Tissue-Specific Stem Cells:
Lessons from the Skeletal Muscle Satellite Cell

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In 1961, the satellite cell was first identified when electron microscopic examination of skeletal muscle demonstrated a cell wedged between the plasma membrane of the muscle fiber and the basement membrane. In recent years it has been conclusively demonstrated that the satellite cell is the primary cellular source for muscle regeneration and is equipped with the potential to self renew, thus functioning as a bona fide skeletal muscle stem cell (MuSC). As we move past the 50th anniversary of the satellite cell, we take this opportunity to discuss the current state of the art and dissect the unknowns in the MuSC field.

Introduction
The observation that skeletal muscle has the capacity to regenerate after injury was well documented by microscopic examination in the mid-19th century, primarily in German literature (Scharner and Zammit, 2011). Still, the cellular basis of this regenerative potential remained elusive for a century until 50 years ago when Alexander Mauro detected a mononucleated cell, which he termed a “satellite cell,” closely apposed to mature myofibers in electron micrographs of skeletal muscle (Mauro, 1961). Without any functional evidence, he hypothesized that this could represent a kind of muscle progenitor cell akin to those present in the developing embryo, capable of forming new muscle in response to injury. This turned out to be a very accurate prediction: five decades of research on satellite cells have demonstrated that they have the characteristics that Mauro surmised. In modern parlance, the satellite cell is considered a muscle stem cell distinguished from the plethora of adult tissue-specific stem cells that have been described by the fact that it was identified anatomically before it was characterized functionally; most adult stem cells have been first demonstrated to exist in functional assays, which are then followed by a hunt for the cells histologically.

The history of the satellite cell has been the subject of several recent reviews (Scharner and Zammit, 2011; Yablonka-Reuveni, 2011), and the regulation and contribution of satellite cell progenitors during lineage progression, differentiation, and contribution to muscle repair has also been extensively documented (Chargé and Rudnicki, 2004; Wang and Rudnicki, 2012; Zammit et al., 2006). In this review, we focus on the current status of satellite cell research through the lens of stem cell biology. We highlight recent studies illustrating that satellite cells are essential for maintenance of the stem cell pool and repair of the differentiated muscle tissue in which they reside. In addition, we discuss the properties that satellite cells possess in common with other stem cell populations and the mechanisms that regulate satellite cell functions.

Satellite Cell Identification and Stem Cell Properties
After anatomical identification of satellite cells in 1961, their behavior in response to growth and regeneration was investigated. It was noted that in regenerating muscle, undifferentiated cells increase in abundance and align with the periphery of damaged fibers. As regeneration progresses, immature myogenic progenitors are replaced with more mature myoblasts (Allbrook, 1962). At later stages of repair, undifferentiated cells begin to appear in association with the regenerated fibers (Shafiq and Gorycki, 1965). A series of studies using tritiated thymidine confirmed that satellite cells were mitotically dormant in mature muscle and the source for regenerating muscle (Reznik, 1969; Schultz et al., 1978; Snow, 1977) and that daughters of satellite cells contributed to both the satellite cell compartment and differentiated nuclei in growing muscle (Lipton and Schultz, 1979; Moss and Leblond, 1970, 1971; Schultz, 1996). Thus with the evidence that satellite cells were capable of asymmetric divisions and endowed with self-renewal properties, a new era was born, in which satellite cells were considered bona fide MuSCs.

Cell transplantation was becoming more commonplace in regenerative biology to test cellular contribution to tissue repair and renewal of progenitor populations. The grafting of committed satellite cell progeny (myoblasts) between mice with different isoenzyme subtypes confirmed that donor cells could fuse with host cells or myofibers (Partridge et al., 1978; Watt et al., 1982), providing evidence of a renewable cell source with regenerative capacity. However, identification and isolation of the self-renewing cells, MuSCs, remained elusive for many years.

Eventually, immunotypic analysis identified the paired box 7 transcription factor Pax7 as a uniform marker of satellite cells (Seale et al., 2000). In response to injury, Pax7+ satellite cells enter cycle and differentiate and a subset returns back to quiescence to replenish the dormant satellite cell pool (Abou-Khalil and Brack, 2010). A body of work during the late 1980s and early...
of satellite cells has demonstrated that adult Pax7+ cells self-renew and therefore function as MuSCs (Rocheteau et al., 2012). In their quiescent state, adult satellite cells express, along with Pax7, the Myf5 transcript but lack Myod. During activation and lineage progression, satellite cells turn on Myod and lineage potential of satellite cells. Based on studies using inducible genetic strategies to ablate adult Pax7+ cells have demonstrated that satellite cells are essential for muscle repair and replenishment of the MuSC pool (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). Cre/lox technology has also enabled the assessment of lineage potential of satellite cells. Based on studies with Pax7CreERTM and MyodCre, it appears that most if not all satellite cells are restricted to the myogenic lineage, arguing in favor of unipotency of adult satellite cells (Lepper et al., 2009; Shea et al., 2010; Starkey et al., 2011). However, it appears that at least a subset of satellite cells are driven out of lineage, becoming fibrogenic in pathological conditions or during aging (Amini-Nik et al., 2011; Brack et al., 2007).

Although the contribution of satellite cells to muscle repair is indisputable, their role in other aspects of skeletal muscle biology is less certain (Figure 1). During normal daily activity, muscle fiber size and myonuclear content remain relatively constant throughout most of adult life (Hughes and Schiaffino, 1999), and satellite cells may exhibit a modest decline depending upon the specific muscle and species examined (Brack and Rudnicki, 2004). Demonstration of a tractable muscle-resident cell that contributed to muscle fiber repair and repopulation of the niche was achieved through transplantation of a FACS-isolated subset of muscle-resident cells selected via cell surface receptor expression (such as CD31+, CD45+, Sca1+, CXCR4+, CD31+, Integrin β1+) (Montarras et al., 2005; Sherwood et al., 2004).

Engrafting a single myofiber with its satellite cells marked by a nuclear Myf5lacZ reporter provided the first definitive demonstration that satellite cells possess stem cell activity (Collins et al., 2005). In recent years, satellite cells have been proven to self-renew at the single-cell level (Sacco et al., 2008) and to retain stem cell function over seven rounds of serial transplantation and therefore function as MuSCs (Rocheteau et al., 2012). With the advent of conditional Cre/lox systems, lineage tracing of satellite cells has demonstrated that adult Pax7+ cells self-renew and differentiate in their endogenous environment in response to injury (Lepper et al., 2009; Shea et al., 2010), suggesting that adult Pax7+ satellite cells are sufficient for both MuSC pool maintenance and myofiber repair. Moreover, studies using inducible genetic strategies to ablate adult Pax7+ cells have demonstrated that satellite cells are essential for muscle repair and replenishment of the MuSC pool (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). Cre/lox technology has also enabled the assessment of lineage potential of satellite cells. Based on studies using Pax7CreERTM and MyodCre, it appears that most if not all satellite cells are restricted to the myogenic lineage, arguing in favor of unipotency of adult satellite cells (Lepper et al., 2009; Shea et al., 2010; Starkey et al., 2011). However, it appears that at least a subset of satellite cells are driven out of lineage, becoming fibrogenic in pathological conditions or during aging (Amini-Nik et al., 2011; Brack et al., 2007).

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cells from a muscle, gross histological appearance is normal and fiber size changes negligibly (Cheung et al., 2012). Together, the data suggest that satellite cell contribution to myofiber maintenance is minimal during homeostasis. It would be informative to determine whether ablation of the adult pool would exacerbate muscle atrophy in contexts of higher cellular demand such as exercise or aging.

Adult skeletal muscle is capable of hypertrophy, and the question as to satellite cell participation in that process has been debated for many years. Recent studies have provided direct evidence of hypertrophy without any satellite cell contribution. Using a genetic strategy to ablate the adult Pax7+ cell pool, mice deficient in satellite cells were able to rapidly increase muscle fiber size during a 2 week hypertrophic stimulus (McCarthy et al., 2011). This occurred in the absence of satellite cell-derived myonuclear accretion, suggesting that satellite cells are dispensable for increase in muscle mass. However, it is possible that over longer periods of time, additional differentiated nuclei, and hence a maintained myonuclear domain size in hypertrophic muscle, would be required to maintain a larger muscle fiber (Hughes and Schiaffino, 1999). This is supported by the fact that loss of differentiated nuclei in aged muscle fibers precedes muscle fiber atrophy (Brack et al., 2005).

**Stem Cell Characteristics**

**Developmental Origins**

Based on anatomical position and expression of Pax7, satellite cell precursors first appear during late fetal stage (Relaix et al., 2004, 2005). Lineage tracing approaches demonstrated that the majority of adult Pax7+ satellite cells are formed from a somitic origin of cells that transition through a Pax7+/Myod+ state (Kanisicak et al., 2009; Lepper and Fan, 2010; Schienda et al., 2006). It is clear that the number of Pax7+ cells declines during development and postnatal growth (Schultz, 1989; White et al., 2010). Therefore, not all embryonic Pax7+ cells will form the self-renewing adult pool, but instead some will primarily contribute to the growing myofiber. This suggests that some fetal Pax7+/Myod+ cells differentiate and other Pax7+/Myod+ cells form the adult satellite cell pool. Myod is considered a master regulator of myogenesis (Weintraub et al., 1991). Based on germline mutants, Myod is required for satellite cell differentiation but not for the formation of the satellite cell pool (Chargé et al., 2008; Rudnicki et al., 1992). In vitro experiments demonstrate that subsets of myogenic progenitors lose Myod and return to a quiescent state (Halevy et al., 2004; Olguin and Olwin, 2004; Yoshida et al., 1998; Zammit et al., 2004). Moreover, self-renewing adult satellite cells transiently express Myod prior to niche repopulation during repair (Shea et al., 2010). Therefore, Pax7+/Myod+ precursors are able to form the satellite cell pool providing that Myod is repressed. Based on studies with a Myf5CreYFP reporter, it was demonstrated that the majority (90%) of the adult satellite cell pool is formed from a Myf5+ precursor (Kuang et al., 2007). However, the formation of the satellite cell pool in germine Myf5 nulls suggests that Myf5 is not required for satellite cell formation; compensation by Myod cannot be excluded (Gensch et al., 2008; Haldar et al., 2008). While there are multiple caveats to consider when interpreting these studies, including the contribution of non-Cre-recombined cell populations, these results suggest that the adult satellite cell pool is formed from precursors that are under different transcriptional control. Contribution from the extrinsic environment may also participate in differential regulation of satellite cell precursors.

While the adult satellite cell pool is formed from Pax7+ embryonic precursors, this does not preclude a model whereby an alternative source of cells express Pax7+ and form the satellite cell niche. Using immunotypic markers (CD34+/Sca1+) in combination with donor cell marking for transplantation studies, it has been demonstrated that a subset of PICs (PW1+ Pax7+ interstitial cells) self-renew and contribute to myofibers and Pax7+ satellite cells in injured adult muscle (Mitchell et al., 2010). Moreover, myogenic contribution of PICs was Pax7 dependent and temporally restricted to postnatal growing muscle. Based on lineage tracking, a subset of alkaline phosphatase (AP)-expressing pericytes indelibly marked in postnatal mice contributed to the formation of the adult Pax7+ satellite cell compartment (Dellavalle et al., 2011). Therefore the origin of the adult satellite cell pool may be heterogeneous (Figure 2). In the future, it will be important to dissect the unique properties and lineage relationship between pericytes and PICs that express Pax7 relative to Pax7-derived satellite cells.

**Asymmetric Cell Divisions**

While asymmetric cell divisions are one of the fundamental characteristics of stem cells underlying the process of self-renewal, stem cells may undergo self-renewal by virtue of symmetric divisions (Tajbakhsh et al., 2009). As long as only one daughter of a symmetric cell division is instructed to differentiate, the stem cell pool can be maintained while differentiated progeny are produced. Historically, there were no seminal observations that provided evidence to either support or refute asymmetric cell divisions in the satellite cell lineage, and it was only in the past decade that this has been the focus of studies in the MuSC field.

The first evidence of asymmetric cell divisions came from studies of the role of Notch signaling during satellite cell activation and proliferative amplification. It was found that the Notch inhibitory protein Numb was asymmetrically segregated in progenitors undergoing cell division and that Numb was localized...
to one pole of dividing cells, perpendicular to the plane of cell division (Conboy and Rando, 2002). This asymmetric Numb localization is reminiscent of what had been previously described in specific lineages during Drosophila development (Petersen et al., 2002; Zhong et al., 1996). The result of such an asymmetric segregation in the satellite cell lineage is the inheritance of Numb by one daughter and not the other, the former being destined to differentiate and the latter remaining an undifferentiated progenitor (Conboy and Rando, 2002). The asymmetric segregation of Numb was subsequently investigated during satellite cell activation, and asymmetric segregation of Numb was also detected in cells prior to the onset of differentiation (Shinin et al., 2009). Other proteins, such as Myod (Zammit et al., 2004) and Dek (Cheung et al., 2012), are asymmetrically segregated in activated satellite cells, and those proteins are more highly expressed in cells that have progressed further along the myogenic lineage.

Asymmetric cell divisions have also been suggested in relation to the aforementioned distinction of satellite cell subsets based on developmental history of Myf5 expression (Kuang et al., 2007). In this model, satellite cells undergo asymmetric cell divisions, giving rise to two daughters, one being another stem cell (still with no history of Myf5 gene expression) and the other becoming part of the larger pool of satellite cells (and expressing the Myf5 transcript). In this case, there is no direct evidence as yet of an asymmetric segregation of any specific protein or transcript in the mother cell, and the asymmetry may instead be a divergent cell fate dictated by the different environments of the two daughters. This is particularly likely given the observation that daughters exhibiting divergent fates, one self-renewing and the other committing toward differentiation, were much more frequent when the plane of division was perpendicular to the axis of the myofiber (Kuang et al., 2007). By contrast, divisions that occurred parallel to the myofiber axis were much more likely to give rise to two daughters with identical characteristics as opposed to divergent fates, suggesting that the local environment of the satellite cell niche might be dictating the fate of satellite cell progeny.

Yet another asymmetry that has been described is the asymmetric segregation of sister chromatids as satellite cells divide and the population expands (Conboy et al., 2007; Shinin et al., 2006). Recent evidence suggests that a subset of satellite cells, in fact those with characteristics of long-term stem cells, are more likely to exhibit nonrandom sister chromatid segregation (Rocheteau et al., 2012). This further supports the notion that asymmetric segregation of sister chromatids might be a property of stem cells as originally proposed (Figure 3; Cairns, 1975). Although the functional significance of this remains theoretical, the proposed function is to segregate DNA strands that have been damaged during replication to the more differentiated daughter and reserve for the self-renewing stem cells those strands that served as templates during the previous round of cell division, thus acquiring fewer replication-induced errors (Charville and Rando, 2011). Although this teleological function has not been demonstrated and there may be detrimental consequences of template strand segregation during aging (Charville and Rando, 2011), it is supported by the fact that the oldest template strands segregate with the less differentiated satellite cell daughter (Conboy et al., 2007; Shinin et al., 2006).

