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Traumatic muscle fibrosis: From pathway to prevention

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Abstract

Muscle fibrosis, the disruption, of functional parenchyma by stromal elements, is an often overlooked sequela of traumatic muscle injury, ageing, and congenital disease. The remarkable regenerative capacity of skeletal muscle is dependent on the interaction of myogenic progenitors and the same stromal connective tissue elements responsible for fibrosis generation and propagation. The coordination of effective therapeutic strategies to mitigate muscle fibrosis following injury requires a clear understanding of the prominent cellular progenitors, extracellular constituents, and signaling mechanisms involved in muscle healing. Recent studies have begun to elucidate the critical cellular processes that delineate physiologic regeneration and dysregulated healing resulting in muscle fibrosis. This review presents the salience of these novel findings in the context of the current treatment paradigms for muscle fibrosis.

I. Introduction

Skeletal muscle is a dynamic organ that mediates voluntary movement and aids in the protection of vital structures. The unique functional demands placed upon skeletal muscle confer susceptibility to frequent and persistent injury. Accordingly, skeletal muscle exhibits significant regenerative capacity, and is capable of repair and replacement of injured or damaged myofibers with little to no decrement in function (1). Structurally, muscle is composed of longitudinally oriented contractile elements, surrounded and compartmentalized by stromal connective tissue and anchored to bony structures through

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tendinous insertions. Despite a remarkable capacity for regeneration, fibrotic replacement of functional muscle by stromal elements has been well documented in response to trauma, heritable disease, and aging. Indeed, muscle fibrosis poses a significant clinical problem for patients following radiation treatment, crush injury, laceration, and amputation, resulting in progressive loss of function and significant morbidity. Recent studies have begun to elucidate the biomolecular mechanisms underlying the balance of muscle regeneration and muscle fibrosis, and the critical departures in cellular processes that delineate the two. While the deposition of extracellular elements, and proliferation of stromal cells following injury are thought to underlie the pathogenesis of fibrosis formation, those same elements have proven indispensable for successful regeneration after injury, suggesting a critical and well-orchestrated balance between functional muscle tissue and the connective tissue that surrounds it. A clear understanding of the prominent cellular progenitors, extracellular constituents, and signaling mechanisms involved in muscle healing are essential to address skeletal muscle fibrosis in the clinical setting for the purposes of optimizing functional recovery following injury.

II. Clinical Manifestations

A. Traumatic Musculoskeletal Injury and Repair

Traumatic injury is often sufficient to disrupt the regenerative capacity of skeletal muscle, and the resulting fibrosis remains an often overlooked sequelae of pathologic healing. During the current armed conflicts in Iraq and Afghanistan (2005–2009), musculoskeletal injuries comprised 77% of military casualties (2). In the civilian setting, fibrotic accumulation has also been well documented following less severe injuries including muscle strain, laceration, and contusion of muscle tissue. Muscle strain proves the most frequent sports injury whereby the extent of muscle fibrosis corresponds to the severity of injury (3). In addition to physical trauma, thermal and ionizing radiation are sufficient to disrupt muscle regeneration. Fibrosis of skeletal muscle and overlying cutaneous tissue is a well-documented and debilitating consequence of radiation treatment in cancer patients, resulting in loss of function of critical structures (4). The extent and severity of muscle injury correlates with functional disability and loss of function. In athletes, the extent of connective tissue accumulation in injured hamstring muscle correlated with days lost from competition(5). An assessment of functional outcomes using the comprehensive Quality of Well Being (QWB), reveals persistent functional limitation following traumatic injury, of these 85% were blunt trauma affecting the extremities(6).

When precipitated by acute injury, the generation and propagation of muscle fibrosis follows distinct and well-orchestrated stages (7) (Fig. 1). Successful muscle regeneration begins with an initial inflammatory infiltration resulting in muscle degeneration, phagocytosis of injured myofibers, and deposition of extracellular elements including tenascin-C, fibronectin, and hyaluronic acid (8). Fibroblasts and other stromal cells recruited to the site of injury subsequently deposit type I, III, and VI collagens. Inciting trauma concurrently activates resident myogenic satellite cells, which subsequently proliferate and fuse. The transient extracellular matrix is gradually remodeled and replaced as viable muscle tissue matures.

Histologically, regenerating myofibers can be identified by centrally located nuclei and the mature muscle by the characteristic migration of nuclei to the periphery of the cells.

