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Making Skeletal Muscle from Progenitor and Stem Cells: Development versus Regeneration

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Abstract

For locomotion, vertebrate animals use the force generated by contractile skeletal muscles. These muscles form an actin/myosin-based bio-machinery that is attached to skeletal elements to effect body movement and maintain posture. The mechanics, physiology, and homeostasis of skeletal muscles in normal and disease states are of significant clinical interest. How muscles originate from progenitors during embryogenesis has attracted considerable attention from developmental biologists. How skeletal muscles regenerate and repair themselves after injury by the use of stem cells is an important process to maintain muscle homeostasis throughout lifetime. In recent years, much progress has been made towards uncovering the origins of myogenic progenitors and stem cells as well as the regulation of these cells during development and regeneration.

Introduction

In humans, more than 600 skeletal muscle groups are anatomically defined. Despite their complexity in shape and function, each muscle group is made up of hundreds to thousands of fundamental structural units called myofibers. The myofiber is unique in its constitution as it is a multi-nucleated syncytium containing tens to hundreds of nuclei resulting from cellular fusion of differentiated single muscle cells, the myocytes. Progenitors that give rise to these differentiated myocytes are a subject of this review. Stem cells that repair damaged myofibers or regenerate new myofibers after trauma in the adult are also evaluated. In particular, we contrast similarities and differences of cellular and molecular events that orchestrate muscle development and regeneration.

I. Cell origin and lineage of myogenic progenitors and stem cells

The embryonic origin of skeletal muscles and their progenitors—The entire trunk and limb skeletal muscles arise from a transient embryonic mesodermal structure called the somite (Fig. 1). Somites are segmented mesodermal units flanking both sides of the spinal cord that were first visualized by Marcello Malpighi in the chick embryo[1]. It is therefore fitting that chick embryos have been a primary experimental system for investigating skeletal muscle development since the 1970s[2]. In particular, chick-quail chimera experiments[3], in which surgically combined host and donor cells can be distinguished by nucleolar morphology or quail-specific antigen, were performed to demonstrate a somitic origin of the limb musculature[4,5]. The dorsal epithelial portion of the somite, the dermomyotome, contains the myogenic progenitors[6]. Furthermore, limb and ventral body wall muscles only come from the ‘lateral’ half of the somite, while the dorsal axial muscles derive from the ‘medial’ half[7]. Focal labeling of somitic cells with fluorescent

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dyes was also used to evaluate the morphology of emerging myogenic cells[8,9]. Live imaging of such labeled cells revealed that cells near the medial and lateral borders (or 'lips') of the dermomyotome, represent the primary wave of myogenic cells [10]. The myogenic contribution of the central portion of the dermomyotome was not addressed in these studies.

En1 is specifically expressed in the central dermomyotome of the mouse. Using loxP-recombination-based LacZ reporter expression for cell marking/tracing via *En1* gene-directed *Cre* (*En1-Cre*) activity, Atit et al.[11] showed that marked central dermomyotome cells (at embryonic day 9.5; E9.5) become a triangular domain underneath the dermomyotome a day later (at E10.5). Descendant cells become incorporated not only into dorsal trunk muscles, but also interscapular brown fat and dorsal dermis at E16.5[11] (Table 1). A chick-quail chimera study has long since determined that embryonic muscles and their progenitors have the same somitic origin[12]. A recent revisit of this issue agrees with trunk muscle and dermis fates deriving from central dermomyotome cells[13], though chick has no brown fat for comparison. Gros et al.[13] further showed by live imaging that GFP-expressing central dermomyotome cells divided vertically (relative to the epithelial plane) with one daughter cell moving inward, explaining the triangular domain observed in *En1-Cre* marked somites. Using reporter gene knock-in alleles of two dermomyotome-expressing genes, *Pax3* and *Pax7* (encoding related transcription factors), Relaix et al.[14] concluded that the vertically dividing cells were indeed $Pax3^{+}Pax7^{+}$ central dermomyotome cells that give rise to a new population of inner cells. As *Pax3;Pax7* double mutants failed to generate additional myogenic cells after the primary wave of myogenesis, $Pax3^{+}Pax7^{+}$ cells represent the secondary progenitors for continuous expansion of muscle mass (Fig. 1).

Central dermomytome cells do not contribute to ventral body wall or limb muscles. These two populations originate from the lateral half of the somite[7], presumably the lateral dermomyotome. This region expresses high levels of Pax3 and mice mutant for *Pax3* alone lack these muscles[15]. Because *Pax3* is also expressed in the presomitic mesoderm[16,17], *Pax3*-Cre-mediated lineage tracing precludes assignment of limb and ventral body wall muscle progenitors specifically to the lateral dermoymotome[18] (Table 1). A transgenic line called *M-Cre* was used to help define the lateral dermomyotome as a source of limb muscle progenitors[18]. However, constitutive Cre mediated lineage-tracing marks all cells expressing Cre 'at any one time' prior to the assay time point, thus negating temporal specificity. As a gene often possesses a dynamic expression pattern, analysis of constitutive Cre-based lineage tracing must include all expression patterns prior to the assay time point for accurate interpretation.

