

Muscle reconstitution by muscle satellite cell descendants with stem cell-like properties

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Summary

Recent studies have demonstrated that a distinct subpopulation with stem cell-like characteristics in myoblast culture is responsible for new muscle fiber formation after intramuscular transplantation. The identification and isolation of stem-like cells would have significant implications for successful myogenic cell transfer therapy in human muscle disorders. Using a clonal culture system for mouse muscle satellite cells, we have identified two cell types, designated 'round cells' and 'thick cells', in clones derived from single muscle satellite cells that have been taken from either slow or fast muscle. Clonal analysis of satellite cells revealed that the round cells are immediate descendants of quiescent satellite cells in adult muscle. In single-myofiber culture, round cells first formed colonies and then generated progeny, thick cells, that underwent both myogenic and osteogenic terminal

differentiation under the appropriate culture conditions. Thick cells, but not round cells, responded to terminal differentiation-inducing signals. Round cells express Pax7, a specific marker of satellite cells, at high levels. Myogenic cell transfer experiments showed that round cells reconstitute myofibers more efficiently than thick cells. Furthermore, round cells restored dystrophin in myofibers of mdx nude mice, even when as few as 5000 cells were transferred into the gastrocnemius muscle. These results suggest that round cells are satellite-cell descendants with stem cell-like characteristics and represent a useful source of donor cells to improve muscle regeneration.

Key words: Myoblast transfer therapy, Muscle regeneration, Muscle satellite cell

Introduction

Transplantation of myoblasts is a potential therapeutic approach to human muscle disorders that exploits their differentiation capacity. However, the majority of transplanted myoblasts quickly die after intramuscular injection (Beauchamp et al., 1999; Fan et al., 1996; Hodgetts et al., 2000; Qu et al., 1998), which might be due to the host immune response (Smythe et al., 2001). A previous study has suggested that only a minority of transplanted myoblasts survive and contribute to new myofiber formation (Beauchamp et al., 1999). This minority retains stem cell-like characteristics: self-renewal for new stem cell-like cells and generation of cells committed to terminal differentiation. These cells may represent a population previously referred to as reserve cells (Schultz, 1996). The efficiency of myogenic cell transfer might be improved by using a distinct population with stem cell-like properties. Thus, the identification and isolation of an undifferentiated subpopulation with stem cell-like characteristics from postnatal skeletal muscle would certainly improve the myogenic cell transfer therapy for human muscle diseases.

Skeletal muscle exhibits a great capacity for regeneration after injury. Muscle stem cells, also known as muscle satellite cells, are responsible for the repair and regeneration of adult

skeletal muscle tissue. Histopathological analysis has shown that satellite cells differentiate into myotubes and myofibers exclusively (Saito and Nonaka, 1994). However, the multipotentiality of satellite cells was suggested by studies of both primary cultured mouse myoblasts and an immortalized mouse myoblastic cell line (Asakura et al., 2001; Katagiri et al., 1994; Teboul et al., 1995; Yamamoto et al., 1997). Furthermore, our previous clonal analyses indicated that mouse and human muscle satellite cell-derived clones actually preserve the ability to undergo myogenic, osteogenic and adipogenic terminal differentiation in vitro (Wada et al., 2002).

It has been suggested that satellite cells are highly heterogeneous in nature. Satellite cells have been shown to exhibit diversity in relation to the type of fibers in which they reside. Satellite cells originating from slow or fast muscles form myotubes in vitro that express different isoforms of myosin heavy chains (Qu et al., 2000; Rosenblatt et al., 1996). After transplantation, myoblasts fuse with host myofibers expressing the same type of myosin heavy chain (Qu et al., 2000). In addition, clonal analysis suggests that satellite cells within the same muscle proliferate at different rates in vitro (Molnar et al., 1996; Schultz et al., 1985; Yablonka-Reuveni et al., 1987). However, whether multipotentiality is a common

property of all satellite cells or is preserved in only a limited fraction of satellite cells remains unknown.

Two populations of satellite cell-derived myogenic cells have been identified in rat muscle (Rantanen et al., 1995; Schultz, 1996): a minority of cells divide slowly and represent a population with stem cell-like properties, whereas the majority divide rapidly and are committed to terminal differentiation. The former remain undifferentiated when cultured under differentiation-inducing conditions, and are involved in the generation of rapidly dividing cells. Thus, these stem-like cells were designated reserve cells (Schultz, 1996). The two populations were also identified in myogenic cultures derived from a single cell (Baroffio et al., 1996). Myogenic cells with stem cell-like properties might be necessary for muscle to regenerate on transplantation (Beauchamp et al., 1999). However, their ability to reconstitute skeletal muscle has not been determined because they have not been identified and purified from the primary myogenic culture prior to transplantation.

We have established a unique primary culture system of mouse and human myogenic cells, in which muscle satellite cells located on isolated single myofibers are able to grow clonally and form colonies containing only their descendants (Wada et al., 2002). This culture system enables us to follow the sequence of events occurring in each colony derived from a single satellite cell. Using this culture system, the present study indicates that the majority of mouse muscle satellite cell clones retain both myogenic and osteogenic differentiation potential *in vitro*. We have identified two distinct cell types in single-satellite cell-derived colonies: 'round cells', which divide slowly, and 'thick cells', which divide rapidly. Round cells, representing activated satellite cells that are the immediate descendants of quiescent satellite cells, are capable of self-renewal and the generation of rapidly dividing progeny, thick cells. We showed that round cells can efficiently contribute to muscle regeneration when transplanted into injured adult mouse host muscle. The identification and isolation of activated satellite cells with stem cell-like properties from adult muscles shown here has significant implications for successful myogenic cell transfer in human muscle diseases.

Materials and methods

Cell culture

C57Bl/6 mice, or GFP transgenic mice whose genetic background is congenic with C57Bl/6 (Okabe et al., 1997), were used as donor mice. Single fibers were isolated from the gastrocnemius muscles of 8- to 12-week-old male mice essentially as described previously (Bischoff, 1986; Rosenblatt et al., 1995; Wada et al., 2002); they were cultured in dishes coated with type I collagen (Sumilon, Tokyo, Japan) at 37°C, under 10% CO₂, in primary cultured myocyte growth medium (pmGM) (Pinset and Montarras, 1998; Wada et al., 2002). Except for preparation of the thick cell population for cell transfer experiments, we avoided replating cells in order to exclude a possible alteration of cell characteristics in the experiments. To obtain a thick cell population containing no round cells, satellite cell-descendant cells in the single fiber culture on day 6 were passaged several times every two or three days to convert round cells to thick cells.

To enhance clonal growth of round cells, single fibers were cultured in pmGM supplemented with human basic fibroblast growth factor (bFGF; 10 ng ml⁻¹; PeproTech EC, London, UK) and mouse leukemia

inhibitory factor (LIF; 1000 U ml⁻¹; AMRAD Biotech, Victoria, Australia).

