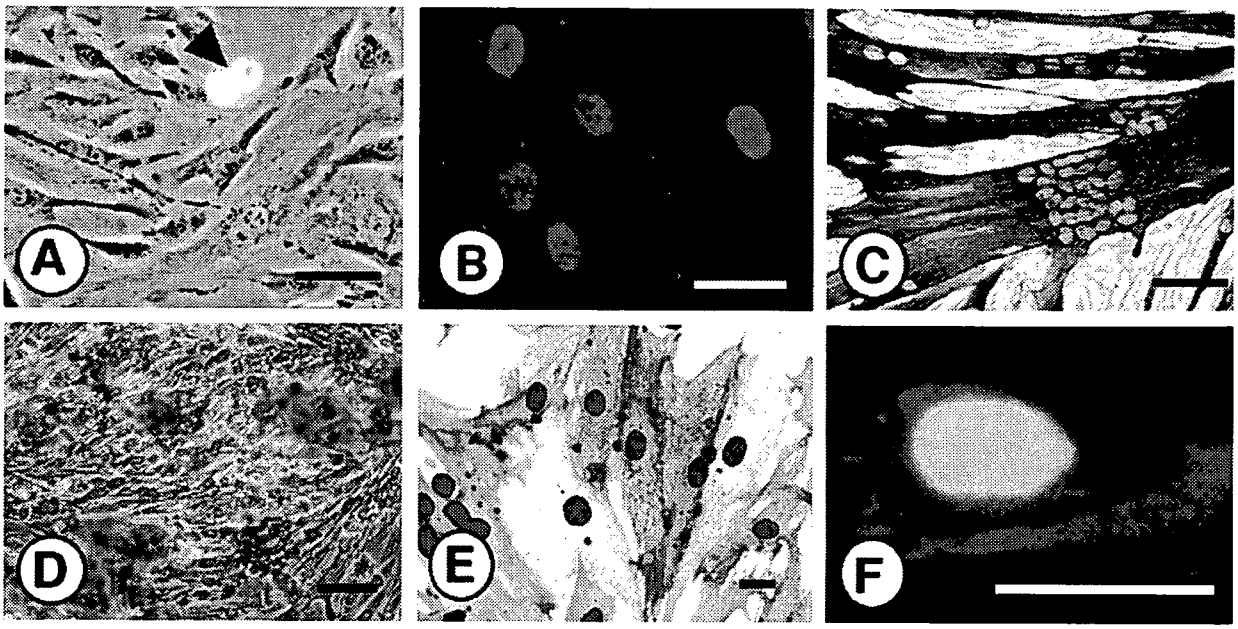

Fig. 2 – Multipotentiality of parental primary human myogenic cells. (A) The primary human myogenic cell clone Hu5 was grown in pmGM and examined by phase-contrast microscopy. The arrowhead indicates dividing cells. (B) Undifferentiated Hu5 cells (passage 6) were subjected to immunofluorescence analysis with antibodies to Pax7. (C) Primary Hu5 was grown to confluence in pmGM to trigger spontaneous differentiation into myotubes. The cells were then subjected to immunocytochemical analysis with antibodies to sarcomeric MHC (immune complexes were detected with the peroxidase substrate TrueBlue). (D) Primary Hu5 cells were cultured for 6 days in medium containing serum (10%) and βGP (10 mM), after which calcium deposition was detected by staining with Alizarin Red S. The cells were then examined by phase-contrast microscopy. (E) Undifferentiated Hu5 cells were subjected to immunohistochemical detection of ALP. Nuclei were stained with DAPI superimposed. (F) Undifferentiated Hu5 cells were subjected to immunofluorescence analysis with antibodies to Pax7 and to bone-specific ALP. Pax7 (green) was present in nuclei, whereas ALP (red) was localized to the plasma membrane. Scale bars: 20 μm (A, B, D, and F) and 50 μm (C and E).

and bone-specific ALP were expressed in both parent Hu5 and E18 cells (Table 2). Taken together with the results here, the immortalized line E18 largely retains both the myoblast- and osteoblast-specific properties that are preserved in the parent Hu5 cells.

E18 differentiated into myotubes under conditions that promote myogenic differentiation (Fig. 3A), and MyoD was highly expressed in the nuclei of E18 myotubes (Fig. 3B). The number of nuclei in myotubes was sometimes varied and mainly depended on cell density. E18 also underwent ossification when cultured in medium containing βGP alone (Fig. 3C). Because the immortalized line E18 preserved both the properties and the multipotentiality that the parent Hu5 cells possess (Fig. 2C and E) (Hashimoto et al., 2006), we consequently studied the myogenic and osteogenic differentiation kinetics of E18.

2.4. *Down-regulation of osteogenic properties during myogenesis in human myogenic progenitor cells*

Culture of E18 under conditions that promote myogenic differentiation induced the expression of myogenic differentiation markers, including sarcomeric myosin heavy chain (MHC) and muscle creatine kinase (MCK), as well as the formation of myotubes (Fig. 4Aa through d, and C). The MyoD protein level was reduced in undifferentiated E18 cells under low cell density culture conditions (Hashimoto et al., 2006), but its expression was induced during myogenic differentiation culture (Fig. 4Ae through h); in contrast, parent Hu5 cells expressed MyoD throughout the culture (Fig. 4Aq through t). Although the level of MyoD expression in E18 cells under low cell density culture conditions was reduced, it is possible that the high level of expression of *Myf5*, another member of the MyoD family, compensated for the loss of MyoD function (Fig. 4C). Both Hu5 and E18 cells were able to express MyoD and Runx2 simultaneously (Fig. 4An, q, and r). The expression of Runx2 at both the mRNA and protein levels decreased whereas the amount of myogenin mRNA increased during myogenic differentiation culture of both Hu5 and E18 cells (Fig. 4Ai through l, q through t, and C). The expression of ALP was also down-regulated in terminally differentiated E18 myotubes (Fig. 4B) as well as parent Hu5 myotubes (data not shown). Expression of the ectopic *hTert* and *E7* genes persisted in E18 cells throughout myogenic terminal differentiation (Fig. 4C). Together, these results thus indicate that expressions of both *Runx2* and *bone-specific ALP* were down-regulated in primary and immortalized human myogenic progenitor cells during myogenic differentiation.

Table 1 – Sequences of PCR primers and amplification conditions

Target gene	Primer	Sequence (5′ → 3′)	Annealing temperature (°C)	Amplification cycles (bp)	Product size
Telomerase reverse transcriptase	hTert-F	GGAAGCAGAGGTCAGGCAGC	58	28	719
	hTert-R	AGAGCAGCGTGGAGAGGATG			
Human papilloma virus-16 E7	HPV16 E7-F	GATGGTCCAGCTGGACAAGC	53	24	143
	HPV16 E7-R	GTGCCCATTAACAGGTCTTC			
MyoD	hMyo D-F	GCAAGCGCAAGACCACCAAC	58	30	678
	hMyo D-R	GGCGGCCACCATCCCCTCAG			
Myf5	hMyf5-F	AATTTGGGGACGAGTTTGTG	52	30	642
	hMyf5-R	GAGGCTGTGAATCGGTGCTG			
Myogenin	hmgn-F	ACCCTGCTCAACCCCAACCA	53	33	395
	hmgn-R	CTCCCCACTGCCTTTATCTT			
Muscle creatine kinase	hMCK-F	GGACCCTAACTACGTGCTCAG	58	26	327
	hMCK-R	GTTCACCCACACCAGGAAGC			
Runx2	hRunx2-F	GTCTTACCCCTCCTACCTGA	53	30	184
	hRunx2-R	TGCCTGGCTCTTCTTACTGA			
Osteocalcin	hOSC-F	AGCCACCGAGACACCATGAGA	58	30	434
	hOSC-R	TGGGGACCCCACATCCATAG			
BMP2	hBMP2-F1	ACTCGAAATTCCCCGTGACC	54	30	319
	hBMP2-R1	CGCTGTTTGTGTTTGGCTTG			
BMP4	hBMP4-F1	ACTCTGCTTTTCGTTTCCTC	52	30	340
	hBMP4-R1	TGGTGGGTCCGAGTCTGATG			
BMP7	hBMP7-F1	CCACCCACGCTACCACCATC	58	30	530
	hBMP7-R1	TGCTGCTGTTCTCTGCCACG			
myostatin	hGDF8-F1	TATTTGAGACCCGTCGAGAC	54	30	527
	hGDF8-R1	CCTCTGGGGTTTGCTTGGTG			
Glyceraldehyde-3-phosphate dehydrogenase	hGAPDH-F	GGGCTGCTTTTAACTCTGGT	56	20	702
	hGAPDH-R	TGGCAGGTTTTTCTAGACGG			

Table 2 – Expression of both myogenic and osteogenic lineage markers in primary and immortalized human myogenic cells

Cell lineage	Myogenic lineage			Osteogenic lineage	
Lineage marker	Desmin	Nestin	MyoD	Runx2	ALP
Hu5 (primary)	+	+	+	+	+
E18 (immortalized)	+	+	+[a]	+	+

Expression of both myogenic and osteogenic lineage markers were determined by immunofluorescence analyses.
a MyoD expression was up-regulated in a cell density-dependent manner (Hashimoto et al., 2006).

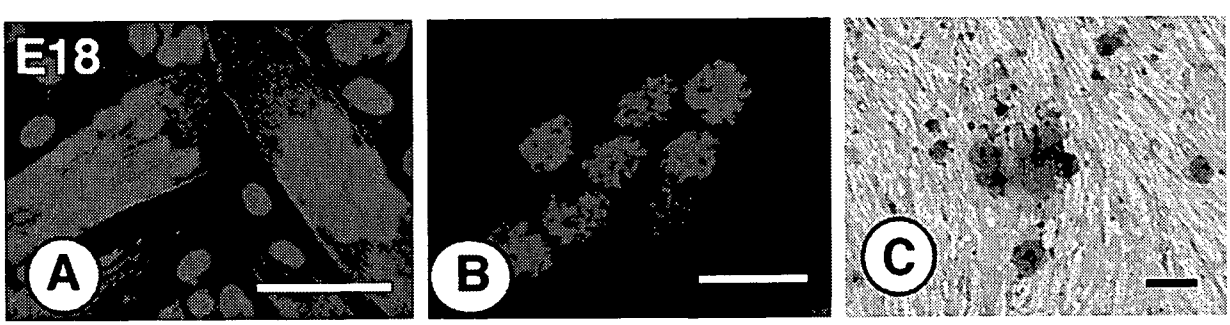

Fig. 3 – Multipotentiality of an immortalized human myogenic progenitor cell clone E18. (A and B) The Hu5-derived clone E18 differentiated into myotubes after culture for 6 days in pmDM. The cells were then subjected to immunofluorescence analysis with antibodies (red) to MHC in (A) or MyoD in (B). Nuclei were detected by staining with DAPI (blue) in (A). (C) E18 cells were cultured for 6 days in medium containing serum in the presence of BMP2 (500 ng/ml) plus βGP (10 mM). The cells were then stained with Alizarin Red S. Scale bar, 100 μm.

