The Molecular Regulation of Muscle Stem Cell Function

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Muscle satellite cells are responsible for the postnatal growth and robust regeneration capacity of adult skeletal muscle. A subset of satellite cells purified from adult skeletal muscle is capable of repopulating the satellite cell pool, suggesting that it has direct therapeutic potential for treating degenerative muscle disease. Satellite cells uniformly express the transcription factor Pax7, and Pax7 is required for satellite cell viability and to give rise to myogenic precursors that express the basic helix-loop-helix (bHLH) transcription factors Myf5 and MyoD. Pax7 activates expression of target genes such as *Myf5* and *MyoD* through recruitment of the Wdr5/Ash2L/MLL2 histone methyltransferase complex. Extensive genetic analysis has revealed that Myf5 and MyoD are required for myogenic determination, whereas myogenin and MRF4 have roles in terminal differentiation. Using a Myf5-Cre knockin allele and an R26R-YFP Cre reporter, we observed that in vivo about 10% of satellite cells only express Pax7 and have never expressed Myf5. Moreover, we found that Pax7⁺/Myf5⁻ satellite cells give rise to Pax7⁺/Myf5⁺ satellite cells through basal-apical asymmetric cell divisions. Therefore, satellite cells in skeletal muscle are a heterogeneous population composed of satellite stem cells (Pax7⁺/Myf5⁻) and satellite myogenic cells (Pax7⁺/Myf5⁺). Evidence is accumulating that indicates that satellite stem cells represent a true stem cell reservoir, and targeting mechanisms that regulate their function represents an important therapeutic strategy for the treatment of neuromuscular disease.

In adult skeletal muscle, satellite cells reside beneath the basal lamina of muscle closely juxtaposed to muscle fibers and comprise 2–7% of the nuclei associated with a particular fiber. Satellite cells are normally mitotically quiescent, but they are activated (i.e., enter the cell cycle) in response to stress induced by weight bearing or by trauma such as injury (Bischoff 1994). The descendants of activated satellite cells, called myogenic precursor cells (MPCs), undergo multiple rounds of division before fusion and terminal differentiation. Satellite cells are distinct from their daughter myogenic precursor cells as defined by biological, biochemical, and genetic criteria (Charge and Rudnicki 2004). Activated satellite cells also generate progeny that restore the pool of quiescent satellite cells (Kuang et al. 2008).

Our laboratory discovered that satellite cells express the transcription factor Pax7 (Seale et al. 2000). Satellite cells also express vascular cell adhesion molecule 1 (VCAM-1) (Rosen et al. 1992), *c-met* (receptor for hepatocyte growth factor [HGF]), M-cadherin protein (Irintchev et al. 1994; Cornelison and Wold 1997), neural cell adhesion molecule 1 (NCAM1) (Bischoff 1994), Foxk1 (Garry et al. 1997), CD34 (Beauchamp et al. 2000), and syndecans 3 and 4 (Cornelison et al. 2001). Our laboratory also recently identified several additional novel genes including *IgSF4*, *neurtin*, *Hoxc10*, *TcR-β*, *Klra18*, *Itm2α*, *G0S2*, and *MEGF10* that are expressed in satellite cells in vivo but are not expressed by primary myoblasts (Seale et al. 2004a). Additionally, numerous growth factors such as fibroblast growth factor 6 (FGF6), HGF, bone morphogenetic proteins (BMPs), and nitric oxide (NO) have been suggested to have roles in stimulating satellite cell activation (Charge and Rudnicki 2004). Nevertheless, the precise molecular mechanisms regulating satellite cell function remain poorly understood.

Early experiments using quail-chick chimeras suggested that satellite cells were derived from the somite (Armand et al. 1983). Recent experiments support this work and indicate that the progenitors of satellite cells originate in embryonic somites as Pax3/Pax7-expressing cells (Kassar-Duchossoy et al. 2005; Relaix et al. 2005). However, studies by De Angelis et al. (1999) provided evidence that satellite cells may also be derived from cells associated with the embryonic vasculature including the dorsal aorta. In the adult, results from several laboratories support the notion that under conditions of severe tissue damage and ischemia, satellite cells can be derived from so-called adult stem cells during regeneration (Asakura et al. 2002; LaBarge and Blau 2002; Polesskaya et al. 2003). However, under physiological conditions, satellite cells are the primary source of myogenic progenitors (Parise et al. 2008).

