in aged muscle. Although aged muscle displayed a significant increase between 5 and 7 days following damage in aged muscle ( $p<0.05$ ), it was not the same degree as young muscle. This suggests a delayed regenerative response exhibited by aged muscle following CTX-induced muscle damage.



**Figure 6.** Regeneration is impaired in aged skeletal muscle. (A) Regenerating myofibres were identified via eMHC (red) and DAPI (blue) at 5 and 7 days following damage. No expression of eMHC was found at 3 days, indicating the lack of regenerating myofibres in both groups. (B) Regenerating myofibre CSA was found to increase from 5 to 7 days in young mice, and there was a significant difference at 7 days between young and aged muscle (interaction and simple main effect:  $p < 0.05$ ). (C) Relative regenerating myofibre area was significantly lower at 5 and 7 days in aged muscle compared to young (interaction:  $p < 0.05$ ). Additionally, relative regenerating myofibre area increased significantly in aged muscle from 5 to 7 days following damage. The \* indicates significant differences detected by a simple main effects analysis ( $p < 0.05$ ). Scale bar represents 50  $\mu\text{m}$ .

H&E stained muscle cross-sections were inspected to assess the proportion of necrotic area relative to total damaged area. Necrotic regions are characterized by the presence of degenerating myofibres and an absence of newly regenerated myofibres containing centrally located nuclei (20, 108, 109). Furthermore, areas containing uninjured myofibres were not included in the analysis. The area observed at 3 days following damage was entirely necrotic in both young and aged muscle (Figure 7A, B). Between 3 and 5 days following damage, young muscle exhibited a significant decline in necrotic area ( $p < 0.05$ ); however, the same was not observed in the aged muscle ( $p > 0.05$ ). Additionally, at 5 and 7 days following damage, young muscle was found to have significantly

less necrotic area compared to aged muscle ( $p<0.05$ ). Due to the difference in the necrotic and regenerating area, all subsequent analyses were characterized with respect to these distinct regions.



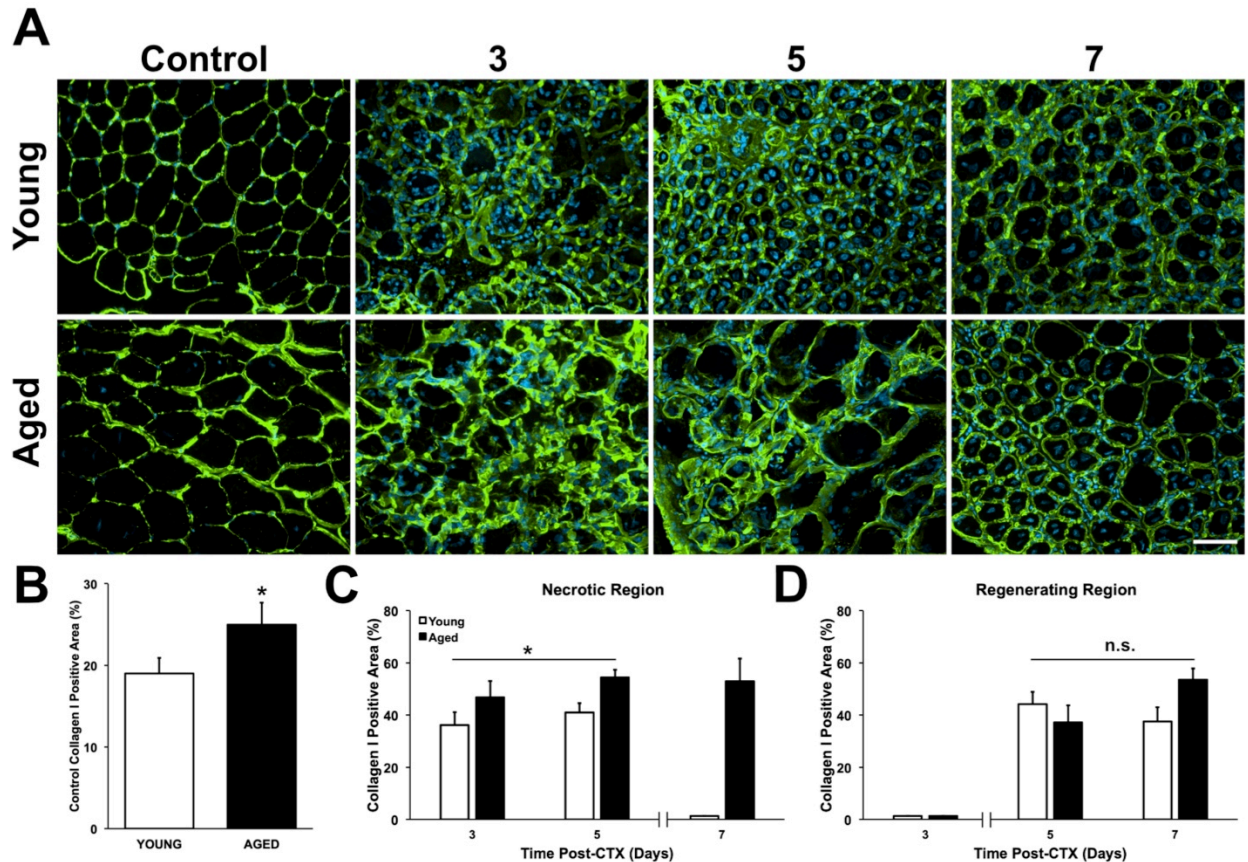
**Figure 7.** Necrotic regions persist throughout the regeneration process in aged muscle. (A) Representative images of whole muscle sections undergoing regeneration at their respective time points. (B) The entire TA cross section was necrotic at 3 days following damage in young and aged muscle, however, necrotic area decreased significantly young muscle only at 5 days following damage (interaction:  $p<0.05$ ). Young muscle displayed significantly lower necrotic regions 5 days following damage, and virtually no necrotic regions by 7 days following damage. The \* indicates significant differences detected by a simple main effects analysis ( $p<0.05$ ). Scale bar represents 500  $\mu\text{m}$ .

### Assessment of ECM Remodeling

The aberrant changes leading to fibrosis has been previously shown to be a result of the accumulation of structural extracellular protein content, specifically collagen (110–112).

However, a limitation of the past studies is that collagen isoforms were not differentiated, thus, it remains unknown whether changes in the ECM is due to one specific or multiple collagen isoforms. Different isoforms of collagen serve different roles in skeletal muscle. For instance, collagen I is the most abundant form of collagen within the body, and is distributed across the entire muscle tissue, providing structural support to the myofibres and the entire muscle group. Collagen IV is specific to the basement membrane surrounding individual myofibres, and as a result, only provide structural support for individual myofibres while also forming a critical component of the satellite cell niche (74,113,114). Although several other collagen types are expressed in skeletal muscle tissue, collagen I and IV were selected for study because of their well-characterized and canonical roles in skeletal muscle. To assess the effect of age on collagen I and IV levels following muscle damage, percent area positive for these collagen isoforms was quantified in both the necrotic and regenerating regions.