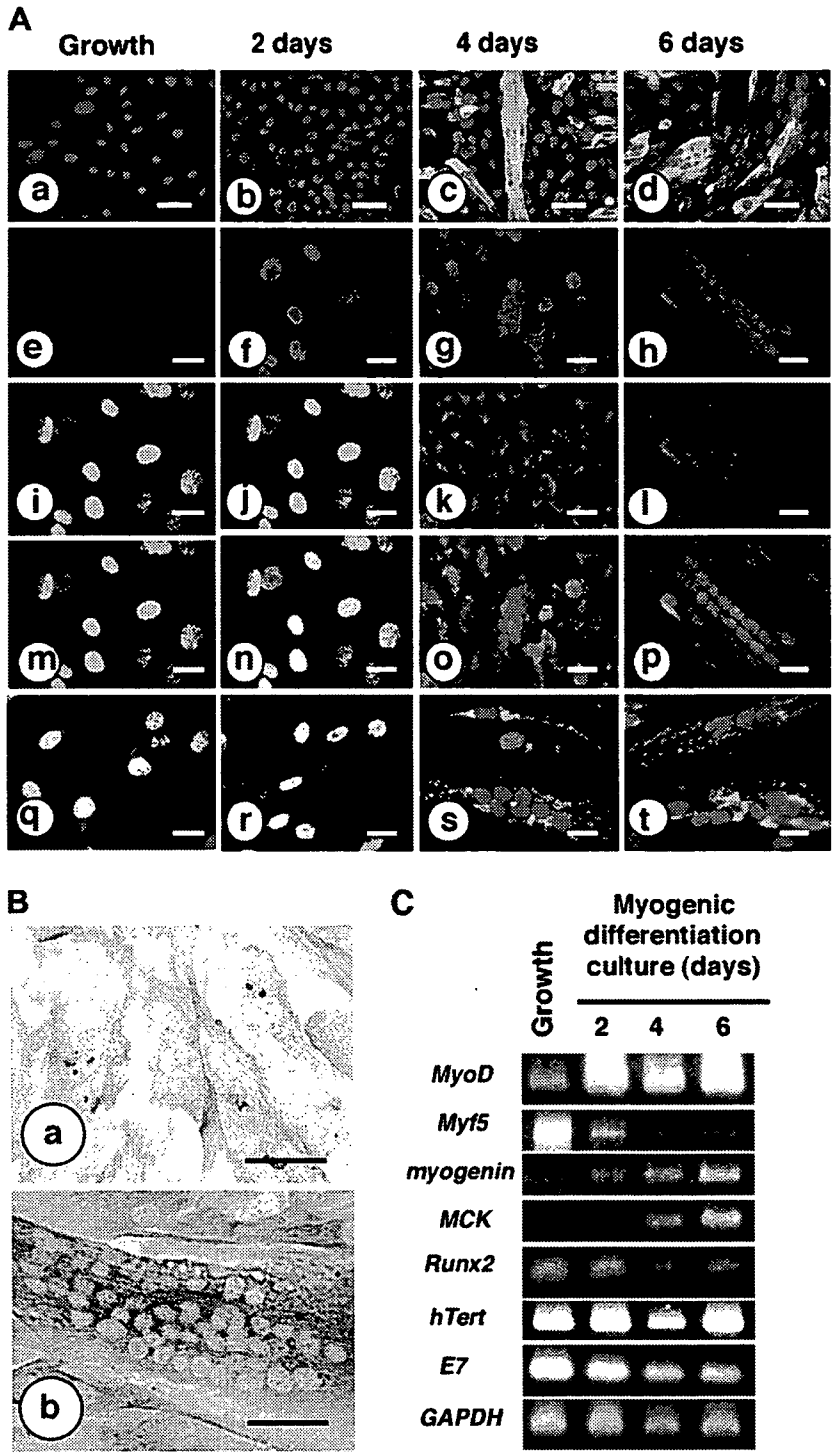

Fig. 4 – Myogenic differentiation of the immortalized human myogenic progenitor cell clone E18. (A) Immortalized Hu5-derived clone E18 cells were cultured under growth conditions or in pmDM for 2, 4, or 6 days (to induce myogenic terminal differentiation), as indicated. Expression of myogenic- or osteogenic-specific proteins was then examined by immunofluorescence analysis with antibodies to MHC (panels a through d), MyoD (e through h), or Runx2 (i through l). Nuclei were stained with DAPI (blue) in panels a through d. Merged images of panels e through h and panels i through l are shown in panels m through p, respectively. Expression of MyoD (red) and Runx2 (green) was also examined in parent Hu5 cells cultured under the same conditions (panels q through t); merged images are shown. Scale bars, 50 μm (panels a through d) or 20 μm (panels e through t). (B) Panel a, expression of ALP activity, as revealed by staining with Fast Blue RR, in E18 under growth conditions. Panel b, expression of ALP activity (blue) and MHC (brown) in myotubes formed by differentiated E18 cells cultured in pmDM. Scale bars, 50 μm. (C) RT-PCR analysis of the expression of *MyoD*, *Myf5*, *myogenin*, *MCK*, *Runx2*, *hTert*, *HPV-16 E7*, and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* genes in Hu5/E18 cells under growth conditions or after culture in pmDM for 2, 4, or 6 days.

2.5. *Osteogenic differentiation of human myogenic progenitor cells without BMP2-stimulation*

E18 cells expressed bone-specific ALP and were able to undergo osteogenic terminal differentiation in the absence of BMP2 (Figs. 3C, 4Ba). To determine the role of BMP2 in the osteogenic differentiation of E18 cells, we cultured these cells in medium containing βGP, BMP2, or both for 6 days. Whereas βGP alone induced calcification in E18 as well as parent Hu5 cells (Figs. 2E, 3C), BMP2 alone did not (Fig. 5A); BMP2 did, however, markedly enhance the effect of βGP during the early stage of osteogenic terminal differentiation (Fig. 5A). BMP2 simultaneously inhibited myogenic differentiation (Fig. 5B and C). The enhancement of ossification by BMP2 was likely independent of its inhibition of myogenesis, given that bFGF inhibited myogenesis without enhancing ossification (Fig. 5A and B). In addition, exogenous BMP2 inhibited *osteocalcin* gene expression (Fig. 5B, lanes 4 and 6; also see Fig. S2 online). Osteocalcin is a late marker of osteogenic differentiation and inhibits ossification (Ducy et al., 1996). Therefore, the results suggest that exogenous BMP2 enhances ossification in human myogenic progenitor cells through the inhibition of *osteocalcin* gene expression.

Expression of osteocalcin was induced in confluent E18 cells cultured in hDMEM supplemented with 10% FBS in the absence of BMP2 and βGP (Fig. 5B, lane 2). The result shows that E18 cells retain the ability to undergo osteogenic differentiation spontaneously in vitro. Then, to determine whether human myogenic progenitor cells express *BMPs* by themselves, expression of *BMPs* was determined by RT-PCR analysis (Fig. 5D). Both Hu5 and E18 cells expressed *BMP4* but not *BMP7*. Hu5 cells also expressed *BMP2* at trace levels, whereas E18 cells did not. In addition, both Hu5 and E18 cells also expressed another TGFβ super-family member, *myostatin*. To determine whether the action of autocrined BMPs is required for maintenance of ALP expression, E18 cells were cultured in pmGM supplemented with a BMP inhibitor, noggin, for up to 3 days. However, even an excess of noggin did not inhibit ALP expression in E18 cells (Fig. 5E). Thus, it is unlikely that expression of ALP in E18 cells depends on autocrined BMPs.

Whereas βGP alone markedly inhibited spontaneous myogenesis in confluent cultures of clone E18, the combination of βGP and BMP2 prevented it (Fig. 5B and C). Immunofluorescence analysis revealed that the number of MyoD-positive cells decreased, but that a substantial proportion of them remained after culture of E18 cells with βGP alone or with βGP plus BMP2 (Fig. 5C). In contrast, the expression of MHC, which is encoded by a target gene of MyoD, was inhibited greatly by βGP alone and completely by both βGP and BMP2. These results suggest that *MyoD* expression is partially down-regulated during 6 days of culture but that MyoD function is almost completely suppressed during ossification induced by βGP.

2.6. *Modulation of Rho signaling alters the differentiation program of immortalized human myogenic progenitor cells*

Rho family GTPases are possible determiners in the switch in cell fate (McBeath et al., 2004; Sordella et al., 2003). Therefore, to explore the role of Rho signaling in the myogenesis–osteogenesis switch of human myogenic progenitor cells, we examined the effects of modulating this pathway in either the myogenesis or osteogenesis of E18 cells. The amounts of both total and activated Rho proteins increased under myogenic differentiation-inducing conditions (Fig. 6A, left panels). In contrast, total Rho protein levels remained constant and the amount of active Rho slightly declined during osteogenic differentiation-inducing culture (Fig. 6A, right panels). Treatment of E18 cells with a specific inhibitor of Rho, C3 transferase (Aktories and Hall, 1989), completely blocked activation of Rho proteins under both myogenic and osteogenic differentiation-inducing conditions (Fig. 6B). C3 transferase also prevented myogenic differentiation of E18 cells, although it did not inhibit ALP expression in them (Fig. 6C and D). In contrast, C3 transferase did not affect calcification of E18 cells in the presence of βGP alone (Fig. 6E). The results suggest that Rho functioning is essential for myogenic but not osteogenic terminal differentiation of E18 cells.

3. Discussion

We have shown that the human myogenic progenitor cell exhibits both osteoblast- and myoblast-specific properties both in vivo and in vitro. All human myogenic progenitor cell clones obtained in the present study retain dual cell lineage-specific properties. Thus, isolated human myogenic cells acquire their osteogenic properties cell-autonomously. The present study shows that at least one distinct subset of myogenic progenitor cells derived from human skeletal muscle preserves the dual lineage-specific properties although it remains to be determined whether all human myogenic progenitor cells retain both properties or not.

Previous studies of primary cultured mouse myogenic cell clones have identified multipotent myogenic progenitor cells that are able to give rise to myotubes, osteoblasts, and adipocytes in vitro (Wada et al., 2002). Their osteogenic differentiation is completely dependent on stimulation with exogenous BMP2. In the present study, adult human myogenic progenitor cells were able to express bone ALP and osteocalcin, and form bone matrix spontaneously without exposure to any exogenous growth factor. FBS is assumed to contain BMPs at levels sufficient to induce differentiation in astrocytes (Kondo and Raff, 2004). Previous studies suggest that BMPs prevent myogenesis of myogenic progenitor cells and also irreversibly induce osteogenesis (Wada et al., 2002). However, human myogenic progenitor cells preserve muscle differentiation potential even though they already express an early osteogenic differentiation marker, ALP. In addition, the osteoblast-specific genes are down-regulated during terminal muscle differentiation. Myogenin is a possible candidate that suppresses *Runx2* expression and other osteoblastic characteristics, as occurs in mouse myogenic cells (Wada et al., 2002). Therefore, it is unlikely that the osteogenic properties of human myogenic progenitor cells are induced by exogenous BMPs, such as those derived from FBS, even if these cells are hypersensitive to BMPs.

The human myogenic progenitor cells analyzed here express BMP4 in a cell-autonomous way. However, it is unlikely that autocrined BMP4 is of importance to maintaining ALP

