Hindawi Publishing Corporation
Advances in Biology
Volume 2014, Article ID 612471, 16 pages
http://dx.doi.org/10.1155/2014/612471

Review Article

The Basis of Muscle Regeneration

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Received 27 March 2014; Revised 28 May 2014; Accepted 18 June 2014; Published 9 July 2014

Academic Editor: Shihuan Kuang

Muscle regeneration recapitulates many aspects of embryonic myogenesis and is an important homeostatic process of the adult skeletal muscle, which, after development, retains the capacity to regenerate in response to appropriate stimuli, activating the muscle compartment of stem cells, namely, satellite cells, as well as other precursor cells. Moreover, significant evidence suggests that while stem cells represent an important determinant for tissue regeneration, a "qualified" environment is necessary to guarantee and achieve functional results. It is therefore plausible that the loss of control over these cell fate decisions could lead to a pathological transdifferentiation, leading to pathologic defects in the regenerative process. This review provides an overview about the general aspects of muscle development and discusses the cellular and molecular aspects that characterize the five interrelated and time-dependent phases of muscle regeneration, namely, degeneration, inflammation, regeneration, remodeling, and maturation/functional repair.

1. Muscle Regeneration Recapitulates Many Aspects of Development

Regenerative potential, robust in lower vertebrates, is gradually lost in higher vertebrates such as mammals [1–5]. Nevertheless, mammalian tissues, including skeletal muscle, are capable of homeostasis and regeneration, partially recapitulating the embryonic developmental program.

Muscle development and regeneration share common features because the molecular program that underlines prenatal development is reactivated for tissue reconstruction after injury [6–8] (Figure 1). Regenerative medicine has therefore gained important insights through the study of developmental biology.

Skeletal muscles are derived from somites, which receive signals, including Wnts, Sonic hedgehog, BMP-4, insulin-like growth factor-1 (IGF-1), and noggin, from the neighboring tissues that induce primordial muscle precursor cells to be committed in a myogenic fate and to subsequently differentiate into mature muscle [6] (Figure 1(a)).

The molecular basis of muscle development has been successfully studied thanks to the gene targeting approach in which the function of one or more myogenic factors was altered either by producing animals lacking one or more of the factor-encoding genes or by producing transgenic animals in which a specific gene is overexpressed under the control of a muscle-specific promoter.

The paired-domain transcription factors Pax3 and Pax7 act upstream of the primary myogenic basic helix-loop-helix (bHLH) transcription factors (MyoD, Myf5, myogenin, and MRF4), which are responsible for the induction of the myogenic program. Pax3 is expressed in the presomitic mesoderm and early epithelial somites [9, 10]. Gene targeting approaches (Table 1) revealed that Pax3-deficient mice lack the limb and diaphragm muscles [11–13], whereas Pax7, a paralogue of Pax3, is induced during somite maturation, is nonessential for embryonic muscle formation, and plays a critical role for postnatal muscle formation [14–16]. Pax3 plays also a critical role in the migration of muscle precursors, thus regulating the expression of c-Met [17], a factor involved

