Cell & Tissue Research | Springer

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Journal:	*Cell and Tissue Research*
Manuscript ID	CTR-18-0193
Manuscript Type:	Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Mahdy, Mohamed A. A.; South Valley University, Anatomy and Embryology; University of Pretoria, Anatomy and physiology
Keywords:	Animal models, fibrosis, muscle injury, muscular dystrophy, regeneration

Skeletal muscle fibrosis: an overview

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Abstract

Extracellular matrix (ECM) is an essential component of skeletal muscle. It provides a framework structure that hold myofibers, and blood capillaries and nerves supplying the muscle. In addition, it has a principal role in force transmission, maintenance and repair of muscle fibers. Excessive accumulation of ECM components, especially collagens, either due to excessive ECM production, alteration in ECM-degrading activities, or a combination of both is defined as fibrosis. Skeletal muscle fibrosis impairs muscle function, negatively affects muscle regeneration after injury, and increases muscle susceptibility to re-injury, therefore, it considered as a major cause of muscle weakness. Fibrosis of skeletal muscle is a hallmark of muscular dystrophies, aging, and severe muscle injuries. Thus, better understanding of the mechanisms of muscle fibrosis will help to advance our knowledge of the events that occur in dystrophic muscle diseases and develop innovative antifibrotic therapies to reverse fibrosis in such pathologic conditions. This paper explores an overview of the process of muscle fibrosis, as well as the different murine models for studying fibrosis in skeletal muscles. In addition, factors regulating fibrosis, and strategies to inhibit muscle fibrosis are discussed.

Keywords: Animal models, fibrosis, muscle injury, muscular dystrophy, regeneration.

1. Introduction

Skeletal muscle consists of myofibers, the contractile part of the muscle, connective tissue or extracellular matrix (ECM), and the blood capillaries and nerves supplying the muscle (Jarvinen et al., 2005). The myofibers are the basic units of skeletal muscle structure (Sambasivan and Tajbakhsh, 2015), while the ECM is the framework structure that hold myofibers, blood vessels and nerves (Grounds, 2008, Jarvinen et al., 2005). ECM forms up to 10% of the skeletal muscle weight (Kjaer, 2004) and it plays a principal role in force transmission, maintenance, and repair of muscle fibers following injury (Gillies and Lieber, 2011).

Skeletal muscle subjected to different types of injuries undergoes degeneration with inflammatory cellular infiltration (Karalaki et al., 2009, Mahdy, 2018, Mann et al., 2011). The quiescent satellite cells (SCs) activate, proliferate, and differentiate forming new myotubes with production of new ECM, blood vessels and nerves (Laumonier and Menetrey, 2016, Mann et al., 2011, Saclier et al., 2013, Yin et al., 2013). The myotubes mature into myofibers and ECM undergoes remodelling (Alameddine and Morgan, 2016, Lei et al., 2013). The regenerated muscle in normal conditions resembles undamaged muscle in morphological, as well as, functional states (Charge and Rudnicki, 2004).

Despite the high regeneration ability of skeletal muscle, it is compromised in several conditions by excessive deposition of ECM resulting in muscle fibrosis (Gillies and Lieber, 2011, Mahdy, 2018). The excessive deposition of fibrous tissue impairs muscle function (Delaney et al., 2017, Jarvinen et al., 2002, Uezumi et al., 2014), affects muscle fiber regeneration after injury (Murphy and Ohlendieck, 2016), and increases muscle susceptibility to re-injury (Prazeres et al., 2018). Fibrosis of skeletal muscle is a characteristic feature of muscular dystrophies (Pessina et al., 2014), myopathies and severe injuries, such as lacerations and contusions (Uezumi et al., 2014).

It is essential to understand the mechanisms of muscle fibrosis (Prazeres et al., 2018). This will help to advance our knowledge of the events that occur in dystrophic muscle diseases and develop innovative antifibrotic therapies to reverse fibrosis in such pathologic conditions. Several studies have investigated the mechanism of muscle fibrosis and identified a variety of factors that regulate muscle regeneration and fibrosis. In addition, several *in vivo* models have been developed to study muscle fibrosis and develop therapies to combat fibrosis and enhance muscle regeneration. This review highlights the process of muscle fibrosis, different *in vivo* models available for studying fibrosis, recent strategies to enhance fibrosis in these models, and methods to inhibit muscle fibrosis.

2. Composition of muscle ECM

ECM consists of collagen fibrous networks in an amorphous matrix of hydrated proteoglycan (PG) (Purslow, 2014). Its main components can be divided into 4 classes: collagenous and non-collagenous glycoproteins, proteoglycans, and glycosaminoglycans (GAGs) (Velleman et al., 2012). ECM acts as a scaffold to support blood vessels and nerves (Grounds, 2008, Wang and Tang, 2016). In addition, ECM has a principal role in force transmission, maintenance, and repair of muscle fibers (Gillies and Lieber, 2011, Wang and Tang, 2016). The ECM is arranged in the muscle in three levels (Fig.1); the endomysium, perimysium, and epimysium (de Rezende Pinto et al., 2015).

The endomysium is a thin delicate membrane surrounds each myofiber (Purslow, 2010). It is located directly in contact with the sarcolemma (Turrina et al., 2013). The endomysium maintains muscle integrity and promotes myogenesis and muscle regeneration (Campbell and Stull, 2003). The endomysial ECM conveys tension between overlapping muscle fibers (Chapman et al., 2016).

In addition, the endomysium contains small diameter blood vessels and finest neurons (Ross and Pawlina, 2010). The endomysium is composed mainly of collagen type I, type III, and type V (Table.1) (Kovanen, 2002).

The perimysium surrounds number of myofibers combined in the form of fascicles, the functional units of the muscle fibers. The perimysial network joins with the epimysium at the muscle surface (Purslow, 2010). The perimysium transmits the force from the myofibers to tendons, in addition, it contains larger blood vessels and nerves (Ross and Pawlina, 2010). The perimysium is composed mainly of collagen type I and type III (Kovanen, 2002).

