Skeletal muscle satellite cells can spontaneously enter an alternative mesenchymal pathway

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Summary
We show that muscle satellite cells, traditionally considered as committed myogenic precursors, are comprised of Pax7-expressing progenitors that preserve a mesenchymal repertoire extending beyond a mere myogenic potential. Mouse satellite cells from freshly isolated single myofibers, cultured individually in serum-rich growth medium, produced myogenic and non-myogenic clones. Only the myogenic clones expressed muscle-specific transcription factors and formed myotubes. Pax7 was initially expressed in all clones, but subsequently was associated only with the myogenic clones. Some cells in the non-myogenic clones expressed \(\alpha\)-smooth muscle actin and nestin whereas others differentiated into mature adipocytes. This type of cell composition mirrors characteristics of mesenchymal stem cell progeny. Overall, individual myofibers persistently gave rise to both clonal phenotypes, but the ratio of myogenic to non-myogenic clones randomly varied among fibers. This randomness indicates that clonal dichotomy reflects satellite cell suppleness rather than pre-fated cell heterogeneity. We conclude that satellite cells possess mesenchymal plasticity, being able to commit either to myogenesis or to a mesenchymal alternative differentiation (MAD) program.

Key words: Skeletal muscle satellite cells, Mesenchymal stem cells, Myoblasts, Adipocytes, Pax7 protein

Introduction
The growth and repair potential of adult skeletal muscle relies on a reservoir of myogenic precursors termed satellite cells that reside at the periphery of myofibers beneath their basement membrane (Mauro, 1961). In a mature muscle, satellite cells are quiescent but can re-enter the cell cycle in response to a variety of stimuli, ranging from work overload to massive trauma. Once activated, satellite cells divide and give rise to daughter myoblasts that eventually form new myofibers, or fuse with existing muscle fibers when more localized repair is required (Hawke and Garry, 2001).

Since they were first identified, satellite cells have been considered unipotent myogenic precursors. Indeed, studies of isolated myofibers unequivocally establish the myogenic potential of satellite cells (Bischoff, 1975; Rosenblatt et al., 1995; Yablonka-Reuveni and Rivera, 1994). Upon activation, satellite cells express the muscle-specific transcription factors Myf5 and MyoD, followed by the expression of myogenin upon differentiation (Cooper et al., 1999; Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994; Yablonka-Reuveni et al., 1999). Myf5 may already be expressed in quiescent satellite cells, although at a lower level than in the proliferating progeny (Beauchamp et al., 2000; Cooper et al., 1999). The paired-box transcription factor Pax7 is expressed in both quiescent satellite cells and their proliferating progeny (Seale et al., 2000; Yablonka-Reuveni, 2004; Zammit and Beauchamp, 2001).

Earlier studies have shown that myogenic cultures, already executing the myogenic program, can be diverted from this path when treated with bone-morphogenensis proteins or adipogenic-inducing agents (Katagiri et al., 1994; Teboul et al., 1995). Based on such studies, the possibility that satellite cells could also be diverted from a myogenic fate has begun to erode the classic convention that satellite cells are unipotent myogenic precursors. Indeed, multipotency of satellite cells was recently enforced by studies showing that cells within cultures emanating from single myofibers expressed some osteogenic and adipogenic markers (Asakura et al., 2001; Csete et al., 2001). Nonetheless, this expression of markers associated with non-myogenic cell lineages might reflect gene stimulation within the context of the myogenic progeny rather than implying multipotency of satellite cells.

The appearance of adipogenic cells within cultures emanating from single myofibers may indeed reflect the capacity of satellite cells to assume an adipogenic fate (Asakura et al., 2001; Csete et al., 2001). Nevertheless, the latter studies have not provided data regarding the incidence of myofiber cultures yielding adipogenic cells, nor did they investigate the lineal relationships between the adipogenic and myogenic cells or rule out the possibility that the adipogenic cells could have originated from remnant connective tissue cells adhering to the myofibers. Therefore, to prove multipotency of satellite cells it is necessary to clone these progenitors from freshly isolated myofibers and to assess their capacity to give rise to different cell lineages.

The present study, aimed at determining whether one common or separate satellite cells give rise to myogenic and adipogenic populations, provides the first direct evidence that true satellite cells can spontaneously (i.e. without treatment with lineage-inducing additives) give rise to non-myogenic descendants. Our results show a complete execution of both myogenic and adipogenic differentiation in all cultures.
emanating from individual myofibers. Clonal analysis of satellite cells from freshly isolated myofibers demonstrates that myogenic and adipogenic cells develop in separate myogenic and non-myogenic clones, respectively. The non-myogenic clones contain adipocytes along with non-skeletal muscle contractile cells, a composition characteristic of descendants of mesenchymal stem cells (Dennis and Charbord, 2002; Minguell et al., 2001). We conclude that satellite cells can enter a mesenchymal alternative differentiation (MAD) program: a developmental route that culminates with formation of cells other than myogenic ones.

