Adult Skeletal Muscle Stem Cells

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Abstract Skeletal muscles in vertebrates have a phenomenal regenerative capacity. A muscle that has been crushed can regenerate fully both structurally and functionally within a month. Remarkably, efficient regeneration continues to occur following repeated injuries. Thousands of muscle precursor cells are needed to accomplish regeneration following acute injury. The differentiated muscle cells, the multinucleated contractile myofibers, are terminally withdrawn from mitosis. The source of the regenerative precursors is the skeletal muscle stem cells—the mononucleated cells closely associated with myofibers, which are known as satellite cells. Satellite cells are mitotically quiescent or slow-cycling, committed to myogenesis, but undifferentiated. Disruption of the niche after muscle damage results in their exit from quiescence and progression towards commitment. They eventually arrest proliferation, differentiate, and fuse to damaged myofibers or make de novo myofibers. Satellite cells are one of the well-studied adult tissue-specific stem cells and have served as an excellent model for investigating adult stem cells. They have also emerged as an important standard in the field of ageing and stem cells. Several recent reviews have highlighted the importance of these cells as a model to understand stem cell biology. This chapter begins with the discovery of satellite cells as skeletal muscle stem cells and their developmental origin. We discuss transcription factors and signalling cues governing stem cell function of satellite cells and heterogeneity in the satellite cell pool. Apart from satellite cells, a number of other stem cells have been shown to make muscle and are being considered as candidate stem cells for amelioration of muscle degenerative diseases. We discuss these "offbeat" muscle stem cells and their status as adult skeletal muscle stem cells vis-a-vis satellite cells. The ageing context is highlighted in the concluding section.

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1 Discovery of Satellite Cells: Skeletal Muscle Stem Cells

Skeletal muscle tissue is made of long tube-like multinucleated cells called myofibers. The myofibers harbour the specialised actin–myosin apparatus that enables the muscle to perform contractile function generating force for locomotion or maintenance of posture. During development the myofibers are formed by fusion of hundreds of nuclei. Apart from the contractile fibers, the connective tissue, vasculature, and the innervating motor and sensory nerves constitute the muscle tissue. Although the cellular turnover in skeletal muscle is low, the wear and tear caused by the mechanical nature of its function necessitates homeostatic cellular replacement. Importantly, the tissue has a robust regenerative potential. For example, skeletal muscle damaged by injury regenerates within a span of 2–3 weeks to achieve form and function equivalent to the original. The regenerative/repair potential of skeletal muscle was recognized in the nineteenth century (see Scharner and Zammit 2011). Evidence for the existence of skeletal muscle stem cells to enable regeneration came almost a century later. Independently, Alexander Mauro and Bernard Katz noticed mono-nucleated cells in close association with myofibers in frog and rat muscles using electron microscopy (Katz 1961; Mauro 1961). Mauro speculated these cells to be the stem cells of skeletal muscle that help accomplish repair and regeneration. As these cells were outside the plasma membrane of the myofiber, yet contained within the extracellular matrix enclosing the myofiber, these cells were called "satellite cells" (Fig. 1). When single myofibers were isolated and placed in cell culture, the satellite cells migrated out and generated proliferating muscle precursor cells (myoblasts), which then fused to form multinucleated myofibers in vitro (Bischoff 1975; Konigsberg et al. 1975). These and several other similar studies strongly pointed to satellite cells as skeletal muscle stem cells, and this notion had been widely accepted in the field. In a more recent study, single fibers from mouse hindlimb muscles with an average of seven satellite cells were transplanted into injured muscles. The extensive contribution of the small number of donor satellite cells to host muscle tissue as well as to new satellite cells in the regenerated muscle tissue is one of the most convincing evidences in vivo that satellite cells are stem cells (Collins et al. 2005). Transplanted single satellite cells, isolated on the basis of expression of a combination of positive and negative surface markers, also contribute significantly to differentiated muscle cells during muscle regeneration as well as generated new satellite cells establishing stem cell function (Sacco et al. 2008). A stringent measure of stem cell function is the long-term potential to generate differentiated cells as well as self-renew. This capacity has been demonstrated for haematopoietic stem cells by serial transplantations (see Perry and Li 2010). A similar experiment has been done for satellite cells (Rocheteau et al. 2012). A pool of satellite cells isolated by FACS were grafted in injured muscle and allowed to participate in the regeneration process. At the end of 3 weeks, when regeneration was complete, donor satellite cells marked by GFP were sorted again and grafted into injured muscle of another host. This cycle was repeated up to six times successfully. Given the low cellular turnover of the muscle

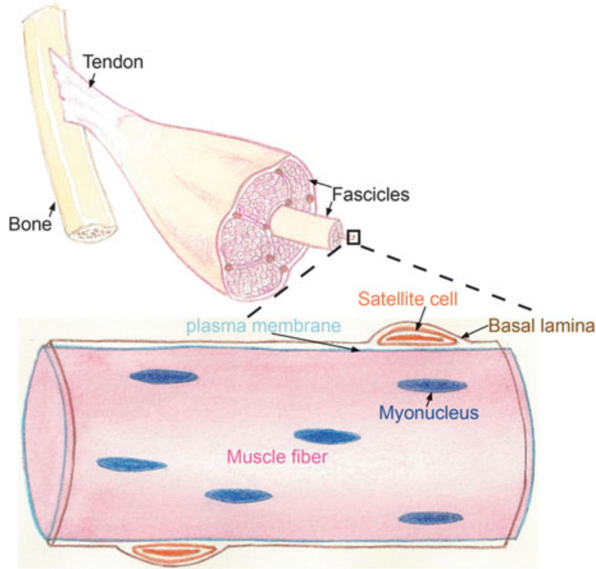

Fig. 1 Anatomical location of satellite cell. Illustration of a skeletal muscle cross-section revealing the "bundles of bundles" organization. Muscles are made of bundles of fascicles, which are in turn bundles of muscle fibers. *Bottom panel* zooms in on a portion of the muscle fibre. The nuclei of the multinucleated muscle fibre, myonuclei, are located in the periphery of the fibre within the plasma membrane. Satellite cells are sandwiched between the plasma membrane of host fibre and the basal lamina of the basement membrane enveloping the fibre

tissue and the extent of cellular loss and replenishment involved in the injury-induced regeneration experiments, this experiment demonstrates robust long-term regenerative capacity of satellite cells and attests to its "stemness". To date, many lines of evidence have been generated by studies indicating that the satellite cells are skeletal muscle stem cells. Some of these studies will be cited in this chapter in different contexts of satellite cell biology.