**Satellite Cell Heterogeneity**

It is becoming more apparent that adult stem cell populations are heterogeneous. Heterogeneity in the adult satellite cell pool has been demonstrated based on multiple criteria, such as expression profile (Beauchamp et al., 2000; Rocheteau et al., 2012), proliferation kinetics in vitro (Day et al., 2009), self-renewal potential (Kuang et al., 2007), and molecular regulation (Kitamoto and Hanaoka, 2010; Kuang et al., 2007; Shea et al., 2010). Functional heterogeneity within a pool of adult stem cells can arise from differentially specified subsets that retain distinct properties during cellular division or evolution of a subset of cells derived from a single homogeneous cell population that will emerge during cellular division.

It is widely accepted that Pax7 expression declines in differentiating progenitors (Zammit et al., 2004). Evidence for varied Pax7 levels within the satellite cell pool was recently demonstrated via a transgenic GFP reporter of Pax7 transcript (Rocheteau et al., 2012). Functional analysis of satellite cells at extreme ends of the Pax7 expression distribution demonstrated that Pax7lo cells had reduced metabolic activity and slower cell cycle entry kinetics, whereas Pax7hi cells were primed to differentiate in vitro. Although cells with lower Pax7 expression appeared to be more prone to differentiation, all Pax7+ cells could support more than six serial transplantsations, demonstrating that both subsets of satellite cells possess remarkable regenerative capacity and the ability to self-renew. Using an inducible Cre/lox system, Pax7 was deleted from satellite cells in growing postnatal muscle and adult muscle prior to injury. The results

**Figure 3. Nonrandom Segregation of Chromosomes and Asymmetric Fate Determination**

The satellite cell pool is composed of primitive (Pax7hi, green) and lineage primed (Pax7lo, gray) subsets. Pax7lo cells are in a dormant (less metabolically active) state. Pax7hi satellite cells are lineage primed. After cytokinesis, satellite cell daughters can each inherit some chromosomes bearing newer (purple) or older (dark green) template strands. Pax7th subsets undergo nonrandom segregation of chromosomes, whereby one daughter contains exclusively chromosomes bearing older template strands, while its sister contains only chromosomes bearing newer template strands. In contrast, Pax7th cells undergo random segregation of chromosomes ensuring that each daughter cell inherits an older and newer template strand. Subsets of Pax7hi and Pax7lo are capable of multiple rounds of self-renewal (curved arrows) and differentiation (blue cells), but Pax7th cells are endowed with enhanced self-renewal capability. Daughter cells containing newer template strands express high levels of Numb, Myod, and Dek1 and are biased to differentiate (blue cell). Pax7th cells can give rise to Pax7lo, but not vice versa, suggesting a hierarchical relationship in the satellite cell pool based on Pax7 expression. In addition to cell fate determination, nonrandom segregation of chromosomes may act to protect genome integrity during cell proliferation. Because of low metabolic output of primitive satellite cells, they may be protected from oxidative damage. Lineage-primed satellite cells are capable of self-renewal but may be acutely sensitive to proliferative demands and other forms of genotoxic insults.
demonstrated that Pax7 is essential for satellite cell function during early postnatal growth and is dispensable for adult muscle tissue repair (Lepper et al., 2009). Given that a subset of postnatal Pax7+ cells seeds the adult Pax7+ pool, it supports the hypothesis that there is a small subset of postnatal Pax7+ cells that evolve during the selective pressure of muscle growth to propagate satellite cells that function independently of Pax7. It raises the possibility that functionally distinct satellite cell subsets can interconvert for selective advantage under conditions of high cellular demand as observed in spermatogonial stem cells (Nakagawa et al., 2007, 2010).

Adult stem cells with limited proliferative history are endowed with greater stem cell capability than more frequently dividing counterparts (Foudi et al., 2009; Wilson et al., 2008), suggesting that stem cell self-renewal capacity may decline in proportion with the number of divisions a stem cell has undergone in its history. Interestingly, subsets of presumptive satellite cells in growing postnatal muscle divide with slower proliferation kinetics, based on tritiated thymidine uptake (Schultz, 1996). These data suggest that one level of functional heterogeneity in the satellite cell pool may exist based on their proliferative history. In dystrophic muscle, disease progression correlates with proliferative capacity of satellite cells, suggesting that restricting proliferative output of stem cells relative to their downstream progeny may extend self-renewal capacity and ameliorate disease pathogenesis (Sacco et al., 2010). Whether functional heterogeneity across a pool of satellite cells is stable once specified or adaptive based on cellular demand, it is clear that the satellite cell pool is functionally heterogeneous. The challenge in the future is to determine how molecular heterogeneity at the cell-intrinsic and -extrinsic levels instructs functional diversity within the satellite cell pool.

**Regulation of Satellite Cell Function**

**Stem Cell Niche**

The stem cell niche refers to the microenvironment that maintains “stemness” (Schofield, 1978). The attributes of the niche as originally conceptualized were (1) a defined anatomical site, (2) a location where stem cells could reproduce, (3) a place where differentiation is inhibited, and (4) a space that also limits the numbers of stem cells. Therefore, the niche is a protector of stem cell number and function, restraining proliferation and differentiation of stem cells, and maintaining a quiescent phenotype.

The location of the satellite cell, i.e., residing in a depression in the plasmalemma and beneath the basal lamina of the muscle fiber, provided a defined anatomical site of the putative MuSC (Mauro, 1961). The hypothesis that the muscle fiber is a satellite cell niche is supported by evidence generated from single muscle fiber experiments. Removal of the myofiber plasmalemma drives quiescent satellite cells into cycle, suggesting a role in inhibition of mitogen-induced cell cycle entry (Bischoff, 1986a). Quiescence is a conserved property of stem cells mediated by the niche (Orford and Scadden, 2008). Studies on freshly isolated single muscle fibers have shown that a subset of quiescent satellite cells can proliferate and return back to quiescence when in contact with the single muscle fiber, whereas a larger subset commits to differentiation but their fusion is inhibited (Bischoff, 1986a; Olguin and Olwin, 2004; Zammit et al., 2004). Therefore the muscle fiber fulfills three of Schofield’s criteria of a stem cell niche. Whether the muscle fiber constrains stem cell numbers is to be determined.

In response to injury, the muscle fiber degenerates, which probably leads to niche destruction and a loss of inhibitory signals. Besides losing inhibitory factors that restrict proliferation and differentiation from the degenerated niche, muscle injury also promotes the release of stimulatory factors present at the basal lamina of the muscle fiber that drive proliferation and differentiation (Bischoff, 1986b; Sheehan and Allen, 1999). The observation that microenvironment stiffness is a regulator of stem cell potential and fate decisions suggests that maintenance of stemness from the niche is regulated at many levels (Engler et al., 2006; Gilbert et al., 2010). Resolving the signals that drive self-renewal versus differentiation will require a careful characterization of the basal and apical aspects of the niche (Figure 4A).

The satellite cell niche may be composed of different cell types, as observed for other stem cell compartments (Morrison and Spradling, 2008). Besides the muscle fiber, there are other muscle-resident cells in close proximity to the satellite cell, such as Ang1-expressing endothelial cells (Abou-Khalil et al., 2009; Christov et al., 2007). It is becoming clear that other cell types, such as fibro-adipogenic progenitors (FAPs) and TCF4+ fibroblasts, influence satellite cell behavior in contexts of regeneration and growth (Joe et al., 2010; Murphy et al., 2011). It remains to be determined whether such cell types constitute the niche and therefore regulate stemness or function as paracrine agents involved in proliferation and differentiation of satellite cell progeny (Figure 4A). As cell-specific Cre drivers become more readily available, it will be possible and essential to determine the cell types and mode of regulation that contribute to a functional satellite cell niche.

**Signaling Pathway Regulation of Satellite Cell Function**

A balance between extrinsic cues and intracellular signals converge to preserve stem cell function. Over the past 50 years, many excellent studies have demonstrated that multiple extrinsic signaling pathways, such as IGF, FGF, Wnt, Notch, BMP, and TGF-β, function in the activation of satellite cells, their downstream progeny, and their lineage progression (Kuang et al., 2008). More recently, the signaling pathways that modulate functions specific to stem cells, such as maintenance of quiescence during homeostasis, reversible quiescence and self-renewal after proliferation, asymmetric fate decisions, and symmetric expansion of stem cells, are becoming more defined (Figure 4B).

It was recently shown that active Notch signaling is required to maintain satellite cells in the quiescent state, implying a role of niche-derived Notch ligand that binds to a Notch receptor on the quiescent satellite cell (Bjornson et al., 2011; Mourikis et al., 2011). In these studies, Rbp-j, the downstream transcriptional coactivator in the Notch pathways, was deleted from adult satellite cells in uninjured muscle. This led to activation and ectopic differentiation of satellite cells, possibly in the absence of cell cycle entry and therefore bypassing the transient amplifying (TA) progenitor stage (Bjornson et al., 2011; Mourikis et al., 2011). Therefore, not only is RBP-J important for restricting cell cycle entry, it also argues that in nonquiescent satellite cells, RBP-J, possibly as a mediator of Notch1 signaling, may be required for normal step-wise lineage progression (Conboy
The satellite cell pool is lost when the TA stage is bypassed, implying implications for understanding the relationship between self-renewal and differentiation potential of downstream progenitors within the stem cell hierarchy. To replenish the satellite cell pool after injury, stem cells return back to quiescence after proliferating. Our understanding of reversion back to quiescence in vivo is limited. Based on in vitro experiments, it is becoming apparent that multiple signaling pathways are actively involved in promoting satellite cell quiescence. Activation of the Ang1/Tie2 signaling complex promotes the return to quiescence of myoblasts in vitro (Abou-Khalil et al., 2009). P38/MAPK pathway has also been implicated in regulating quiescence of satellite cells (Jones et al., 2005). Finally, Myostatin, a secreted growth factor belonging to the TGF-β family, was shown to negatively regulate quiescence and the return to quiescence of satellite cells in vitro (McCroskery et al., 2003; McFarlane et al., 2008). Together these data illustrate a critical role of growth factor signaling in the return of cycling satellite cell progenitors back to a quiescent state. Further in vivo analysis and genetic approaches that directly target satellite cells will clarify the signaling cascades that regulate the maintenance of quiescence and return to quiescence after proliferation.

Recently, signaling through Notch-3 was found to negatively regulate satellite cell pool size in regenerating muscle (Kitamoto and Hanaoka, 2010). Whether this is regulated at the cell-autonomous level of the satellite cell or by a niche-based mechanism will require cell-specific genetic mutants. Using a satellite cell-specific mutant for Spry1, it was demonstrated that Spry1, an inhibitor of growth factor signaling, is required to restore satellite cell pool size after injury (Shea et al., 2010). This suggests that a balance between inhibitory and stimulatory cues from the microenvironment is required for quiescence to be achieved. One can theorize that Spry1 and other intracellular growth factor inhibitors help to dampen the stimulatory factors at the basal lamina to direct fate toward quiescence and the repression of differentiation. The challenge in the future is to identify niche-derived inhibitory signals that promote and retain stemness after injury.

Stem cells possess a unique ability to divide symmetrically or asymmetrically depending on tissue requirements (Tajbakhsh et al., 2009). Noncanonical Wnt signaling through the Wnt7A-Frz7-Vangl2 cascade was recently implicated in driving symmetric expansion of satellite cells located on single muscle fibers in vitro (Le Grand et al., 2009). It will be interesting to unravel the relationship between asymmetric divisions via Numb and symmetric divisions via Wnt7A-Vangl2 that occur to control stem cell expansion and differentiation.

In general, the cellular output within a pool of cells in response to manipulation of signaling cascades is not equivalent. Through loss-of-function approaches, it has been demonstrated that satellite cells differentially respond to manipulation of signaling molecules such as Spry1 and Notch-3 (Kitamoto and Hanaoka, 2010; Shea et al., 2010). It will be important to decipher whether heterogeneous cell signaling requirements are due to extrinsic differences, such as localization of satellite cells in discrete niches, or to cell-intrinsic differences, based on lineage relationships.

While knowledge of the signaling cascades that regulate stem cell properties of satellite cells is progressing, it is likely that more
pathways participate than have been identified. During cell division and lineage progression, signaling molecules are used reiteratively. For example, Numb is partitioned to initially allow asymmetric fate of neural progenitors and subsequently to maintain progenitors (Petersen et al., 2004). Likewise, Numb may be important for satellite cell self-renewal and also, at a later stage of myogenic lineage progression, for regulating the divergent fates of proliferating progenitors (Conboy and Rando, 2002; Jory et al., 2009; Shinin et al., 2006). Therefore, while canonical Wnt, FGF, and BMP signaling cascades participate in myogenic lineage progression and differentiation (Kuang et al., 2008), it may be that they are also deployed in some fashion to direct MuSC properties.

**Epigenetic Regulation of Satellite Cell Function**

Based on what we know about extrinsic cues controlling stem cell function, the role of the stem cell niche cannot be understated. However, more stable modes of regulation may also maintain key functions of satellite cells. Control of the function of stem cells, particularly embryonic stem cells (ESCs), has focused on regulatory mechanisms at the level of epigenetics, namely the ensemble of heritable changes in gene function that occur without modifications of the primary DNA sequence (Bird, 2007). Such changes include DNA methylation and chromatin structural modifications mediated by histone modifications and nucleosome positioning. Not too long after the identification of the satellite cell, electron micrographs demonstrated that there are changes in chromatin organization in satellite cells from adult muscle and growing muscle consistent with their transition from a quiescent to a proliferative state (Church, 1969). As our knowledge in the field of epigenetics becomes more sophisticated, it is becoming appreciated that the regulators of epigenetic states in stem cells include mediators of DNA methylation and demethylation, microRNAs and other noncoding RNAs, and modifiers of histones, including acetylases, deacetylases, methylases, and demethylases. This field is redefining cellular states at a molecular level that are much more varied and complex and probably reflect the dynamic interaction of stem cells with their environment resulting in greater functional heterogeneity than was previously envisioned.

It is likely that epigenetic states will better define such key satellite cell features as prolonged quiescence and lineage fidelity. It is also likely that DNA and histone modifications will underlie many of the changes in aged satellite cells that account for age-related declines in functionality and rejuvenation through exposure to the systemic environment (Brack et al., 2007; Conboy et al., 2005). Although there has been extensive research on the epigenetic control of myogenicity, primarily using the C2C12 myoblast cell line (Juan et al., 2011), studies in satellite cells have been limited because of the need for large numbers of cells for such analysis. Advances in both satellite cell purification and methodologies for genome- and epigenome-wide analyses of limited cell numbers will allow for rapid advances in these areas in the coming years.

There have, however, been studies of regulators of epigenetic states in satellite cells. It was shown that Pax7 functions with the Wdr5-Ash2L-MLL2 histone methyltransferase (HMT) complex to direct methylation of histone H3 lysine 4 (H3K4). Binding of the Pax7-HMT complex resulted in H3K4 trimethylation of chromatin at the Myf5 locus (McKinnell et al., 2008), suggesting that Pax7 may act through chromatin modifications to stably and hereditarily maintain the myogenic program. Conversely, the regulation of Pax7 expression may be determined by the regulation of the repressive Polycomb repressive complex 2 (PRC2) and its enzymatic component, EZH2 (Palacios et al., 2010), suggesting a network providing stability of the myogenic program related to Pax7 expression.