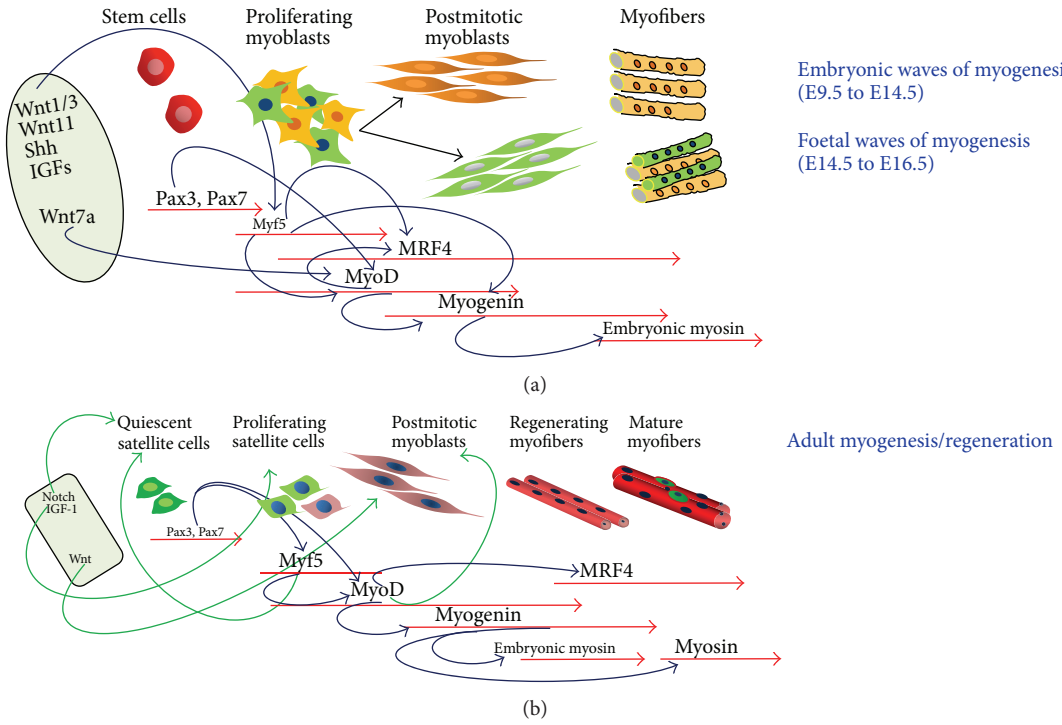

Figure 1: Schematic representation of muscle formation during embryonic development and adult regeneration. (a) Developmental myogenesis occurs in two distinct waves of differentiation that are characterized by a specific and sequential pattern of muscle-related gene expression (red arrows). Skeletal muscles are derived from somites, which receive signals from the neighboring tissues, namely, axial structures (neural tube and notochord), dorsal ectoderm, and lateral mesoderm that in turn induce the activation (blue arrows) of muscle regulatory factors. Shh (from the notochord) and Wnt1/3 and Wnt11 and IGFs (from dorsal neural tube) signaling have been demonstrated to regulate the expression of Myf5. Pax3 and Myf5 independently regulate MyoD expression, whereas Myf5 regulates the transient expression of MRF4. Myf5 and MyoD independently activate the expression of Myogenin, which promotes the expression of Myosin. (b) Illustration of the lineage progression of adult myogenesis during muscle regeneration, which recapitulates many of the cellular and molecular aspects of muscle development illustrated in panel (a). Environmental cues (Notch, IGF-1, Wnt, etc.) influence the activity of satellite cells (green arrows). Notch and IGF-1 signaling stimulate the proliferation of satellite cells, whereas Wnt signaling is involved in the transition from proliferation to the differentiation phase of myoblasts. Notch signaling is also necessary for the maintenance of the quiescent state of satellite cells. Depending on MyoD activity, satellite cells can follow one of two fates: they may maintain Myf5 expression while downregulating MyoD and self-renewing; alternatively, they maintain MyoD expression and differentiate. Quiescent satellite cells express Pax3 and Pax7. Pax7 regulates MyoD and Myf5 expression (blue arrows). Myf5 regulates the expression of MyoD, which in turn promotes the expression of myogenin and MRF4 (blue arrows). Myogenin promotes the expression of Myosin (blue arrows).

in the delamination and migration of limb-muscle precursors [18].

The four myogenic regulatory factors (MRFs) MyoD, Myf5, myogenin, and MRF4 orchestrate an entire program of muscle-specific gene expression when ectopically expressed in nonmuscle cell types [19]. Functionally, the myogenic bHLH factors act as heterodimers, interacting with ubiquitous bHLH proteins, known as E-proteins, and with other myogenic transcription factors, such as the members of MEF family, which act in a combinatorial fashion to activate muscle gene expression (reviewed in [20]).

Myf5 is activated first in the medial and epaxial somite progenitors, which give rise to the deep back muscles; Myf5 is later activated in the lateral hypaxial somite cell progenitors, which give rise to the limb, diaphragm, and body wall muscles [21–24]. Muscle progenitor lineages that give rise to hypaxial and limb muscles also activate the expression of MyoD [25].

Gene targeting experiments (summarized in Table 1 and reviewed in [19]) have revealed the specific contribution of MRFs to muscle development and the establishment of skeletal muscle cell commitment and differentiation. The activation of MyoD/Myf5 represents the key step for the commitment of multipotential somite cells to the myogenic lineage; the disruption of both genes in the double knockout MyoD-null/Myf5-null mouse results in the absence of skeletal myoblasts (Table 1) [19, 26]. Moreover, these studies indicate that Myf5 has regulatory functions in muscle progenitor specification, and MyoD has subsequent functions in muscle differentiation (Figure 1(a)). In contrast, myogenin appears to function downstream of Myf5 and MyoD and plays a critical role in the terminal differentiation of myoblasts (Figure 1(a)); however, myogenin is also dispensable for establishing the myogenic lineage (Table 1) [19, 27, 28].

The specific role of MRF4 during myogenesis is somewhat more complex; however, its temporal expression pattern

Table 1: Gene-targeting of factors involved in muscle development.

Gene knockout	Phenotype	References
Pax3	Absence of the limb and diaphragm muscles	[11–13]
Pax7	(i) Normal muscle development (ii) Satellite cells are progressively lost postnatally because of apoptosis accompanied by cell cycle defects	[15, 16, 140]
MyoD	(i) Viable without obvious defects (ii) Increased expression of myf-5 (iii) Normal expression of myogenin (iv) Impaired regeneration	[182–184]
Myf5	(i) Muscle develops relatively normally (ii) Normal expression of myogenin (iii) Hypertrophy and fibrosis in adult life	[21, 29, 185–187]
Myogenin	(i) Die perinatally (ii) Prevents muscle differentiation (despite the continued expression of MyoD) (iii) Reduced muscle	[19, 27, 28, 188, 189]
MRF4	(i) Variable viability dependent on targeting construct (ii) No obvious muscle defects	[19, 20, 29, 190, 191]
MyoD and Myf5	(i) Die perinatally (ii) Absence of skeletal muscle cells (iii) Absence of myogenin (iv) No markers of differentiation	[19, 26]

suggests potential roles in both muscle determination and terminal differentiation [19, 20, 29]. The muscles of adult mice express high levels of MRF4, whereas myogenin, MyoD, and Myf5 expression are reduced during postnatal life [30]. Interestingly, the expression of MyoD and myogenin mRNA and protein are reactivated in the skeletal muscles of aged animals [30, 31], and their expression pattern is similar to that recorded in young adult denervated muscles, suggesting that muscle aging could involve the denervation of myofibers.

The myogenic program, as well as muscle regeneration, is also regulated at epigenetic level. In proliferating myoblasts, the ability of the MRFs to activate the differentiation program is countered by the association of muscle regulatory regions with histone deacetylases (HDACs) and corepressor complexes, including YY1 and polycomb proteins, which preclude premature muscle-gene expression by promoting histone modifications [32]. The class II histone deacetylases (HDAC4 and HDAC5) interact with MEF2 proteins and repress the activation of transcription from promoters containing MEF2 sites [33]. The class I histone deacetylase HDAC1 associates directly with MyoD, is capable of deacetylating MyoD in vitro, and inhibits the ability of PCAF to enhance MyoD-dependent transcription in cell culture experiments [20]. In the adult, class IIa histone deacetylases together with PGC-1α (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha) and NFAT (nuclear factor of activated T-cells) control the slow myofiber gene expression program [34]. In particular, signaling by calcium/calmodulin-dependent protein kinase (CaMK) and protein kinase D (PKD) induces the phosphorylation of class IIa HDACs, which creates docking sites for the 14-3-3 chaperone protein, resulting in nuclear export with the consequent activation of slow myofiber genes [34]. Additionally, HDAC9 has been shown to modulate the response of skeletal muscle to motor innervations [34].

Recent studies have also revealed that myogenic transcription factors control the expression of a group of microRNAs (miRNA or miR), which act through multiple mechanisms to modulate muscle development and function [35–37].

miRNAs are endogenous and ~22 nucleotides long and inhibit translation or promote mRNA degradation by annealing to complementary sequences in the 3′ untranslated regions (UTRs) of specific target mRNAs [38]. MicroRNA expression profiles are highly dynamic during embryonic development and in adulthood. The misexpression of microRNAs can perturb embryogenesis, organogenesis, tissue homeostasis, and the cell cycle [39].