Dysregulation at any of these stages may compromise muscle healing, and by doing so permit fibrosis formation (Fig. 2). The cellular composition of the inciting inflammatory infiltrate helps to orchestrate the progression through each stage of healing. An initial predominance of 'inflammatory' M1 macrophages, which secrete high levels of the cytokines TNF α , IL-1 β , and IL-12, drives phagocytosis of debris and induces apoptosis of fibrotic progenitors (9,10). Inflammatory cells present at the site of injury, specifically monocytes, subsequently shift to an 'alternatively activated', M2, anti-inflammatory regulatory cell phenotype. M2 macrophages clear apoptotic cells, secrete high levels of anti-inflammatory cytokines IL-1ra, IL-10 and TGF β , and while designated as 'pro-fibrotic', promote tissue repair wound healing and muscle regeneration (11,12). Severe and persistent injury, manifested by chronic inflammation, disrupts this paradigm, resulting in fibrosis formation (13).

B. Hereditary Disorders

The hereditary causes of muscular disorders are widespread and varied, resulting from critical mutations in the costamere apparatus responsible for force transduction from muscle to skeletal muscle extracellular matrix (ECM), to metabolic storage disorders, and over activation of osteogenic signaling pathways in soft-tissue. The debilitating impact of skeletal muscle fibrosis can be seen most prominently in the heritable dystrophies. Duchenne's muscular dystrophy is an X-linked recessive disorder characterized by progressive proximal muscular weakness, which affects the pelvic girdle more commonly and earlier in life than in the distal appendages and shoulder girdle. Duchenne's and Becker's, the phenotypically milder form of the disease are caused by loss of function deletion and alteration of the dystrophin gene. Together these heritable dystrophies account for more than 80% of all muscular dystrophies, with an incidence of 1 in 3500 newborn males(14). The dystrophin protein is a component of the structural complex responsible for transmitting force from myofibers to the surrounding extracellular matrix. The progressive accumulation of fibrosis eventually involves vital muscular organs, including the heart and diaphragm. By the third decade of life, DMD patients usually exhibit established cardiac diseases, and demise is usually due to ventricular dysfunction, heart block, or arrhythmias.

C. Aging/Metabolic Disorders

Environmental and modifiable risk factors are also sufficient to compromise muscle regeneration and engender fibrosis. Fat infiltration in skeletal muscle has been well described in patients with obesity (15), Type II diabetes mellitus (16), and spinal cord injury (17). The presence of adipocytes in the connective tissue septa of gastrocnemius muscles in healthy adults(18,19), suggests disease need not be a prerequisite for stromal infiltration despite a strong correlation between metabolic disease and intra-muscular ectopic fat. Multiple studies have indicated that aging induces fibrotic accumulation within muscle (20, 21) Muscle loss with aging is progressive and exacerbated by weight loss. However, even in weight stable individuals, the deposition of ectopic fat leads to progressive loss of muscle quality as determined by cross-sectional area and force generation (20). This can be

attributed in part to the depletion of a viable stem cell pool within the skeletal muscle niche (21). Studies assessing molecular and cellular changes within aged muscle reveal a number of age-related homeostatic perturbations, including decreased Notch signaling, increased activation of the TGF β signaling pathway, and reduced proliferative potential of myogenic progenitors which predispose to fibrosis formation (22,23).

Nutritional, energy balance, and other metabolic factors play a unique and critical role in muscle regeneration. In particular, the branched chain amino-acid leucine upregulates downstream protein synthesis essential for muscle regeneration through direct interaction with the mammalian target of rapamycin (mTOR) pathway. (24). The mTOR/AKT pathway is the downstream mediator of multiple anabolic growth factors including insulin-like growth factor (IGF), and regulates cellular processes including proliferation and protein synthesis. Specifically regarding the activation and proliferation of satellite cells, a recent study in a murine model demonstrated a TGF β -independent decrement in myogenic differentiation markers, and a compromise of myofiber size (25)

D. Current and Novel Therapeutics

Excessive fibrosis and disruption of physiologic regeneration engenders a connective tissue scar that compromises muscle function. Current treatment paradigms are oriented to reduce scar formation and support muscle regeneration. The rationale for current treatment, although supported by experimental findings gleaned from basic science and translational studies, often lack definitive evidence due to the paucity of standardized case-control studies regarding muscle healing. Consistent with most soft-tissue injuries, immediate care for traumatic muscle injury focuses on the RICE paradigm (rest, ice, compression, and elevation), to reduce blood flow and subsequent inflammation to the site of injury (26). Immediate immobilization of the injured muscle has been shown to minimize deformation at the injury site and extent of connective tissue scar formation (27). Continued immobilization, however, has been implicated in atrophic and compromised healing, and conversely, mobilization of healing muscle is essential for myofiber growth and orientation (3, 28). The ideal timing of mobilization remains the subject of debate, and consensus is further compounded by variability of location and extent of injury.