The tamoxifen inducible forms of Cre, the Cre-ER fusion and its successive improved versions Cre-ER^{T} and Cre-ER^{T2}, offer an opportunity for temporally controlled cell marking[19]. Using a *Pax7-Cre-ER^{T2}* allele for inducible lineage tracing, it was found that *Pax7*-expressing cells marked at different time points contribute to different sets of cell fates and muscle groups[20] (Table 1). When central dermomyotome cells were marked at E9.5, they contribute to the three fates revealed by the *En1-Cre* and *En1-Cre-ER^{T}* study[11]. $Pax7^{+}$ cells marked at E10.5 contribute to ventral and proximal forelimb muscles, and brown fat, but less so to dermis. E11.5 marked cells do not contribute to dermis, but they can be traced to distal fore- and hind-limb muscles and some brown fat. By E12.5, *Pax7*-descendant cells become restricted to the myogenic lineage and are found even in the most distal limb muscles. How three fates become segregated from a simple epithelium (the dermomyotome) and how $Pax7^{+}$ cells become fate restricted to the myogenic lineage are fundamental cell fate determination issues that have yet to be experimentally explored.

Myogenic specification and differentiation—Myogenic differentiation has been extensively studied and reviewed[21,22]. Following is a brief summary. Cloning of *MyoD*

changed the landscape of the myogenic field[23]. Forced expression of this transcription factor can convert various cultured cell types to the myogenic fate, earning its reputation as the master regulator of myogenesis. *MyoD* has three related family members, *Myf5, Myf6* (also called *Mrf4*), and *Myogenin*[24]. Their expression is largely restricted to the myogenic lineage. *MyoD* and *Myf5* expression prefigures the differentiated myocytes and defines the myogenic domain (Fig. 1). *Myogenin* is turned on in the myocytes prior to their fusion into myofibers. *Myf6* expression is the last to be detected. *Myf5* mutants lack the initial myotome, but this defect is compensated and mutants eventually develop with negligible muscle defects[25]. Single mutants for *MyoD* or *Myf6* are also viable with no severe muscle defects at birth[26,27]. However, *Myf5/Myf6/MyoD* triple mutants have drastically reduced embryonic muscles[28]. *Myogenin* mutants have no defects in myogenic specification and differentiation, but a defect in myocyte fusion in the trunk and limbs[29]. *MyoD;Myf6* double mutants are also defective in myocyte fusion[30]. Importantly, *MyoD;Myf6;Myogenin* triple mutants are devoid of any myocyte fusion, despite normal *Myf5* expression[30]. It therefore appears that *Myf5* acts primarily at the early step of specification and *Myogenin* at the late step of myocyte fusion, while *MyoD* and/or *Myf6* can participate in both steps in a semi-redundant fashion. These four genes' function and regulation are complex and not necessarily agreed upon by all investigators in the field. Nevertheless, embryonic myogenesis involves intricate interplays between these myogenic regulatory factors (MRFs).

Surprisingly, *Myf5-Cre* marked cells contribute to brown fat[31], in addition to muscles and committed myogenic progenitors[32], suggesting that its expression does not commit cells to myogenic differentiation (Table 1). However, the *Myf5-Cre* knock-in construct used a constitutive Cre and an exogenous polyA signal, which precludes endogenous 3'UTR usage and miRNA regulation. It is therefore not completely clear whether there was 'aberrant' or 'unintended' expression of Cre in an earlier progenitor stage. For example, *Myf5-Cre* is expressed in the presomitic mesoderm, where Myf5 protein is not detected[33]. Therefore, not surprisingly, *Myf5-Cre* also directs lineage labeling in cartilage and dermis in this study. If a subpopulation of Myf5$^+$ cells is indeed multi-potential, it will be important to determine their temporal and spatial distribution. By contrast, *MyoD-Cre*[34] and *Myogenin-Cre*[33] have thus far only been reported to mark the myogenic lineage (Table 1).

Muscle progenitors in the peri-natal period—The Pax7$^+$ cell source represents a major contributor to myofibers during the first 3 weeks of birth based on temporally controlled labeling and tracing of hind limb muscles using the *Pax7-Cre-ER^{T2}* allele[35]. In other muscle groups, notably most trunk and the diaphragm muscles, myogenic progenitors also express *Pax3*[36]. Whether all Pax7$^+$ myogenic progenitor cells detected in the perinatal period are descendants of embryonic Pax7$^+$ cells has not been conclusively determined as tamoxifen-induced labeling efficiency in the embryo is low[20]. There is a recent report that peri-natal Pax7$^+$ cells can arise from PW1$^+$Pax7$^-$ muscle interstitial cells[37]. Using CD34 and Sca1 surface antigens for fluorescent-activated cell sorting (FACS), the investigators described ~56% of the CD34$^+$Sca1^{high} fraction expresses PW1 but not Pax7, and these cells can generate muscles and Pax7$^+$ cells after transplantation into adult animals. Because these cells never express *Pax3* during embryogenesis (not marked in *Pax3-Cre* mice), they are presumably not of somitic origin. However, at least some embryonically and perinatally marked Pax7$^+$ cells can directly contribute to adult muscle stem cells[20,35]. The relative contributions of embryonic versus peri-natal *de novo* Pax7$^+$ cells to muscle fiber formation and adult muscle stem cells are currently unclear.

