Myogenic specification of side population cells in skeletal muscle

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Skeletal muscle contains myogenic progenitors called satellite cells and muscle-derived stem cells that have been suggested to be pluripotent. We further investigated the differentiation potential of muscle-derived stem cells and satellite cells to elucidate relationships between these two populations of cells. FACS® analysis of muscle side population (SP) cells, a fraction of muscle-derived stem cells, revealed expression of hematopoietic stem cell marker Sca-1 but did not reveal expression of any satellite cell markers. Muscle SP cells were greatly enriched for cells competent to form hematopoietic colonies. Moreover, muscle SP cells with hematopoietic potential were CD45 positive. However, muscle SP cells did not differentiate into myocytes in vitro. By contrast, satellite cells gave rise to myocytes but did not express Sca-1 or CD45 and never formed hematopoietic colonies. Importantly, muscle SP cells exhibited the potential to give rise to both myocytes and satellite cells after intramuscular transplantation. In addition, muscle SP cells underwent myogenic specification after co-culture with myoblasts. Co-culture with myoblasts or forced expression of MyoD also induced muscle differentiation of muscle SP cells prepared from mice lacking Pax7 gene, an essential gene for satellite cell development. Therefore, these data document that satellite cells and muscle-derived stem cells represent distinct populations and demonstrate that muscle-derived stem cells have the potential to give rise to myogenic cells via a myocyte-mediated inductive interaction.

Introduction

Myogenic satellite cells reside beneath the basal lamina of adult skeletal muscle closely juxtaposed against skeletal muscle fibers and account for 2–5% of sublaminal nuclei in adult muscle. Satellite cells are normally mitotically quiescent, but are activated (initiate proliferation) to mediate the postnatal growth and regeneration of muscle (Seale and Rudnicki, 2000). The progeny of activated satellite cells, termed myogenic precursor cells, undergo multiple rounds of cell division before terminal differentiation. The number of quiescent satellite cells in adult muscle remains relatively constant over multiple cycles of degeneration and regeneration, suggesting a capacity for self-renewal within the satellite cell compartment. Satellite cells express specific genes such as M-cadherin, CD34, and Pax7, and after activation give rise to a large number of daughter myoblasts in addition to repopulating the satellite cell pool (Beauchamp et al., 1999, 2000; Seale et al., 2000). Therefore, satellite cells represent a unique population of committed myogenic progenitors that are distinct from their daughter myoblasts by both biochemical and biological criteria (Seale and Rudnicki, 2000).

Satellite cells have long been considered monopotent stem cells with the ability to only give rise to cells of the myogenic lineage. However, recent experiments have identified the existence of adult stem cells present in most (if not all) tissues that appear to exhibit the ability to differentiate into many different cell types after reintroduction in vivo. This work has raised important questions regarding the developmental potential of stem cells derived from diverse tissues including muscle, bone marrow, and brain (Seale et al., 2001). For example, hematopoietic stem cells (HSCs),* in addition to their ability to produce all blood cell lineages, also exhibit developmental plasticity when introduced into different tissues. HSCs can differentiate into hepatic cells (Lagasse et al., 2000), cardiac muscle and vascular endothelium (Jackson et al., 2001), and several epithelial cell types (Krause et al., 2001) after transplantation. Therefore, many or all tissues appear to contain a population of adult stem

*Abbreviations used in this paper: CMV, cytomegalovirus; HSC, hematopoietic stem cell; IL, interleukin; LTR, long-term repeat; MHC, myosin heavy chain; MP, main population; SP, side population; TA, tibialis anterior; WP, whole population.
cells that differentiate in a context-specific manner, presumably in response to growth factors and signals provided by their host tissues (Seale et al., 2001).

Satellite cells are believed to be the committed stem cell of the myogenic cells responsible for the postnatal growth and regeneration of muscle (Seale et al., 2001). The notion that satellite cells exclusively accomplish the regeneration of adult muscle has been challenged by the demonstration that muscle also contains a population of adult stem cells, called muscle-derived stem cells. Muscle-derived stem cells exhibit the capacity to reconstitute the entire hematopoietic repertoire after intravenous injection into lethally irradiated mice (Jackson et al., 1999; Kawada and Ogawa, 2001). Muscle-derived stem cells isolated by FACS® of side population (SP) cells, on the basis of Hoechst dye exclusion, exhibit the capacity to give rise to hematopoietic cells after intravenous injection into lethally irradiated mice. In addition, transplanted SP cells isolated from bone marrow or muscle actively participate in the formation of skeletal myotubes during regeneration (Gussoni et al., 1999). Importantly, Pax7-deficient muscle entirely lacks myogenic satellite cells, yet contains muscle SP cells that exhibit a high level of hematopoietic progenitor activity (Seale et al., 2000). Together, these data demonstrate that muscle-derived stem cells represent a distinct cell population from satellite cells. These studies suggested the hypothesis that induction of Pax7 in muscle-derived stem cells induces satellite cell specification by restricting alternate developmental programs. However, direct proof that muscle-derived stem cells represent the progenitors of satellite cells and any understanding of the mechanisms that regulate this developmental step remains lacking.

To investigate the relationship between muscle-derived stem cells and myogenic satellite cells, we used a variety of cell culture assays as well as in vivo intramuscular transplantation to explore their developmental potential. Our experiments demonstrate that the ability of muscle-derived stem cells to undergo myogenic specification is a regulated process that appears to involve cell-mediated inductive interactions.