The regulation of myogenesis by microRNAs has likewise focused mainly on developmental myogenesis and the regulation of myogenic differentiation (Braun and Gautel, 2011). Recent studies have also examined the role of miRNAs in the regulation of quiescent satellite cells. miR-206 was recently shown to target Pax3 in quiescent satellite cells, accounting for the differential expression in Pax3 in cellular subsets (Boutet et al., 2012). This study also revealed that alternate polyadenylation of the Pax3 transcript is an important determinant not only of satellite cell heterogeneity but also of the susceptibility of the Pax3 transcript to regulation by any miRNA (Boutet et al., 2012). Another microRNA, miR-489, was recently shown to be highly expressed in quiescent satellite cells and to regulate specifically the quiescent state by targeting the transcript of the Dek gene, a gene known to be important for cell cycle regulation and alternative splicing (Cheung et al., 2012). Knockdown of miR-489 in vivo led to spontaneous activation of quiescent satellite cells, thus demonstrating the importance of a stable miRNA network for maintaining the quiescent state.

**Conclusions and Perspectives**

The last fifty years have led to a remarkable understanding of the satellite cell. It is widely accepted that the satellite cell is essential for regenerative myogenesis and can maintain itself after injury. It has been demonstrated that the muscle fiber constitutes a major functional component of the satellite cell niche. More recently, experiments have illustrated the functional heterogeneity within the satellite cell pool. As we move forward, what will the next fifty years bring?

Remarkably, we do not know whether satellite cells maintain themselves or differentiate to maintain muscle tissue during normal daily wear and tear. This information is critical as biologists devise strategies to harness skeletal muscle strength of the ever-expanding human aged population.

To date, direct experimental evidence is lacking to determine whether stem cell number is limiting. If MuSCs have a finite capacity, then a quorum will be required to functionally repair muscle tissue and maintain homeostasis. In the context of regenerating a tissue, such as skeletal muscle, it may be too simplistic to consider MuSC number as an independent entity. Rather, there is probably a cooperative relationship between MuSC number and function in response to a regenerative insult. For example, in the presence of few MuSCs, a greater functional demand will be imposed on those rare cells, leading to their exhaustion. In contrast, increasing the number of MuSCs in a muscle will lessen the burden on each cell imposed by the regenerative insult. In scenarios of limiting satellite cell number, other MuSCs may participate, contributing as facultative stem cells. Other cell types such as PIsCs, mesangioblasts, and pericytes are able to contribute to muscle tissue repair (Péault et al., 2007). Further analysis is
needed to determine whether such cell types function as facultative stem cells that are dependent on cellular context. Understanding whether their contribution is dependent on the presence of a functional pool of satellite cells may reveal further complexities of the regulation of skeletal muscle repair. Development of genetic tools to ablate fractions of satellite cells will illustrate the intimate cooperative relationship between satellite cell number and function and their interdependency with other contributing cell types.

Technological advances in recent years have provided greater resolution of stem cell heterogeneity. With the advent of fluorescent reporters of proliferative history, it is now possible to isolate and compare such heterogeneous cells to dissect their function and molecular regulation (Foudi et al., 2009; Tumbar et al., 2004). Marking satellite cells based on Cre drivers has proven invaluable for tracking them and their contribution to growth and repair (Lepper and Fan, 2010; Lepper et al., 2011; Shea et al., 2010). More recent advances through the use of multicolor Cre reporters or genetic barcoding utilized in other stem cell compartments have provided improved cellular resolution that facilitates the tracking of clonally diverse cells in a population to be studied (Lu et al., 2011; Snippert et al., 2010). Such techniques reveal that the level of stem cell heterogeneity is more dynamic and context dependent than previously appreciated (Lu et al., 2011). Information gleaned from such approaches will allow the relationship between molecular and functional heterogeneity to be resolved.

The anatomical location of the satellite cell provides a landmark for the satellite cell niche. Based on its conceptual framework, although the niche impacts stem cell properties of the satellite cell, the mechanism by which this is achieved remains a mystery. In the future, functional components of the satellite cell niche will be identified through technological advances in live imaging, microdissection, and biochemical analysis. As our understanding of satellite cell heterogeneity evolves, it will be interesting to identify whether there are distinct niches that, in some way, establish satellite cell heterogeneity. Advances in our knowledge of cell-intrinsic mechanisms that regulate stem cell function, such as epigenetic regulation of satellite cells, will illustrate the communication between the extrinsic environment and intrinsic effectors to specify and maintain stem cell states.

In conclusion, the seminal observations of the satellite cell in its niche made by Alexander Mauro almost fifty years ago have spawned many great advances in our understanding of this tissue-specific stem cell. It is apparent that there is greater heterogeneity in the satellite cell pool in terms of cellular subsets that specify the pool, their function, and the signaling cascades that regulate them. We are just beginning to unravel the microenvironmental influences that mediate stem cell properties and how epigenetic regulation governs stable properties of satellite cells during homeostasis and repair.

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REFERENCES


Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells

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Skeletal muscle damaged by injury or by degenerative diseases such as muscular dystrophy is able to regenerate new muscle fibers. Regeneration mainly depends upon satellite cells, myogenic progenitors localized between the basal lamina and the muscle fiber membrane. However, other cell types outside the basal lamina, such as pericytes, also have myogenic potential. Here, we discuss the main properties of satellite cells and other myogenic progenitors as well as recent efforts to obtain myogenic cells from pluripotent stem cells for patient-tailored cell therapy. Clinical trials utilizing these cells to treat muscular dystrophies, heart failure, and stress urinary incontinence are also briefly outlined.

Introduction

It has been known for more than a century that skeletal muscle, the most abundant tissue of the body, has the ability to regenerate new muscle fibers after it has been damaged by injury or as a consequence of diseases such as muscular dystrophy (1). Muscle fibers are syncytial cells that contain several hundred nuclei within a continuous cytoplasm. Therefore, whether the process of regeneration depends upon the fusion of mononucleated precursor cells or upon the fragmentation of dying muscle fibers, which release new cells, remained controversial for a long time, even after the demonstration by Beatrice Mintz and Wilber Baker (2) that multinucleated fibers are formed by the fusion of single cells. In 1961, Alexander Mauro (3) observed mononuclear cells between the basal lamina that surrounds each muscle fiber and the plasma membrane of the muscle fiber and named them satellite cells (SCs) (Figure 1). SCs were later accepted to be, and are still considered for a group of inherited disorders characterized by progressive muscle wasting and weakness leading to a variable degree of mobility limitation, including confinement to a wheelchair and, in the most severe forms, heart and/or respiratory failure (5). Many muscular dystrophies arise from loss-of-function mutations in genes encoding cytoskeletal and membrane proteins, the most common and severe being Duchenne muscular dystrophy (DMD), which is caused by mutations in the gene encoding dystrophin, an integral part of a complex that links the intracellular cytoskeleton with the extracellular matrix in muscle. Muscular dystrophies are some of the most difficult diseases to treat, as skeletal muscle is composed of large multinucleated fibers whose nuclei cannot divide. Consequently, cell therapy has to restore proper gene expression in hundreds of millions of postmitotic nuclei (6).

In this Review, we discuss recent work indicating the possible existence of a stem/progenitor cell compartment in adult muscle (see also ref. 7) as well as studies related to the derivation of myogenic cells from embryonic and induced pluripotent stem cells (PSCs) for the development of new cell therapy strategies for diseases of skeletal muscle. An overview of clinical trials based upon transplantation of skeletal muscle stem cells is also provided. Neither the role of SCs in aging skeletal muscle nor the SC niche are discussed here due to space constraints, and readers are directed to excellent recent reviews on these topics by Suchitra Gopinath and Thomas Rando (8) and Michael Rudnicki and colleagues (9), respectively.

SCs

Identification and characterization. The most stringent way to classify cells as SCs remains by determining their anatomical location: SCs are found underneath the basal lamina of muscle fibers, closely juxtaposed to the plasma membrane (3). SCs originate from somites (10, 11), spheres of paraxial mesoderm that generate skeletal muscle, dermis, and axial skeleton, but the exact progenitor that gives rise to SCs remains to be identified. SCs are present in healthy adult mammalian muscle as quiescent cells and represent 2.5%–6% of all nuclei of a given muscle fiber. However, when activated by muscle injury, they can generate large numbers of new myofibers within just a few days (12). Quiescent SCs (13) express characteristic (although not unique) markers. In the mouse, the most widely used of these markers is the transcription factor paired box 7 (Pax7) (14), which is essential for SC specification and survival (15). In contrast, Pax3 is expressed only in quiescent SCs in a few specific muscle groups such as the diaphragm (16). The basic helix-loop-helix (bHLH) gene myogenic regulatory factor 5 (Myf5) is expressed in the large majority of quiescent SCs, and for this reason, mice expressing nuclear LacZ under the control of the Myf5 promoter (Myf5LacZ/+) mice have...
been useful for identifying and characterizing SCs (17). Many other markers (18–29) have been identified and are listed in Table 1. Some of these surface markers are useful for isolating “purified” SC populations by cell sorting, but since each marker is not exclusively expressed on SCs, a combination of different markers must be used. Alternatively, transgenic mice such as those expressing GFP under the control of promoters that drive the expression of genes encoding SC markers— for example, the Pax3 promoter—can be used to isolate SCs (29–31). In humans, markers of both quiescent and activated SCs do not fully correspond to those in the mouse, and relatively little is known about them due to the difficulty of obtaining human tissue. For example, although CD34 is a marker of SCs in mice, it does not mark SCs in human muscle (32); and M-cadherin is not as consistent a marker of SCs in humans as it is in mice. Among the more reliable markers of SCs in human muscle is CD56, although it also marks natural killer lymphocytes (33).

Activation. In response to a muscle injury, SCs are activated and start to proliferate; at this stage, they are often referred to as either myogenic precursor cells (mpc) or myoblasts (34, 35). Several signals, deriving both from damaged fibers and infiltrating cells, are involved in SC activation, including HGF (36), FGF (37), IGF (38), and NO (39).

The progression of activated SCs toward myogenic differentiation is mainly controlled by Myf5 and myogenic differentiation 1 (MyoD) (17) and is followed by fusion into regenerating fibers. The whole process takes approximately 7 days in the mouse (40), during which time SCs undergo different fates, giving rise to a few Pax7+MyoD− cells, which return to quiescence (to maintain the progenitor pool), and many Pax7+MyoD+ cells, which are committed to differentiation (41) (Figure 1). Notch signaling is thought to regulate this process through promotion of asymmetric divisions, although there is not agreement on the role of Numb (a Notch inhibitor and cell-fate determinant) in inducing differentiation (42) and sustaining self renewal (43). The occurrence of asymmetric cell division is also supported by the identification of a subpopulation of SCs able to retain BrdU after pulse-chase labeling, with some cells displaying selective template DNA strand segregation during mitosis (43, 44). In addition, Rudnicki and colleagues validated the label-retention model of SCs and demonstrated that approximately 10% of Pax7+ mouse SCs had never expressed Myf5 and that these cells remain adherent to the basal lamina during asymmetric mitosis, generating one Pax7−Myf5− satellite “stem cell” and one Pax7+Myf5+ SC “progenitor,” eventually destined to differentiate (25) (Figure 1). The same group also elegantly described Wnt7a as regulating the symmetric expansion of Pax7−Myf5− SCs (45).

Transplantation. Because of their features, SCs were considered obvious candidates for a cell-therapy approach to treating muscular dystrophy. Pioneer studies demonstrated that intramuscular injection of normal myoblasts (46) into mdx mice, which lack dystrophin and are a model for DMD, resulted in fusion with host fibers and extensive dystrophin production. These studies led to several clinical trials in the early 1980s (see Muscular dystrophies section for details) that failed for a number of reasons, including poor survival and migration of donor cells after transplantation and rejection of the donor cells due to an immune response by the patients (47).

Many subsequent preclinical studies aimed to improve the survival, proliferation, and differentiation of the SCs after engraftment. For example, transplantation in dystrophic mouse mus-
cles of a single muscle fiber that contained as few as seven SCs led to an increasing number of new SCs that in turn generated more than 100 new muscle fibers and could also be activated after injury (48). This is a much more efficient way to generate dystrophin-expressing fibers in mdx mice than the same number of donor-derived new fibers that are generated is several orders of magnitude less than the number of injected cells. Unfortunately, this method would be difficult to translate into clinical protocols.

In the past few years, several groups have succeeded in prospectively isolating “pure” populations of SCs by using a combination of different markers (Table 1), such as Pax3-GFP (30), CXCR4 and β1 integrin (49), αv integrin and CD34 (50), or syndecan-3 and -4 (51). It is still unknown whether the different protocols allow isolation of the same cell population, enriched to different extents for a more primitive “stem-like” fraction. However, all these studies revealed that freshly isolated cells have a much greater capacity to generate dystrophin-expressing fibers in mdx mice than the same cells after in vitro expansion (30); the simplest explanation for this is that the “stem” fraction either dies in culture or generates differentiation-committed SCs. Importantly, a short culture period of 3–4 days, without subculture, allowed lentiviral-mediated transduction and thus genetic correction of SCs freshly isolated from mdx mice, without compromising myogenic potency in vivo (52). Despite these encouraging results, previous unsolved problems still prevent the use of SCs to systemically treat patients with muscular dystrophy: in particular, the inability of these cells to cross the endothelial wall makes systemic delivery impossible and prevents possible healing of the diaphragm and cardiac muscles, both critical for patient survival (34).

**Other myogenic progenitors**

The availability of cell-autonomous, tissue-specific transgenic markers allowed the unequivocal demonstration of the existence of myogenic progenitors originating from tissues other than skeletal muscle (53). Upon transplantation (either BM transplantation [BMT] or direct injection into skeletal muscle), these cells, identified by transgene expression, participate in muscle regeneration in wild-type and/or dystrophic mice (Figures 1 and 2; Table 2) and eventually enter the SC pool. The possibility that myogenic differentiation may depend upon fusion (and hence exposure to the dominant activity of MyoD) remains, but for skeletal muscle, this would be part of the physiological mechanism that creates the tissue. Below, we describe briefly some examples of these unorthodox myogenic cells.