Although the *Pax7* mutant has no discernable embryonic muscle defect (unless combined with the *Pax3* mutation), *Pax7* is essential for perinatal myogenesis. *Pax7* mutants are born with a normal number of cells actively transcribing the *Pax7* locus in hind limb muscles (scored by knock-in reporter expression or by *Pax7-Cre-ER^{T2}*-mediated reporter

expression[35]). This number declines in ensuing weeks; consequently, surviving mutant adults have barely any satellite cell and are extremely compromised in injury induced muscle regeneration[38,39]. Mitotic defect[38], cell death[40], and reduced myogenic potential[39] have been implicated in the loss of satellite cells. Inducible lineage tracing of mutant cells showed that they do incorporate into myofibers more readily and over a longer period relative to control cells[35]. Thus, *Pax7* likely maintains the progenitor pool size by counter-balancing the tendency to differentiate. How *Pax7* acts, how its expression is regulated, and how the number of $Pax7^+$ cells is controlled during this period are critical questions pertaining to the establishment of a normal pool of muscle stem cells for use throughout the active life of a vertebrate animal.

As $Pax7^+$ cells continuously incorporate into myofibers during the perinatal period, one predicts that their differentiation depends on the *MyoD* gene family. However, as mentioned above, *Myf5, MyoD*, and *Myf6* single mutants can develop to adulthood with relatively normal skeletal muscles, presumably due to redundancy between these genes. Intriguingly, *MyoD-Cre* was shown to direct lineage labeling of perinatal $Pax7^+$ muscle progentiors at a high rate[34]. *Myf5-Cre* also directs marking of postnatal $Pax7^+$ cells[32]. Since neither protein has been detected in muscle stem cells, these knock-in *Cre* genes must be transcribed in muscle progenitor/stem cells sometime in the history of their making. Whether these two genes play a redundant role in this process is unknown. Systematic combinations of conditional gene inactivation among *Myf5, MyoD, Mrf4*, and *Myogenin* during the perinatal period will help to define their roles, relative to those defined in embryogenesis by the respective germ line mutants.

Adult myogenic stem cells in injury-induced skeletal muscle regeneration—In adulthood, skeletal muscle possesses a tremendous capacity to repair itself after extensive injury, which is exemplified by full tissue restoration after mincing of an entire skeletal muscle[41]. The tissue's regenerative potential is maintained over up to 50 toxin-induced injury/regeneration cycles[42]. Such regenerative capacity has been largely attributed to muscle stem cells endogenous to the skeletal muscle. One such cell type is the satellite cell, which was first identified by Alexander Mauro exactly fifty years ago using transmission electron microscopy (TEM) performed on frog tibialis anticus (i.e. tibialis anterior) muscle[43]. These cells are named after their physical location: attached to the sarcolemma and beneath the basal lamina 'orbiting' the muscle fiber. In the same year, *in vitro* culture experiments with myoblasts obtained by enzymatic digestion of skeletal muscle tissue established that mononucleated muscle progenitor cells could give rise to multi-nucleated myofibers via fusion[44,45]. Whether *in vitro* isolated myoblasts corresponded to TEM-defined satellite cells *in vivo* was not certain at the time. Years later, elegant transplantation experiments of muscles, in which satellite cell nuclei pulse-labeled with tritiated thymidine were chased into myonuclei, demonstrated that satellite cells could proliferate and potentially differentiate into muscle fibers after transplantation-induced injury[46]. Further progress in the satellite cell field was impeded in large part due to a lack of satellite cell specific molecular markers and consequently, satellite cell research was limited to laborious ultra-anatomical studies by TEM.

Identification of *Pax7* being specifically expressed in skeletal myofiber associated cells that resemble satellite cells has greatly accelerated research in the field[47]. An EM study of the frog leg muscle has detected Pax7 immuno-reactivity in the satellite cell[48]. Similarly, we found Pax7 immuno-reactivity and β-gal enzymatic activity driven by *Pax7-Cre-ER^{T2};LacZ* reporter in satellite cells of mouse tibialis anterior muscles by TEM (Fig. 2). These findings provided evidence that $Pax7^+$ cells are bona fide satellite cells as described by Alexander Mauro. However, Pax7 is not the only marker used for identifying satellite cells. For example, the *Myf5^{nLacZ}* knock-in allele directs β-gal activity in a large proportion of

satellite cells. Transplantation of single muscle fibers along with their myofiber-associated cells from *Myf5*$^{+/nLacZ}$ mice into immuno-compromised and irradiated dystrophic muscles gave rise to hundreds of genetically marked myonuclei and satellite cells[49]. Montarras et al.[36] obtained similar results via transplantation of Pax3^{+} cells isolated by FACS on the basis of fluorescent reporter gene expression from the *Pax3* locus. More recently, it has become possible to isolate adult muscle progenitor cells via FACS using a combination of positive and negative selections of cell surface markers[32,50,51]. When transplanted in bulk into dystrophic muscles of immune-deficient mice, these various populations of cells proficiently contributed myonuclei to myofibers, as well as satellite cells to the host muscle fiber niches. Importantly, Sacco et al.[50] found all cells sorted by CD45^{-}Sca1^{-}CD34^{+}β1integrin^{+} criteria positive for *Pax7* by single cell RT-PCR assay, and transplantation of such single cells results in the generation of new muscles and stem cells, fulfilling the 'gold standard' for demonstrating their stemness[52]. It remains to be determined whether all or only some *in vitro* defined transplantation competent muscle stem cell populations contain Pax7^{+} cells.