Results
Adult muscle is a rich source of hematopoietic progenitors
Adult skeletal muscle contains muscle-derived stem cells that efficiently reconstitute the entire hematopoietic repertoire after intravenous injection into lethally irradiated mice (Gussoni et al., 1999; Jackson et al., 1999; Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002). Therefore, a hallmark property of muscle-derived stem cells is the ability to undergo hematopoietic differentiation. To elucidate whether muscle-derived stem cells can differentiate into hematopoietic cells in vitro, we prepared dissociated muscle-derived cells by enzymatic digestion and cultured the cells in methylcellulose medium (Methocult M3434) containing stem cell factor, interleukin (IL)-3, IL-6, and erythropoietin, permitting the clonal growth of myeloid-type hematopoietic cells. By 14 days, cultures of muscle-derived cells efficiently gave rise to high numbers of hematopoietic colonies consisting of large colonies (Fig. 1 A) and small colonies (Fig. 1 B). May-Grunwald’s Giemsa staining revealed that large colonies contained a variety of hematopoietic cells including Mac-1- and Gr-1- positive granulocytes and monocytes (Fig. 1, D–F). The incidence of hematopoietic colonies was 18.8/10^4 muscle-derived cells plated versus 81.2/10^4 bone marrow cells plated (Fig. 2 E), which is consistent with previously reported works (Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002). By contrast, no hematopoietic colonies were formed after plating of 10^4 nucleated peripheral blood cells (Fig. 2 E), ruling out the possibility that hematopoietic progenitors were derived from circulating peripheral blood cells contaminating the muscle preparation. Similar results were also reported previously (Kawada and Ogawa, 2001).

To test whether muscle-derived stem cells can differentiate into lymphoid cells in vitro, muscle-derived cells were cultured in methylcellulose medium (Methocult M3630) containing IL-7, permitting the clonal growth of pre-B cell lineage. By 10 days, cultures of muscle-derived cells gave rise to hematopoietic colonies consisting of small pre-B cells as confirmed by immunohistochemistry with B220 antibodies (Fig. 1, C and G). Therefore, these results indicate that adult skeletal muscle contains a rich source of hematopoietic progenitors for both myeloid and lymphoid lineages.

Satellite cells do not exhibit hematopoietic potential
Satellite cells have long been considered monopotent stem cells, with the ability to only give rise to cells of the myogenic lineage. However, recent experiments have indicated that satellite cells readily differentiate into different cell types such as osteocytes and adipocytes (Asakura et al., 2001). To investigate whether satellite cells have the potential to differentiate...
into hematopoietic cells, we cultured single muscle fibers from heterozygous Myf5-nlacZ mice in Methocult M3434. The expression of Myf5-nlacZ recapitulates the expression of the endogenous Myf5 mRNA both during embryogenesis (Tajbakhsh et al., 1996) and in adult muscle (Beauchamp et al., 2000). Isolated muscle fibers maintained in culture for 14 d gave rise to colonies uniformly composed of Myf5-nlacZ–positive multinucleated, contractile myotubes (Fig. 2, A–D). Importantly, hematopoietic colonies were never detected in these long-term cultures of isolated muscle fibers (Fig. 2 E; n = 180 fibers). By contrast, 10–30 hematopoietic colonies were formed after plating of $1 \times 10^6$ unfractionated muscle cell suspension. The inability of satellite cells to undergo hematopoietic differentiation and the preferential differentiation of Myf5-nlacZ–expressing cells into muscle (Fig. 2 C) strongly support the hypothesis that satellite cells are restricted in their developmental potential within the mesenchymal range of cell lineages.

**Figure 2.** Satellite cells do not exhibit hematopoietic potential. Isolated single muscle fibers prepared from Myf5-nlacZ transgenic mice were cultured in Methocult M3434. (A) Satellite cells expressing Myf5-nlacZ (arrowheads) were detected on freshly isolated muscle fibers (day 0). (B) Migrating satellite cells derived from muscle fibers were detected after 4 d of culture (arrowheads). (C) By day 10, the migrating cells formed colonies containing multinucleated myotubes that expressed Myf5-nlacZ (arrows). (D) By 14 days, differentiated myotubes (arrow) were detected adjacent the original muscle fiber (indicated by F). (E) Single muscle fibers (total fibers; n = 180 for three independent experiments) or $1 \times 10^4$ cells prepared from whole muscle, bone marrow, or peripheral blood were cultured in Methocult M3434. By day 14, hematopoietic colonies were counted. The error bars represent the SEM from three independent experiments. Bar: (A–C) 50 μm; and (D) 100 μm.

**Figure 3.** Muscle SP cells express Sca-1 and CD45 but do not express Myf5-nlacZ or MD6.0-lacZ. (A) FACS® fractionation of SP cells from bone marrow and muscle was used for the isolation of adult stem cells by Hoechst dye exclusion. Both muscle and bone marrow SP cells stained with Hoechst dye were sensitive to verapamil. (B) x- and y-axes indicate intensity of CD45-FITC and Sca-1-PE, respectively. The percentage of cells in each quadrant is indicated in the panel. (C) Unsorted (WP) or sorted cells prepared from Myf5-nlacZ or MD6.0-lacZ transgenic mouse muscle were stained with X-gal. The error bars represent the SEM from three independent experiments. At least a total of 1,000 cells were counted in each fraction.