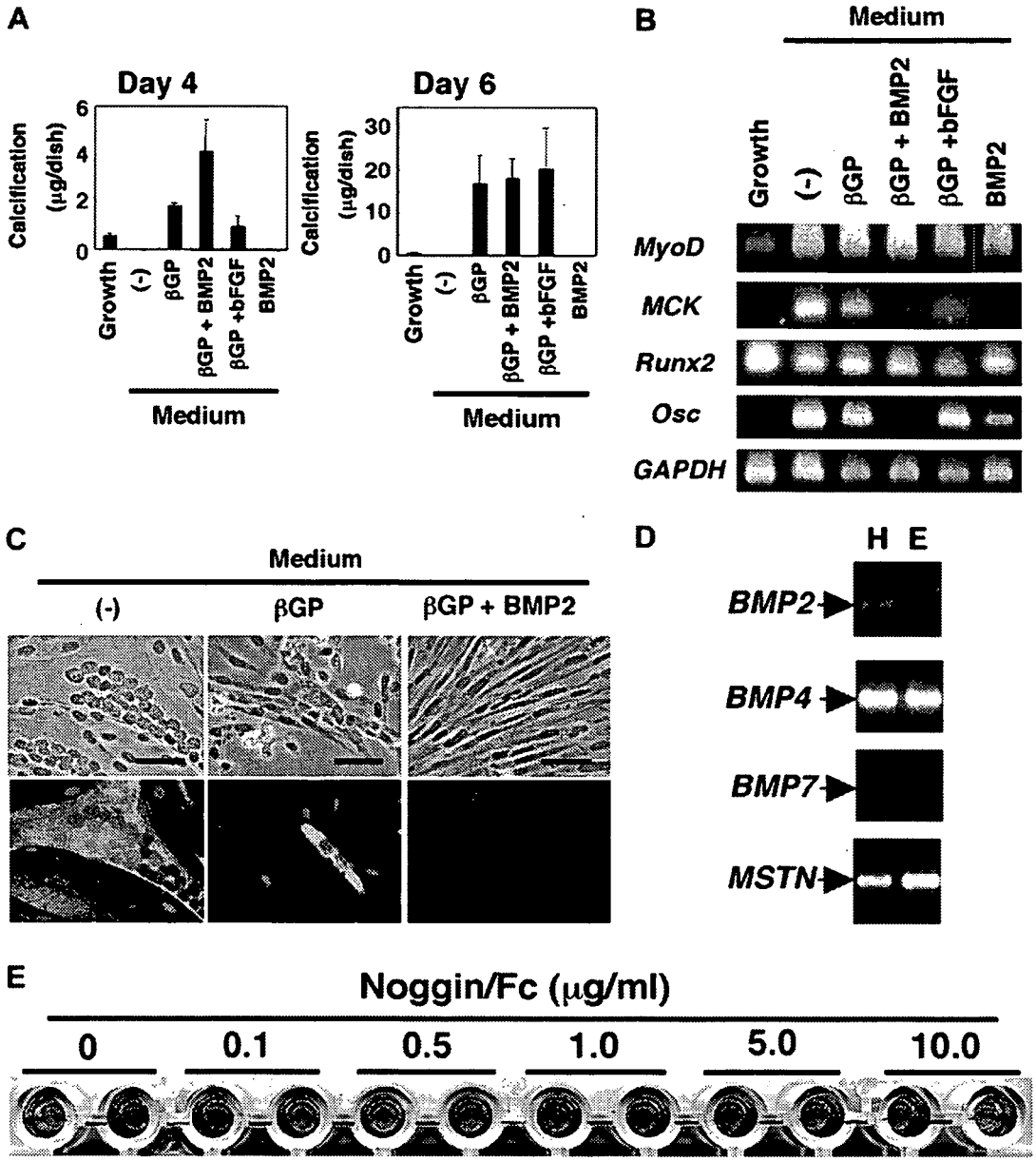

Fig. 5 – Osteogenic terminal differentiation of an immortalized human myogenic progenitor cell clone E18. (A) E18 cells were cultured for 4 or 6 days in growth medium or serum-containing medium supplemented (or not) with either βGP (10 mM) alone, βGP plus BMP2 (500 ng/ml), βGP plus bFGF (10 ng/ml), or BMP2 alone. The deposited calcium was then quantified. Data are expressed as micrograms of calcium per 35 mm culture dish and are means ± SD of values from three experiments. (B) E18 cells were cultured for 6 days under the conditions described in (A) and were then subjected to RT-PCR analysis of the expression of *MyoD*, *MCK*, *Runx2*, *osteocalcin* (*Osc*), and *GAPDH* genes. The number of amplification cycles for *MyoD* was determined to detect the low level expression of *MyoD* under growing conditions (lane 1) although it might be somewhat excess for the other samples (lanes 2–6). (C) E18 cells were cultured for 6 days in serum-containing medium in the absence (left panels) or presence of βGP alone (middle panels) or of both βGP and BMP2 (right panels). They were then subjected to immunofluorescence analysis with antibodies to MyoD (red) and to MHC (green) (lower panels); phase-contrast images of the same fields are shown in upper panels, respectively. Scale bars, 50 μm. (D) Hu5 and E18 cells were cultured in pmGM and then subjected to RT-PCR analysis of the expression of *BMP2*, *BMP4*, *BMP7* and *myostatin* genes. (E) E18 cells were cultured in pmGM with or without noggin/Fc for up to 3 days and then subjected to staining for ALP activity with Fast Blue RR. A macroscopic view of duplicated samples in a 96-well plate is shown.

expression of human myogenic progenitor cells because even an excess of noggin does not reduce expression levels of ALP. Nonetheless, we cannot exclude the possibility that autocrined BMP4 is involved in triggering, but not in maintaining, ALP expression in human myogenic cells. If that is indeed the case, the mode of BMP4 action should be quite different from those that have been shown in previous works. It is conceivable that prolonged exposure to BMP4,

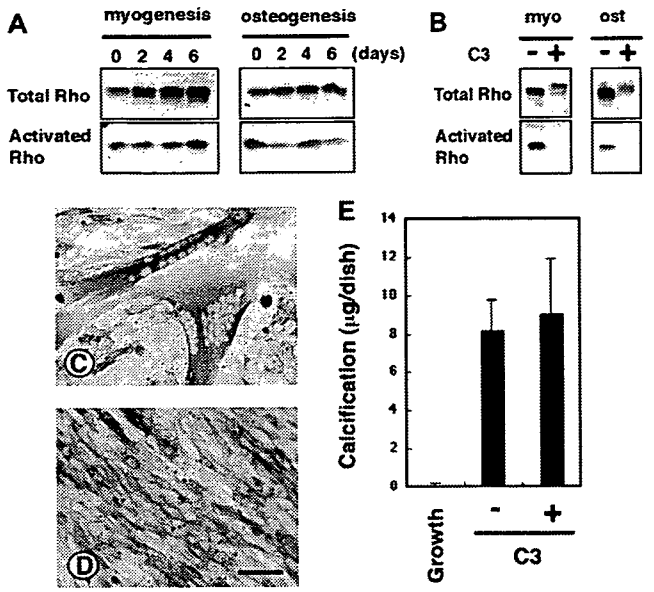

Fig. 6 – Involvement of Rho signaling in the regulation of myogenensis but not osteogenesis of human myogenic progenitor cell E18. (A) E18 cells were cultured for up to 6 days in pmDM (left panels) or medium containing 10% FBS and 10 mM βGP (osteogenesis-inducing medium) (right panels). Cellular proteins were extracted and then activated Rho protein was affinity-purified. Twenty micrograms of total cellular proteins (upper panels) and purified activated Rho from 300 μg of the cell lysate (lower panels) were subjected to immunoblotting analysis with antibodies against Rho. This experiments is representative of three independent experiments. (B) E18 cells were cultured for 6 days in pmDM (left panels) or osteogenesis-inducing medium (right panels) in the absence (–) or presence (+) of 40 μg/ml C3 transferase. Levels of total and activated Rho proteins were determined as in (A). (C and D) E18 cells were cultured for 6 days in pmDM in the absence (C) or presence (D) of 40 μg/ml C3 transferase. Expressions of ALP activity (blue) and MHC (brown) were determined as described in Section 4. Scale bars, 50 μm. (E) E18 cells were cultured for 6 days in growth medium (growth), or osteogenesis-inducing medium in the absence (–) or presence (+) of C3 transferase (40 μg/ml). The deposited calcium was then quantified. Data are expressed as micrograms of calcium per 35 mm culture dish and are means ± SD of values from nine experiments.

even at very low concentrations, induces osteogenic properties in human myogenic progenitor cells. In addition, we should note a possibility that autocrined myostatin modulates BMP binding to the BMP type I receptor ACVR1/ALK2 (Rebbapragada et al., 2003). Therefore, the physiological role of autocrined BMP4 on human myogenic progenitor cells should be determined.

Myogenic progenitor cells derived from adult muscle satellite cells are fundamentally tissue-specific progenitor cells that are tonically restricted to the muscle lineage by the local parenchymal environment. Our results, however, suggest that human myogenic progenitor cells used do not require specific phenotypic reprogramming for osteogenic terminal differentiation and can act as "determined osteoprogenitor cells" under certain circumstances. The osteoblastic properties of E18 cells are not acquired during immortalization in vitro because their parent primary Hu5 cells also underwent osteogenic terminal differentiation without exogenous growth factor exposure.

Recently, human muscle pericytes expressing ALP have been shown to retain the capacity to regenerate skeletal muscle (Dellavalle et al., 2007). In contrast to Hu5 cells, pericyte-derived myogenic cells express myogenic markers only in differentiated myotubes. Therefore, pericyte-derived myogenic cells may be distinct from myogenic progenitor cells used here. Nonetheless, we cannot exclude a possibility that both pericyte-derived myogenic cells and Hu5 cells belong to the same cell lineage because a minor subset of pericyte-derived myogenic cells contributes to the resident satellite cell pool (Dellavalle et al., 2007).

Fibrodysplasia ossificans progressiva (FOP; OMIM 135100) is a heritable disorder characterized by progressive ossification of skeletal muscles (Kocyigit et al., 2001; Mahboubi et al., 2001). The osteoprogenitor cells responsible for ectopic bone formation have not been identified in muscle of individuals with FOP. Previously, candidates for osteoprogenitor cells expressing smooth muscle markers and Runx2 were identified in the muscle of FOP patients (Hegyi et al., 2003; Levy et al., 2001), but their ability to form ectopic bone remains unknown. We have now shown that human myogenic progenitor cells form bone matrix in vitro. We therefore propose that myogenic progenitor cells are responsible for the ectopic ossification observed in human skeletal muscle. Previous studies have proposed that BMP2/4 or their antagonist noggin is responsible for the symptoms of FOP. However, dysfunction of BMPs and noggin in individuals with FOP remains to be elucidated (Cohen, 2002; Warman, 2002; Xu et al., 2000). The involvement of BMPs in ectopic bone formation in skeletal muscle has not been shown in human disorders. Our finding that human myogenic progenitor cells do not require inductive agents to express osteogenic phenotypes has significant implications for ectopic bone formation in human skeletal muscle. FOP is thus a candidate for a disease caused by multipotent muscle progenitor cells that are misled down a heterotopic osteogenic differentiation pathway. Recently, a mutation in the BMP type I receptor ACVR1 was found in individuals with FOP, although how the mutation in ACVR1 perturbs BMP signaling is unknown (Shore et al., 2006). It should be determined whether the mutation in ACVR1 switches the fate of human myogenic progenitor cells. The E18 cells established in the present study would contribute to further study on the function of mutated ACVR1 in human myogenic cells.

We previously proposed a "stock options" model for the generation of different fates from multipotent muscle stem cells (Wada et al., 2002). This model proposes that stem cells can be activated and then induced to express multiple determination genes and thereby give rise to multipotent progenitor cells (multiblasts). Multiblast is defined as a multipotent progenitor cell derived from dormant muscle satellite cell. Multiblasts proliferate and increase in number during early

period of muscle regeneration. Depending on the differentiation-inducing conditions, determination genes or proteins not relevant to the induced terminal differentiation pathway are then down-regulated or functionally inactivated (Fig. 7A). In addition, we assume that a minor subset of multiblasts contributes to the resident satellite cell pool. The present study indicates that human myogenic progenitor cells expressing multiple determination genes select an option

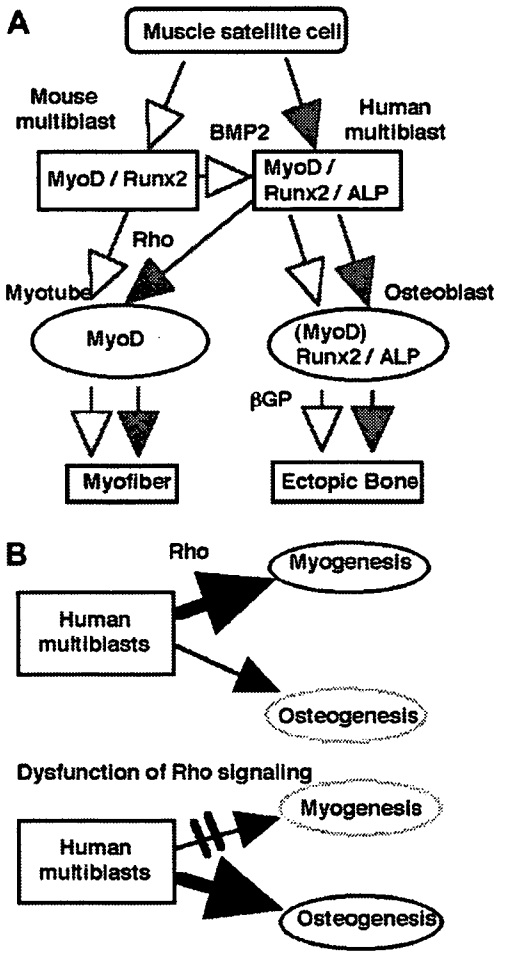

Fig. 7 – Differentiation pathways of human myogenic progenitor cells (multiblasts). (A) Similar but distinct myogenic (red arrows) and osteogenic (blue arrows) differentiation pathways of human (filled arrows) and mouse (open arrows) myogenic cells. Human myogenic progenitor cells (multiblasts) express bone-specific ALP as well as MyoD and Runx2, whereas mouse multiblasts express ALP only after exposure to BMP2. Under conditions that promote osteogenic terminal differentiation, MyoD persists but is inactivated [indicated by (MyoD)] in human myogenic cells; in contrast, its expression is completely down-regulated in mouse myogenic cells. (B) Rho function is essential for terminal myogenic but not osteogenic differentiation of human myogenic progenitor cells (multiblasts) (upper panel). Impairment of a Rho signaling pathway would turn multipotent human myogenic progenitor cells into osteoprogenitors (lower panel).

for the terminal differentiation pathway according to this model. However, in contrast to mouse myogenic progenitor cells, human myogenic progenitor cells used here have the ability to undergo osteogenic differentiation without exogenous BMPs.