The above evidence for various sources of muscle stem cells is based upon *in vitro* isolation followed by transplantation, a long time tradition for studying stem cells. Using the *Pax7-Cre-ER*^{T2} mice for adult-specific cell marking and lineage tracing, Lepper et al.[35] provided direct *in vivo* evidence for Pax7^{+} satellite cells being the major source of muscle stem cells: after toxin-induced injury to the tibialis anterior muscle, lineage-labeled Pax7^{+} descendant cells robustly contributed to all regenerative myofibers and self-renewed to replenish the pool of quiescent satellite cells[35]. While this unequivocally demonstrates that Pax7^{+} cells are a major source of muscle stem cells in acute injury induced muscle regeneration, it will be important to determine the role of these cells in other physiological contexts, including long-term skeletal muscle tissue maintenance, exercise-induced muscle growth, and muscle wasting diseases. Moreover, a critical question concerns whether Pax7^{+} cells are the exclusive muscle stem cell source. As new myofibers form as a syncytium by fusion of multiple myocytes, lineage marking of the Pax7^{+} cell population alone cannot be used to exclude the input from other cell sources within the same myofiber due to diffusion of the lineage tracer. Indeed, numerous transplantation-based studies have reported several different Pax7^{-} cell types contributing to both myofibers as well as the satellite cell compartment[53]. To determine the physiological relevance of these clinically relevant cells for curing muscle degenerative diseases, it will be important to ascertain the role of them via direct lineage-tracing and/or genetic cell ablation of Pax7^{+} satellite cells to reveal if there is any remaining capacity for adult myogenesis.

Quite unexpectedly, tamoxifen-induced adult-specific inactivation of *Pax7* alone (via *Pax7-Cre-ER*^{T2} allele[35]) or *Pax3* and *Pax7* together (via a widely expressed *Cre-ER*^{T} allele[54]) does not cause an obvious defect in injury-induced muscle regeneration. The conditional mutant *Pax7* cell is not only able to regenerate myofibers effectively, but also proliferate, re-occupy the architectural satellite cell niche, and support an additional round of muscle regeneration. One could not help asking what is the function of *Pax7* in adult satellite cells? Whether other transcription factors compensate for or replace its role in adult satellite cell function is an open question. Along the same line of comparing gene function at different stages of myogenesis, the *MyoD* mutant develops with embryonic muscles relatively normally, but in adulthood muscle regeneration is compromised due to defects in myoblast proliferation and differentiation[55,56]. *Myf5* mutants also develop muscles normally, but are defective in muscle regeneration due to compromised myoblast proliferation[57,58]. Conversely, while *Myogenin* is required for embryonic myocyte fusion, this function of *Myogenin* is dispensable post-natally[59]. Instead Myogenin controls a distinct transcriptional program[59], in particular the response to denervation-induced muscle atrophy[60]. The

significance of such temporal switching of myogenic gene functions may lie in the fundamental differences between development and regeneration.

II. Signaling during myogenesis

Myogenic induction by Wnt proteins—In the embryo, the spinal cord and surface ectoderm surrounding the somite are necessary and sufficient for inducing myogenesis[17,61-63]. Several *Wnt* genes are expressed in these two tissues. Co-cultures of presomitic cells and cells producing selective Wnt proteins induce expression of *Pax3* and *Pax7*[64], as well as *Myf5* and *MyoD*[65,66]. In accordance, *Wnt1;Wnt3a* (both normally expressed in the dorsal spinal cord) double mutant embryos have reduced expression of *Pax3* and *Myf5* in the medial dermomyotome[67]. The myogenic inducing potential of Wnt genes expressed by the surface ectoderm has not been tested genetically. *In vitro* pharmacological studies suggest that Wnts activate the Gα - adenylyl cyclase - protein kinase A signaling cascade to phosphorylate the transcription factor CREB, which in turn activates myogenic genes. Consistently, both germ line inactivation of *Creb* and dominant negative *Creb* over-expression in the somite lead to severely reduced expression of *Pax3, Myf5*, and *MyoD in vivo*[66]. In addition, the PKC pathway, which can be activated via Gα signaling, has also been implicated in myogenic induction by modulating Pax3 activity and *MyoD* expression[68].

The canonical Wnt/β-catenin pathway also plays a role in myogenesis. Inactivation of *β-catenin* in presomitic mesoderm cells leads to disorganized somites due to segmentation defects[69], disallowing a firm conclusion about its role in the primary wave of myogenesis[70]. *En1-Cre* directed *β-catenin* inactivation in the central dermomyotome leads to an increase in the domain of myogenic cells at the expense of dermal cells, indicating an inhibitory role of *β-catenin* during the secondary wave of myogenesis[11]. By contrast, *β-catenin* inactivation by *Pax7-Cre* caused only a modest defect in muscle architecture, represented by a change in muscle fiber sub-type distribution[70]. Since both *Pax7* and *En1* are expressed in the central dermoymtome, the discrepancies likely reflect different timing of expression and/or efficiency of Cre between the two alleles. Analysis of the role of *β-catenin* via gene inactivation is further complicated by an accompanying cell adhesion defect in mutant cells. Inactivation of *BCL9*, which regulates β-catenin's nuclear activity but not its adhesion function, should in principle eliminate this issue. Yet, conditional inactivation of *BCL9* by *Myf5-Cre*, causes no developmental defects[71]. The timing of *Myf5-Cre* activity and BCL9 protein perdurance may obscure a potential role of the canonical pathway in embryonic myogenesis.