For induction of osteogenic terminal differentiation, cells were cultured in pmGM supplemented with recombinant human bone morphogenetic protein 2 (BMP2; 250 ng ml⁻¹; PeproTech EC), for the indicated period.

Time-lapse recording

For time-lapse observation, single myofibers isolated from gastrocnemius muscles of an 8-week-old C57Bl/6 mouse were plated on a 35-mm collagen-coated dish and cultured in pmGM for 8 days. Cells were then placed in a humid chamber (Olympus, MI-MIBC), maintained at 37°C, and observed under a microscope (Olympus, IX-81) with a 10× Plan Apo Fluor objective lens. Time-lapse images were taken by a CCD camera (CoolSnap HQ; Roper Scientific, Atlanta, GA), with MethaMorph image analysis software (Roper Scientific).

Immunofluorescence and immunocytochemical analyses

For immunofluorescence or immunocytochemical analysis, sections and paraformaldehyde-fixed cultured cells were incubated for 12 to 36 hours at 4°C with mouse monoclonal antibodies to Pax7 (Ericson et al., 1996) (Developmental Studies Hybridoma Bank, Iowa City, IA), sarcomeric myosin heavy chain (MHC) (Bader et al., 1982) or dystrophin (Sigma, St Louis, MO), or rabbit polyclonal antibodies to green fluorescence protein (GFP; Medical Biological Laboratory, Nagoya, Japan) or myogenin (Hashimoto et al., 1994). Cy3-labeled antibodies to mouse immunoglobulin G or biotinylated-labeled antibodies to rabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME) were used as secondary antibodies. The biotinylated antibodies were detected with streptavidin-conjugated Alexa488. Nuclei of cells were visualized by staining with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) (0.5 μg ml⁻¹; Sigma, St Louis, MO).

Alkaline phosphatase (ALP) activity was detected as described previously (Wada et al., 2002).

Intramuscular cell transplantation

Round cells were detached from the culture vessel without enzymatic treatment because they are loosely attached to the substratum. Round cells were washed with Dulbecco's modified phosphate buffered saline [PBS: 0.8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na₂HPO₄, 0.28 g/l KH₂PO₄ (pH 7.4)] and detached by gentle pipetting in PBS supplemented with 0.02% EDTA. Cells were collected by centrifugation, suspended in hDMEM supplemented with 10% FBS, and centrifuged again. Then the cells were resuspended in 100 μl Leibovitz-15 (L-15; Sigma) and an aliquot used for counting cell numbers. Finally, cells were centrifuged again, resuspended in L-15 at 1000 cells μl⁻¹, and kept on ice until transfer.

Thick cells were detached by incubation in 0.05% trypsin and 0.53 mM EDTA (Invitrogen, San Diego, CA), collected by centrifugation, and then resuspended in L-15 at 1000 cells μl⁻¹ for the transfer of 5000 cells, or at 5×10⁴ cells μl⁻¹ for the transfer of 10⁶ cells. Each gastrocnemius muscle of 8- to 10-week-old host C57Bl/6 or mdx-nude (kindly provided by Drs Takahashi and Partridge) mice was injected with 50 μl of 10 μM cardiotoxin (CTX; Wako Pure Chemical Industries, Osaka, Japan), on the day before transplantation to induce muscle regeneration. Five microliters of L-15 containing 5000 cells were injected longitudinally into each gastrocnemius muscle of the host mice using a gel-loading tip (Sorensen BioScience, Salt Lake City, UT). To transfer a large number of cells into a muscle, 20 μl of L-15 containing 10⁶ thick cells was injected using a Hamilton syringe with a 27 G needle. Host mice were sacrificed at 14 or 28 days after cell transfer, and gastrocnemius muscles were isolated. Isolated muscles were fixed with 4% paraformaldehyde overnight, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen, and sectioned at a thickness of 12 μm with a cryostat.

Scanning electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate/HCl buffer overnight at 4°C, and were then treated as described previously (Takeuchi et al., 1995). Ion-coated samples were viewed with a scanning electron microscope (S4500, Hitachi).

Results

Satellite cell clones from both slow and fast muscles spontaneously undergo myogenic terminal differentiation with a distinct timing

To determine the myogenic differentiation potential of each muscle satellite cell clone derived from slow and fast muscle, we isolated single myofibers from extensor digitorum longus (EDL; fast muscle) and soleus (slow muscle) muscles, and cultured them in pmGM for up to 8 days. Muscle satellite cells were activated to proliferate, and migrated onto the bottom of culture vessels. Myogenic cells derived from satellite cells grew clonally on a collagen type I-coated substratum. Within 4 days of culture, the myogenic cells from both EDL and soleus muscles displayed a rounded shape, suggesting a weak attachment to the substratum (Fig. 1A,C). These cells are designated 'round cells' in the present study. Later, more flattened cells, designated 'thick cells', appeared in culture, and the number of round cells decreased in each colony (Fig. 1B,D). Thick cells were easily distinguished from fibroblastic cells, which appeared much more flattened. Terminal myogenic differentiation into myotubes occurred spontaneously after the appearance of thick cells (Fig. 1B-D). Satellite cells derived from both soleus and EDL muscles underwent the same proliferation/differentiation processes, although thick cells and myotubes appeared earlier in soleus myofiber cultures than in EDL cultures (Fig. 1C-E). Round cells continuously migrated, as suggested by their numerous filopodia, (Fig. 1F), and formed 'burst'-type colonies in which cells kept their distance from each other (Fig. 1A). The number of colonies containing MHC-expressing cells increased with time in both fast and slow muscle fiber cultures (Fig. 1E). On the eighth day of culture, all satellite cell-derived clones underwent myogenic terminal differentiation, regardless of their origin. Thus, all satellite cell-derived clones kept myogenic differentiation potential under our isolation and culture conditions.