BMP2 has previously been shown to stimulate the expression of *Runx2* (Lee et al., 2000; Takazawa et al., 2000), which in turn induces the expression of osteogenic lineage-specific genes, including those for osteocalcin. However, our results indicate that BMP2 suppressed the transactivation of *osteocalcin* gene by Runx2 in confluent human myogenic cells expressing bone ALP. Although osteocalcin is a late marker of osteogenesis induced by Runx2 (Xiao et al., 1999), it functions as an inhibitor of ossification (Ducy et al., 1996). In addition, Runx2 inhibits osteogenic differentiation at a late stage (Liu et al., 2001). We therefore propose that BMP2 inhibits the expression of *osteocalcin* gene by inactivating Runx2, resulting in enhancement of ossification. BMP2 may therefore activate *Runx2* gene expression at an early stage of osteogenesis and subsequently inactivate Runx2 function at a late stage. A similar biphasic effect of BMP2 on *osteocalcin* gene expression was also apparent in mouse myogenic cells (Fig. S2 online).

Rho family GTPases have been shown to be involved in the regulation of terminal differentiation in muscle (Bryan et al., 2005; Carnac et al., 1998; Gallo et al., 1999; Meriane et al., 2000; Takano et al., 1998). In addition, Rho GTPase signaling regulates a switch in both the adipogenesis-myogenesis decision of embryonic mouse mesenchymal precursors (Sordella et al., 2003) and the adipogenesis–osteogenesis decision of human mesenchymal stem cells (McBeath et al., 2004). C3 transferase inhibits myogenic but not osteogenic differentiation of human myogenic progenitor cells. Therefore, Rho GTPase signaling might be involved in a myogenesis–osteogenesis switch of human myogenic progenitor cells. Human myogenic progenitor cells preferentially undergo myogenic differentiation both in vivo and in vitro. However, accidental inactivation of a Rho signaling pathway would turn multipotent human myogenic progenitor cells into osteoprogenitors that have lost the ability to undergo myogenic differentiation (Fig. 7B).

DMD is an X-linked human disease caused by dystrophin deficiency (Bonilla et al., 1988; Hoffman et al., 1987). The mdx mouse, which harbors a mutated dystrophin gene, serves as an animal model of DMD (Ryder-Cook et al., 1988; Sicinski et al., 1989). However, unlike in humans with DMD, regeneration overcomes the inherent muscle degeneration in the mdx mouse. Dystrophin deficiency thus causes progressive muscular dystrophy in a human-specific manner. Progressive ossification in FOP patients is also likely to be triggered in a human-specific manner, given that human myogenic cells retain species-specific properties (Fig. 7A). To understand the mechanisms that cause human muscle diseases, the features of human myogenic progenitor cells must be elucidated. Taken together, the immortalized human myogenic cell lines established in the present study should provide a means with which to characterize further the human-specific properties of myogenic progenitor cells that might be critical for symptoms of human muscle diseases, including DMD and FOP.

4. Materials and methods

4.1. Cell culture

The human myogenic cell clone Hu5 was isolated from normal subcutaneous muscle tissue (Wada et al., 2002; Hashimoto et al., 2006). Hu5 and its derivatives were maintained at 37 °C under 10% CO_2 in dishes coated with type I collagen (Sumilon, Tokyo, Japan) and containing primary cultured myocyte growth medium (pmGM), consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France), and glucose (4.5 mg/ml). The immortalized human myogenic cell clone E18 is available from RIKEN BioResorce Center (http://www.brc.riken.go.jp).

For induction of myogenic differentiation, cells were cultured in primary cultured myocyte differentiation medium (pmDM) consisting of the chemically defined medium TIS (Hashimoto et al., 1995; Hashimoto et al., 1994) supplemented with 2% FBS. For induction of osteogenic terminal differentiation, cells were cultured in DMEM supplemented with 10% FBS, glucose (4.5 mg/ml), and the indicated combinations of 10 mM β-glycerophosphate (βGP) (Sigma, St. Louis, MO), recombinant human bone morphogenetic protein (BMP2) (500 ng/ml) (Strathman Biotech, Hamburg, Germany, or PeproTech EC, London, UK), and human basic fibroblast growth factor (bFGF) (10 ng/ml) (PeproTech EC). For inhibition of BMPs, 2×10^3 Ric10 cells per well were plated in type I collagen-coated 96-well plate and cultured in pmGM supplemented with recombinant mouse noggin fused to the Fc region of human immunoglobulin chimeric protein (noggin/Fc, 0.1–10 mg/ml) (719-NG; R&D Systems, Minneapolis, MN).

4.2. Detection of calcification and ALP activity

Paraformaldehyde-fixed cultured cells were stained with the calcium-specific dye Alizarin Red S (0.01%, Sigma) for 30 min. Calcium deposited by cells in a 35 mm dish was extracted with 0.5 M HCl and then measured with a calcium quantification kit (Sigma).

ALP activity in cells fixed with 4% paraformaldehyde was detected by incubation of the cells for 20 min in a solution containing 100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 50 mM $MgCl_2$, 0.01% naphthol AS-MX, and Fast Blue RR (0.5 mg/ml). Cell nuclei were visualized by staining with 2, 4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) (0.5 μg/ml, Sigma).

4.3. Immunofluorescence and immunochemical analyses

Cultured cells were grown on collagen-coated coverslips (Iwaki, Tokyo, Japan) for immunofluorescence or immunocytochemical analysis. Muscle biopsy specimens were obtained with informed consent from individuals at the Kanagawa Cancer Center Research Institute or the National Center of Neurology and Psychiatry; they were frozen and sectioned at a thickness of 6 or 8 μm with a cryostat. The sections and cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature or placed on ice, respectively, and were then incubated with primary antibodies. Primary antibodies included those to Runx2 (kindly provided by Y. Ito) (Zhang et al., 2000), mouse MyoD (Novocastra, Newcastle, UK), sarcomeric MHC (Bader et al., 1982) (kindly provided by T. Endo and T. Masaki), Pax7 (Ericson et al., 1996) (DSHB, Iowa City, IA), bone-specific ALP (Lawson et al., 1985) (B4-78 and B4-50; DSHB or kindly provided by T. Kimlinger), laminin (Sigma), dystrophin (Sigma), nestin (Arimatsu et al., 1999) (kindly provided by Y. Arimatsu), or desmin (Progen, Heidelberg, Germany). Secondary antibodies included biotinylated or Cy3-labeled antibodies to mouse or rabbit immunoglobulin G and Cy5-labeled antibodies to mouse immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME). The biotinylated antibodies were detected with streptavidin-conjugated horseradish peroxidase, Cy3, or fluorescein isothiocyanate. The peroxidase reaction was performed with 3,3-diaminobenzidine (Sigma) or TrueBlue substrate (KPL, Gaithersburg, MD). The antibodies to Runx2 were detected with biotinylated antibodies to mouse immunoglobulin G and a TSA Direct kit (New England Nuclear, Boston, MA). Cell nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI). Samples were visualized using an upright microscope (model BX50; Olympus, Tokyo, Japan) and a CCD camera (DP50; Olympus). Images were also acquired using a confocal microscope (LSM 5 PASCAL; Carl Zeiss, Oberkochen, Germany) and an upright microscope (Axioplan 2; Carl Zeiss) equipped with a 40x Plan-NEOFLUAR objective lens. Images were post processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

4.4. RT-PCR

Total RNA was extracted from cultured cells with TRIzol-LS (Life Technologies, Rockville, MD), treated with RNase-free DNase (RQ-1; Promega, Madison, WI), and then reverse transcribed with the use of a Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) and random hexamers as primers. Targeted genes were amplified by PCR with the primers listed in Table 1.

4.5. Rho activation assay

The activation of Rho was monitored with an affinity purification assay (Ren et al., 1999) performed with the Rho Activation Assay Biochem Kit (Cytoskeleton, Denver, CO) using 300 μg of each cell lysate. Immunoblotting analysis was done as described previously (Hashimoto et al., 2004; Hashimoto and Ogashiwa, 1997; Hashimoto et al., 1995). Immune complexes were detected with the use of chemiluminescence reagents (Amersham Pharmacia Biotech).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2007.11.004.

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Autologous Transplantation of SM/C-2.6$^+$ Satellite Cells Transduced with Micro-dystrophin *CS1* cDNA by Lentiviral Vector into *mdx* Mice

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Duchenne muscular dystrophy (DMD) is a lethal muscle disorder caused by mutations in the dystrophin gene. Transplantation of autologous myogenic cells genetically corrected *ex vivo* is a possible treatment for this disorder. In order to test the regenerative efficiency of freshly isolated satellite cells, we purified quiescent satellite cells from limb muscles of 8–12-week-old green fluorescent protein-transgenic (GFP-Tg) mice using SM/C-2.6 (a recently developed monoclonal antibody) and flow cytometry. Freshly isolated satellite cells were shown to participate in muscle regeneration more efficiently than satellite cell-derived myoblasts passaged *in vitro* do, when transplanted into tibialis anterior (TA) muscles of 8–12-week-old cardiotoxin-injected C57BL/6 mice and 5-week-old dystrophin-deficient *mdx* mice, and analyzed at 4 weeks after injection. Importantly, expansion of freshly isolated satellite cells *in vitro* without passaging had no detrimental effects on their regenerative capacity. Therefore we directly isolated satellite cells from 5-week-old *mdx* mice using SM/C-2.6 antibody and cultured them with lentiviral vectors expressing micro-dystrophin *CS1*. The transduced cells were injected into TA muscles of 5-week-old *mdx* mice. At 4 weeks after transplantation, the grafted cells efficiently contributed to regeneration of *mdx* dystrophic muscles and expressed micro-dystrophin at the sarcolemma. These results suggest that there is potential for lentiviral vector-mediated *ex vivo* gene therapy for DMD.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by mutations in the dystrophin gene.[1] Dystrophin is a 427 kd large sub-sarcolemmal protein that forms the dystrophin/glycoprotein complex at the sarcolemma with α- and β-dystroglycans, α-, β-, γ-, and δ-sarcoglycans, and other molecules, and links the cytoskeleton with the basal lamina.[2,3] The lack of dystrophin in the sarcolemma causes instability of the muscle membrane, leading to muscle degeneration and myofiber loss. Although there is no effective treatment for the disease at present, cell therapy could be a promising approach. Satellite cells are quiescent mononucleated cells located external to the muscle membrane but internal to the basal lamina in adult skeletal muscle.[4] On muscle damage, they activate, proliferate, and then exit the cell cycle either to differentiate into mature myofibers or to renew the quiescent satellite cell pool. Because satellite cells have robust regenerative capacity,[5,6] they are expected to be a feasible source for cell therapy in DMD. Indeed, transplantation of myoblasts successfully restored dystrophin expression in dystrophin-deficient muscle under immunosuppression.[7,8] Nevertheless, in the early 90s, transplantation of satellite cell-derived myoblasts failed to improve muscle force in DMD patients.[9–11] The failure has been ascribed to poor survival[12–14] and limited distribution of the transplanted cells after injection.[15] The latter problem could possibly be partly overcome by using high-density injections of myoblasts.[16,17] On the other hand, the mechanisms by which grafted myoblasts are rapidly lost after injection have not been fully addressed.[12–14]

Many studies have employed crude cell preparations containing both satellite cells and non-myogenic cells[18,19] or satellite cell-derived myoblasts extensively amplified *in vitro*.[15,20–22] In a recent study, Montarras *et al.* directly isolated (Pax3)green fluorescent protein (GFP)-expressing satellite cells from the diaphragm of adult *Pax3*$^{GFP/+}$ mice by flow cytometry.[23] These cells constituted a homogeneous population and the majority were quiescent. When grafted into irradiated muscles of immunodeficient *nu/nu* dystrophin-deficient *mdx* mice, the freshly isolated satellite cells efficiently contributed to both fiber repair and the muscle satellite cell compartment,[23] thereby suggesting that fresh satellite cells are a potential source for cell therapy in DMD.