2 Developmental Origin of Satellite Cells

Most of the studies on satellite cell biology, traditionally, have focussed on limb muscle or trunk muscle stem cells. Using quail–chick chimaera to trace derivatives of donor quail embryonic tissues in chick hosts (Armand et al. 1983), the origin of trunk muscle satellite cells was shown to be the somites. Somites are epithelial segments of mesoderm on either side of the body axis, which harbour the developmental founder muscle stem cell population. Somitic origin of limb muscle satellite cells was also demonstrated by genetic labelling and tracing in chick as well as mice (Schienda et al. 2006; Tajbakhsh 2009; Murphy and Kardon, 2011). Pax3 and Pax7

are paired-box transcription factors that mark developmental muscle progenitors in the somites. Several independent studies, documenting the persistence of a population expressing Pax3 and later Pax7 throughout developmental and post-natal muscle formation, had established the continuity between developmental muscle founder cells and adult muscle satellite cells (Ben-Yair and Kalcheim 2005; Gros et al. 2005; Kassar-Duchossoy et al. 2005; Relaix et al. 2005). Musculature in the head derives from non-somitic cranial mesoderm with a distinct regulatory program (Tajbakhsh et al. 1997). The satellite cells of head muscles also derive from cranial mesoderm (Harel et al. 2009; Sambasivan et al. 2009). Moreover, the satellite cells continue to have a gene expression signature reminiscent of their cranial mesoderm origin. Thus, it appears that all satellite cells share their embryonic origin with their host muscles and the subset of progenitors that assure continued muscle development eventually assume the role of adult stem cells. The contribution to the satellite cell pool from an originally non-muscle progenitor pool has also been reported, but this appears to be minor (Dellavalle et al. 2011). Remarkably, satellite cells isolated from extraocular muscles, a head muscle group governing eye movements, participates effectively in limb muscle regeneration upon heterotopic transplantation (Sambasivan et al. 2009). This suggests that in spite of varied origin, satellite cells of all muscles have universal potential to effect myogenic stem cell function.

3 Regulatory Mechanisms Governing Satellite Cell Function

Unlike blood, skin, or intestine, skeletal muscle tissue is reported to exhibit low cellular turnover. The average age of intercostal muscle fibers in humans is estimated to be 15 years (Spalding et al. 2005). Hence, in adult homeostatic muscle, constant high stem cell activity is not required. As the nuclear addition to the growing muscle ceases in juveniles, satellite cells enter a mitotically quiescent G_0 state in adult muscles (White et al. 2010). When the muscle tissue needs repair or an injury warrants a regenerative response, satellite cells are awakened from quiescence. They proliferate, commensurate to the extent of repair/regenerative demand, commit further to muscle lineage becoming myoblasts, cease expansion, differentiate and either fuse with damaged muscle fibre or make new myofibers to restore the muscle structure and function (Fig. 2). Inflammation plays a key role in the regenerative response (see Kharraz et al. 2010) and connective tissue, vasculature as well as innervation ought to be restored in a coordinated fashion for functional regeneration of the tissue. Globally, at the cellular and molecular level myofiber regeneration largely recapitulates muscle lineage progression during development (Fig. 2). Here, we will focus on skeletal muscle stem cell function and highlight the role of transcription factors and signalling cues that regulate generation and maintenance of quiescent stem cell state of satellite cells and their self-renewal.

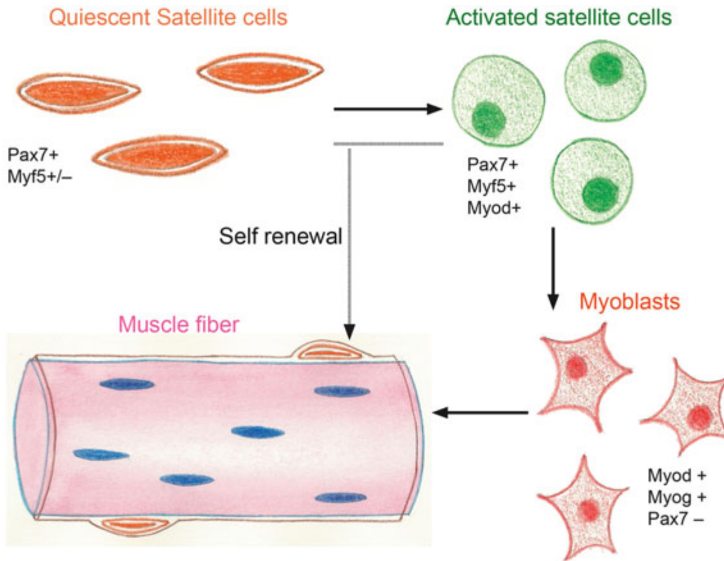

Fig. 2 Lineage progression during muscle differentiation. During muscle regeneration, mitotically quiescent satellite cells are activated. All satellite cells express Pax7, whereas the muscle regulatory factor Myf5 protein expression is seen in a proportion of satellite cells. The activated progenitors proliferate; they continue to express the satellite cell marker Pax7, but also induce *Myod* expression and are referred to as myoblasts. When Myog is induced, they commit to differentiate. Myoblasts fuse with each other to make the muscle fibre. The *gray-line arrow* points to self-renewal. The precise regulatory state of the cells that renew satellite cell pool in vivo is not known. Renewal is possibly accomplished mainly by the progenitor population