Depending on the specific injury sustained, operative treatment may be warranted. In cases where muscle is nearly or completely lacerated, and near the musculo-tendinous junctions, suture repair proves beneficial. Previous studies in mouse laceration models demonstrate that surgically repaired muscles have better functional results than immobilized limbs (29). In addition, studies on torn biceps in military service subjects showed that surgically repair biceps had better functional, satisfaction and cosmetic outcomes over long-term follow-up (30). The type of surgical stitch employed may have a significant effect on the outcome, satisfaction score, and failure rate (30). The Kessler stitch and Mason-Allen stitch are commonly used suture patterns in intra-substance repairs. Direct comparison of strength and failure of the Kessler stitch and a combination of the Mason-Allen stitch with perimeter suturing in a porcine muscle model showed the combination to have higher load to failure (30). Careful patient selection, stitch employment, and post-operative planning are critical for a successful outcome.

Other medical treatments are widely used to mitigate inflammation following injury, and other prophylactic treatments are in development. Non-steroidal anti-inflammatory drugs (NSAIDs), particularly cyclooxygenase (COX) inhibitors are commonly used in the acute post-traumatic setting due to their anti-inflammatory and analgesic effects. Use of these agents, however, should be employed with discretion. Use of NSAIDs within the first 48 hours following injury has been shown to interrupt the initial inflammatory infiltrate necessary for muscle regeneration and subsequent activation of myogenic cells in response to injury (31, 32). Further, NSAIDs have been shown to increase the rate of aseptic nonunion in long bone fractures by an odds ratio of 2.51, and thus should be avoided in the setting of concomitant fracture or polytrauma (33).

The identification of mesenchymal fibro-adipogenic progenitors (mFAPs) responsible for fibrotic accumulation has led to renewed interest in small molecule tyrosine kinase inhibitors (TKIs), and their selective inhibition of fibrogenesis. Recent clinical studies have demonstrated efficacy of TKIs in the treatment of multiple fibrotic diseases including idiopathic pulmonary fibrosis and systemic sclerosis (34). Specifically, studies in mouse models demonstrate a reduction in MFAP proliferation when treated with nilotinib, a next generation TKI which targets both the TGF β and PDGFR receptor pathways, and has an improved side-effect profile than imatinib (10, 35). Though the effects of nilotinib appear to be cell-autonomous to mesenchymal FAPs, the drug diminishes proliferation of myogenic cells through reduction of trophic support provided by the former(35). The documented clinical success of TKIs in related pathologies, and recent studies in murine models suggest these class of drugs have great therapeutic potential for treating muscle injury.

More novel therapeutic approaches include biologics such as members of the fibroblast growth factor (FGF) and the insulin-like growth factor (IGF) family of proteins are subjects of on-going investigation and show promise in accelerating the regeneration process and myogenic proliferation(36). Growth factor supplementation, however, causes unwanted effects including joint pain and swelling (37). Targeted inhibition of TGF β , a well-known potentiator of fibrosis through the SMAD phosphorylation pathway, is another strategy to delay the fibrotic response in favor of functional myofibril proliferation and recovery. Suramin, a heparin sequestration compound, antagonizes TGF β function on fibroblast proliferation (38). Relaxin has also been show to inhibit fibrosis in a dose-dependent manner(39). Continued efforts to identify molecular targets to selectively inhibit TGF β will provide novel strategies for controlling this central regulator of fibrosis. Tissue engineering through stem cell technologies provide another potential avenue for healing. Recent reports using muscle-derived and adipose-derived stem cells have shown success in mouse models to improve muscle regeneration, strength, angiogenesis, and reduction in fibrosis. Incorporation of stem cells in novel biomaterial vehicles, including atellogenic scaffolds has also shown significant promise. However, the availability/harvesting of these autologous stem cells in relation to the timing of the injury remains a significant hurdle (40). An alternative approach of cell-derived therapy demonstrates an improved regenerative response to the transplantation of M1 polarized macrophages in the setting of muscle injury, potentially attributable to increased cell recruitment and levels of insulin-like growth factor 1 (IGF-1) at the site of injury (41).

Skeletal muscle is a tissue innately responsive to mechanical force. The specificity of directionality and magnitude of mechanical forces perceived by muscle tissue modulate multi-factorial responses including attenuation of inflammatory response, glucose uptake, and activation of satellite cells (42–44). It is no surprise then, that mechanical manipulation of injured and regenerating muscle can augment healing; the use of massage and other therapeutic manipulation of muscle following injury has been shown to improve outcomes(45). Further, the advent of novel biomaterials, including implantable ferrogels have been demonstrated to improve muscle regeneration following regular pulsatile activation of the implant (46). Taken together, the use of focused mechanical manipulation, uncomplicated by biologic and, can serve as a translatable treatment modality to improve muscle healing following trauma.