**Muscle SP cells in vitro undergo hematopoietic but not myogenic differentiation**

The expression of Myf5-nlacZ was readily detected in the nuclei of satellite cells on freshly isolated muscle fibers (Fig. 2 A; Beauchamp et al., 2000). A similar expression profile of lacZ within myonuclei and satellite cell nuclei was also ob-
served in single muscle fiber cultures prepared from MD6.0-
lacZ transgenic mice carrying myoD upstream–driving lacZ gene (Asakura et al., 1995; unpublished data). Therefore, if purified muscle SP cells (a fraction of muscle-derived stem cells) contained a fraction of satellite cells, Myf5-nlacZ– or MD6.0-lacZ–expressing cells should be detected within the SP compartment. To test this possibility, we used the FACS®/Hoechst method (5 μg/ml Hoechst 33342) to fractionate muscle cell suspensions into SP and main population (MP). Compared to bone marrow–derived SP cells (a fraction of HSCs), increased numbers of SP cells were detected in muscle (0.2 vs. 2.3% of total cells; Fig. 3 A). Similar results were obtained when skeletal muscle cells were stained with 12.5 μg/ml Hoechst 33342 (unpublished data) as described previously (Gussoni et al., 1999). Both bone marrow and muscle SP cells stained with Hoechst dye were sensitive to verapamil, which is consistent with reported results (Jackson et al., 1999). Recent studies have demonstrated stem cells derived from skeletal muscle that exhibit hematopoietic potential express the hematopoietic marker CD45 and Sca-1 (Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002). To investigate the origin of the hematopoietic progenitors in muscle SP cells, FACS® analysis was used to subfractionate muscle SP cells with CD45 and Sca-1 antibodies. Most of the muscle SP cells (92%) expressed Sca-1, which is consistent with previous observation (Gussoni et al., 1999). 16% of muscle SP cells expressed CD45 consisting of both Sca-1–positive (9.2%) and –negative (6.8%) fractions (Fig. 3 B). Next, sorted cells (MP and SP) and unfractonated whole population (WP) cells prepared from Myf5-nlacZ or MD6.0-lacZ mouse muscle were stained with X-gal. Both WP and MP cells contained a similar proportion of lacZ-expressing cells (Fig. 3 C). By contrast, the SP fraction did not contain any cells that expressed Myf5-nlacZ or MD6.0-lacZ. Desmin and Pax7 are also known to be expressed in both quiescent satellite cells and derivative myoblasts in adult muscle (Seale et al., 2000). Importantly, no desmin or Pax7 expression was detected in cells from the muscle SP fraction (unpublished data). Therefore, satellite cells and their daughter myoblasts strictly reside within the MP fraction of cells by FACS®/Hoechst analysis.

We have demonstrated that putative muscle-derived stem cells differentiate into hematopoietic cells in vitro. However, to confirm that these hematopoietic progenitors were derived from the SP compartment, cells prepared from muscle and bone marrow were fractionated by FACS® into SP and MP populations. The purified fractions were cultured in Methocult M3434 or in myoblast growth medium to score hematopoietic (Fig. 4 A) and myogenic (Fig. 4, B and C) colony formation. In Methocult M3434, both bone marrow and muscle SP cells efficiently formed numerous hematopoietic colonies. By con-
trast, WP or MP fractions gave rise to markedly fewer hematopoietic colonies (Fig. 4 A). Importantly, CD45-positive muscle SP cells exclusively gave rise to hematopoietic colonies; CD45⁻:Sca-1⁺ muscle SP cells efficiently gave rise to hematopoietic colonies, whereas CD45⁺:Sca-1⁻ muscle SP cells gave rise to a lower number of hematopoietic colonies. Therefore, these results indicate that muscle SP cells that exhibit hematopoietic potential are CD45 positive. In addition, most of the SP population was negative for hematopoietic lineage markers such as B220, CD3, Gr-1, MAC1, and Terr119 (Gussoni et al., 1999; unpublished data), suggesting that muscle SP cells contain primitive hematopoietic progenitors.

Importantly, by day 10 after being plated, muscle SP cells did not form differentiated muscle colonies, and instead formed colonies that displayed fibroblastic morphology (Fig. 4 C). By contrast, the WP and MP cell fractions prepared from muscle gave rise to 8–30 differentiated muscle colonies per plate under both culture conditions (Fig. 4, B and C). None of the cells derived from bone marrow differentiated into muscle colonies under these conditions. Importantly, most bone marrow SP cells, in contrast to muscle SP cells, did not attach to the plate and failed to survive in myoblast growth medium. Therefore, muscle SP cells undergo preferential hematopoietic differentiation in vitro and do not appear capable of forming myogenic progenitors under the culture conditions tested.

**Sca-1⁺-positive cells are located outside of muscle fibers**

To further confirm that muscle SP cells are distinct cell population from satellite cells, we first examined detection of HSC markers CD34, Sca-1, and CD45, in satellite cells on freshly isolated muscle fibers. Immunohistochemical detection clearly demonstrated that CD34 was expressed in satellite cells on muscle fibers (Fig. 5 A). About three to four CD34-expressing satellite cells were normally detected per muscle fiber (unpublished data), which is consistent with previous observation (Beauchamp et al., 2000). By contrast, satellite cells on muscle fibers were never observed to express Sca-1 or CD45 (n = 20 fibers; Fig. 5 A). Previously, it has been reported that the majority of muscle SP cells express Sca-1, an important cell surface marker for HSCs (Gussoni et al., 1999; Jackson et al., 1999). To examine where Sca-1-expressing cells are located within muscle, double immunostaining was performed. Clearly, Sca-1⁺-positive cells were located outside of the muscle fiber basal lamina as revealed by staining with anti–laminin antibodies (Fig. 5 B). In addition, Sca-1-expressing cells were frequently detected closely juxtaposed to blood vessels as well as endothelium that expressed PECAM (Fig. 5 B). Similar results were reported in previous works in which Sca-1⁺-positive cells were detected in endothelium and outer layer of blood vessels, but were not detected beneath the basal lamina of skeletal muscle fibers (Zammit and Beauchamp, 2001). These results further confirm that muscle-derived stem cells are distinct cell population from satellite cells and may be associated with the vasculature within muscle.