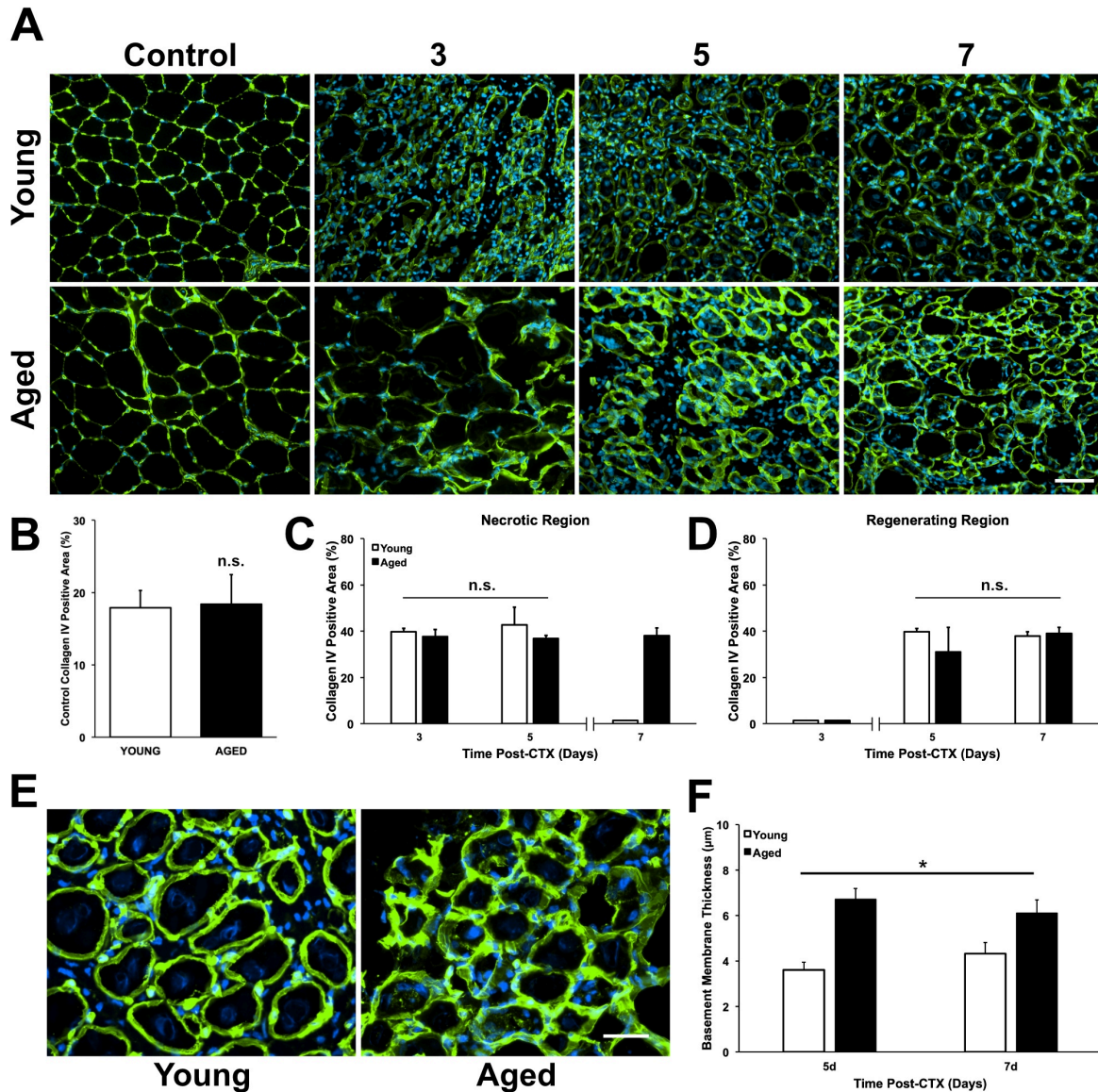
In control TA muscles, collagen I content was found to be greater in the aged group compared to young ( $p < 0.05$ ; Figure 8A, B). Following CTX-induced muscle damage, collagen I content increased in both young and aged muscle, however, collagen content within aged muscle remained significantly greater in the necrotic regions (Figure 8C). No statistically significant differences were observed in the regenerating region ( $p > 0.05$ ). Nonetheless, the accumulation of collagen I at rest and acutely following damage is consistent with previous studies examining collagen isoforms (67, 110). Due to the selected time points, it is uncertain whether this trend would continue beyond 7 days, potentially leading to the accumulation of collagen I in the regenerating region in aged muscle.



**Figure 8.** Collagen I deposition is greater in the necrotic regions of aged muscle. (A) Immunostaining of collagen I (green) and DAPI (blue) at each time point following damage. (B) Collagen I positive area in the undamaged contralateral leg was observed to be greater in aged muscle compared to young ( $p < 0.05$ ; indicated by \*). (C) Percent area of collagen I was greater in the necrotic region of aged muscle at 3 and 5 days following damage. (D) No significant differences were observed in the regenerating regions, 5 and 7 days following damage. \* denotes a significant main effect of age (young vs. aged) ( $p < 0.05$ ). Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 50  $\mu\text{m}$ .

Collagen IV was investigated to determine the integrity of the basement membrane following CTX-induced muscle damage. Area analysis of collagen IV showed no difference in control or damaged TA muscles (Figure 9A-D) between young and aged mice. Although no differences in percent area were found, notably thicker and irregularly structured collagen IV bands were found surrounding regenerating myofibres in aged muscle (Figure 9E). Analysis was undertaken to determine the thickness of the collagen IV bands around regenerating myofibres. To

assess this, five equally spaced lines were drawn around each regenerating myofibre spanning the distance of the basement membrane. Results showed a significantly thicker collagen IV bands around aged regenerating myofibres compared to young ( $p < 0.05$ ; Figure 9F). The thicker and irregularly shaped collagen IV bands may be a result of the incomplete breakdown and/or accumulation of collagen IV.

