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Review

Skeletal muscle stem cell birth and properties

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Abstract

Development and maintenance of an abundant tissue such as skeletal muscle poses several challenges. Curiously, not all skeletal muscle stem cells are born alike, since diverse genetic pathways can specify their birth. Stem and progenitor cells that establish the tissue during development, those that maintain its homeostasis, as well as participate in its regeneration have generated considerable interest. The ability to distinguish stem cells from more committed progenitors throughout prenatal and postnatal life has guided researchers to identify stem cell properties and characterise their niche. These properties include markers that influence cell behaviour and mode of division during normal development, after trauma and cell transplantations. This review addresses these issues from a developmental perspective.

Keywords: Embryonic and postnatal myogenesis; Pax7; Muscle regulatory factors; Satellite cell; Asymmetric cell division

Contents

1. Introduction

Skeletal muscle, like skin, is found throughout the body of the organism. In higher vertebrates skeletal muscles form an integrated network with a prominent skeletal system via tendons, resisting gravity and facilitating mobility. Lower vertebrates and particularly those that are aquatic, have a proportionally greater muscle mass. The anatomical origin of stem cells of a tissue in the embryo is a key question in developmental biology, as it helps understand the microenvironment in which the tissue is established, and the molecular signals that regulate its development. Although all skeletal muscles in vertebrates originate from mesoderm, distinct genetic regulatory networks govern the birth of skeletal muscle stem cells. Here we refer to "stem cell" as the most upstream cellular ancestor of muscle, and "progenitor" as its more committed daughter (see Fig. 1B). In some contexts, we

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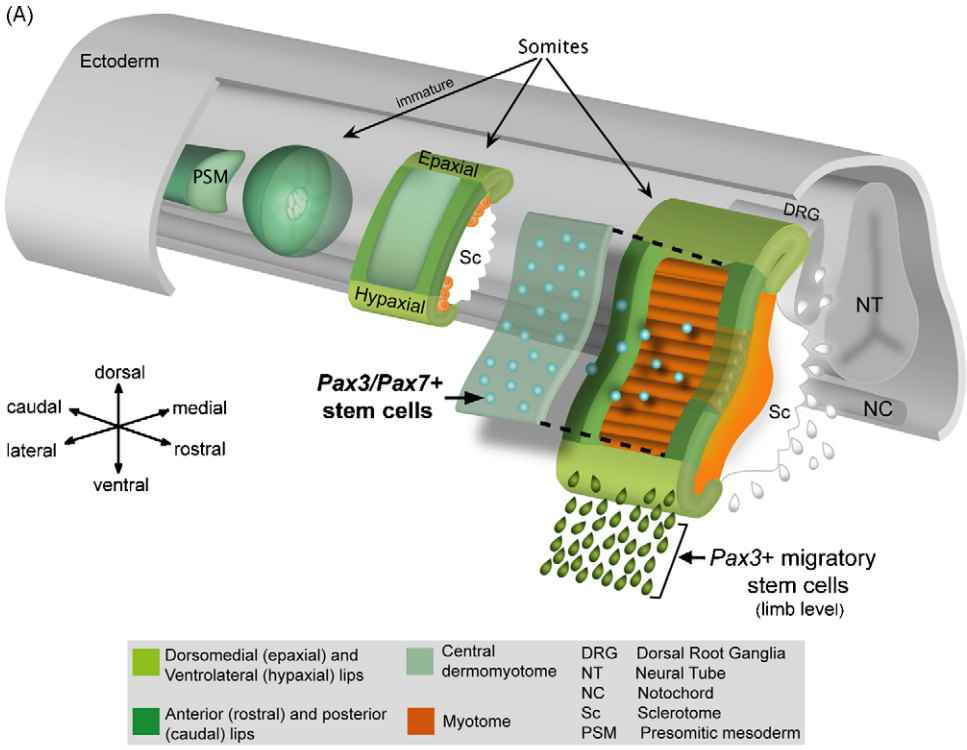

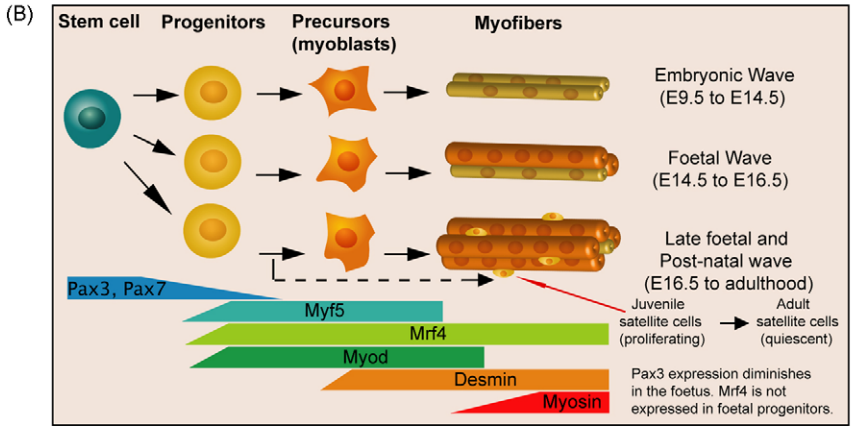

Fig. 1. A model of skeletal muscle formation from stem and progenitor cells in the mouse embryo. (A) Presomitic (paraxial) mesoderm (PSM) segments into epithelial somites. Dorsal portion of somites—dermomyotome (DM) harbours muscle stem/progenitor cells. The progenitors in the dorsomedial lip of the DM are the first to commit to myogenesis. They undergo an epithelial to mesenchymal transition, migrate underneath the DM to form the myotome where they differentiate into mononucleated myocytes that are attached to the anterior (rostral) and posterior (caudal) edges of the somite. The progenitors from the other three lips follow suit and contribute to the growth of myotome. Pax3/Pax7 expressing stem/progenitors from central portion of the DM (represented as an overlying layer, displaced) "parachute" into the underlying differentiated myotome to assure muscle growth. Myotomes are referred to here as the anlagen of trunk muscles. Progenitors from the ventrolateral lip of limb level somites migrate to establish limb muscles. In the mouse, these express *Pax3* but not *Pax7*, and *Pax3* null mutants are deficient in limb (as well as diaphragm and tongue) muscles. Note that not all DRGs are indicated; only representative somites along the rostral-caudal axis are illustrated; the nascent spherical epithelial somite buds from the mesenchymal PSM located more caudally; the myotome and sclerotome extend the full width of each somite; once the somites dissociate, myofibres fuse along the rostral-caudal axis across previous somite borders. (B) Illustration of lineage progression and the multiple waves of developmental myogenesis. The expression patterns indicated at the bottom represent primarily the onset during the embryonic wave. Pax3 is not expressed in head muscle progenitors and in the body its expression declines in the foetus. Mrf4 is not expressed in head and foetal progenitors. Desmin is an intermediate filament protein expressed in muscle and Myosin is a component of the contractile apparatus. Myogenin, which is required for muscle differentiation from myoblasts, is not indicated here. The lineage relationship between the stem cell from the dermomyotome and the progenitors within each wave of myogenesis is yet to be resolved. Around E16.5, proliferating, Pax7+ cells appear in a satellite cell position (see also Fig. 3). A subset of these cells will become the future adult quiescent satellite cells.

employ the term "progenitor" as the generic ancestor if "stem cell" is too restrictive.