III. Translational Study of Fibrosis and Cellular Mediators

A. Satellite Cells

The series of events leading to muscle fiber regeneration following injury begin with activation of resident myogenic stem cells, referred to as satellite cells. Injured myofibers are replenished by resident myogenic progenitors, most faithfully identified by markers Pax7, a paired box transcription factor, and M-Cadherin (47, 48). These cells lie within the basal lamina and sarcolemma of adult myofibers are capable of self-renewal (49, 50), and differentiate into myotubule progenitors which fuse with other differentiating cells or previously damaged myofibers following injury (51). The transcription factor pax7 in turn activates downstream myogenic regulatory factors (MRFs) including Myf5 and MyoD, which induce myogenic differentiation in myoblasts (52, 53). Expression of these MRFs indicates a committed myogenic fate, and loss of Pax7 positivity indicates a loss of quiescence and the capacity for symmetric differentiation. Using lineage restricted susceptibility to diphtheria toxin, Sambivisian et. al, successfully demonstrated the necessity of Pax7⁺ cells for muscle regeneration following injury and exercise (Fig. 4) (54). Exclusive ablation of this population was sufficient to abrogate neomyogenesis regardless of contribution of other candidate cell types. The regenerative potential of muscle decreases with age, and this decrement has been attributed in part to an increase of Wnt signaling in the systemic environment (21). While the functions of replenishment of the stem cell pool and myogenic differentiation capacity have been well characterized, recent studies have demonstrated other functions of this population including production of ECM elements to supplement that of surrounding fibroblasts. Satellite cells are capable of synthesizing collagens I and VI, the relative amounts of which change upon “activation” and subsequent myogenic differentiation (55).

Given the remarkable regenerative capacity of skeletal muscle, and stem-cell characteristics of Pax7⁺ satellite cells, many hypothesize whether fibrotic accumulation in muscle tissue with aging or trauma may also arise from satellite cells indicating a failure of differentiation. Indeed previous experiments have demonstrated spontaneous mesenchymal conversion of Pax7 lineage cells when cultured (56). The purification of satellite populations, however, are dependent on long culture times, and staged replating in order to separate faster growing myogenic cells from slower growing mesenchymal cells. A pure lineage of satellite cells

then are influenced by the stress of culture conditions, and may be fundamentally different from their analogues *in vivo* (57). Nonetheless, this subset represents only 10% of the population, suggesting fibrotic accumulation in the setting of regenerative failure is likely not due to impaired differentiation of this cellular population.

B. The Satellite Cell Niche

The behavior and regenerative capacity of resident satellite cells are dependent on the well characterized “niche”, located between the sarcolemma of individual myofibers, and enveloping basal lamina of the endomysium. This conformation provides polarity to satellite cells, orienting the apical aspect of those cells toward the sarcolemma (58). The structural components of ECM change considerably over the course of regeneration following trauma. The unique structural components of the transitional ECM, including hyaluronic acid, tenascin-C, fibronectin, and membrane associated proteins with which they interact coordinate cues for myogenic cell migration and differentiation (8, 59). The deposition of extracellular elements following trauma is thought to be an essential component of wound healing for this reason. Particularly in skeletal muscle tissue, where force must be transmitted from contractile elements to surrounding connective tissue structures, the interplay of parenchyma and stroma is critical to normal function. As such, the normal course of healing invariably follows initial collagen deposition at the site of injury, which is subsequently remodeled, and finally replaced by proliferating myotubules. Murphy et al. demonstrated the loss of resident connective tissue fibroblasts confers impaired regeneration of myofibers despite persistence of Pax7 populations, suggesting that the interaction between satellite and mesenchymal cells is crucial to muscle healing (60). Of particular significance in this stem niche is collagen VI, a prominent component of the endomysial basal lamina, and essential for connective tissue function (61). Congenital deficiency of collagen VI, known as Ullrich’s and Bethlem’s muscular dystrophy, results in an early-onset and phenotypically variable compromise of muscle function, fibrosis, joint laxity, and dystrophic changes. The majority of collagen VI synthesis is provided by interstitial fibroblasts resident in muscle tissue (62). The work of Urciuolo et. al, demonstrated that the genetic knockout of collagen VI leads to impairment of muscle regeneration through premature differentiation and depletion of the satellite cell pool following injury in a mouse model (55). Interestingly, collagen VI deficient mice possessed significantly decreased elasticity of muscle tissue suggesting mechanical forces play a regulatory role in satellite cell propagation and differentiation.