Both in vitro and in vivo models have helped understand regeneration and satellite cell function. The de novo formation of myofibers from myoblasts in vitro, and undifferentiated stem-like "reserve cells" formed in differentiating cultures (Kitzmann et al. 1998) have been used as a model system. In vivo, hindlimb muscles of mice, especially, the easy-to-access *Tibialis anterior*, are injured using a range of "traumatic" insults such as myotoxic snake venoms (notexin, cardiotoxin), glycerol, barium chloride, or physical injury by placing a cryofrozen metal rod over exposed muscle for studying satellite cell behaviour/function. Strenuous exercise on a running wheel and swimming are "physiological" injury methods, wherein several muscle groups could be studied (Gayraud-Morel et al. 2009).

Pulse-chase experiments to detect DNA synthesis by thymidine analogue incorporation found satellite cells to be quiescent and activated to proliferate upon muscle injury (Snow 1977). Quiescent satellite cells express Pax7, arguably the most reliable marker to identify muscle stem cells from mid-embryogenesis to aged mice (Tajbakhsh 2009). *Pax7* is required for generation of satellite cells as *Pax7* null muscle is almost devoid of satellite cells (Seale et al. 2000). *Pax7* null mice are considerably smaller and this could at least in part be due to failure of muscle

growth, although craniofacial abnormalities and defects in the central nervous system could also contribute to this phenotype (Mansouri et al. 1996). In muscle, accelerated differentiation as well as apoptotic loss of satellite cells in the absence of *Pax7* function has been reported (Gunther et al. 2013; Lee et al. 2012; Lepper et al. 2009; Relaix et al. 2006). In the majority of muscles, the paralogue of *Pax7*, *Pax3* is downregulated during foetal development from embryonic day E14.5 (Goulding et al. 1991; Horst et al. 2006) and only a subset of satellite cells express *Pax3* (Montarras et al. 2005). However, *Pax3* fails to rescue satellite cells in *Pax7* mutants (Relaix et al. 2006). Both of these paired-box transcription factors harbour a homeodomain. Pax7 was found to exhibit higher binding affinity to homeodomain-binding sites than Pax3 and this could explain nonredundancy (Soleimani et al. 2012). *Pax7*, however, is not necessary for the specification of juvenile satellite cells as these cells are found in perinatal *Pax7* mutant muscle (Kuang et al. 2006; Lepper et al. 2011; Oustanina et al. 2004; Relaix et al. 2006). Conditional knockout of *Pax7* revealed critical requirement for satellite cells around 2 weeks after birth (Lepper et al. 2011) and indispensability of its function for maintenance of the adult satellite cell pool (Gunther et al. 2013; von Maltzahn et al. 2013). Importantly, inactivation of *Pax7* in adult satellite cells abrogates muscle regeneration (Gunther et al. 2013; von Maltzahn et al. 2013). Pax7 has been shown to recruit histone methyl transferase complex to the regulatory sequences of *Myf5* and thus positively regulate muscle gene expression (McKinnell et al. 2008). A role for Pax7 as a pioneer transcription factor was reported recently in pituitary (Budry et al. 2012), thereby extending the regulatory repertoire of this transcription to chromatin modelling. However, its molecular function in maintenance of quiescent satellite cell pool remains unknown.

Of the four muscle regulatory basic-helix-loop-helix transcription factors (bHLH; MRFs) Myf5, Myod, Mrf4 (Myf6), and Myog (myogenin), only Myf5 is widely expressed in quiescent satellite cells. *Myf5*^{nlacZ} reporter (Beauchamp et al. 2000) as well as Myf5 protein is expressed in satellite cells (Gayraud-Morel et al. 2012). Muscle regeneration in *Myf5* null mutants is deficient, likely due to potential Myf5 requirement in expansion of myoblasts or in balancing self-renewal and differentiation (Gayraud-Morel et al. 2012; Ustanina et al. 2007). *Myf5* may function to help maintain myogenic commitment of satellite cells. However, satellite cells in *Myf5* heterozygous mice are more committed to differentiation (Gayraud-Morel et al. 2012). Thus the molecular function of *Myf5* in quiescent satellite cells remains unclear.

Once satellite cells are activated in response to repair/regenerative stimuli, *Myf5*, *Myod*, and eventually *Myog* are activated to drive differentiation. While some satellite cells induce these factors within a few hours of an activation cue, the majority express these factors a day after the injury. Though Myod protein is not detected in quiescent adult satellite cells, it appears to play a key role in balancing differentiation and stem cell generation during development. In *Myod* null mice differentiation of activated satellite cells is delayed as they continue to proliferate indicating *Myod* function in balancing differentiation and self-renewal (Cornelison et al. 2000; Megeney et al. 1996; White et al. 2000; Yablonka-Reuveni et al. 1999). In transplantation experiments, *Myod* null satellite cells survive better as apoptosis

is suppressed (Asakura et al. 2007). This raises the possibility that under physiological conditions Myod might control the number of myoblasts by regulating cell survival as well. Although *Myog* regulates differentiation of muscle cells and plays a key role in the embryo, it appears to be dispensable for differentiation in the adult (Hasty et al. 1993; Meadows et al. 2008, 2011; Moncaut et al. 2013; Nabeshima et al. 1993).