2. Muscle stem cells originate in distinct anatomical locations

2.1. Somitic origin of muscle stem cells: trunk and limbs

Trunk musculature, involved in locomotion and posture maintenance, derives from presomitic (paraxial) mesoderm (PSM), which runs parallel to the body axis, flanking the neural tube (Fig. 1). In the mouse embryo, beginning from about E8 (embryonic day 8), epithelial somites bud from the anterior PSM in a head to tail fashion such that the youngest somites are located at the posterior end. Somitogenesis is regulated by a combination of a maturation gradient which includes FGF, Wnt and retinoic acid pathways, and a segmentation clock that includes molecular oscillators involving Notch and Wnt pathways [1]. The somites harbour stem/progenitor cells that give rise to cartilage, endothelial cells, tendon, connective tissue, dermis of the back as well as skeletal muscle [2,3]. The ventral portion of the somite forms the sclerotome which gives rise to the axial skeleton whilst the dorsal somite, called the dermomyotome (DM) retains an epithelial morphology for several days, and it harbours skeletal muscle stem and progenitor cells of the trunk, limbs and some head muscles. The DM is a highly regionalised sheet of epithelial cells that can be divided into a central portion and four edges, the dorsomedial (epaxial), ventrolateral (hypaxial), anterior (rostral) and posterior (caudal) lips (Fig. 1). The signals emanating from the surrounding embryonic structures such as the overlying ectoderm (Wnt7a, Wnt6), neural tube (Shh, Wnt1), notochord (Shh) and lateral mesoderm (BMP4) impinge upon the cells in the DM to regulate precisely the emergence of muscle progenitor cells in consecutive waves [4–7]. These signalling molecules act on different parts of the DM to varying degrees depending on the distance from the signalling source, thereby setting up the regionalisation. Muscle stem/progenitor cells in the different regions of the DM differ in the programme that commits them to myogenesis depending on the local signals that instruct them. The progenitors migrate underneath the DM, differentiate into mononucleated myocytes to form the myotome. Myocytes fuse across somite boundaries at later stages to form multinucleated fibres that act as scaffolds for further fibre addition. Studies have shown that cells from the central portion of the DM "parachute" into the underlying myotome to assure the continued growth of skeletal muscles during embryonic and foetal stages [8–11]. Evidences for the existence of a similar pool of stem cells in lower vertebrates have been reported recently highlighting the conserved developmental mechanisms with respect to the generation of this pool [12,13].

At the level of the limbs, the progenitors from the ventrolateral lip delaminate, migrate a relatively longer distance compared to those contributing to the myotome, to found limb musculature [14,15]. Progenitors of the diaphragm and tongue muscles also originate from the ventrolateral lip of DM at the cervical and occipital levels respectively [16]. The migratory progenitors for some of the neck muscles also come from occipital (anterior-most) somites. These have received less attention in the literature.

2.2. Paraxial head and prechordal mesoderm origin of muscle stem cells: head

In terms of development, the muscle stem and progenitor cells in the head experience a very different environment compared to those in the trunk. First, the progenitors of head muscles have multiple origins. The occipital somites deliver the stem cells into the tongue, as well as the posterior branchial arches that make posterior neck muscles [17–19]. The paraxial head mesoderm (PHM) gives rise to the bulk of head muscles, whilst the prechordal mesoderm (PCM) contributes to some extraocular muscles (see Fig. 2; [18]). The evidence for this dual origin comes from cell tracing studies done in chick and little to no information is available for the relative contributions of the PHM and PCM to extraocular muscles in mammals.

Secondly, the PHM is apparently unsegmented unlike its trunk counterpart [17,20]. Thus, the stem and progenitor cells of most of the head musculature reside in an embryonic tissue whose organisation is distinct from that of the trunk muscle progenitors.

Thirdly, the signals that regulate myogenesis in the head vary compared to those that operate in somites. The head mesoderm grafted at the location of somitic mesoderm fails to activate myogenic marker expression, but somitic mesoderm transplanted at head mesoderm position activates the myogenic program [21,22]. This suggests that the head environment contains cues that are permissive for myogenesis for the mesoderm from trunk. In fact, Shh, Wnt1, Wnt3a and Wnt13 are produced by tissues surrounding the PHM [23]. But, the head mesoderm does not respond to the cues in the trunk indicating that these signals play distinct roles. This indeed is the case since Wnt and Shh signals that play a positive role in myogenic induction in somitic progenitors inhibit the onset of the myogenic programme in head muscle progenitors [23]. Furthermore, within the head itself, the signals that influence the muscle progenitors of various muscle groups, for example extraocular and branchiomeric, vary [24].

Though the focus so far has been on the local environment, it is also likely that the stem/progenitors themselves are intrinsically distinct at different levels of the body axis thus influencing the way they interpret the cues from their surroundings. That Hox genes predispose the somitic muscle progenitors at different axial levels to particular fates has been reported as well [25,26]. However, an investigation of muscle progenitors in the head and trunk in this context is lacking.

Finally, as the local environments that influence muscle stem cells are different between head and trunk, the gene regulatory networks that are downstream to the signalling pathways and that govern myogenic induction are also distinct. This aspect is elaborated below.