The epimysium surrounds the entire muscle, it is the thickest and strongest sheath (de Rezende Pinto et al., 2015, Jarvinen et al., 2005). The epimysium is continuous with the tendons that connect the muscles to the bones (Purslow, 2010), and thickens at both muscle origin and insertion (McCormick and Phillips, 1999). The major blood vessels and nerve supplying the muscle penetrate the epimysium (Ross and Pawlina, 2010). The epimysium is composed mainly of collagen type I (Kovanen, 2002) and minor amounts of collagen type III (Riso et al., 2016).

3. Role of ECM in muscle repair following injury

ECM has a principal role in muscle repair following injury (Gillies and Lieber, 2011). Muscle injury induces formation of hematoma and infiltration of inflammatory cell populations (Serrano and Muñoz-Cánoves, 2010, Tidball, 2005). Fibrin and fibronectin extravasate into the injury site and bind with collagen type I and type III, and proteoglycans produced by fibroblasts, forming a new and temporary ECM (Mann et al., 2011, Serrano et al., 2011, Serrano and Muñoz-Cánoves, 2010). In addition, fibrocytes, as well as, fibroadipogenic progenitors (FAPs), mesenchymal progenitor cells present in the interstitium of healthy muscle and express platelet-derived growth

factor receptor (PDGFR)-α, proliferate and share in the formation of temporary ECM (Juban and Chazaud, 2017, Lemos et al., 2015, Munoz-Canoves and Serrano, 2015, Wang et al., 2016). The ECM deposition is under control of some growth factors as transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), and the rennin-angiotensin system (RAS) (Serrano and Muñoz-Cánoves, 2016). The temporary ECM provides a suitable environment for myoblast differentiation (Osses and Brandan, 2002) and acts as a scaffold for the regenerating myofibers (Delaney et al., 2017, Mann et al., 2011). In addition, the components of temporary ECM, as well as the basement membrane, guide the formation of neuromuscular junctions (Serrano et al., 2011). Moreover, collagen VI, a major component of ECM, regulates SCs function and maintains SC pool during muscle regeneration (Urciuolo et al., 2013). Furthermore, there is a positive interaction between SCs and fibroblasts during muscle regeneration following injury (Murphy et al., 2011).

Remodeling of the ECM, by the proteolytic enzymes, matrix metalloproteinases (MMPs), is essential for efficient skeletal muscle regeneration (Alameddine and Morgan, 2016, Laumonier and Menetrey, 2016). MMPs are produced from both damaged myofibers and infiltrating cells, they have the ability to degrade ECM components. In addition, FAPs population decline and return to the pre-damage level which is important to prevent excessive deposition of ECM (Lemos et al., 2015, Mann et al., 2011). ECM degradation facilitates the recruitment of inflammatory cells (Serrano et al., 2011) and migration of SCs to repair the injured muscle (Chen and Li, 2009). In addition, ECM remodelling provides protection for the damaged muscle from future injury through strengthening of the deposited ECM (Kim and Lee, 2017). The balance between ECM production and degradation is essential for efficient regeneration.

4. Muscle fibrosis following injury

Fibrosis is defined as excessive accumulation of ECM components, especially collagens, either due to excessive ECM production, alteration in ECM-degrading activities, or a combination of both (Alameddine and Morgan, 2016, Gillies and Lieber, 2011). This abnormal accumulation of ECM is apparent in the endomysium and perimysium of skeletal muscle (Wang and Tang, 2016).

Muscle fibrosis is closely associated and overlapping with inflammation. In response to muscle injury, neutrophils are recruited to the injury site to phagocytose damaged cells and initiate regeneration (Bersini et al., 2018, Tidball, 2005, Tidball and Welc, 2015). The recruited neutrophils release chemoattractant cytokines (Soehnlein et al., 2009) which promote further infiltration of monocytes and macrophages (Fig. 2) (Mann et al., 2011, Saclier et al., 2013, Serrano et al., 2011). Macrophages have two heterogeneous phenotypes, they play active role in muscle fibrosis. The classically activated M1 phenotype produces proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 (Ogle et al., 2016, Saclier et al., 2013, Wang et al., 2018) which activates fibroblasts proliferation (Bersini et al., 2018, Pedersen et al., 2001), while the alternatively activated M2 subtype produces TGF-β1 and fibronectin (Saclier et al., 2013, Wang et al., 2018). Disturbance of the balance between M1 and M2 macrophage activation increases the expression of TGF-β1 (Arnold et al., 2007, Perandini et al., 2018). TGF-β1 activates resident fibroblasts (Bersini et al., 2018, Braga et al., 2015), and inhibits FAPs apoptosis, as well as induces their differentiation into fibrogenic lineage leading to excessive ECM deposition and fibrosis (Lemos et al., 2015). In addition, PDGFRβ^{+} cells, mesenchymal pro-fibrotic cells that show overlap with the PDGFR-α^{+} cells in fibrotic muscle, proliferate in response to injury and transdifferentiate to myofibroblast via the activation of αv integrins (Murray et al., 2017).

Muscle fibrosis commonly appears in muscular dystrophies (Serrano and Muñoz-Cánoves, 2010), aging and following muscle injury (Wang and Tang, 2016). Fibrosis deteriorates both functional and structural properties of skeletal muscle (Delaney et al., 2017, Jarvinen et al., 2002) and affects muscle fiber regeneration after injury (Murphy and Ohlendieck, 2016). In addition, fibrosis increases muscle susceptibility to re-injury (Prazeres et al., 2018).

5. Muscular dystrophies

Muscular dystrophies are a group of inherited skeletal muscle diseases caused by mutations in genes controlling stability and viability of muscle fibers (Bersini et al., 2018). There are nine major types of muscular dystrophies, among them; Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, limb girdle muscular dystrophy, myotonic muscular dystrophy, and congenital muscular dystrophies (reviewed in (Smith and Barton, 2018)). They share common characteristics, such as progressive weakness due to progressive cycles of myofiber degeneration and regeneration with progressive replacement of muscle tissue with fibrous and fat tissue (Kang and Kunkel, 2006, Serrano and Muñoz-Cánoves, 2010, Živković and Clemens, 2015). DMD is caused by mutation of the dystrophin gene and loss of dystrophin protein (Chen and Li, 2009, Živković and Clemens, 2015) which connects the cytoskeleton and ECM (McGreevy et al., 2015). Loss of dystrophin protein decreases myofiber's sarcolemmal stability which renders myofibers to be weak and eventually break upon contraction (Yucel et al., 2018).