### Table 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>SC expression</th>
<th>Localization</th>
<th>Function</th>
<th>Prospective isolation</th>
<th>Expression in other tissues/cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax7</td>
<td>100% of quiescent and activated SCs</td>
<td>Nucleus</td>
<td>Transcription factor</td>
<td>Pax7-GFP</td>
<td>Absent</td>
<td>14</td>
</tr>
<tr>
<td>Pax3</td>
<td>Quiescent SCs (only in a subset of muscles)</td>
<td>Nucleus</td>
<td>Transcription factor</td>
<td>Pax3-GFP</td>
<td>Melanocyte stem cells</td>
<td>16</td>
</tr>
<tr>
<td>Myf5</td>
<td>Most quiescent SCs and all proliferating SCs and myoblasts</td>
<td>Nucleus</td>
<td>Transcription factor</td>
<td>Myf5-nLacZ</td>
<td>Absent</td>
<td>17</td>
</tr>
<tr>
<td>Syndecan-3 and -4</td>
<td>98% of quiescent and activated SCs</td>
<td>Membrane</td>
<td>Transmembrane heparan sulfate proteoglycan</td>
<td>Cell sorting</td>
<td>Brain, dermis, BM, bone, smooth muscle, tumors</td>
<td>18</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Quiescent and activated SCs</td>
<td>Membrane</td>
<td>Adhesion molecule</td>
<td>Cell sorting</td>
<td>Activated endothelial cells</td>
<td>19</td>
</tr>
<tr>
<td>c-met</td>
<td>Quiescent and activated SCs</td>
<td>Membrane</td>
<td>HGF receptor</td>
<td>Not used</td>
<td>Many tissues and tumors</td>
<td>20</td>
</tr>
<tr>
<td>Foxk1</td>
<td>Quiescent and activated SCs</td>
<td>Nucleus</td>
<td>Nuclear factor</td>
<td>Not used</td>
<td>Neurons</td>
<td>21</td>
</tr>
<tr>
<td>Cd54</td>
<td>Quiescent and activated SCs</td>
<td>Membrane</td>
<td>Membrane protein</td>
<td>Cell sorting</td>
<td>Hematopoietic, endothelial, mast, and dendritic cells</td>
<td>13</td>
</tr>
<tr>
<td>M-cadherin</td>
<td>Quiescent and activated SCs; myoblasts</td>
<td>Membrane</td>
<td>Adhesion protein</td>
<td>Not used</td>
<td>Endothelial fibrous and adipose tissue</td>
<td>22</td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>Quiescent and activated SCs; myoblasts</td>
<td>Membrane</td>
<td>Membrane protein</td>
<td>Not used</td>
<td>Vessel-associated cells</td>
<td>23</td>
</tr>
<tr>
<td>αβ Integrin</td>
<td>Quiescent and activated SCs; myoblasts</td>
<td>Membrane</td>
<td>Adhesion protein</td>
<td>Cell sorting</td>
<td>Many tissues</td>
<td>24</td>
</tr>
<tr>
<td>β1 Integrin</td>
<td>Quiescent and activated SCs; myoblasts</td>
<td>Membrane</td>
<td>Adhesion protein</td>
<td>Cell sorting</td>
<td>Glia, neurons, and natural killer cells</td>
<td>25</td>
</tr>
<tr>
<td>Cd56</td>
<td>Quiescent and activated SCs; myoblasts</td>
<td>Membrane</td>
<td>Homophilic binding glycoprotein</td>
<td>Cell sorting</td>
<td>Unknown</td>
<td>26</td>
</tr>
<tr>
<td>SM/C2.6</td>
<td>Quiescent and activated SCs; myoblasts</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Cell sorting</td>
<td>Unknown</td>
<td>27</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>Subset of quiescent SCs</td>
<td>Membrane</td>
<td>SDF1 receptor</td>
<td>Cell sorting</td>
<td>HSCs, vascular endothelial cells, and neuronal cells</td>
<td>28</td>
</tr>
<tr>
<td>Nestin</td>
<td>Around 98% of quiescent SCs and myoblasts</td>
<td>Intermediate filament</td>
<td>Intermediate filament protein</td>
<td>Nestin-GFP</td>
<td>Neuronal precursor cells</td>
<td>29</td>
</tr>
</tbody>
</table>

*Prospective isolation: direct isolation of cells from tissue, usually based upon cytofluorimetric sorting with antibodies directed against cell surface markers.

*Novel monoclonal antibody directed against an unknown antigen present on SCs. Foxk1, forkhead box k1.*
Cells from ectoderm: neural stem cells. To date, neural stem cells (both murine and human) are the only ectoderm-derived stem cells that have been shown to differentiate into skeletal muscle when cocultured with skeletal myoblasts or transplanted into regenerating skeletal muscle (54). Interestingly, cells expressing Myf5 exist in the brain and spinal cord, suggesting a cryptic potency that becomes apparent in vitro (55).

Hematopoietic cells. The first evidence of in vivo generation of skeletal muscle from BM cells was reported in 1998 (56) in a study that used transgenic mice expressing a nuclear LacZ under the control of the striated muscle promoter myosin light chain 1/3 fast (MLC3f). After transplantation of BM from the transgenic mice and subsequent injury to the host muscle, unequivocal β-gal–positive nuclei were detected in regenerated fibers, demonstrating that murine BM contains transplantable progenitors that can be recruited to an injured muscle through the circulation, where they participate in muscle repair (56). This opened the possibility of treating muscular dystrophy by BMT, but work in mice indicated that, unfortunately, the frequency of this event was too low, even in a chronically regenerating dystrophic muscle and even if the side population (SP) progenitor-enriched fraction was transplanted (57, 58). To address this issue, subsequent experiments were directed to identifying a rare, potentially highly myogenic progenitor, but those studies have so far had modest success. The hematopoietic, CD45+ fraction of the BM has been identified as the cell population with myogenic potential (59), and retrospective analysis in a DMD patient that had undergone BMT confirmed the persistence of donor-derived skeletal muscle cells over a period of many years, again at very low frequency (60). Together, these data suggested that HSCs or a yet-to-be-identified cell that expresses several markers in common with true HSCs has myogenic potential. More recent approaches confirmed that hematopoietic cells have myogenic potential but disagreed on the stage at which myogenic differentiation would occur. One study reported that the progeny of a single mouse hematopoietic progenitor cell can both reconstitute the hematopoietic system and contribute, at low frequency, to muscle regeneration (61). However, a similar study showed that in response to injury, CD45+ hematopoietic progenitors contribute to regenerating mouse skeletal muscle through fusion of mature myeloid cells rather than fusion of the HSCs (62).

A subpopulation of circulating cells expressing CD133 (also known as Ac133), a well-characterized marker of HSCs, also expresses early myogenic markers (63). When injected into the circulation of dystrophic scid/mdx mice, CD133+ cells have been found to contribute to muscle repair, recovery of force, and replenishment of the SC pool. The group that discovered this also isolated a population of muscle-derived stem cells (MDSCs) expressing CD133 (64). Furthermore, when CD133+ cells from DMD patients were genetically corrected by lentivirus-mediated exon skipping for dystrophin exon 51, these cells were able to mediate morphological and functional recovery in scid/mdx mice (64). Thus, different subpopulations of hematopoietic cells, whose characterization is still incomplete, seem to possess myogenic potency, but none of these exhibit this property at high frequency.

Cells derived from mesoderm (other than hematopoietic cells). Many different types of mesoderm stem/progenitor cells have been shown to exhibit myogenic potential, usually after drug treatment, genetic modification, or coculture with SCs or myoblasts. In some cases, evidence of in vivo myogenesis has been documented. The list of such cells includes mesenchymal stem cells (MSCs), multipotent adult progenitor cells (MAPCs), MDSCs, CD133+ cells, mesangioblasts (MABs), endothelial progenitor cells (EPCs), and adipose-derived stem cells, all of which are briefly described below or in Table 2. More details can be found in previous reviews (6, 32).
MSCs have been shown to be capable of skeletal myogenesis (65). However, recently, Perlingeiro and colleagues demonstrated that although Pax3 activation enabled the in vitro differentiation of murine and human MSCs into MyoD+ myogenic cells, these cells failed to cause functional muscle recovery in mdx mice, despite good engraftment (66). The reason for this failure remains unclear.

MABs are vessel-associated progenitors (67) that express early endothelial markers when isolated from the embryo and pericyte markers when isolated from postnatal tissues. Since MABs are able to cross the vessel wall and are easily transduced with lentiviral vectors, they have been used in preclinical models of cell therapy for muscular dystrophy. Intraarterial delivery of either wild-type or genetically corrected MABs morphologically and functionally ameliorated the dystrophic phenotype of mice lacking α-sarcoglycan (Sgca), which model limb-girdle muscular dystrophy 2D, a muscular dystrophy caused by mutations in the SGCA gene (68). In addition, intraarterial delivery of wild-type postnatal canine MABs resulted in extensive recovery of dystrophic expression and ameliorated pathologic muscle morphology and function in golden retriever dogs that model DMD (69). Similar cells have been isolated from human postnatal skeletal muscle and shown to represent differentiated from adipose tissue, differentiate into adipocytes, osteoblasts, and myoblasts (74). Recently, the myogenic and muscle repair capacities of hMADS cells have been enhanced by transient expression of MyoD (75). The easy availability of their tissue source, their strong capacity for expansion ex vivo, their multipotent differentiation, and their immune-privileged behavior suggest that hMADS cells could be an important tool for cell-mediated therapy for skeletal muscle disorders.

### PSCs for muscle regeneration

PSCs can give rise to all cell types. Among the various PSCs, we limit our discussion to ES cells (76, 77) and induced pluripotent stem (iPS) cells (78), as they are, in practice, the two types of PSC most commonly used to direct differentiation toward a given cell type, skeletal muscle, for the purpose of this Review. PSCs hold tremendous hopes for the cell therapy of degenerative diseases; and iPS cells further offer the possibility of deriving patient-specific PSCs (79) to study diseases in vitro (80) and the potential for genetic correction for autologous cell therapy.

Turning PSCs into skeletal muscle. A critical step in establishing the potential of PSCs as a therapeutic for skeletal muscle diseases is the development of techniques for the differentiation of these cells into tissue-specific progenitors suitable for transplantation. The most elegant way to obtain specific transplantable cell types is by exposing them in vitro to the same molecules that control their in vivo commitment during embryogenesis (reviewed in ref. 81), although the empirical testing of molecules and substrates could generate equally useful cells.

Seminal studies from the mid-1990s described how ES cell-derived embryoid bodies (EBs), tridimensional structures formed when ES cells are grown in the absence of an embryonic fibroblast feeder layer, contained multinucleated muscle fibers that express skeletal muscle myosin heavy-chain genes (82, 83). Ten years later, it was documented for the first time in vivo that intramuscular injection of mouse EBs cocultured with mdx muscle-derived progenitors in mdx mice led to the production of a few clusters of donor-derived dystrophin-positive fibers (84). Recently, Studer and coworkers have derived transplantable myoblasts from human ES cells (85), while Chang and colleagues have generated transplantable satellite-like cells from mouse ES cells (86). Upon transplantation into mdx mice, the latter cells have been found to regenerate acutely and chronically injured muscle and could also be secondarily transplanted (86). MyoD-mediated myogenic conversion of ES cell-derived cells is another intriguing approach,
whose proof of principle dates to the early 1990s (87). On this front, Perlingeiro and colleagues recently achieved in vivo skeletal muscle differentiation from purified PDGFRα+Flk1− progenitors isolated from EBs generated from mouse ES cells containing an inducible Pax3 gene (88). At the time of writing, there are no reports on the generation of myogenic progenitors from iPSCs, but assuming that the present protocols for ES cells can be adapted for iPSCs, we believe that in the next months, papers on this topic are likely to appear.

Muscle regeneration from ES/iPS cells via mesodermal progenitors. There is a large body of evidence indicating the existence of non-conventional muscle progenitors (see Other myogenic progenitors). Thus, the possibility of deriving mesodermal myogenic progenitors (89) from ES/iPS cells offers an alternative route for cell therapy for skeletal muscle regeneration. This approach has the advantage of producing myogenic progenitors that could be delivered systematically through the circulation.

In 2005, Studer and colleagues described the derivation from ES cells of mesenchymal precursors (90) that differentiated in vitro into different mesodermal lineages, including skeletal muscle. A recent article described the derivation from mouse ES cells of PDGFRe− mesodermal progenitors that, after in vivo transplantation, expressed markers of SCs and contributed to muscle regeneration (91).

Issues to be solved. Despite the excitement for these novel strategies for treating degenerative muscular conditions, a number of safety and efficacy issues, some common to other cells (immunogenicity, survival, and differentiation), some prominent for ES/iPS cells, such as tumor formation, still need to be solved. For example, cytofluorimetric purification of committed progenitors (88) may dramatically decrease the possibility of transplanting undifferentiated tumorigenic cells. The use of standardized protocols for generating iPSCs (92), together with stringent tumorigenic assays for the derived cell types, will certainly be a fundamental step toward their clinical application.

Skeletal muscle stem cells: past and ongoing clinical trials

Until now only SCs, and, to a very minor extent, CD133+ cells have been used in human clinical trials. The pathologies treated include forms of muscular dystrophy, heart failure associated with myocardial infarction (HDMI), and stress urinary incontinence (SUI).

Muscular dystrophies. In 1990, Peter Law and collaborators reported the first SC transplant in a 9-year-old boy affected by DMD, showing safety and dystrophin production (93). Soon after, 11 clinical trials in DMD patients were conducted using intramuscular injection of SCs (Table 3) (94–104). Although there were no adverse effects, new dystrophin production was demonstrated in many but not all cases and clinical benefit in none (6, 105). This is not surprising considering that intramuscular injection in several locations of a single muscle (or at most a few muscles) cannot elicit a general effect, although improved strength of the injected muscles was detected in 15% of the patients treated. Treating muscular dystrophies by intramuscular injection of myoblasts presents several problems that have not been solved yet. First, intramuscular injected cells distribute locally, implying that a huge number of SCs would be needed. Second, immune responses toward the injected SCs have been described, even in the case of major histocompatibility complex class I-lacking SCs (93). Third, muscular dystrophies are chronic diseases that require continuous cell supplementation. Last, a sustained expression of dystrophin persists for only a limited period of time (6, 105). Subsequent experimentation has been devoted to solving these problems, and a phase I clinical trial has been completed.
completed (104). Although encouraging results have been obtained, this method is still limited by the impossibility of delivering myoblasts systematically through the circulation. Recently, Torrente and colleagues reported the first CD133+ cell transplant (108). They designed a phase I double-blind trial with an autologous transplant of unmodified, and thus still dystrophic, muscle-derived CD133+ cells in 8 boys affected by DMD exclusively to test safety; and indeed, no adverse events were reported.

Heart failure. Among different options to treat heart infarction, skeletal muscle–derived myoblasts were considered an optimal cell therapy, as they can be easily obtained from the same patient (avoiding the need for long-term immune suppression), rapidly expanded in vitro, and transplanted back in the patient heart. Preclinical experiments performed in animal models demonstrated their ability to engraft correctly, survive in postinfarction scars, differentiate into contractile skeletal muscle cells, and improve heart function (109–111), possibly also because they release angiogenic factors. Unfortunately, in these models, myoblasts were not able to differentiate to cardiomyocytes and did not integrate electrically with the host cardiomyocytes (112, 113). Despite this significant problem, several nonrandomized clinical trials using myoblasts to treat the infarcted heart demonstrated thickening of the LV, an increase in LV ejection fraction, and prevention of LV dilatation, with clinical improvement in some patients (114–120). Histological analysis showed the presence of new myofibers in the scar zone expressing skeletal muscle–specific myosin heavy chain (121). In general, the transplantation procedure was clinically well tolerated, but a high incidence of arrhythmias, some of which were tolerated, but a high incidence of arrhythmias, some of which were fatal, was reported. In 2007, the results of the MAGIC study, an international phase II double-blind trial were published (122). Ninety-seven patients affected by HFMI were randomized to receive placebo, a low dose of myoblasts (400 × 10^6 cells), or a high dose of myoblasts (800 × 10^6 cells). LV end-diastolic volumes decreased substantially in patients receiving myoblasts, supporting a role for myoblasts in remodeling of the heart muscle. The incidence of secondary events, including arrhythmias, was not different between the groups, although all patients received the antiarrhythmic agent amiodarone and were implanted with a cardioverter defibrillator. The results of the CAUsMIC (123) and SEISMIC trials (124) have demonstrated safety and some clinical improvement. Ongoing trials include the MARVEL study, a phase II/III randomized, double-blind, placebo-controlled trial for which results are expected during the fall of 2009.