The maintenance of satellite cell numbers in aged muscle after repeated cycles of degeneration and regeneration has been interpreted to support the notion that satellite cells possess an intrinsic capacity for self-renewal (Bischoff 1994). Asymmetric distribution of Numb protein in daughters of satellite cells in cell culture has been implicated in the asymmetric generation of distinct daughter cells for self-renewal or differentiation (Conboy and Rando 2002). However, whether satellite cells are true stem cells or are dedifferentiated myoblasts (Zammit et al. 2004) has remained unresolved. Notably, recent work from our laboratory has defined a subset of satellite cells that form a stem cell reservoir for the satellite cell compartment (Kuang et al. 2007).

PAX7 AND REGENERATIVE MYOGENESIS

The Pax Family of Developmental Control Transcription Factors

The paired-box family of transcription factors (Pax1–9) has important functions in the regulation of the develop-

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ment and differentiation of diverse cell lineages during embryogenesis (Mansouri et al. 1999). *Pax7* and the closely related *Pax3* gene are paralogs with almost identical amino acid sequences and partially overlapping expression patterns during mouse embryogenesis (Jostes et al. 1990; Goulding et al. 1991). Notably, *Pax3* has an essential role in regulating the developmental program of *MyoD*-dependent migratory myoblasts during embryogenesis (Maroto et al. 1997; Tajbakhsh et al. 1997). More recently, *Pax3*⁺/*Pax7*⁺ progenitors originating in the embryonic somite have been suggested to be the precursors of satellite cells in adult muscle (Kassar-Duchossoy et al. 2005; Relaix et al. 2005).

Pax7 and *Pax3* proteins bind similar if not identical sequence-specific DNA elements, suggesting that they regulate similar sets of target genes (Schafer et al. 1994). Furthermore, increased expression and gain-of-function mutations in both *Pax3* and *Pax7* are associated with the development of alveolar rhabdomyosarcomas, indicating that both molecules regulate similar activities in the myogenic program (Bennicelli et al. 1999). Although the *Pax3* and *Pax7* proteins are structurally similar, analysis of null mutations in mice indicates that they are required for the development of a number of distinct cell lineages (Mansouri et al. 1996; Conway et al. 1997; Tremblay et al. 1998; Seale et al. 2000) and appear to have nonredundant roles in myogenesis (Seale et al. 2000, 2004b; Oustanina et al. 2004; Kassar-Duchossoy et al. 2005; Relaix et al. 2005).

Spotch (*Sp*) mice, lacking a functional *Pax3* gene, do not survive to term and fail to form limb muscles due to impaired migration of *Pax3*-expressing cells originating from the somite (Daston et al. 1996; Tremblay et al. 1998). Compound mutant *Sp/Myf5*^{−/−} mice do not express *MyoD* in their somites, suggesting that *Myf5* and *Pax3* function upstream of *MyoD* in myogenic determination (Tajbakhsh et al. 1997). Forced expression of *Pax3* induces *MyoD* expression and subsequent myogenesis in nonmuscle tissues in avian embryos (Maroto et al. 1997). However, ectopic expression of *Pax3* in C2C12 myoblasts efficiently inhibits myogenic differentiation (Epstein et al. 1995). Coexpression of *MyoD* and *Pax3* is not observed in the mouse myotome (Williams and Ordahl 1994). Therefore, *Pax3* was suggested to function as an indirect upstream factor that induced migration or other cellular changes to facilitate subsequent induction of *MyoD* transcription (Borycki and Emerson 1997). Contrary to this notion, a *Pax3-FKHR* fusion was observed to activate many muscle regulatory genes including *Myf5* following expression in NIH-3T3 cells (Khan et al. 1999). Notably, *Pax7* was recently demonstrated to bind a 57-kb regulatory element upstream of the *Myf5* transcription start site (Bajard et al. 2006). These data together with the coexpression of *Pax3* and *Pax7* in somite-derived pro-satellite cells (Kassar-Duchossoy et al. 2005; Relaix et al. 2005) suggest the hypothesis that *Pax3* mediates the migratory phase of the lineage, whereas *Pax7* is required to achieve their myogenic potential.