Conversion of round cells to thick cells occurs prior to myogenic terminal differentiation

The observation that the number of thick cells rapidly increased in colonies simultaneously with a rapid decrease in the number of round cells (Fig. 1A,B) suggests the conversion of round cells to thick cells. To reveal the sequence of events that occur during myogenic terminal differentiation in the single fiber culture, the behavior of round cells derived from gastrocnemius muscle was examined by phase-contrast, time-lapse microscopy. Fig. 2 shows the images taken between 1 and 108 minutes from time-lapse movies on day 7 of the culture. The round cell (arrowhead) migrated continuously during the culture (Fig. 2A-C). When a round cell contacted another cell, its rounded morphology flattened (Fig. 2D-I); thus, the nucleus became visible under a phase-contrast microscope (Fig. 2H-J). Then, the newly formed thick cell fused with another thick cell and differentiated into a myotube (Fig. 2K,L). In addition,

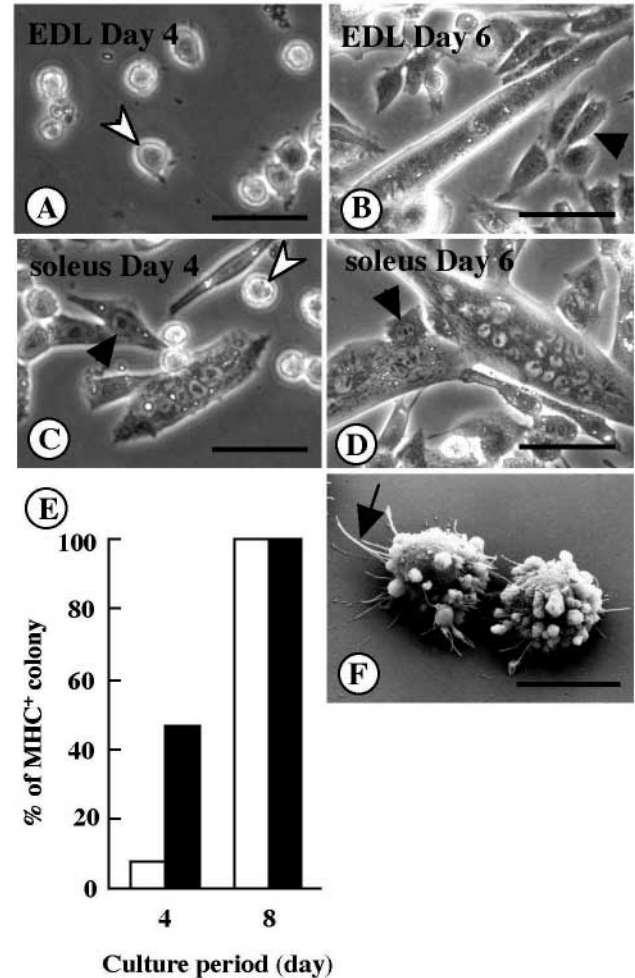


Fig. 1. Two distinct subpopulations appear in satellite cell-derived clones. (A-D) Single myofibers were isolated from EDL (A,B) or soleus (C,D) muscle and cultured for 4 (A,C) or 6 days (B,D). Two distinct subpopulations, including cells designated round cells (white arrowheads) with a rounded shape and more flattened cells designated thick cells (black arrowheads), appeared in either EDL- or soleus-fiber culture. Images were obtained by phase-contrast microscopy. Scale bars: 50 μ m. (E) The ability to undergo myogenic differentiation of satellite cell clones derived from EDL (white column) and soleus muscles (black column). Single myofiber cultures were fixed on day 4 or 8, and then subjected to immunofluorescence for MHC. Expression of MHC was determined in 45-80 colonies. (F) Ultra-microscopic view of round cells. Round cells derived from gastrocnemius muscle were observed under a scanning electron microscope. The arrow indicates a filopodium. Scale bar: 10 μ m.

expression of myogenin (Wright et al., 1989), an early marker of myogenesis, was induced in a fraction of the thick cells but in none of the round cells (Fig. 3A-D) in colonies containing myotubes. These results indicate that round cells are converted to thick cells without cell division, and that thick cells, but not round cells, undergo myogenic terminal differentiation.

Satellite cell clones from both slow and fast muscles retain the ability to respond to BMP2

To determine whether each muscle satellite cell-derived clone

Fig. 2. Round cells are converted to thick cells prior to myogenic terminal differentiation. The sequence of events that occur during myogenic terminal differentiation of satellite cell-descendants was observed by phase-contrast, time-lapse microscopy. (A-L) The images were taken at the indicated time points. Arrowheads indicate the position near the nucleus of the same cell in A through L. Scale bar: 10 μ m.

retains the ability to undergo osteogenic differentiation or not, we exposed the single fiber culture to BMP2 for 2 days during either an early (days 2-4) or a late (days 7-9) period in vitro. ALP, an early marker of osteogenic differentiation, was detectable in only 3.6% of EDL-derived colonies that were exposed to BMP2 on days 2-4 (Fig. 4A,C). By contrast, ALP expression occurred in 86.4% of EDL-derived colonies that were exposed to BMP2 on days 7-9 (Fig. 4B,C). BMP2 markedly inhibited myogenic terminal differentiation but did not affect the conversion of round cells to thick cells.

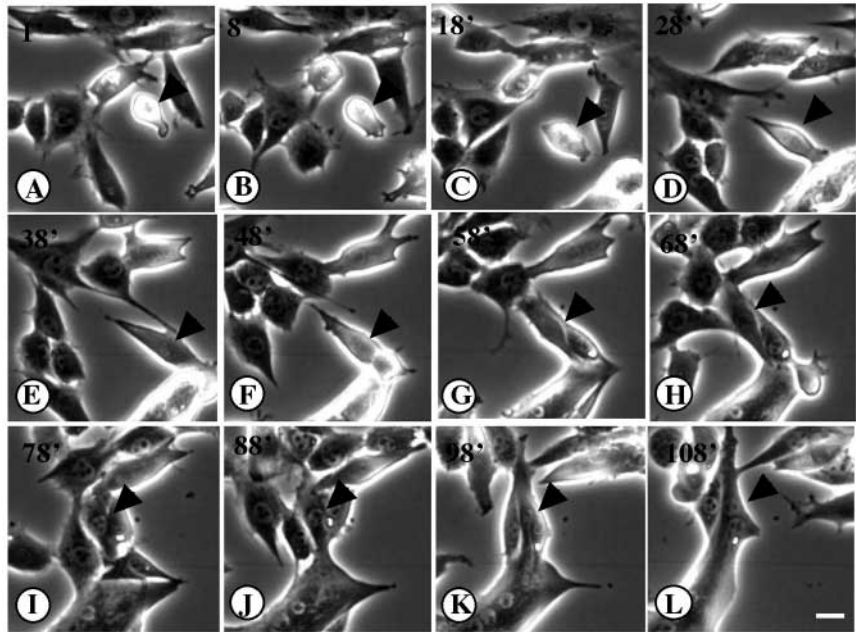
The ability of soleus-derived clones to respond to BMP2 developed similarly, although the incidence of ALP-positive colonies in the soleus fiber culture was lower than that in the EDL fiber culture following BMP2 exposure at 7-9 days in vitro (Fig. 4D). Given that the ability of myogenic cells to respond to BMP2 is lost when they undergo myogenic terminal differentiation (Wada et al., 2002), the relatively low incidence of osteogenic differentiation (62.3% versus 86.4% of colonies) in soleus myofiber culture may be due to the more rapid progression of myogenesis (Fig. 1E). Indeed, continuous exposure to BMP2 from day 0 to day 9 in vitro prevented myogenic differentiation and increased the incidence of osteogenic differentiation of soleus-derived colonies up to 86.2% of 87 independent colonies.