The cues from the milieu that act in concert with the intrinsic factors discussed above have also been studied extensively. A role of the myofiber in regulating quiescence and activation of satellite cells was revealed by a simple experiment. On isolated single myofibers in culture, satellite cells proliferated better when the myofiber was selectively killed using a toxin (Bischoff 1990). Since then, a role for Notch signalling has been shown in regulating the quiescent state of satellite cells. In fact, conditional removal of *Rbpj*, an effector of Notch, during homeostasis causes loss of quiescent satellite cells. While some are activated and enter the cell cycle, the majority of satellite cells directly differentiate from G_0 without undergoing S-phase (Bjornson et al. 2012; Mourikis et al. 2012b). By contrast, if *Rbpj* activity is abrogated after cell cycle entry, the majority of the myogenic cells undergo S-phase. These findings underscore the diverse contextual roles of Notch signalling during muscle homeostasis and regeneration. Overexpression of NICD, the constitutively active intracellular domain of Notch receptor, in primary myoblasts inhibits DNA synthesis and cell proliferation. In vivo, when overexpressed specifically in satellite cells, NICD increases the number of *Pax7* expressing satellite cells but regeneration is impaired (Wen et al. 2012). This study also showed that Rbpj directly regulates *Pax7* expression. Intriguingly, however, loss of Notch3 receptor results in increased number of satellite cells and mutant muscle mass increases after multiple rounds of muscle injury (Kitamoto and Hanaoka 2010). It has been reported that Notch3 antagonizes Notch1. At this juncture, it is relevant to note that Notch signalling is critical for sustaining the $Pax7^+$ muscle progenitor population during development. Loss of Delta like-1 (Dll1; a Notch ligand) accelerates muscle differentiation and premature depletion of somitic muscle progenitors (Schuster-Gossler et al. 2007), whereas NICD overexpression abrogates differentiation resulting in expansion of these progenitors (Mourikis et al. 2012a). Notch is also key for the emergence of satellite cells and occupation of sub-laminar niche (Brohl et al. 2012; Fukada et al. 2011). Though, Notch and Delta signalling has been reported to activate satellite cells upon muscle injury, such a role is unclear given that the loss of function of Rbpj does not compromise transit amplification of myogenic cells (Mourikis and Tajbakhsh 2014). Together, these evidences show that Notch signalling through distinct receptors regulates the maintenance of $Pax7^+$ muscle progenitors throughout development, the emergence of undifferentiated quiescent satellite cells postnatally, and maintenance of stem cell state in the adults.

Tie-2/angiopoietin signalling through ERK 1/2 has also been shown to promote renewal of satellite cells by favouring mitotic quiescence and blocking muscle differentiation (Abou-Khalil et al. 2009). In addition, calcitonin signalling has been shown to maintain satellite cells in quiescence. Calcitonin receptor is expressed in a quiescent-specific manner and proliferation of satellite cells is inhibited by elcatonin, an exogenous agonist (Fukada et al. 2007).

Wnt signalling has also been proposed to play a role in maintenance of quiescence. When isolated myofibers are co-cultured with cells expressing *Wnt4* and *Wnt6*, satellite cell proliferation is inhibited (Otto et al. 2008). Moreover, Wnt pathway was shown to be active in quiescent myogenic cells in vitro (Subramaniam et al. 2013). However, recent genetic studies in the mouse have modified our view on the role of Wnt (see below). Wnt through Frizzled receptors activates either the β-catenin pathway or the planar cell polarity (PCP) pathway. Both these pathways are implicated in regulating satellite cell proliferation. The Wnt PCP pathway activated by Wnt7a and Frizzled 7 (Fzd7) plays a role in self-renewing divisions of satellite cells (Le Grand et al. 2009). On single myofibers in culture, recombinant Wnt7a promotes symmetric expansion of satellite cells, whereas silencing *Fzd7* or the planar cell polarity effector Vangl2 had the opposite effect on satellite cell expansion. In mutant mice lacking *Wnt7a*, satellite cell number reduced following a round of experimentally induced regeneration (Le Grand et al. 2009). Thus, Wnt PCP pathway promotes self-renewing cell divisions of satellite cells. The mechanism proposed is that of PCP pathway orienting cytokinesis such that both daughter cells contact the basal lamina. The majority of first divisions observed ex vivo were planar and not all divisions with planar orientation are self-renewing (Kuang et al. 2007). Notably, Sprouty1, an antagonist of FGF pathway is key for activated satellite cells to revert to and maintain quiescence during self-renewal (Shea et al. 2010). Thus, multiple pathways finely control generation and maintenance of stem cell state.

A number of signals are known to activate satellite cells. Following expansion of the satellite cell pool, cessation of proliferation and onset of differentiation must occur to achieve efficient repair/regeneration. One of the early factors identified to activate satellite cells out of quiescence and promote proliferation is the hepatocyte growth factor/scatter factor (Tatsumi et al. 1998). HGF activates the MAPK/ERK signalling pathway via Met receptor tyrosine kinase (Day et al. 1999) and as revealed by studies on cultured muscle cells, suppresses myogenic differentiation to allow expansion of progenitors (Halevy and Cantley 2004; Jones et al. 2001). Proliferation of satellite cells is also supported by cytokines such as fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs). Quenching endogenous basic FGF (FGF2) by injecting specific antibody attenuated myoblast expansion. Activation/proliferation of satellite cells is compromised in *Fgf6* null mutant muscle, which displays reduced numbers of myoblasts and defective regeneration (Floss et al. 1997). Sphingosine-1-phosphate (S1P) is a signalling sphingolipid, derived from the membrane phospholipid sphingomyelin. S1P acts to induce satellite cell proliferation. Exogenous S1P acts a mitogen for quiescent muscle “reserve cells” in culture, whereas inhibition of S1P biosynthesis reduces “reserve cell” proliferation as well as impairs muscle regeneration in vivo (Nagata et al. 2006). Other factors from the niche such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and nitric oxide (NO) also regulate satellite cell activation and expansion. For a comprehensive discussion of the various signalling pathways involved in these processes, see (Yin et al. 2013).