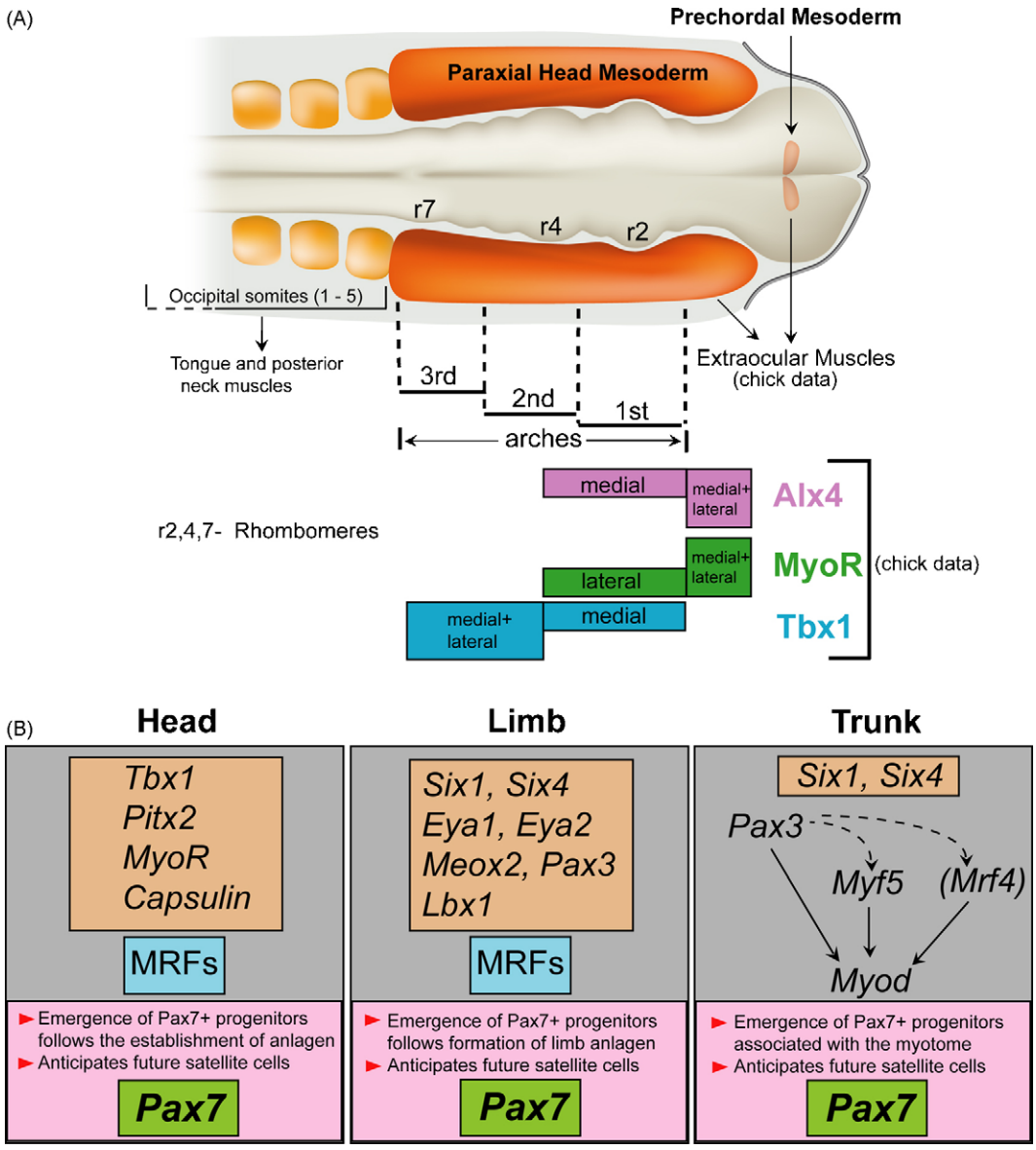

Fig. 2. A model for skeletal muscle formation in the head, and regulatory networks in the organism. (A) The anterior most somites at the level of the uppermost spinal cord—occipital somites, provide progenitors for tongue and posterior neck muscles. Anterior neck, upper and lower jaw, and other facial muscles develop from progenitors that migrate from paraxial head mesoderm (PHM) into the anterior branchial arches. Extraocular muscle progenitors face a non-branchiomeric surrounding and derive from both PHM and prechordal mesoderm (chick data; [18]). In, chick, expression of *Alx4*, *Tbx1* and *MyoR* were shown to delineate distinct regions of the PHM [20]. (B) Regulatory networks in muscle stem/progenitor cells from observations in mouse mutants. Mrf4 acts in embryonic progenitors and not in those of the head or the foetus. Pax7 marks specifically those progenitors that accomplish muscle growth once myogenesis had been initiated and an "anlagen" established. In this model, Pax7 expression in these progenitors is the unifying theme of all muscle stem/progenitors of the body, and thus future satellite cells. *Note: Pax7* is expressed in cells migrating from the somite to the limb in chick, but not the mouse.

3. Molecules that regulate muscle stem/progenitor cell function

3.1. Hierarchy of regulatory factors: trunk progenitors

As a result of their intrinsic potential and signalling from their microenvironment, cells in the epithelial dermomyotome are specified to generate at least three distinct cell types: muscle, dermis, and endothelial. For muscle, the signals impinge mainly on two kinds of transcription factor networks—paired-box transcription factors, Pax3 and Pax7, and a family of basic-Helix–Loop–Helix (bHLH) transcription factors known as myogenic regulatory factors (MRFs). Signals emanating from the overlying ectoderm, and to some extent fibronectin [27], are necessary for maintaining the dermomyotome as an epithelium and *Pax3/7* expression. The bHLH gene *Paraxis* plays a key role here since null mice lose epithelial integrity, however, some muscle gene expression occurs [4,28,29].

Pax genes play critical roles in cell specification and organogenesis during development. In mammals, there are 9 Pax genes (*Pax1–9*) that are expressed in a variety of tissues during embryogenesis. Mutations in these genes are often associated with

diseases in humans [30]. Pax genes are characterised by a Paired domain that binds DNA. They are categorized into four groups of paralogues as defined by their sequence and structural homology as well as functional similarities. Early chordates have four evolutionary prototypes that underwent duplications to generate the four subgroups [30,31]. *Pax3/7* comprise one subgroup. *Pax3/7* have overlapping expression in the central nervous system, neural crest cells and somites. Together, *Pax3* and *Pax7* are necessary for the emergence and survival of muscle stem progenitors since double mutants display near complete loss of these cells [11,30,32]. *Pax3/7* expression, induced by Wnt signalling, marks the progenitors of both dermis ([8]; unpublished observations) and muscle. The pathways that regulate the cell fate choice of these progenitors remain to be elucidated.