SUI. SC-derived myoblasts have also been used as cell therapy for individuals with SUI, which is characterized by the loss of small amounts of urine upon coughing, laughing, sneezing, exercising, or other movements that increase intraabdominal pressure. Myoblasts may represent an interesting approach for the treatment of this disease (125), as the main cause of SUI is impaired tone of the urethral smooth and striated muscle, which is associated with atrophy of the supporting structures of the uretha, the mucosa, and vascular submucosa (126, 127). Treatment of SUI with SC-derived myoblasts has been performed using two different strategies: injection of autologous myoblasts to improve the sphincter tone; and injection of myoblasts together with fibroblasts (isolated by differential adhesion from the same muscle biopsy) to both improve sphincter tone and treat mucosa atrophy. Until now, all published studies have been nonrandomized, open studies, demonstrating a remarkable clinical improvement in most of the patients treated (127, 128). Moreover, structural and functional techniques have demonstrated thickening of the urinary sphincter and an increase in maximum urethral closure pressure. The onset of improvement is not immediate and may be delayed up to six months after cell injection; however, the benefit, once obtained, lasts for a long period of time, at least up to 12 months (126). Cystoscopic studies have not demonstrated overgrowth of myoblasts nor obstruction of the lower urinary tract (129). Unfortunately, these successful results have not been confirmed yet in a randomized study.

Conclusions

This is an exciting period for those studying the biology of skeletal muscle stem cells and seeking to harness the information for clinical applications. By learning more about SCs and other mesodermal skeletal muscle progenitors, we can learn how to better use them to repair muscle. The main limitations of SCs are loss of “stemness” upon culture and an inability to cross the vessel wall for systemic delivery. Limitations for other cell types are incomplete characterization and their overall minor myogenic potency. Nevertheless, a phase I clinical trial with donor-derived MABs is planned for the end of 2010, given the fact that, because of extensive preclinical work, these cells appear at the moment as the best candidates for the cell therapy of muscular dystrophy. Moreover, the terrific prospective of deriving countless autologous, genetically corrected iPS cells from patients certainly will set the stage for future cell therapies. Finally, we should remember that SCs have already been used for clinical trials for DMD, myocardial infarction, and SUI. The last seems to be a case of success for this approach and a situation from which we may also learn in order to redirect efforts toward therapies for myocardial infarction and muscular dystrophies, which have thus far been less successful.

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Satellite cells, the engines of muscle repair

Yu Xin Wang and Michael A. Rudnicki

Abstract | Satellite cells are a heterogeneous population of stem and progenitor cells that are required for the growth, maintenance and regeneration of skeletal muscle. The transcription factors paired-box 3 (PAX3) and PAX7 have essential and overlapping roles in myogenesis. PAX3 acts to specify embryonic muscle precursors, whereas PAX7 enforces the satellite cell myogenic programme while maintaining the undifferentiated state. Recent experiments have suggested that PAX7 is dispensable in adult satellite cells. However, these findings are controversial, and the issue remains unresolved.

It has been 50 years since Mauro first postulated that satellite cells could be resident progenitor cells involved in skeletal muscle regeneration. Satellite cells were initially characterized according to their anatomical position as sublaminar mononuclear cells ‘wedged’ between the basal lamina and the plasma membrane (also known as the sarcolemma in this context) of myofibres. Within this niche, satellite cells are commonly adjacent to a myonucleus of their host myofibre and an endothelial cell of a nearby capillary. The close proximity of satellite cells to myofibres suggested that they may have a role in muscle regeneration.

Since the discovery of satellite cells, evidence has accumulated showing that they are the primary contributors to the postnatal growth, maintenance and repair of skeletal muscle. In adult muscle, satellite cells express the transcription factor paired-box 7 (PAX7) (FIG. 1a) and remain quiescent under normal physiological conditions. Readily responsive to molecular triggers from exercise, injuries or disease, satellite cells have a remarkable ability to self-renew, expand, proliferate as myoblasts or undergo myogenic differentiation to fuse and restore damaged muscle (FIG. 1b). Most importantly, satellite cells are maintained through repeated cycles of growth and regeneration, which supports the notion that they are a heterogeneous population containing stem cells that sustain their self-renewal.

Recent studies using transgenic mice have further examined the role of satellite cells during postnatal regeneration and in hypertrophy. In this Opinion article, we focus on the evidence that satellite cells are essential during these processes, describe the mechanisms maintaining their homeostasis through many rounds of regeneration and discuss findings showing that PAX7 is required to specify this lineage.

Myogenesis

Many similarities between the activation of satellite cells and myogenesis in the somite (BOX 1) have reinforced the idea that adult muscle regeneration to a certain extent recapitulates embryonic development through analogous, but not necessarily identical, mechanisms. Below, we briefly introduce the genetic networks involved in myogenesis — for recent, comprehensive reviews on myogenesis, see REFS 4–7.

Myogenic regulatory factors. Similarly to the establishment of embryonic progenitors in the myotome (BOX 1), the activation of satellite cells into myoblasts involves the upregulation of the basic helix–loop–helix (bHLH) transcription factors myogenic factor 5 (MYF5) and myoblast determination protein (MYOD) (FIG. 1b). Together with muscle-specific regulatory factor 4 (MRF4; also known as MYF6) and myogenin, which are upregulated during myoblast differentiation, these myogenic regulatory factors (MRFs) transcriptionally and epigenetically determine the myogenic capacity of muscle progenitors.

Mice lacking MyoD have seemingly normal muscle but express about fourfold higher levels of MYF5 (REF. 8). MYF5-deficient animals also have normal muscle. However, mice lacking both Myf5 and MyoD are totally devoid of myoblasts and myofibres, indicating that these genes are required for the determination of myogenic precursors. Although MRF4 has some capacity to function as a determination factor, it has not been established that it does so in the presence of MYF5 and MYOD.

As a downstream target of MYOD, myogenin regulates the transition from myoblasts into myocytes and myotubes (FIG. 1b) and, although myogenin-knockout mice show proper compartmentalization of muscle groups, they almost completely lack myofibres and accumulate undifferentiated myoblasts. Mice lacking both MyoD and Mrf4 display a phenotype that is similar to the myogenin-null phenotype, suggesting that, for myoblasts only expressing MYF5, the differentiation into myocytes requires MRF4 (REF. 15). Thus, MYF5 and MYOD are required for the determination of myogenic precursors and act upstream of myogenin and MRF4, which are required for terminal differentiation. Together, these findings suggest that various overlapping regulatory networks control myogenic differentiation.

PAX3 and PAX7 specify myogenic progenitors. The expression of MRFs is regulated by PAX3 and PAX7, both of which have been shown to directly bind proximal promoters of MyoD and distal enhancer elements of Myf5, thereby regulating their expression. PAX3 and PAX7 play key parts in maintaining the proliferation of progenitors and preventing early differentiation. Moreover, ectopic expression of PAX3 or PAX7 in mouse embryonic stem cells showed that either one is sufficient to promote a myogenic fate.
Belonging to the PAX family of transcription factors, PAX3 and PAX7 are paralogues with conserved amino acid sequences and have almost identical sequence-specific DNA-binding motifs. Despite this homology, studies using knockout mice suggest that PAX3 and PAX7 have overlapping roles only in myogenic specification; the distinct phenotypes of these mice indicate that PAX3 has unique functions during embryonic development and that PAX7 is involved in the specification of satellite cells. PAX3-null mice (also known as splotch mice) have many developmental defects, including a lack of limb muscles and reduced MYOD expression in the myotome (BOX 1). Interestingly, mice lacking both PAX3 and MYF5 cannot upregulate MYOD to compensate for the lack of MYF5 and thus lack muscle, which further indicates that PAX3 activates MYOD. Even though PAX3 is expressed at an earlier embryonic stage than PAX7, PAX7 is upregulated in PAX3-null mice, presumably to compensate for the loss of PAX3 (REF. 22). In fact, muscle formation requires PAX3 or PAX7, as progenitor cell populations from mice lacking both PAX3 and PAX7 undergo apoptosis, and these mice do not form muscle.

Although Pax7 knock-in at the Pax3 locus can rescue the Pax3-null phenotype during somitogenesis, it does not fully rescue the lack of delamination and long-range migration of muscle progenitors to the limb bud, which requires the activation of hepatocyte growth factor receptor (HGFR; also known as MET) by PAX3 (REFS 21, 25) (BOX 1). This suggests that PAX3 is solely responsible for the migration of progenitors to establish myogenic compartments within the limb.

By contrast, PAX7 seems to be uniquely required in satellite cells (discussed in detail below). Indeed, PAX3 is downregulated postnatally in most muscle progenitors, except for the satellite cells of a subset of muscle groups such as the diaphragm, whereas PAX7 expression is maintained in all satellite cells. Furthermore, PAX7-null mice have normal embryonic myogenesis but lack functional satellite cells. Together, these findings argue that PAX3 and PAX7 have overlapping but non-redundant roles in the myogenic programme.

**Specification of satellite cells**

It has long been postulated that satellite cells are the remnants of embryonic muscle development. Somitic progenitors that eventually give rise to satellite cells express PAX3 and/or PAX7 and do not express MRFs. These progenitor PAX3/PAX7+ cells upregulate MYF5 and MYOD when they enter the myogenic differentiation programme, or remain as satellite cells during late fetal myogenesis without upregulating MRFs. PAX3/PAX7+ cells that do not express MRFs are first found to align with nascent myotubes at embryonic day 15.5 and then become satellite cells by taking a sublaminar position. Notably, once they arrive at the nascent myotubes, most satellite cells rapidly upregulate MYF5 and downregulate PAX3 (REF. 26).

Lineage-tracing studies suggest that PAX3+ cells contribute to embryonic myoblasts and the endothelial lineage, whereas PAX7+ cells contribute to fetal myoblasts, supporting the notion that these cells represent distinct myogenic lineages. Therefore, satellite stem cells (PAX7+MYF5+) see below) in adult muscle may represent a lineage continuum of the embryonic PAX3+PAX7+MRF+ progenitors. However, these studies have not conclusively ruled out the possibility that satellite cells are a distinct myogenic lineage, independent from those that give rise to fetal myoblasts, or that they arise from atypical myogenic stem cells in postnatal muscle (BOX 2).

**Relative roles of PAX3 and PAX7 in satellite cells**

PAX7 expression is maintained in all satellite cells and proliferating myoblasts but is sharply downregulated before differentiation. PAX3 and PAX7 co-expression in adult satellite cells seems to be limited to...
only a few muscle groups — mostly the diaphragm and body wall muscles\(^2\). Although PAX3 expression is robust in the embryo, its downregulation after birth suggests that it has little function in most satellite cells. Prolonged expression of PAX3, by preventing its proteosomal degradation, results in the inhibition of terminal differentiation\(^{26}\), consistent with the ability of ectopically expressed PAX7 to inhibit differentiation\(^2\). This result has been used to argue that PAX3 regulates satellite cell activation. Although studies have indicated a role for PAX3 in satellite cell development and maintenance\(^{30,31,32,33}\), this currently remains controversial.

By contrast, PAX7 is required for the development and maintenance of satellite cells. Indeed, cells found in the satellite cell positions of PAX7-null mice are not myogenic and do not express satellite cell markers (such as syndecan 4 and CD34; see below)\(^{31}\). Importantly, although rare PAX3\(^+\) myogenic cells are observed in PAX7-null mutants, PAX3 cannot compensate for the loss of PAX7 in the establishment of the satellite cell lineage. Moreover, although satellite cells in the diaphragm normally express abundant PAX3, the phenotype of PAX7-null satellite cells that express PAX3 in the diaphragm is identical to other muscle groups: they cannot maintain the undifferentiated state, they fuse into myofibres early or they have reduced survival, ultimately compromising their capacity for muscle growth and regeneration\(^2,25,31,34,35\). This suggests that PAX3 alone is insufficient to rescue these cells\(^2,32\).

Two possible functions have been proposed for PAX7 in the specification of satellite cells: the specification of the myogenic identity and the maintenance of the undifferentiated state. Ectopic overexpression of PAX7 in C2C12 myoblasts and primary myoblasts suggests that PAX7 drives the expression of Myf5 but not MyoD\(^{29,32}\). Furthermore, PAX7 has been shown to directly activate Myf5 by promoting the recruitment of the ASH2-like (ASH2L)–MLL2 histone methyltransferase complex at a regulatory element 57 kb upstream of the Myf5 coding region\(^4\), which indicates that PAX7 can epigenetically specify the myogenic identity of satellite cells. However, PAX7 expression correlates with an undifferentiated but committed myogenic state\(^6\), which suggests that PAX7 in the early postnatal period probably functions to prevent the transition into terminal differentiation while maintaining the myogenic identity by promoting MYF5 expression. Both functions together allow PAX7 to specify the satellite cell lineage.

**Box 1 | Embryonic myogenesis**

Early embryonic myogenic precursors giving rise to body wall muscles are marked by paired-box 3 (PAX3) expression in the paraxial mesoderm\(^{26}\). At this early stage, PAX3\(^+\) progenitors are multipotent and give rise to the dorsal dermis and vascular progenitors of the aorta\(^{26}\). Morphogens expressed by surrounding embryonic structures eventually determine the cellular commitment within the somites (mesodermal structures found on either side of the neural tube in vertebrate embryos that eventually give rise to muscles, skin and vertebrae)\(^{30}\). Sonic hedgehog signalling from the notochord acts on the ventral medial segment of the somite and leads to the formation of the sclerotome, which contains precursors to bone and cartilage\(^{30}\). WNT signalling in the dorsal portion of the somite leads to the retention of an epithelial morphology and the formation of the dermomyotome\(^{31}\). Combined expression of PAX3 and PAX7 in the dermomyotome further specifies progenitor cells towards the muscle lineage, but transient Notch activation from neural crest cells is required before the expression of myogenic regulatory factors (MRFs; which define muscle progenitors)\(^{22,24,37}\). Notch signalling is essential for retaining myogenic progenitors in a proliferative state; however, its downregulation is required to allow progression into terminal differentiation. MRF\(^+\) progenitors migrate and proliferate to give rise to the myotome, in which cells downregulate PAX3 and PAX7 to undergo differentiation and form primitive nascent myofibres\(^{22,24,37}\). These primitive myofibres act as templates for the formation of additional myofibres during postnatal growth.

The development of limb muscles requires the establishment of myogenic compartments in the limb bud. This process involves the delamination and migration of progenitors from the dermomyotome\(^{32}\). Induction of hepatocyte growth factor (HGF) in the limb bud activates myogenic progenitors expressing HGF receptor (HGF\(R\); also known as MET), which delamine from the ventrolateral lip of the dermomyotome. PAX3, and not PAX7, is able to induce the expression of HGF in embryonic progenitors\(^{38}\). Thus, PAX3 is required to activate the long-range migration of progenitors to the limb bud. This is consistent with the lack of limb muscles in PAX3-null mice\(^{32}\).