The role of Wnt signaling in adult regenerative myogenesis is multifaceted (Fig. 3). A selective combination of Wnt5a, 5b, 7a, and 7b can convert muscle resident $CD45^+Sca1^+$ cells (i.e. the side-population cells) to express $Pax7$[72], suggesting that $CD45^+Sca1^+$ cells are a source of *de novo* $Pax7^+$ stem cells for muscle regeneration. Although these *Wnt* genes were originally reported to be up-regulated during regeneration[72], subsequent re-evaluation did not substantiate such claim[73]. Thus, the physiological significance of the side-population cells and whether their conversion to $Pax7^+$ cells occurs *in vivo* are not yet known. On the other hand, FGF6 and FGFR4 are expressed during the early phase of the regenerative response[73,74], and both *Fgf6* and *Fgfr4* mutants display compromised muscle regeneration[74,75], supporting the importance of FGF signaling pathway for proficient muscle repair.

For symmetric division of $Pax7^+$ muscle stem cells, Wnt7a has been shown to employ the planar cell polarity (PCP) pathway: *Wnt7a* gene delivery into muscles via electroporation causes increased muscle stem cell number, and consequently muscle hypertrophy, and the PCP component Vangl-2 is a likely effector mediating this function[76]. Although β-catenin

nuclear localization has been documented in cultured proliferating myoblasts, it may act in the transit amplifying progenitors rather than in the self-renewing stem cells[77]. β-catenin nuclear localization is also detected in differentiating myogenic cells[71]. Importantly, *BCL9* inactivation or pharmacological intervention of β-catenin activity *in vivo* caused delayed myogenic differentiation but did not disturb progenitor proliferation during regeneration, evidence that the canonical pathway participates in the differentiation step[71,78]. Wnt3a has also been shown to employ the canonical pathway to accelerate myocyte fusion using the *in vitro* C2C12 myoblast model[79]. As myocyte fusion immediately follows differentiation, separating Wnt/β-catenin functions in these two steps in unsynchronized populations of cells may prove to be difficult.

Lastly, Wnt/β-catenin pathway activation also induces muscle fibrosis and thus, is implicated in the decline of muscle function in aging. Gene delivery of *Wnt3a* via intramuscular eletroporation leads to fibrotic accumulation[76]. *In vitro*, recombinant Wnt3a causes direct conversion of myoblasts into fibroblasts[78]. Importantly, a β-catenin transcriptional reporter is activated in aging but not in young muscles after injury, and depletion of Wnt proteins via soluble Frz receptors can rejuvenize aged myoblasts to prevent fibrogenesis[71]. While Wnt acts locally to divert aging muscle stem cells to the fibrogenic fate, TGFβ1 has been reported to be a candidate systemic factor that may attenuate muscle stem cell activation[80,81] as serum levels of TGFβ1 increase in aging humans and mice[82]. Most of the above cited effects of Wnt and TGFβ1 in adult and aging muscle regeneration are primarily based on *in vitro* culture or *in vivo overexpression*, pharmacology and RNAi studies; the genetic evidence remains to be obtained.

Maintenance of myogenic progenitors and stem cells by Notch signaling—The Notch-Delta receptor-ligand mediated signaling pathway has been shown to be instrumental in the regulation of various progenitor/stem cells and their differentiated daughters, including the myogenic progenitor and their differentiating progeny. Delta like 1 (Dll-1) mutant embryos initially have a normal number of $Pax3^+$ and $Pax7^+$ cells; over time, these cells become diminished[83]. Similarly, somitic conditional inactivation of RBP-Jκ, a transcriptional mediator of Notch signaling, also leads to a gradual depletion of $Pax7^+$ cells[84]. Consequently, by mid- to late embryogenesis, both Dll-1 and conditional RBP-Jκ mutants have much reduced musculature. These data reinforce the positive role of Notch signaling in controlling the myogenic progenitor pool size.

One prominent negative regulator of Notch signaling is Numb. Asymmetric distribution of Numb during cell division helps determine distinct developmental paths of daughter cells by affecting differential Notch activities[85]. Curiously, forced expression of a Numb-GFP fusion protein in mouse embryos[86] resulted in an increased progenitor pool, seemingly contradicting the positive role for Notch signaling in progenitor maintenance. Because this particular *Numb-GFP* transgene did not cause down-regulation of Notch reporter expression, it is still unresolved how Numb acts in regulating embryonic muscle progenitor cell number. When Numb protein distribution was followed by *in vitro* live imaging of post-natal myoblasts, either isolated in bulk[87] or from dissociated myofibers[88], it was found to be distributed both asymmetrically and symmetrically during cell division. The proportion of asymmetrically segregated Numb in daughter cells declined over successive divisions, possibly reflecting the absence of *in vivo* regulators in culture. While Shinin et al.[88] proposed that Numb co-segregates with the progenitor, Conboy et al.[87] suggested that Numb associates with the differentiating daughter cell. As of yet, the precise role of Numb in the myogenic lineage is not resolved.