It is known that the progenitors are determined as muscle precursors (myoblasts) upon activation of the myogenic regulatory factors. In vertebrates, there are four MRFs, namely *Myf5*, *Myod*, *Mrf4* and *Myogenin*. Induction of MRFs in Pax3/7 expressing cells, *i.e.*, progenitor to myoblast progression, can be regulated by Shh (in dorsomedial DM lip; [33,34]) and BMP pathways as well as the Pax3/7 transcription factors. *Pax3* directly regulates *Myf5* expression [35,36]. In combination with *Pax3*, *Myf5* is essential for trunk myogenesis [36]. *Myf5* and *Myod* commit progenitors to a muscle fate whereas *Myogenin* is required for differentiation of committed precursors. The role of *Mrf4* is more complex. It can determine progenitors as myoblasts in the embryo, but not in the foetus, and also promote differentiation by activating *Myogenin*. Mice triple mutant for *Myf5*, *Myod* and *Mrf4* totally lack skeletal muscle and myoblasts [37,38]. The muscle progenitors, however, are present and they either enter another differentiation programme, or are lost by apoptosis after mid-gestation [39]. Muscle differentiation involves withdrawal from the cell cycle, and fusion of myoblasts to form multinucleated myofibres. Here *Myogenin* plays an essential role since differentiation is severely compromised in mice mutant for this gene. Surprisingly, however, conditional inactivation of *Myogenin* at foetal stages does not result in a loss of the differentiation programme [40], suggesting that *Myogenin*-independent mechanisms sustain muscle development after the embryonic period. Mrf4 is also expressed in myonuclei of mature fibres and may maintain their differentiation status, however the inactivation of this gene has failed to reveal a muscle phenotype in the adult [41,42].

Myogenesis occurs in multiple waves during development to achieve growth of the musculature to adult size. Earliest is the embryonic wave, followed by a foetal wave (E14.5 onwards, in mouse) and finally postnatal growth (see Fig. 1). This implies many phases of progenitor to precursor and precursor to differentiated cell transitions during development while maintaining the stem cell pool. Indeed, the *Pax3/7* expressing stem/progenitor population derived from the central dermomyotome is maintained throughout development. Moreover, this pool also generates satellite cells—the postnatal progenitors that assure growth and regeneration throughout life [9–11]. Maintenance of this reservoir during development necessitates a tight regulation of progenitor to precursor lineage progression. Recent reports implicate a role for Notch signalling in this regulation. Disruption of the Notch pathway depletes the progenitor pool by accelerating their commitment to myogenesis thereby compromising muscle development. This ultimately results in severe muscle hypotrophy and loss of the Pax3/7 stem/progenitor pool [43,44].

The myoblast to differentiated cell transition is also regulated so as to expand the precursor population to a threshold level. This is likely brought about by tight regulation of the MRF gene regulatory network. Multiple mechanisms are known to influence Myod function that could be implicated in this process [45,46]. In this context, it is important to recall earlier work in the field which showed that a threshold level of MRFs is necessary for the commitment switch to be engaged [47], akin to an action potential in nerve cell signal transmission. Removal of inductive signals after MRF transcript accumulation can result in the loss of expression and failure to progress in the lineage [48]. This is a critical notion particularly when expression is observed in ancestral cells. Resolving whether this expression equates with commitment will be a major challenge in distinguishing stem, progenitor and myoblast cell states. In cell marking studies using genetic tools, for example, the expression of *Cre* recombinase under the regulatory control of a MRF locus could result in a recombination event and expression of a reporter gene. In this scenario, the reporter gene marks the cell for MRF expression, but that cell may not necessarily reside in the lineage if the requisite protein level was not reached, and the cell did not progress to commitment. Therefore, the interpretation of genetic recombination events in the context of lineage requires detailed analyses of the population to assess if the read out obtained is indeed faithful.

Thus, the origin of trunk muscle stem/progenitor cells that assure successive waves of myogenesis and contribute to adult muscle satellite cell pool could be traced to the dermomyotome. Another aspect yet to be explored is that of lineage relationship among the stem/progenitor population of each wave of myogenesis. For example, is there a subset of the stem cell pool that contributes exclusively to muscle precursors of each wave or a common pool whose daughters contribute to all of these waves to varying degrees? Since *Mrf4* can direct embryonic, but not foetal muscle progenitor cell commitment [37], one can argue that embryonic and foetal progenitors are distinct, possibly originating from a common stem cell ancestor. It is also possible that embryonic progenitors down regulate *Mrf4* expression and "change hats" to adopt a foetal progenitor status. In both cases, we know that *Mrf4* regulatory elements do not respond to the foetal signalling environment for progenitor cell expression. This question needs to be resolved to evaluate how the lineage is structured prenatally.

3.2. Hierarchy of regulatory factors: limb progenitors

Stem/progenitor cells of limbs are characterised by their dependence on Pax3 function. In *Pax3* null mice, migration of muscle stem cells from the ventrolateral DM lip is abrogated. *Pax7* is not normally expressed in these cells and when ectopically expressed from the *Pax3* locus, it fails to rescue

this phenotype revealing the functional divergence between these closely related genes [49]. A paracrine system involves Hepatocyte growth factor/Scatter factor (HGF/SF)—a ligand expressed along migratory routes, and Met, a receptor tyrosine kinase expressed by the stem/progenitors. In mutant mice lacking either *Met* or *HGF/SF* these progenitors are present but fail to migrate [50,51]. Apart from this paracrine influence, cell-autonomous mechanisms also govern the migration of these progenitors. Mice deficient for the homeodomain transcription factor Lbx1 show impaired migration of progenitors [52–54]. Only a small number of migratory progenitors reach the targets and their expansion is critical to make the muscles. Another homeodomain factor Six1 controls the proliferation of these progenitors in a complex with Eya1 and Dach through regulation of *c-myc* expression. *Six1: Eya1* double mutants display complete absence of limb musculature [55]. *Six1/4* and *Eya1/2* influence *Pax3* expression and *Myf5* is a direct transcriptional target of *Six1/4* [56-58]. *Meox2* also regulates limb muscle progenitors [59,60]. Once at the target site, lineage progression leads to muscle formation while self-renewal mechanisms assure maintenance of the stem cell pool.