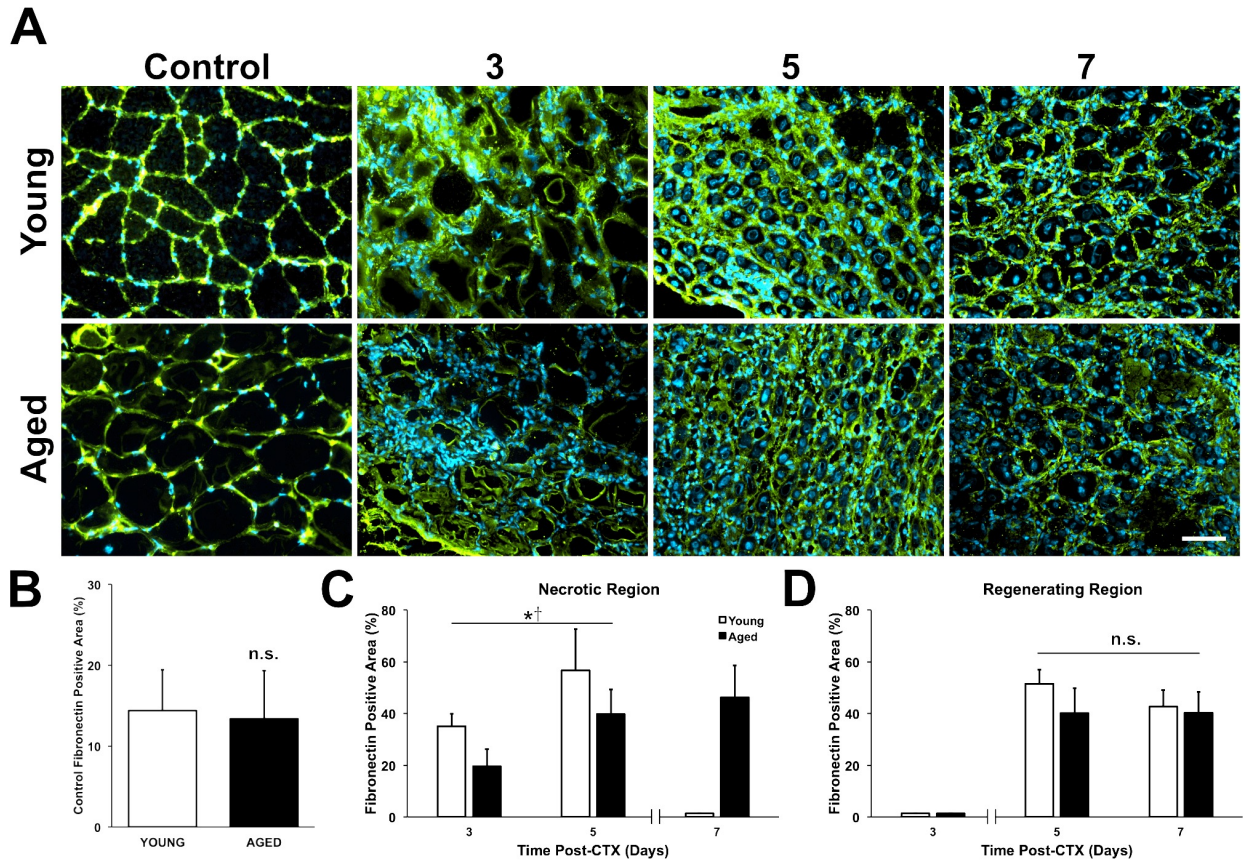


**Figure 9.** Collagen IV deposition does not change following CTX-induced muscle damage. (A) Immunostaining of collagen IV (green) and DAPI (blue) at each time point following damage. (B) Collagen IV positive area in the undamaged contralateral leg was not significantly difference in young and aged muscle ( $p > 0.05$ ). (C) and (D) shows no significant changes in collagen IV content

*between groups and through the regeneration process. (E) Magnified of collagen IV area in young and aged muscle. (F) Aged muscle was shown to have a significantly greater basement membrane thickness compared to young. Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 50  $\mu\text{m}$ .*

In addition to the collagen isoforms, fibronectin was also investigated. This protein serves as a link between collagen IV and integrins on the cell surface. Previously, fibronectin has been shown to play an important role in satellite cell expansion, migration, and fusion (115, 116). Given the delay in regeneration characterized by low eMHC-expressing myofibres (Figures 5 and 6), it was speculated that aberrant fibronectin expression might contribute to impaired regeneration in aged muscle. Percent area analysis of fibronectin showed no differences between groups in control TA muscle (Figure 10A, B). In the damaged TA muscles, fibronectin expression was found to be greater in the necrotic regions of young muscle during all time points ( $p < 0.05$ ; Figure 10C). Additionally, the expression of fibronectin increased between 3 and 5-days following damage in the necrotic regions of both aged and young muscle ( $p < 0.05$ ; Figure 10C). No changes in fibronectin expression were observed in the regenerating regions between groups ( $p > 0.05$ ; Figure 10D). This may suggest the potential role of fibronectin in modulating the activation of satellite cells, enabling young muscle to regenerate to a higher degree at 5 days following damage.



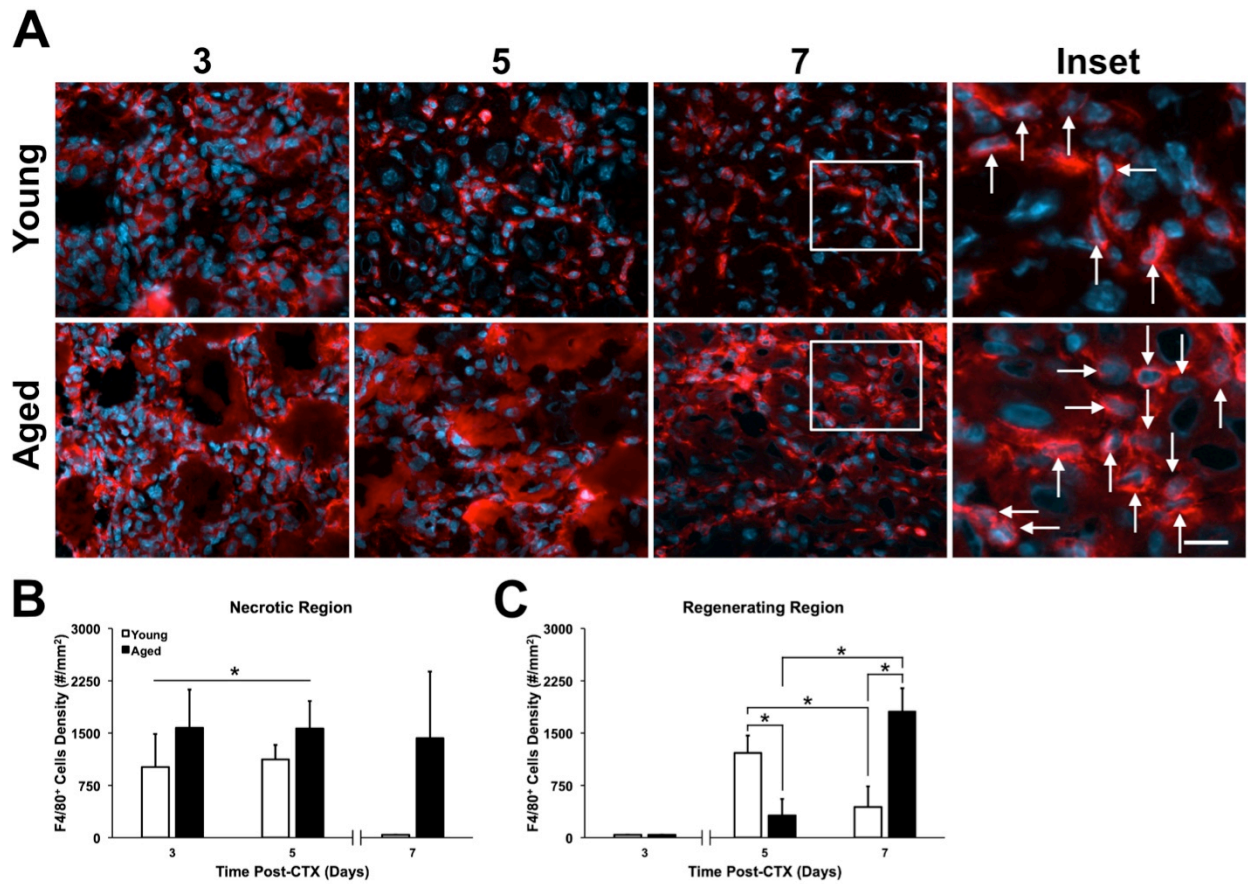


**Figure 10.** Fibronectin content is acutely greater in the necrotic region of young muscle. (A) Immunostaining of fibronectin (green) and DAPI (blue) at each time point following damage. (B) Fibronectin positive area in the undamaged contralateral leg was not significantly different in young and aged muscle ( $p > 0.05$ ). (C) Fibronectin content increases over time from 3 to 5 days following damage in the necrotic region. Additionally, fibronectin was greater in the young groups during these acute time points. (D) No statistically significant changes were observed in fibronectin content in the regenerating region of young and aged muscle. \* denotes a significant main effect of age (young vs. aged) ( $p < 0.05$ ). † denotes a significant main effect of recovery time point following damage ( $p < 0.05$ ). Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 50  $\mu\text{m}$ .

### Macrophage Density Following Damage

To determine if the impaired regenerative capacity of aged muscle was concomitant with prolonged inflammation, immunostaining of the macrophage specific F4/80 marker was completed and quantified within the necrotic and regenerating regions of muscles (Figure 11A). In aged TA muscles, macrophage density was found to be greater in the necrotic regions at 3 and

5 days following damage ( $p < 0.05$ ; Figure 11B) with no changes observed between these time points ( $p > 0.05$ ). In the necrotic regions, macrophages were localized around the edge of necrotic myofibres or entering the necrotic myofibres as they begin to degrade. In the regenerating regions, 5 days following damage, there was a significantly greater macrophage density in young muscle, while at 7 days following damage; macrophage density was greater in aged muscle (Figure 11C). Between 5 and 7 days, macrophage density in young muscle declined significantly, whereas in aged muscle, macrophage density increased (Figure 11C).