Pax7 Is Required for the Myogenic Specification of Satellite Cells

Our laboratory discovered that satellite cells express the transcription factor *Pax7* and that *Pax7* has a critical

role in regulating the function of satellite cells (Seale et al. 2000; Kuang et al. 2006). *Pax7* is specifically expressed in satellite cells in adult muscle, and their daughter myogenic precursor cells in vivo, and primary myoblasts in vitro. Cell culture and electron microscopic analysis indicated ablation of satellite cells in *Pax7*^{−/−} skeletal muscle. Fluorescence-activated cell sorting (FACS)/Hoechst analysis demonstrated that the proportion of muscle-derived side population (SP) cells, a putative adult stem cell population, was unaffected. These results demonstrate that satellite cells and muscle-derived SP cells represent distinct cell populations and revealed an essential role for *Pax7* in specifying the satellite cell myogenic lineage functioning upstream of the *MyoD*-family of bHLH factors (Seale et al. 2000, 2004b; Poleskaya et al. 2003).

We performed an extensive analysis of *Pax7*^{−/−} mice and have confirmed the progressive ablation of the satellite cell lineage in multiple muscle groups (Seale et al. 2000; Oustanina et al. 2004). Small *Pax7*-deficient cells do survive in the satellite cell position, but these cells arrest and die upon entering mitosis. *Pax7*^{−/−} muscles are reduced in size, the fibers contain approximately 50% the normal number of nuclei, and fiber diameters are significantly reduced. Together, these data confirm an essential role for *Pax7* in regulating the myogenic potential of satellite cells (Kuang et al. 2006).

In previous studies, we investigated the potential of atypical nonsatellite cell progenitors to participate in muscle regeneration. CD45⁺/Sca1⁺ cells purified from regenerating wild-type muscle express *Pax7* and give rise to skeletal myoblasts. In contrast, CD45⁺/Sca1⁺ cells from regenerating *Pax7*^{−/−} muscle do not undergo myogenic progression unless exposed to Wnts (Poleskaya et al. 2003). Retroviral expression of *Pax7* in CD45⁺/Sca1⁺ cells from uninjured muscle induced the formation of myogenic progenitors expressing *Myf5* and *MyoD*, which differentiated into myogenin and myosin-heavy-chain-expressing myocytes (Seale et al. 2004b). Together, these results demonstrate that *Pax7* is required for the myogenic specification of muscle-derived adult stem cells during regenerative myogenesis (Poleskaya et al. 2003; Seale et al. 2004b). It is important to note that although atypical myogenic cell progenitors have the proven potential to participate to some degree in muscle regeneration under conditions of severe trauma (Peault et al. 2007), our experiments strongly support the contention that under physiological conditions, the growth and regeneration of skeletal muscle are mediated largely if not exclusively by muscle satellite cells (Parise et al. 2008).

In summary, satellite cells arise from a novel population of muscle progenitor cells that originate in the central domain of the dermomyotome. These progenitors express *Pax3* and *Pax7* (Gros et al. 2005; Relaix et al. 2005), and although neither their emergence nor their maintenance requires *Pax3* function (Kassar-Duchossoy et al. 2005), recent studies have demonstrated that *Pax7* is uniquely indispensable (Bajard et al. 2006). In the absence of *Pax7*, satellite cells die and thus fail to repopulate their niche (Relaix et al. 2005, 2006; Kuang et al. 2006). *Pax7* is therefore essential for the formation and maintenance of a population of functional satellite cells (Fig. 1).

Figure 1. Transcriptional hierarchy regulating the developmental program of satellite cells in skeletal muscle. Progenitors of satellite stem cells originate in the somite as Pax3- and or Pax7-expressing progenitors. Satellite stem cells express Pax7, whereas satellite myogenic cells have additionally activated Myf5 transcriptional competence, as revealed by expression of *Myf5-lacZ* and *Myf5-cre* knockin alleles. Following activation and entrance into the cell cycle, myogenic precursor cells express Myf5 and MyoD. Induction of myogenin and Mef2c together with down-regulation of Myf5 and then MyoD mark withdrawal from the cell cycle and entrance into the terminal differentiation program.