Histochemical analysis indicated that ALP expression was induced exclusively in thick cells but not in round cells (Fig. 4A,B). The temporal change of BMP2 responsiveness of satellite cell descendants in the myofiber culture paralleled the time course of the round cell to thick cell conversion (Fig. 4C,D). The conversion occurred earlier in the single-fiber culture of soleus muscle (Fig. 1B). Actually, even when exposed to BMP2 on days 2-4, ALP was induced in certain soleus colonies containing thick cells (13.3%; Fig. 4D).

The present results suggest that round cells represent activated satellite cells, whereas thick cells represent multipotent progenitor cells (multiblasts) (Wada et al., 2002). Taken together, the results suggest that round cells are converted to thick cells prior to both myogenic and osteogenic terminal differentiation.

Basic FGF and LIF synergistically suppress both round cell to thick cell conversion and myogenic terminal differentiation

Differences in responsiveness to differentiation signals between round cells and thick cells support the notion that the efficiency of their contributions to muscle regeneration in vivo on transplantation differs in the two cell types of satellite cell



descendants. However, we were unable to obtain a sufficient number of round cells to test this because round cells are spontaneously converted into thick cells during culture. To achieve continuous clonal expansion of round cells without conversion to thick cells, we cultured isolated myofibers from gastrocnemius muscles of adult mice in pmGM supplemented with various growth factors. Basic FGF (bFGF) or LIF markedly inhibited the expression of MHC (Fig. 5A-C). When combined, bFGF and LIF synergistically inhibited myogenic

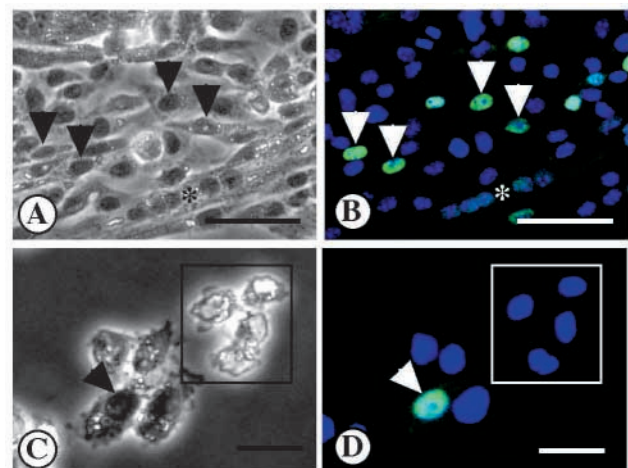


Fig. 3. Thick cells, but not round cells, express myogenin. Myofibers obtained from gastrocnemius muscle were cultured in pmGM for 7 days. Then, satellite cell-derived colonies were subjected to immunostaining with anti-myogenin antibody. (A,B) A fraction of thick cells (arrowheads) around a myotube (asterisk) expressed myogenin (green in B). Scale bar: 50 μ m. (C,D) One of the thick cells (arrowhead), but none of the round cells (within the square), expressed myogenin (D). Scale bar: 20 μ m. Nuclei were visualized with DAPI (blue in B and D). (A,C) Phase contrast microscopy images of the same fields as those shown in B and D.

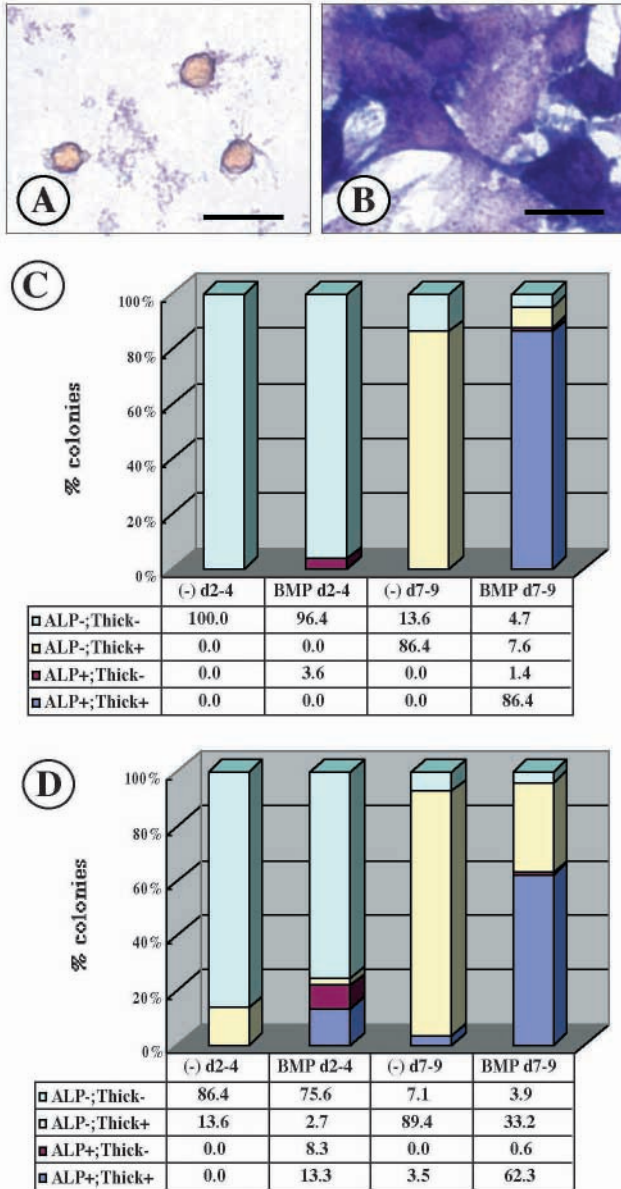


Fig. 4. A distinct subpopulation responds to BMP2 and undergoes osteogenic differentiation. (A,B) Satellite cell-derived colonies obtained in EDL-fiber culture were exposed to BMP2 on days 2-4 (A) or days 7-9 (B). They were then subjected to staining for ALP. Scale bar: 20 μ m. (C,D) Analysis of the ability to respond to BMP2 by satellite cell-descendants derived from EDL- (C) or soleus- (D) fiber cultures. Satellite cell-derived colonies were exposed to BMP2 on days 2-4 (BMP d2-4) or days 7-9 (BMP d7-9), or were cultured for the same periods in the absence of BMP2. Light blue indicates colonies that contain neither thick cells nor ALP-positive cells (ALP-; Thick-). Yellow indicates colonies that contain thick cells but no ALP-positive cells (ALP-; Thick cell+). Dark red indicates colonies that contain ALP-positive cells but no thick cells (ALP+; Thick cell-). Dark blue indicates colonies that contain both ALP-positive cells and thick cells (ALP+; Thick cell+). The numbers of colonies examined were 52-109 in control cultures without exposure to BMP2 and 107-199 in cultures exposed to BMP2.

into myotubes, resulting in small colonies (Fig. 6B,C). By contrast, round cells continued to proliferate for at least 7 days without conversion to thick cells in medium containing both bFGF and LIF (Fig. 6A,B; compare the size of the colony in A with that in B at the same magnification). The inhibition of the round cell to thick cell conversion by the combination of bFGF and LIF resulted in extensive growth of round cells. We routinely obtained at least 10,000 purified round cells when 120-160 myofibers from a pair of gastrocnemius muscles were cultured under such conditions. However, the present culture conditions cannot prevent the conversion during a prolonged culture period.