Dystrophic muscles are characterized by accumulation of growth factors and cytokines which mediate the progression of fibrosis in dystrophic muscles (Ciciliot and Schiaffino, 2010, Serrano and Muñoz-Cánoves, 2010). Among these growth factors, the profibrotic factors, TGF-β and CTGF (Morales et al., 2018, Smith and Barton, 2018) and osteopontin (Zanotti et al., 2011) are

highly expressed in dystrophic muscles. The enhanced muscle fibrosis due to accumulation of fibrosis-related factors is considered the endpoint of severe dystrophies (Fig. 2) (Bersini et al., 2018, Wang and Tang, 2016).

6. Aged muscle

Aged muscle is characterized by loss of muscle mass, sarcopenia (Ciciliot and Schiaffino, 2010). Sarcopenia is associated with decreased muscle force and endurance together with increased fibrosis. Age-related fibrosis is mediated though different factors, such as defects in cell populations, alteration in cell signaling, and changes in growth factors regulation (Serrano et al., 2011) which in turn lead to change in muscle microenvironment (Zhou et al., 2017).

The myogenic ability of SCs decreases in aged muscles due to increased level of IL-6 (reviewed in (Forcina et al., 2018)). On the other hand, aged SCs , as well as, myoblasts have an increased tendency to convert to a fibrogenic lineage (Brack et al., 2007, Ciciliot and Schiaffino, 2010, von Maltzahn et al., 2012). This myogenic-fibrogenic conversion is under the control of the Wnt signaling pathway (Biressi et al., 2014, Brack et al., 2007), as well as, the compositional changes of ECM in aged muscle (Parker, 2015, Stearns-Reider et al., 2017). The Wnt signaling pathway mediates the myogenic-fibrogenic conversion of SCs through upregulation of TGF-β2 expression (Biressi et al., 2014). Interestingly, intramuscular injection of Wnt3a enhances the proliferation of muscle resident stromal cells into collagen-producing cells (Trensz et al., 2010).

A cross talk between Wnt/β-catenin signaling and TGF-β signaling has been shown in the pathogenesis of fibrosis. TGF-β signaling upregulates the expression of Wnt/β-catenin and vice versa ((Guo et al., 2012) for review). It has been shown that TGF-β is upregulated in aged myogenic cells together with increased level of phosphorylated Smad2/3, β-catenin, and collagen

I (Rajasekaran et al., 2017, Serrano et al., 2011). On the other hand, fibroblasts isolated from aged muscle show an increased level of TGF-β, collagen IVa2, laminin (Thorley et al., 2015), and tissue inhibitors of metalloproteinase (TIMP)-1 and 2, inhibitors of ECM degradation (Stearns-Reider et al., 2017). Therefore, collagen deposition increases in intact muscle with advancement of age (Serrano et al., 2011).

7. Factors controlling muscle fibrosis

Several growth factors have been reported to play a role in enhancing muscle fibrosis, such as TGF-β1 (Burks and Cohn, 2011, Serrano et al., 2011), CTGF, myostatin, Wnt signaling, PDGF family, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) (Fig. 3) (Laumonier and Menetrey, 2016, Mann et al., 2011).

7.1 TGF-β1

TGF-β1 is a multifunctional cytokine that has been reported as a potent profibrotic factor playing a central role in fibrosis development in different organs (Delaney et al., 2017). TGF-β1 is produced by different types of cells including inflammatory, mesenchymal, and epithelial cells (Bersini et al., 2018). The activating effect of TGF-β1 occurs through phosphorylation of Smad2/3 proteins (Lieber and Ward, 2013), which consequently activates Smad4 forming a protein complex. This complex activates transcription factors leading to the expression of target genes, such as fibronectin, CTGF and plasminogen activator inhibitor-1 (PAI-1) (reviewed in (Cisternas et al., 2014a)). TGF-β1 stimulates the fibroblasts to produce ECM proteins (Delaney et al., 2017, Kim and Lee, 2017). In addition, TGF-β1 enhances the secretion of TIMPs (Bersini et al., 2018, Kim and Lee, 2017) and PAI-1 enzymes which in turn inhibit MMP-2 and MMP-9-induced ECM

degradation (Kim and Lee, 2017). On the other hand, TGF-β1 induces the transdifferentiation of several resident cell types into myofibroblasts (Bersini et al., 2018, Darby et al., 2016). The transdifferentiated myofibroblasts express α-smooth muscle actin (α-SMA) protein (Darby et al., 2016, Mahdy et al., 2017), and synthesize a large amount of ECM (Braga et al., 2015).

7.2 CTGF

CTGF is a profibrotic cytokine. Its expression level is upregulated in dystrophic muscles (Morales et al., 2018, Sun et al., 2008). The increased expression of CTGF is correlated with necrotic-regenerative foci (Morales et al., 2018) and the degree of muscle fibrosis (Sun et al., 2008). CTGF is expressed in response to TGF-β1 (Sun et al., 2008, Vial et al., 2008). CTFC increases the expression of ECM molecules, such as collagen I α2 chain, integrins, and fibronectin (Vial et al., 2008). Overexpression of CTGF in normal skeletal muscle induces a strong increase in the fibrotic protein levels; collagen type III and fibronectin, as well as decorin and α-SMA (Morales et al., 2011). Genetic reduction of CTGF level, as well as, blocking of CTGF activity by a neutralizing antibody attenuates muscular dystrophy in *mdx* mice through reduction of muscle fibrosis and fibrotic proteins (Morales et al., 2013). However, the reduction of CTGF levels is not associated with a decrease in pSmad3, pERK1/2, and p38 signalling pathway suggesting that the fibrotic effect of CTGF is independent of the increased levels of TGF-β activity in dystrophic muscles of *mdx* mice (Morales et al., 2013).