**Myogenic specification of muscle SP cells after intramuscular injection**

Culture of purified muscle SP cells in Methocult M3434 or myoblast growth medium did not give rise to any SP-derived myoblasts or myocytes (Fig. 4, B and C). To exam-
Figure 6. Muscle SP cells undergo myogenic specification and satellite cell differentiation in vivo after intramuscular injection. Myf5-nlacZ muscle SP cells were injected into regenerating tibialis anterior (TA) muscle of scid/bg mice. (A) By day 14, whole-mount staining of the TA muscle showed clusters of lacZ-positive nuclei. (B and C) By day 14, cross-sections showed that muscle SP cell–derived myocytes that expressed lacZ in the nucleus were integrated into desmin (B) or MHC-positive (C) regenerating muscle fibers (arrows). By 1 month, TA muscles were used for histochemical (D–K) and culture (L) experiments to detect muscle SP cell–derived satellite cells. (D–F) The lacZ-expressing satellite cell was detected between the basal lamina (laminin; red) and sarcolemma (dystrophin; green) of muscle fiber (arrows). The lacZ-expressing satellite cells were positive for Pax7 (G–I) in the nucleus and desmin (J and K) in the cytoplasm (arrows). (E, F, I, and K) DAPI staining indicates nuclei (blue). Arrowheads indicate myonuclei. Dissociated cells prepared from the TA muscle contained a lacZ-positive myoblast cluster (L). Bar: (A) 50 μm; (B and C) 10 μm; (D–F) 7 μm; (G–K) 5 μm; and (F) 40 μm.

In brief, cardiotoxin was injected into TA muscles to induce muscle damage, cells were injected after a further 24 h, and muscle was allowed to heal for an additional 2 wk–1 mo. By day 14, whole-mount staining of the TA muscle showed clusters of lacZ-positive nuclei. By day 14, cross-sections showed that muscle SP cell–derived myocytes that expressed lacZ in the nucleus were integrated into desmin (B) or MHC-positive (C) regenerating muscle fibers (arrows). By 1 month, TA muscles were used for histochemical (D–K) and culture (L) experiments to detect muscle SP cell–derived satellite cells. (D–F) The lacZ-expressing satellite cell was detected between the basal lamina (laminin; red) and sarcolemma (dystrophin; green) of muscle fiber (arrows). The lacZ-expressing satellite cells were positive for Pax7 (G–I) in the nucleus and desmin (J and K) in the cytoplasm (arrows). (E, F, I, and K) DAPI staining indicates nuclei (blue). Arrowheads indicate myonuclei. Dissociated cells prepared from the TA muscle contained a lacZ-positive myoblast cluster (L). Bar: (A) 50 μm; (B and C) 10 μm; (D–F) 7 μm; (G–K) 5 μm; and (F) 40 μm.

muscle SP cells undergoing myogenic specification in vivo, we intramuscularly injected FACS®-Hoechst-purified muscle SP cells derived from Myf5-nlacZ or ROSA26 mice into regenerating tibialis anterior (TA) muscle of adult scid/bg immunodeficient mice. In brief, cardiotoxin was injected into TA muscles to induce muscle damage, cells were injected after a further 24 h, and the muscle was allowed to heal for an additional 2 wk–1 mo. By day 14, whole-mount staining of the TA muscle injected with Myf5-nlacZ muscle SP cells showed clusters of lacZ-positive nuclei (Fig. 6 A). Examination of frozen sections revealed the presence of lacZ-expressing myocytes as confirmed by immunohistochemistry with antibodies reactive with desmin (Fig. 6 B) or myosin heavy chain (MHC; Fig. 6 C). The total number of Myf5-nlacZ–expressing nuclei counted in serial sections was 1.4% (n = 3) of the total muscle SP cells injected. The efficiency of engraftment is similar to previous results after intramuscular cell injections into regenerating muscle (Beauchamp et al., 1999; Galli et al., 2000). Desmin/lacZ double-positive regenerating muscle fibers were also detected in cross sections prepared from TA muscle after injection of ROSA26 muscle SP cells (unpublished data).