Round cells contribute to reconstruction of myofibers more efficiently than thick cells

After development of the culture system that enables clonal expansion of round cells, we transferred round cells to regenerating gastrocnemius muscles to determine their possible contribution to muscle reconstruction *in vivo*. We routinely obtained 10,000 round cells in a myofiber culture derived from a pair of mouse gastrocnemius muscles, which avoided physiological damage by minimizing the time spent in cell preparation. Five thousand round cells obtained from a single-fiber culture of GFP-transgenic mice were injected into gastrocnemius muscles of C57Bl/6 mice that had received an intramuscular injection of CTX on the day before transplantation. Both round cells and thick cells had formed few GFP-positive myofibers at 14 days after transplantation to host muscles (Table 1). After 28 days, more GFP-positive myofibers were found in cryosections of host muscles injected

differentiation (Fig. 5D,E). In addition, bFGF, but not LIF, markedly enhanced the proliferation of round cells (Fig. 5E).

In the absence of additional bFGF and LIF, round cells were spontaneously converted to thick cells and then differentiated

Table 1. In vivo myofiber formation by myogenic cells at different stages of maturation/differentiation

Cell type	Number of transplanted cells	Days after transplantation	Number of GFP(+) fibers	
			Gastrocnemius	Required cell number GFP(+) fiber
Round cell	5000	14	4.8 \pm 7.5* (5) ^{†‡}	1041 [§]
	5000	28	15 \pm 5 (4)	333
Thick cell	5000	14	2.7 \pm 4.3 (6) [‡]	1851
	5000	28	0.3 \pm 0.5 (6)	16667
	10 ⁶	28	138 \pm 46 (4)	7246

*Average and standard deviation.

[†]Number of samples examined.

[‡]Expression level of GFP in GFP-positive myofibers after 14 days was relatively low compared with that apparent in GFP-positive myofibers after 28 days.

[§]Estimated values based on [number of transplanted cells]/[number of GFP-positive fibers].

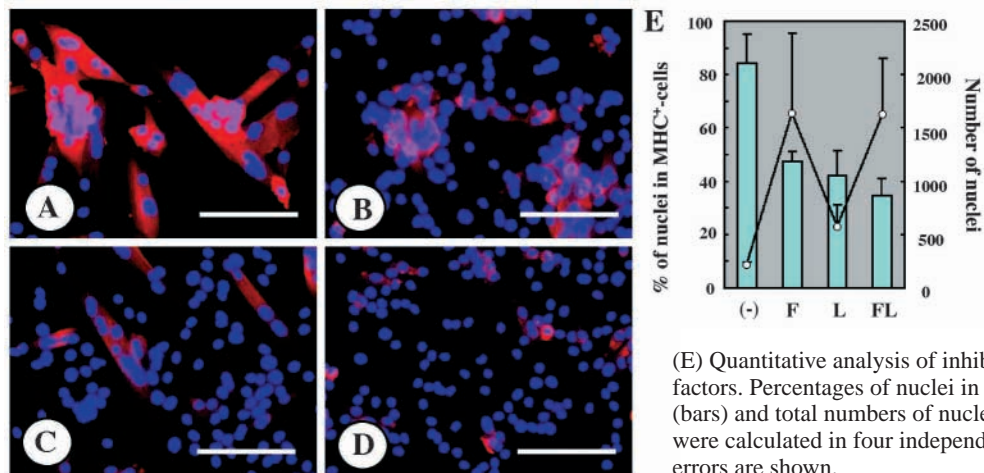


Fig. 5. Basic FGF and LIF synergistically suppress the expression of MHC. (A-D) Single myofibers isolated from gastrocnemius muscle were cultured in pmGM (A), or in pmGM supplemented with bFGF alone (B), LIF alone (C), or bFGF plus LIF (D), for 6 days. Cells were then subjected to immunostaining for MHC (red). Nuclei were visualized by DAPI. Scale bars: 100 μ m.

(E) Quantitative analysis of inhibition of MHC expression by growth factors. Percentages of nuclei in MHC-expressing cells to total nuclei (bars) and total numbers of nuclei in each field (0.55 mm²) (open circles) were calculated in four independent samples. Averages and standard errors are shown.

with 5000 round cells (Fig. 7A, Table 1). Peripherally located nuclei in several GFP-positive myofibers suggest the completion of myofiber reconstruction (Fig. 7B). By contrast, very few, if any, GFP-positive myofibers were found at 28 days after transplantation in host muscles injected with 5000 thick cells (Fig. 7C). However, when 1,000,000 thick cells were transferred, they were capable of greatly contributing to myofiber formation (Fig. 7D, Table 1). Therefore, thick cells still retain the capacity to reconstitute myofibers in vivo, although their efficiency in myofiber formation is comparatively much lower than that of round cells (approximately 2-5%). We cannot exclude the possibility that a thick cell culture contains a small number of round cells or cells similar to round cells. However, round cells will be easily lost from the thick cell culture through successive passages because these cells divide slowly.

To compare the ability of quiescent satellite cells to form new myofibers with that of their cultured descendant cells, we transplanted ten or thirty single myofibers isolated from gastrocnemius muscles of GFP-transgenic mice into host gastrocnemius muscles that were pre-treated with CTX. The fact that GFP-positive myofibers were not observed 24 hours after transplantation implies that the transplanted single myofibers were physically damaged during the process of transplantation. Transplanted quiescent satellite cells located on single myofibers formed GFP-positive myofibers within two weeks: the average number of GFP-positive myofibers from three independent experiments was 0.52 ± 0.16 /transplanted myofiber. Single myofibers of mouse gastrocnemius muscle were associated with 3.3 ± 1.3 satellite cells (T.M. and N.H., unpublished). Thus, only seven quiescent satellite cells are sufficient for the formation of a single new myofiber by intramuscular transplantation. These results suggest that