C. Fibrotic Progenitors

A population of resident mesenchymal progenitors has been recently discovered and shown to be responsible for fibro-fatty deposition in response to muscle trauma (63,64). These cells are embryologically and spatially distinct from muscle satellite cells, and invariably express platelet derived growth factor receptor-alpha (PDGFR α), a tyrosine kinase family receptor. This cellular population resides outside the basal lamina of myofibers, but remains distinct from pericytes and endothelial cells. As the name suggests, this cellular population is multi-potent *in vitro*, and capable of differentiation into adipocytes and fibroblasts. *In vivo*, these cells mediate mesenchymal accumulation of fat and collagenous fibrosis dependent on the type of injury (63, 64) (Fig. 4). Significantly, the induction of mesenchymal differentiation

of this population when subjected to TGF β resulted in near 100% conversion in PDGFR α ⁺ cells (64). Myoprogenitor cells, in contrast, exclusively formed myofibers with little to no contribution to fibrotic accumulation *in vivo*, and exhibited no decrement in myogenicity when subjected to the same levels of TGF β ligand. The designation, mFAP, is misleading, and potentially reductive in regards to tissue fate, as osteogenic capacity was initially assessed with *in vitro* conditions that did not include exogenous BMP-2 ligand used in standard osteogenic differentiation assays (63). Indeed, this cell population also been described to form bone when engrafted on a BMP (bone morphogenic protein) laden scaffold *in vivo* (65). The work of Lemos et. al., has elucidated the pathway by which a temporal switch in predominate cytokines secreted by recruited monocytes determines the apoptosis of this cellular population, and permits proliferation of myogenic progenitors, facilitating physiologic regeneration. The initial predominance of TNF α signaling is necessary to induce apoptosis in FAPs, without which PDGFR cells are allowed to proliferate, compromise physiologic muscle regeneration, and progressively deposit fibrotic elements leading to outright fibrosis. Interestingly, the effect of TGF β supersedes the apoptotic effect of TNF signaling.

The work of Dularoy et. al. further described a subset of PDGFR α ⁺ cells, and demonstrated, through lineage tracing experiments, the contribution of ADAMS12⁺ cells responsible for collagen deposition following cardiotoxin injury (66). Despite the transient nature of collagen deposition in this particular study due to effective myofiber regeneration, this population still serves as an intriguing candidate for a responsible mediator in fibrotic pathology. The protein fibroblast activation protein-alpha (FAP) similarly describes a lineage of mesenchymal progenitor cells located in muscle tissue, but also in multiple tissues including bone marrow and pancreas (67). This population is responsible for the majority of follistatin secretion within muscle tissue, and when ablated results in the impaired regeneration of muscle tissue following injury (67). These cells share many of the same phenotypic markers used to identify the mesenchymal progenitors implicated in fibrosis generation including PDGFR, Sca-1 and CD90. This finding underscores the necessity of the mesenchymal progenitor population in muscle healing following injury. FAP is a membrane associated dipeptidyl peptidase initially identified on cells in different malignancies and other chronic inflammatory conditions (68), (69). When present in tumors, stromal cells identified by FAP provide exemption from immune-surveillance (70). This provides further evidence of the importance of the inflammatory milieu in the determination of fibrosis following injury in skeletal muscle. Interestingly, the polarization of the inflammatory setting to the Th2 innate immunity predominance of IL-4/IL-13 cytokines increases the ability of resident mesenchymal progenitor cells to facilitate muscle regeneration (71). This effect is corroborated by the differential phenotype observed in response to muscle injury that correlates with the level of IL-4/IL-13 signaling conferred by genetic background (72).

Analogous to the interrogation of cellular mediators responsible for the generation of fibro-fatty infiltration in muscle tissue, studies in trauma-induced ectopic endochondral bone development (heterotopic ossification=HO) have revealed a similar population of connective tissue cells resident to skeletal muscle and distinct from myoprogenitors. Multiple lineage tracing experiments initially identified Tie-2⁺ cells reliably contributed to all stages of ectopic endochondral ossification (73). The work of Woszcna et al. describes a similar

population, using cells of a Tie-2 lineage, later shown to be restricted to a mesenchymal population demonstrating PDGFR α positivity, which contribute to HO formation in an *in vivo* murine model. Within this spectrum of pathologic wound healing, from endochondral bone to adipocyte accumulation, non-myogenic mesenchymal precursors have been characterized by the same phenotype, CD45⁻, CD31⁻, Sca-1⁺, PDGFR α ⁺. Taken together the responsible cellular mediators of intramuscular HO, and fibrosis likely represent the same, or closely related, cell populations. Stromal cells possessing this list of phenotypic markers have been identified in other cellular populations such as the FAP⁺ cells lining the bone marrow cavity. Ablation of these cells results in compromised hematopoiesis, underscoring the critical interaction of these supporting mesenchymal cells with the surrounding parenchyma, and the affect they may have on impaired healing (67).