7.3 Myostatin

Myostatin is a member of the TGF-β protein family; it was reported as inhibitor to skeletal muscle development (McCroskery et al., 2005). Myostatin stimulates TGF-β1 synthesis and

induces connective tissue formation, suggesting co-regulatory relationships of different members of TGF-β family (Zhu et al., 2007). In addition, myostatin induces the proliferation of fibroblasts *in vivo* through binding to its transmembrane receptor, activin type IIB receptors (ActRIIB). This binding activates Smad3 phosphorylation and delays p38 MAPK and PI3K-Akt activation pathway (Li et al., 2008). Inhibition of myostatin signaling induces apoptosis of fibroblasts in dystrophic muscles (Li et al., 2012). In addition, myostatin-null mice develop less fibrous tissue deposition following laceration-induced injury (Zhu et al., 2007).

7.4 Wnt signaling pathway

Wnt/β catenin is a pro-fibrotic signaling pathway responsible for fibrosis development in different organs. The canonical Wnt signaling pathway increases following injury (Cisternas et al., 2014b), aging muscle (Lieber and Ward, 2013) and dystrophic muscle (Trensz et al., 2010). The activation of the Wnt/β-catenin signaling stimulates the transformation of fibroblasts into myofibroblasts which in turn increases TGF-β expression (Cisternas et al., 2014a). Treatment with recombinant Wnt3a significantly enhances collagen deposition and upregulates expression of fibrosis-related genes, collagen I and III (Trensz et al., 2010).

7.5 PDGF family

The PDGF family, polypeptides produced by different cell types, such as macrophages, platelets, smooth muscle cells, and endothelial cells, plays important role in inflammation, wound healing, and fibrosis (reviewed in (Kendall and Feghali-Bostwick, 2014)). PDGF induces proliferation and differentiation of fibroblasts and mesenchymal cells (Kendall and Feghali-Bostwick, 2014)

through binding and activation of two different receptors, α and β ((Rosenbloom et al., 2013) for review).

7.6 VEGF

VEGF is an angiogenic factor, intramuscular gene transfer of VEGF to ischemic muscle enhances neovascularization, as well as ECM deposition resulting in muscle fibrosis (Karvinen et al., 2011). Fibroblasts isolated from dystrophic muscles treated with VEGF showed upregulation of fibronectin protein expression, as well as transformation into myofibroblasts expressing α-SMA (Gutpell and Hoffman, 2015). Interference with VEGF signaling reduces collagen deposition and inhibits pro-fibrotic gene expression (Chaudhary et al., 2007).

7.7 FGF

FGF signaling has been shown to regulate different cellular pathways and behaviors, such as cell proliferation, differentiation and survival, and wound healing (reviewed in (Lieu et al., 2011)). FGF treatment induces proliferation of fibroblasts *in vitro* in a dose-dependent manner (Yu et al., 2012). Inhibition of FGF signaling attenuates fibrosis through reducing collagen deposition and inhibiting the expression of pro-fibrotic genes (Chaudhary et al., 2007).

7.8 EGF

EGF treatment enhances fibroblast proliferation (Yu et al., 2012), as well as viability *in vitro* through the upregulation of phospho-(Ser) kinase substrates expression (You and Nam, 2013). In addition, EGF treatment of cultured fibroblasts enhances their motility and contractility (Iwabu et

al., 2004), as well as upregulates fibronectin expression through activation of protein kinase C delta (PKC δ) signaling pathway (Mimura et al., 2004).

8. Murine models of muscle fibrosis

Among mammalian models of DMD, several murine models have been used to investigate muscle fibrosis due to ease of breeding and genetic engineering, and lower cost compared to using large animal models, such as dogs and pigs (Rodrigues et al., 2016). These models can be categorized into two main groups; dystrophic and normal non-dystrophic models (Pessina et al., 2014). Using these models are very essential to understand the mechanism of muscle fibrosis and develop novel therapeutics against fibrosis (Bersini et al., 2018).

8.1 Dystrophic models

There are several dystrophic animal models, either naturally occurring or laboratory-generated ones. These models are useful for better understanding of the pathobiology of DMD disease in order to develop novel therapeutic strategies for treating DMD (McGreevy et al., 2015).

The *mdx* mouse (C57BL/10 background) is the most common experimental model for studying DMD (Ameen and Robson, 2010, Bersini et al., 2018). This mouse model lacks the dystrophin protein as in DMD patients (Chen and Li, 2009). However, the *mdx* mouse does not exhibit the exact pathogenesis and progression of DMD patients. The *mdx* mouse shows limited fibrosis in the limb muscles of aged mice (Gutpell et al., 2015, Pessina et al., 2014) while fibrosis is developed in diaphragm muscle only (Bersini et al., 2018, Gutpell et al., 2015), which makes evaluation of the effectiveness of fibrosis treatment much difficult.

There are several attempts that have been done to enhance muscle fibrosis in *mdx* mouse strain to mimic human muscular dystrophy. These trials aim to overcome the limited fibrosis in the limb muscles of aged *mdx* mice which is considered a major disadvantage of this model (Gutpell et al., 2015, Pessina et al., 2014). These attempts include crossing the *mdx* strain with either different genetic backgrounds or other gene-knockout strains to enhance the progression of dystrophic phenotype in *mdx* mouse (McGreevy et al., 2015). However, crossing *mdx* strain with different genetic backgrounds, such as albino, BALB/c, C3H, C57BL/6, DBA/2, and FVB shows different phenotypic variations that differ with different backgrounds (Fukada et al., 2010, McGreevy et al., 2015). Among these crossing strains, the DBA/2-*mdx* mouse strain shows more comparable features of the human condition, such as hind limb muscles with lower muscle weight, muscle weakness, fewer myofibers, and increased fat and fibrous tissue deposition (Fukada et al., 2010, Rodrigues et al., 2016). However, the DBA/2 strain have various mutations in different genes resulting in hearing loss and eye abnormalities (McGreevy et al., 2015). On the other hand, the Dmd^{mdx} mice (D2-mdx) show earlier dystrophic phenotype, more severe disease phenotype, and early onset of cardiac function deficit suggesting that this model is more suitable than *mdx* mice (Coley et al., 2016).