The study that had implicated Wnt4 and 6 on satellite cell quiescence also showed that the β-cat pathway was active in proliferating satellite cells and that

Wnt1, Wnt3a, and 5a promoted expansion of satellite cells (Otto et al. 2008). Furthermore, Wnt signalling has been suggested to control the switch from proliferation to differentiation. Exogenous Wnt3A treated regenerating muscle in mice manifested accelerated differentiation and inhibition of Wnt signalling lead to impaired regeneration (Brack et al. 2008). Notably, muscle regeneration was susceptible to Wnt inhibition only at later stages when myogenic differentiation occurs. The study proposed a late requirement for Wnt, in contrast to early requirement for Notch. Those studies also suggested that activation of GSK3β by Notch and inhibition by Wnt could provide the switch from proliferative phase to differentiation. Thus, these studies suggested that Wnt signalling plays multiple roles in satellite cell biology—in quiescence, self-renewal, proliferation, and differentiation. Surprisingly, however, there is no apparent requirement of Wnt-β-catenin signaling in satellite cell function. Specific abrogation of Wnt-β-catenin signaling in satellite cells has no observable impact on quiescence / self-renewal of satellite cells or on muscle regeneration (Murphy et al. 2014). However, Wnt pathway is active in proliferating satellite cells and might play a role in their expansion. It is worth noting that different Wnts, as discussed above, have different effects on satellite cells (Otto et al. 2008). Furthermore, different levels of Wnt activation have been suggested to produce distinct cell fate output (Subramaniam et al. 2013). It is likely that the diversity in Wnt ligands and/or strength of Wnt pathway activation could underlie the pleiotropic function of Wnt signalling in satellite cell biology. The relationship with the Notch pathway, however, remains elusive given the recent reports that Notch activity is suppressed during the early stages of satellite cell activation (Mourikis et al. 2012b).

Insulin-like growth factors (IGFs) are key signalling molecules that act in the final step of lineage progression of satellite cells. They play a central role in muscle growth by promoting differentiation and hypertrophy. For reviews see (Vinciguerra et al. 2010; Zanou and Gailly 2013).

In essence, a complex regulatory network comprising transcription factors and signalling molecules tightly orchestrate satellite cell function by controlling quiescence, activation, proliferation, and finally differentiation and self-renewal. One of the approaches to ameliorate muscle-wasting diseases is by modulating the pathways regulating satellite cell function. However, more in vivo studies are required to unveil the precise roles of these pathways as in vitro studies have in some cases been misleading. A concerted effort is required to make this extensive knowledge of the regulatory network clinically applicable.

4 Heterogeneity and Asymmetric Divisions in Satellite Cell Population

All satellite cells are not equal. Differences exist in the expression of markers, extent of commitment into the muscle lineage, in the expression of traits associated with stem cells as well as stem cell potential. A number of molecular markers

identify satellite cells. Markers such as CD34, SM/C-2.6, and α7-integrin have been used for enrichment by fluorescence activated cell sorting (FACS) of satellite cells. However, these markers are not unique to satellite cells in the muscle, and hence hematopoietic and endothelial cells ought to be eliminated by using markers such as CD45, Ter111, and CD31 (Tedesco et al. 2010). Human muscle satellite cells express CD56, an isoform of neural cell adhesion molecule, and it is widely used for enrichment by FACS (Illa et al. 1992). Markers such as Met, m-Cadherin, Caveolin1, Calcitonin receptor, sphingomyelin-binding lysenin, Nestin, SM/C-2.6, Syndecan1/4, and others have been used to identify satellite cells in vivo and heterogeneity in the satellite cell population has been observed based on the expression of some of these markers (Kuang and Rudnicki 2008). For example, quantitative PCR analysis of single cells revealed the heterogeneity in satellite cells with respect to M-Cadherin expression (Cornelison and Wold 1997). How is this heterogeneity related, if at all, to stem cell function remains to be explored.

Pax7 marks all quiescent satellite cells and continues to mark its activated progeny until the onset of differentiation. Pax7-nGFP (transgenic as well as knock in) mice, allow efficient isolation of mouse satellite cells by FACS (Bosnakovski et al. 2008; Gayraud-Morel et al. 2012; Sambasivan et al. 2009). Satellite cells fractionated for high Pax7-nGFP ($Pax7^{Hi}$) manifest several stem cell features. Firstly, they express lower levels of muscle-specific genes such as Myod and Myog relative to low Pax7 ($Pax7\text{-}nGFP^{Lo}$) cells (Rocheteau et al 2012). Secondly, they appear to be dormant, i.e. take longer to exit mitotic quiescence when activated in culture as well as have low mitochondrial activity. Rare expression of Pax3 in adult satellite cells also identifies heterogeneity in the population within a muscle and across different muscle groups (Montarras et al. 2005).

Initial reports of heterogeneity were based on expression of $Myf5^{nlacZ}$. The *nlacZ* knock-in, encoding the β-galactosidase reporter, is expressed under the control of endogenous *Myf5* regulatory elements (Tajbakhsh et al. 1996). In the embryo, Myf5 controls the expression of muscle-specific genes and the cells expressing this factor are fated to differentiate into muscle. The majority of satellite cells express the *Myf5* reporter, while a minority of 5–10 % do not (Beauchamp et al. 2000). Apparently, this heterogeneity in contemporary expression from the *Myf5* locus could reflect the developmental heritage of satellite cell population. Employing the Cre-lox system of genetic tracing, using $Myf5^{Cre}$ and $R26R^{stop\text{-}YFP}$ mouse lines, the embryonic progenitors expressing *Myf5* and all their descendants were permanently genetically marked (Kuang et al. 2007). This experiment revealed 5–10 % of satellite cells that never expressed *Myf5* unlike the rest. When activated to divide, *Myf5*-negative cells could generate *Myf5* expressing and nonexpressing progeny unlike the *Myf5*-positive counterparts, placing them upstream in the muscle lineage. Remarkably, in the context of transplantation, *Myf5*-negative cells were able to generate more satellite cells, i.e. self-renew more efficiently, than *Myf5*-positive population (Kuang et al. 2007). This experiment links the heterogeneity in marker expression to that in stem cell function among satellite cells. However, a similar strategy with $Myod^{Cre}$ and $Mrf4^{Cre}$ revealed no such heterogeneity in satellite cells with respect to history of expression of these MRFs (Kanisicak et al. 2009;

Sambasivan et al. 2013). Furthermore, *Myf5*^{Cre} crossed to a Rosa reporter mouse showed considerably less *Myf5*-negative cells after several months indicating that all satellite cells experience *Myf5* expression over time (Comai et al. in press). Though these observations appear to contradict the *Myf5*^{Cre} data, it should be noted that Cre expression only indicates the activity of corresponding MRF loci and not that of functional protein expression from these loci. Therefore, it remains plausible that the regulatory state of satellite cells and their developmental precursors with respect MRF expression could potentially influence their stem cell function.