By 1 mo after injection of My5-nlacZ muscle SP cells, a few lacZ-positive nuclei were detected at the basal lamina (laminin*) and sarcolemma (dystrophin*) of TA muscle fibers (Fig. 6, D–F). The lacZ-positive nuclei were also positive for Pax7 (Fig. 6, G–I), a satellite cell marker in resting muscle (Zammit and Beauchamp, 2001). Cytoplasm surrounding the lacZ-positive nuclear-expressed desmin and the plasma membrane was also evident, indicating that the lacZ-positive nuclei are within a satellite cell. By contrast, desmin protein was not located surrounding myonuclei (Fig. 6, J and K). Next, we cultured dissociated cells prepared from TA muscle 1 mo after injection of cells. After 4 d of culture, Myf5-nlacZ–expressing myoblast clusters were occasionally detected (Fig. 6, F and L). These results strongly suggest that intramuscularly transplanted muscle SP cells are competent to give rise to satellite cells within the recipient muscle fibers. In addition, these SP cell–derived satellite cells have a normal proliferative potential in vitro. Together, these data indicate that muscle SP cells have the capability to undergo myogenic specification and suggest that muscle-derived stem cells undergo myogenic specification via a myocyte-mediated inductive interaction. In addition, the in vivo potential of muscle SP cells to give rise to satellite cells supports our hypothesis that at least some portion of satellite cells originates from muscle-derived stem cells.

Muscle SP cells undergo myogenic specification after co-culture with primary myoblasts

Intramuscular transplantation experiments suggest that muscle-derived stem cells undergo myogenic specification via a myocyte-mediated inductive interaction. To investigate whether cell-mediated interactions could influence the developmental fate of muscle SP cells in vitro, we co-cultured FACS®-Hoechst-purified muscle SP cells from ROSA26 mice, expressing lacZ protein ubiquitously (Zambrowicz et al., 1997), with equal numbers of myoblasts or fibroblasts. Immunohistochemistry for desmin was performed to confirm the differentiation of lacZ-expressing ROSA26 cells into myocytes. Culture of muscle SP cells alone resulted in
the formation of any lacZ-expressing myogenic cells (Fig. 7 A). By contrast, co-culture of muscle SP cells with primary myoblasts resulted in the formation of multinucleated myotubes that coexpressed lacZ and desmin. The number of nuclei of myogenic cells that coexpressed desmin and lacZ was 3.8% ($n = 4$) of the total lacZ-expressing ROSA26 cell nuclei. We also observed myogenic conversion in co-cultures of muscle SP cells with C2C12 myoblasts but not with C3H10T1/2 fibroblasts or conditioned medium from primary myoblast cultures (unpublished data). We also co-cultured $1 \times 10^4$ FACs/Hoechst-purified muscle SP cells from TgN(GFP)5-Nagy (GFP) mice, expressing a GFP ubiquitously (Hadjantonakis et al., 1998), with equal numbers of primary myoblasts. Immunohistochemistry for MHC was performed to confirm the differentiation of GFP-expressing muscle SP cells into muscle cells after co-culture with primary myoblasts (Fig. 7 B). Finally, co-culture of muscle SP cells isolated from Myf5-nlacZ mice with primary myoblasts resulted in the formation of Myf5-nlacZ–expressing multinucleated myotubes and mononuclear myocytes (Fig. 7 C), indicating that muscle SP cells did not simply form heterokaryon myotubes fused with primary myoblasts. By contrast, we failed to detect any muscle differentiation of bone marrow SP cells after co-culture with primary myoblasts (unpublished data), suggesting that there is a biological difference between muscle and bone marrow SP cells. Together, these data unequivocally demonstrate that some portion of muscle SP undergoes myogenic specification after co-culture with primary myoblasts.

To investigate the myogenic potential of CD45-negative (CD45$^-$ mSP) and CD45-positive (CD45$^+$ mSP) muscle SP cells, preparations from GFP mice were co-cultured with primary myoblasts. Immunostaining with anti-MHC antibody revealed the presence of GFP-expressing multinucle-
ated myotubes in both cultures (Fig. 8). The number of nuclei of myogenic cells that coexpressed MHC and GFP was 5.1% (CD45− mSP; n = 3) and 8.8% (CD45+ mSP; n = 3) of the total GFP-positive cell nuclei. These data demonstrate that some portion of both CD45− mSP and CD45+ mSP undergo myogenic differentiation after co-culture with primary myoblasts.

Myogenic differentiation of muscle SP cells is Pax7 independent

Recently, we demonstrated that mice lacking the Pax7 gene display complete absence of satellite cells (Seale et al., 2000). To test whether muscle differentiation of muscle SP cells requires Pax7, we co-cultured Pax7−/− derived muscle SP cells with myoblasts. To distinguish muscle SP cells from myoblasts, GFP retrovirus containing virus long term repeat (LTR) and cytomegalovirus (CMV) promoter-driven enhanced GFP gene was used to infect purified muscle SP cells before initiating co-culture with myoblasts. Immunostaining with anti-MHC antibody revealed the presence of GFP-expressing multinucleated myotubes in both cultures (Fig. 9). The number of nuclei of myogenic cells that coexpressed MHC and GFP was 7.6% (Pax7+/+; n = 3) and 4.7% (Pax7−/−; n = 3) of the total GFP-positive cell nuclei. These data demonstrate that some portion of muscle SP cells undergoes terminal myogenic differentiation after co-culture with primary myoblasts and that Pax7 is not required for this step.

The muscle-specific transcription factor MyoD readily induces the myogenic program when ectopically expressed in many nonmuscle cell types (Weintraub et al., 1991). To investigate whether MyoD expression was sufficient to induce SP myogenic conversion, purified SP cells from wild-type and Pax7−/− muscle were infected with retrovirus expressing an LTR/CMV promoter–driven MyoD gene. Notably, SP cells from wild-type and Pax7−/− muscle differentiated into MyoD/desmin-positive myoblasts (Fig. 10, D, F, J, and L). The efficiencies of retrovirus infection and myogenic conversions in all cultures were >50% of the total cells based on the expression of MyoD and desmin (Fig. 10, D and J). Importantly, Pax7 protein could not be detected in the MyoD-induced myoblasts derived from wild-type muscle SP cells (Fig. 10 E). Therefore, MyoD-induced myogenic differentiation of muscle SP cells is Pax7 independent. In addition, the lack of Pax7 induction in MyoD-expressing myoblasts suggests that Pax7 is not regulated by MyoD and is therefore likely upstream of MyoD during satellite cell development.