**Figure 11.** Macrophage response is delayed in aged muscle following CTX-induced damage. (A) Immunostaining of F4/80 (red) and DAPI (blue) used to identify macrophages. White arrow indicates F4/80+ cells (macrophages) (B) Macrophage density was greater in the necrotic region of aged muscle and remains steady between 3 to 5 days following damage. \* denotes significant main effect of age ( $p < 0.05$ ). (C) A significant interaction between age and recovery time point following damage was observed ( $p < 0.05$ ). Simple main effect analyses demonstrated a significant difference in macrophage density at 5 days following damage, with young muscle having greater

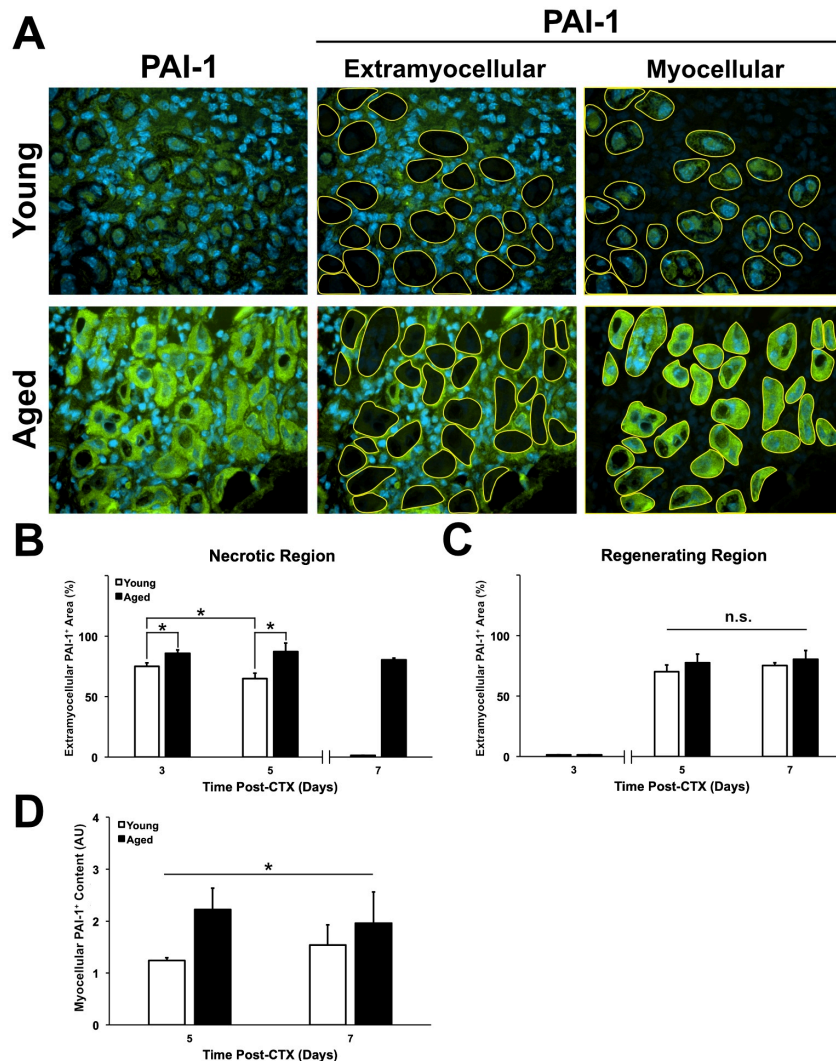
density. However, at 7 days following damage, macrophage density in aged muscle was greater than young ( $p<0.05$ ). A significant drop in macrophage density between 5 and 7 days in young muscle was observed, while the opposite was observed in aged muscle ( $p<0.05$ ). \* in (C) indicates significant differences detected by simple main effects analyses ( $p<0.05$ ). Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 50  $\mu\text{m}$ .

### **Expression Pattern and Localization of PAI-1**

Throughout the regeneration process, the modulation of the ECM is needed for the activation, migration, and differentiation of satellite cells (73, 74, 116). PAI-1 serves as the upstream inhibitor of the plasminogen system, and has been shown to cause fibrosis, cell senescence, and an impairment in the regeneration process (18, 117, 118). Further investigation into the expression pattern and localization of PAI-1 during the acute stages of the regeneration process was undertaken to better understand the role of this regeneration suppressor in aged muscle.

Due to the extracellular effect of PAI-1 in delaying the breakdown of the ECM, immunostaining of this protein was completed and quantified via area analysis in the distinct necrotic and regenerating regions of the muscle. Extramyocellular PAI-1 was determined through the selection of the area not occupied by myofibres (Figure 12A). Aged muscle had a significantly greater extramyocellular PAI-1 content at 3 and 5 days in the necrotic region compared to young muscle ( $p<0.05$ ; Figure 12B). Interestingly, extramyocellular PAI-1 content decreased significantly between 3 and 5 days in young muscle ( $p<0.05$ ), while in aged muscle, PAI-1 content remained constant. No differences were found in the regenerating regions in aged muscle ( $p>0.05$ ; Figure 12C). This was consistent with elevated collagen I and macrophage content in the necrotic regions of aged muscle, as previously demonstrated.

During the investigation of extramyocellular PAI-1, regenerating myofibres appeared to be expressing PAI-1. PAI-1 expression within the regenerating myofibre was assessed via analysis of signal intensity. A significant main effect of age ( $p<0.05$ ) with aged muscle displaying greater PAI-1 within the regenerating myofibre was found (Figure 12D). This result suggests a potential role of myocellular PAI-1 in modulating the aberrant changes in the regenerative capacity of skeletal muscle, potentially causing senescence in these myofibres (18).



**Figure 12.** Aged muscle displayed a greater extramyocellular localization of PAI-1. (A) Immunostaining of PAI-1 (green) and DAPI (blue) at 5-days following damage. (B) A significant interaction between age and recovery time point following damage was observed in the necrotic regions ( $p<0.05$ ). Simple main effect analyses demonstrated a significant greater extramyocellular PAI-1 expression within aged muscle at 3 and 5 days following damage ( $p<0.05$ ).

*Extramyocellular PAI-1 within the necrotic regions of young muscle declined significantly between 3 and 5 days following damage ( $p < 0.05$ ). \* in (B) indicates significance from the simple main effects analyses ( $p < 0.05$ ). (C) No significant differences in extramyocellular PAI-1 were observed in the regenerating region of young and aged muscle ( $p > 0.05$ ). (D) Brightness analysis of PAI-1 within regenerating myofibres showed significantly greater PAI-1 content within aged regenerating myofibres compared to young (main effect of age:  $p < 0.05$ ; indicated by \*). Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 50  $\mu\text{m}$ .*