**Requirement for PAX7 in satellite cells**

Recently, a study by Lepper et al.\(^{30}\) using an inducible knockout mouse strain lacking PAX7 specifically in satellite cells when injected with tamoxifen challenged the proposed requirement for PAX7 expression in adult satellite cells. Interestingly, when postnatal time points were examined, the phenotype of mice in which PAX7 had been deleted between postnatal day 7 (P7) and P21 was the same as that of the previously characterized PAX7-null mice\(^{22,31,34,35}\). This result confirms a crucial function for PAX7 during postnatal growth, and this is further supported by the observed early fusion of progenitors in PAX7-null mice and the conversion of fetal cells from conditional PAX7–knockout mice into fibroblasts\(^{35}\).

Surprisingly, induced ablation of both PAX3 and PAX7 after P21 did not lead to any deficiency in muscle regeneration, satellite cell number or primary myoblast growth or differentiation\(^{39}\). This suggests a rather dramatic change in the genetic requirement of muscle regeneration around P21, the time point when myonuclear accretion is completed (see below) and when postnatal growth is thought to end and satellite cell numbers to reach a steady state\(^{37}\). The distinct regeneration phenotypes obtained by PAX7 deletion between P7–P21 and adulthood challenge the notion that PAX7 is required throughout adulthood to activate downstream MRFs. It remains unclear whether satellite cells are poised for activation and extrinsic signalling is sufficient to initiate MRF expression or whether temporal specification by PAX7 is sufficient to maintain satellite cell identity after P21. The temporal specification hypothesis is supported by the finding that tamoxifen-induced knockout of PAX7 leads to loss of myogenic identity in fetal cells but not adult cells\(^35\). One possibility is that the epigenetic function of PAX7 in recruiting ASH2L–MLL2 to target genes has been completed by P21 and that target genes that define myogenic identity are capable of maintaining their epigenetic memory in the absence of PAX7.

However, the ability of satellite cells to function after P21 without PAX7 is difficult to reconcile with the published literature. For example, in vitro knockdown of PAX7 in any aged myoblast or satellite cell has been shown to result in growth arrest and marked reduction in MYF5 expression\(^{30,34}\). Why would a knockdown of PAX7 but not a deletion affect satellite cell maintenance? The notion that proliferating myogenic cells have become ‘addicted’ to PAX7 might be considered; that is, that continued PAX7 expression might be required to keep the cells in a proliferative state. Another interesting possibility is that the floxed allele analysed by Lepper et al. is a hypermorph that is expressing a truncated but functional PAX7 lacking a paired domain.
Box 2 | Atypical muscle stem cells

Several laboratories have observed that stem cell populations other than satellite cells also have myogenic capacity. These include pericytes, mesangioblasts, side population cells, CD45~SCA1~ cells and even bone marrow–derived haematopoietic stem cells (HSCs)40–44. Their myogenic specification requires activation by injury or co-culturing with myoblasts; however, even under such conditions, only small fractions of these cells adopt myogenic fates. Paired-box 7 (PAX7) is required for the myogenic specification of CD45~SCA1~ cells during regeneration45, whereas the myogenic identity of pericytes, side population cells and HSCs can be activated by alternative pathways. Limited engraftment of these cells to muscle is observed in transplantation experiments, but the extent of their contribution during in vivo muscle regeneration will require further lineage-tracing studies.

PW1~ interstitial cells (PICs) were recently described as non-satellite cell myogenic progenitors during postnatal muscle growth that can also adopt either vascular or myogenic fates46. The myogenic specification of PICs depends on PAX7 and is enhanced by the presence of satellite cell–derived myoblasts, suggesting that the recruitment of PICs depends on community effects. As most newly specified fetal satellite cells also express PW1, and because lineage-tracing experiments suggest that PICs are not derived from satellite cells, it was speculated that PICs could be a postnatal source of satellite cells.

However, in recent analyses of satellite cell–depleted muscle47–50, atypical muscle stem cells could not promote muscle regeneration or replenish the satellite cell pool. This challenges the previous evidence and suggests either that these atypical myogenic stem cells have very limited myogenic potential or that they are not recruited during regeneration in this model owing to a lack of paracrine signals from satellite cells (FIG. 5). The functional participation of atypical myogenic stem cells will need to be studied further. Moreover, the paracrine factors secreted by satellite cells to induce the recruitment of atypical muscle stem cells could be an interesting therapeutic target to modulate regeneration kinetics.

If PAX7 is required only in the specification of new satellite cells, such a defect would become apparent only after several rounds of regeneration, when satellite cell pools have become exhausted. Moreover, this defect can be masked by alternative mechanisms of self-renewal in satellite cells, such as reversible quiescence (see below).

Nevertheless, Lepper et al.46 challenged the paradigm that PAX7 is crucial for satellite cells in adults. Thus, further analysis is needed to elucidate the requirement and function of PAX7 in adult satellite cells.

Satellite cells in postnatal life

After postnatal growth (~P21), maturation of skeletal muscle slowly reaches a homeostasis of rest and regeneration. Satellite cells also enter quiescence as their numbers decrease dramatically from ~30% of sublaminar nuclei to <5% by 8 weeks, which reflects fusion into myofibres, a process known as myonuclear accretion51,52. This entry into quiescence was recently shown to depend on Notch signalling, as mice lacking two Notch targets, Hairy and Enhancer of Split–related 1 (HESR1) and HESR3, fail to generate quiescent satellite cells53. Inhibition or disruption of Notch signalling in quiescent satellite cells leads to their early differentiation and fusion40,54,55, similarly to what is observed in PAX7–null mice.

Originally defined by their anatomical position to the host myofibre1, quiescent satellite cells are reliably identifiable by the expression of molecular markers, such as PAX7, α7β1 integrin, CD34, syndecan 3, syndecan 4, myotubule cadherin (M–cadherin), caveolin 1 and CXC chemokine receptor 4 (CXCRI4; also known as fusin and CD184)44. The identification of these markers, as well as the generation of transgenic reporter mouse lines, has only recently allowed the prospective isolation and characterization of satellite cells in their quiescent state28,43.

Satellite cells are heterogeneous. All adult satellite cells express PAX7, and this expression is conserved through evolution52. However, subpopulations of satellite cells express a mixture of different surface markers as well as altered expression levels of MYF5 and MYOD45. Because satellite cells are self-renewing, within this heterogeneity there must be a stem cell–like population that is resistant to differentiation and maintains the satellite cell pool through many rounds of regeneration. Experiments have shown that small fractions (~20%) of satellite cells have lower proliferation kinetics and can resist differentiation46. Furthermore, satellite cells from different muscle groups also display differential engraftment potential after transplantation46. In these transplantation experiments, only a small fraction of satellite cells shows stem cell–like properties and engrafts into the satellite cell compartment.

PAX7~MYF5~ satellite stem cells. Using lineage tracing in a mouse strain in which cells that have expressed MYF5 are permanently labelled with yellow fluorescent protein (YFP), we identified that ~10% of adult satellite cells express PAX7 but not YFP, indicating that they have never expressed MYF5 (PAX7~MYF5~ cells)39 (FIG. 1a). Compared with their committed YFP+ counterparts, PAX7~MYF5~ satellite cells were able to engraft as satellite cells, self-renew more efficiently, give rise to committed YFP+ (PAX7~MYF5+) satellite cells and better resist differentiation. Because of these stem cell characteristics and their uncommitted status, we concluded that these PAX7~MYF5~ satellite cells were in fact satellite stem cells.

Similarly to other adult stem cells reviewed in REF. 47, satellite stem cells follow traditional stochastic (symmetric) and asymmetric paradigms of self-renewal48 (FIG. 2a). The asymmetric nature of the satellite cell niche, with the basal lamina acting as the basement membrane and the sarcolemma of the host myofibre as the apical surface, determines the cell fate of stem cell divisions according to the orientation of the mitotic axis in relation to the host fibre. During in vivo regeneration, satellite stem cell divisions in the apicobasal orientation give rise to a committed MYF5~ satellite cell, which is pushed into the apical surface, and a daughter cell, which remains attached to basal lamina and retains the satellite stem cell identity. By contrast, planar divisions of satellite stem cells occur along the host myofibre, after which both daughter cells remain in contact with the basal lamina, symmetrically expand, giving rise to two PAX7~MYF5~ cells.

It remains unclear what intrinsic mechanism is responsible for the orientation of divisions and how subsequent events lead to commitment of the apical daughter cell. However, insights into extrinsic regulators revealed that satellite stem cells are similar to embryonic PAX7~MYF5+ cells in the dermomyotome (BOX 1), in that inhibition of Notch signalling drives satellite stem cells to commitment and differentiation49,50.

Furthermore, WNT signalling is thought to promote symmetric cell divisions by acting through the planar cell polarity pathway50. Specifically, WNT7A signalling through Frizzled 7 markedly stimulates the symmetric expansion of satellite stem cells but does not affect the growth of myoblasts or the kinetics of their differentiation (FIG. 2b). This induces the polarized distribution of the planar cell polarity effector VANG-like 2 (VANGL2), promoting a planar division plane and
therefore symmetric division of the daughter cells. This pathway is thought to regulate the regenerative potential of muscle. In support of this, WNT7A is upregulated during regeneration and increases both the number of satellite cells and the muscle mass, and muscle lacking WNT7A exhibits a decrease in satellite cell number following regeneration.

Reversible quiescence. Replenishment of the satellite cell pool requires stem cells to expand but then return to quiescence, a common feature of adult stem cells. This process is very elusive and only applies to a subpopulation of satellite cells, so our current understanding is minimal. In vitro observations of satellite cells on cultured myofibres suggest that a population of MYOD+ reserve cells appear in late regeneration and can return to quiescence by sustaining PAX7 expression and resisting differentiation49–52. This could arise from the expansion of a MYOD+ population of uncommitted satellite cells or the downregulation of MYOD in proliferating myoblasts. Most PAX7+MYF5+ satellite stem cells correlate with this population and do not express MYOD or myogenin in culture53. One known mechanism regulating the reversible quiescence of satellite stem cells is mediated by the receptor Tyr kinase TIE2 (also known as TEK)54. TIE2 activation by binding of the hormone angiopoietin 1, which is secreted from neighbouring fibroblasts and vascular cells, activates extracellular signal-regulated kinase (ERK) signalling downstream of TIE2. This enhances PAX7 expression and thereby sustains the undifferentiated state, ultimately increasing the number of stem cells reverting to quiescence.

Reversible quiescence of committed myoblasts challenges the paradigm that MYOD, as a determination factor, is an irreversible epigenetic checkpoint in the myogenic programme. For a subpopulation of myoblasts, this reversion was reported to require the activation of Sprouty homologue 1 (SPRY1), a negative regulator of ERK signalling55. However, myoblasts unaffected in SPRY1 deletion can maintain a reduced, but homeostatic, number of satellite cells through subsequent injuries. This indicates that alternative mechanisms of reversion into quiescence exist. Although contradictory, TIE2–ERK and SPRY1–ERK signalling apply to two distinct cell types at different time points during regeneration. This suggests that temporal regulation of the ERK signalling pathway controls the number of satellite cells returning to quiescence and reveals the possibility of modulating this homeostasis with extrinsic factors.

Satellite cells in regeneration

The main function of satellite cells is to proliferate and differentiate into muscle cells to regenerate muscle during exercise or injury. Satellite cells are integral to regeneration. The ability for satellite cells to contribute to myogenesis has been demonstrated in vitro and in vivo. In a series of diphtheria toxin–induced ablation experiments, it was revealed that muscle failed to regenerate when PAX7+ satellite cells were depleted56–58. Two different transgenic alleles were used to eliminate PAX7+ satellite cells from muscle, and both led to the same conclusion: no other cell types can rescue the myogenic function of PAX7+ cells during exercise or injury59,60 (BOX 2). Instead of the formation of myofibres, adipocyte infiltrates expanded in place of muscle61. This genetic ablation result resembles the phenotypes of PAX7-null mice and supports the notion that satellite cells are the primary, if not the sole, contributors to muscle regeneration.

Healthy adult muscle has a slow turnover rate, so satellite cells remain mostly quiescent (see above) unless activated by exercise or injury5. Consistent with this, ablation of satellite cells in resting conditions does not result in progressive muscle mass loss56. However, in degenerative muscle diseases, such as Duchenne muscular dystrophy, chronic regeneration of muscle triggers higher proliferative demand on satellite cells, leading to their eventual depletion62. Interfering with the function of satellite cells in a mouse model for Duchenne muscular dystrophy (mdx mice), by either inducing reduced myogenic differentiation (for example, in MYOD-null mice) or reducing the telomere lengths (for example, in mice lacking telomerase mRNA), results in an exaggerated dystrophic phenotype63,64.

Together, these experiments suggest that satellite cells are absolutely necessary for regeneration and that satellite cells lost in disease are not replaced by other stem cell sources.

Interactions with other cell types during regeneration. Although satellite cells are the main players in repairing muscle, various other cell types are also recruited during regeneration and can modulate the behaviour of satellite cells by secreting cytokines (FIG. 5). During the initial phase of regeneration, secretion of pro-inflammatory cytokines by macrophages can induce myoblast entry into terminal differentiation through the p38 kinase-mediated repression of the Pax7 locus65. Expansion of resident fibroadipogenic progenitors (FAPs) during regeneration also promotes the terminal differentiation of myoblasts66.

However, not all signals during regeneration are pro-myogenic. The presence of muscle connective tissue (MCT) fibroblasts expressing T cell factor 4 (TCF4) seems to prevent early differentiation of myoblasts by creating a transitional niche. Reciprocally, myoblasts promote MCT fibroblast...
proliferation. Late in regeneration, newly formed myotubes prevent the adipogenic differentiation of mesenchymal progenitors expressing platelet-derived growth factor receptor-α (PDGFRα). These interactions give rise to a complex interaction network between satellite cells and other cell types, which allows the extrinsic regulation of satellite cells in regeneration.

Satellite cell requirement in hypertrophy. Hypertrophy of muscle (that is, an increase in myofibre size resulting from the production of additional contractile proteins) caused by overloading or exercise requires additional myonuclei for protein synthesis. Because myonuclei are mitotically arrested and cannot replicate within the myofibre to meet such a demand, hypertrophy is commonly associated with the activation of satellite cells, which are thought to proliferate and fuse into existing myofibres. Indeed, classic studies using γ-radiation to inhibit the proliferation of satellite cells found a dependence on satellite cell activation in overloading-induced hypertrophy. However, controversy over the specificity of γ-radiation to satellite cells, as well as a better understanding of intrinsic pathways leading to the hypertrophy of myotubes, such as calcineurin and AKT signalling, indicated that further examination is needed.

A recent study provided evidence that satellite cells are not required for overloading-induced hypertrophy and that intrinsic mechanisms within the myofibres can compensate for protein synthesis without additional myonuclei. Specifically, significant hypertrophy was observed in satellite cell-depleted muscles that were exposed to overloading conditions. However, in controls, activation of satellite cells and fusion into myofibres were observed. This indicates that, in a physiological context, satellite cells indeed participate in hypertrophy. It is not surprising that myofibres can undergo short-term hypertrophy without additional myonuclei, but the addition of myonuclei should reduce the ‘workload’ placed on the existing myonuclei. Importantly, productive hypertrophy resulting from the participation of satellite cells would be expected to couple tissue mass with appropriate numbers of stem and progenitor cells. Thus, satellite cells are essential to achieve tissue homeostasis and allow growth and repair over the long term.