8.2 Non-dystrophic models

Due to the fact that the course of inflammation at early stages of *mdx* dystrophy is similar to the innate immune response following acute muscle injury (Tidball and Villalta, 2010). Normal non-dystrophic muscles following induction of degeneration/regeneration events could be used as models for fibrosis (Pessina et al., 2014). ECM accumulation has been reported in nearly all models of muscle injury. However, this accumulation is transient in some injury models (Lieber and Ward,

2013, Mahdy et al., 2015). Developing fibrosis in normal non-dystrophic muscles is an easy method to perform and will help therapeutic purposes to test different therapies to limit muscle fibrosis (Pessina et al., 2014). These models could be obtained either following traumatic-induced injuries, surgical-induced injuries or chemical-induced injuries.

8.2.1 Traumatic-induced models

Traumatic injuries include laceration and crushing. Muscle laceration induces extensive inflammatory response with upregulation of TGF-β1 which in turn induces the differentiation of myoblasts into fibrotic cells (Li et al., 2004). The lacerated muscle develops collagen tissue formation up to 12.8% at 4 weeks after injury (Chan et al., 2003). However, regeneration in this model is challenging: suturing muscle laceration results in functional healing but decreases fibrosis, while, immobilization does not provide good environment for muscle regeneration (Menetrey et al., 1999).

Crushing-induced injury is performed through applying a clamp or dropping a mass on a superficial muscle (Speck et al., 2013, Takagi et al., 2011). As a result of crushing injury, collagen fibers accumulate around the myofiber bundles (Filippin et al., 2011) with increased levels of TGF-β1 and insulin-like growth factor (IGF-I) (Takagi et al., 2011, Zimowska et al., 2009). However, fast-twitch muscle regenerates better with less fibrosis compared to slow-twitch muscle following crush-induced injury (Zimowska et al., 2009).

8.2.2 Surgical-induced models

Tenotomy, resection of muscle tendon, activates interstitial fibrosis (Akpulat et al., 2016, Valencia et al., 2017) due to increased levels of MMP-2, TIMP-2, and TGF-β1 (Hirunsai et al., 2015). However, this model lacks myofiber necrosis, inflammatory events, and regenerating myofibers

(Akpulat et al., 2016). In addition, tenotomy induces myofiber atrophy which results in loss of the contractile function of muscle (Valencia et al., 2017).

Denervation, resection of the nerve supplying the muscle, induces progressive increase in collagen I deposition in the endomysium and perimysium (Fanbin et al., 2011, Faturi et al., 2016, Madaro et al., 2018). The increased fibrosis is associated with a rapid increase in TGF-β1 (Fanbin et al., 2011, Liu et al., 2016) which subsequently increases CTGF and α-SMA expression levels (Liu et al., 2016). Muscle fibrosis persists following denervation and is associated with progressive accumulation of FAPs and activation of signal transducer and activator of transcription 3 (STAT3) and IL-6. However, denervation-induced model is associated with myofiber atrophy (Madaro et al., 2018).

8.2.3 Chemical-induced models

Injection of biological toxins, such as cardiotoxin and botulinum toxin, or chemical agents, such as barium chloride and glycerol, into skeletal muscle, induces muscle injury followed by regeneration with connective tissue deposition (Lieber and Ward, 2013, Mahdy, 2018).

8.2.3.1 Biotoxins

Intramuscular injection of biotoxins, such as cardiotoxin and botulinum toxin is widely used method to induce muscle regeneration. Cardiotoxin is a biotoxin isolated from cobra venoms (Czerwinska et al., 2012). It is used to induce muscle degeneration and regeneration (Mahdy et al., 2016). Cardiotoxin induces accumulation of fibrous tissue at early regenerative stage, at day 7 after injury, then subsequently decreases and return to the non-injured state at two weeks after injury (Mahdy et al., 2015, Pessina et al., 2014). Muscle fibrosis following cardiotoxin injection is

associated with transient accumulation of FAPs, STAT3 and IL-6 (Madaro et al., 2018). This model is suitable to investigate early fibrosis. However, the major disadvantage of this model is the mild and transient fibrosis following injury (Mahdy, 2018, Pessina et al., 2014).

8.2.2.2 Chemical agents

Chemical agents are used to induce muscle damage and regeneration, such as barium chloride (Pessina et al., 2014) and glycerol (Kawai et al., 1990, Mahdy et al., 2016). Fibrous tissue accumulation differs between the two models. Single intramuscular injection of barium chloride induces a very mild and transient fibrosis. While repeated injections of barium chloride induce significant fibrosis (Pessina et al., 2014). These results suggest that repeated myotoxin injections, one injection/week for six weeks, could be an alternative to induce fibrosis in non-dystrophic muscle. However, this method requires up to eight weeks, from the first injection, to develop significant fibrosis as fibrosis becomes clear two weeks after last injection (Pessina et al., 2014).

Collagen deposition following glycerol-induced injury differs according to the animal. Glycerol injection is used mainly to induce muscle adiposity in mice, meanwhile, it induces mild accumulation of fibrous tissue at day 4 after injury which increases progressively with regeneration to become apparent at late stage of regeneration, at 2 weeks after injury (Mahdy et al., 2015, Uezumi et al., 2010). However, this model lacks significant accumulation of fibrous tissue at early stages following injury. In contrast to mice, glycerol injury in rats induces significant accumulation of fibrous tissue at early stages following injury which increases up to day 14 after injury. Interestingly, glycerol-induced injury in rats is not accompanied by adipocytes infiltration as in mice (Mahdy et al., 2018). This difference is suggested to be due to persistent inflammatory cells, up to day 14 after injury in rats, which secret various inflammatory cytokines and in turn alter

muscle environment and enhance muscle fibrosis (Gosselin and McCormick, 2004). These difference between mice and rat in response to glycerol injury suggest that glycerol-injured rat muscle is more suitable for studying fibrosis than that of mice.