For transplanting autologous cells, which are expected to evade the host immune response to grafted cells, the lentiviral vector is a potential tool for introducing the therapeutic gene

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because it integrates into the host genome in a variety of dividing and non-dividing cells. Because the dystrophin complementary DNA is too large to be incorporated into a lentiviral vector, a truncated but fully functional version of the dystrophin complementary DNA[24-27] has to be used instead of the full-length one. When compared with conventional transfection of myogenic cells with large dystrophin-coding plasmids[28] or nucleofection in combination with ϕC31 integrase,[29] transfection by lentiviral vectors led to much more efficient expression of mini- or micro-dystrophin in *mdx* mice,[30] in non-human primate cells, and in human myogenic cells.[31] Lentiviral vectors were also used for introducing the therapeutic genes into other types of stem cells. Bachrach *et al.* reported expression of human micro-dystrophin in *mdx*^{5cv} muscles after systemic delivery of autologous side population cells modified with lentiviral vectors expressing micro-dystrophin.[32] Sampaolesi *et al.* reported intra-arterial delivery of autologous mesoangioblasts corrected by lentiviral vectors expressing α-sarcoglycan (α-SG), resulting in many α-SG-positive fibers, and morphological and functional recovery in downstream muscles of α-SG-null dystrophic mice.[33] A more recent study reported the autologous transplantation into skeletal muscle, of monkey muscle precursor cells transduced with micro-dystrophin by lentiviral vectors.[31] However, whether *ex vivo* gene therapy using lentiviral vectors expressing micro-dystrophin is indeed beneficial in large animal models such as dystrophic dogs, is still subject to controversy.[34]

Previously, Fukada *et al.* established a method of direct purification of quiescent satellite cells from adult mouse skeletal muscles, using fluorescence activated cell sorting (FACS) and a novel monoclonal antibody named SM/C-2.6.[35] The method is simple, and is expected to be applicable to the isolation of satellite cells from dystrophic (autologous) muscles for cell therapy.

In this study, we first directly isolated satellite cells from *mdx* mice using the SM/C-2.6 antibody and FACS. We showed that *mdx*-SM/C-2.6$^+$ cells transduced with lentiviral vectors expressing micro-dystrophin efficiently contributed to regeneration of *mdx* muscles and expressed micro-dystrophin at the sarcolemma when grafted. Our results indicate that the autologous satellite cell isolated by the SM/C-2.6 antibody and genetically corrected by a lentiviral vector is a feasible tool for cell therapy of DMD or of localized forms of muscular dystrophy.

RESULTS

Passaged SM/C-2.6$^+$ satellite cells show reduced regenerative capacity

We isolated satellite cells from the limb muscles of 8–12-week-old C57BL/6 mice using FACS and a novel monoclonal antibody, SM/C-2.6.[35] A previous study has shown that satellite cells are highly enriched in the SM/C-2.6$^+$ fraction.[35] Immediately after isolation by FACS, SM/C-2.6$^+$ cells expressed Pax7, but not MyoD, myogenin, or Ki67 (**Table 1**). After 4 days of culture, more than 95% of the cells expressed MyoD and Ki67 (data not shown). Pax7 marks quiescent, activated satellite cells and their progeny, myoblasts,[36] whereas MyoD marks activated satellite cells and myoblasts.[37,38] Ki67 is a marker of proliferating cells. It follows, therefore, that SM/C-2.6$^+$ cells are highly purified satellite cells in the G_0 phase immediately after isolation from muscle tissues.

Table 1 Expression of myogenic and proliferative markers of freshly isolated SM/C-2.6$^+$ cells from limb muscles of C57BL/6 or *mdx* mice

Marker	B6-SM/C-2.6$^+$ cells (%)	*mdx*-SM/C-2.6$^+$ cells (%)
Pax7	95 ± 1.4	94 ± 2.1
MyoD	0 ± 0	19 ± 2.8
Myogenin	0 ± 0	7 ± 1.3
Ki67	0.6 ± 1.0	34 ± 1.8

The expression level of each marker is shown as the percentage of positive cells in total cells stained with 4',6-diamidino-2-phenylindole in three randomly selected fields. Data are represented as mean values ± SD.

To investigate the regenerative efficiency of SM/C-2.6$^+$ satellite cells when grafted into mouse skeletal muscles, three kinds of cells were prepared from limb muscles of 8–12-week-old GFP-Tg mice: (i) quiescent SM/C-2.6$^+$ cells freshly isolated by FACS (**Figure 1a**), (ii) expanded SM/C-2.6$^+$ cells *in vitro* with or without passaging (**Figure 1a**), and (iii) cultured primary myoblasts isolated by a conventional pre-plating method.[39] These cells were injected at 2×10^4 cells per muscle into the tibialis anterior (TA) muscle of 8–12-week-old C57BL/6 and 5-week-old dystrophin-deficient *mdx* mice. Twenty four hours before cell transplantation the recipient C57BL/6 muscles were injected with cardiotoxin (CTX) so as to induce regeneration. Four weeks after the injection, we investigated the contribution of each cell population to muscle regeneration by immunodetection of GFP-positive fibers. Freshly isolated SM/C-2.6$^+$ cells (**Figure 1b**) produced many more GFP-positive fibers than those produced by the same number of cultured SM/C-2.6$^+$ cells passaged once *in vitro* (**Figure 1c**, passage 1). We next examined the effects of expansion, without passaging and with repeated passaging, on the regenerative capacity of the cells. The number of GFP-positive myofibers derived from GFP-Tg SM/C-2.6$^+$ cells dropped considerably after first passage *in vitro* and then gradually decreased with subsequent passages in both CTX-injected C57BL/6 mice (**Figure 1d**) and in *mdx* mice (**Figure 1e**). Primary myoblasts prepared by the pre-plating method[39] also showed low regenerative capacity (**Figure 1d**). Surprisingly, the regenerative efficiency of cells expanded *in vitro* without passaging was comparable to that of freshly isolated cells (**Figure 1d and e**). These results suggested to us that it is possible to genetically correct dystrophin-deficient satellite cells *ex vivo* before transplantation without causing a reduction in their regenerative capacity.

In order to know why fresh or "expansion" cells gave rise to more myofibers when compared with cells passaged *in vitro*, we compared the colony formation ability of fresh satellite cells with that of passaged myoblasts (passage 1). The results showed that fresh satellite cells formed larger colonies than passage 1 cells, when plated at a density of 1 cell/well on 96-well plates, although the rate of colony formation was not significantly different between these two cells (fresh, 26% versus passage 1, 23%) (**Supplementary Figure S1**). In contrast, there was no difference in fusion index between fresh satellite cells and passaged myoblasts (data not shown). Collectively, a reduction in the proliferative ability of passaged myoblasts *in vitro* might partly explain their lower regenerative capacity *in vivo*.

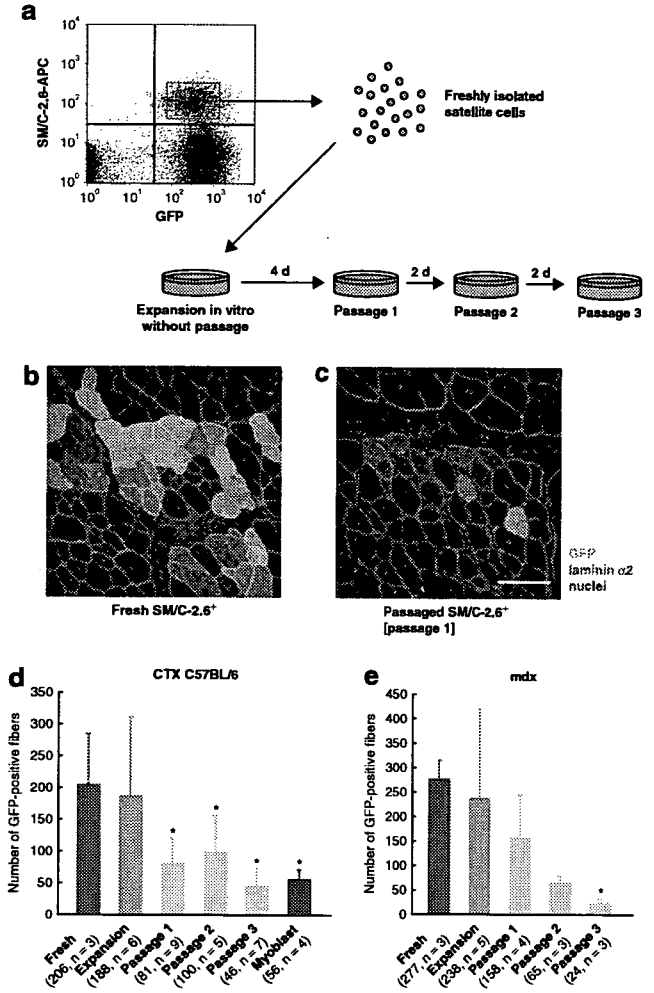
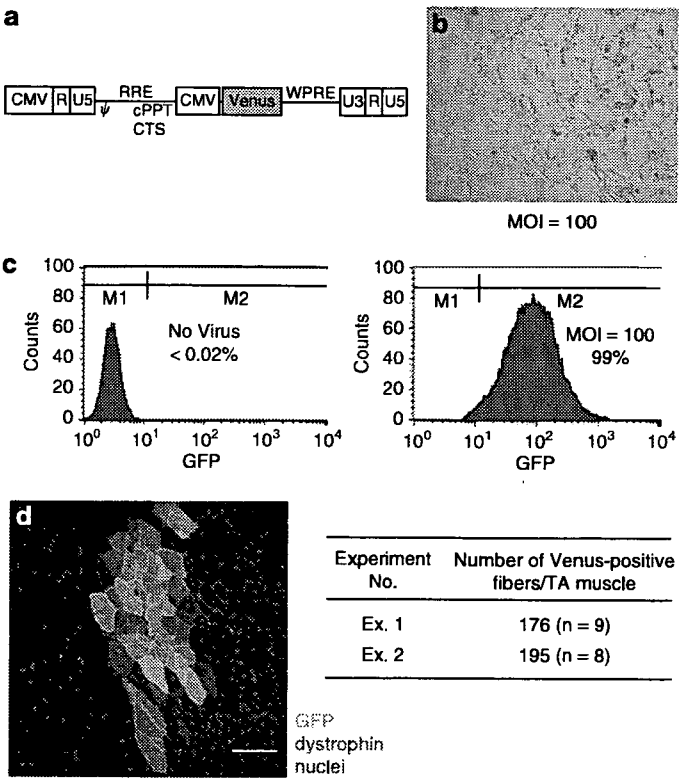


Figure 1 The regenerative capacity of SM/C-2.6$^+$ satellite cells isolated from adult mouse skeletal muscles by fluorescence activated cell sorting (FACS). (**a**) Flow cytometry of mononucleated cells derived from limb muscles of green fluorescent protein-transgenic (GFP-Tg) mice after staining with SM/C-2.6 antibody and culture conditions of sorted cells. SM/C-2.6$^+$ GFP$^+$ cells (red square) were sorted as the satellite cell fraction. These cells were cultured in proliferation medium for 4 days (expansion *in vitro* without passage) and then passaged up to three times at 2-day intervals. (**b**) Freshly isolated and (**c**) passaged SM/C-2.6$^+$ cells (passage 1) from GFP-Tg mice were injected into C57BL/6 tibialis anterior (TA) muscles. The muscles were treated with cardiotoxin (CTX) 24 hours before cell transplantation and then injected with 2×10^4 cells per TA muscle. Four weeks after the injection, cross-sections were stained with anti-GFP (green) and laminin α2 (red) antibodies. Nuclei were stained with TOTO3 (blue). Bar: 80 μm. (**d**, **e**) Comparison of muscle regenerative efficiencies of three kinds of cells prepared from GFP-Tg mice: (i) quiescent SM/C-2.6$^+$ cells freshly isolated by FACS (red bars), (ii) expanded SM/C-2.6$^+$ cells *in vitro* without passaging (orange bars) or passaged SM/C-2.6$^+$ cells (yellow bars with passage numbers), and (iii) primary myoblasts isolated by the preplating method (blue bar in **d**). The same numbers of cells (2×10^4 cells) were grafted into TA muscles of CTX-treated C57BL/6 (d) and *mdx* mice (**e**). The number of GFP-positive fibers per cross-section was counted after staining with anti-GFP antibody. Error bars represent SD. $^*P < 0.05$ compared with freshly isolated SM/C-2.6$^+$ cells.