Materials and Methods

Animals
Male and female 4-6 month-old C57BL/6 wild-type mice (ATL, Kent, WA) and C57BL/6-EGFP transgenic mice (Jackson laboratories) were used in this study (Okabe et al., 1997). GFP mice express enhanced green fluorescent protein under the control of the chicken β-actin promoter and cytomegalovirus. All tissues in these mice, with the exception of erythrocytes and hair, express GFP. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Isolation and culture of EDL myofibers
Single muscle fibers with associated satellite cells were isolated from the extensor digitorum longus (EDL) muscle as previously described (Rosenblatt et al., 1995) and further adopted for our studies (Shefer and Yablonka-Reuveni, 2004). In brief, the hind limb EDL muscles were digested for 60 minutes at 37 °C in 0.2% (w/v) collagenase type I (Sigma-Aldrich). Collagenase was reconstituted in DMEM (high glucose, with L-glutamine, 110 mg/l sodium pyruvate, and pyridoxine hydrochloride; supplemented with 50 U/ml penicillin and 50 mg/ml streptomycin; GIBCO-Invitrogen). Following digestion, the muscle was triturated with a wide-bore pipette to release single myofibers that were then cultured individually in 24-well plates (BD Biosciences). Wells were pre-coated with 1 mg/ml reduced growth factor Matrigel (BD Biosciences) diluted in DMEM. A total of 0.5 ml growth medium (DMEM containing 20% fetal bovine serum (Sigma-Aldrich), 10% horse serum (HyClone), 1% chicken embryo extract (Shefer and Yablonka-Reuveni, 2004; Yablonka-Reuveni, 1995)) was added to each well. Myofibers were incubated at 37.5 °C, 5% CO2 in a humidified tissue culture incubator. Collectively, we utilized GFP mice to investigate the clonal identity during early time points.

Fixation of cultures
Cultures were fixed at various time points as indicated in the Results. Fixative solution consisted of 4% paraformaldehyde in 0.1 M phosphate buffer, containing 0.03 M sucrose, pH 7.2-7.4. The fixative was warmed to 37 °C and added to the cultures for 10 minutes, maintaining a 1:1 ratio between culture medium and fixative. Cultures were then rinsed with Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl, pH 7.4) and permeabilized with 0.5% Triton X-100 in TBS at room temperature for 5 minutes. Cells were then incubated in TBS containing 1% normal goat serum (TBS-1%NGS) for one hour at room temperature, followed by overnight at 4°C to block nonspecific antibody binding. When fiber cultures were analyzed, the blocking solution contained 20% normal goat serum (TBS-20%NGS).

Immunofluorescence
Wild-type cultures were analyzed by standard single and double immunofluorescence as described (Yablonka-Reuveni et al., 1999). Additionally, in some instances, cultures were co-labeled with isoform-specific mouse monoclonal antibodies and reactivity was monitored with isoform-specific secondary antibodies. Fixed cultures were rinsed with TBS containing 0.05% Tween 20 (TBS-TW20) and reacted with the primary antibodies for one hour at room temperature followed by overnight at 4°C. Cultures were then rinsed with TBS-TW20, reacted with secondary antibodies for 90 minutes at room temperature and rinsed with TBS-TW20. After exposure to the secondary antibodies, cultures were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) to label nuclei. Cultures were then rinsed with TBS-TW20 followed by a final rinse with TBS. Each well received 40 µl Vectorshield mounting medium (Vector Laboratories) plus an additional 90 µl of 25% glycerol in TBS. Controls consisted of cultures reacted with one of the two primary antibodies followed by the two secondary antibodies and of cultures reacted with secondary antibodies alone.

GFP clones were analyzed exclusively with secondary antibodies emitting red fluorescence. To allow detection of coexpressed proteins, cultures underwent sequential labeling rounds. Cultures were first labeled with a mouse monoclonal antibody (reactivity detected with an IgG1-specific secondary antibody) followed by labeling with a polyclonal antibody (reactivity detected with anti-rabbit IgG secondary antibody). In instances of three sequential labeling rounds, for the third cycle, cultures were reacted with a second monoclonal antibody (reactivity detected with an IgG2a-specific secondary antibody). To strip each preceding antibody labeling, cultures were extensively rinsed then agitation in TBS-TW20 for 5 minutes followed by blocking in TBS-20%NGS for 90 minutes at room temperature. Complete signal stripping was ensured by fluorescence microscopy. Final steps of DAPI counter staining and mounting were performed as described above for wild-type cultures. To verify that the multiple
Primary antibodies
Primary antibodies were either monoclonal, produced in mouse or rat (mouse mAb and rat mAb, respectively), or polyclonal, produced in rabbit (rabbit pAb). Antibodies were diluted in the blocking solution (TBS-1%NGS).

The following primary antibodies were used: Anti-Pax7, mouse mAb (IgG1, ascites fluid, Developmental Studies Hybridoma Bank (DSHB); 1:2000 dilution); -MyoD, mouse mAb (IGG1, clone 5.8A, BD Biosciences; 1:800); rabbit pAb (M-318, Santa Cruz Biotechnology; 1:500); -Myf5, rabbit pAb (1:400) (Smith et al., 1993) produced and provided by Stephen Konieczny, Purdue University; -myogenin, mouse mAb (IGG1, clone F5D, hybridoma supernatant, DSHB; 1:2); anti-desmin, mouse mAb (clone D33, Dako; 1:200); anti-sarcomeric myosin, mouse mAb (IGG2b, clone MF20, hybridoma supernatant, DSHB; 1:20); anti-laminin B2, rat mAb (clone 1914, Chemicon; 1:250); anti- -smooth muscle actin, mouse mAb (IGG2a, clone 1A4, Sigma-Aldrich; 1:3000); anti-nestin, clone MF20, hybridoma supernatant, DSHB; 1:20); anti-laminin B2, rat mAb (clone 1914, Chemicon; 1:250); anti- -smooth muscle actin, mouse mAb (IGG2a, clone 1A4, Sigma-Aldrich; 1:3000); anti-nestin, clone MF20, hybridoma supernatant, DSHB; 1:20); Anti-MyoD, mouse mAb (IgG1, clone A4, Sigma-Aldrich; 1:3000); anti-nestin, clone MF20, hybridoma supernatant, DSHB; 1:20); Anti-c/EBP, rabbit pAb (14AA, Santa Cruz Biotechnology; 1:250), anti-c/EBP, mouse mAb (H-7, IgG2a, SCBT; 1:250), anti-c/EBP, rabbit pAb (M-17, SCBT; 1:250) and anti-PPARY, mouse mAb (IGG1, E-8, SCBT; 1:250) were provided by Denis Guttridge, Ohio State University. Anti-CD45, rat mAb (clone M1/9, hybridoma supernatant, American Tissue Culture Collection; 1:2) was provided by Andrew Farr, University of Washington. Anti-CD11a, rat mAb (clone M17/4, BD Biosciences; 1:200) was provided by Karin Bornfeldt, University of Washington. Anti-CD31, rat mAb (CBL1337 clone 390, Chemicon; 1:100), anti-MOMA2, rat mAb (PN IM2897, Immunotech; 1:100) and anti-F4/80, rat mAb (CL:A3-1, Serotech; 1:50) were provided by Stephen Schwartz, University of Washington.