Without examining Numb localization, symmetric and asymmetric satellite cell divisions can be observed in cultured myofibers and their associated satellite cells isolated from

Myf5-Cre;YFP reporter mice[32]. In this experimental paradigm, YFP was used as an indicator for a sub-population of satellite cells at one point in time expressing *Myf5* ($Pax7^+YFP^+$), relative to those expressing only Pax7 ($Pax7^+YFP^-$)[32]. Two types of cell division were found. Type 1, planar symmetric cell division (along the plane of the myofiber) resulted in the majority of daughter cells either both maintaining the $Pax7^+YFP^-$ stem cell fate or both displaying *Myf5* expression ($Pax7^+YFP^+$). Type 2, asymmetric division along the apical-basal plane mostly gave rise to cells with different fates: the cell that divided away from the basal lamina gained *Myf5* expression ($Pax7^+YFP^+$), while the basal sister stayed in the satellite cell compartment and did not express *Myf5* ($Pax7^+YFP^-$). Furthermore, the cell that remained basally displayed elevated levels of Notch3 while the apical cell expressed the Dll-1, suggesting that activation of Notch3 by Dll-1 positively selects the basal cells to maintain the stem cell fate. However, in response to repetitive muscle injury, *Notch3*-deficient mice regenerated better and had more satellite cells than control animals, suggesting that *Notch*3 is instead a negative regulator of the muscle stem cell pool[89]. By contrast, over-expression of an intracellular domain of Notch1 (NICD, an activated form of Notch1) increased proliferation of cultured myoblasts[87], supporting its positive role in stem cell expansion. It is interesting to note that while Notch1 is expressed in nearly all satellite cells, Notch3 is expressed only in a subset. One may therefore speculate that the Notch pathway serves to regulate the number of satellite cells through the balanced activity between Notch1 (positive) and Notch3 (negative). Whether different Delta like ligands are involved in mediating differential Notch activities is yet to be explored.

Although Dll-1 expression is up-regulated in injured muscles of young adults[87], aging muscles lose the ability to activate Dll-1 after injury and are compromised in the regenerative process[80]. Pharmacological inhibition of Notch signaling can indeed block muscle regeneration in young adult mice, while forced activation of Notch signaling is sufficient to restore efficient muscle regeneration in old mice[80]. These results provide strong support for the continuous participation of Notch signaling in the maintenance of the skeletal muscle stem cell activity throughout lifetime. For studying the aging process, gene over-expression or functional blocking reagents, rather than genetic means, are often used. As such, the nuances of differential roles between gene family members may be overlooked. Restricting knockouts of each Delta and Notch gene to the muscle progenitors versus their surrounding cells will help to delineate the specific role of each gene member.

III. Regeneration recapitulates development?

Studies of embryonic muscle development and the muscle regeneration have primarily been two distinct disciplines. It has been largely by inferential extensions that these processes were compared. From the above descriptions, it should be clear that in many cases, the molecular mechanisms that control adult muscle regeneration do not recapitulate those that control embryonic myogenesis. During skeletal muscle progenitor and stem cell expansion and maintenance, there is a progressive change of genetic requirements for *Pax3* and *Pax7*, to *Pax7* only, to neither, from embryo, to neonate, to adult, respectively. For muscle differentiation, there is also a temporal shift of genetic contributions among the *MRF* members, for example, the roles of MyoD and Myf5 during embryonic muscle development versus adult muscle regeneration reviewed above. The most striking shift of function is exemplified by *Myogenin, which is* required for fusion of embryonic myocytes but dispensable for fusion of regenerating adult myocytes[90]. Lastly, the Wnt/β-catenin pathway also appears to act differently during embryonic myogenesis, adult muscle regeneration, and aging muscle fibrosis. The pathway that is thus far continuously required for maintaining embryonic muscle progenitors and adult muscle stem cells is Notch signaling. Yet, Notch3 is only required for maintaining muscle stem cell pool size after repeated injuries, but not for regulating the embryonic myogenic progenitor pool. There are many other signaling and

transcriptional regulators of muscle development and regeneration that have only been examined in one context but not in the other, but they are not covered here due to space. A comprehensive evaluation of these regulators in both contexts will help to conceptualize the differences between these two processes. Because embryonic development relies on active progenitors cycling continuously but adult muscle stem cells are relatively quiescent in the absence of injury, these two processes are fundamentally distinct. In particular, the muscle regenerative process involves stem cell activation and a return to a homeostatic state in a chaotic inflammatory environment not seen during embryogenesis. Therefore, it is only reasonable that these adult stem cells utilize different strategies from those employed by embryonic progenitors to adapt to unpredictable circumstances such as injury-induced regeneration.