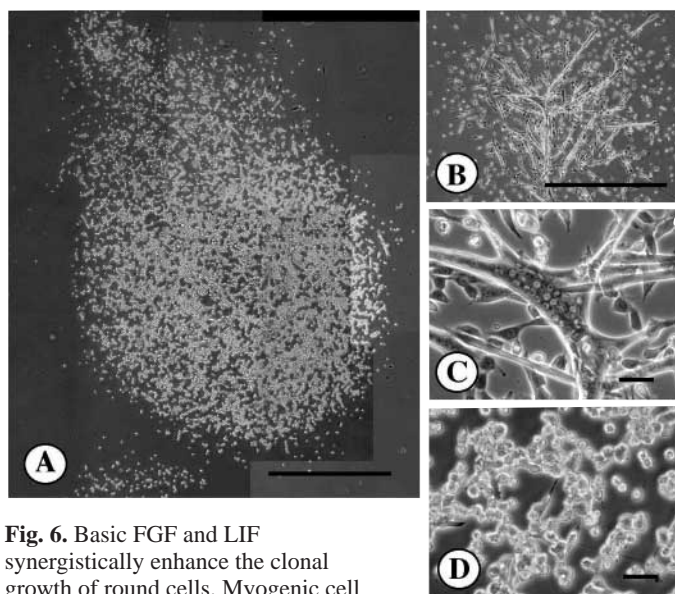


Fig. 6. Basic FGF and LIF synergistically enhance the clonal growth of round cells. Myogenic cell clones derived from satellite cells in gastrocnemius muscle were cultured in pmGM alone (B,C), or in pmGM supplemented with bFGF plus LIF (A,D), for 7 days. Images were obtained by phase-contrast microscopy. Scale bars: 1 mm in A,B, 100 μ m in C,D.

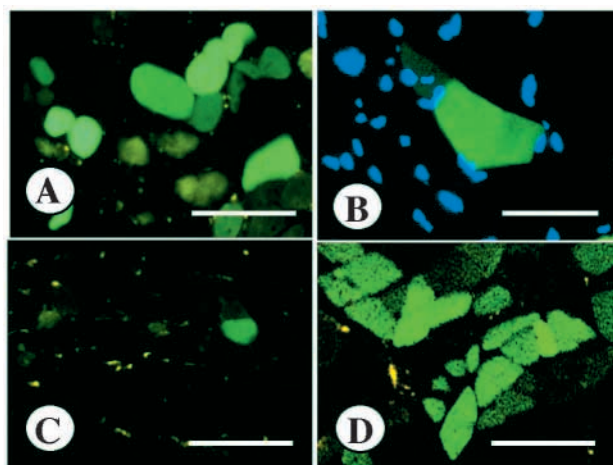


Fig. 7. Round cells enhance muscle regeneration more efficiently than thick cells. Five thousand round cells (A,B) or thick cells (C), or 1,000,000 thick cells (D) derived from gastrocnemius muscles of GFP-transgenic mice were transplanted into gastrocnemius muscles of congenic C57Bl/6 mice pre-treated with CTX. The muscles were removed and cryosectioned 28 days after transplantation. Images in A-D were obtained by epifluorescence microscopy with a GFP filter. Scale bars: 100 μ m in A,C,D; 50 μ m in B.



Fig. 8. Round cells retain the capacity to restore dystrophin in myofibers of mdx nude mice. Five thousand round cells derived from GFP-transgenic mice were transplanted into gastrocnemius muscles of mdx nude mice pre-treated with CTX. The muscles were removed 28 days after transplantation and subjected to immunofluorescence analysis with antibodies to GFP (B) and dystrophin (C). Immunofluorescence analysis with antibodies to dystrophin revealed the absence of revertant myofibers in muscles that were treated with CTX alone (A). Scale bars: 20 μ m.

quiescent satellite cells have an extremely high ability to reconstitute myofibers *in vivo*. The efficiency of muscle regeneration by cells belonging to the satellite cell lineage might be altered as a result of isolation and/or tissue culture (Smythe et al., 2001).

Round cells have the ability to restore dystrophin in myofibers of mdx mice

To determine whether round cells have the capacity to restore dystrophin in the muscle of mdx nude mice, which lack functional dystrophin due to a genetic mutation (Sicinski et al., 1989), 5000 round cells derived from GFP-transgenic mice were injected into gastrocnemius muscles of mdx nude mice that had received an intramuscular injection of CTX on the day before transplantation. We did not find any revertant fibers in the gastrocnemius muscles in this series of experiments (Fig. 8A). After 28 days, GFP-positive myofibers (approximately 10–30/gastrocnemius muscle) were identified in cryosections of host muscles (Fig. 8B). Immunofluorescence analysis shows that dystrophin was restored in approximately 10% of GFP-positive myofibers (Fig. 8C). Thus, round cells representing immediate descendants of quiescent satellite cells have the capacity to restore dystrophin in the muscle of mdx nude mice.

Round cells retain stem cell-like properties and express Pax7 at high levels

The high ability of round cells to form new myofibers after transplantation suggests that these cells correspond to a stem-like subpopulation in myoblast culture (Beauchamp et al., 1999). Time-lapse recording revealed that round cells grew more slowly than thick cells (Fig. 9A). The maximum growth rate of round cells, which is estimated from the slope of their growth curve, was significantly lower than that of thick cells (9.3 ± 0.7 versus 16.7 ± 1.5 cells/hour). A serial recording also showed a single round cell generating two round-shaped daughter cells that migrated away soon after cell division (Fig. 9B). These results indicate that round cells retain stem cell-like characteristics: slow division, self-renewal for new stem-like cells, and generation of a progeny committed to terminal differentiation.

To clarify the nature and diversity of round and thick cells, the expression of satellite cell lineage markers and stem cell markers were determined by immunofluorescence analysis. Both round cells and thick cells expressed MyoD, M-cadherin, desmin and nestin, but neither Sca1 nor CD34 (data not shown). Furthermore, all round cells expressed Pax7, an essential transcription factor for satellite cell specification (Seale et al., 2000), at high levels (Fig. 10A,B). Undifferentiated thick cells also expressed Pax7, but at a lower level than that apparent in round cells (Fig. 10C,D), whereas differentiating thick cells expressed myogenin, but not Pax7 (Fig. 10E,F). The high level of Pax7 expression in round cells suggests that Pax7 is a possible molecular marker for identification of stem-like cells in myoblast culture.