9. Strategies for enhancing muscle fibrosis in the available models

Several approaches have been done to overcome the disadvantages of *mdx* mice. Pessina et al. (2014) develops novel strategies to enhance muscle fibrosis in *mdx* mice through inducing damage into the limb muscles of young *mdx* mice. These strategies include chronic exercise, injection of myotoxic agents, and delivery of profibrotic growth factors into muscles of young *mdx* mouse. In addition, combining treatments in normal non-dystrophic muscles provides a promising approach to enhance muscle fibrosis that can be applied in genetically modified animal models (Mahdy et al., 2017, Pessina et al., 2014).

Recently, we have shown that combination of the profibrotic cytokine, TGF-β1, with glycerol-induced injury provides a simple method to enhance collagen deposition at the early stages of regeneration in glycerol-injured model, however, it inhibits adipocyte infiltration (Mahdy et al., 2017). It was reported that TGF-β1 inhibits the differentiation of mesenchymal progenitors into adipocytes *in vitro* as well as *in vivo* (Arrighi et al., 2015) and enhances the expression of fibrosis-related genes (Uezumi et al., 2011).

10. Strategies for inhibition of muscle fibrosis

Strategies for combating fibrosis basically depends on targeting TGF-β signaling pathway as an important mediator for fibrosis development (reviewed in (Burks and Cohn, 2011, Garg et al.,

2015, Walton et al., 2017)). In this respect, several factors have been used to limit muscle fibrosis development due to their anti-fibrotic effect (Fig. 3).

10.1 TGF-β inhibitors

Suramin

Suramin is an anti-parasitic and anti-neoplastic agent approved by the Food and Drug Administration (FDA). It acts as anti-fibrotic agent through competitive binding to the receptors of several growth factors including TGF-β1 (reviewed in (Garg et al., 2015)). Suramin treatment reduces muscle fibrosis following contusion-induced injury (Nozaki et al., 2008). In addition, suramin inhibited muscle fibrosis following laceration-induced injury *in vivo* and reduces fibroblast proliferation, as well as fibrotic proteins, α-SMA and vimentin, *in vitro* (Chan et al., 2003).

Decorin

Decorin is a member of small leucine-rich proteoglycan family. It consists of a protein core and a glycosaminoglycan chain (reviewed in (Chen and Birk, 2013)). Decorin treatment inhibits muscle fibrosis following laceration-induced injury and reduces the fibrosis-related proteins through binding to TGF-β1 legend (Fukushima et al., 2001, Hwang et al., 2006, Li et al., 2004). In addition, decorin suppresses the inhibitory effect of myostatin through upregulation of follistatin (Zhu et al., 2007). *In vitro* studies revealed that decorin up-regulates follistatin expression and down-regulates myostatin and TGF-β1 expression (Li et al., 2007). Furthermore, decorin inhibits the fibrotic activity of CTGF (reviewed in (Brandan and Gutierrez, 2013)).

Losartan

Losartan, an FDA-approved anti-hypertensive medication that blocks angiotensin II type 1 (AT1) receptor, is recently used as an anti-fibrotic treatment (Hwang et al., 2016, Kim et al., 2017). Losartan treatment significantly reduced fibrosis in dystrophic muscle (Elbaz et al., 2012), as well as after contusion and laceration-induced injuries (Bedair et al., 2008, Kobayashi et al., 2013). Losartan inhibits AT1 activation which indirectly blocks TGF-β1 activation (reviewed in (Garg et al., 2015)). Losartan reduces fibrotic markers; TGF-β1 and CTGF, in addition to collagen type I and III expression. Although losartan treatment reduced fibrosis development following volumetric muscle loss, it neither improved muscle regeneration nor function (Garg et al., 2014).

INF-γ

INF-γ is a TGF-β1 pathway inhibitor, it significantly reduces muscle fibrosis and improves physiological properties of injured skeletal muscles (Chen et al., 2008, Foster et al., 2003). INF-γ upregulates Smad7 expression (reviewed in (Garg et al., 2015)). Interestingly, Cheng et al. (2008) showed upregulation of mRNA expression and protein levels of endogenous INF-γ at early stages following cardiotoxin-induced injury. This endogenous INF-γ which is produced by inflammatory cells and myoblasts is essential for efficient regeneration (Cheng et al., 2008). This elevation of INF-γ could explain the transient fibrosis and efficient regeneration following cardiotoxin-induced injury which was reported in the previous studies (Mahdy et al., 2015, Pessina et al., 2014).

Halofuginone

Halofuginone is the analog of febrifugine, an alkaloid isolated from the plant *Dichroa febrifuga*. It is used as antimalarial, as well as antiprotozoal drug, against coccidiosis and cryptosporidiosis in poultry and cattle, respectively (reviewed in (Pines and Spector, 2015)). Halofuginone has

antifibrotic properties, it blocks TGF-β-mediated Smad phosphorylation (Turgeman et al., 2008) which significantly reduces collagen production in *mdx* mice (Huebner et al., 2008). In addition, halofuginone treatment induces apoptosis of fibroblasts isolated from *mdx* muscles (Bodanovsky et al., 2014).

10.2 Myostatin inhibitors

Follistatin

Follistatin belongs to the tissue growth factor-β family, it antagonizes several members of the family, such as myostatin, activin, and growth differentiation factor 11 (Gilson et al., 2009, Yaden et al., 2014). Follistatin treatment significantly downregulates CTGF gene expression and Smad2/3 protein in cardiotoxin-injured muscle (Yaden et al., 2014). Follistatin-overexpressing mice develop a significantly decreased level of fibrosis in response to laceration-induced injury compared to wild-type mice (Zhu et al., 2011). Gene therapy using follistatin in combination with replacing the defective gene, dystrophin, has been used as a treatment for muscular dystrophy. This combination increases muscle strength, as well as reduces muscle fibrosis (Reviewed in (Rodino-Klapac et al., 2009)).