Interestingly, a strong correlation between the heterogeneity and various types of asymmetric division in the satellite cell pool has been observed. One of the ways stem cells perform the double act of self-renewal and generation of differentiating progeny is by asymmetric cell division. Thus, asymmetric division can be linked to stem cell activity. Asymmetric partitioning of potential cell fate determining factors as well as that of template DNA strands has been reported in satellite cells (see Yennek and Tajbakhsh 2013). However, the role of such asymmetric divisions in vivo in balancing self-renewal and differentiation is yet to be elucidated.

Remarkably, satellite cells on single myofibers, enzymatically isolated and placed in culture, divide either parallel to host myofiber such that both daughters in contact with the myofiber or perpendicular to the myofiber (one daughter in contact with myofiber and another away from it). Moreover, asymmetric induction of *Myf5* occurs uniquely in the perpendicular divisions, wherein daughter contacting the myofiber acquires this factor (Kuang et al. 2007). The assumption is that if such divisions occur in vivo, the Myf5^{+} daughter proximal to the fibre will differentiate and fuse with the fibre and the Myf5^{-} daughter will replenish the satellite cell pool. Numb, an endocytic adapter shown to antagonize Notch signalling in invertebrates, is apportioned unequally to daughters of satellite cells (Conboy and Rando 2002; Shinin et al. 2006). A function of Numb in favouring self-renewal or differentiation is disputed in vertebrates as Numb does not appear to inhibit Notch activity in myogenic cells either in the embryo or adult (George et al. 2013; Jory et al. 2009; Le Roux and Tajbakhsh unpublished) and therefore, the functional outcome of this asymmetry is unclear.

Template DNA strand co-segregation is an intriguing type of asymmetric cell division. DNA strands that act as template for DNA replication (i.e. parental strands) of all chromosomes are segregated to one of the daughter cells. It has been proposed that since parental strands are free of mutations arising during replication, TDSS is a mechanism to protect stem cell genome from potentially deleterious mutations. This speculation is known as immortal strand hypothesis and remains to be tested. It is likely that differential co-segregation of parental and newly synthesized strands harbouring distinct epigenetic codes might determine the asymmetric fate outcome of the daughters (Evano and Tajbakhsh 2013; Yennek and Tajbakhsh 2013). Interestingly, a higher incidence of TDSS is observed in Pax7-nGFP^{Hi} cells (higher expression level of Pax7) 5 days post-injury of muscle underscoring distinct behaviours of these cells in the proliferation state. The functional significance of the TDSS divisions in proliferating satellite cells remains an open question. During muscle homeostasis, however, Pax7-nGFP^{Hi} and

PaxnGFP^{Lo} cells exhibit similar engraftment and long-term self-renewal capacity after serial transplantation indicating that in the quiescent state, these cells have similar potentials in spite of exhibiting diverse properties when isolated from the niche.

In summary, while satellite cells appear to be unequal on several criteria, the link between the heterogeneity by various markers is not fully understood. Importantly, a direct correlation between the heterogeneity and stem cell function of satellite cells is yet to be demonstrated in vivo.

5 Unconventional Muscle Stem Cells

Attempts at stem cell therapy for muscular dystrophies have been gaining momentum. Muscular dystrophies are a diverse group of congenital muscle wasting diseases characterised by muscle degeneration and caused by a variety of mutations. Cell therapy to regenerate the affected musculature with genetically corrected myogenic stem or progenitor cells is a much-attempted strategy. Satellite cells when isolated activate the myogenic programme and expand as myoblasts. Several attempts at transplantation failed due to poor survival of myoblasts in the host. This spurred the search for alternative stem cells with high myogenic capacity that could be genetically modified and expanded in culture and systemically delivered. A variety of cell types have been discovered to have the capacity to differentiate into muscle (Fig. 3). They range from non-satellite cells derived from muscle to neural stem cells of ectodermal origin. Studies on cell fate plasticity have also added to this list (see Tedesco et al. 2010).

When cells without muscle fate commitment fuse with the myofiber, they will come under the influence of the MRFs and could change fate to induce muscle-specific genes. Thus, fate conversion in a heterokaryon is one mechanism by which non-muscle cells could participate in myogenesis (Blau et al. 1983). Another route for myogenically uncommitted cells is to first acquire muscle progenitor identity and follow the muscle lineage progression to make differentiated muscle cells. Either way, unorthodox stem cells (non-satellite cells) apparently uncommitted to muscle fate have been shown to differentiate as muscle during regeneration. Remarkably, some of these cell types also exhibit a potential to occupy the satellite cell compartment. The capacity to make satellite cells indicates commitment occurring at progenitor level rather than accidental or controlled fusion as the mechanism. This section will describe the various non-satellite cell types reported to have myogenic capacity in the context of adult muscle regeneration (Tedesco et al. 2010) and discuss their significance vis-à-vis satellite cells in muscle stem cell function.