3.3. Hierarchy of regulatory factors: head progenitors

For all muscle stem/progenitor cells in the organism, the signal inputs converge on the MRFs. However, distinct upstream regulatory pathways distinguish those in the head from the body proper. Those that derive from the anterior occipital somites will be excluded from the present discussions of head progenitors as these are trunk derivatives. To begin with, *Pax3* is not expressed in head muscle progenitors [21,61], and its function is dispensable in this location [36]. In mice, *Pax7* expression in the anlagen of head muscles follows, not precedes, that of *Myf5* expression ([61]; unpublished observations). Interestingly, in mice deficient for upstream regulators Six1 and Six4, that play key roles in the establishment of body muscles, PHM derived muscles are spared [58]. Since *Pax3* and *Pax7* are not expressed in the early head muscle progenitors, the signalling cues from the head environment are likely to impinge on MRFs either directly or through other mediators. Here, the picture in the head is complicated. Even within the branchiomeric group, the progenitors of different sub groups differ in their programme. In mouse, transcription factors MyoR and Capsulin play a critical role in mandibular arch (first branchial arch) muscle progenitors, as the combined loss of their function leads to specific ablation of muscles derived from this arch [62]. Loss of a T-box transcription factor Tbx1 affects only some of the branchiomeric muscle groups [63]. A homeobox transcription factor Pitx2 also plays a major role in progenitors of extraocular muscles [64–67]. In chick, anlage of the extraocular muscle lateral rectus expresses *Pax7*, *Lbx1* and *Paraxis*—markers of somitic muscle progenitors [20,22]. Therefore considerable diversity in the programme upstream of the MRFs among progenitors of various head muscle groups might reflect their respective microenvironment and the diversity in their origin (prechordal vs. paraxial head mesoderm).

Thus, stem/progenitor cells have multiple ways to make muscle. Such diversity might in fact be necessary for the appropriate patterning and innervations of the muscles by conferring distinct identities and this could be crucial for their development.

Strikingly, however, in all three locations (head, trunk, limbs) muscle anlagen are established first and then a population of $Pax7^+$ cells. This population assures development of muscles to their adult size and importantly, anticipates the emergence of future satellite cells—the adult muscle stem/progenitors (see Fig. 2B). Therefore, in contrast to the different modes to initiate myogenesis, continued growth of muscle and generation of satellite cells appear to rely on a similar programme throughout the body. Furthermore, satellite cells express Pax7 both in the trunk and in the head. The following section will focus on satellite cells.

4. Stem/progenitor cells of the adult muscle

4.1. Satellite cell origin

Post-natal growth of the muscles is accomplished by satellite cells. This is also true for lower vertebrates [68]. In adults, satellite cells maintain homeostasis of the muscle tissue and they are the major cell type to contribute to muscle regeneration following damage by injury. Satellite cells owe their name to their anatomical location on muscle fibre periphery. They are located between the plasmalemma of the muscle fibre and the basement membrane ensheathing it (Fig. 3A). The remarkable capacity of muscle tissue to regenerate following injury highlights the potential of these quiescent cells. Satellite cells are activated upon damage to muscle, proliferate and fuse to make myofibres thereby regenerating the tissue. Evidences that questioned the centrality of satellite cells in muscle regeneration and its recent re-emergence as the uncontested principal cell type that contributes to the process was discussed previously [69,98].

The embryological origin of satellite cells was first addressed in a chick-quail chimera study. Satellite cells of quail origin were found when chick somitic mesoderm was replaced by that of quail supporting a somitic origin for satellite cells [70], but the issue of endothelial cells which also migrate to the limb remained unresolved. Electroporation of the central dermomyotome in the trunk with a molecular marker showed that virtually all of these marked cells gave rise to $Pax7^+$ satellite cells after hatching, thereby establishing the dermomyotome origin of satellite cells, in chick [9]. Compelling evidences that satellite cells originate from the $Pax3/7^+$ cells in the mouse somite have also been reported [10,11]. It is not formally excluded, however, if other regions of the dermomyotome can also give rise to satellite cells. The ventral lips of limb level somites give rise to both migratory endothelial progenitors expressing *Vascular Endothelial Growth Factor Receptor2* (*VEGFR2*) and muscle progenitors marked by *Pax3* expression [71,72]. This raises the possibility of an endothelial progenitor contribution to the satellite cell pool [73], however, satellite cells in the limb were shown to arise almost exclusively from the $Pax3^+$ population in the hypaxial somite [74].

For head muscles of non-occipital somite origin, the source of satellite cells is unknown. One can assume that the embryonic tissue that provides progenitors for developmental myogene-