Molecular Regulation of Pax7 Function

The mechanisms by which Pax7 activates downstream target genes were difficult to address experimentally due to the relatively weak *trans*-activation properties of Pax7 (Bennicelli et al. 1999). We therefore undertook a multi-level approach to investigate the molecular determinants regulating Pax7 function in satellite cells (McKinnell et al. 2008). We used gene expression analysis to identify several novel and strongly regulated candidate target genes. Together with several other target genes (*Plagl1*, *Lix1*, *Syne2*, *Cipar1*, *Trim54*, and *Mest*), and *Myf5* was confirmed as an important Pax7 target gene using small interfering RNA (siRNA), FACS of Pax7-deficient satellite cells, and chromatin immunoprecipitation (ChIP) of Pax7 over regulatory sequences. Tandem affinity purification (TAP) and mass spectrometry were used to purify Pax7 together with associated protein complexes. This analysis revealed that Pax7 associates with the Wdr5/Ash2L/MLL2 histone methyltransferase (HMT) complex (McKinnell et al. 2008), which directs methylation of histone H3 lysine 4 (H3K4).

Pax3 binds at approximately 57.5 kb upstream of the transcriptional start of the *Myf5* gene (Bajard et al. 2006). Pax7 similarly binds and recruits the Wdr5/Ash2L/MLL2 HMT complex to binding sites in the regulatory sequences of target genes (McKinnell et al. 2008). Importantly, binding of the Pax7-HMT complex results in H3K4 trimethylation of surrounding chromatin (McKinnell et al. 2008). Together, these experiments indicate that Pax7 enforces satellite cell commitment by recruiting an HMT complex to Myf5, resulting in transcriptional activation. Notably, Pax family genes are essential for the embryonic specification of diverse tissues; thus, Pax recruitment of HMT complexes could be a conserved mechanism for seeding lineage-specific gene expression programs during development.

MYOGENIC bHLH REGULATORY FACTORS

The MyoD Family of Transcription Factors and Myogenesis

The myogenic regulatory factors (MRFs) form a group of bHLH transcription factors (MyoD, Myf5, myogenin,

and MRF4) that regulate the skeletal muscle developmental program (Perry and Rudnick 2000). MRF proteins contain a conserved basic DNA-binding domain that binds the E box, a DNA motif that contains the core E-box sequence CANNTG (Weintraub et al. 1991). The HLH domain mediates dimerization with other HLH-containing proteins (e.g., factors encoded by the E2-2 and E2-5 genes: E12, E47, HEB, and ITF2) (Barndt and Zhuang 1999). Myf5 and MyoD are expressed in proliferating myoblasts and are subject to distinct cell cycle regulation (Kitzmann et al. 1998). MyoD is up-regulated shortly after induction of differentiation followed by myogenin. Myf5 and MyoD levels progressively decrease after this point. The levels of myogenin increase through differentiation, followed by up-regulation of MRF4 several days after the induction of differentiation as myogenin levels decrease (Megeney and Rudnicki 1995).

The Mef2 class of transcription factors also has an important regulatory role in the control of muscle-specific transcription. There exists four alternatively spliced *Mef2* genes: *Mef2A*, *Mef2B*, and *Mef2D* are expressed ubiquitously, whereas *Mef2C* is restricted to muscle, brain, and spleen. Mef2 sites are found in the promoters of many muscle-specific genes including *myogenin*, suggesting that the MRFs and Mef2s form an autoregulatory network (Berkes and Tapscott 2005). In addition, Mef2 proteins and MRFs synergistically coactivate E-box- and Mef2-site-containing promoters (Kaushal et al. 1994; Molkentin et al. 1995; Naidu et al. 1995). During development, Mef2 expression follows myogenin expression, suggesting that Mef2 proteins likely act following differentiation (Edmondson et al. 1994). Moreover, mice lacking myogenin contain virtually no mature myotubes but express normal levels of MyoD and Mef2C, suggesting that expression of Myf5 or MyoD induces myogenin and MEF2 expression upon initiation of terminal differentiation (Venuti et al. 1995). Importantly, Mef2d recruits the Ash2L histone methyltransferase complex to target genes, and this recruitment is regulated by p38 signaling (Rampalli et al. 2007).

The introduction of null mutations in the MyoD family into the germ line of mice revealed the hierarchical relationships existing among the MRFs and established that functional overlap is a feature of the MRF regulatory network. Newborn mice lacking a functional MyoD gene display no overt abnormalities in muscle but express about fourfold higher levels of Myf5 (Rudnicki et al. 1992). Newborn Myf5-deficient animals are also viable and display apparently normal muscle (Braun et al. 1992; Kaul et al. 2000). Muscle development in the trunk of embryos lacking Myf5 is delayed until the onset of MyoD expression, which occurs with somewhat delayed kinetics (Braun et al. 1992; Tajbakhsh and Cossu 1997). Strikingly, newborn mice deficient in both Myf5 and MyoD are totally devoid of myoblasts and myofibers. Thus, Myf5 and MyoD are required for the determination of myogenic precursors and act upstream of myogenin and MRF4 (Rudnicki et al. 1993).