SM/C-2.6$^+$ satellite cells transduced with lentiviral vectors efficiently contribute to muscle regeneration

Successful gene and cell therapy for DMD requires sustained expression of the therapeutic gene in striated muscle. The



Experiment No.	Number of Venus-positive fibers/TA muscle
Ex. 1	176 (n = 9)
Ex. 2	195 (n = 8)

Figure 2 Lentiviral vector-mediated gene transfer into SM/C-2.6$^+$ satellite cells and transplantation of transduced cells into *mdx* mouse muscles. (**a**) Structure of the lentiviral vector expressing Venus under the control of a cytomegalovirus (CMV) promoter. (**b**) Fluorescence of Venus-expressing satellite cell-derived myoblasts. Freshly isolated SM/C-2.6$^+$ cells from C57BL/10 limb muscles were transduced with lentiviral vectors expressing Venus at a multiplicity of infection (MOI) of 100 for 16 hours, and cultured in proliferation medium for 3 days. (**c**) Flow cytometric analysis of non-transduced (left panel) and transduced (right panel) SM/C-2.6$^+$ cells 3 days after the transduction. M2 denotes the area of Venus-expressing cells. At a MOI of 100, 99% of the cells expressed Venus. (**d**) Venus- and dystrophin-positive fibers formed by SM/C-2.6$^+$ cells transduced with lentiviral vectors *in vitro*. Transduced cells (2×10^4) were injected into tibialis anterior (TA) muscles of *mdx* mice. Four weeks after the injection, cross-sections were stained with anti-GFP (green) and dystrophin (red) antibodies. Nuclei were stained with TOTO3 (blue). The number of Venus-positive fibers per cross-section was counted. Bar: 80 μm. cPPT, central polypurine tract; CTS, central termination sequence; GFP, green fluorescent protein; RRE, rev responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

lentiviral vector can carry a relatively large transgene and integrate it into the genome of non-dividing cells such as quiescent satellite cells. We therefore attempted lentiviral vector–mediated gene transfer into satellite cells. For this purpose, we used a human immunodeficiency virus-1–based lentiviral vector pseudotyped with vesicular stomatitis virus-G glycoprotein.[40] To start with, we used a vector that expresses Venus, a variant of yellow fluorescent protein[41] under the control of a cytomegalovirus (CMV) promoter (**Figure 2a**). Freshly isolated satellite cells from limb muscles of 8–12-week-old C57BL/10 mice, which are syngenic to *mdx*, were transduced with the lentiviral vectors at a multiplicity of infection (MOI) of 100 for 16 hours. After removal of free viral vectors and *in vitro* expansion of the cells for 3 days, numerous Venus-positive cells were detected (**Figure 2b**). Flow

cytometric analyses revealed that 99% of the SM/C-2.6$^+$ satellite cell–derived myoblasts expressed Venus when transduced at a MOI of 100 (**Figure 2c**, right panel). These transduced cells were injected into TA muscles of 5-week-old *mdx* mice at 2×10^4 per muscle. Four weeks after the injection, the muscle regeneration capacity of cells transduced with lentiviral vectors was investigated by immunodetection of Venus- or dystrophin-positive fibers. As in the case of the non-transduced cells (**Figure 1d** and **e**), grafting of transduced cells too led to many Venus- and dystrophin-positive fibers (**Figure 2d**). This serves to show that SM/C-2.6$^+$ satellite cell-derived myoblasts transduced with lentiviral vectors contribute efficiently to muscle regeneration.

Direct isolation of SM/C-2.6$^+$ satellite cells from dystrophic muscles of *mdx* mice

In order to test whether autologous myogenic precursor cells genetically corrected to express a dystrophin gene represent a possible tool in DMD therapy, we next attempted to directly isolate SM/C-2.6$^+$ cells from limb muscles of 5-week-old *mdx* mice. Numerous inflammatory and fibroblastic cells reflecting the active cycles of the degeneration–regeneration process are found in dystrophic muscles. SM/C-2.6 antibody reacts with activated fibroblastic cells (Fukada *et al.*, unpublished data). Because satellite cells are negative for both Sca-1 and CD31,[35] we stained *mdx* muscle-derived mononuclear cells with a cocktail of CD45, CD31, Sca-1, and SM/C-2.6 antibodies and collected SM/C-2.6$^+$ CD45$^-$ CD31$^-$ Sca-1$^-$ cells as the satellite cell fraction (**Figure 3a**). When these cells were cultured in proliferation medium for 4 days, more than 95% of them expressed MyoD (**Figure 3b**). These results indicate that, using the SM/C-2.6 antibody, a pure population of satellite cells can be isolated, not only from wild-type muscle but also from dystrophic muscle of *mdx* mice. Immediately after isolation, the majority of satellite cells from C57BL/6 mice were negative for MyoD, myogenin, and Ki67. On the other hand, 19% of *mdx*-satellite cells were positive for MyoD and 34% of the cells were positive for Ki67 (**Table 1**). There was no difference between *mdx*- and B6-SM/C-2.6$^+$ cells with respect to expression of Pax7 (**Table 1**). This proves that a considerable fraction of satellite cells are in an activated, proliferative state in skeletal muscles of *mdx* mice.

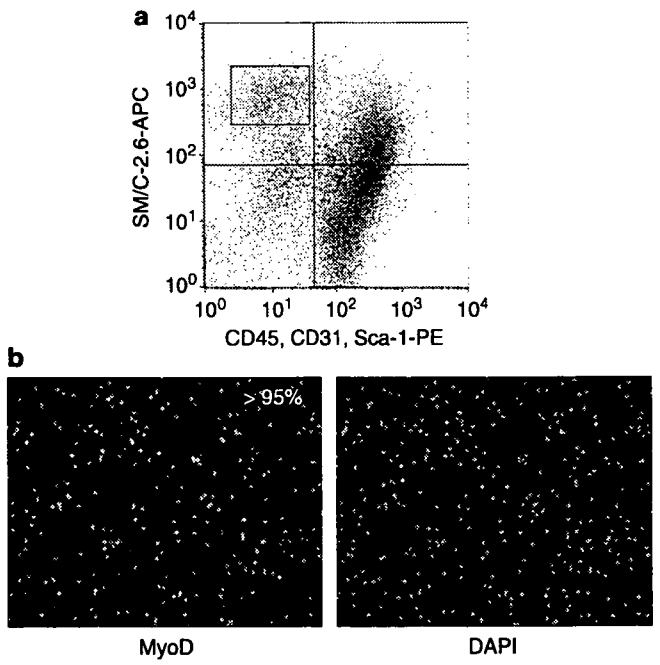


Figure 3 Direct isolation of SM/C-2.6$^+$ satellite cells from dystrophic muscles of *mdx* mice. (**a**) Flow cytometry of mononucleated cells derived from *mdx* mice, and stained with a cocktail of CD45, CD31, Sca-1, and SM/C-2.6 antibodies. SM/C-2.6$^+$ CD45$^-$ CD31$^-$ Sca-1$^-$ cells (red square) were sorted as the satellite cell fraction. (**b**) Sorted *mdx*-satellite cells were cultured in proliferation medium for 4 days and stained with anti-MyoD antibody (green) and 4′,6-diamidino-2-phenylindole (DAPI) (nuclei, blue). More than 95% of them expressed MyoD.

Successful micro-dystrophin gene transfer into *mdx*-SM/C-2.6$^+$ satellite cells

The full-length dystrophin complementary DNA, at 14 kilobase (kb), is too large to be incorporated into a lentiviral vector. In previous studies, we constructed a rod-truncated micro-dystrophin *CS1* and demonstrated that it effectively rescued

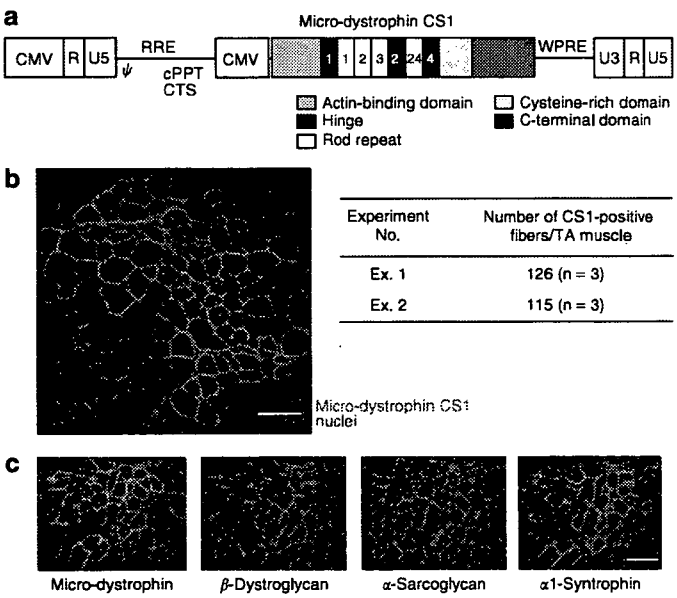


Figure 4 Lentiviral vector-mediated micro-dystrophin *CS1* gene transfer into *mdx*-SM/C-2.6$^+$ cells and transplantation of transduced cells into *mdx* muscles. (**a**) Structure of the lentiviral vector expressing micro-dystrophin *CS1*. *CS1* complementary DNA was inserted downstream of the cytomegalovirus (CMV) promoter. *CS1* has the N-terminal domain, a shortened version of the central rod domain with four rod repeats and three hinges, the cysteine-rich domain, and the C-terminal domain. The numbers of rod repeats and hinges are also shown. (**b**) Freshly isolated SM/C-2.6$^+$ cells from *mdx* dystrophic muscles were transduced with lentiviral vectors expressing micro-dystrophin *CS1* at a multiplicity of infection of 200 for 16 hours and cultured in proliferation medium for 2 days. Transduced cells (2×10^4) were injected into *mdx* tibialis anterior (TA) muscles. Four weeks after the injection, cross-sections were stained with anti-dystrophin antibody (red) and TOTO3 (nuclei, blue). The number of micro-dystrophin *CS1*-positive fibers per cross-section was counted. Bar: 80 μm. (**c**) Restoration of dystrophin-associated proteins at the sarcolemma of micro-dystrophin-positive fibers. Serial cross-sections were stained with anti-dystrophin, β-dystroglycan, α-sarcoglycan, and α1-syntrophin antibodies (red), and TOTO3 (nuclei, blue). Bar: 80 μm. cPPT, central polypurine tract; CTS, central termination sequence; RRE, rev responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

the dystrophic phenotypes of *mdx* mice when introduced as a transgene[24] or by adeno-associated viral vectors.[25] We therefore inserted a 4.9 kb micro-dystrophin *CS1* into the lentiviral vector as a therapeutic gene. Freshly isolated *mdx*-SM/C-2.6$^+$ cells were transduced with lentiviral vectors expressing micro-dystrophin *CS1* under the control of a CMV promoter (**Figure 4a**) at a MOI of 200 for 16 hours. In this condition, 97% of the cells expressed micro-dystrophin *CS1* (data not shown). These transduced cells were injected into TA muscles of 5-week-old *mdx* mice at 2×10^4 cells per muscle. Four weeks after the injection, the muscle regeneration capacity of the cells was investigated by immunodetection of micro-dystrophin-positive fibers. Many myofibers expressed micro-dystrophin *CS1* on the sarcolemma at an average of 120 fibers per muscle (**Figure 4b**). Further, we examined the restoration of the dystrophin-associated protein complex in micro-dystrophin-positive fibers by immunodetection of α-SG, β-dystroglycan, and α1-syntrophin. As shown in **Figure 4c**, all these proteins were expressed at the sarcolemma of micro-dystrophin *CS1*-positive myofibers, thereby suggesting the recovery of dystrophin-associated protein complex by the introduction of micro-dystrophin. These results indicate that *mdx*-SM/C-2.6$^+$ cells transduced with lentiviral vectors expressing micro-dystrophin *CS1* efficiently contribute to regeneration of dystrophic muscles of *mdx* mice and restore the expression of the dystrophin/dystrophin-associated protein complex. It therefore follows that transplantation of autologous myogenic precursor cells prepared using the SM/C-2.6 antibody and genetically corrected by a lentiviral vector, is a possible approach for cell therapy in DMD or in localized forms of muscular dystrophy.

DISCUSSION

In vitro passaging reduced the regenerative capacity of satellite cells: In the present study, we directly isolated satellite cells from skeletal muscles of wild-type and *mdx* mice using SM/C-2.6, a novel monoclonal antibody,[35] and flow cytometry. Almost all satellite cells prepared from normal muscle are negative for MyoD, myogenin, and Ki67 immediately after isolation, thereby indicating that they are in a quiescent state. In contrast, approximately 20% of *mdx*-satellite cells are positive for MyoD and 35% are positive for Ki67 (**Table 1**). This result indicates that a fraction of *mdx*-satellite cells are already in an activated state.