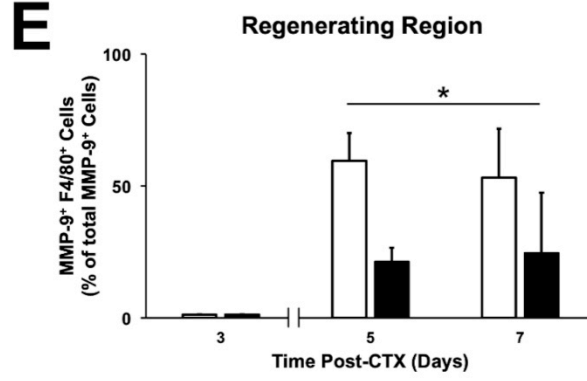
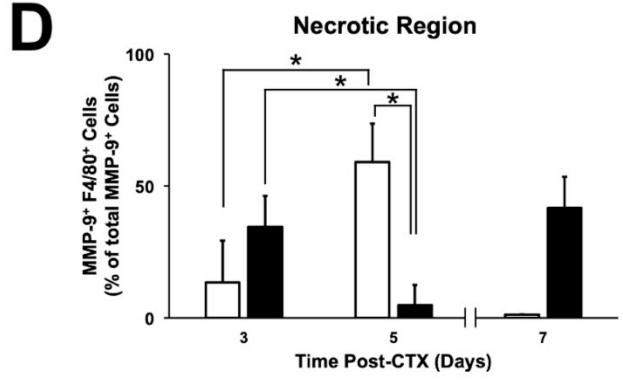
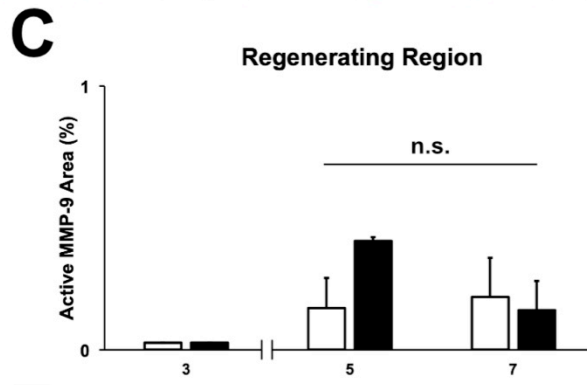
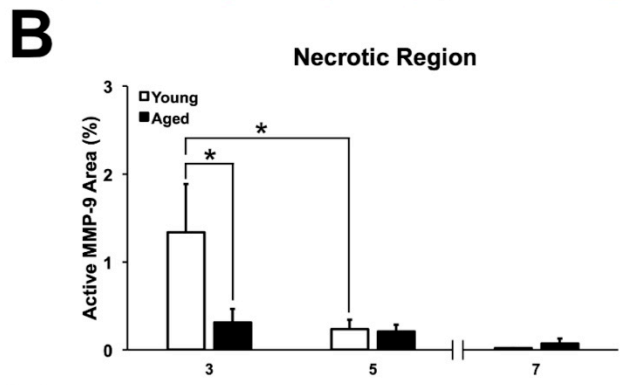
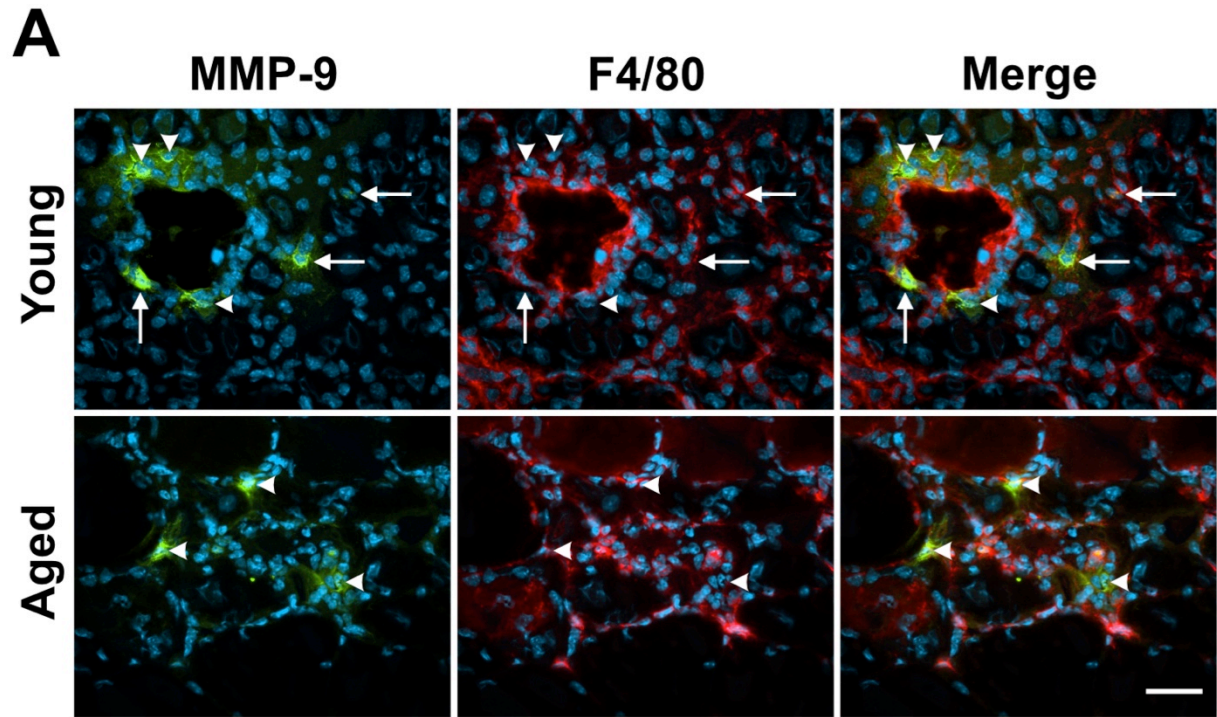
### **Active MMP-9 Localization and Expression Pattern**

Although PAI-1 serves as the upstream inhibitor of the plasminogen system, its downstream effectors are proteases which degrade the ECM when activated or allow ECM accumulation when non-active. In skeletal muscle, the inducible enzyme MMP-9 has been shown to play a central role in ECM degradation, thus enabling satellite cell migration and normal regeneration to occur (44, 99, 119). For this project, active MMP-9 was investigated to better understand the expression pattern and localization of this proteolytic enzyme, and its effects on ECM remodeling.

An area analysis was performed to determine the extracellular distribution of active MMP-9 within the necrotic and regenerating regions of muscles. Active MMP-9 was greater 3 days following damage in the necrotic regions of young compared to aged muscles ( $p < 0.05$ ; Figure 13A, B). Active MMP-9 decreased from 3 to 5 days following damage in the young muscle only. No significant differences in active MMP-9 content was found in the regenerating region ( $p > 0.05$ ; Figure 13C). This is consistent with previous findings suggesting the role of MMPs during the early inflammation stages to enable the degradation of the ECM, and facilitate satellite cell migration (44, 99, 119).

Interestingly, immunostaining of active MMP-9 showed its presence around specific nuclei (Figure 13A). Previously, MMP-9 mRNA was found to be localized primarily in macrophages and polymorphonuclear leukocytes (i.e. neutrophils) in skeletal muscle (44, 95, 99). This study

demonstrated delayed macrophage infiltration and the inability to degrade the ECM following damage in aged muscle. As a result, it was speculated that aged muscle would display a depression in macrophage-specific MMP-9 expression attributing to the aberrant changes in the ECM. To determine the contribution of macrophages in secreting MMP-9, combination staining of F4/80 and active MMP-9 were completed. Total MMP-9+ cells and macrophage (F4/80+) cells colocalized with MMP-9 were counted and analyzed. Results from this analysis showed a significantly greater percentage of macrophage-specific MMP-9 cells 5 days following damage in the necrotic region of young muscle ( $p<0.05$ ; Figure 13D). Between 3 and 5 days, percent of macrophage-specific MMP-9 cells decreased significantly in the aged muscle, while the opposite was found in young muscle ( $p<0.05$ ). Young muscle displayed a greater amount of macrophage-specific MMP-9 cells in the regenerating regions compared to aged muscle ( $p<0.05$ ; Figure 13E). Taken together, young muscle acutely displays greater active MMP-9 protein and a greater amount of macrophage-specific MMP-9 compared to aged muscle.