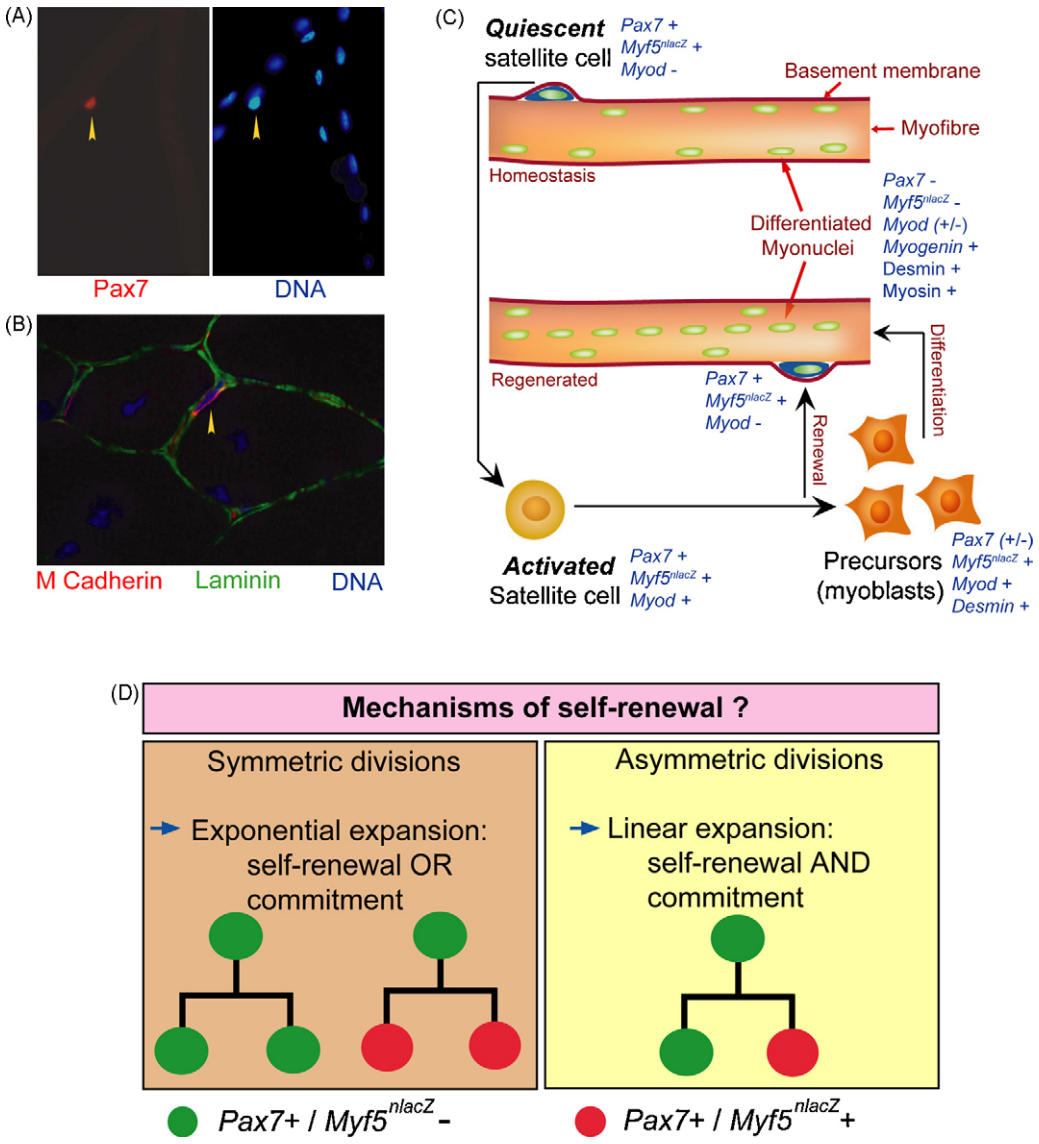

Fig. 3. Satellite cell activation and self-renewal in the adult. (A) Pax7 positive satellite cell indicated on an isolated myofibre. (B) M-cadherin antibody staining indicates a satellite cell located between the basement membrane (laminin staining) and the muscle fibre on cross-section. (C) Plasmalemma of the host myofibre and its basement membrane are components of the satellite cell niche. Quiescent satellite cells in adult muscles are Pax7+. Activation of satellite cells, upon injury, is accompanied by induction of *Myod* expression. Once activated, they enter cell cycle, proliferate and differentiate to accomplish regeneration. A subset of cells downregulates *Myod* but retains *Pax7* expression and these cells are thought to renew the satellite cell pool. (D) Choices of a satellite cell. Satellite cell self-renewal can be achieved by symmetric or asymmetric cell divisions. Symmetric cell divisions can result in the exponential expansion of the satellite cell or myoblast pools, whereas asymmetric cell divisions result in the maintenance of satellite cells, whilst generating myoblasts. Asymmetric divisions of activated satellite cells, involving cosegregation of template DNA strands or Myod or Numb have also been reported (see text).

sis also doubles up as the source of satellite cells. It is likely that the Pax7$^+$ cells detected in muscle anlagen following Myf5 induction could eventually supply satellite cells. In this regard, some questions remain unaddressed. Do *Pax7* expressing cells in head muscle anlagen represent 'naïve' cells that never expressed *Myf5*, or *Myf5* expressing precursors, or once committed cells that had expressed *Myf5*? The possibility of a committed precursor 'retrogressing' in lineage to become a progenitor is a significant query in the light of a recent report on the presence of a 'naïve' subset among the satellite cell pool that is low in Myf5 expression or Myf5-negative [75].

In newborn animals the proportion of satellite cells in muscle is higher and they proliferate to add nuclei to the growing muscle. Once growth is achieved satellite cells become quiescent and they represent a very small proportion of nuclei in adult muscles [76]. The time window in which quiescent satellite cells appear and the mechanism by which this quiescent pool is set apart from the progenitors that participate in post-natal growth is not known. Similar questions remain to be addressed for satellite cells in head muscles. In *Pax7* mutant mice satellite cells are born but their numbers drop dramatically after birth and consequently muscle growth is severely stunted ([77,78], but see also ref. [79]). This loss of *Pax7* null satellite cells is at least in part due to apoptosis implying this factor in survival of satellite cells [10,80]. Dependence on *Pax7* function for survival distinguishes the post-natal muscle stem/progenitor cells from those

that assure prenatal development. *Pax7* had been implicated in quiescence of satellite cells using over expression studies [81], but other studies do not support this hypothesis as neither proliferation nor muscle differentiation is overtly affected by constitutive expression of *Pax7* comparable to physiological levels [80,82]. These overexpression studies have also suggested that Pax7 regulates *Myod* expression in cultured cells [80,81,83]. Recent findings of molecular markers that specifically identify quiescent satellite cells will help in addressing these questions [84,85].

4.2. Satellite cell self-renewal

A remarkable attribute of muscle tissue is its capacity to regenerate following repeated injuries. This indicates replenishment of the satellite cell pool during each bout of regeneration. Replenishment could be accomplished either by a mechanism of self-renewal or by external reinforcement. Stem cells derived from bone marrow were found to occupy a satellite cell position, express satellite cell markers and also contribute nuclei to myofibres upon injury [86,87]. Mesenchymal stem cells from synovial membrane of human origin when transplanted into nude mice become incorporated in muscle as functional satellite cells [88]. Progenitors derived from developing vasculature—mesoangioblasts [89] and side population cells isolated on the basis of vital DNA dye efflux from muscle tissue [90,91] also 'become' satellite cells. However, Sherwood et al. [92] showed that cells in satellite cell compartment derived from elsewhere unlike 'original' satellite cells do not have intrinsic myogenic capacity indicating that cell autonomous mechanisms are important for satellite cell function. Thus, external reinforcement as a mechanism of satellite cell pool replenishment is unlikely. Further, these experiments raise the important issue of whether the nucleus of these exogenous cells is fully reprogrammed to a satellite cell state. Christov et al. [93] showed that bone marrow derived cells occupy a satellite cell position several months after bone marrow transplantation, and in the absence of injury. Some of these are Pax7-positive, however their morphology suggested that they were not fully reprogrammed. Furthermore, Pax7-positive bone marrow-derived cells were observed outside the basement membrane suggesting that the muscle environment can activate this gene under certain circumstances. Evaluating the epigenetic signature of these cells will require definition to assess the cell state and equate it with resident satellite cells.

Grafting of purified satellite cells or satellite cell derived myogenic cells gives rise to satellite cells of donor origin [94–96]. However, it is possible that the grafted cells occupy a satellite cell compartment without undergoing any division and hence does not provide an evidence for self-renewal. In an elegant study, using isolated single fibre transplantation, Collins et al. [97] showed that as few as 7 satellite cells grafted along with the fibre contributes extensively to muscle regeneration as well as new functional satellite cells. Since the input satellite cells were so few and the myonuclei and satellite cells generated were so substantial, it is interpreted as a result of self-renewal [97,98].