Secondary antibodies
The following secondary antibodies (Molecular probes, diluted 1:1000 in TBS-1%NGS blocking solution) were used in a variety of combinations as indicated in the Results section: Alexa568-conjugated goat anti-mouse IgG; Alexa647-conjugated goat anti-mouse IgG1; Alexa568-conjugated goat anti-mouse IgG2a; Alexa647-conjugated goat anti-mouse IgG2b; Alexa488-conjugated goat anti-rabbit IgG; Alexa488-conjugated streptavidin.

Lipid staining with oil-red-O soluble dye
To facilitate labeling of cultures with oil-red-O alone or in combination with immunofluorescence, we followed a published procedure (Koopman et al., 2001). Cultures were fixed, permeabilized and blocked as described above. For colabeling, cultures were stained with oil-red-O after the secondary antibody was rinsed away. Stock solution of 0.5% (w/v) oil-red-O was prepared in 60% triethyolphosphate (Sigma-Aldrich) and filtered through Whatmann paper to remove dye aggregates. A working solution of oil-red-O was freshly prepared by diluting five volumes of the stock solution with three volumes of water followed by filtration through a 0.45 μm filter. Cultures were stained with oil-red-O for 30 minutes at room temperature, and then rinsed with TBS. Cultures were counterstained with DAPI to identify nuclei, and covered with mounting solution as in immunofluorescent labeling.

Microscope and imaging system
Observations were made with an inverted phase-contrast microscope equipped for immunofluorescent analysis (Nikon eclipse, TE2000-S). Images were acquired with a Qimaging Retiga 1300i Fast 1394 monochrome CCD camera. Camera and color acquisition were controlled by MetaView Imaging System (Universal Imaging Corporation). Composites of digitized images were assembled using Adobe Photoshop software.

Confocal microscopy
For confocal analysis, single fibers were cultured in 8-unit glass chamber tissue culture slides (Lab-Tek), coated with Matrigel as described above. Myofibers were fixed 4 hours after culturing. Myofibers were reacted with an antibody against laminin (reactivity monitored with a green fluorescent secondary antibody), and counterstained with 1 ng/ml propidium iodide for 15 minutes to highlight nuclei. Myofibers were viewed using a Zeiss PASCAL laser-scanning confocal microscope. Images were scanned and 0.32-μm serial optical sections were acquired. Composite images of myofibers were assessed for the presence of nuclei above or beneath the basement membrane.

Results
Spontaneous formation of fat-accumulating cells within single fiber cultures
In cultures of viable single myofibers from the EDL muscle, satellite cells first migrated to the vicinity of the myofiber and then underwent extensive proliferation followed by fusion into myotubes (Fig. 1). Myofibers fixed at T0 exhibited Pax7+ nuclei (i.e., satellite cells) on their surface (Fig. 1A, A'). By 19-36 hours, mononucleated cells were detected just emerging from, or adjacent to the myofiber. These cells were always positive for Pax7 (Fig. 1B-C'). Over the next three days, there was an increase in the total number of cells near the myofiber. By the first week, most cells coexpressed Pax7 and MyoD (Fig. 1F-F'), as well as Myf5 and desmin (data not shown). In addition to myotubes, development of cells containing large fat droplets was observed within 2-3 weeks (Fig. 1G-H'). These latter cells were identified as adipocytes based on their morphology and staining with oil-red-O, a reagent that stains neutral lipids (mainly triglycerides) with a dark red color (Fig. 2A,B). Typical large, spread-out cells with large nuclei were also present in the adipogenic areas; these cells were barely noticeable by phase-contrast microscopy (Fig. 1G,G'). The fine cytoplasmic details of these spread-out cells were easily detectable when analyzing cultures from EGFP mice or when the cells were immunostained with a variety of antibodies as detailed below.