Discussion

Satellite cells are committed myogenic progenitors that mediate muscle growth during the postnatal period as well as regeneration after injury (Seale and Rudnicki, 2000). The observation that muscle SP cells introduced intravenously participate in the formation of new myofibers and potentially give rise to satellite cells (Gussoni et al., 1999) raised the possibility that muscle-derived stem cells were in fact satellite cells (Jackson et al., 1999). However, our data reported here that muscle-derived stem cells and satellite cells displayed distinct developmental potentials and were not copurified during FACS®/Hoechst isolation confirms that they are distinct cell populations. Consistent with these data, mutant mice lacking Pax7 display a complete absence of satellite cells, yet contain a normal proportion of SP cells in mus-
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Myogenic specification of muscle SP cells efficiently formed hematopoietic colonies in culture but never gave rise to muscle cells. These muscle SP cells with hematopoietic potential were observed to express the hematopoietic marker CD45. Interestingly, isolated muscle SP cells efficiently formed hematopoietic colonies in culture but never gave rise to muscle cells. These muscle SP cells with hematopoietic potential were observed to express the hematopoietic marker CD45. By contrast, satellite cells from cultured myofibers were capable of differentiation into myocytes, adipocytes, and osteocytes (Asakura et al., 2001), but not into hematopoietic cells. Gussoni et al. (1999) suggested that muscle SP cells converted into myoblasts after 2 wk in culture, but did not document this observation. It remains possible that muscle SP cells undergo spontaneous myoblast differentiation after a long-term culture. However, in numerous experiments, we have never noted the presence of SP-derived myogenic progenitors. Together, these data strongly support the notion that muscle-derived stem cells and satellite cells represent distinct cellular populations within adult skeletal muscle.

Importantly, myogenic specification of muscle SP cells and the formation of mononuclear myoblasts were observed to occur in vitro after co-culture with primary myoblasts. In addition, muscle SP cells contain at least two distinct fractions with myogenic potential as CD45^− mSP and CD45^+ mSP. Both exhibit the potential to give rise to myogenic cells after co-culture with primary myoblasts. Recent work demonstrated that both intramuscular and intravenous transplantation of bone marrow and intravenous transplantation of bone marrow SP cells contributed to skeletal muscle fiber formation (Ferrari et al., 1998; Gussoni et al., 1999; Fukada et al., 2002). More recently, it has been demonstrated that muscle-derived CD45-positive cells integrate into regenerating muscle fibers after intramuscular injection (McKinney-Freeman et al., 2002). By contrast, we have observed that CD45^− mSP cells have the potential to give rise to adipocytes and osteocytes after induction (unpublished data). Therefore, it is interesting to hypothesize that the CD45^− mSP fraction of cells contains progenitor cells that are similar to multipotential mesenchymal stem cells (Pittenger et al., 1999).

Interestingly, muscle SP prepared from Pax7^+/− mice, when co-cultured with primary myoblasts, also resulted in the formation of multinucleated myotubes. In addition, forced expression of MyoD could induce myogenic differentiation of Pax7^+/− muscle SP cells. Furthermore, Pax7 could not be induced by MyoD in myoblasts derived from wild-type muscle SP cells. Therefore, these data suggest that terminal myogenic differentiation of muscle-derived stem cells is a Pax7-independent process and that Pax7 functions upstream of MyoD during satellite cell development.

Muscle SP cells differentiated into myocytes and formed new myofibers after intramuscular injection into regenerating muscle. Therefore, the observed Pax7-independent myogenic differentiation of muscle SP cells suggests that Pax7 is required for the specification of satellite cells, but not for terminal myogenic differentiation. Together, these data indicate that muscle-derived stem cells are competent for induction of myogenic specification as well as for direct myogenic differentiation. The inability of muscle SP cells to undergo myogenic specification except in the presence of myogenic cells suggests that the process is subject to regulation via a mechanism that involves cell-mediated inductive interactions.

Primary myoblasts derived from neonatal mice readily give rise to satellite cells after intramuscular injection (Heslop et al., 2001). Muscle SP cells have been suggested to differenti-
ate into satellite cells based on their location after intravenous injection into irradiated mice (Gussoni et al., 1999). Furthermore, fetal liver and bone marrow cells have a potential to differentiate into satellite cells based on the location after injection into busulphan-treated neonatal mice (Fukada et al., 2002). Here, we demonstrate that muscle SP cells have the potential to differentiate into satellite cells after intramuscular injection into regenerating muscle. These satellite cells express satellite cell markers Myf5-nlacZ, Pax7, and desmin, and are proliferative in vitro, suggesting that muscle SP cells can differentiate into functional satellite cells. The in vivo potential of muscle SP cells that give rise to satellite cells supports our hypothesis that at least some portion of satellite cells originates from muscle-derived stem cells. However, it remains unclear whether both CD45<sup>+</sup> mSP and CD45<sup>+</sup> mSP have the potential to give rise to satellite cells after intramuscular transplantation. In addition, it remains to be elucidated whether Pax7 is required for muscle SP cells to differentiate into satellite cells after intramuscular transplantation. We are currently investigating these questions.