IV. Signaling pathways in fibrosis development

The heterogeneous group of cytokines in the TGF β superfamily has been implicated as a major mediator for a number of disease and fibrotic processes in multiple tissues (74). A comprehensive delineation of TGF β signaling resides outside the scope of this review. In brief, TGF β signaling promotes conversion of fibrosis effector cells (resident and circulating fibrotic progenitors) into myofibroblasts, including increased expression of extracellular matrix elements, pro-fibrotic growth signals, and receptors (75). Significant expression in the muscle of Duchenne's muscle dystrophy patients has been shown to correlate with a fibrotic phenotype (76). TGF β has been implicated as the dominant pro-fibrotic cytokine, characteristically secreted by M2 polarized macrophages, thought to coordinate muscle healing following injury. The predominance of TGF β signaling begins 3–5 days following (77)traumatic insult and persists for days to weeks as multiple cell populations, including resident fibro-progenitors, and circulating fibrocytes (78) are recruited to the site of injury. Accordingly, targeted inhibition of TGF- β to delay the fibrotic response in favor of functional myofibril proliferation and recovery has been used to augment muscle healing. Suramin, a heparin sequestration compound, and relaxin, have been shown to antagonize TGF β function on fibroblast proliferation. (38,39). Continued efforts to identify molecular targets to selectively inhibit TGF β will provide novel strategies for controlling this central regulator of fibrosis.

The BMP family of ligands are another, highly conserved member of the TGF β superfamily. BMPs are upregulated in response to traumatic injury, although the heterogeneity of this superfamily corresponds with the phenotypic variance observed in pathologic healing. Studies have demonstrated upregulation of BMP-4 signaling in muscle tissue of patients with Fibrodysplasia ossificans progressive (FOP), as well as in myoblasts from dystrophic mice (77). Interestingly, this cytokine does not show significant upregulation in patients that subsequently develop HO following significant blast injury. Instead, cytokine profile of trauma induced HO matches that of fibrotic muscle, suggesting a related etiology distinct from hyper-activation of the BMP-2, ALK-2 signaling pathway (79). BMP signaling also maintains the regenerative niche of Satellite cells, and prevents premature myogenic differentiation (80). As mentioned previously, however, when subjected to increased amounts of BMP-2 signaling, mesenchymal progenitors marked by PDGFR α positivity mediated intramuscular bone deposition. The differential effects of BMP signaling on the

course of muscle regeneration underlies the complex coordination of this signaling pathway in the inflammatory milieu. Interestingly, BMP cytokines also mediate the trophic maintenance of muscle mass; Sartori et. al. demonstrated the essential role of SMAD1/5/8 by salvaging the atrophic phenotype of denervated skeletal muscle, and, conversely, the prevention of muscle hypertrophy through pharmacologic inhibition of the same pathway (81).

Myostatin is a 8–13 kDa secreted protein that shares structural similarities to Activin A, another member of the TGF β superfamily of cytokines (82). Similar to the TGF β signaling pathways, the Activin family of receptors mediates its downstream effects through the SMAD2/3 pathway, and augments the proliferation and ECM secretion of myofibroblasts (83). Myostatin and TGF β act in concert, and have been shown to induce expression of the other in fibrotic muscle tissue (84). Myostatin is a well characterized inhibitor of muscle hypertrophy, and animals deficient in the protein develop systemic hypertrophy and hyperplasia of muscle tissue as well as decreased adipose deposition (85). *In vitro* studies have demonstrated a dose-dependent inhibitory effect of myostatin on cell-cycle progression in myoblasts suggesting a differential effect of the cytokine on different cellular populations (86). Furthermore, a direct relationship between progression of fibrosis and levels of myostatin signaling has been established in multiple studies (83, 87). Inhibition of myostatin signaling improves muscle healing after injury, and reduces the fibrotic phenotype of dystrophic mice (88). Follistatin, in contrast, is a direct antagonist of the myostatin receptor, responsible for increasing muscle mass, preventing muscle atrophy in response to disuse, and increases proliferation and differentiation of myogenic progenitors (89).