A more detailed study was designed to follow up the time-dependent events associated with adipocyte differentiation in myofiber cultures (Fig. 2). Single myofiber cultures (n=100, from two independent experiments) were grown for six weeks and were inspected bi-weekly for adipocyte formation. Parallel cultures (n=4 per time point) were harvested weekly for oil-red-O staining. Additional myofiber cultures were fixed at various time points for immunolabeling with antibodies against skeletal myogenic markers. By days 10-14, 47 of the 100 myofiber-cultures that were followed morphologically exhibited enlarged cells containing minuscule droplets, confirmed by oil-red-O staining to contain fat (Fig. 2A). Such cells, typically associated with earlier phases of adipocyte differentiation, are referred to...
as multilocular cells (Mersmann et al., 1975). These cells were negative for Pax7, MyoD, Myf5, myogenin and desmin as determined by immunofluorescence combined with oil-red-O staining (data not shown). The number of fat-accumulating cells, as well as the number of fat deposits, gradually increased with time. After 2-3 weeks, multilocular cells were present in 33 fiber cultures, whereas 63 additional cultures featured fusion of the miniscule fat deposits, forming cells with either a few large droplets (paucilocular cells) or one large droplet (unilocular cells) (Fig. 2B). Formation of paucilocular cells preceded the development of unilocular cells. By week six, all cultures contained adipogenic cells with pauci- and unilocular cells present in 97 out of 100 cultures. Like multilocular cells, pauci- and unilocular cells did not express myogenic markers. Large oil droplets were never detected in myogenic cells, although small droplets, representing basal level of triglycerides, could be detected by oil-red-O staining in some myogenic cells (Fig. 2C,D). Fig. 2E depicts a time-course quantification of the aforementioned myofiber cultures as they accumulate multi-, pauci- and unilocular cells.

Analysis of single myofibers for the presence of adhering cells

Before a firm conclusion regarding the origin of the adipocytes formed in myofiber cultures could be made, it was critical to evaluate whether myofibers were isolated with adhering connective tissue cells. As the connective tissue surrounding myofibers contains microvasculature and resident immune cells (Pimorady-Esfahani et al., 1997), we examined the expression of the following panel of well-recognized antigens characteristic of the latter cells: (a) CD45, expressed by all hematopoietic cells excluding circulating erythrocytes and platelets (Trowbridge and Thomas, 1994) but not by satellite cells (Asakura et al., 2002); (b) F4/80, expressed by the majority of mature macrophages and dendritic cells (Austyn and Gordon, 1981); (c) MOMA-2, expressed by mature murine tissue macrophages not necessarily identified by F4/80 expression (Kraal et al., 1987); (d) CD11a, expressed by all leukocytes (Sanchez-Madrid et al., 1982); and (e) CD31, expressed by endothelial cells and reproducibly recognizes the microvasculature (DeLisser et al., 1994). CD34 immunolabeling also recognizes the muscle microvasculature (Zammit and Beauchamp, 2001) but was not used in the present study as CD34 is also expressed by myofiber-associated satellite cells (Beauchamp et al., 2000). Freshly isolated myofibers as well as myofiber cultures were consistently negative for all the above markers (data not shown). Furthermore, a three-dimensional analysis of myofibers conducted using confocal microscopy demonstrated the absence of cells outside the myofiber basement membrane. Collectively, the absence of adhering cells indicated that the adipogenic cells, developing in myofiber cultures at later time points, were indeed derived from satellite cells.

Clonal analysis of satellite cells from single myofibers identifies myogenic and adipogenic cells in different clones

We followed the fate of clones derived from freshly isolated
myogenic and adipogenic cells accumulating during a 6-week period.

Fig. 2. Formation of adipocytes in mouse myofiber cultures. (A-D) Representative images of myofiber-derived cultures co-stained with DAPI (blue) and oil-red-O (red). (A) Multilocular cells (indicated with arrows) in a non-myogenic area within a 10-day-old culture. (B) Multi-, pauci- and unilocular cells (indicated by the upper right, middle and lower left arrows, respectively) in a non-myogenic area of a 3-week-old culture. (C,D) Myoblasts and myotubes exhibiting minimal staining with oil-red-O in 10- and 21-day-old cultures; myoblasts are indicated with arrows in panel C and a myotube is indicated with an arrowhead in panel D; a multilocular cell present in panel D is indicated with an arrow. Bar, 20 μm. (E) Graph depicting the number of fiber cultures containing adipogenic cells accumulating during a 6-week period.

myofibers in order to determine whether the observed myogenic and adipogenic cells were produced by a common or distinct satellite cells. Clones were derived from 242 individual myofibers prepared from both wild-type and GFP mice (n=17). The observed range of the number of clones per myofiber as shown in Fig. 4A is in agreement with that reported for satellite cells in freshly isolated EDL myofibers (Zammit et al., 2002). Two distinct clonal types were identified as early as day 4 following clonal establishment: a) One type of clone consisted of flat cells, with extensive filamentous cytoplasm and one or two large nuclei; mononucleated cells with irregular edges or triangular shape were also present (Fig. 3A). Many of the cells were multilocular by day 7. Subsequently, by day 14-21 pauci- and unilocular cells developed (Fig. 3C). The cells in this type of clone neither expressed myogenic-specific markers nor formed myotubes for up to two months after cloning. This type of clone was classified as non-myogenic. b) The second type of clone initially included mononucleated, round-edged cells with a small volume of cytoplasm surrounding small round nuclei. By day 7, this type of clone spontaneously formed multilocule myotubes (Fig. 3B) whereas multi-, pauci- or unilocular cells never formed, even when the clone was maintained for two months. This type of clone was classified as myogenic.

Of the 949 clones produced from the 242 myofibers summarized in Fig. 4A, 37% were myogenic. 58% were non-myogenic and 5% included both myotubes and adipocytes in the same well. The mixed-culture wells had two foci of clonal growth, well separated from each other, that were seen from the first days in culture. Immunofluorescent labeling of these cultures revealed the expression of myogenic characteristic markers was restricted to only one focal growth. Hence, the bifocal cultures probably developed from two separate progenitors that landed in the same well. Fig. 4B depicts the distribution of myogenic, non-myogenic and mixed clones per each fiber that contributed to the collective clonal analysis shown in Fig. 4A. Each inset in Fig. 4B depicts fibers grouped according to the total number of clones they produced. No correlation was found between the number of clones produced per fiber and the distribution of clones to myogenic and non-myogenic types.