Mice lacking myogenin are immobile and die perinatally due to deficits in myoblast differentiation, as evidenced by an almost complete absence of myofibers (Hasty et al. 1993; Nabeshima et al. 1993). However, normal numbers of MyoD-expressing myoblasts are present and these are

organized in groups similar to wild-type muscle. Myogenin-deficient embryos form primary myofibers normally but appear unable to form secondary myofibers (Venuti et al. 1995). Therefore, myogenin has an essential *in vivo* role in the terminal differentiation of myoblasts.

Mice carrying different targeted MRF4 mutations display a range of phenotypes consistent with a late role for MRF4 in the myogenic pathway (Zhang et al. 1995; Rawls et al. 1998). Interestingly, mice lacking both MyoD and MRF4 display a phenotype similar to the myogenin-null phenotype (Rawls et al. 1998). Therefore, MRF4 function may be substituted by the presence of myogenin but only in the presence of MyoD. Notably, MRF4 appears to have a role as a determination factor in a subset of myocytes in the early somite and as a differentiation factor in later muscle fibers (Kassar-Duchossoy et al. 2004).

Gene targeting and expression analysis have therefore suggested the functional classification of the MRFs into two groups: In the first group, Myf5 and MyoD act as determination factors, and in the second group, myogenin and MRF4 act as differentiation factors.

Myf5 and MyoD Regulate the Development of Distinct Myogenic Lineages

The temporal-spatial patterns of myogenesis in Myf5- and MyoD-deficient embryos provide strong evidence for unique roles of Myf5 and MyoD in the development of epaxial and hypaxial musculature (Kablar et al. 1997, 1998, 2003; Kablar and Rudnicki 1999). Embryos lacking MyoD display normal development of paraspinal and intercostal muscles in the body proper, whereas muscle development in limb buds and branchial arches is delayed by about 2.5 days. In contrast, embryos lacking Myf5 display normal muscle development in limb buds and branchial arches and a marked delay in development of paraspinal and intercostal muscles. Although MyoD mutant embryos exhibit delayed development of limb musculature, the migration of Pax-3-expressing cells into the limb buds and subsequent induction of Myf5 in myogenic precursors occur normally. These results indicate that Myf5 expression in the limb is insufficient for the normal progression of myogenic development.

The phenotype of Myf5- and MyoD-deficient animals strongly supports the notion that the putative myogenic lineages that give rise to epaxial and hypaxial musculature have different requirements for Myf5 or MyoD for appropriate development. Importantly, analysis of primary myoblasts expressing either MyoD (wild type) or Myf5 (MyoD^{-/-}) has revealed striking differences in gene expression, morphology, and differentiation potential and indicate that Myf5 and MyoD are not functionally equivalent (Sabourin et al. 1999; Ishibashi et al. 2005). Indeed, recent studies using conditional ablation in mice have confirmed that Myf5 determines a distinct myogenic cell population (Gensch et al. 2008; Halder et al. 2008). Therefore, these experiments clearly support the hypothesis that Myf5 and MyoD have important and unique roles in the development of epaxial and hypaxial musculature (Kablar et al. 1997, 1998, 1999, 2003).

Molecular Regulation of Myogenic Factor Function

Initiation of myogenic differentiation is characterized by cell cycle withdrawal, stimulation of MyoD transcriptional activity, and sequential induction of myogenin and Mef2 expression (Berkes and Tapscott 2005). However, at odds with this simplistic model are data from gene expression and ChIP-on-Chip studies revealing that MyoD directs multiple subprograms of gene expression, each of which is uniquely regulated (Bergstrom et al. 2002; Blais et al. 2005; Cao et al. 2006). For example, activation of a subset of late-activated MyoD target genes requires p38 α / β kinases, whereas expression of an immediate-early target, myogenin, requires formation of a MyoD-Pbx1 complex (Berkes et al. 2004; Penn et al. 2004). Genes expressed early in differentiation, such as *myogenin*, are induced primarily by MyoD (Cao et al. 2006). In contrast, MyoD initiates regional histone modifications at late-expressed targets, such as *MyHC* and *MLC*. However, full expression of *MyHC* and *MLC* requires myogenin transcriptional activity. Thus, MyoD recruits different complexes to unique binding sites on specific genes at different times to orchestrate gene expression during myogenic differentiation.