Notch, a transmembrane receptor, and Wnt, a secreted glycoprotein, are signal transducers implicated in patterning and development. The two pathways interact in an established paradigm where WNT initiates pre-patterning of cell fate, and Notch providing inhibitory regulation. This paradigm is maintained in response to muscle regeneration following injury. Satellite cell activity is dependent on the exquisitely coordinated cytokine switch and receptor signaling to alternate between satellite cell quiescence and differentiation during healing (22,90). Indeed, increased Notch signaling has been demonstrated to increase muscle regeneration in aged muscle tissue (22). A decrement in Notch signaling results in depletion of the satellite cell pool via premature differentiation of that cellular population, and loss of symmetric proliferation (91). Notch signaling proves antagonistic to the effects of TGF- β and downstream SMAD3 signaling via disinhibition of cyclin dependent kinases (CDKs) required for satellite cell proliferation (23). Conversely, WNT signaling has been implicated in the differentiation of myogenic progenitors, and proves necessary for successful regeneration of muscle tissue in response to injury (90). This signaling paradigm establishes a balance for successful muscle regeneration whereby initial Notch signaling is necessary to sustain an adequate population of myogenic stem cells capable of coordinated activation and differentiation in response to trauma, and subsequent Wnt signaling to ensure progression through myogenic differentiation to ensure appropriate functional tissue.

The p38 mitogen-activated protein kinase pathways are ubiquitous in cellular functioning, and intricately coordinate proliferation and quiescence in response to upstream stimuli. In particular, the MAPK pathway has been shown to mediate exit of quiescence of satellite

cells, and is necessary for myogenic differentiation(92, 93). Studies in aged mice demonstrate the upregulation of the p38 MAPK pathway independent of upstream fibroblast growth factor receptor1 (FGFR1) signaling. This upregulation correlated with diminished quiescence, and predisposition to myogenic differentiation of myogenic stem cells (94). Interestingly, the same pathway is upregulated in response to Myostatin signaling, and mediates proliferation and ECM secretion in the fibroblasts responsible for fibrogenesis (83).

The establishment of synaptic communication at the neuromuscular junction is an essential event in successful muscle generation, and upon closer examination, reveals necessary molecular interactions, which orient the healing response. Denervation of muscle not only leads to fatty and atrophic degeneration, but also induces premature differentiation of myogenic progenitors through accumulation of myogenic regulatory transcription factors in the nucleus (95). These myogenic signals upregulate expression of nicotinic acetylcholine receptors in turn, highlighting the reciprocal dependence of myogenic regeneration of the nervous system (96). Recent studies attribute the coordination of the electrical signaling and myogenic differentiation induction to the actions of p21 activated kinase (PAK1), and corepressor (C-terminal binding protein (97). Similarly, the nervous system modulates the growth and regeneration of skeletal muscle through humoral factors, most notably via beta-adrenergic signaling, as demonstrated by the compromised regenerative phenotype of beta-adrenergic receptor knockout mice (98). The beta-adrenergic agonist, clenbuterol has been demonstrated in multiple studies to augment satellite cell activation, mitigate the expression of atrophic genes (67, 98–100).

V. Conclusion

Advances in the delivery of acute care and traumatic surgery have increased the burden of patients living with severe musculoskeletal injuries, the fibrotic sequelae of which prove a persistent and often overlooked pathology. Understanding of intramuscular pathogenesis that drives the accumulation of fibrotic deposition and limits the normally robust healing response of skeletal muscle remains incomplete. Studies utilizing genetic and traumatic models have recently shed light on the complex orchestration of muscle regeneration and its dysregulation. As such, development of novel pharmacologic and tissue engineered therapeutic solutions has accelerated in recent years. Furthermore, increased knowledge into the cellular mechanisms that drive fibrosis can be used to optimize treatment protocols for patients suffering from fibrotic muscle, and guide specific prophylactic therapies for those who have suffered traumatic injury. More generally, the elucidation of the cellular effectors and responsible signaling pathways in the development of muscle fibrosis will not only help to develop nascent therapy following traumatic injury, but will also establish a paradigm through which the balance of stromal and parenchymal healing following injury can be better understood.

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Figure 1.

H&E stained sections of human skeletal muscle tissue. (a) uninjured muscle with preserved cytoarchitecture of muscle fascicles. (b) acute injury demonstrating smaller diameter of injured and regenerating myofibers and surrounding inflammatory infiltrate. (c) fibrotic muscle with interstitial collagenous deposition and chronic inflammatory cells.