Discussion

Transplantation of normal myoblasts into postnatal skeletal muscle is a potential therapeutic approach to the treatment of myopathies such as Duchenne muscular dystrophy (DMD) (Partridge et al., 1989; Smythe et al., 2001; Vilquin et al., 1996). Transplanted myoblasts have the ability to form myofibers through cell fusion *in vivo*. Although myoblast transfer therapy has been found capable of ameliorating the dystrophic symptoms of mdx mice (Partridge et al., 1989),

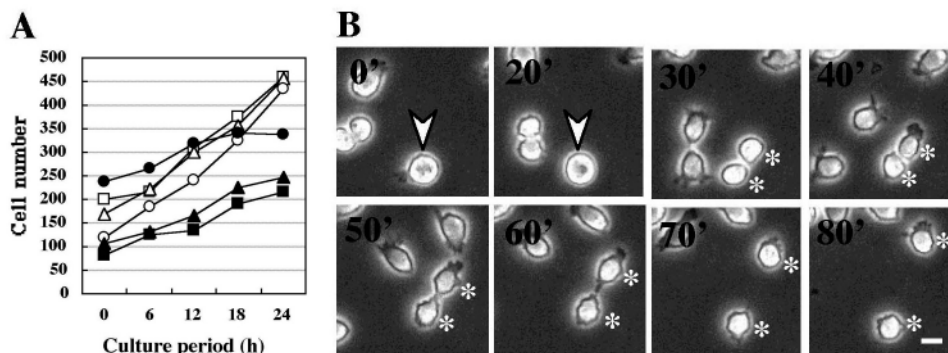


Fig. 9. Round cells divide slowly and generate new round cells. (A) The behavior of round cells on day 6, 7 or 8 of myofiber cultures (black symbols), and thick cells at early passages (white symbols), was recorded by time-lapse microscopy. Cell numbers in the same fields were counted every 6 hours. Three independent cultures of round cells or thick cells were analyzed. (B) A round cell on day 6 of the fiber culture (arrowhead) generated two daughter cells (asterisks) displaying a rounded shape. The images were taken at the indicated time points. Scale bar: 10 μ m.

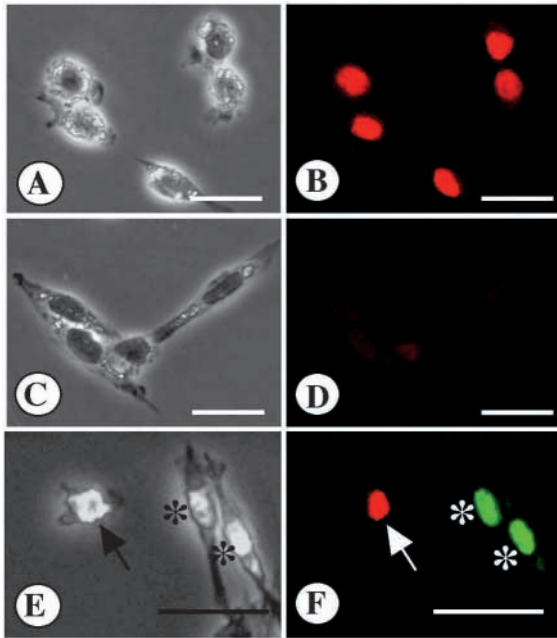


Fig. 10. Round cells express Pax7 at high levels. Myofibers from EDL muscle were cultured in pmGM for 6 days. (A,B) Round cells expressed Pax7 at high levels. (C,D) Undifferentiated thick cells expressed Pax7 at a reduced level. (E,F) Differentiating thick cells (asterisks) expressed myogenin (green in F) but not Pax7 (red in F). A round cell (arrow) expressed Pax7 but not myogenin. (A,C,E) Phase contrast microscopy images of the same fields as those shown in B, D and F. Scale bars: 20 μ m.

attempts to develop this approach have met with little success because of both host and donor factors that limit the delivery of exogenous myoblasts into host muscle. The major limiting factor in myoblast transfer is that the vast majority of donor myoblasts undergo rapid death. Many studies attribute this death to the inflammatory and immunological responses of the host (reviewed by Smythe et al., 2001). A recent study suggests a role of natural killer cells in the rapid death of transplanted myoblasts (Hodgetts et al., 2003). By contrast, the properties of donor myogenic cells have been suggested to be involved in this death (Beauchamp et al., 1999; Lee et al., 2000; Qu et al., 1998). Muscle fibers of donor origin are derived from only a discrete minor fraction of myoblasts that can survive after transplantation (Beauchamp et al., 1999). This finding supports the notion that the cells that give rise to muscle after transplantation constitute a distinct subpopulation with stem cell-like properties. The identification and isolation of the minority that represent stem-like cells should have significant implications for myoblast transfer therapy. Neonatal or prepubescent muscle-derived cells with stem cell-like properties that are enriched by pre-plate techniques survived well when injected into adult muscle (Lee et al., 2000; Qu et al., 1998), although they are assumed to be a distinct population from quiescent satellite cells in adult muscle. In the present study, we identified similar stem-like cells, designated round cells, as immediate descendants of quiescent satellite cells in adult muscle. Round cells with stem cell-like properties are easily distinguished from their multipotent progeny, thick cells or nonmyogenic cells, by their rounded shape under our

culture conditions. Therefore, we can identify and isolate these cells for transplantation.

In many previous studies, more than 10^5 myoblasts have been transferred intramuscularly because only a minor fraction with stem cell-like characteristics contributed to muscle reconstruction. Thus, myoblast cultures must be expanded to collect sufficient numbers of the stem-like cells, but the stem-like cells can be lost during extensive culture because they divide slowly, whereas the other cells divide rapidly. Round cells, however, can efficiently regenerate skeletal muscle after intramuscular injection even when the transferred cell number is as small as 5000. Therefore, the isolation of cells with stem cell-like properties is required to obtain sufficient numbers of these cells in culture.

The presence of two subpopulations of myogenic cells has been demonstrated in postnatal muscle: a majority of rapidly dividing cells and a minority of slowly dividing reserve cells (Rantanen et al., 1995; Schultz, 1996). The latter stem-like subpopulation has been identified within primary myogenic cultures derived from single cells (Baroffio et al., 1995; Baroffio et al., 1996) and the myogenic cell line C2C12 (Hashimoto and Ogashiwa, 1997; Yoshida et al., 1998). These results suggest that the term 'myoblast' has been used simply to describe a mononucleated, undifferentiated cell with the potential to undergo myogenic terminal differentiation (Beauchamp et al., 1999). We identified round cells in our single-fiber culture system that can grow satellite cells clonally, excluding contamination of non-myogenic cells through careful isolation of single myofibers (Wada et al., 2002). Round cells divide slowly, whereas thick cells divide rapidly. In addition, we found that round cells generate thick cells after self-renewal. Thus, round cells retain the stem cell-like properties suggested in previous studies: self-renewing, slowly cycling, and generating a rapidly dividing progeny that undergo terminal differentiation. Stem-like cells in human myogenic cells decreased in number with successive passages (Baroffio et al., 1996). We also found that round cells are gradually lost during prolonged culture and successive passages. Round cells have the capacity of regenerating skeletal muscle *in vivo*, a capability that is attributed to a subpopulation with stem cell-like characteristics in myoblast culture (Beauchamp et al., 1999). Taken together, round cells correspond to the stem-like subpopulation described previously both *in vitro* (Baroffio et al., 1996) and *in vivo* (Beauchamp et al., 1999).