Transplantation experiments showed that freshly isolated SM/C-2.6$^+$ satellite cells possess a higher capacity for muscle reconstitution when compared with SM/C-2.6$^+$ myoblasts passaged *in vitro* prior to transplantation. This result indicates that passaging and subsequent proliferation of satellite cells in culture reduce their intrinsic capacity for muscle reconstitution. In order to clarify the mechanisms of low myogenicity of passaged cells, we performed a colony-forming assay of freshly isolated satellite cells and passaged satellite cell-derived myoblasts (passage 1). When the cells were seeded at a density of 1 cell/well on 96-well plates, fresh satellite cells formed larger colonies than "passage 1" myoblasts (**Supplementary Figure S1**). In contrast, there was no difference in fusion index between these two cell populations (data not shown). Collectively, reduced efficiency of muscle fiber regeneration by passaged myoblasts can be partly explained by gradual loss of proliferative ability during passaging.

Importantly, we also found that satellite cells that were expanded *in vitro* without passaging showed regenerative capacity comparable to freshly isolated satellite cells. We therefore hypothesized that it might be possible to introduce therapeutic genes into satellite cells *in vitro* by a lentiviral vector before transplantation without causing any reduction in their regenerative capacity.

Comparison of regenerative capacity of SM/C-2.6$^+$ satellite cells with other reports: Previously, Montarras *et al.* directly isolated (Pax3) GFP-expressing satellite cells, which constitute a homogeneous population of small, non-granular, CD34$^+$ CD45$^-$ Sca-1$^-$ cells, from diaphragms of adult *Pax3*$^{GFP/+}$ mice by flow cytometry, and examined their regenerative capacity.[23] The researchers concluded that *in vitro* expansion of freshly isolated satellite cells for a few days prior to transplantation is a disadvantageous approach, because such satellite cell-derived myoblasts displayed considerably lower muscle regenerative efficiency than fresh satellite cells. In contrast, we observed no reduction in regenerative capacity as a result of *in vitro* expansion of freshly isolated satellite cells without passaging, although their capacity was remarkably reduced after passaging (**Figure 1d** and **e**). The discrepancy between the results of Montarras *et al.* and our results may be due to differences in the culture conditions employed. One possible explanation could be that our culture medium contained basic fibroblast growth factor. It has been reported that addition of basic fibroblast growth factor to culture medium improves transplantation efficiency.[42,43] The modification of culture conditions may enable maintenance of the intrinsic muscle regenerative capacity of satellite cells.

Previous muscle transplantation experiments utilized the progeny of satellite cells enzymatically dissociated from myofibers and extensively cultured to increase their numbers.[15,20–22] When 5×10^5 to 1×10^6 myoblasts taken from normal mice and prepared by the pre-plating method were transplanted into non-irradiated muscles of *mdx* mice, it resulted in fewer than 100 dystrophin-positive myofibers per muscle.[21] On the other hand, when 5×10^5 cells were injected into muscles of immunodeficient *mdx nu/nu* mice that had been pre-irradiated to ablate endogenous satellite cell function, they formed an average of 328 dystrophin-positive fibers.[18] Furthermore, grafting of 2×10^4 satellite cells freshly isolated from *Pax3*$^{GFP/+}$ mice into pre-irradiated TA muscles of *mdx nu/nu* mice led to dystrophin expression in an average of 587 fibers.[23] In our experiment, the same number (2×10^4) of satellite cells freshly isolated from adult normal mice gave rise to an average of only 277 myofibers in non-irradiated *mdx* muscles (**Figure 1d** and **e**). This shows that grafted muscle precursor cells form a far greater number of dystrophin-positive fibers in irradiated muscle than in non-irradiated muscle. The use of immunosuppressants such as FK506 also greatly improves the efficiency of transplantation.[7] In the present study, we injected myogenic cells into non-irradiated TA muscles of immunocompetent mice without any immunosuppressant. Therefore, in our experimental

conditions, the intrinsic function of SM/C-2.6$^+$ satellite cells may be underestimated.

The use of the Lentiviral vector is feasible for ex vivo *gene transfer*: In this study we showed that, at a MOI of 200, lentiviral vectors can introduce the rod-truncated micro-dystrophin gene *CS1* into more than 97% of *mdx*-satellite cells without detrimental effects on cell viability and regenerative capacity. But at a MOI of 300 we observed cell toxicity, whereas at a MOI of 100, the transduction efficiency was below 80% (data not shown). When we injected the transduced autologous myoblasts into *mdx* muscle, the cells contributed to regeneration of myofibers and expressed micro-dystrophin and dystrophin-associated proteins at the sarcolemma. Our results therefore suggest that *ex vivo* gene transfer into autologous myogenic cells by a lentiviral vector is feasible. On the other hand, direct intramuscular injection of vesicular stomatitis virus-G glycoprotein–pseudotyped lentiviral vectors led to relatively low expression of the transgene in mouse skeletal muscles.[30,44] Because the lentiviral vector genome is inserted into the host genome, the transduction of cells other than the target cell could introduce the risk of mutagenesis. Further, *in vivo* administration could induce undesirable immune responses to exogenous viral proteins. In effect, direct *in vivo* administration of lentiviral vectors poses a safety problem for clinical application. In contrast, *ex vivo* gene transfer has the merit of minimizing the risks of introducing free lentiviral vectors into the host. Transduced cells can efficiently proliferate and differentiate *invitro* (data not shown).

Limitations of ex vivo *gene therapy in DMD, using satellite cells*: One of the demerits of our procedure, as compared to *in vivo* gene transfer, is that only a part of the genetically modified myogenic precursor cells contributes to regeneration of the host muscle, given the poor survival rate of these cells. In fact, by using real-time polymerase chain reaction on transcripts from the transgenic enhanced GFP gene, we found that more than 90% of the injected cells were lost within the first 24 hours after injection (data not shown). In addition, migration of the surviving cells is limited in the host muscle after injection. Furthermore, because *in vitro* passaging greatly reduces their myogenicity, it is difficult to obtain a sufficient number of satellite cells or their progeny from a small muscle biopsy of a DMD patient. Therefore current myoblast transfer might be more realistic for localized forms of muscular dystrophy, such as oculo-pharyngeal muscular dystrophy or facio-scapulo-humeral muscular dystrophy.[45] Surprisingly, however, Collins *et al.* transplanted a single intact myofiber into irradiation-ablated muscles and demonstrated that as few as seven satellite cells associated with one transplanted myofiber can generate over 100 new myofibers containing thousands of myonuclei.[5] Their observations suggest that proper isolation and handling of satellite cells might greatly improve their myogenic potential.

In this study we have demonstrated transplantation of autologous satellite cells genetically corrected by a lentiviral vector *ex vivo* into *mdx* muscle. For treating DMD patients, however, it is necessary to find the optimum *in vitro* culture condition that will enable human muscle precursor cells to maintain their intrinsic myogenic potential. It would also be useful to identify the factors that support survival and/or proliferation of transplanted cells in the host muscle.

MATERIALS AND METHODS

Animals. All procedures used on the experimental animals were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight-to-twelve-week-old C57BL/6 mice were purchased from Nihon CLEA (Tokyo, Japan). C57BL/6-GFP Tg mice were kindly provided by Dr. Okabe (Osaka University, Japan). C57BL/10 mice and C57BL/10-*mdx* mice were maintained in our animal facility and propagated by allowing mating.

In order to induce muscle regeneration, 50 µl of CTX (10 µmol/l in saline; Wako Pure Chemical Industries, Tokyo, Japan) was injected into the TA muscle 24 hours before cell transplantation.

Cell preparation and FACS analysis. Freshly isolated muscle-derived cells were prepared from 8–12-week-old GFP-Tg mice, C57BL/6 mice, C57BL/10 mice, or 5-week-old *mdx* mice as previously described.[35] Hind-limb and fore-limb muscles were isolated and digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37 °C. The muscle slurries were filtered through 100 µm nitrex mesh (BD Biosciences, Franklin Lakes, NJ) and subsequently through 40 µm nitrex mesh (BD Biosciences, Franklin Lakes, NJ). Erythrocytes were eliminated by treatment with 0.8% NH_4Cl in Tris-buffer. Mononucleated cells were stained with biotinylated SM/C-2.6 monoclonal antibody,[35] and labeled by allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA). Mononucleated cells derived from *mdx* muscles were stained with antibodies to additional surface markers, phycoerythrin-conjugated anti-CD45 antibody (clone 30-F11; BD PharMingen, San Diego, CA), phycoerythrin-conjugated anti-CD31 antibody (clone 390; BD PharMingen, San Diego, CA), and phycoerythrin-conjugated anti-Sca-1 antibody (clone D7; BD PharMingen, San Diego, CA). After being washed, stained cells were re-suspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (Trace Biosciences, New South Wales, Australia) and 2 µg/ml propidium iodide (BD PharMingen, San Diego, CA). Cell sorting was performed on a FACS VantageSE flow cytometer (BD Biosciences, Franklin Lakes, NJ). Debris and dead cells were excluded by forward scatter, side scatter, and propidium iodide gating. We used only propidium iodide-negative fractions for further experiments. We usually obtained approximately 1.5×10^5 sorted cells from 1 g of muscle of 8–12-week-old female C57BL/6 mice.

Cell culture and intramuscular transplantation. Freshly isolated SM/C-2.6$^+$ cells from GFP-Tg mice were seeded at a density of 1×10^5 cells per 35-mm dish coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) in a growth medium, Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum and 2.5 ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA), and expanded for 4 days. Further, these cells were passaged up to three times at 2-day intervals. Primary myoblasts isolated by the pre-plating method[39] from GFP-Tg mice were also cultured in growth medium. Freshly sorted cells, expanded and passaged cells, or cultured primary myoblasts were injected into TA muscles of 8–12-week-old CTX-treated C57BL/6 mice or 5-week-old *mdx* mice that show active cycles of the degeneration–regeneration process. The number of injected cells was 2×10^4 per TA muscle. Four weeks later, the injected muscles were isolated and fixed in 4% paraformaldehyde for 30 minutes, immersed sequentially in 10% sucrose/PBS and 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry. Frozen muscle tissues were sectioned (6 µm) using a cryostat. The sections were blocked with 5% goat serum (Cedarlane, Hornby, Canada) in PBS and then reacted with anti-GFP antibody (1:100; Chemicon International, Temecula, CA) and/or anti-laminin $\alpha 2$ antibody (1:100; Alexis, San Diego, CA), or anti-$\alpha 1$-syntrophin antibody

(1:500)[46] at 4 °C overnight. Dystrophin (1:20; NCL-DYSB or DYS2; Novocastra, Newcastle, UK), α-SG (1:50; NCL-α-SARC; Novocastra, Newcastle, UK), and β-dystroglycan (1:50; NCL-β-DG; Novocastra, Newcastle, UK) were detected using monoclonal antibodies after blocking with a MOM kit (Vector Laboratories, Burlingame, CA). The sections were incubated with appropriate combinations of Alexa 488-, Alexa 568-, and Alexa 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 30 minutes. The nuclei were counterstained with TOTO-3 (1:5,000; Molecular Probes, Eugene, OR). The stained sections were observed under the confocal laser scanning microscope system TCSSP (Leica, Heidelberg, Germany).

Immunocytochemistry. Cells sorted using FACS were collected by Cytospin3 (Thermo Fisher Scientific, Waltham, MA). After being fixed with 4% paraformaldehyde for 10 minutes, the cells were blocked with 5% goat serum in PBS and then reacted with anti-Pax7 antibody (1:2; Developmental Studies Hybridoma Bank, Iowa, IA), anti-MyoD antibody (1:200; Dako, Glostrup, Denmark), anti-myogenin antibody (1:200; Developmental Studies Hybridoma Bank, Iowa, IA), and anti-Ki67 antibody (1:2, Ylem, Rome, Italy) at 4 °C overnight. Primary antibodies were detected by Alexa 488- or Alexa 568-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 30 minutes. Stained cells were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and observed with fluorescence microscopy IX70 (Olympus, Tokyo, Japan).