Figure 2. Picrosirius Red staining of rat skeletal muscle tissue two weeks following blast injury, demonstrating interstitial deposition of collagen around injured myofibers.

Figure 3. Picrosirius red staining of mouse skeletal muscle from hindlimb ten days following myotoxin injury. Robust collagen deposition as demonstrated by corresponding red, and polarized staining.

Figure 4.
Fluorescent immunostaining for mononuclear Pax7+ cells in myotoxin injured skeletal muscle from mouse hindlimb.

Figure 5. Fluorescent immunostaining for PDGFR α in myotoxin injured skeletal muscle from mouse hindlimb. Areas of fibrotic accumulation enriched for cells expressing mesenchymal marker PDGFR α .

Table 1

Cellular populations following muscle injury		
Cell Marker	Role in Muscle Homeostasis	Citations
Pax7 ⁺	<ul style="list-style-type: none"> Muscle satellite cell Marks mononuclear stem cells located between sarcolemma and basal lamina. Pax7⁺ cells capable of symmetric and asymmetric differentiation. Activates downstream coordinators of myogenesis Myf5 and MyoD1 	<ul style="list-style-type: none"> Seale, 2000. Collins, 2005. Sacco, 2008. Kuang, 2007. Sambasivan, 2011.
MyoD1 ⁺	<ul style="list-style-type: none"> “Master Regulator” of myogenic differentiation. Marker of committed myogenic progenitors. 	<ul style="list-style-type: none"> Cooper, 1999. Yablonka-Reuveni, 1999.
PDGFR- α^+	<ul style="list-style-type: none"> Surface Tyrosine Kinase receptor characterizing embryonic mesenchymal tissue. Defines a population of resident mesenchymal cells in muscle tissue, distinct from perivascular and endothelial cells. 	<ul style="list-style-type: none"> Lemos, 2015. Joe, 2010. Uezumi, 2010. Uezumi, 2011. Oishi, 2013.
ADAMS12 ⁺	<ul style="list-style-type: none"> Subset of PDGFR+ cells, responsible for collagen deposition following muscle injury. 	<ul style="list-style-type: none"> Dulauroy, 2012.

Table 2

Cell Signaling Pathways in Fibrogenesis			
Signaling Pathway	Mechanism of Action	Affect on Muscle Regeneration	Citations
TGF- β Superfamily: TGF- β , Activin, Myostatin	<ul style="list-style-type: none"> Canonical SMAD2,3 TAK1 PI3K/Akt/mTOR 	<ul style="list-style-type: none"> Activation and differentiation of fibrotic cells. Upregulation of ECM protein transcription. Prevent proliferation of myogenic progenitors via downstream transcription of CDK inhibitors. Preservation of Satellite stem cell pool via TAK1 signaling. Myostatin induces muscular atrophy. 	<ul style="list-style-type: none"> Blobe, 2000. Massague, 2012 Li, 2004 Ogura, 2015. Carlson, 2008. Bernasconi, 1995. Quan, 2006. McCroskery, 2005. Zimmers, 2002. Wagner, 2002.
Follistatin	<ul style="list-style-type: none"> TGF-β Superfamily Antagonist 	<ul style="list-style-type: none"> Increases muscle mass, prevents muscle atrophy. Increases proliferation and differentiation of myogenic progenitors 	<ul style="list-style-type: none"> Thompson, 2005. Zhu, 2011.
BMP2,4,7	<ul style="list-style-type: none"> Canonical SMAD1,5,8 	<ul style="list-style-type: none"> Necessary for maintenance of satellite cell niche. 	<ul style="list-style-type: none"> Jackson, 2011 Ono, 2011.
WNT	<ul style="list-style-type: none"> Canonical: β-Catenin/Axin Complex. Non-Canonical: PCP Pathway 	<ul style="list-style-type: none"> Canonical pathway necessary for differentiation of Myogenic progenitors. Excessive Wnt signaling, especially in older patients Noncanonical pathway allows symmetric stem cell proliferation. 	<ul style="list-style-type: none"> Brack, 2007. Hayward, 2008. Conboy, 2002. Brack, 2008. Le Grand, 2009.
Notch	<ul style="list-style-type: none"> Nuclear translocation via NICD 	<ul style="list-style-type: none"> Maintenance of Satellite stem cell pool, prevents premature and asymmetric differentiation. 	<ul style="list-style-type: none"> Conboy, 2003. Brack, 2007. Carlson, 2008. Bjornson, 2012.

Cell Signaling Pathways in Fibrogenesis			
Signaling Pathway	Mechanism of Action	Affect on Muscle Regeneration	Citations
			<ul style="list-style-type: none">Hindi, 2012.

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