The stem cell-like subpopulation has been identified only retrospectively by its ability to survive *in vivo* and its apparent lack of distinct phenotypic traits under differentiation-inducing conditions *in vitro*. In contrast to the previous studies, we can identify round cells by their rounded appearance, and can follow their fate progressively both *in vitro* and *in vivo*. Using this culture system, we found a recapitulation of the ability to respond to terminal differentiation-inducing signals in a muscle satellite cell lineage. Round cells do not differentiate even when cultured under myogenic or osteogenic differentiation-inducing conditions, whereas their progeny, thick cells, respond to these conditions and undergo myogenic or osteogenic terminal differentiation. Therefore, round cells and thick cells are distinct cell types at different stages of maturation/differentiation in a muscle satellite cell lineage: round cells are activated satellite cells that preserve

multipotentiality but do not differentiate; their progeny, thick cells, are multipotent progenitors, previously designated multiblasts (Wada et al., 2002), that retain the ability to respond to differentiation-inducing signals.

Our clonal culture system reveals that round cells with stem cell-like properties appear in fiber culture independently of type of fiber origin. The present study also indicates that multipotentiality is preserved in the majority of satellite cells despite their type of fiber origin. In culture, muscle satellite cell descendants derived from slow muscle spontaneously undergo myogenic terminal differentiation earlier than those from fast muscle, although the processes of myogenesis, colonization of round cells, conversion of round cells to thick cells, and myotube formation by thick cells, are conserved among them. The finding that the number of round cells in each colony declines earlier in cultures originating from slow muscle suggests that round cells derived from slow muscle retain relatively low potential for self-renewal. It is well known that severe atrophy is induced in slow muscle rather than fast muscle under unloaded conditions such as low gravity and bed rest. Differences in the self-renewal activity of round cells between slow and fast muscles might be involved in the symptoms of atrophy.

Various factors in donor cells may affect the myofiber reconstruction upon transplantation: cell type of muscle-derived cells, stage of cell differentiation, cell modification during culture, and number of transplanted cells. Pre-treatment of host muscle (injection of CTX, irradiation, or none) also affects muscle reconstruction. In the present study, we compared the efficiency of muscle regeneration produced by cells at different stages of maturation/differentiation in a satellite cell lineage under basically identical conditions. Although the survival rate of the transplanted cells was not monitored, the number of GFP-positive myofibers and the expression level of GFP should indicate the extent of survival, proliferation and differentiation of the transplanted cells *in vivo*. Five thousand round cells had the capacity to reconstitute myofibers expressing GFP at a high level in host muscles efficiently at 28 days after transplantation, whereas these cells had formed only a few myofibers expressing GFP at a reduced level at 14 days. The results imply that transplanted round cells continue to proliferate and provide myonuclei after transplantation.

Quiescent satellite cells actually preserve a prominent capacity for muscle regeneration, as was also suggested by whole or sliced muscle transplantation (Schultz et al., 1988; Smythe et al., 2001). The required number of transplanted cells for the formation of a single myofiber *in vivo* is estimated to be 6.6 quiescent satellite cells. Round cells also preserve an ability to reconstruct host muscle that, although much lower than that of quiescent satellite cells, is approximately 20-50 times that of thick cells. The number of quiescent satellite cells is approximately 3-5% of nuclei in skeletal muscle (Gibson and Schultz, 1983) (R. Umeda and N.H., unpublished). Thus, it does not seem possible to collect a sufficient number of quiescent satellite cells from skeletal muscle directly. Transplantation of a large number of thick cells is another strategy to improve muscle repair by cell transplantation. However, numbers of transplanted cells are limited by host muscle capacity and by the culture scale for cell preparation. The present study suggests that transplanted round cells contribute to new myofibers for a prolonged period *in vivo*.

Thus, round cells may be a possible cell source of myogenic cell transfer therapy in the future.

The restoration of dystrophin was observed in approximately 10% of GFP-positive fibers after transplantation of round cells to mdx nude mice. Incorporated donor nuclei may supply only a limited amount of dystrophin that covers the limited area designated the nuclear domain of a host myofiber (Kong and Anderson, 2001). Therefore, incorporation of a large number of donor nuclei into host myofibers is required to produce amounts of dystrophin adequate to be distributed over the whole area of the plasma membrane of a host fiber. By contrast, incorporation of a relatively small number of donor nuclei from GFP-round cells may be sufficient to produce fluorescence along the whole length of a host myofiber because GFP is a very diffusible protein and is highly expressed in donor nuclei.

The present results indicate that control of the conversion from round cell to thick cell is crucial to expand the number of round cells with stem cell-like properties in culture. We found that bFGF and LIF synergistically enhance the proliferation of round cells and suppress their conversion to thick cells. However, the present culture conditions cannot prevent the conversion during a prolonged culture period and successive passages. Further improvement of culture conditions is required to obtain sufficient numbers of round cells for cell transfer therapy. Time-lapse recording of round cell behavior reveals that round cells are converted to thick cells without cell division and that the conversion is triggered by cell-to-cell contact (Fig. 2). These observations suggest that culture conditions capable of avoiding cell-to-cell contact may inhibit the conversion.

In a clinical trial of human myoblast transfer on a patient with DMD, many nuclei of transplanted myoblasts were incorporated into host myofibers but did not express dystrophin (Gussoni et al., 1997). The clinical trial, however, suggested that donor factors, including the ability of cells to proliferate, differentiate, and function *in vivo*, may be crucial for successful myoblast transfer therapy. Rapid death of transferred cells is a serious problem in DMD muscle. Therefore, isolation and maintenance of a subpopulation of human muscle satellite cell descendants equivalent to the mouse round cells presented here could greatly enhance the potential of myogenic cell transfer therapy. Pax7 is an essential transcription factor for muscle satellite cell specification (Seale et al., 2000) and is exclusively expressed in adult satellite cells (Wada et al., 2002). The high level of Pax7 expression in mouse stem-like cells has significant implications for the identification and isolation of human satellite cell descendants with stem cell-like properties.

Our findings raise a simple question. Why did many previous studies not describe the stem-like cells that are similar to round cells? Only a few studies have reported results showing the presence of myogenic cells with a morphology similar to that of round cells (Praud et al., 2003; Torrente et al., 2001). Based on the present results, both high cell density and a relatively low concentration of growth factors may enhance the conversion of round cells to thick cells in early passages. It is conceivable that most 'myoblast' cultures reported previously had lost round cells in early passages.

Taking the above results together, we conclude that round cell transfer has significant potential for the improvement of myogenic cell transfer therapy in human muscle disorders.

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