Generation of lentiviral vectors and* in vitro *transduction. The third-generation self-inactivated human immunodeficiency virus-1–based lentiviral vector, pCSII-CMV-IRES2-Venus, has been described previously.[47] The vector contains a CMV promoter; an internal ribosomal entry site (IRES) followed by *Venus*, which is a variant of yellow fluorescent protein[41]; and a woodchuck hepatitis virus post-transcriptional regulatory element. A rod-truncated micro-dystrophin *CS1* complementary DNA (four rod repeats, 4.9 kb) was excised from pCAG-*CS1*[24] and cloned into pCSII-CMV-IRES2-Venus, generating pCSII-CMV-*CS1*-IRES2-Venus. The lentiviral vectors expressing Venus only, or micro-dystrophin *CS1* followed by Venus, were generated by transient cotransfection of the pCSII-CMV-IRES2-Venus or pCSII-CMV-*CS1*-IRES2-Venus, respectively, with the packaging construct (pCAG-HIVgp), vesicular stomatitis virus-G protein, and Rev-expressing construct (pCMV-VSV-G-RSV-Rev) into 293T cells, using the calcium phosphate transfection method.[47–49] Two days after transfection, the vector-containing supernatant was collected, filtered through a 0.45-μm-pore-size filter (Thermo Fisher Scientific, Waltham, MA), and concentrated by centrifugation twice at 50,000*g* for 2 hours at 20 °C. The virus pellet was re-suspended in Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) and stored at −80°C until use. The titer of the concentrated virus was 5×10^8 to 1×10^9 infectious units/ml when assayed on 293T cells, and infectivity was determined by Venus expression as analyzed on a FACS VantageSE (BD Biosciences, Franklin Lakes, NJ).

Sixty thousand freshly isolated SM/C-2.6$^+$ cells in 300 μl growth medium were seeded in each well of 24-well plates and cultured for 16 hours with viral vectors expressing Venus or micro-dystrophin *CS1* at MOI of 100 or 200, respectively. After removal of free viral vectors by changing the medium, the transduced cells were cultured for 2 or 3 days and trypsinized. A cell suspension containing 2×10^4 cells in 20 μl of PBS was injected into the TA muscles of *mdx* mice. The infection efficiency of the injected cells was evaluated using a FACS VantageSE (BD Biosciences, Franklin Lakes, NJ).

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SUPPLEMENTARY MATERIAL

Figure S1. Freshly isolated satellite cells give rise to larger colonies than passaged myoblasts *in vitro*.

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STEM CELLS
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Molecular Signature of Quiescent Satellite Cells in Adult Skeletal Muscle

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ABSTRACT

Skeletal muscle satellite cells play key roles in postnatal muscle growth and regeneration. To study molecular regulation of satellite cells, we directly prepared satellite cells from 8- to 12-week-old C57BL/6 mice and performed genome-wide gene expression analysis. Compared with activated/cycling satellite cells, 507 genes were highly upregulated in quiescent satellite cells. These included negative regulators of cell cycle and myogenic inhibitors. Gene set enrichment analysis revealed that quiescent satellite cells preferentially express the genes involved in cell-cell adhesion, regulation of cell growth, formation of extracellular matrix, copper and iron homeostasis, and lipid transportation. Furthermore, reverse transcription-polymerase chain reaction on differentially expressed genes confirmed that calcitonin receptor (CTR) was exclusively expressed in dormant satellite cells but not in activated satellite cells. In addition, CTR mRNA is hardly detected in nonmyogenic cells. Therefore, we next examined the expression of CTR in vivo. CTR was specifically expressed on quiescent satellite cells, but the expression was not found on activated/proliferating satellite cells during muscle regeneration. CTR-positive cells reappeared at the rim of regenerating myofibers in later stages of muscle regeneration. Calcitonin stimulation delayed the activation of quiescent satellite cells. Our data provide roles of CTR in quiescent satellite cells and a solid scaffold to further dissect molecular regulation of satellite cells. STEM CELLS *2007;25:2448–2459*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Muscle satellite cells, which account for 2%–5% of the total nuclei in adult skeletal muscle, play a major role in muscle regeneration [1, 2]. Under normal conditions, satellite cells are found external to the myofiber plasma membrane and beneath the muscle basal lamina [3] and are mitotically quiescent in the adult skeletal muscle [4, 5]. When activated by muscle damage, they proliferate, differentiate, fuse with each other or injured fibers, and eventually regenerate mature myofibers under the influence of innervation [6]. Recently, it was clearly demonstrated that the proliferation capacity of satellite cells in vivo is robust and that the contribution of interstitial cells or bone marrow-derived cells to muscle fiber regeneration is limited [7]. Importantly, a small fraction of activated satellite cells exit the cell cycle and return to the quiescent satellite state during muscle regeneration to maintain their numbers and the regenerative capacity of muscle.

Besides muscle fiber repair, satellite cells are also responsible for postnatal growth [8] and hypertrophy of skeletal muscle [9], and impairment of their functions is related to several pathological conditions, for example, muscular dystrophies and aging-related muscle atrophy [10]. Moreover, several studies showed that satellite cells differentiate into adipogenic cells or osteocytes in vitro [11–13], implying that they contribute to the fatty infiltration seen in Duchenne muscular dystrophy. Thus, normal functioning of satellite cells is indispensable for the integrity of skeletal muscle, and the cells themselves are an important source of cells for cell therapy of muscle diseases, making it valuable to clarify the molecular regulation of maintenance, activation/proliferation, and differentiation in satellite cells.

Like hematopoietic stem cells, most satellite cells are in a quiescent and undifferentiated state in the adult. Although quiescence is important to retain the proliferative and differentiative potential of satellite cells throughout the lifetime, the molecular regulation of quiescence remains poorly defined. Recent studies suggested that myostatin, a skeletal muscle-specific transforming growth factor-β superfamily member, suppresses the activation of satellite cells [14]. Myostatin has been shown to induce a potent cyclin-dependent kinase inhibitor, p21(Cdkn1a), in vitro [15]. Other in vitro studies suggested that the decrease of MyoD protein and induction of another cyclin-dependent kinase inhibitor, p27(Cdkn1b) [16], and a Rb-related pocket protein, p130 [16, 17], are involved in the attainment of quiescence by proliferating myoblasts.

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We previously reported a method to purify quiescent satellite cells from adult skeletal muscle using the fluorescence-activated cell sorting (FACS) technique and a novel antibody named SM/C-2.6 [18]. In this study, to clarify the molecular regulation of quiescent satellite cells, we performed genome-wide gene expression profiling of quiescent satellite cells isolated from C57BL/6 mice. Expression analysis of individual genes identified several candidate genes that regulate dormancy of satellite cells. Gene set enrichment analysis (GSEA) revealed that the gene sets involved in cell-cell adhesion, cell growth, copper and iron ion homeostasis, lipid transport, and formation of extracellular matrix were coordinately upregulated in quiescent satellite cells. Furthermore, we demonstrate that calcitonin receptor (CTR) is expressed specifically on quiescent satellite cells in vivo and that calcitonin significantly attenuates the activation of satellite cells. Our study is the first report of in-depth gene expression analysis of quiescent satellite cells and will greatly facilitate the investigation of molecular regulation of satellite cells in both physiological and pathological conditions.

MATERIALS AND METHODS

Animals

All procedures using experimental animals were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. C57BL/6 mice were purchased from Nihon CLEA (Tokyo, http://www.clea-japan.com).

Preparation of Satellite Cells and Nonmyogenic Cells from Mouse Limb Muscles

Mononuclear cells were prepared from fore- and hindlimb muscles of 8- to 12-week-old female C57BL/6 mice as described [19] and incubated on ice for 30 minutes in the presence of a 1:200 dilution of phycoerythrin-conjugated anti-CD45 (clone: 30-F11) and biotinylated SM/C-2.6 [18]. Cells were then incubated with streptavidin-labeled allophycocyanin on ice for 30 minutes and resuspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 2 μg/ml propidium iodide (PI). Cell sorting was performed on a FACSVantage SE flow cytometer (BD Biosciences, San Diego, http://www.bdbiosciences.com). Dead cells were excluded by PI gating. All antibodies and reagents for FACS analysis were purchased from BD Pharmingen (San Diego, http://www.bdbiosciences.com/index_us.shtml).

Cell Culture

Satellite cells were cultured in growth medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing 20% fetal calf serum (FCS; Trace Biosciences, New South Wales, Australia), 2.5 ng/ml basic fibroblast growth factor (Invitrogen), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gibco-BRL, Gaithersburg, MD, http://www.gibcobrl.com) on culture dishes coated with Matrigel (BD Biosciences). Single living myofibers were prepared as described [20] and transferred to Matrigel-coated 24-well culture dishes (one fiber per well). After a 2-day culture in growth medium with or without elcatonin, satellite cells that had detached from muscle fibers were counted.

Immunocytochemical Analysis

FACS-sorted cells were collected on glass slides by Cytospin 3 (Thermo Shandon Inc., Pittsburgh, http://www.thermo.com) and immunostained as described [19]. Cultured cells were fixed on 8-well Lab-Tek Chamber Slides (Nunc, Rochester, NY, http://www.nuncbrand.com) and stained as described [19, 21] with mouse anti-Pax7 (1:100; clone: Pax7; Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), mouse anti-MyoD (1:200; clone: 5.8A; NeoMarkers; Lab Vision, Fremont, CA, http://www.labvision.com), mouse anti-myogenin (1:100; clone: F5D; Developmental Studies Hybridoma Bank), rabbit anti-Ki67 (1:2; Ylem, Rome), or rabbit anti-p57 antibodies (1:50; GeneTex, San Antonio, http://www.genetex.com) at 4°C overnight and then reacted with secondary antibodies conjugated with Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Images were photographed using a phase-contrast and fluorescence microscope IX70 (Olympus, Tokyo, http://www.olympus-global.com) equipped with a Quantix air-cooled CCD camera (Photometrics, Kew, VIC, Australia, http://www.photometrix.com.au) and IP Lab software (Scanalytics, Rockville, MD, http://www.scanalytics.com).

Immunohistochemistry

Immunostaining of muscle cryosections was performed as previously described [21] using rat anti-laminin α2 (1:200; clone 4H8-2; Alexis Biochemical, Lausen, Switzerland, http://www.axxora.com), rabbit anti-M-cadherin [21], rabbit anti-human CTR (1:200; Serotec Ltd., Oxford, U.K., http://www.serotec.com), goat anti-Notch 3 (1:100; R&D Systems Inc., Minneapolis, http://www.rndsystems.com), or mouse anti-Pax7. Rabbit anti-mouse HeyL polyclonal antibody was produced in our laboratory. In brief, the DNA fragment corresponding to amino acids 220–287 of mouse HeyL (GenBank: NM_013905) was fused to glutathione *S*-transferase in the pGEX-1 Lambda T vector (GE Healthcare, Uppsala, Sweden, http://www.gehealthcare.com). The purified fusion protein was used to immunize New Zealand White rabbits. The obtained serum was affinity-purified. For Pax7 staining, an M.O.M. kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) was used to block endogenous mouse IgG. For CTR staining, horseradish peroxidase-conjugated anti-rabbit IgG donkey secondary antibody (1:100; GE Healthcare) and Alexa 568-conjugated Tyramid (Molecular Probes) were used to amplify the signal. Nuclei were counterstained with TOTO-3 (1:5,000; Molecular Probes) or DAPI. The images were recorded using a confocal laser scanning microscope system TCSSP (Leica, Heerbrugg, Switzerland, http://www.leica.com) or Axiophot microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Cell Cycle Analysis

Muscle-derived mononucleated cells or cultured SM/C-2.6 positive cells were suspended at 10^6 cells per milliliter in DMEM (Invitrogen) containing 2% FBS (Trace Biosciences), 10 mM Hepes, and 10 μM Hoechst 33342 (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and incubated for 45 minutes at 37°C. An additional incubation was performed in the presence of 10 μg/ml Pyronin Y (Sigma-Aldrich) for 45 minutes at 37°C. Cells were then washed with PBS containing 2% FCS. Muscle-derived mononucleated cells were stained with SM/C-2.6 antibody and analyzed by FACSVantage SE flow cytometer.

Cell Proliferation Assay

After cell sorting, quiescent satellite cells were plated on 96-well culture plates at a density of 3,000–8,000 in the absence or presence of elcatonin (0.01–0.1 U/ml) (Asahi Kasei Pharma Corporation, Tokyo, http://www.asahi-kasei.co.jp/asahi/en) and cultured for 1–2 days. Then 5-bromo-2′-deoxyuridine (BrdU) (10 μM) was added to the culture. To examine the effects of elcatonin on activated satellite cells, satellite cells were cultured for 3 days and then elcatonin was added to the culture 24 hours before addition of BrdU. Twenty-four hours later, BrdU uptake was quantified by cell proliferation enzyme-linked immunosorbent assay, BrdU Kit (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com), and lumi-Image F1 (Roche). In Figure 6B, cells were exposed to elcatonin for 30 minutes and washed twice with PBS and then plated on culture dishes.