4.3. Self-renewal and asymmetric cell divisions

A major question in stem cell biology is the role of asymmetric vs. symmetric cell divisions, both of which can direct self-renewal and lineage progression. Asymmetric cell divisions are generally associated with tissue homeostasis and a linear mode of cell expansion whereas symmetric cell divisions result in exponential cell growth and are assumed to be employed during a crisis, *i.e.*, regeneration after trauma and also during development when the establishment of the tissue is of primary importance (Fig. 3B). Unfortunately, however, little to no evidence is available for when these strategies are deployed over time in the organism. Interestingly, some recent evidence suggests that regulators of asymmetric cell divisions tend to be tumour suppressors, whereas those regulating symmetric divisions belong to the oncogene class [99], suggesting that asymmetric cell divisions may be the preferred mode of cell division during extended periods. This notion remains untested.

Identifying tissue specific stem cells is a formidable challenge in the field of stem cell biology, within a developmental context, as well as in the clinic. It is appropriate therefore to define stem cells as entities endowed with particular properties that distinguish them from their more committed progenitors. Individual properties may be shared between both, for example, haematopoietic stem cells as well as progenitors can self-renew. It would be erroneous, then, to assume that properties generally attributed to stem cells are exclusive to them. Thus, there is a necessity to distinguish stem and progenitor cells "states" using multiple criteria.

One property that has been used to identify stem cells and their niche is the ability of slowly dividing cells to retain administered nucleotide analogues such as BrdU for extended periods, thus the notion of "label retaining cells" (LRCs) [100,101]. This approach is based on the premise that stem cells divide less frequently than their daughters, the latter diluting rapidly the label by random segregation of labelled DNA strands after a pulse-chase experiment with BrdU. Although some stem cell niches have been identified using this approach, some researchers have challenged the view that LRCs truly identify the stem cell niche. This experimental approach requires more scrutiny. As against slow-division, the adult stem cells can retain nucleotide analogues for extended periods by co-segregating "old" template DNA strands of all chromosomes in a non-random fashion exclusively to one daughter cell. This hypothesis, which involves the co-segregation of "immortal" DNA strands proposed 3 decades ago [102], has stimulated the imagination as well as generated controversy [103,104]. If stem cells had the ability to retain template DNA strands over extended periods, they could avoid errors arising during DNA replication by excluding newly replicated DNA strands to more committed daughter cells during cell division thereby retaining the more pristine DNA strands. This phenomenon has been reported for the cells in the small intestine, mammary epithelial cells, cultured neuronal cells, p53 deficient cells transfected with p53, and skeletal muscle satellite cells [104-109]. For the small intestine, a double labelling strategy was employed where the first label (^{3}H-Thymidine) was retained for extended periods in cells near the bottom of the

crypt. Labelled cells in this location subsequently incorporated the second label (BrdU), and after a second chase period, the $BrdU^+$ DNA was segregated to daughter cells and lost whereas the ^{3}H-Thymidine DNA strands continued to be retained [110]. These studies provided compelling evidence that selective retention of DNA strands occurs in the gut. In skeletal muscle, label-retaining cells were cultured and shown to continue to retain template DNA strands. The frequencies reported for this phenomenon vary from a conservative figure of at least 7% [104] to a higher value of about 50% [105] for satellite cells *in vivo*. This phenomenon may be related to the escape from DNA replication errors as previously postulated [102,111]. Alternatively, it was proposed that the concerted segregation of BrdU labelled chromatids might be a read-out of the epigenetic state of the daughter cells that would acquire distinct fates [104]. This notion was addressed in more detail recently [103]. In either case, the mechanism that guides this phenomenon presently defies the imagination. More detailed studies are required to determine if this property is related to other types of asymmetry discussed here.

Satellite cells have been shown to segregate the cell determinant Numb [104,112], and its co-segregation with template DNA strands was also reported [104] suggesting that a common mechanism governs these asymmetries. The precise role of Numb in satellite cell regulation remains enigmatic. Asymmetric segregation of Numb protein was reported to occur from the first cell division, several days prior to cell differentiation [104]. Since Numb has been reported to inhibit Notch activity in other systems [113], and down regulation of Notch has been associated with myogenic differentiation [114,115], it is not obvious why Numb asymmetry during the first days after satellite cell activation does not result in differentiation. Notably, loss of Numb function perturbs the stem/progenitor pool size in the CNS indicating that this protein may play a role in self-renewal. Perhaps a sub-population is responsive to Numb activity. This highlights once again the notion of satellite cell heterogeneity likely related to the competence to differentiate at any given time. More recent studies have indicated that Numb plays a role in maintaining cell–cell contacts by interacting with Cadherins [116,117]. Its up regulation during differentiation therefore may be a consequence rather than a cause. High-resolution single cell fate analysis should help determine what are the precise functions of Numb in satellite cell lineage progression.

Though asymmetric DNA strand segregation and Numb segregation during mitosis are suggestive of self-renewal, is this mechanism common for all self-renewing divisions? Though this question remains open, these reports add strong evidence to the heterogeneity in the satellite cell pool [98]. Further evidence for heterogeneity in satellite cells associated with their self-renewal comes from Kuang et al. [75]. Using *Myf5^{Cre}* in combination with a *ROSA-Stop-YFP* reporter mouse, they detect a subset of *Pax7* expressing satellite cells that never expressed *Myf5* (*i.e.*, YFP-negative; about 10%). Upon isolation of fibres, Pax7+/Myf5− cells divide perpendicular to the fibre generating Pax7+/Myf5+ daughters adjacent to the myofibre and Pax7+/Myf5− daughters adjacent to the basement membrane [75,118]. Furthermore, Myf5+ (YFP+) satellite cells prospectively isolated using positivity for α7 and β1 integrins and negativity for Lin, Sca1, CD31 and CD45 have a propensity to differentiate when grafted whereas Myf5− (YFP−) satellite cells self-renewed efficiently in the injected muscle. These experiments suggest that a small fraction of satellite cells are more "stem-like" whereas the majority are more committed progenitors. The link between the label-retaining subset and the Myf5− subset is yet to made.

Asymmetric distribution of the transcription factors Myod [119] and Pax7 has also been reported [75]. In a cultured myofibre explant model, activated satellite cells express Pax7 as well as Myod and then divide to generate a cluster of cells that express both of these markers. After several rounds of division, cells that are Pax7+/Myod− were detected among double positive cells in the cluster. Moreover, rare satellite cells can distribute Myod asymmetrically after division ([119], unpublished observations), suggesting that the Myod-negative cells could be self-renewing satellite cells. A role for the basal lamina of the basement membrane has been proposed [100] and mitotic spindle orientation probably plays a determinant role in the distribution of cell fate regulators selectively to daughter cells.