Interestingly, many miRNAs are expressed in a tissue-specific manner, and several miRNAs, such as miR-1, miR-133, miR-214, miR-181, and miR-206, are specifically expressed in skeletal muscles [35–37]. miR-214 is expressed during somitogenesis and modulates the response of muscle progenitors to Hedgehog signaling [35]. miR-1 and miR-133 modulate muscle growth and differentiation by regulating serum response

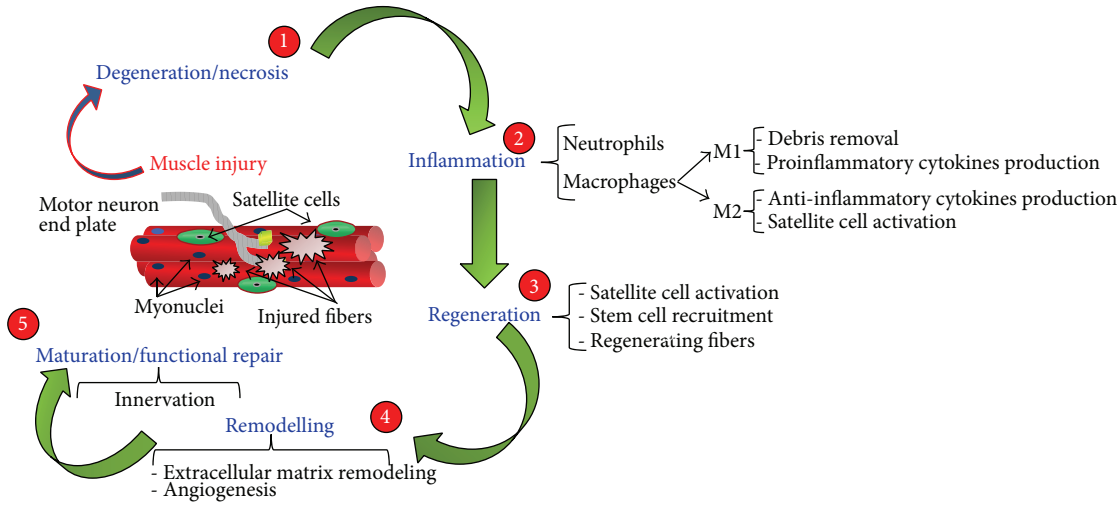

FIGURE 2: Schematic model outlining the different phases of muscle regeneration. Muscle regeneration occurs in five interrelated and time-dependent phases (text in blue). The necrosis of muscle fibers activates a transient muscle inflammation, which is necessary for the removal of necrotic cellular debris. Inflammation is followed by a regeneration phase that is characterized by the activation of stem cell populations (satellite cells and other nonmuscle stem cells), which replace damaged myofibers. The fourth phase involves the remodeling of extracellular matrix and angiogenesis. The muscle regeneration is completed by the reinnervation of regenerating fibers.

factor (SRF) and MEF2 activity and thus have distinct roles in modulating skeletal muscle proliferation and differentiation in cultured myoblasts in vitro [35–37]. miR-133 enhances myoblast proliferation by repressing SRF [36, 40]. In contrast, miR-1 promotes myogenesis by targeting HDAC4, a transcriptional repressor of muscle gene expression [36, 40]. Similar to miR-1, miR-206 also promotes skeletal muscle differentiation through the negative regulation of DNA polymerase α (polA1) translation, repression of cyclin-dependent kinase 2 (Cdk2) activity and downregulation of connexin 43 [35]. miR-181 is upregulated upon differentiation and stimulates muscle growth [35]. The activity and expression pattern of different myo-miR suggests that miRNAs can play roles in establishing a differentiated phenotype and suggests a potential role for miRNAs in skeletal muscle regeneration and diseases.

2. Muscle Regeneration: The Phase of Necrosis and Inflammatory Response

Notably, most of the factors involved in muscle development are activated during muscle regeneration (Figure 1(b)) [7, 8]. Muscle regeneration occurs in five interrelated and time-dependent phases, namely, degeneration (necrosis), inflammation, regeneration, remodeling, and maturation/functional repair (Figure 2). Although the phases of muscle regeneration are similar in different organisms (e.g., mouse, rat, and human) and after different types of damage/trauma, the kinetics and amplitude of each phase are different in each organism and may depend on the extent of damage and the damage model used.

One of the most frequently used and the easiest and most reproducible methods to induce muscle regeneration is based on cardiotoxin (CTX) injection, a peptide that is isolated from snake venoms and acts as protein kinase C-specific inhibitor. CTX produces a local myonecrosis and stimulates muscle regeneration [8, 41–44].

Necrosis involves the influx of calcium ions, the loss of the plasmalemma, myonuclear, contractile material, and cellular organelles dissolution, leading to amorphous debris. Necrotic fibers appear pale and enlarged with altered internal architecture and with the presence of internal nuclei, which may reflect invasion by macrophages. Evans Blue Dye is an in vivo evaluable marker of necrotic myofibers and can be administered as an intravital dye into the tail vein of mice and identified macroscopically by the striking blue colour within tissue, or observed by red autofluorescence in tissue sections examined by fluorescence microscopy [45].

Necrotic cell death stimulates a host inflammatory response (Figure 2).

The inflammatory response of injured skeletal muscle plays an important and critical role in muscle homeostasis and regeneration and involves the recruitment of specific myeloid cell populations within the injured area [46–50].

Neutrophils represent the first inflammatory myeloid cells that invade the site of muscle injury; the number of neutrophils usually drops 24 hours after damage; neutrophils are normally no longer detectable after 36–48 hours after injury [51, 52] (Figures 2 and 3).

Neutrophils enter into damaged tissue by interacting with adhesion molecules of vascular endothelial cells [53–57], a process that is mediated by the interaction of a β2 integrin on neutrophils with vascular ligands such as intracellular adhesion molecule-1 [53, 55].