Cells in both myogenic and non-myogenic clones express transcription factors associated with adipogenesis

Two families of transcription factors are prominent in the control of adipogenesis: peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer binding proteins (c/EBPs) (Kirkland et al., 2002). Early events include up-regulation of c/EBPα and c/EBPβ, followed by the induction of PPARγ and c/EBPβ (Lin and Lane, 1994; Tontonoz et al., 1994). To study the expression dynamics of adipogenic-associated transcription factors in both clonal types, the nuclear localization of ‘early’ and ‘late’ myogenic and adipogenic transcription factors was analyzed by immunofluorescence (Fig. 3E-H). GFP clones, sequentially immunostained, were used to study early time points. Standard double immunostaining was performed when wild-type clones were analyzed at later time points. Four-day-old clones were classified as myogenic or non-myogenic based on MyoD expression (MyoD-expressing clones also expressed Myf5 and desmin). Regardless of classification, all clones initially expressed PPARγ and c/EBPs (α, β, γ and δ forms) at comparable levels. By day 7, all clones continued to express PPARγ and c/EBPs, with the exception that myonuclei expressed low (if any) levels of PPARγ (Fig. 3E-H) and c/EBPα. Within the non-myogenic clones, the formation of pauci- and unilocular cells coincided with elevated levels of PPARγ (Fig. 3E) and c/EBPα in their nuclei.

Cells expressing α-smooth muscle actin accumulate in non-myogenic clones in parallel with adipocyte development

Spread-out filamentous cells present in non-myogenic clones (e.g., Fig. 5A) resembled non-skeletal muscle contractile cells
(Serini and Gabbiani, 1999). To evaluate these cells, clones were tested for the expression of $\alpha$-smooth muscle actin ($\alpha$SMA), nestin and desmin, cytoskeletal proteins characteristic of skeletal and non-skeletal muscle contractile cells (Graves and Yablonka-Reuveni, 2000; Kachinsky et al., 1994; Springer et al., 2002; Treutelaar et al., 2003). Indeed, the non-myogenic clones were comprised of cells expressing $\alpha$SMA (Fig. 5A) and nestin (Fig. 5C) however desmin (Fig. 5E,F) was exclusively expressed in the myogenic clones. Nestin was expressed in all cells within the non-myogenic clones, excluding adipocytes whereas $\alpha$SMA was expressed only in flat filamentous cells containing large nuclei. During the first week, only 1-2 $\alpha$SMA-expressing cells were present in the non-myogenic clones, but their number increased with time until a network of $\alpha$SMA positive cells was formed, engulfing the accumulating multilocular cells that expressed PPAR$\gamma$ (Fig. 5G). Differentiated myogenic cells coexpressed $\alpha$SMA and sarcomeric myosin (Fig. 5B,B$'$) whereas in the non-myogenic clones, cells expressing $\alpha$SMA were negative for sarcomeric myosin (Fig. 5A,A$'$).

**Pax7 is initially expressed in both myogenic and non-myogenic clones**

The single-fiber analyses detailed above demonstrated that satellite cells initially express Pax7 (Fig. 1A-E$'$). Here we investigated the expression of Pax7 in myogenic and non-myogenic clonal descendants of satellite cells. Clones were examined for the expression of Pax7 and MyoD (Fig. 6 and Table 1). Four-day-old clones (Table 1A) were derived from GFP mice, whereas 7-, 10-, and 14-day-old clones (Table 1B) were derived from both GFP and wild-type mice. As elaborated earlier, monitoring of GFP clones using fluorescent microscopy facilitated detection of all clones as early as culture day four. GFP and wild-type clones were analyzed by sequential and double immunostaining, respectively.

Table 1A summarizes the distribution of Pax7- and MyoD-expressing cells in 4-day-old clones derived from GFP mice. Of the 86 clones analyzed, 34 (40%) were Pax7$^+$/MyoD$^-$, 27 (31%) were Pax7$^+/\!/\!$MyoD$^+$, 25 (29%) were Pax7$^{-}/\!/\!$MyoD$^{-}$ and 0 (0%) were Pax7$^{-}/\!/\!$MyoD$^+$. Of the 61 clones that expressed Pax7 (i.e. Pax7$^+/\!/\!$MyoD$^-$ and Pax7$^+/\!/\!$MyoD$^+$), 56% expressed MyoD. Collectively, all MyoD-expressing clones were also positive for Pax7, whereas of the MyoD-negative clones 52% were positive for Pax7. The existence of Pax7$^+/\!/\!$MyoD$^-$ clones was also confirmed when clones were first labeled for MyoD antibody and then with Pax7 (data not shown). Pax7 immunoreactivity in the MyoD$^-$ clones was less intense than in the MyoD$^+$ clones regardless of staining order (Fig. 6B,D). We were unable to discern the status of Pax7 and MyoD expression of clones prior to day 4 in culture for two reasons:

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**Fig. 3.** Cell morphology and expression of adipogenic-associated markers in non-myogenic (left column) and myogenic (right column) clones. (A,B) Fluorescent images of 7-day-old clones derived from one GFP-myofiber; the clone depicted in panel A shows multiple cell morphologies and the clone depicted in panel B shows myotubes. (C,D) Representative merged images of 2-week-old cultures visualized with DAPI (blue) and oil-red-O (red); adipogenic cells are detected only in the non-myogenic clone. (E,F) Images of 2-week-old cultures labeled with anti-PAR$\gamma$ (red) and DAPI (blue) merged with the phase-contrast image; intense nuclear staining of PPAR$\gamma$ was evident only in the adipogenic cells (E); a very low level of PPAR$\gamma$ was detected in some of the myonuclei (F). (G,H) Double immunostaining with c/EBP$\delta$ (green) and MyoD (red), image of each stain was merged with the phase-contrast image; the non-myogenic clone was additionally stained with oil-red-O; both clones exhibit similar levels of nuclear expression of c/EBP$\delta$ (G and H) but only the myogenic clone expresses MyoD (G$'$,H$'$). Note that the two mononucleated cells in panel H$'$ that seem to be negative, in fact express low levels of MyoD. However, this weaker staining is lost when the MyoD immunostaining image and the phase-contrast image are merged. The variations in the level of MyoD probably reflect different phases of the cell cycle as previously reported (Kitzmann et al., 1998). Of particular interest is the pair of cells (top of micrograph in H$'$) that seem to be just separating from each other following cell division with only one cell expressing a high level of MyoD. Bar, 20 $\mu$m.
Plasticity of satellite cells

first, the characteristically meager volume of the cytoplasm of cells in such early cultures does not permit an unbiased distinction of nuclear-specific versus non-specific immunosignal and second, cells in such early clones frequently detach when subjected to the immunostaining procedure.

Table 1B summarizes the distribution of Pax7- and MyoD-expressing clones at different time points after culturing. Data shown for the 4-day-old clones are a summary of the data presented in Table 1A. In contrast to the finding that during the earlier time point not all Pax7-expressing clones are also positive for MyoD, from 1 week in culture onwards, the expression of Pax7 strictly coincided with MyoD expression (Table 1B).

Importantly, clone-per-fiber distribution, as well as the frequency of myogenic/non-myogenic clones was consistent for the clones at all four time points summarized in Table 1B and in Fig. 4.

Discussion

The clonal analyses of satellite cells presented in this study demonstrate that Pax7-expressing progenitors can commit to mutually exclusive myogenic or non-myogenic differentiation pathways. Although some satellite cells follow the 'classic' myogenic route that culminates with myotube formation, others enter an alternative non-myogenic program that culminates with adipogenic differentiation. Commitment of satellite cells to either program occurred prior to the initial round of cell proliferation as all cells within a given clone were uniformly myogenic or non-myogenic. Once commitment to a pathway has commenced, no transdifferentiation between the two lineages occurred within the progenitor’s descendants. Individual myofibers persistently gave rise to both clonal phenotypes. However, the ratio of myogenic and non-myogenic clones varied among fibers. This randomness of satellite cell fate commitment led us to conclude that clonal dichotomy does not reflect the existence of pre-fated subpopulations of satellite cells but rather points toward plasticity/multipotency of the satellite cell.

The non-myogenic clones were comprised of αSMA-expressing cells alongside mature adipocytes: a composition that mirrors characteristics of mesenchymal stem cell progeny (Allen and Dexter, 1983; Dennis and Charbord, 2002; Minguell et al., 2001). Moreover, cells in the non-myogenic clones expressed nestin, a protein associated with a variety of progenitor cells possessing a broad developmental plasticity (Kania et al., 2003; Vogel et al., 2003). In view of the
aforementioned features of the non-myogenic clones, we propose that satellite cells can enter either a myogenic differentiation program or a mesenchymal alternative differentiation (MAD) program. This dual aptitude of satellite cells is reminiscent of developmental events during somite diversification when the dermomyotome establishes the myogenic lineage (Buckingham et al., 2003), whereas somitic cells facing the notochord develop into mesenchyme, giving rise to loose, migratory cells and to adipogenic progenitors (Tajbakhsh, 2003). Unlike cultures derived from fibers or from whole muscle, the cloning of satellite cells permits insight into their mesenchymal suppleness. Specifically, when satellite cells are cloned they are removed from the muscle niche where they typically belong and which provides lineage-restricting cues. This ‘un-homing’ of satellite cells exposes their expanded mesenchymal potential as well as their tissue-directed default myogenic fate.

Several studies have identified multipotential cells (termed MSCs/MDCSs and SP cells) in cell isolates prepared from whole muscles. In all cases, these multipotential cells expressed the stem cell antigen Sca-1 (Asakura et al., 2002; Gussoni et al., 1999; Qu-Petersen et al., 2002; Polesskaya et al., 2003). The intramuscular location of these multipotential cells has not yet been fully resolved. Nevertheless, immunolabeling of muscle cross sections and isolated myofibers demonstrated the absence of Sca-1+ cells in the satellite cell niche (Asakura et al., 2002; Dreyfus et al., 2004; Tamaki et al., 2002; Zammit and Beauchamp, 2001). In view of our experiments demonstrating the absence of cells adhering to the surface of the myofibers and the aforementioned studies ruling out the presence of Sca-1+ cells underneath the myofiber basement membrane, we conclude that MSCs/MDCSs or SP cells cannot be considered as possible progenitors of the myogenic or non-myogenic descendants derived from myofibers.

For each time point, clones were pooled from three independent experiments. No statistical difference was found between the different experiments with regard to clonal distribution (ANOVA, P>0.05).