ActRIIB blockade

ActRIIB is the transmembrane receptor for myostatin (Burks and Cohn, 2011). ActRIIB is expressed in muscles of *mdx* mouse with higher level in the fast-twitch than the slow-twitch muscles (Morine et al., 2010). Targeting myostatin is done by using the soluble form of ActRIIB (ActRIIB/FC) which traps myostatin and prevent its binding to its receptor, ActRIIB ((Amthor and Hoogaars, 2012) for review). ActRIIB/FC significantly reduces the proliferation of dystrophic

muscle fibroblasts and induces their apoptosis *in vitro,* as well as in *mdx* mice *in vivo* (Li et al., 2012). Therefore, ActRIIB/FC treatment is used to inhibit progressive fibrosis in dystrophic muscles. However, blocking of myostatin using ActRIIB blockade exacerbates heart failure in canine models of DMD (Amthor and Hoogaars, 2012).

10.3 Other cytokines inhibitor

Nintedanib

Nintedanib, a tyrosine kinase inhibitor, is primarily used as a clinical anti-fibrotic treatment of idiopathic pulmonary fibrosis (Rosenbloom et al., 2013). It inhibits inflammation and fibrosis in lung fibrosis models (Wollin et al., 2014). Nintedanib targets the receptor kinase receptors of FGF, PDGF and VEGF. A recent study showed that nintedanib significantly reduces fibroblast proliferation *in vitro*, as well as, muscle fibrosis and ECM proteins in *mdx* mouse *in vivo.* Furthermore, nintedanib treatment improves muscle functional tests in *mdx* mice suggesting its potential use for treatment of DMD patients (Piñol-Jurado et al., 2018).

11. Concluding remarks

Muscle fibrosis is a characteristic feature of muscular dystrophies, myopathies, and severe injuries. Excessive deposition of ECM impairs muscle function, as well as muscle regeneration after injury. In addition, persistent fibrosis hinders gene- and cell-based therapies for muscular dystrophies. Therefore, understanding the mechanisms through which the profibrotic factors activate muscle fibrosis is an essential step toward developing anti-fibrotic treatments. Despite the existence of different types of models to study fibrosis, these models are not similar to the severe form of human dystrophies. Therefore, it is important to enhance fibrosis in the available genetic models through delivery of profibrotic growth factors and develop novel wild-type models with severe fibrosis

through combination of injuries and/or delivery of profibrotic growth factors into injured muscles. Enhancing fibrosis models will help to develop effective anti-fibrotic therapies as a promising approach for treating muscular dystrophies.

Acknowledgements:

The author is supported by a Postdoctoral Fellowship from University of Pretoria, South Africa. The author thanks Hamza I. Isa, Ahmadu Bello University, Zaria, for editing the draft of this manuscript.

Conflict of interest:

The authors declare that they have no conflict of interest

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Figure legends:

Fig. 1: Schematic diagram showing arrangement of extracellular matrix (ECM) in skeletal muscle in three levels; the endomysium, perimysium, and epimysium. Figure was produced using Servier Medical Art (https://smart.servier.com).

Fig. 2: ECM deposition in regenerating/fibrotic muscle. Upper panel (A-E) shows basic histological structure of normal regenerating muscle with transient infiltration of inflammatory cells after injury followed by appearance of newly formed myotubes with central nuclei and transient deposition of ECM. Lower panel (F-J) shows fibrotic muscle due to chronic injury/muscular dystrophy with persistent inflammatory cells and progressive replacement of muscle tissue with fibrous tissue. HE stain. * indicates ECM deposition. Figure modified from (Mann et al., 2011).

Fig. 3: Schematic diagram showing factors enhancing muscle fibrosis and inhibitors for muscle fibrosis. Figure was produced using Servier Medical Art (https://smart.servier.com).

Fig.1

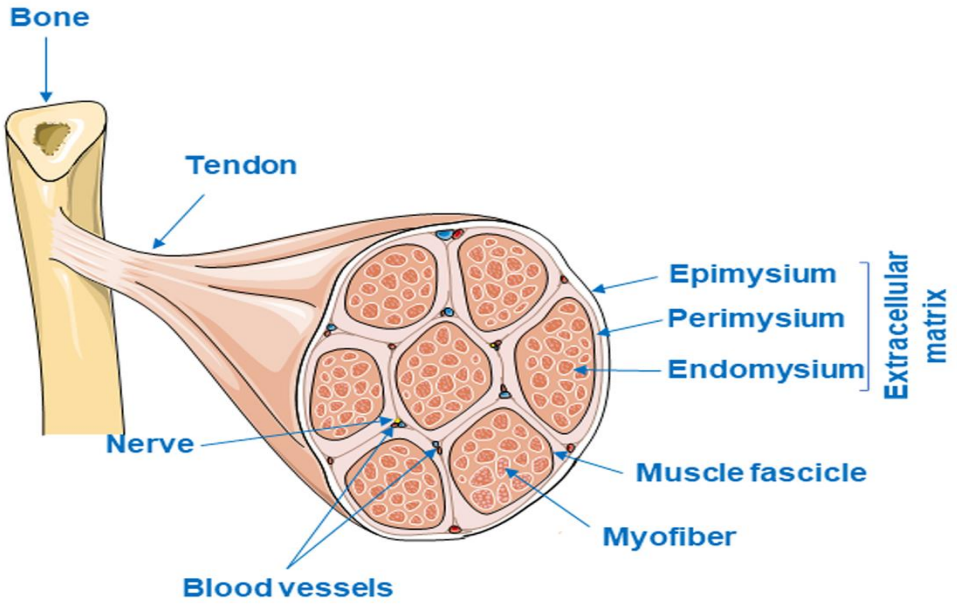
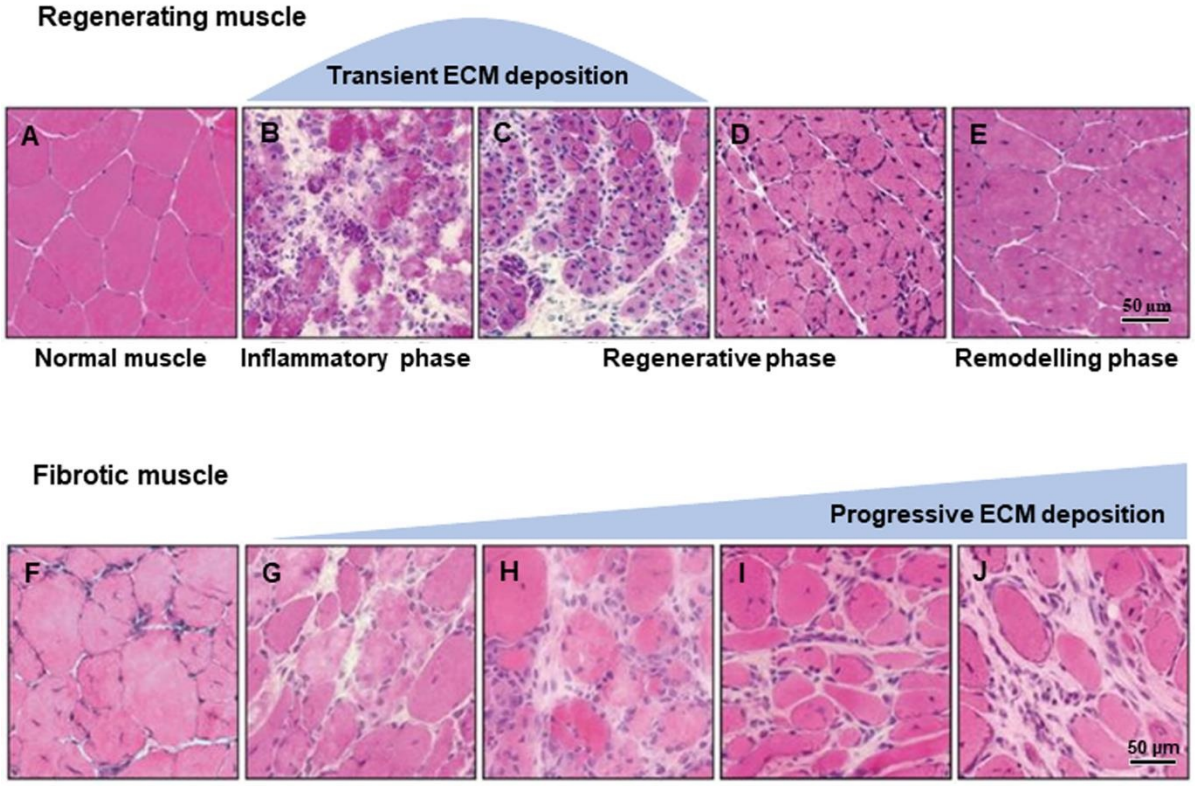