The phagocytic activity of neutrophils involves the release of high concentrations of free radicals and proteases, as well as the secretion of proinflammatory cytokines that stimulate

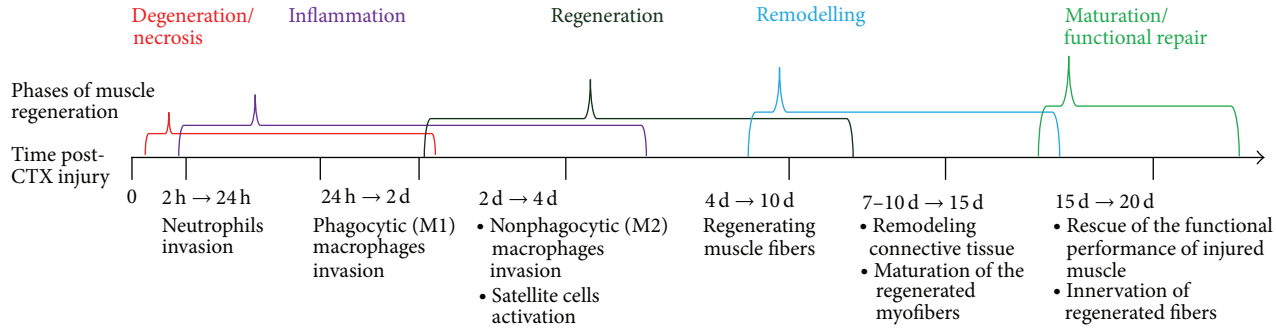

Figure 3: Schematic diagram of time-dependent cellular response following cardiotoxin (CTX) injection. The relevant biological responses, activated after CTX injection, are indicated. A few hours after cardiotoxin treatment, neutrophils infiltrate between the necrotic fibers, and, by 24 hr after injection, the inflammatory response is characterized by neutrophil and macrophage infiltration. At this stage, the phagocytosis of necrotic myofibers is very active. During the second day after treatment, spindle-shaped mononucleated cells mixed with necrotic debris, leukocytes, and different classes of macrophages appear. New myofibers appear during the following day, and, by 7–10 days after injection, the overall architecture of the muscle is restored, although most regenerated myofibers are smaller and display centrally located myonuclei. The progressive maturation of regenerating myofibers occurs by 15 days.

the homing of other inflammatory cell populations, namely, monocytes and macrophages [49] (Figure 2).

The production of soluble interleukin-6 receptor (sIL-6R) by neutrophils regulates the change from a neutrophilic to a mononuclear population.

Macrophages rapidly increase within 24 hours after injury (Figure 3). They are the predominant inflammatory cell type within the injured area and are detectable at the levels of the perimysium and epimysium. Macrophages remove tissue debris and activate stem cell populations [58–63] (Figure 2). In a pioneering study, McLennan [61] described different populations of macrophages with potentially differing activities: ED2+ and ED3+ resident macrophages, which presumably do not possess phagocytic activity, are abundant in uninjured muscles but are not present within the degenerating fibers, while ED1+ cells are rarely observed within the undamaged regions of the muscles but are abundant in the perimysium between arterioles and degenerating fibers. Recent evidence supports a model according to which the tissue macrophage subtype specification is distinct from that of circulating monocytes [64]. Circulating monocytes can be classified into at least two populations, the $CX_3CR1^{lo}CCR2^{+}Gr1^{+}$ "inflammatory" subset monocyte, which is actively recruited to inflamed tissues, and $CX_3CR1^{hi}CCR2^{-}Gr1^{-}$, which display an anti-inflammatory function and invade the damaged tissue in a second wave of infiltration to support tissue repair [65, 66]. Other evidence supports the hypothesis that the inflammatory monocytes are actually the only monocyte population that is recruited in injured skeletal muscle; then they switch to anti-inflammatory macrophages to support myogenesis [59, 67]. M1 and M2 nomenclature is usually used to refer to the two extremes of a spectrum of possible forms of macrophage activation. In particular, it has been proposed that macrophages develop into either type 1 inflammatory (M1) or type 2 anti-inflammatory (M2) subsets and that macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences (Table 2) [68]. The M1 phenotype expresses CD68 and is typically interleukin-(IL-)12^{high} and IL-10^{low} [69–71], whereas M2 macrophages express CD163 and are typically IL-10^{high} and IL-12^{low} [58, 69, 72] (Table 2). M1 macrophages are activated by the T-helper (Th) 1 cytokines interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), IL-1, and IL-6 [69] (Table 2). The M2 population is divided into three possible subtypes: M2a, M2b, and M2c, each with diverse physiological roles (Table 2). M2a are induced by exposure to IL-4 and IL-13; M2b are induced by combined exposure to immune complexes and toll-like receptors (TLR) or IL-1R agonists; M2c macrophages are induced via IL-10 [69] (Table 2). M2a and M2b macrophages exert immunoregulatory functions and drive type II responses, whereas M2c are more related to the suppression of immune responses and tissue remodeling [69] (Table 2).

What are the factors/signaling processes that mediate macrophage polarization?

It has been demonstrated that IL-10 plays a central role in regulating the switch of muscle macrophages from a M1 to M2 phenotype in injured muscle in vivo, and this transition is necessary for proper and efficient muscle growth and regeneration [73]. The ablation of IL-10 amplified the inflammatory response, causing increases in IL-6 and CCL2 while preventing the switch to CD163-positive and arginase-1-positive M2 macrophages. This resulted in the accumulation of muscle fiber damage and altered muscle regeneration and growth [73].

Macrophage polarization is mediated by STAT protein activity. STAT1 is an essential mediator of M1 macrophage polarization in the presence of IFN-γ, whereas STAT6, which is activated by IL-4 and/or IL-13, is required to drive M2 macrophage activation [74]. The mutual exclusivity of these signaling pathways could be a crucial factor in M1 versus M2 polarization and represents a potential target for

Table 2: Characteristics of macrophage subtypes.