Bone marrow derived stem cells were one of the first non-satellite cell types shown to make muscle. When marrow-derived cells from a donor encoding muscle-specific β-gal transgene was transplanted into recipient's regenerating muscle, β-gal$^+$ donor nuclei were detected in regenerated muscle fibre. Later a subset of

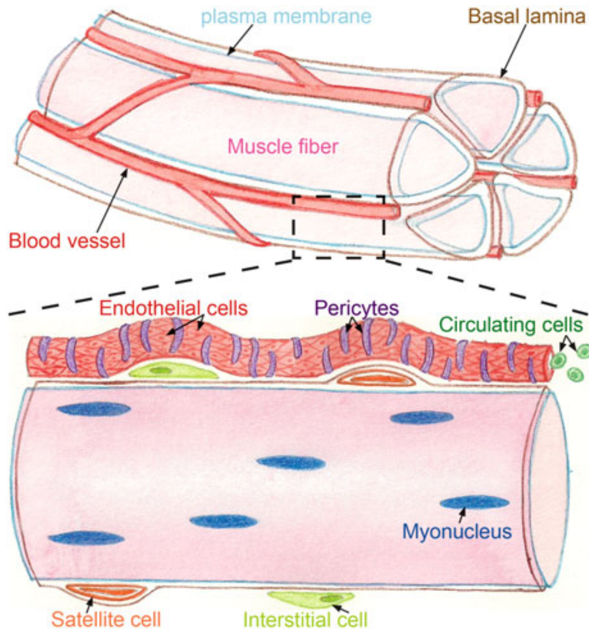

Fig. 3 The "off-beat" stem cells with muscle differentiation potential. *Top panel* shows a bundle of few fibers with associated blood vessels. In the *bottom panel*, a close-up illustration of a portion of this bundle reveals some of the non-satellite cell muscle stem cells, in relation to the muscle fibre and satellite cells. Unlike satellite cells, the interstitial cells are excluded by the basement membrane ensheathment of the muscle fibre. In particular, *Pw1* expressing interstitial cells make muscle when grafted on to regenerating muscle. Pericytes have also been shown to have myogenic capacity. Circulating stem cells, such as those with hematopoietic stem cell signature also rarely fuse into muscle when transplanted

bone marrow-derived/muscle-derived cells was shown to contribute to myofibers following transplantation. Owing to the presence of specific export channels (ABC), this subset excludes live DNA stain Hoechst and is separable as a "side population" on FACS (Ferrari et al. 1998; Gussoni et al. 1999). Muscle-derived stem cells with hematopoietic marker signature (CD45+ fraction) has similar myogenic potential. Bipotential stem cells have also been reported. Single HSC from c-Kit^{+}, Sca1^{+}, and blood lineage marker negative (Lin^{-}) subset of side population from bone marrow of CD45^{-}GFP^{+} mice reconstituted the blood of irradiated host and at the same time contributed spontaneously (without stimulated by injury) to *Panniculus carnosus* skin muscle. Such spontaneous contribution was found at a low frequency, which increased upon damage to muscle (Corbel et al. 2003). An independent study with a similar cell experimental approach showed HSC generate myeloid derivatives, which then fuse with myofibers to contribute to muscle (Camargo et al. 2003). While fusion into muscle fibre induces some muscle gene expression in grafted bone marrow-derived donor cells, they appear not to fully reprogram, as they fail to express sarcoglycan (Lapidos

et al. 2004). Though these studies have provided evidence for HSC plasticity, experiments using HSC-specific Cre recombinase driver mouse lines in combination with Cre-inducible reporter lines to trace HSC contribution to muscle during development or regeneration are needed to reveal the significance of such contribution. Nevertheless, with current evidence, the frequency of such events appears low and cannot be generalized to all muscle groups.

Mdx mice model to a certain extent human Duchenne muscular dystrophy (DMD) caused by loss of function mutations in Dystrophin, a component of membrane glycoprotein complex. Amelioration of muscle wasting could be achieved by delivering functional dystrophin into dystrophic muscle fibers. To date, the cell type found most suitable for cell therapy and has been taken to clinical trials to treat DMD, is the mesoangioblast (Sampaolesi et al. 2006; Sampaolesi et al. 2003). Blood vessel wall-associated pericytes are the source of mesangioblasts (Dellavalle et al. 2007). In fact, a Cre-loxP mouse genetic tracing study using pericyte specific TNAP-Cre (tissue non-specific alkaline phosphatase) driver has assessed the developmental plasticity of pericyte lineage towards skeletal muscle. TNAP^{+} cells of pericyte lineage contribute to perinatal muscle growth by differentiating into muscle at low frequency and also contributing to Pax7^{+} satellite cells (Dellavalle et al. 2011).

Other notable cell types shown to contribute to regenerating muscle upon transplantation are cells expressing a combination of endothelial (CD144 or vascular endothelial cadherin) and CD56 from human muscle (Zheng et al. 2007) as well as PW1^{+} cells in the interstitial space between myofibers outside the basement membrane from mice (Fig. 3). PW1^{+} cells have also been suggested to replenish the satellite cell pool (Mitchell et al. 2010). Whereas the potential of unorthodox muscle stem cells is promising and could be harnessed to treat muscular dystrophies, the discovery of the myogenic potential and especially their ability to replenish the satellite cell pool have raised a number of questions. What is the relative contribution of the various stem cells during normal muscle regeneration? What is the main mode of renewal of satellite cells? Are there muscle stem cells upstream of satellite cells in the lineage hierarchy? Four independent studies have addressed these questions by specifically ablating satellite cells. Diphtheria toxin is a bacterial protein, which selectively kills cells engineered to express active diphtheria toxin peptide (DTA) or cells expressing the human diphtheria toxin receptor (DTR). In mice carrying *Pax7-CreErt2* as well as ROSA-stop *DTA* alleles, *DTA* expression is activated in satellite cells when tamoxifen is injected (Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011). Intramuscular injection of DTA in *Pax7*^{DTR} mice uniquely targets Pax7^{+} satellite cells (Sambasivan et al. 2011). Both these strategies have been used to eliminate the majority of, or all satellite cells in adult musculature of mice. When satellite cell depleted muscle is challenged with muscle injury or strenuous exercise, it collapses owing to a dramatic failure of regeneration and restitution of myofiber regeneration occurs by transplantation of heterologous satellite cells (Sambasivan et al. 2011). These studies proved the indispensability of satellite cells to muscle regeneration and firmly established satellite cells as the major endogenous muscle stem cells.