MyoD specifically forms an E-box-associated complex with histone acetyltransferases (HATs) p300 and p300/CBP (CREB-binding protein)-associated factor (PCAF) *in vitro* (Puri et al. 1997). These observations suggest that MyoD recruits p300 and PCAF to muscle-specific promoters, which induces histone acetylation and transcriptional activation (Berkes and Tapscott 2005). The capacity of MyoD to stimulate myogenic differentiation genes is partially correlated with its ability to associate with acetyltransferases and to reorganize chromatin. In addition, an *in vitro* transcription system has identified specific roles for p300/CBP and PCAF (Dilworth et al. 2004).

HATs stimulate MyoD-dependent transcription by acetylating nucleosomal histones surrounding E boxes and directly acetylating MyoD. Nucleosomal histones H3 and H4 are acetylated following recruitment by MyoD to its promoter. Subsequently, p300/CBP recruits PCAF that then acetylates three MyoD lysine residues adjacent to its bHLH domain: Lys-99, -102, and -104 (Sartorelli et al. 1999; Poleskaya et al. 2000). This PCAF-mediated acetylation increases the interaction among these acetylated lysines of MyoD and the bromodomain of CBP, which ultimately enhances transcriptional activity (Poleskaya et al. 2001). By interacting simultaneously with the basal transcription machinery and with upstream transcription factors, p300/CBP functions as a physical bridge that stabilizes the transcription complex.

Histone deacetylases (HDACs) negatively regulate myogenic gene expression through interactions with MyoD and Mef2. HDAC-4/5 interact with Mef2 proteins to repress transcriptional activation from promoters containing Mef2 sites (McKinsey et al. 2001). In addition, these HDACs inhibit MyoD-activated transcription in promoters that contain an Mef2 site and an E box, suggesting that this class II histone deacetylase inhibition is

mediated indirectly through Mef2 (Lu et al. 2000). In contrast, the class I deacetylase HDAC-1 is able to directly associate and deacetylate MyoD in vitro. Cell culture experiments demonstrate that in undifferentiated myoblasts, but not differentiating myotubes, HDAC-1 is complexed with MyoD and inhibits PCAF-permissive MyoD-dependent transcription (Mal et al. 2001). Following the induction of myogenic differentiation, HDAC-1 dissociates from MyoD following displacement by the retinoblastoma (Rb) protein, permitting MyoD to function in transcriptional activation and associate with PCAF (Puri et al. 2001; Mal and Harter 2003).

MyoD can also repress transcription in proliferating myoblasts by recruiting Suv39h1 to promoters of genes such as *myogenin* that are activated during differentiation (Mal 2006). Suv39h1 is a histone H3 lysine 9 (H3-K9)-specific methyltransferase (HMT) that silences genes by modifying chromatin (Schotta et al. 2003). Silencing of *myogenin* transcription requires sustained methylation of H3-K9 on the *myogenin* promoter as well as a stable interaction between Suv39h1 and MyoD (Mal 2006).

The MyoD family functions as heterodimers with members of the E-protein family to induce myogenic gene activation. Our laboratory found that the switch from α to β alternative splicing forms of HEB have an important role in regulating the switch from growth to differentiation. Upon induction of differentiation, a MyoD-HEB β complex bound the E1 E box of the *myogenin* promoter, leading to transcriptional activation. Importantly, forced expression of HEB β with MyoD synergistically led to precocious myogenin expression in proliferating myoblasts. However, after differentiation, HEB α and HEB β synergized with myogenin, but not MyoD, to activate the *myogenin* promoter. Therefore, HEB α and HEB β have novel and central roles in orchestrating the regulation of myogenic factor activity through myogenic differentiation (Parker et al. 2006).