**Figure 13.** Active MMP-9 expression was elevated acutely following damage in young muscle. (A) Immunostaining of active MMP-9 (green), F4/80 (red), and DAPI (blue). The arrow indicates F4/80+ and MMP-9+ cells. The arrowhead indicates F4/80- and MMP-9+ cells. (B) A significant interaction between age and recovery time point in MMP-9+ area following damage was observed within the necrotic regions ( $p < 0.05$ ). Simple main effect analyses demonstrated a significant

greater active MMP-9 expression within young muscle at 3 days following damage ( $p < 0.05$ ). Active MMP-9 expression declined significantly from 3 to 5 days following damage ( $p < 0.05$ ) to match the expression of aged muscle. (C) No significant differences of active MMP-9 positive area was observed in the regenerating regions of young and aged muscle ( $p > 0.05$ ). (D) A significant interaction between age and recovery time point on MMP-9+ F4/80+ cells were detected following damage ( $p < 0.05$ ). A simple main effects analysis showed significantly greater macrophage-specific (F4/80+) MMP-9 expression in the necrotic regions of young muscle 5 days following damage ( $p < 0.05$ ). Additionally, macrophage-specific MMP-9 expression increased significantly between 3 and 5 days in young, however, the opposite was observed in aged muscle (both  $p < 0.05$ ). (E) A significant main effect of age was found in the regenerating regions, with young muscle displaying a greater percentage of macrophage-specific MMP-9 compared to aged muscle ( $p < 0.05$ ). \* in (B) and (D) indicates significance from the simple main effects analyses ( $p < 0.05$ ). \* in (E) indicates a main effect of age. Note that the 7-day time point in the necrotic region and the 3-day time point in the regenerating regions were not used in the statistical analysis due to insufficient instances of those regions depending on age and time point. Scale bar represents 100  $\mu\text{m}$ .



## DISCUSSION

Aging has been shown to cause several negative physiological changes to skeletal muscle, including reductions in muscle strength, size, and impaired regeneration (7, 8, 112). The purpose of this study was to investigate and characterize the physical changes in the extracellular environment during the regeneration process in young and aged muscle. To date, there are limited studies investigating the regenerative response in aged muscle during the acute stages following damage (120). Therefore, this project was designed to determine the severity of the impaired regenerative response observed in aged muscle up to 7 days following CTX-induced muscle damage, and the role of the extracellular environment in potentially modulating the regenerative response. Characterization of the regenerative capacity and the changes in the extracellular environment provides a basis for further investigation into the regulatory role of ECM and various cell types during skeletal muscle regeneration.

This study characterized the severity of the impaired regenerative response in aged muscle using several histochemical/immunohistochemical techniques. It was originally hypothesized that the regenerative response to damage would be impaired in aged muscle (Hypothesis #1). Results from this analysis demonstrated that aged muscle was unable to regenerate to the same capacity as young muscle, as indicated by fewer and smaller regenerating myofibres compared to young, thus supporting Hypothesis #1. The inability of aged muscle to regenerate to full capacity was particularly apparent at the 7-day time point where virtually all of the myofibre area within young muscle was regenerated, however, only half of the myofibre area in aged muscle was regenerated. The other half of the myofibre area in aged muscle remained occupied by necrotic myofibres. The greater necrotic area observed in aged muscle is consistent with previous studies that found a greater degree of cell death and an inability to initiate the regeneration process (120–123).

However, only one of these studies (120) investigated the aged skeletal muscle following damage but did not quantify the extent or degree of necrosis. Therefore, the present study provides important information regarding the extent and degree of aged muscle necrosis following damage.

The impaired regenerative potential of aged muscle was hypothesized to be related to aberrant accumulation of the ECM (Hypothesis #2), therefore, characterization of the changes in the ECM was undertaken. Two collagen isoforms (I and IV) were investigated in the present study to provide a better understanding of the changes occurring in the ECM following muscle damage. Previous findings have demonstrated an aberrant accumulation of collagen in aged muscle (67, 110), however, these studies examined total collagen expression (i.e.: no detection of specific isoforms). Results from the present study showed that aged muscle displayed greater collagen I deposition acutely following damage in the necrotic regions, however, no differences were observed in the regenerating regions between 5 and 7 days following damage. The acute accumulation of collagen I was in support of Hypothesis #2. The findings from the present study were able to determine the specific accumulation of collagen I isoform acutely following damage in aged muscle.

Collagen IV is a major structural component of the basement membrane where it aggregates with various other ECM proteins to provide structural stability (114, 124). In aged muscle, it is unknown whether collagen IV – as a networking protein – accumulates in the basement membrane and contributes to fibrosis. Investigation of collagen IV from the present study found no differences in the presence of this protein between groups. This suggests that the integrity of the basement membrane does not change as a result of aging. However, the basement membrane was found to be thicker and irregular in structure around regenerating myofibres in aged muscle compared to young muscle at the 5 and 7-day time points. Thus, in aged muscle,

collagen IV-positive area did not increase (contrary to Hypothesis #2), yet thickness of the basement membrane increased (supporting Hypothesis #2). The increased thickness and irregular structure may be due to incomplete breakdown of existing collagen IV and/or accumulation of newly synthesized collagen IV in aged muscle (44), while the absence of an increase in overall collagen IV-positive area may be a result of smaller and less numerous *de novo* myofibres. The irregular structure of collagen IV may also be a result of improper linking of the basement membrane to the sarcolemma. Investigation of an important sarcolemmal-ECM linking protein, fibronectin, was completed to determine the role of this protein in modulating regeneration and the irregular structure in collagen IV.

Fibronectin binds to collagen IV and other ECM molecules to provide signals to the cells about physical changes to their environment (125). Fibronectin is critical in regulating satellite cell function during the regeneration process (115, 116). For example, fibronectin is essential to satellite cell proliferation by initiating Wnt signaling (115). Examination of fibronectin expression in the present study found a reduction of this protein within aged muscle during the early phase of the regeneration process. This is consistent with a recent study that found the genetic deletion of fibronectin in young muscle causing the loss of satellite cell number (126). Given the importance of fibronectin on satellite cell function, findings from the current study suggest that reduced expression of fibronectin (Figure 10) in aged muscle acutely following damage could be the cause of its impaired myogenic response (Figure 6, 7). No differences were observed in fibronectin expression during the latter portion (5 and 7 days following damage) of the regeneration process, and therefore, it was concluded that fibronectin was likely not contributing to the irregular organization of collagen IV. The lower levels of fibronectin observed in aged muscle was opposed to Hypothesis #2.

Macrophages play a critical role in the initiation and progression of the regeneration process (34, 37, 39) and, thus, were primary candidates for examination in the current study. In aged muscle, macrophages have been shown to contribute to chronic inflammation, eventually leading to muscle loss (127, 128). These cells have also been shown to play a role in ECM remodeling during the regeneration process in healthy and dystrophic skeletal muscle through the secretion of proteolytic enzymes (44). In the present study, macrophage density was measured throughout the regeneration process to determine the differences in the presence of these cells in young and aged muscle. Macrophage density was hypothesized to be greater in aged muscle throughout the time course of the study (Hypothesis #3). In aged muscle, macrophage density was found to be greater than young muscle in the necrotic regions, while in the regenerating regions, macrophage density increased between 5 and 7 days following damage (Figure 11). The opposite trend was observed in regenerating young muscle, with macrophage density declining in the regenerating regions between 5 to 7 days following damage. This suggests that in aged muscle, although there are plenty of macrophages in necrotic regions, there is a delay in their infiltration into the regenerating regions. This is likely a contributing factor to the delay in the regenerative response considering the well-defined importance of macrophages in stimulating the proliferation and differentiation of satellite cells (39, 129, 130). On the other hand, the prolonged presence of macrophages in aged muscle may be the cause of the persistent necrotic regions observed in aged muscle. Overall, the macrophage analysis was in support of Hypothesis #3.