Fig.2


Regenerating muscle
Transient ECM deposition
A
B
C
D
E
50 µm
Normal muscle
Inflammatory phase
Regenerative phase
Remodelling phase
Fibrotic muscle
Progressive ECM deposition
F
G
H
I
J
50 µm

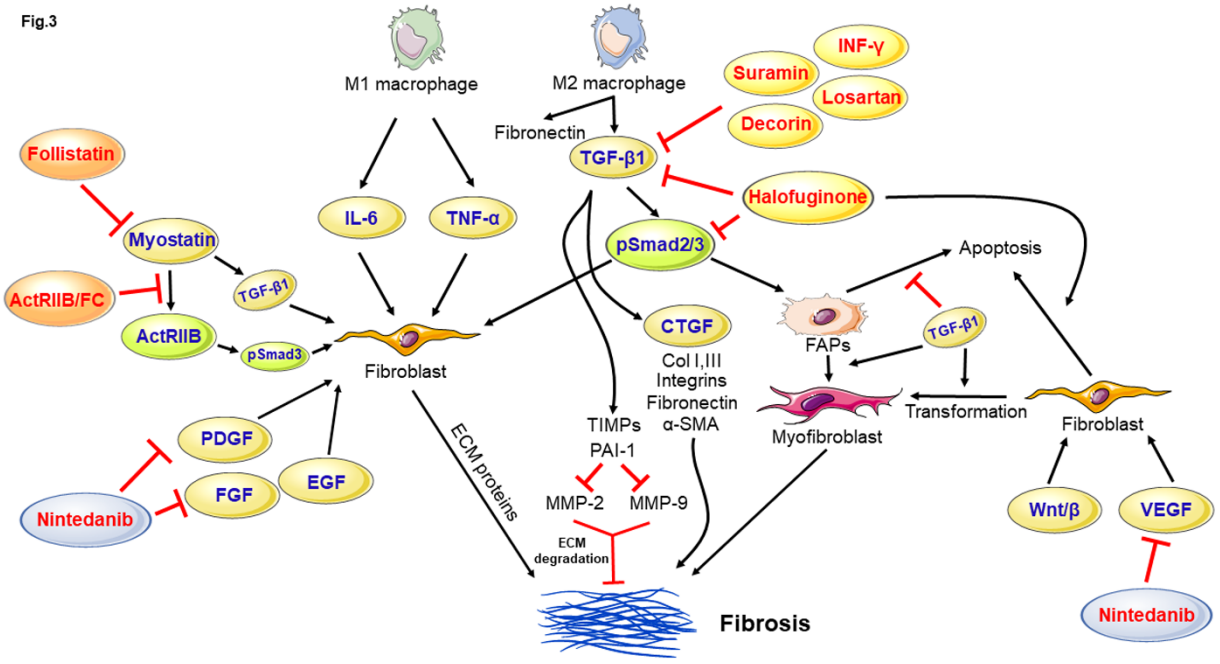


Table.1: Extracellular components of skeletal muscle.

	Endomysium	Perimysium	Epimysium
Level	- Surrounds each myofiber	- Surrounds group of myofibers	- Surrounds entire muscle
Function	- Maintains muscle integrity -Transmits tension between muscle fibers - Contains small diameter blood vessels and finest neurons	- Transmits the force from myofibers to tendons - Contains larger blood vessels and nerves	- Continuous with the tendons connecting muscles to bones - Thickens at both muscle origin and insertion - Major blood vessels and nerve supplying the muscle penetrate the epimysium
Composition	- Collagen type I, type III, and type V	- Collagen type I and type III	- Collagen type I