Concluding remarks

50 years after their discovery, we have solidified the absolute requirement for muscle satellite cells during muscle regeneration and in hypertrophy. Composed primarily of a nucleus and very little cytoplasm, the regenerative capacity of satellite cells can only be described as remarkable. Indeed, whereas the requirement for satellite cells has been questioned for short-term hypertrophy, it is clear that maintenance of muscle and productive hypertrophy absolutely require satellite cells.

We have only scratched the surface of a complex genetic network that requires intrinsic and extrinsic activation to regulate the myogenic fate of satellite cells. We know that PAX3 and PAX7 regulate the expression of MRFs and are crucial for muscle development, and that early postnatal specification of the satellite cell lineage requires PAX7 to establish an undifferentiated but myogenic state. However, further work is needed to definitively confirm whether PAX7 is required in adult satellite cells.

Within the past two decades, our understanding of satellite cell biology has formed new paradigms for the maintenance and genetic regulation of adult stem cells in general. Without question, our advancing knowledge of satellite cell biology has opened new doors for the development of therapeutics targeting stem cell growth, in vitro expansion of satellite cells and even induction of embryonic stem cells for transplantation as promising avenues for the treatment of patients suffering from degenerative muscle diseases.

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Cellular and Molecular Signatures of Muscle Regeneration: Current Concepts and Controversies in Adult Myogenesis

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Adult skeletal muscle generates force in a controlled and directed manner through the contraction of highly specialized, postmitotic, multinucleated myofibers. Life-long muscle function relies on maintenance and regeneration of myofibers through a highly regulated process beginning with activation of normally quiescent muscle precursor cells and proceeding with formation of proliferating progenitors that fuse to generate differentiated myofibers. In this review, we describe the historical basis and current evidence for the identification of satellite cells as adult muscle stem cells, critically evaluate contributions of other cells to adult myogenesis, and summarize existing data regarding the origins, genetic markers, and molecular regulation of satellite cells in normal, diseased, and aged muscle.

Satellite Cells as Adult Muscle Precursor Cells and Candidate Muscle Stem Cells

Adult skeletal muscle possesses remarkable regenerative capacity, and large numbers of new myotubes normally are formed in only a few days after acute muscle damage (Bintliff and Walker, 1960; LeGros Clark, 1946; Walton and Adams, 1956; Weber, 1863). Early hypotheses proposed that new myofibers were generated via budding of myotubes from existing, injured fibers (LeGros Clark, 1946; Volkman, 1893); however, further study has demonstrated instead that this rapid repair occurs through the differentiation and subsequent cell fusion of myogenically specified mononuclear precursor cells contained within a population of “satellite cells” positioned between the plasma membrane and the surrounding basal lamina of mature, differentiated muscle fibers (Mauro, 1961; Snow, 1978). Alexander Mauro first proposed in 1961 that satellite cells might represent “dormant myoblasts” left over from embryonic muscle development and capable of recapitulating the developmental program of skeletal myogenesis in response to muscle damage (Mauro, 1961). Subsequent studies, in both the chick (Konigsberg, 1963) and mouse (Yaffe, 1969), demonstrated that multinucleated myotubes could indeed be generated in vitro from single myogenic precursor cells, and that these precursors ultimately derived from muscle satellite cells (Bischoff, 1975). Furthermore, in vivo-labeled (Snow, 1978), and clonally cultured in vitro-labeled (Lipton and Schultz, 1979), satellite cells were shown to participate in the regeneration of damaged muscle when transplanted in vivo, contributing almost exclusively via fusion with pre-existing myofibers. Pulse-chase experiments using a single dose of tritiated thymidine to label dividing cells indicated that DNA synthesis among sublaminar nuclei was limited to satellite cell nuclei, and that true muscle nuclei do not undergo mitosis (Moss and Leblond, 1970). Although these methods labeled satellite cells relatively infrequently, indicating the relative quiescence of satellite cells (Schultz et al., 1978), in some cases DNA label from satellite cells marked during the pulse phase eventually appeared in myonuclei during the chase phase, demonstrating the capacity of at least some satellite cell progeny to incorporate into existing myofibers, even in the absence of acute tissue injury (Moss and Leblond, 1970). Such studies formed the basis for our current view of muscle satellite cells as the primary mediators of postnatal muscle growth and repair. These cells respond to regenerative cues, such as injury or exercise, by proliferating to form myoblasts, which divide a limited number of times before terminally differentiating and fusing to form multinucleated myotubes (reviewed in Morgan and Partridge, 2003; Sloper and Partridge, 1980) (see Figure 1A). The life-long regenerative capacity of satellite cells implies that they can be robustly and perpetually renewed while maintaining the ability to generate differentiated progeny and suggests that they may represent an adult stem cell population for skeletal muscle.

Stem cells are thought to exist in many adult tissues capable of regeneration and are defined by their unique capacity to both self-renew and differentiate. Adult stem cells have been best characterized in the mammalian blood-forming system, where clonogenic, multipotent hematopoietic stem cells (HSC) have been prospectively isolated from bone marrow and demonstrate at the single-cell level the capacity to regenerate the entire hematopoietic system (reviewed in Kondo et al., 2003). While it is clear that satellite cells likewise contain unspecifed precursor cells capable of extensive proliferation and differentiation to generate mature myofibers, to formally establish that they indeed function as adult stem cells, it will be necessary to demonstrate that individual cells within the satellite cell pool maintain both self-renewal and differentiation potential in vivo. Although this clonal analysis is still lacking, as outlined below, multiple lines of evidence from in vitro and in vivo studies do suggest that muscle stem cells are contained within at least a subset of satellite cells. First, as noted above, satellite cell number and regenerative capacity normally remain nearly constant through multiple cycles of injury and repair, suggesting satellite cell self-renewal. In addition, studies of isolated single myofibers and primary satellite cells maintained in culture have revealed that some cultured sat-
Taken together, these data strongly suggest that satellite cells represent self-renewing adult muscle stem cells and alone are sufficient for muscle repair. This conclusion likely will be strengthened by ongoing studies of the capacity for and regulation of satellite cell self-renewal, particularly those testing the continued regenerative potential of single or clonally marked satellite cells in serial transplantations.

Developmental Origins of Skeletal Muscle and Satellite Cells

Myogenic precursors are specified during development by signals emanating from neighboring cells of the notochord, neural tube, and dorsal ectoderm. This specification depends critically on the function of myogenic transcription factors, such as Pax-3 and Pax-7 (Borycki et al., 1999; Cossu et al., 1996a, 1996b; Goulding et al., 1994). Once committed, somite-derived cells migrate to multiple sites of embryonic myogenesis, begin to express the myogenic basic helix-loop-helix transcription factors Myf-5 and MyoD (Birchmeier and Brohmann, 2000), and differentiate into muscle fibers. Somite-derived myogenic progenitors that do not differentiate into myofibers at this time have been suggested instead to be retained into adulthood as muscle satellite cells (Armand et al., 1983; Mauro, 1961; Yablonka-Reuveni et al., 1987). This concept recently has been confirmed in two papers in which myogenic reporter strains and cell-lineage tracing experiments demonstrated that avian (Gros et al., 2005) and mouse (Relaix et al., 2005) embryonic and fetal myogenic progenitor cells arise from the central dermomyotome, following generation of the primary myotome. Importantly, neonatal Pax-7+ progenitor cells found in the satellite cell position were shown to originate also from the avian embryonic dermomyotome, indicating that some of the precursor cells that contribute to embryonic and fetal muscle are retained after birth as satellite cells (Gros et al., 2005). In rapidly growing neonatal muscle, nuclei of satellite cells and myoblasts comprise ~30% of myofiber-associated nuclei, but after cessation of muscle growth, quiescent satellite cells represent only ~5% of adult myofiber nuclei (Cardasis and Cooper, 1975). It remains to be determined, however, whether the quiescent satellite cells in adult muscle have the same developmental origin as embryonic, fetal, and neonatal cells, or, alternatively, if proliferating dermomyotome-derived myofibers-associated progenitors are exhausted during the muscle growth, and subsequent regeneration of the adult muscle invokes a distinct lineage of precursor cells. Additional analysis of myogenic reporter markers (Relaix et al., 2005) in older animals, and after repeated rounds of muscle injury and regeneration, will be particularly informative to address this issue and also may give further insight into the self-renewal potential of embryonically specified dermomyotome-derived satellite cells.

Phenotypic and Functional Heterogeneity of Satellite Cells

Satellite cells are classically defined by their position beneath the basal lamina and by their ability to undergo myogenic differentiation (Beauchamp et al., 2000; Mauro,
However, accumulating evidence suggests that the satellite cell compartment contains cells of distinct ontogeny and function. First, although several markers have been associated with satellite cells, no single marker defines all satellite cells. For example, while most satellite cells express the surface protein CD34 (Beauchamp et al., 2000; Conboy and Rando, 2002; Sherwood et al., 2004a), they can variably express other surface markers (Sherwood et al., 2004a), as well as myogenic transcription factors, such as Pax-7, MyoD, and Myf-5 (Beauchamp et al., 2000; Cornelison and Wolf, 1997; Zammit et al., 2004). CD34 and Pax-7 identify quiescent satellite cells, and Pax-7, M-Cadherin, MyoD, and Myf-5 are actually upregulated with differentiation of satellite cells into myoblasts (Morgan and Partridge, 2003; Seale et al., 2000, 2004). This heterogeneity of marker expression may reflect functional differences among satellite cells or distinct stages of myogenic lineage specification or may distinguish myogenic from nonmyogenic cell types within myofiber compartment. In this regard, it is interesting that in vitro studies have shown that some cells emerging from explanted single myofibers can express markers of osteocytes or adipocytes, rather than myogenic markers (Asakura et al., 2001; Csete et al., 2001). Recent studies from our group (Sherwood et al., 2004a) and others (Shefer et al., 2004) have indicated that single cells from within the satellite-cell compartment exhibit mutually exclusive abilities to generate either myogenic or fibroblastic and adipogenic colonies in clonal in vitro assays. Importantly, activated immune or inflammatory cells may also populate the satellite-cell compartment, infiltrating beneath the basal lamina of damaged muscle fibers (Stauber et al., 1988), although such infiltrating hematopoietic cells display no myogenic activity (Sherwood et al., 2004a). Finally, intrinsic differences in proliferation (Beauchamp et al., 1999; Rantanen et al., 1995; Rouger et al., 2004; Yablonka-Reuveni et al., 1987), differentiation (Rantanen et al., 1995; Zammit et al., 2004), and fusogenic capacity (Rouger et al., 2004) among individual satellite cells have been reported. More detailed studies employing prospective isolation and/or clonal marking to further delineate the precise lineage relationships, cellular interaction, and myogenic capacities of these distinct populations of myofiber-associated cells will greatly enhance our understanding of their normal roles and relative importance during muscle regeneration.

Adult Myogenic Cells Distinct from Satellite Cells
Recent reports have suggested that adult skeletal muscle progenitors distinct from satellite cells may function in some models of muscle injury and repair. For example, muscle-resident side population (muSP) cells, defined by their ability to exclude Hoechst 33342 (Asakura et al., 2002), have been shown to contribute to myofibers when injected intramuscularly (McKinney-Freeman et al., 2002) or when cocultured with myoblasts (Asakura et al., 2002), although these cells lack myogenic activity when cultured alone (Asakura et al., 2002). Similarly, although they do not generate myogenic cells when cultured alone, interstitial muscle-resident CD45+Sca-1+ cells reportedly acquire myogenic activity when cocultured with primary myoblasts or in response to muscle injury or Wnt signaling (Polesskaya et al., 2003). Finally, cells with high proliferative potential and surprisingly broad differentiation capacity, and thus termed “muscle-derived stem cells” (MDSC) or “multipotent adult progenitor cells” (MAPC), have been isolated following prolonged culture of cells from muscle (Cao et al., 2003; Jiang et al., 2002; Qu-Petersen et al., 2002). Descriptions of these distinct subsets of myogenic cells have raised the possibility that multiple mechanisms may support adult skeletal muscle regeneration. Yet, as compared to conventional satellite cells, many of these populations, with the notable exception of cultured MDSC (Qu-Petersen et al., 2002), display vanishingly small myogenic potential. In addition, in all cases, the contribution of these cells to the maintenance or repair of skeletal muscle under physiologic conditions is uncertain, and their therapeutic potential has not been clearly established. Moreover, recent data suggest that satellite cells are alone sufficient to mediate extensive regeneration of damaged adult skeletal muscle in vivo (Collins et al., 2005). Thus, in further assessing the intrinsic myogenic function of these populations, it will be essential to exclude the possibility that their activity derives from “contamination” with a small subset of highly myogenic satellite cells or their progeny or from a change in developmental potential induced by cell culture. The use of lineage-tracing methods (Gros et al., 2005; Relaix et al., 2005) and single myofiber transplantation (Collins et al., 2005) will be a key first step in determining the myogenicity of these populations in relationship to conventional satellite cells.

Bone marrow cells (Ferrari et al., 1998; Fukada et al., 2002; Gussoni et al., 1999; LaBarge and Blau, 2002), and even single hematopoietic stem cells (Camargo et al., 2003; Corbel et al., 2003; Sherwood et al., 2004b), also have been reported to contribute to myofibers when injected directly into injured muscle or intravenously into irradiated injured or dystrophic animals. The frequency with which these unexpected contributions to skeletal muscle have been detected has varied widely and, while generally quite low (<1% of total myofibers; Camargo et al., 2003; Corbel et al., 2003; Ferrari et al., 1998; Gussoni et al., 1999; Sherwood et al., 2004b), has been reported in some cases to reach levels of 5%–12% of differentiated cells (Abedi et al., 2004; LaBarge and Blau, 2002). In this regard, it is worth noting that the method of detection of donor-derived myofibers can significantly influence the estimation of donor contributions, and assay systems employing highly diffusible markers, such as GFP (Jockusch and Voigt, 2003), cannot accurately quantify the numbers of donor myonuclei incorporated due to spread of the marker throughout the myofiber. In any case, such observations initially generated a great deal of excitement, suggesting that bone marrow could represent a reasonably accessible, novel source of regenerative cells for muscle repair; however, as with other nonsatellite cell populations, the physiological significance of bone marrow contributions to skeletal muscle remains uncertain (Ferrari et al., 2001; Gussoni et al., 2002), and accumulating evidence suggests that these events may in fact represent an accidental or even pathological re-
sponse elicited by severe muscle damage and inflammation (see below).

Interestingly, while early efforts aimed at eliciting myogenic activity from bone marrow cells focused largely on the hematopoietic lineages, recent studies have suggested that nonhematopoietic elements isolated from adult bone marrow or from embryonic sites of hematopoietic development might eventually be harnessed for therapeutic skeletal muscle regeneration. For instance, cultured “mesangioblasts” (Minasi et al., 2002), a subset of blood vessel-associated cells originating from the embryonic dorsal aorta region, which have been shown to generate multiple mesodermal cell types following in vivo transplantation, can, when injected in aortic arches into dystrophic α-sarco-glycan knockout mice, contribute to myofiber formation and significantly improve muscle function (Sampaiolesi et al., 2003). In addition, clonal, cultured marrow-derived stromal cells, lacking expression of CD34, c-Kit, and CD45, have been shown to participate in myogenesis in vitro and in vivo, regenerating myofibers and sublaminar Pax-7+ satellite cells in immunocompromised dystrophic mice (Dezawa et al., 2005). Significantly, the myogenic contributions from marrow stromal cells observed in this study were much more robust than those typical of BM-derived HSC or hematopoietic progenitor cell subsets; however, it is important to note that the activation of a myogenic program by these stromal cells absolutely required in vitro induction, first by culturing the cells with certain growth factors and then by ectopic expression of constitutively active Notch-1 (Dezawa et al., 2005). Thus, while this work suggests a promising therapeutic potential of marrow stromal cells, lacking expression of CD34, c-Kit, and CD45, these data do not necessarily suggest that analogous populations of hematopoietic progenitor cells is clearly of interest in synthesizing recent literature and developing a further understanding of skeletal muscle biology and regeneration. At present the molecular mediators, as well as the overall significance, of these rare events remain unclear. Future studies using coculture or transplantation models will be required to identify the secreted factors, cell-surface receptors, and signaling pathways involved in blood-cell/muscle-cell fusion. Knowledge of these mechanisms ultimately should allow experiments to block its occurrence and thereby test its physiologic importance.