During the induction of myoblast differentiation, p38 α and p38 β kinases are progressively phosphorylated, promoting the myogenic differentiation pathway (Cuenda and Cohen 1999; Zetser et al. 1999; Wu et al. 2000). Furthermore, myoblasts treated with the inhibitor SB203580, specific to the p38 α and β isoforms, fail to fuse into myotubes or induce muscle-specific genes. Within the *myogenin* promoter, it has been proposed that MyoD interacts with the Pbx transcription factor in a p38 α -dependent manner (Berkus et al. 2004; de la Serna et al. 2005). Constitutively bound Pbx associates with MyoD and recruits the SWI/SNF chromatin-remodeling complex, facilitating the direct binding of MyoD to an E box within the *myogenin* promoter. p38 α / β phosphorylation permits targeting of SWI/SNF to muscle promoters, in addition to increased Mef2 transcriptional activity, whose interaction with MyoD contributes to the induction of muscle-specific gene transcription (Black and Olson 1998; Zetser et al. 1999; Wu et al. 2000; Simone et al. 2004). The mammalian SWI/SNF enzyme is a multiprotein chromatin-remodeling complex that actively alters nucleosomal structure by catalyzing a shift in the histone octamer along the DNA, thus exposing previously condensed DNA to transcriptional complexes (Simone

2006). These studies indicate a crucial and specific role for p38 α / β in the recruitment of SWI/SNF to muscle gene promoters, providing an additional mechanism to account for the positive effect of p38 α / β in myogenesis (Simone et al. 2004).

SATELLITE STEM CELLS AND REGENERATIVE MYOGENESIS

The Stem Cell Niche

Recent advances have provided important insights into the role that the microenvironment has in regulating stem cell function (Fuchs et al. 2004). The stem cell niche directs the maintenance of stem cell identity as well as the asymmetric generation and issue of committed daughter cells from the niche. The stem cell niche was originally described in studies of *Drosophila* oogenesis. Germ stem cells adhere to cap cells in contact with the basal lamina. Daughter cells that lose cap cell contact become cytotoblasts that are destined to differentiate into oocytes (Song et al. 2002). In mammals, analogous stem cell niches have been described for neural stem cells in the brain (Zhong et al. 2000), hematopoietic stem cells in bone marrow (Calvi et al. 2003; Zhang et al. 2003), stem cells in the crypt of the intestinal villus (Sancho et al. 2003), and stem cells in the hair follicle (Tumbar et al. 2004).

Cell polarity has been hypothesized to be established within the niche by cell–cell interactions mediated by cadherins and cell–extracellular matrix interactions mediated by integrins (Fuchs et al. 2004). Stem cell polarity and spindle orientation relative to the basal lamina determine whether a stem cell division will be symmetric or asymmetric. Planar divisions (parallel to the basal lamina) are symmetrical, generating identical daughter cells. In contrast, apical-basal divisions (90° to the basal lamina) are asymmetrical, with one daughter cell remaining a stem cell at the basal surface and a committed daughter cell destined for differentiation on the apical surface (Fuchs et al. 2004; Tumbar et al. 2004). Therefore, the discovery of the stem cell niche provides an additional means to establish the identity of a stem cell population within a particular tissue.

Satellite Stem Cells Maintain the Satellite Cell Compartment

Previous studies of satellite cell activation have used individual myofibers in cell culture (Zammit et al. 2004). However, we have noted that in cultured myofibers, satellite cells uniformly and immediately melt through the basal lamina and either proliferate on the outside matrix of the myofiber or migrate onto the culture plate. In contrast, in vivo satellite cell divisions on a living fiber are uniformly beneath the basal lamina (our unpublished data). The existence of a polarized environment for muscle satellite cells is evidenced by expression of M-cadherin on the apical surface facing the myotube (Irintchev et al. 1994) and by expression of α 7/ β 1-integrin on the basal surface facing the basal lamina (Blanco-Bose et al. 2001).

In recent work from our laboratory, we discovered that the satellite cell population is heterogeneous based on the

Figure 2. The satellite cell population is heterogeneously composed of satellite stem cells and satellite myogenic cells. Using *Myf5-Cre* and *ROSA26-YFP Cre* alleles, we observed that, in vivo, 10% of sublamina Pax7-expressing satellite cells have never expressed *Myf5*. Moreover, we found that Pax7⁺/Myf5⁻ satellite cells gave rise to Pax7⁺/Myf5⁺ satellite cells through apical-basal-oriented divisions that asymmetrically generated a basal Pax7⁺/Myf5⁻ cell and an apical Pax7⁺/Myf5⁺ cell. Prospective isolation and transplantation into muscle revealed that, whereas Pax7⁺/Myf5⁻ cells exhibited precocious differentiation, Pax7⁺/Myf5⁺ cells extensively contributed to the satellite cell reservoir throughout the injected muscle. Therefore, satellite cells are a heterogeneous population composed of stem cells and committed progenitors.