They also indicate a possible paracrine role for satellite cells in recruiting the unorthodox stem cells to muscle lineage. Notably, freshly isolated satellite cells, not cultured in vitro, graft much more efficiently than cultured progeny of satellite cells (Ikemoto et al. 2007; Montarras et al. 2005). Recently, satellite cells cultured in Forskolin, an adenylyl cyclase activator, have been reported to retain their engraftment potential (Xu et al. 2013). This brings satellite cells back in the race as potential therapeutic cell type.

Whereas the muscle fibre is the central functional component of the tissue, skeletal muscle regeneration will be incomplete without regeneration of connective tissue. In disease states muscle function is diminished by pathological fibrosis and fat infiltration. The potential cellular source for this regeneration and pathology, fibro-adipogenic progenitors (FAPs), was recently identified using different markers; PDGFRα (Uezumi et al. 2010; Uezumi et al. 2011), $CD45^{-}$, $CD31^{-}$, $Sca1^{+}$, $CD34^{+}$ (Joe et al. 2010) and Tcf4 (Murphy et al. 2011). Apparently, the FAPs identified by these various means represent the same population of progenitors. Importantly, these progenitors also expand during regenerative response and support muscle repair through paracrine influence on satellite cells (Joe et al. 2010) and are necessary for robust muscle regeneration (Murphy et al. 2011). Thus, in addition to satellite cells and unconventional stem cells with capacity/plasticity to make muscle, muscle tissue is also host to non-myogenic interstitial progenitor cell population that impacts on satellite cell behaviour.

In short, apart from satellite cells, a number of stem cell types influence or have the potential to influence muscle regeneration. In the current scenario of clinical trials for Duchenne muscular dystrophy using non-satellite stem cells and the demonstration of indispensability of satellite cells in muscle regeneration, the significance of understanding the role of satellite cells vis-à-vis other stem cells is accentuated and needs to be explored.

6 Ageing and Regenerative Capacity of Satellite Cells

Ageing is characterized by decline in homeostatic maintenance of tissue structure and function as well as blunted regenerative response. Affected stem cell function is partially responsible for this decline. Sarcopenia, age-related muscle loss, is a major health concern. The impact of ageing on satellite cell function has been studied extensively. The effect on satellite cell function is due to changes in the milieu [reviewed in Hikida (2011)], local and systemic, as well as cell intrinsic changes. The result is poor activation/proliferation and also defective differentiation.

Although, depletion of satellite cells with age has been reported, there is controversy in the field on age-related reduction of satellite cell number (Garcia-Prat et al. 2013; Tajbakhsh 2013). This is likely due to differences in the experimental approaches such as markers used for enumeration as well as age of the animals used for the study. Poor self-renewal capacity owing to changes in cues from niche affects maintenance of satellite cell number. Increased FGF2 in aged

muscle downregulates Sprouty 1, a self-renewal promoting factor (Shea et al. 2010), and thus compromises satellite cell quiescence leading to a decline in the number of satellite cells (Chakkalakal et al. 2012).

Increased levels of TGFβ in aged muscle causes reduced regenerative potential of satellite cells. The effector of TGFβ, phospho-Smad3, induces cell cycle inhibitors, an action countered by Notch signalling, to limit satellite cell proliferation (Carlson et al. 2008). Notch signalling is also directly affected in aged muscle. The failure to induce Delta1 in ageing muscle fibers could be one of the reasons for reduced activation of satellite cells and therefore, poor regenerative response of aged muscle (Conboy et al. 2003). Similarly, systemic increase in Wnt has been implicated in defective differentiation of satellite cells, i.e. myogenic to fibrogenic switch (Brack et al. 2007). The role of "old", local, as well as systemic, milieu in blunting satellite cell function has been demonstrated by heterochronic transplants and heterochronic parabiosis experiments. Transplantation into young muscle bed supports efficient regeneration of old donor-derived satellite cell (Garcia-Prat et al. 2013). In parabionts, wherein the circulation of two mice an old and another young are joined, the regenerative potential of the old satellite cells is dramatically improved (Conboy et al. 2005). Altered inflammatory response, local and systemic, upon ageing could also impact satellite cells (Shavlakadze et al. 2010). These key experiments showed that the intrinsic changes in the satellite cells notwithstanding, amelioration of cues in the local and systemic environment could rejuvenate satellite cell response to regenerative demand. These findings have raised therapeutic hopes for patients suffering from sarcopenia.

Concluding Remarks

Since their discovery in 1960s, satellite cells have been studied extensively. Propelled by their possible therapeutic potential for treatment of muscle degenerative diseases such as dystrophies and sarcopenia, satellite cell properties have been investigated in detail. Consequently, they have emerged as an excellent model to understand adult tissue-specific stem cell biology. The hunt for cells better suited for stem cell therapy of muscle wasting diseases has revealed the plasticity of several non-satellite cells to make skeletal muscle. However, the recent findings of the indispensability of satellite cells for muscle regeneration and of inability of other stem cells to replenish the experimentally depleted satellite cell pool, firmly establishes satellite cells as the major adult stem cell compartment of the skeletal muscle tissue. The failure of satellite cell-derived myogenic cells to transplant due to limited migratory capacity in the muscle bed had deterred their application in regenerative medicine. However, the successful transplantation of freshly isolated, non-cultured satellite cells has underlined the need to further understand the mechanisms controlling satellite cell "stemness" and activation. These studies would help to turn the therapeutic dream into a reality.

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