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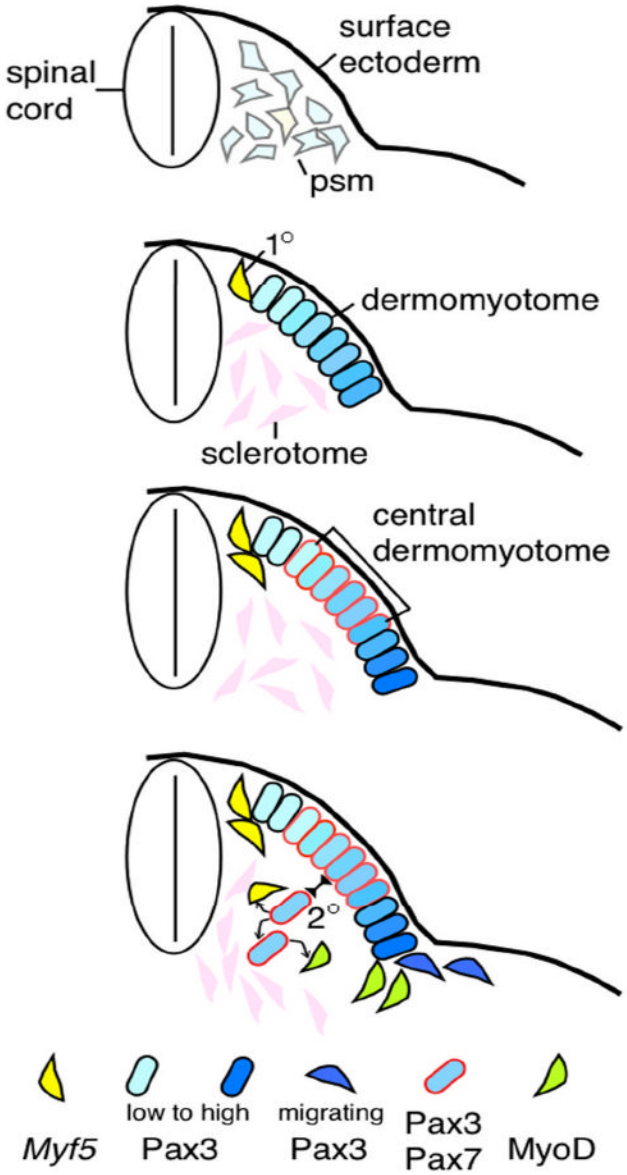

Figure 1. Developmental progression of myogenesis and myogenic gene expression

Top panel: presomitic mesoderm cells express *Pax3* (pale blue) and low levels of *Myf5* (pale yellow) transcripts. Epithelial somite stage is omitted. Second panel: appearance of sclerotome, dermomyotome ($Pax3^+$), and primary (1°) myogenic cells ($Myf5^+$) at the dorsal medial edge. Pax3 expression is at a higher level at the lateral edge. Third panel: Pax7 expression emerges and overlaps with $Pax3^+$ cells in the central dermomyotome in more mature somites. Bottom panel: Vertical division of the central dermomyotomal cells, which give rise to the secondary (2°) myogenic progenitors. These cells are presumed to give rise to more progenitors and $Myf5^+$ or $MyoD^+$ myogenic cells. The lateral $Pax3^+$ cells give rise to migrating myoblasts entering the ventral body wall and limbs. Keys to cells with specific gene expression are at the bottom.

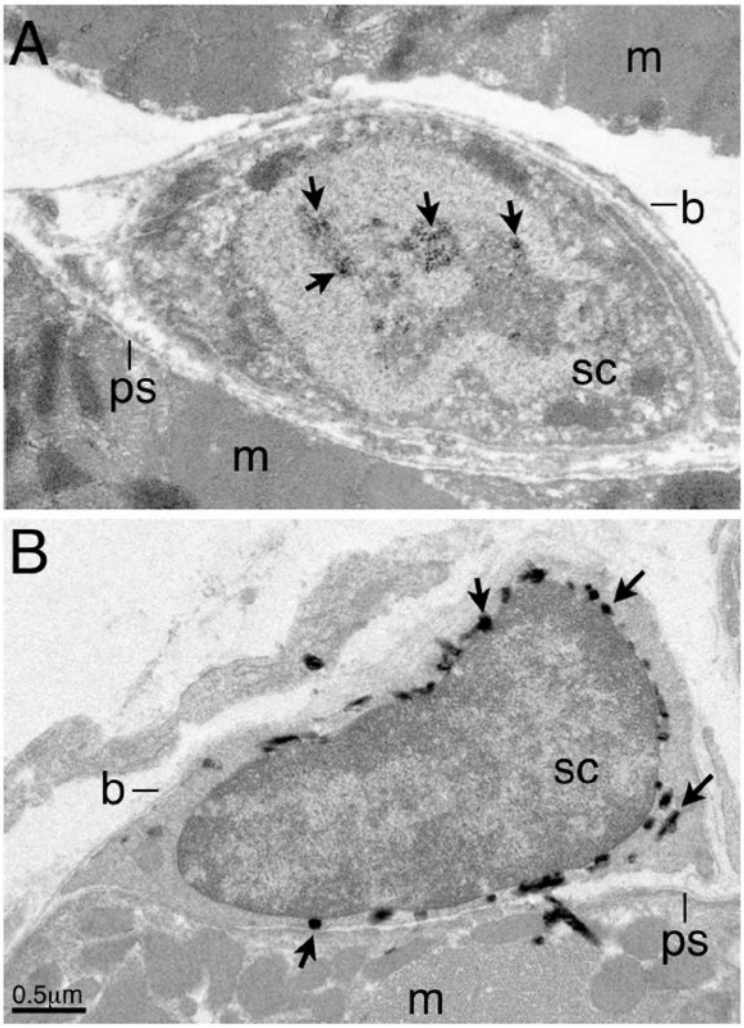

Figure 2. Pax7 expression is detected in satellite cells of the mouse tibialis anterior muscle