expression of two transcriptional factors, Pax7 and Myf5 (Fig. 2) (Kuang et al. 2007). Genetic analysis using the Cre-LoxP system (*Myf5-Cre/R26R-YFP*) revealed that about 10% of satellite cells have never previously expressed *Myf5-cre*. However, time-lapse videography of cultured myofibers demonstrated up-regulation of *Myf5-Cre* and induction of yellow fluorescent protein (YFP) expression in satellite daughter cells. Moreover, we observed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis following prospective isolation that YFP⁺ cells expressed *Myf5* mRNA, whereas YFP⁻ cells did not. We therefore hypothesized that the Pax7⁺/YFP⁻ satellite cells represent a novel stem cell population and that the majority of Pax7⁺/YFP⁺ satellite cells represent committed myogenic progenitors.

During muscle regeneration, both YFP⁺ and YFP⁻ satellite cells proliferate. Both types of satellite cells underwent planar divisions, with the polarity of cell division parallel to the basal lamina entirely symmetrical, generating two identical daughter cells that both remained either YFP⁺ or YFP⁻. Strikingly, basal-apical divisions, with the polarity of cell division at right angles to the basal lamina, were typically asymmetrical, with the basal cell remaining YFP⁻ and the apical cell up-regulating YFP. Prospective isolation and transplantation into muscle revealed that whereas Pax7⁺/Myf5⁺ cells undergo terminal differentiation, Pax7⁺/Myf5⁻ cells extensively contributed to the satellite cell reservoir throughout the injected muscle. Repression of Notch signaling resulted in reduced numbers of satellite cells due to inhibition of satellite stem cell self-renewal. We therefore conclude that Pax7⁺/Myf5⁻ satellite cells represent a novel population of stem cells that actively maintain the homeostatic composition of

Figure 3. Hypothetical scheme describing the generation of different myogenic lineages from satellite stem cells during regenerative myogenesis. Satellite stem cells would enter different developmental programs depending on whether Myf5 or MyoD expression predominates. Predominance of MyoD would drive the program toward early differentiation, analogous to the behavior of *Myf5*⁻ myoblasts, whereas predominance of Myf5 would drive the program toward enhanced proliferation and delayed differentiation. Myoblasts coexpressing Myf5 and MyoD would exhibit the intermediate growth and differentiation program of primary cultures of typical satellite-cell-derived myoblasts.

stem cells and committed progenitors within the satellite cell niche (Fig. 2) (Kuang et al. 2007).

The induction of expression of MyoD during satellite cell activation has not been well characterized relative to Myf5 induction. Interestingly, early studies suggested that Myf5 and MyoD were up-regulated independently of each other during satellite cell activation (Cornelison and Wold 1997). Notably, Myf5 and MyoD appear to direct the developmental program of distinct myogenic lineages during embryogenesis (Kablar et al. 1997, 1998, 1999, 2003; Gensch et al. 2008; Halder et al. 2008). Therefore, these data suggest a hypothesis whereby satellite stem cells can enter different developmental programs depending on whether Myf5 or MyoD expression predominates (Fig. 3). Predominance of MyoD would drive the program toward early differentiation, analogous to the behavior of *Myf5*⁻ myoblasts (Montarras et al. 2000). In contrast, predominance of Myf5 would drive the program toward enhanced proliferation and delayed differentiation, analogous to the behavior of *MyoD*⁻ myoblasts (Sabourin et al. 1999). Myoblasts coexpressing Myf5 and MyoD would exhibit the intermediate growth and differentiation program of primary cultures of typical satellite-cell-derived myoblasts. This hypothesis also explains the spectrum of differentiation observed in clonal cultures of myogenic cells derived from skeletal muscle. Future studies using lineage markers should elucidate these phenomena as well as the molecular control of the lineage choice.

CONCLUSIONS

Recent experiments have established that a subset of satellite cells, termed satellite stem cells, can be purified

from skeletal muscle and become capable of repopulating the satellite cell pool following transplantation. Significant challenges remain regarding transplantation as a therapeutic approach, including expansion of cells and delivery to affected muscles. Notably, studies are well under way to molecularly characterize satellite stem cells and define the regulatory pathways that govern their self-renewal and commitment to differentiate. Clearly, the development of drugs or biologics that specifically target these mechanisms represents an important therapeutic strategy for the treatment of neuromuscular disease.

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