Table 1. Expression of Pax7 in myogenic and non-myogenic clones

| Clonal identity* † | Number of clones | Number of Pax7+ clones‡ | % Pax7+ clones |
|-------------------|-----------------|------------------------|---------------|
| Myogenic (MyoD+)  | 34              | 34                     | 100           |
| Non-myogenic (MyoD–) | 52              | 27                     | 52            |
| Total (MyoD+ & MyoD–) | 86              | 61                     | 71            |

A. Distribution of Pax7+ cells in 4-day-old myogenic and non-myogenic clones

B. Distribution of Pax7+ or MyoD+ clones after various days in culture

For each time point, clones were pooled from three independent experiments. No statistical difference was found between the different experiments with regard to clonal distribution (ANOVA, P>0.05).

*All cells within a given clone were uniform with regard to MyoD expression or lack of expression. Analyses of parallel clones showed that MyoD+ clones were also positive for Myf5 and desmin, while MyoD– clones were negative for the latter protein markers.

†All clones were stained first with anti-Pax7, next with anti-MyoD and finally with anti-c/EBPβ. All cells in all clones were positive for c/EBPβ.

‡All cells within a given clone were uniform with regard to Pax7 expression or lack of expression. Representative images of GFP cells immunostained with Pax7 and MyoD are shown in Fig. 6.

Fig. 5. Expression of cytoskeletal proteins in parallel non-myogenic (left column) and myogenic clones (right column). (A–B′) Double immunostaining for αSMA (green) and sarcomeric myosin (red); both non-myogenic cells and myotubes express αSMA (A,B), however only myotubes also express sarcomeric myosin (A′,B′).

(C,D) Images of immunolabeled cultures showing nestin expression in both clonal types. (E,F) Images of immunolabeled cultures showing desmin expression only in the myogenic clone.

(G,H) Merged images of clones double immunostained for αSMA (green) and PPARγ (red); both αSMA and PPARγ were expressed in the non-myogenic clone, whereas only αSMA was expressed in myotubes. Bar, 20 μm.
Plasticity of satellite cells

Myogenic and adipogenic differentiation programs are mutually exclusive

The absence of adipocytes in myogenic clones, at any time point of clonal analysis, indicates that myogenesis and adipogenesis are mutually exclusive under our culture conditions. Indeed, when a cell line that can form both myotubes and adipocytes was cloned and treated concomitantly with inducers for both muscle and fat, colonies underwent either myogenic or adipogenic differentiation (Davis et al., 1987; Weintraub et al., 1989).

The acronym MAD was previously introduced for ‘mesenchymal adipose-like default’ cells in connection with age-dependent accumulation of fat in non-adipose tissues including muscle (Kirkland et al., 2002; Taylor-Jones et al., 2002). Here, we use the MAD acronym in the context of satellite cells assuming a mesenchymal alternative (non-myogenic) differentiation pathway. Unlike the latter two studies discussing accumulation of fat within myogenic cells, we show fat formation through a complete adipogenic differentiation program, initiated when satellite cells commit to the MAD pathway. Progeny of satellite cells undertaking this route sequentially express transcription factors associated with adipogenesis and display characteristic morphological changes. At first, fat accumulated in deposits of various sizes, forming multilocular cells. This step was followed by the formation of increasingly larger deposits, forming pauci- and unilocular cells that exhibited enhanced nuclear expression of c/EBPα and PPARγ. Interestingly, during the first week, all cells expressed similar levels of the various c/EBPs and PPARγ regardless of clonal identity. Hence, this ‘basal’ expression level of adipogenic-associated transcription factors does not interfere with myogenesis.

The identity of myogenic and MAD clones remained unchanged throughout the extended times they were followed. Once a pathway had commenced, no transdifferentiation between the lineages occurred within descendants of the progenitor. Although 5% of the clonal cultures exhibited both myogenic and non-myogenic cells, these phenotypes emerged in two separate foci. The bifocal growth pattern within one culture well and the low frequency of such an event indicates that the bifocal cultures resulted from occasional co-seeding of two progenitors. Our conclusion, that each satellite cell commits exclusively to either the myogenic or MAD program, but cannot concurrently execute both programs, apparently conflicts with a previous study proposing that progeny of an individual satellite cell can concomitantly follow myogenic, adipogenic and osteogenic differentiation programs (Wada et al., 2002). However, unlike our direct cloning of satellite cells from single myofibers, the latter study analyzed expanded clonal populations prepared from cells accumulating in 6-day-old myofiber-derived cultures. The sole clone presented in the latter study consisted of cells that spontaneously differentiated into myotubes, yet other differentiation programs were revealed only upon addition of inducers. In light of our observations that satellite cell fate is established prior to the first round of proliferation, we suggest that the founder of the clonal culture analyzed by Wada and colleagues (Wada et al., 2002) was already committed to the myogenic lineage. Application of adipogenic or osteogenic inducers to myogenic cells would be expected to inflict the respective cell phenotype as shown in earlier studies (Katagiri et al., 1994; Teboul et al., 1995).