Subset of macrophages	Factors driving macrophages polarization	Molecules expressed by polarized macrophages	Functional properties of polarized macrophages
M1	IFN-γ + LPS, TNFα	TLR2, TLR4, CD16, CD32, CD64, CD80, CD86, IL-12, IL-23, TNF-α, IL-1, IL-6, Type I IFN, IL-1RI, IL-15Ra, IL-7R, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL16, CCR7, COX2, iNOS, and miR-26-2	Proinflammatory properties, phagocytosis, and cytotoxic and antitumoral properties
M2a	IL-4, IL-13	CD163, CD23, CD302, CD209, IL-10 Decoy IL-1RII, CCL17, CCL22, CCL24, CXCR1, CXCR2, miR-193b, and miR-222	Anti-inflammatory properties and immunoregulatory functions drive type II responses
M2b	IC + TLR	CD163, IL-10, CCL1, miR-27a, miR-222, miR29b-1, and miR-132	Anti-inflammatory properties and immunoregulatory functions drive type II responses
M2c	IL-10	CD163, scavenger receptor A and B, CD14, CCR2, CCL16, CCL18, CXCL13, CD204, and CD206	Suppression of immune responses and tissue remodeling

IC: immune complexes; IFN-g: interferon-g; LPS: lipopolysaccharide; TLR: toll-like receptor; COX2: cyclooxygenase 2; TNFα: tumour necrosis factor; iNOS: inducible nitric oxide synthase; Interleukin-: IL-.

the modulation of macrophage polarization for therapeutic purposes.

In a recent work, Mounier et al. [75] documented the critical role of a master regulator of energy homeostasis, namely, AMPKα1, in macrophage skewing. AMPKα1 is the only catalytic subunit of AMPK that is expressed in macrophages [76]. Using various genetic mouse models, the authors reported that the ablation of AMPKα1 expression resulted in (i) the accumulation of necrotic tissue after damage, (ii) a delay in the disappearance of phagocytosed myofibers, (iii) a deficit in the acquisition of the M2 macrophage phenotype, and (iv) impaired skeletal muscle regeneration [75].

Gene knockout experiments were supported by the pharmacological inhibition of AMPK activity: wild-type macrophages treated with an inhibitor of calcium/calmodulin-dependent protein kinase 2 (CAMKK2), which is an upstream activator of AMPK, also failed to switch to an M2 phenotype following the phagocytosis of apoptotic myoblasts.

Thus, AMPKα1 is crucial for M1 to M2 macrophage skewing, which is necessary for a proper regenerative process [75–78].

Epigenetic mechanisms are also implicated in macrophage polarization [79], and the role of miRNAs in regulating macrophage activation in response to different environmental cues has been defined. It has been reported that M2 macrophages express greater levels of miR-125a-5p than do M1 macrophages [80]. The overexpression of miR-125a-5p diminished the M1 phenotype expression induced by LPS but promoted the M2 marker expression induced by IL-4 [77]. In contrast, the knockdown of miR-125a-5p promoted M1 polarization, while diminished IL-4 induced M2 marker expression [80]. Moreover, miRNA let-7c is expressed at a higher level in M2 than in M1 macrophages [81].

In the context of "functional" regeneration, M2 macrophages appear soon after M1 macrophages and play an important role in deactivating M1 macrophages [59]. Under pathologic conditions, it is possible that the M1 macrophage influx is accompanied by the contemporaneous invasion of M2a macrophages. This is the case for muscular dystrophy [82, 83], in which the simultaneous recruitment of M1 and M2a macrophages may reduce the muscle damage caused by M1 macrophages. Moreover, Villalta and coworkers [83] reported that the selective deactivation of the M1 phenotype in a dystrophic mdx animal model was associated with reduced expressions of IL-6, monocyte chemoattractant protein-1 (MCP-1), interferon-gamma-inducible 10-kDa protein (IP-10), and iNOS, which mediate the cytotoxicity of M1 macrophage population [83]. In contrast, arginase-expressing M2a macrophages can reduce muscle cell damage caused by M1 macrophages in mdx dystrophy, improving satellite cell proliferation [83].

Thus, the inflammatory response is a coordinate process that must be finely regulated to obtain an efficient regenerative process, and the perturbed spatial distribution of inflammatory cells, altered identity of the inflammatory infiltrate (cell type and magnitude of influx), and disrupted temporal sequence result in a persistent rather than resolved inflammatory phase [84].

3. The Phase of Regeneration, Remodeling, and Maturation

The regenerative capacity of the skeletal muscle is guaranteed by an intrinsic mechanism that restores the injured contractile apparatus.

The dominant role in muscle regeneration is played by the muscle stem cells known as satellite cells [85, 86], which reside between the basal lamina and sarcolemma of myofibers and were described as "dormant myoblasts that failed to