The role of macrophages in modulating changes in the ECM is accomplished through the secretion of important proteins of the plasminogen system. Investigation of the upstream inhibitor of the plasminogen system, PAI-1, was of particular interest due to its role in fibrosis, cell senescence, and muscle regeneration (18, 117, 118). PAI-1 expression was hypothesized to be

greater in aged muscle, contributing to the attenuation of ECM breakdown (Hypothesis #4). Extramyocellular PAI-1 content was found to be greater in aged muscle acutely following damage (Figure 12). The presence of extramyocellular PAI-1 is consistent with lower active MMP-9 levels and aberrant accumulation of collagen I, and the impairment in formation of regenerating myofibres following damage. This is also consistent with the established role of MMP-9 having proteolytic specificity for collagen I (131). Additionally, PAI-1 has been previously shown to impair macrophage infiltration in damaged muscle (22, 86, 132, 133), and thus, delay the regeneration process. In the present study, the identification of elevated levels of extramyocellular PAI-1 in aged muscle provides crucial information to better understand the mechanisms impairing aged muscle regeneration.

A previous study has demonstrated PAI-1 as a downstream target of p53-induced senescence, and also a critical mediator of replicative senescence in mouse and human fibroblast cells (134). This was shown by experimental knockdown of PAI-1 via RNA interference causing the restoration of replicative capabilities of the cells, whereas the overexpression of PAI-1 was sufficient to revert the cells back to a senescent state (134). The induction of senescence by PAI-1 is a result of its ability to prevent the breakdown of senescence-inducing molecules in the extracellular environment (134–136). Interestingly, in the current study, PAI-1 was found to be present both extracellularly and within regenerating myofibres (myocellular) in both young and aged muscle. The present findings demonstrate greater PAI-1 in aged regenerating myofibres 5 and 7 days post-damage. The presence of PAI-1 within regenerating myofibres is not understood, however, it may simply represent higher secretion of the protein – consistent with the elevated levels of extramyocellular PAI-1. Taken together, the greater quantities of extramyocellular and

myocellular PAI-1 in aged muscle supports Hypothesis #4, however, the mechanism by which this protein impairs muscle regeneration remains unknown.

Investigation into the downstream effectors of the plasminogen system, specifically active MMP-9, was undertaken to identify potential aberrant changes in the expression and localization of this important proteolytic enzyme capable of degrading collagen I and IV. It was hypothesized that MMP-9 expression would be reduced in aged muscle, leading to the accumulation of the ECM (Hypothesis #5). Findings from the present study supported this hypothesis and demonstrated a lack of active MMP-9 expression in aged muscle acutely following damage, which was consistent with previous findings from Kherif et al. (44) in dystrophic muscle. Additionally, the reduction of active MMP-9 expression in aged muscle was also consistent with the accumulation of PAI-1 and collagen I. Active MMP-9 protein expression was found to be localized primarily around macrophage cells within young muscle, however, in aged muscle, the same was not observed. The reduction in macrophage-specific MMP-9 expression, even though there are greater amounts of macrophages present in aged muscle following damage, suggests dysfunctional macrophages present in aged muscle. It was also speculated that active MMP-9 in aged muscle may arise from a different cell type, with the next likely candidates being polymorphonuclear leukocytes (neutrophils) and fibroblasts (44,95,99,119,137). Moreover, the lack of active MMP-9 in aged muscle acutely following damage and its localization around non-macrophage cell type(s) provides valuable information about the negative physiological changes in aged muscle.

## **CONCLUSION**

Overall, this study characterized notable alterations to the extracellular environment during skeletal muscle regeneration with aging. Data from this study supports the novel hypothesis that the impaired regeneration process observed in aged muscle is a result of poor remodeling of the

ECM. The delayed remodeling of the ECM immediately following damage likely slows the activation of satellite cells, and thus reduces size of regenerating myofibre in aged muscle. These findings demonstrate that the extrinsic changes regulating the remodeling process and the intrinsic changes to aged regenerating myofibre may be attributed to the elevations of extramyocellular and myocellular PAI-1. Additionally, these findings provide novel information about lack of an important downstream enzyme of the plasminogen system: MMP-9. Future work is needed to better understand the mechanism by which extramyocellular and myocellular PAI-1 impairs muscle regeneration, and in doing so, will aid in the development of therapeutic strategies to help mitigate age-related muscle decline.

### **LIMITATIONS**

One consistent theme throughout this study was the occurrences of freeze artifacts (freeze fracture) within the damaged muscle. Freeze fracture pockets were present predominantly in the earlier recovery time points (3 and 5 days) following damage. As previously mentioned, inflammation occurs immediately following muscle damage and involves the buildup of fluids within the muscle. Rapid freezing of the fluid within the muscle may result in the formation of ice crystals, and thus, causing the formation of freeze artifacts within the muscles. Images were carefully taken to avoid the majority of the freeze fracture pockets. In some instances, the freeze fracture pockets were unavoidable. Luckily for this study, the images could still be analyzed, however, future studies may wish to use a different freezing method to prevent freeze fracture.

## FUTURE DIRECTIONS

Future studies may wish to examine the remodeling of the ECM and the expression pattern of important plasminogen system proteins through a longer time course (i.e. 14, 21, 28, 35, and/or 50 days following damage). This will provide a better understanding of the long-term consequences of CTX-induced damage on aged muscle morphology. Protein assays should also be employed for the plasminogen system proteins to validate the findings of the present study.

The result from this study suggests that PAI-1 may be exerting a negative effect on the regeneration process through inhibition of collagen breakdown in the extracellular environment, and senescence within regenerating myofibres. PAI-1 expression was found to be elevated as early as 3 days following damage. Therefore, inhibition of PAI-1 using drugs, such as tiplaxtinin (PAI-039), or knockdown/knockout of PAI-1 at various time points may provide a better understanding on the temporal role of this fibrinolytic inhibitor during muscle regeneration. Overexpression of PAI-1 in young muscle may also provide valuable information on whether PAI-1 alone mediates fibrosis and senescence or in combination with other systemic factors found in aged muscle, such as TGF- $\beta$ .

Additional investigation of uPA:PAI-1 ratio in aged muscle may provide a better understanding of the upstream balance of plasminogen activation, while investigation of MMP:TIMP ratio may aid to better understand the downstream balance of ECM degradation (Figure 3). Localization of these proteins should also be undertaken to determine potential cell type(s) responsible for their secretion. For this study, only macrophage cells were investigated, however, future studies may wish to investigate neutrophils (Ly6G+), fibroblasts (Tcf4+ or vimentin+), quiescent satellite cells (Pax7+ / MyoD-), and/or activated satellite cells (Pax7+ / MyoD+) to identify the origin of these proteins throughout the regeneration process.



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## APPENDICES

### Appendix A

Hematoxylin and eosin (H&E) staining of muscle sections.

- Air dry sections for 15 minutes.
- Apply Harris modified hematoxylin for 3 minutes to each muscle section using a pasteur pipette. Make sure to completely cover each muscle section.
- Rinse slides in beaker of ddH<sub>2</sub>O briefly.
- Place slides in coplin jar and run under cool tap water for 3 minutes.
- Remove slides from coplin jar and rinse slides with ddH<sub>2</sub>O for 2 minutes.
- Wipe excess water off using a Kim wipe.
- Apply eosin solution (1% w/v Eosin Y disodium salt in ddH<sub>2</sub>O + 0.1% v/v glacial acetic acid) for 10 minutes.
- Rinse slides in beaker of ddH<sub>2</sub>O briefly.
- Place slides in coplin jar and run under cool tap water for 3 minutes.
- Remove slides from coplin jar and rinse slides with ddH<sub>2</sub>O for 2 minutes.
- *Dehydration\**: submerge slide into 95% ethanol for 10 seconds, followed by 100% ethanol for 10 seconds.
- *Clear\**: submerge slide into xylene for 10 seconds.
- *Mount\**: wipe off excess xylene using a Kim wipe and apply permount along with the coverslip before xylene completely dries.
- Let slides set on a horizontal surface for at least 15 minutes before proceeding with the analysis.