In summary, three types of asymmetry are observed during satellite stem cell divisions. A detailed study of these pathways will be necessary to understand how self-renewal and lineage progression are related to these events.

4.4. The satellite cell niche

What constitutes a satellite cell niche? The niche is the microenvironment within which stem cells reside and it is thought that extrinsic signals that characterise this geographic address are crucial for maintaining the stem cell state. Stem cells niches for blood, intestine and hair follicle in mammals are all well described. The anatomy of these niches and the nature of the instructive cues from the neighbouring cells that constitute these niches have been studied in some detail in these tissues [120]. Here, we look at what's known about satellite cell biology from the point of view of a niche.

In general, cell–cell contacts and cell–matrix contacts are critical in a niche. Satellite cells in contact with the plasma lemma of live isolated myofibers proliferate less in culture when exposed to mitogens than those that are not [121] suggesting that myofibres attenuate the satellite cell response to mitogenic signals to ensure its quiescence. Interaction between myofibre and satellite cells could be mediated by calcium dependent adhesion molecule M-Cadherin and this molecule is detected in the junction between satellite cells and its host fibre [122]. However, M-Cad expression in adult satellite cells is limited to a subset [123,124] and satellite cell function appears to be unaffected in *M-Cad* null mice [125]. Nevertheless, it is worth noting that Cadherin counterparts in haematopoietic (N-Cadherin) and *Drosophila* germ line systems (DE-Cadherin) are indicated to be key components of the stem cell niche [126]. The mechanism behind the influence of the myofibre on its resident satellite cells remains unexplored.

Candidates for cell–matrix contacts are cell adhesion molecules Syndecan 3 and 4 that also double up as co-receptors

for tyrosine kinases that specifically mark satellite cells [127]. In *Syndecan 3* mutant mice that display a dystrophic phenotype, satellite cells manifest hyperplasia. Loss of *Syndecan 4* results is impaired satellite cell function and hence affects muscle regeneration [128]. CD34, a marker widely used to isolate blood stem cells is also expressed on quiescent satellite cells. It is implicated in cell adhesion and signalling [129] but its function is unknown.

Interestingly, the vasculature has been suggested to be an integral component of the satellite cell niche since over 95% of satellite cells have been reported to be subjacent to an endothelial cell [93]. A vascular component for the neural and haematopoietic niches has also been proposed [130,131].

Growth factors and cytokines released by the neighbouring cells as well as the stem cells also characterise the niche. Soluble factors in the niche that support the quiescent, undifferentiated state of satellite cells are unknown. Factors that activate satellite cells and are likely to be found in the satellite cell niche are discussed here. Hepatocyte growth factor/Scatter factor (HGF/SF) is present in muscle and is likely to be a primary cue for the activation of satellite cells out of quiescence and, along with its cognate receptor Met, has been shown to regulate proliferation and differentiation of myoblasts [132–135]. A role in satellite cell activation is attributed to fibroblast growth factor 2 (FGF2) as well [136]. Myofibres express *FGF6* and different members of the FGF family are present in the muscle tissue; quiescent satellite cells express the receptors *FGFR2* and *FGFR4* [137]. Neighbouring endothelial cells may also secrete survival/growth factors [93]. The obvious question that arises is then how do satellite cells remain quiescent in the presence of these activating factors in the milieu? This is where the basal lamina component of the niche may play a key role. HGF is sequestered in the basal lamina and is released upon injury to activate satellite cells [132]. Thus, the niche supports satellite cell quiescence as well as aids its function by providing activation cues when required.

What is the role of niche in self-renewal of satellite cells? The polarity of the division that generates asymmetrically a daughter with greater potential to self-renew clearly implicates the niche in the mechanism [75]. Interestingly, in one of the well-defined niches, the germ line stem cell niche of *Drosophila*, *dpp* (BMP) signals govern stem and daughter cell behaviour [120]. *Dpp* signalling maintains the stem cell state and one cell diameter is sufficient to create a directed polarity along which the stem cell divides. Consequently, daughter cells leave the niche and progress to contribute to the developing structure. Identification of such instructive cues from the satellite cell niche will shed light on the mechanism of self-renewal.

5. Conclusions and perspectives

In higher vertebrates stem cells fated to make skeletal muscle appear at relatively later stages during development, from somites. In aquatic organisms in which locomotory performance of larvae is key to survival, there is urgency in developing functional musculature. Accordingly, muscle identity is acquired prior to somite formation. Interestingly, more evolutionarily conserved strategies have been reported between fish and higher vertebrates with respect to the emergence of a reservoir of stem cells that assures later growth and regeneration. Expression of *Pax7* marks these stem cells across this phylogenetic spectrum. Surprisingly still, *Pax7* null mutants show no overt prenatal muscle deficit. Accordingly, in *Pax3:Pax7* double mutant mice this population of stem cells is lost prenatally pointing to a degree of functional compensation between these paralogues, *i.e.*, Pax3 playing a predominant role prenatally whilst Pax7 relays this function postnatally. Many groups in the field are now attempting to identify the transcriptional targets and partners of these Pax genes. Advances in this direction will provide more insight into the biology of the muscle stem/progenitor cells.

Efforts are also on to understand the asymmetric division of satellite cells and the consequence of it in terms of choice between self-renewal and commitment to differentiate. More explorations of the satellite cell niche are needed in this context. Compared to niches described for other stem cells, the satellite cell niche appears to be Spartan—consisting of the host fibre and its basement membrane. Endothelial cells of the microvasculature have been suggested to be a component of satellite cell niche. This finding assumes significance in light of the fact that niches of neural and blood stem cells include an endothelial component. Moreover, the niche is not simply a parking space for stem cells—it is believed to be an integral part of stem cell function. Addressing how the niche supports the maintenance of the quiescent, undifferentiated state of satellite cells, what kinds of signals and factors it provides to activate satellite cells at the hour of need and how it influences the daughters of satellite cells to differentiate while at the same time ensuring self-renewal, are the challenges that remain ahead of us in the field. Finally, the knowledge acquired on the biology of muscle stem cells will be of immense help in designing stem cell therapy strategies for muscular dystrophies, however, the fundamental information obtained, in itself, is enough of a driving force to push the limits of our knowledge in this exciting area of research.

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