Signaling pathways induced by oxidative stress were suggested to play a role in directing satellite cells to undertake an adipogenic phenotype (Csete et al., 2001). However, our findings do not implicate oxidative stress pathways as the main underlying mechanism of adipocyte formation in satellite-cell progeny. We analyzed satellite cells from mice whose levels of enzymes involved in processing reactive oxidative species were genetically manipulated. In view of a previous study that demonstrates the importance of catalase and glutathione peroxidase in the response of muscle to oxidative stress (Franco et al., 1999), we specifically focused on mice overexpressing catalase (S. Schriner, N. Linford, W. Ladiges and P. Rabinovitch, unpublished data) or lacking glutathione peroxidase-1 (Ho et al., 1997). Unexpectedly, we found that the formation of mature adipocytes within clones and cultures derived from myofibers of mice overexpressing catalase was

Fig. 6. Images of 4-day-old GFP clones sequentially labeled with anti-Pax7 followed by anti-MyoD. (A–B’) Clone 1 is positive for both Pax7 and MyoD. (C–D’) Clone 2 is positive for Pax7 but negative for MyoD. The use of GFP-labeled cells allowed detailed characterization of the cell body, demonstrating morphological distinctions between the two clonal phenotypes. Bar, 20 μm.
the Pax7– clones seen on day 4 could actually have been cells never express Pax7. In such a scenario, at least some of turned off in individual clones.
Pax7 expression in live cells, as clones develop from single to multiple cells, and identifying the time point at which Pax7 is expression in clones prior to day 4 in culture. Future generation section, at present we are unable to discern Pax7 and MyoD time point onwards. However, as indicated in the Results can infrequently enter the satellite cell niche and acquire myogenic properties.

Based on our observation that satellite cells are positive for Pax7 expression only in myogenic (MyoD+) clones from that week, Pax7 expression exclusively coincided with MyoD expression and from that time point onward clones were either positive or negative for the latter two transcription factors. Based on our observation that satellite cells are positive for Pax7 when situated on the surface of the myofibers, we suggest that all clones initially expressed Pax7, but by day 4 in culture, Pax7 was already downregulated in some of the non-myogenic (MyoD+) clones. This downregulation of Pax7 in non-myogenic clones culminated by one week in culture, resulting in Pax7 expression only in myogenic (MyoD+) clones from that time point onwards. However, as indicated in the Results section, at present we are unable to discern Pax7 and MyoD expression in clones prior to day 4 in culture. Future generation of a transgenic mouse expressing traceable endogenous Pax7 (for example a Pax7-GFP mouse) will enable the monitoring Pax7 expression in live cells, as clones develop from single to multiple cells, and identifying the time point at which Pax7 is turned off in individual clones.

Clearly, we cannot rule out the possibility that some satellite cells never express Pax7. In such a scenario, at least some of the Pax7– clones seen on day 4 could actually have been derived from Pax7+ satellite cells. This would imply satellite cell heterogeneity with respect to Pax7 expression. The Pax7– satellite cells may represent an earlier lineage compartment encompassing progenitors of Pax7+ precursor cells. In any event, the initial higher number of Pax7+ clones compared to MyoD+ clones by day 4, followed by the disappearance of the Pax7+/MyoD+ phenotype combined with an increase in Pax7−/MyoD− clones by day 7, indicates that at least some non-myogenic clones were derived from Pax7+ progenitors. Overall, we conclude that initial expression of Pax7 in myofiber-associated progenitors does not inevitably bestow myogenic lineage commitment. Rather, it is expressed in satellite cells and declines once satellite cells divert to the alternative non-myogenic MAD program.

The role of the myogenic niche in directing satellite cell fate

Three different cell culture systems were used to provide complementary insight into the biology of satellite cells. In standard primary cultures, the myogenic progenitors are extracted from the muscle tissue and cultured at a high density. These culture conditions presumably facilitate inductive myogenic signals between cells, even during early days ex vivo. Indeed, in such cultures, the vast majority of the cells (>90%) express muscle-specific markers (Yablonka-Reuveni, 2004). In the present clonal studies, satellite cells were extracted from their parent myofiber and deprived of inductive signals secreted by neighboring cells that are available in primary cultures. ‘De-homed’ from any restrictive myogenic environment, satellite cells display their mesenchymal suppleness, randomly committing to either a myogenic or a MAD program. The third procedure we used consisted of cultures emanating from single myofibers. In this approach, satellite cells are first in close association with the parent myofiber. However when migrating away from the myofiber, satellite cells initially encounter a
sparse cellular milieu, if any. In this type of culture, execution of the MAD program is apparent, although satellite cells predominantly commit to myogenesis. We propose that, similar to the outcome of satellite cell de-homing ex vivo, MAD commitment prevails in vivo when the skeletal muscle niche is impaired during disuse, aging and myopathies. Thus, understanding the different inductive signals directing satellite cells to enter myogenic or MAD programs can shed light on mechanisms underlying muscle health and disease.

Do MAD cells possess a broad mesenchymal plasticity? In the present study MAD cells spontaneously differentiated to adipocytes and non-skeletal muscle contractile cells. As summarized in the model depicted in Fig. 7 we propose that, depending on the niche that de-homed satellite cells are placed in, MAD cells may give rise to an expanded repertoire of mesodermal lineages similar to that described for mesenchymal stem cells derived from a variety of tissues (Dennis and Charbord, 2002; Grounds et al., 2002; Young et al., 2001). In an analogous manner, the acquisition of myogenic functions by de-homed bone marrow-derived stem cells entering the myofiber sublaminar compartment (Gussoni et al., 1999; LaBarge and Blau, 2002; Dreyfus et al., 2004), demonstrates that a tissue-restricting environment can impose lineage commitment of de-homed promiscuous progenitors. Although the model depicted in Fig. 7 is based on our studies of skeletal muscle satellite cells, we propose this model as a general mechanism for suppleness of tissue specific stem cells when confronted with unfamiliar signaling environment.

In conclusion, the present study provides the first direct evidence that true satellite cells can spontaneously give rise to non-myogenic mesenchymal descendants. This study not only presents novel insight into the biology of satellite cells but also proposes an experimental model for future investigations on mechanisms underlying lineage fate decisions of mesenchymal progenitors.

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