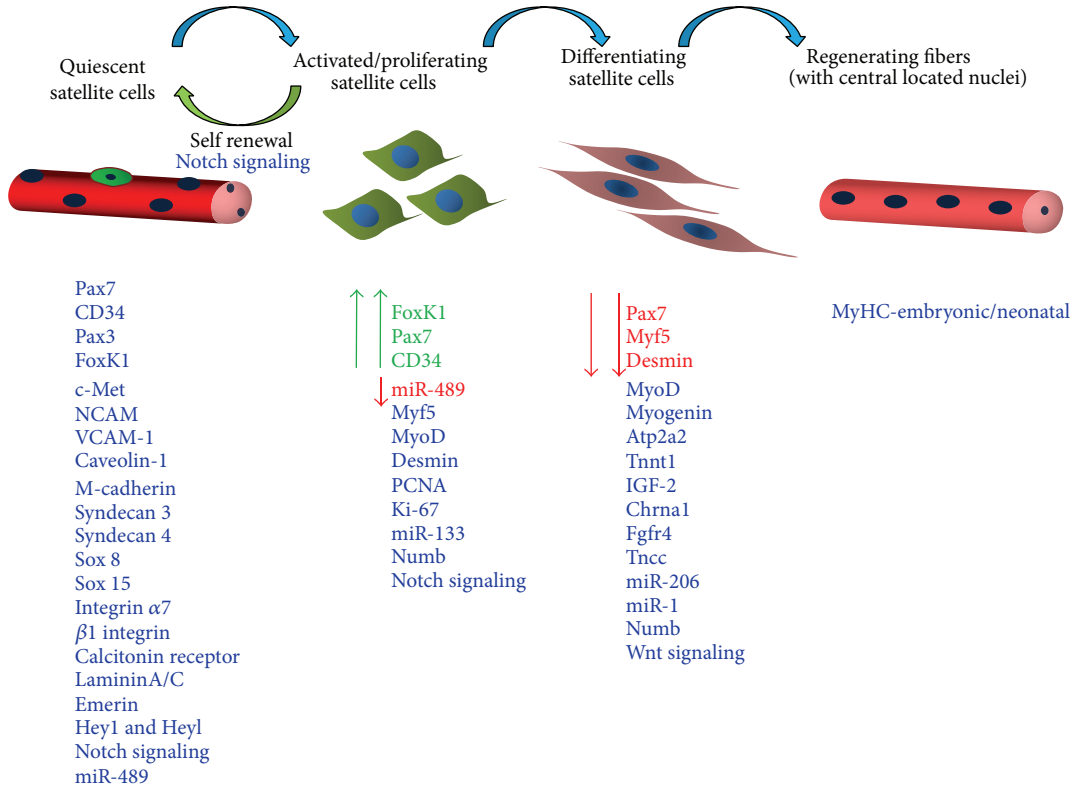

Figure 4: The molecular signature of satellite cells. Satellite cells express specific markers during the different stages of muscle regeneration. The text in blue indicates the molecular markers activated by satellite cells; the text in green indicates the molecular markers that are upregulated by satellite cells; the text in red indicates the markers that are downregulated by satellite cells in a specific stage of differentiation.

fuse with other myoblasts and are ready to recapitulate the embryonic development of the skeletal muscle fibers when the main multinucleate cell is damaged" [85]. Thus, satellite cells are mitotically quiescent until required for growth or repair.

Satellite cells are activated in response to both physiological stimuli (such as exercise) and pathological conditions (such as injury and degenerative diseases) to generate a committed population of myoblasts that can either fuse with existing myofibers, repairing damaged muscle fibers, or alternatively fuse to each other to form new myofibers [87] (Figure 4). A small minority does not differentiate but instead reenters quiescence to maintain the stem cell pool [88].

The discovery of molecular markers selectively expressed by satellite cells but not by muscle fibers has contributed to the characterization of these markers (Figure 4). It has been reported that c-Met [89], M-cadherin [90], FoxK [91], Pax7 [15], NCAM [92], syndecan 3 and 4 [93], CD34 [94], caveolin-1 [95], Sox 8 [96], Sox 15 [97], VCAM-1 [98], integrin $\alpha 7$ [99], $\beta 1$ integrin [100], calcitonin receptor (CTR) [101], lamin A/C [99], emerin [99], Hey1, and Heyl [102] are expressed by quiescent satellite cells, and their expression increases once satellite cells are activated and proliferated [86] (Figure 4). Pax3, the paralog of Pax7, is also expressed in quiescent muscle satellite cells in a subset of muscles. Pax3 plays an important role in regulating the entry of satellite cells into the myogenic program [103, 104].

The relevant markers of proliferating satellite cells, which are silent in quiescent satellite cells, are desmin, Myf5, MyoD, and PCNA [86, 105, 106] (Figure 4). In particular, a Myf5 promoter activity has been demonstrated in resident satellite cells using the knock-in Myf5nLacZ^{pos} [94, 107, 108]. However, Myf5 protein expression has not been detected in quiescent satellite cells, but it is expressed in proliferating progeny and its expression decreases when satellite cells differentiate; thus, the Myf5 protein is not detected upon differentiation and fusion into myotubes [101, 109]. Once activated, satellite cell progeny can follow one of two fates depending on MyoD activity. Satellite cells may downregulate MyoD and self-renew, guaranteeing the maintenance of a pool of quiescent Pax7^{pos} satellite cells. Alternatively, satellite cells maintain MyoD expression but downregulate Pax7 and activate myogenin expression, thus committing to differentiation [110–113] (Figure 2).

The transition from cell proliferation to differentiation involves the downregulation of proliferative-associated genes and cell-cycle withdrawal [107, 109, 114] (Figure 4).

A key role in asymmetrically segregated transit-amplifying cells is played by Notch, Numb, and Wnt. Numb has been demonstrated to promote myogenic differentiation,

thus inhibiting Notch signaling in one daughter satellite cell [115–120]. A recent work revealed that Numb is also necessary for satellite cell-mediated repair because Numb-deficient satellite cells have an unexpected proliferation defect due to an upregulation of myostatin [121].

The onset of differentiation is due to a transition from Notch signaling to Wnt signaling in myogenic progenitors, and this crosstalk occurs via GSK3beta, which is maintained in an active form by Notch but is inhibited by Wnt [122, 123].

Recent studies have uncovered a novel function for Notch signaling in the regulation of cellular quiescence in adult muscle stem cells [118**?**]. Notch signaling has been demonstrated to be necessary for the maintenance of the quiescent state and for muscle stem cell homeostasis, and it has been suggested that Hes1, Hey1, and HeyL, which are downstream targets of Notch signaling, are good candidates to mediate the Notch regulation of satellite cell maintenance by preventing their differentiation [118**?**]. It is likely that Notch signaling can act either to promote or to block the cell cycle progression depending on the cellular context. This divergent behavior of Notch activity could depend on the level of Notch activity and/or the activation of other pathways that may interact with and influence Notch signaling.

The transition from proliferation to differentiation results in the activation of specific markers (Figure 4), including myogenin, neonatal isoform of myosin heavy chain (MyHC), slow-twitch skeletal muscle troponin T (Tnnt1), cardiac and slow-twitch skeletal muscle Ca2+-ATPase (Atp2a2), insulin-like growth factor-2 (IGF-2), fibroblast growth factor receptor 4 (Fgfr4), nicotinic cholinergic receptor alpha polypeptide 1 (Chrna1), and cardiac/slow-twitch skeletal muscle troponin C (Tncc) [124].

Specific epigenetic events are also required to establish and maintain the myogenic identity in quiescent satellite cells and to enable the proper response to external cues once muscle stem cells are activated and exposed to the regenerative environment (for review [125]). miR-1 and miR-206 facilitate satellite cell differentiation by restricting their proliferative potential [40]. In particular, miR-206 is restricted to differentiating satellite cells and plays the crucial role of repressing Pax7 [126]. More recently, quiescence-specific miRNAs have been identified in the satellite-cell lineage [127]. Among these, miRNA-489 is highly expressed in quiescent satellite cells and is quickly downregulated during satellite cell activation, whereas satellite cells that lack a functional miRNA pathway spontaneously exit quiescently and enter the cell cycle [127].