Prospective Isolation of Muscle Precursor Cells

We recently reported a methodology that permits the prospective isolation by fluorescence-activated cell sorting (FACS) of highly myogenic muscle precursor cells from other cell populations contained within the satellite-cell compartment (Sherwood et al., 2004a). Combinatorial analysis of multiple cell-surface markers indicated that autonomously myogenic colony-forming cells (CFC) were highly enriched among the CD45- Sca-1+ Mac-1− CXCR4+ β1-integrin+ (CSM4B) subset of myofiber-associated satellite cells, and that individual CSM4B cells efficiently form myogenic colonies, which express myosin heavy chain (MyHC) upon induction of myogenic differentiation in vitro. CSM4B cells are contained within a population of cells (CD45− Sca-1− CD34+) that also generates myofibers in vivo upon intramuscular injection and expresses mRNA encoding the myogenic transcription factors MyoD, Myf-5, and Pax-7 (Sherwood et al., 2004a).

The precise relationship of the CSM4B subset of myofiber-associated cells to previously described satellite cells is clearly of interest in synthesizing recent literature and developing a further understanding of skeletal muscle biology and regeneration. These cells coexpress CD34 and c-met, previously reported satellite-cell surface markers (Beauchamp et al., 2000; Cornellison et al., 2001), but are not contained in c-kit+, CD13+, CD71+, Flk-1+, CD105+, CD44+, α1-integrin+, or α6-integrin+ populations of myofiber-associated cells. They also fail to express the pan-hematopoietic marker CD45 and the surface protein Sca-1, in either resting or regenerating (2 days following cardiotoxin injection) muscle; both CD45 and Sca-1 have been reported by others (Polesskaya

Mechanisms of Bone Marrow Contribution to Skeletal Muscle: Cell Fusion or Transdifferentiation?

In most studies in which hematopoietic or bone marrow-derived contributions to skeletal muscle have been detected, significant muscle injury has been necessary, except in particular muscles (e.g., panniculus carnosus) (Corbel et al., 2003; Sherwood et al., 2004b). The mechanisms underlying these contributions have been an area of intense research. While initial reports appeared to favor the direct “transdifferentiation” of transplanted BM cells to generate satellite cells (Fukada et al., 2002; LaBarge and Blau, 2002), more recent studies have indicated that donor-marker-expressing myofibers arise via fusion of donor hematopoietic cells with host muscle cells (Camargo et al., 2003; Doyonnas et al., 2004; Sherwood et al., 2004b) (Figure 1B). While the precise cell types involved in these fusion events have not been fully defined, transplantation of BM cells from transgenic mice expressing Cre in a hematopoietic lineage-restricted manner suggests that at least one of the fusion partners is likely to be a committed blood myeloid cell (Camargo et al., 2003; Doyonnas et al., 2004). The stage of differentiation at which muscle-lineage cells participate in heterotypic cell fusions with infiltrating hematopoietic cells remains unknown (Figure 1B). However, when considering the mechanism by which hematopoietic cells fuse with muscle cells, it is important to remember that normally muscle is repaired by fusion of myoblasts with each other, with nascent myotubes, and/or with damaged multinucleated fibers. Thus, macrophages and other inflammatory cells known to infiltrate injured muscle fibers (Stauber et al., 1988) almost certainly are exposed to physiologic fusogenic signals. Significantly, macrophages themselves are known to undergo cell-cell fusion both physiologically, to generate osteoclasts, and pathologically, to generate multinucleated giant cells (Vignery, 2000). Therefore, heterotypic cell fusion between endogenous muscle cells and infiltrating blood cells may occur via mechanisms that normally allow the homotypic fusion of these cells in the context of tissue maintenance and repair. At present the molecular mediators, as well as the overall significance, of these rare events remain unclear. Future studies using coculture or transplantation models will be required to identify the secreted factors, cell-surface receptors, and signaling pathways involved in blood-cell/muscle-cell fusion. Knowledge of these mechanisms ultimately should allow experiments to block its occurrence and thereby test its physiologic importance.
Biochemical Pathways Regulating Muscle Regeneration

Muscle remodeling involves myogenesis, reinnervation, and revascularization and is regulated by multiple biochemical pathways, including those initiated by inflammatory cytokines, growth factors, and the evolutionarily conserved Notch, Wnt, and Sonic Hedgehog (Shh) signaling pathways (Conboy and Rando, 2002; Husmann et al., 1996; Pola et al., 2003; Polesskaya et al., 2003; Tidball, 2005). Muscle repair coincides with injury-induced inflammation, and some inflammatory cytokines, such as IL-4, LIF, TGF-β, IL-6, and TNF-α, regulate myogenic potential (Tidball, 2005). Damaged muscle produces monocyte and macrophage chemo-attractants, and blockade of inflammatory cell infiltration impairs muscle regeneration (Chazaud et al., 2003; Jejurikar and Kuzon, 2003; Lescaudron et al., 1999), possibly due to a reduction in macrophage-secreted factors inducing myoblast proliferation (Bondesen et al., 2004; Robertson et al., 1993). In addition to initiating the inflammatory response, injury promotes the release of growth factors that bind to extracellular matrix (ECM) proteins, such as proteoglycans (Husmann et al., 1996). This process involves the activity of matrix metalloproteinases, recently shown to play a role in muscle repair (Carmeli et al., 2003). The most studied growth factors participating in muscle maintenance and regeneration are FGFs, HGF, IGF-1, and GDF8/myostatin (Heszele and Price, 2004; Husmann et al., 1996; Lee, 2004; Miller et al., 2000). FGF-2 and HGF promote proliferation of myogenic progenitors and delay their differentiation, in part by inhibiting the expression of myogenic regulatory factors, such as MyoD (Maley et al., 1994; Miller et al., 2000). Both growth factors require heparan sulfate proteoglycans for signaling via their receptors, and syndecan-3 and -4 have been identified as the relevant cell-surface proteoglycans expressed by satellite cells (Cornelison et al., 2001). IGF-1 promotes myogenic differentiation and enhances protein synthesis in differentiated myofibers by activating the translation factor 4E-BP and the ribosomal protein S6 kinase (p70S6K) and by inhibiting muscle-specific E3 ligases that promote protein degradation (reviewed in Heszele and Price, 2004). These mechanisms and the antiapoptotic effects of IGF-1 via the suppression of caspases and activation of Akt (Downward, 2004; Lawlor and Rotwein, 2000) likely explain why IGF-1 attenuates experimentally induced muscle wasting (Shavlakadze et al., 2005). On the other end of the proliferative spectrum, the muscle-specific TGF-β family member, GDF8, inhibits cell-cycle progression in myogenic progenitors during embryonic development and in adult muscle via induction of p21 and suppression of the cyclin-dependent kinase CDK25 (McCroskery et al., 2003; McPherron et al., 1997; Thomas et al., 2000; Zimmers et al., 2002).

The appropriate expansion followed by the timely differentiation of myogenic progenitor cells during muscle repair appear to be regulated by the same conserved mechanisms that orchestrate embryonic organogenesis. Namely, proliferation of satellite cells in response to muscle injury is positively regulated by the Notch pathway, while terminal myogenic differentiation of these cells requires the inhibition of Notch by its intracellular antagonist Numb (Conboy and Rando, 2002). Notch receptor is expressed in quiescent satellite cells, and Notch signal transduction responsible for satellite-cell activation is initiated by the upregulation of Notch ligand, Delta, on the fibers adjacent to the damaged muscle and on the satellite cells themselves (Conboy et al., 2003; Conboy and Rando, 2002). The recent demonstration that BM-derived stromal cells become myogenic after stable expression of constitutively active Notch-1 (Dezawa et al., 2005) may suggest that activation of the Notch pathway generally regulates specific differentiation of organ precursor cells toward a myogenic lineage when other myogenic factors, such as FGF-2, are present.

In addition to Notch, Shh mRNA and protein, as well as the Patched receptor, become upregulated in regenerating skeletal muscle (Pola et al., 2003). Moreover, ectopic Shh appears to ameliorate experimentally induced muscle atrophy (Alzghoul et al., 2004), thus demonstrating that yet another classic regulator of embryonic development likely also participates in adult myogenesis. Additionally, Wnt signaling has been reported to be important for the presence of CD45+ cells in regenerating adult muscle, although the physiologic myogenic potential of these cells remains unclear (Polesskaya et al., 2003; Sherwood et al., 2004). Future experiments that decipher how muscle regulates its own inflammatory response and clarify the temporal and spatial crosstalk between Notch, Shh, and Wnt pathways will be instrumental for providing a better understanding of how cell fate is determined during muscle repair.
Satellite-Cell Activity in Diseased Muscle

In certain pathological states, including congenital myopathies, denervation, and muscle atrophy, satellite-cell numbers and proliferative potential may decrease (Jejurikar and Kuzon, 2003). In muscular dystrophy (MD), repeated cycles of muscle regeneration, brought on by repeated loss of differentiated tissue, may lead to an early loss of the proliferative potential of satellite cells in these patients, and a subsequent failure to maintain muscle homeostasis (Luz et al., 2002). Although the underlying mechanism for this loss of satellite-cell responsiveness in diseased muscle has not been fully elucidated, these findings indicate that under particular circumstances satellite cells may be functionally exhausted. Satellite-cell exhaustion may relate, at least in part, to shortening of telomere ends after repeated rounds of DNA replication (Collins et al., 2003; Di Donna et al., 2003), to recurrent exposure to inflammatory conditions and/or oxidative stress (Renault et al., 2002), to an accumulation of mutations in key satellite-cell regulatory genes, introduced during repeated rounds of proliferation, or to a combination of these and other factors. Nonmyogenic cells in the muscle may also contribute to failed muscle regeneration, as fibroblasts in dystrophic patients have been shown to secrete increased levels of IGF-1 binding proteins, which could sequester this cytokine away from myogenic cells (Melone et al., 2000). A better understanding of the dynamic interplay among distinct populations of cells resident in the muscle and recruited by muscle damage will aid in developing a full picture of the complex cellular networks responsible for myogenesis in healthy and diseased muscle and for designing therapeutic strategies to promote muscle repair.

Age-Related Changes in the Molecular Regulation of Satellite Cell Activity

One characteristic of aging is a decline in the ability of organ stem cells to repair damaged tissues. Adult skeletal muscle is a perfect example of a tissue that robustly regenerates throughout adult life but fails to do so in old age (Grounds, 1998). The reason for this decline in regenerative potential is not completely understood and may involve both intrinsic molecular changes in the stem cells themselves and/or alterations in their aged environment.

As mentioned above, muscle repair relies on Notch activity, which is necessary and sufficient for the activation of satellite cells (Artavanis-Tsakonas et al., 1999; Conboy et al., 2003; Conboy and Rando, 2002). Importantly, Notch receptor continues to be expressed in aged satellite cells, while injury-specific induction of the Notch ligand Delta, and therefore subsequent signal transduction, fail with age, resulting in grossly inefficient regeneration of old muscle tissue (Conboy et al., 2003). Strikingly, productive tissue repair can be restored to old muscle by enforced activation of Notch, while the repair of young muscle is severely perturbed when Notch signaling is inhibited (Conboy et al., 2003). Therefore, it seems that Notch is the key age-related determinant of muscle regenerative potential.

Is the age-related decline in satellite cell regenerative potential intrinsic or dependent on the cell environment? In early muscle transplantation studies, small minced or intact muscle from young or old rodents was transplanted into either young or old muscle beds, and the ability of donor muscle pieces to regenerate in the middle of either young or old muscle beds was examined. Amazingly, the efficiency of muscle regeneration was clearly determined in these experiments by the age of the host environment, rather than by the age of the muscle donor (Carlson and Faulkner, 1989; Zacks and Sheff, 1982). In these important studies, the small transplanted muscle was physically isolated from the host satellite cells, revealing the effects of prevalent local and organismal environments on the regenerative potential of the donor satellite cells.

In more recent studies, the age of the systemic environment likewise dominated over the intrinsic age-related regenerative properties of satellite cells when the efficiency of muscle repair was examined in young and old mice with a shared blood circulation (Conboy et al., 2005). Significantly, regeneration-specific Delta-Notch signaling, appropriate activation of satellite cells, and general success in muscle repair were all rejuvenated by the exposure of aged tissue to a young systemic milieu (Conboy et al., 2005). In concert with the above-mentioned pivotal role of satellite cells as muscle stem cells, the aged satellite cells endogenous to the old muscle successfully engaged in tissue repair without any recruitment of young cells from the shared circulation (Conboy et al., 2005). These data strongly suggest that the molecular pathways responsible for muscle repair are regulated by systemic factors and that these factors change with age in ways precluding the activation of satellite cells.

Multiple lines of evidence suggest that many cell-intrinsic changes occur with tissue aging, including the accumulation of oxidative damage, a decline in genome maintenance, and diminished mitochondrial function (Ames, 2004; Golden et al., 2002; Hasty et al., 2003). The rejuvenation of aged stem and progenitor cells by the young extrinsic milieu, even in the presence of these age-related changes, suggests the intriguing possibility that the aging of organ stem cells might be regulated extrinsically and that the molecular changes underlying the loss of the tissue-regenerative potential with age can be reversed or overcome if the stem cell niche is young. Future identification of the relevant extrinsic age-related components will be instrumental for therapies aimed at enhancing the regenerative potential of organ stem cells in aged individuals.

Future Avenues and Perspectives

It is quite clear that endogenous skeletal muscle satellite cells associated with myofibers account for most, if not all, physiologic muscle-regenerative potential and likely represent muscle stem cells. Recent advances have allowed a more refined determination of their origin, position in the myogenic cell lineage, and molecular pathways regulating their function. Other avenues of muscle repair, e.g., by bone marrow-derived cells, may exist; however, unambiguous determination of the precise cell types and specific fusion and reprogramming mechanisms involved in this process will be needed in order to establish whether such cells form muscle tis-
sue under physiologic conditions or can be used therapeutically. Current advances in our understanding of the cellular and molecular mechanisms regulating cell-fate determination and tissue specification during adult muscle repair have indicated a remarkable conservation of developmentally regulated signal transduction pathways, and age-related analysis of these pathways indicates that at least some of them deteriorate in old muscle, causing ineffective tissue repair. The identification of age-related systemic factors that regulate the regenerative capacity of organ stem cells will improve our understanding of aging as a conserved biological process and will help to develop therapies for the enhancement of the regenerative potential often lost in old age or disease.

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