(A) Immuno-EM detection of endogenous Pax7 by a monoclonal antibody (DSHB), followed by a HRP-conjugated goat anti-mouse IgG1 antibody (Molecular Probes) and enzymatic reaction using the DAB substrate (Vecta Lab). The sample was fixed in Zamboni's fixative following the protocol in Chen et al. (2007). (B) TEM of samples from tamoxifen-treated *Pax7-Cre-ER^{T2};LacZ* reporter mice, fixed in 4% paraformaldehyde, reacted with X-gal substrate, following the procedure in Kanisick et al. (2009). Abbreviations: m, muscle fiber, b, basement membrane; ps, plasmalemmal surface; sc, satellite cell. Arrows in (A) indicate reacted DAB deposits in the nucleus, in (B), reacted X-gal precipitates in the cytoplasm. Some X-gal precipitates are often seen next to satellite cells, likely due to substrate diffusion during enzymatic reaction. Scale bar in (B).

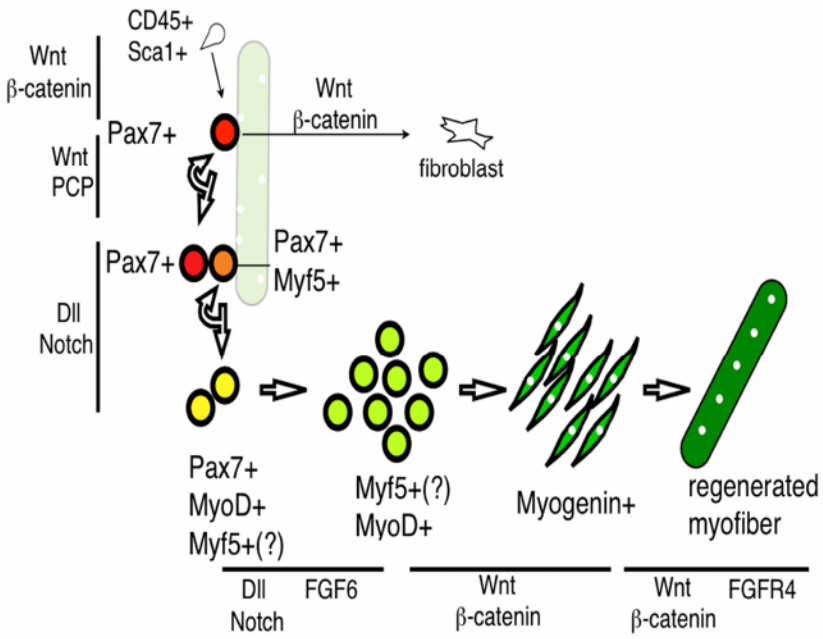

Figure 3. Gene expression and signaling regulation during adult muscle regeneration

$CD34^{+}Sca1^{+}$ side population cells can be converted into $Pax7^{+}$ cells via Wnt/β-catenin signaling. $Pax7^{+}$ satellite cells (red) are associated with the myofiber (pale green) with peripheral myonuclei (white). Upon injury, $Pax7^{+}$ cells either go through symmetric division for self-renewal via the Wnt/PCP pathway or asymmetric division to give rise to committed progenitors ($Pax7^{+}Myf5^{+}$, orange) involving Dll/Notch signaling. These cells expand (as transit amplifying cells) and express MyoD (yellow). Myf5 is presumed (Myf5 (?)) to be expressed in transit amplifying cells and $Pax7^{-}MyoD^{+}$ myoblasts (yellowish green). Myogenin (green) is turned on in differentiated myocytes, which eventually fuse to form the new myofiber (dark green), which has the characteristic of centrally located myonuclei. Wnt/β-catenin can also convert $Pax7^{+}$ cells into fibroblasts. There are at least two steps of muscle regeneration involving FGF signaling: FGF6 affects $MyoD^{+}$ cells, while FGF4R4 affects myofiber number and size. Gene expression for each stage is confined to those covered in the text and does not represent a full list. Wnt, FGF, and Notch pathways that regulate specific steps of myogenic progression are represented by black lines next to the defined steps.

Table 1

Knock-in allele	Cell types labeled	References	Comments
Pax3-Cre	Endothelium	Hutchenson et al.	possible Cre activity in presomitic mesoderm
M-Cre	Trunk and limb muscle	Shienda et al.	
	Limb satellite cells		
	Side population cells		
En1-Cre	Trunk Muscle	Atit et al.	no limb muscle labeled
En1-Cre-ER^{T}	Dorsal dermis		
	Brown fat		
Pax7-Cre-ER^{T2}	Trunk Muscle	Lepper and Fan Keller et al.	cell types labeled dependent on timing of tamoxifen administration
Pax7-IRES-Cre-ER^{T}	Limb muscle		
	Dorsal Dermis		
	Brown fat		
	Fetal muscle progenitors		
	Adult satellite cells		
	Adult muscles		
Myf5-Cre	Trunk muscle	Seale et al.	Cre activty in presomitic mesoderm *also as committed muscle progenitors
	Limb muscle	Kuang et al.	
	Brown fat	Gensch et al.	
	Cartilage		
	Endothelium		
	Adult satellite cells*		
MyoD-Cre	Trunk muscle	Kanisicak et al.	only myogenic cells were characterized
	Limb muscle		
	Perinatal satellite cells		
Myogenin-Cre	Trunk Muscle	Gensch et al.	only muscles are labeled
	Limb muscle		