An important outstanding question concerns the location of muscle-derived stem cells within skeletal muscle. Examination of regeneration in dystrophic muscle has revealed that recruitment of cells via the circulatory system does not provide a substantial source of myoblasts for muscle repair after radiation of mouse limbs (Heslop et al., 2000). It is conceivable that the vascular-associated progenitors of myogenic satellite cells in the embryo persist in close association with the vasculature as adult stem cells in skeletal muscle (De Angelis et al., 1999). Indeed, muscle SP cells express Sca-1, and importantly, Sca-1-positive cells reside between muscle fibers prominently associated with blood vessels. By contrast, satellite cells on freshly isolated muscle fibers express CD34 but not Sca-1. In addition, Sca-1-expressing cells lines isolated from muscle exhibit a high degree of plasticity (Torrente et al., 2001; Qu-Petersen et al., 2002; Tamaki et al., 2002). Together, these observations suggest that muscle-derived stem cells located as vascular-associated cells in skeletal muscle are a progenitor for satellite cells or muscle precursor cells. According to this hypothesis, muscle-derived stem cells and satellite cell populations would co-exist as distinct stem cell tiers in some state of equilibrium within adult muscle. Soon after initiation of muscle regeneration, the number of myogenic precursor cells thought to be derived from satellite cells are much greater than that of the resident satellite cells (Grounds et al., 1992). Therefore, one explanation for this apparent paradox is that muscle-derived stem cells give rise to the myogenic precursor cells. Additional experiments are required to deduce whether the flux between muscle-derived stem cells and satellite cell tiers is an ongoing process or is normally limited to a restricted period during development.

Muscle SP cells clearly possess the ability to form multiple hematopoietic colonies in vitro as well as hematopoietic differentiation in vivo after intravenous injection into irradiated mice (Gussoni et al., 1999). Recently, two groups demonstrated that CD45-positive cells within skeletal muscle contain HSC activity (Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002). We also demonstrated that cells from muscle SP with in vitro hematopoietic potential are CD45-positive (CD45<sup>+</sup>mSP). Bone marrow HSCs cannot be cultured under the conditions used in this work as reported previously (Traycoff et al., 1996). By contrast, muscle cells with hematopoietic potential are readily cultured in myoblast medium and have been suggested to maintain their hematopoietic potential for at least 5 d in vitro (Jackson et al., 1999). HSCs express several surface markers such as Sca-1 and c-kit (Goodell et al., 1997; Gussoni et al., 1999). Moreover, although freshly isolated muscle SP cells express Sca-1, they do not express c-kit (Gussoni et al., 1999). Furthermore, we failed to detect any muscle differentiation of bone marrow SP cells after co-culture with primary myoblasts. Therefore, marker expression and biological assays suggest some important differences between HSCs and muscle-derived cells with hematopoietic potential. In any case, these results strongly suggest that skeletal muscle is an adult organ containing a resident population of adult stem cells with hematopoietic potential. Recent bone marrow transplantation experiments have been interpreted to suggest that hematopoietic progenitors in muscle have a bone marrow origin (Kawada and Ogawa, 2001). However, a clear understanding of the identity and relationship between HSCs in bone marrow and hematopoietic potential cells within muscle remains to be elucidated. For example, it is interesting to speculate that muscle-derived hematopoietic potential cells represent the progenitors for hematopoietic cells such as macrophages and polymorphonuclear lymphocytes, which are mobilized in high numbers in regenerating muscle tissue (Seale and Rudnicki, 2000).

Direct proof that a single stem cell within the muscle SP fraction of cells is indeed capable to undergo hematopoietic, myogenic, or other differentiation remains lacking. It is entirely possible that the muscle SP fraction of cells contains multiple types of progenitors each with a restricted range of potential. It has been suggested recently that a single pluripotent HSC can give rise to many different cell types in a wide range of tissues in mice (Krause et al., 2001). Similar experiments using muscle SP cells should establish the potentiality of muscle-derived stem cells. Nevertheless, our data strongly support the existence of a tier of stem cells in addition to satellite cells in skeletal muscle with the potential to undergo myogenic specification.

**Materials and methods**

**Transgenic mice**

*Myf5-nlacZ* (Tajbakhsh et al., 1996) and *MD6.0-lacZ* (Asakura et al., 1995) transgenic mice were provided by Drs. Shahragim Tajbakhsh (Pasteur Institute, Paris, France) and Stephen J. Tapscott (Fred Hutchinson Research Center, Seattle, WA), respectively. ROSA26 mice and TgN(GFPU)5-Nagy mice were purchased from Jackson Immunoresearch Laboratories (Zambrowicz et al., 1997; Hadjiantonis et al., 1998). Heterozygous mice lacking Pax7 gene, provided by Dr. Peter Gruss (Max-Planck Institute for Biophysical Chemistry, Gottingen, Germany), were maintained by breeding with C57BL/6.