Satellite cells are generally considered to be a homogeneous population of committed muscle progenitors [128]. However, several studies have raised the possibility that satellite cells are a heterogeneous mixture of two populations, namely, slow-cycling stem cells and fast-cycling committed myogenic progenitors [113, 129–132], and have indicated that the asymmetric division in the satellite cell niche is the mechanism for generating these two different populations [116]. In particular, it has been demonstrated that Pax7-positive satellite cells that lack Myf5 expression have a "stem-like" phenotype within the satellite cell population, whereas Pax7-positive satellite cells expressing Myf5 were more frequently committed to a muscle fate [116].

More recently, satellite cells have been demonstrated to divide predominantly via asymmetric chromatid segregation, generating a daughter cell that carries the mother DNA and retains stem cell properties and a daughter cell that inherits the newly synthesized DNA and acquires the myocyte lineage [133]. Using transgenic approaches, Rocheteau et al. [133] identified two different populations of Pax7-positive cells, $Pax7^{Hi}$ and $Pax7^{Lo}$. $Pax7^{Hi}$ quiescent stem cells constitute a metabolically low "dormant" subpopulation and selectively segregate old DNA strands to the renewing stem cell. With asymmetric chromatid segregation, a dividing mother stem cell synthesizes new DNA during S-phase and generates two daughter stem cells, one carrying only the mother DNA, which is the true stem cell, and the other only the newly-synthesized DNA [134].

Different studies also clarified the controversial observations about the dominant role of Pax3 or Pax7 on satellite cell specification and activation [15, 104, 135, 136]. It has become clear that satellite cells are present, although in reduced numbers, in the absence of Pax7 and that these cells retain their myogenic potential [135], suggesting that Pax3 alone is responsible for their initial presence in the Pax7 mutant after birth. However, in the absence of Pax7, satellite cells are progressively lost postnatally because of apoptosis accompanied by cell cycle defects [104], suggesting that Pax7 plays an antiapoptotic function in activated satellite cells.

Recent studies using inducible Cre/loxP conditional gene inactivation have reported contradictory evidence regarding the role of Pax7 in satellite cells and muscle regeneration. It has been demonstrated that Pax7 is only required up to the neonatal period (between 7 and 11 days postnatally) [136]. Surprisingly, when Pax7 was inactivated in adult mice, mutant satellite cells were not compromised in muscle regeneration; they could proliferate and reoccupy the sublaminal satellite niche and were able to support further regenerative processes [136]. This normal regenerative capacity of conditional Pax7 mutants sharply contrasts with the severe defects observed in Pax7 knockout mice [15, 135, 136] and indicates the possibility of adult-specific compensation by Pax3. Surprisingly, muscle regeneration was not impaired in Pax3/Pax7 doubly inactivated muscles, suggesting that, contrary to their essential roles in embryonic myogenesis, neither Pax3 nor Pax7 is required during adult muscle regeneration [136]. Conversely, recent works by von Maltzahn et al. [137] and by Günther et al. [134] demonstrated that Pax7 is an absolute requirement for satellite cell function in adult skeletal muscle because Pax7 null satellite cells and myoblasts exhibit cell cycle arrest and the dysregulation of myogenic regulatory factors.

There could be various reasons for these discrepant results: (a) in the Lepper et al. study [136], muscle regeneration was assayed within 10 days after injury, thus addressing the short-term effects of Pax7 inactivation. (b) It has been observed that the ablation of Pax7, reported by Lepper et al. [136], is incomplete because the $Pax7^{loxP\text{-}Le}$ allele showed

remaining transcriptional activity, which could confer a residual biological activity [134].

In conclusion, muscle regeneration was severely impaired when Pax7 expression was ablated in most satellite cells, causing an alteration in the maintenance of the satellite cell pool, which was most likely due to premature differentiation at the expense of proliferation [134].

It has been suggested that other "nonmuscle" stem cell populations can participate in muscle regeneration and in some way contribute to maintain the pool of satellite cells (reviewed by Messina et al. [138]). These stem cell populations could either reside within muscle or be recruited via the circulation in response to homing signals emanating from the injured skeletal muscle. These populations include endothelial-associated cells [139], interstitial cells [140–145], and bone marrow-derived side population cells [146, 147].

Two recent reports have described the identification of muscle-derived interstitial cells, which are mesenchymal progenitors [148]. These interstitial cells are referred to as fibroadipocyte progenitors (FAPs) based on their high Sca1 expression [149] or PDGF receptor alpha (PDGF-R-alpha) expression [150], respectively.

In resting muscles, the interaction with intact myofibers has been demonstrated to prevent the conversion of FAP into fibroadipocytes [149]. However, under physiologic regenerative stimuli, paracrine factors are produced that promote satellite cell-mediated regeneration [151]. In contrast, in degenerating muscles, such as dystrophic muscles at advanced stages of disease, these cells turn into fibroadipocytes, which mediate fat deposition and fibrosis [149], contributing to the exacerbation of the dystrophic hostile microenvironment.

This suggests that a qualitative microenvironment is necessary to physiologically instruct stem cells and that an altered niche negatively influences stem cell commitment and differentiation.

The remodeling of connective tissue and angiogenesis defines the fourth stage of the regenerative process [44, 150, 152, 153] (Figure 2). This stage is characterized by the activation of the extracellular matrix (ECM), resulting in the overproduction of several types of collagens, fibronectin, elastin, proteoglycans, and laminin [150], which serve to stabilize the tissue, to act as a scaffold for the new fibers, and to guide the formation of neuromuscular junctions (NMJs) [154].

Although the entire process of fibrosis formation is poorly understood, transforming growth factor-β1 (TGF-β1) has been identified to be a key factor in activating the physiological fibrosis cascade in injured skeletal muscle [155]. This fibrotic response is initially beneficial because it is quick, adds support for strength, and helps protect the injury site. However, the overproduction of collagens within the injured area often leads to heavy scarring and the loss of muscular function.