\* *Perform these steps inside the fume hood.*

## Appendix B

*Table 1: List of antibodies and working concentrations.*

	<b>Host</b>	<b>Company</b>	<b>Catalogue</b>	<b>Concentration</b>
Collagen I	Rabbit	Abcam	ab34710	1:200
Collagen IV	Rabbit	Abcam	ab6586	1:300
Fibronectin	Rabbit	Abcam	ab2413	1:300
PAI-1	Rabbit	Abcam	ab66705	1:200
MMP-9	Rabbit	Abcam	ab38898	1:200
eMHC	Mouse	DSHB	F1.652	1:1
F4/80	Rat	Abcam	ab90247	1:100

## Appendix C

Immunostaining using rabbit polyclonal IgG antibodies or rat monoclonal IgG2b antibody.

Day 1:

- Air dry slides at room temperature for 15 minutes.
- Circumscribe sections using a hydrophobic pen.
- Fix sections in ice cold 2% paraformaldehyde (PFA) for 5 minutes in the fridge.
- Rinse 5x (1 minute each) in 1x PBS.
- *Block*: Apply 5% normal goat serum (NGS) + 0.1% Triton-X100 in PBS to each section.  
Incubate at room temperature for 1 hour. Remove block.
- *Primary Antibody*: Apply rabbit antibody at appropriate concentration (see Appendix A) in block (5% NGS + 0.1% Triton-X100 in PBS). Incubate 16-20 hours in a humidified chamber at 4°C (fridge). Apply PBS on negative control sections.

Day 2:

- Remove primary antibody.
- Rinse 5x (1 minute each) in 1x PBS.
- *Secondary Antibody*: Apply 1:1000 goat anti-rabbit IgG secondary antibody and 1:1000 DAPI. Place in a dark chamber for 1 hour at room temperature.
- Remove secondary antibody.
- Rinse 3x (1 minute each) in 1x PBS.
- Apply 1:3 dilution of TrueView (Vector Labs) to quench background fluorescence.
- Rinse 3x (1 minute each) in 1x PBS.
- Apply fluoromount and coverslip. Set for 10-20 minutes on a horizontal surface protected from light.

## Appendix D

Immunostaining using mouse monoclonal IgG antibodies.

Day 1:

- Air dry slides at room temperature for 15 minutes.
- Circumscribe sections using a hydrophobic pen.
- Fix sections in ice cold 2% paraformaldehyde (PFA) for 5 minutes in the fridge.
- Rinse 5x (1 minute each) in 1x PBS.
- *Block 1*: Apply 1-2 drops of Mouse-on-Mouse (MOM) IgG block (Vector Labs) for 1 hour at room temperature. Remove block and rinse 3x (1 minute each) in 1x PBS.
- *Block 2*: Apply 10% NGS + 1.5% bovine serum albumin (BSA) + 0.2% Triton-X100 in PBS to each section. Incubate at room temperature for 1 hour. Remove block.
- *Primary Antibody*: Apply mouse antibody at appropriate concentration (see Appendix A) in block (10% NGS + 1.5% BSA + 0.2% Triton-X100 in PBS). Incubate 16-20 hours in a humidified chamber at 4°C (fridge). Apply PBS on negative control sections.

Day 2:

- Remove primary antibody.
- Rinse 5x (1 minute each) in 1x PBS.
- *Secondary Antibody*: Apply 1:1000 goat anti-mouse IgG secondary antibody and 1:1000 DAPI. Place in a dark chamber for 1 hour at room temperature.
- Remove secondary antibody.
- Rinse 3x (1 minute each) in 1x PBS.
- Apply 1:3 dilution of TrueView (Vector Labs) to quench background fluorescence.
- Rinse 3x (1 minute each) in 1x PBS.

- Apply fluoromount and coverslip. Set for 10-20 minutes on a horizontal surface protected from light.



## Appendix E

Combination immunostaining using mouse monoclonal IgG and rabbit polyclonal IgG antibodies.

Day 1:

- Air dry slides at room temperature for 15 minutes.
- Circumscribe sections using a hydrophobic pen.
- Fix sections in ice cold 2% paraformaldehyde (PFA) for 5 minutes in the fridge.
- Rinse 5x (1 minute each) in 1x PBS.
- *Block 1:* Apply 1-2 drops of Mouse-on-Mouse (MOM) IgG block (Vector Labs) for 1 hour at room temperature. Remove block and rinse 3x (1 minute each) in 1x PBS.
- *Block 2:* Apply 10% NGS + 1.5% bovine serum albumin (BSA) + 0.2% Triton-X100 in PBS to each section. Incubate at room temperature for 1 hour. Remove block.
- *Primary Antibodies:* Apply mouse and rabbit antibodies at appropriate concentration (see Appendix A) in block (10% NGS + 1.5% BSA + 0.2% Triton-X100 in PBS). Incubate 16-20 hours in a humidified chamber at 4°C (fridge). Apply PBS on negative control sections.

Day 2:

- Remove primary antibody.
- Rinse 5x (1 minute each) in 1x PBS.
- *Secondary Antibody:* Apply 1:1000 goat anti-mouse IgG secondary antibody, 1:1000 goat anti-rabbit IgG secondary antibody, and 1:1000 DAPI. Place in a dark chamber for 1 hour at room temperature.
- Remove secondary antibody.

- Rinse 3x (1 minute each) in 1x PBS.
- Apply 1:3 dilution of TrueView (Vector Labs) to quench background fluorescence.
- Rinse 3x (1 minute each) in 1x PBS.
- Apply fluoromount and coverslip. Set for 10-20 minutes on a horizontal surface protected from light.

## Appendix F

Combination immunostaining using rat monoclonal IgG2b and rabbit polyclonal IgG antibodies.

Day 1:

- Air dry slides at room temperature for 15 minutes.
- Circumscribe sections using a hydrophobic pen.
- Fix sections in ice cold 2% paraformaldehyde (PFA) for 5 minutes in the fridge.
- Rinse 5x (1 minute each) in 1x PBS.
- *Block*: Apply 5% NGS + 0.1% Triton-X100 in PBS to each section. Incubate at room temperature for 1 hour. Remove block.
- *Primary Antibody*: Apply rat and rabbit antibodies at appropriate concentration (see Appendix A) in block (5% NGS + 0.1% Triton-X100 in PBS). Incubate 16-20 hours in a humidified chamber at 4°C (fridge). Apply PBS on negative control sections.

Day 2:

- Remove primary antibody.
- Rinse 5x (1 minute each) in 1x PBS.
- *Secondary Antibody*: Apply 1:1000 goat anti-rat IgG2b, 1:1000 goat anti-rabbit IgG secondary antibody, and 1:1000 DAPI. Place in a dark chamber for 1 hour at room temperature.
- Remove secondary antibody.
- Rinse 3x (1 minute each) in 1x PBS.
- Apply 1:3 dilution of TrueView (Vector Labs) to quench background fluorescence.
- Rinse 3x (1 minute each) in 1x PBS.

- Apply fluoromount and coverslip. Set for 10-20 minutes on a horizontal surface protected from light.

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