**FACS®**

Hoechst staining and FACS® analysis was performed as described previously (Jackson et al., 1999). In brief, hindlimb skeletal muscle was digested with collagenase type B and dispase II (Roche). About 2 × 10<sup>5</sup> cells were normally obtained from four adult mice (1- to 2-mo old). Bone marrow was prepared as described previously (Goodell et al., 1997). FACS® analysis was performed on a cell sorter (MoFlo®; Cytomation, Inc.) equipped with dual lasers. Hoechst staining was performed in DME supplemented with collagenase type B and dispase II (Roche). About 2 × 10<sup>5</sup> cells were normally obtained from four adult mice (1- to 2-mo old). Bone marrow was prepared as described previously (Goodell et al., 1997). FACS® analysis was performed on a cell sorter (MoFlo®; Cytomation, Inc.) equipped with dual lasers. Hoechst staining was performed in DME supplemented
with 2% FCS, 10 mM Hepes, 5 μg/ml Hoechst 33342 (Sigma-Aldrich), and with or without 50 μM verapamil (Sigma-Aldrich) at 37°C for 90 min. After Hoechst staining, immunostaining was performed by using antibodies active to Sca-1 or CD45 conjugated with phycoerythrin or FITC (PharMingen), respectively. Hoechst dye was excited at 351 nm by UV laser and its fluorescence was detected at two wavelengths using 424/44 (Hoechst blue) and 775/50 (Hoechst red) filters. FITC or phycoerythrin was excited at 488 nm by argon laser and its fluorescence was detected at FL1 (530/40) or FL2 (580/30) filter. Dead cells and debris were excluded from the plots based on propidium iodide staining (2 μg/ml; Sigma-Aldrich). Sorted cells were characterized by X-gal staining or immunohistochemistry on slide glass.

Cell culture
Single muscle fibers and dissociated muscle cells (muscle-derived cells) were isolated from hindlimb skeletal muscles prepared from 2-month-old heterozygous Myf5-lacZ or MD6.0-lacZ transgenic mice by digestion in 0.4% collagenase type A (Roche) as described previously (Beauchamp et al., 2000). Isolated muscle fibers were immediately fixed or cultured in Methocult M3434 (StemCell Technologies Inc.). 5 × 10^3–10^4 FACS®-fractionated Tg(NGP/FU5–Nagy) (GFPI, ROSA26, or Myf5-lacZ–muscle SP cells were cultured or co-cultured with equal numbers of either primary myoblasts (Sabourin et al., 1999), C2C12 myoblasts, or C3H10T1/2 fibroblasts. Conditioned medium was obtained from growing primary myoblasts for 4 days. Muscle SP cells were also purified from 2-week-old Pax3^+/− and Pax7^−/− mice. PHAN-puro retrovirus vector was used as a control retrovirus vector, and retroviruses were prepared as previously described (Soneoka et al., 1995). PHAN-eGFP and PHAN-MyoD contain viruses LTR and CMV promoter–driving enhanced GFP gene and MyoD gene, respectively. 1 × 10^5 FACS®-fractionated muscle SP cells were cultured in myoblast growth media on collagen-coated dishes for 24 h, followed by infection with retrovirus vectors for 3 h with polybrene. 24 h after retrovirus infection, cultures of muscle SP cells alone or together with equal numbers of primary myoblasts were maintained. Cultures were maintained for 7 d in myoblast growth medium; Ham’s F10 medium supplemented with 20% FCS, 2.5 mg/ml basic FGF (R&D Systems) on collagen-coated dishes, and DME supplemented with 5% horse serum was used for three additional days. Myogenic differentiation of muscle SP cells after co-culture with myoblasts was quantified by number of nuclei in desmin or MHC-stained SP cells by 2 weeks after transplantation was represented by total LacZ expression was detected by X-gal staining or immunohistochemistry on slide glass.

Histochemistry
LacZ expression was detected by X-gal staining overnight as described previously (Beauchamp et al., 2000). Monoclonal anti–rabbit IgG (CHEMICON International) or rhodamine-conjugated anti–rabbit IgG (CHEMICON International), or FITC-conjugated anti–mouse IgG antibodies (CHEMICON International) or rhodamine-conjugated anti–rabbit IgG (CHEMICON International).

Intrasomatic injections
Muscle SP cells (3–6 × 10^5 cells) derived from Myf5-lacZ or ROSA26 mice were injected into regenerating TA muscle of adult scid/bg immunodeficient mice (Buckingham et al., 1998). 24 h after cell injection, retroviral generation was induced by injection of 25 μl of 10 μM cardiotoxin (Latanx). 2 weeks or 1 month after injection of cells, TA muscles were stained with X-gal, and 8-μm frozen sections were prepared for immunohistochemistry. Myf5-lacZ–expressing nuclei were counted in serial sections adjacent to the lacZ–positive region. Myogenic differentiation of muscle cell SP cells by 2 weeks after transplantation was represented by total number of Myf5-lacZ–positive nuclei per each transplanted TA muscle. Three independent transplantation experiments were performed (n = 3). 1 month after injection of cells, TA muscles were also digested for culture experiments as described previously (Sabourin et al., 1999). Cultures were maintained for 4 d in myoblast growth medium on collagen-coated dishes.

Hematopoietic colony assays
Cells prepared from muscle, bone marrow, peripheral blood, and isolated muscle fibers were cultured in Methocult M3434 or M3630 (StemCell Technologies) for 10–14 d. The number of colonies consisting of >50 cells were scored using an inverted microscope (Axiovert 25; Carl Zeiss Micro Imaging, Inc.). Hematopoietic cells were identified by May-Grunwald’s Giemsa (Sigma-Aldrich) and by immunohistochemistry with Mac1 and Gr1 antibodies (PharMingen) for detection of granulocyte/monocyte or B220 antibodies (PharMingen) for detection of pre-B cells following by Vectastain ABC kit (Vector Laboratories).

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