The reparative process is completed when injured myofibers rescue their functional performance and contractile apparatus [44] (Figure 2). Thus, the regeneration of damaged or diseased muscles is only beneficial if the regenerated muscles become effectively innervated. Within two weeks of damage, newly formed NMJs between the surviving axons and the regenerated muscle fibers can be identified. Of note, while the initial phases of muscle regeneration do not necessary depend on neural influence, the subsequent growth and maturation of regenerating muscle fibers require the presence of the nerve because nerve activity can directly influence protein turnover and gene expression within multinucleated regenerating myotubes and indirectly influence the proliferation and differentiation of satellite cells [156–158]. The role of the nerve in controlling the maturation of regenerating innervated myofibers can be monitored by analyzing the expression of different myosin heavy chain (MyHC) isoforms. Regenerating muscle fibers initially express developmental MyHC forms, such as embryonic and neonatal MyHCs (Figure 3), and later on adult fast and slow MyHC forms [159–161].

4. Muscle Regeneration Is Affected in Aging and Muscle Diseases

Muscle regeneration is affected in several pathologic conditions.

The functional performance of skeletal muscle tissues declines during postnatal life and is compromised in different diseases due to an alteration in muscle fiber composition, atrophy, and an overall decrease in muscle integrity as fibrotic invasions replace functional contractile tissue [162–165]. Despite no conclusive evidence, it is becoming more widely accepted that the chronic nature of the inflammatory response to tissue damage is a key driver of the fibrotic response in diverse organs and tissues. Thus, chronic inflammatory response and fibrosis create a hostile microenvironment that inhibits the physiological activity of stem cells and could interfere with muscle regeneration.

The potential critical role of the microenvironment on stem cell biology and therapy is underlined by recent studies, including those by us.

Heterochronic experiments have demonstrated that old muscle successfully regenerates when transplanted in a young animal, whereas the regeneration of young muscle transplanted in an old host is impaired [166, 167]. This hypothesis has been clearly validated in parabiotic experiments, demonstrating the rejuvenation of aged progenitor cells by exposure to a young systemic environment [168]. These results emphasize the importance of the environment, which is created by circulating factors and by the local secretome of factors secreted by satellite cells, the newly differentiating fibers, as well as by the inflammatory cells [169, 170].

In a recent work, we demonstrated that satellite cells display a delayed response to activating stimuli and show a reduced proliferative response to their environment when this environment was suboptimal [171]. We demonstrated that aged satellite cells did not display major defects in the propensity to fuse when differentiating under standard conditions, namely, in DMEM supplemented with 5% horse serum [171]. In contrast, muscle differentiation was dramatically reduced when old satellite cells were cultured in autologous serum (isochronic culture conditions), whereas

the differentiative capacity of aged satellite cells was rescued when differentiated in heterologous/heterochronic serum (from young donors) [171].

Paliwal and coworkers [172] reported that age-dependent increases in the levels of osteopontin (OPN) inhibit skeletal muscle regeneration. The transient upregulation of OPN after muscle injury has been demonstrated to play a positive role in overall regeneration [173]; however, in cultured myoblasts, OPN has been shown to inhibit cell migration and differentiation [174] as well as muscle regeneration in mdx mice [175]. Paliwal et al. [172] demonstrated that OPN is elevated in the blood serum of old mice and only if these animals are injured, suggesting that this cytokine can potentially deregulate regenerative responses and can represent a systemic molecular marker of altered muscle regeneration. Notably, the regeneration of old injured muscle was significantly enhanced by the neutralization of OPN, and young intramuscular CD11b+ macrophage injection also enhanced the myogenic responses of old satellite cells in the presence of old myofibers and old serum, suggesting that the secretome of young inflammatory cells is capable of negating the inhibitory influence of old stem cell niches [172].

In another work, by analyzing both muscle morphology and function in aged and transgenic dystrophic animal models, we observed a significant decrease in myonecrosis and a reduction in fibrosis in mice overexpressing the local form of IGF-1 (mIGF-1) [43, 176]. Moreover, we demonstrated that the local expression of mIGF-1 transgene accelerates the regenerative process of injured skeletal muscle, modulating the inflammatory response and limiting fibrosis [44]. At the molecular level, mIGF-1 expression significantly downregulated proinflammatory cytokines, such TNF-α and IL-1β, and modulated key players of the inflammatory response, such as macrophage migration inhibitory factor (MIF), high mobility group protein-1 (HMGB1), and transcription NF-κB [44]. The rapid restoration of injured mIGF-1 transgenic muscle was also associated with connective tissue remodeling and a rapid recovery of functional properties.

Wehling et al. [177] reported that the depletion of macrophages in the mdx mouse model at the early, acute peak of muscle pathology produced large reductions in lesions in the plasmalemma of muscle fibers, showing that muscle macrophages that are present during the acute, degenerative stage of mdx dystrophy are highly cytolytic and that they play a central role in the pathogenesis of muscular dystrophy [178]. Additionally, Villalta and coworkers [83] reported that arginase-expressing M2a macrophages can reduce the muscle cell damage caused by M1 macrophages in mdx dystrophy, improving satellite cell proliferation.

These data suggest that, by modulating the inflammatory response and reducing fibrosis, it is possible to create a qualitatively different environment for sustaining more efficient muscle regeneration and repair.

However, an important issue is whether aged muscle stem cells are in some way intrinsically defective in responding to regenerative stimuli. This question has been addressed by two recent studies [179, 180]. It has been demonstrated that geriatric age induces intrinsic alterations in the functions of muscle stem cells, which cannot be rejuvenated by a young host environment [179]. The authors found that the dominant factor that induces geriatric processes in satellite cells is the master regulator of senescence p16INK4a (Cdkn2a). Thus, p16INK4a silencing restored satellite cell quiescence, whereas ectopic p16INK4a expression in quiescent young satellite cells prevented their activation [179].

In another study, Cosgrove and collaborators [180] demonstrated that the intrinsically defective capacity of geriatric muscle stem cells to efficiently respond to a young recipient muscle microenvironment depends on the elevated activity of the p38α and p38β mitogen-activated kinase pathway. The transient inhibition of p38α and p38β in geriatric muscle stem cells promoted their rapid expansion and rejuvenated their potential for regeneration [180].

In summary, the alteration in muscle regeneration, observed in different pathologic conditions, can be due to intrinsic alterations that render muscle stem cells defective in responding to regenerative stimuli and to a hostile microenvironment that inhibits stem cell activity [181].

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

Work in the author's laboratory has been supported by Telethon, AFM, PRIN, and ASI.

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