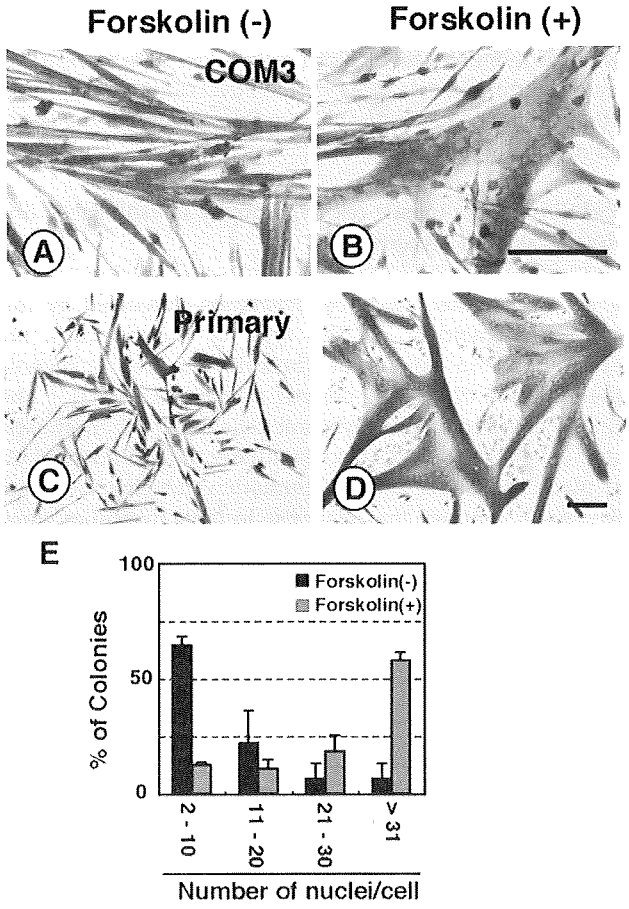

Fig. 7 – Forskolin induces muscle cell hypertrophy in established and primary cultured mouse myogenic cells. COM3, a subline of C2C12 (A and B) and primary cultured mouse myogenic cells (C and D) were cultured in pmDM with (B and D) or without (A and C) stimulation of forskolin (24 μM). MyHC was immunostained with a horseradish peroxidase. Nuclei were counterstained with Mayer's hematoxylin. Images in A–D were obtained by bright field microscopy. (E) Colonies of primary cultured mouse myogenic cells were classified by the size of largest myotube within each colony. Forty-five colonies from three independent dishes were examined in unstimulated (Forskolin (–)) and forskolin-stimulated (Forskolin (+)) cultures, respectively. Histogram represents percentages of colonies that contain the largest myotube with the indicated number of nuclei in the total number of colonies. Averages and standard deviations of three independent cultures are shown.

cultured myogenic cells produced small myotubes during 4 days of differentiation culture (Figs. 7C and E). Forskolin induced myosheet formation in 60.0±6.7% of myogenic cell colonies (Figs. 7D and E). Therefore, the results indicate that the elevation of intracellular cAMP levels by forskolin induced muscle cell hypertrophy in both an established myogenic cell line and primary cultured myogenic cell clones.

Localized PKA activity is also required for cell fusion of mononucleated myogenic progenitor cells

To examine whether localized PKA also plays a pivotal role in fusion of mononucleated myogenic progenitor cells, Ric10 cells were treated with H89 alone. Differentiating mononucleated Ric10 cells were cultured in pmDM for 18 h to avoid the cell detachment caused by the prolonged exposure to H89, and then they were treated with H89 for a further 18 h. H89 markedly inhibited cell fusion of Ric10 cells (69.5±5.6% in control cultures versus 28.5±6.6% in H89-treated cultures) (Fig. 8A, left panel) without significant effects on the

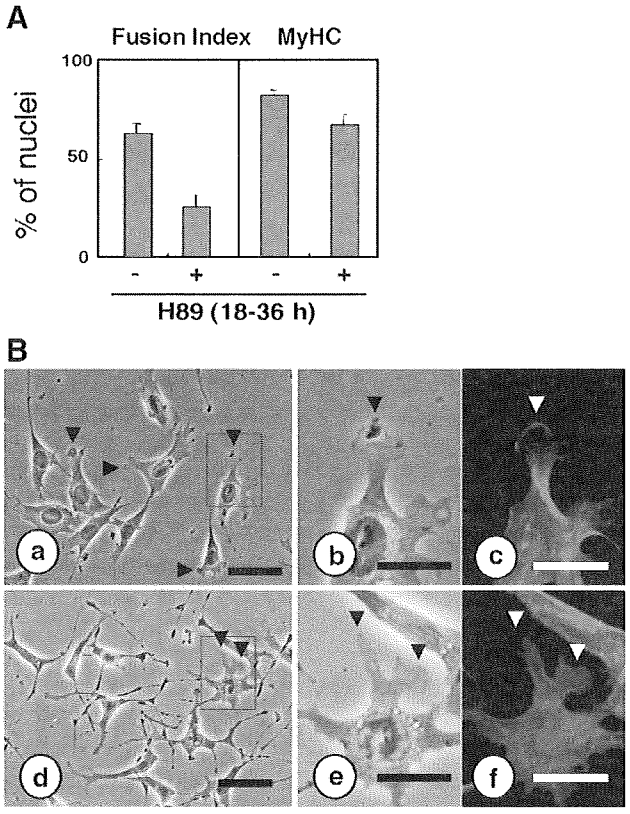

Fig. 8 – Localized PKA activity is required for mononucleated myoblast fusion. (A) Ric10 cells were cultured for 18 h in pmDM. Then the cells were cultured for further 18 h in pmDM supplemented with 0.1% DMSO (–) or 10 μM H89 (+). Fusion index and differentiation potential (MyHC) were analyzed as described in Materials and methods. (B) Ric10 cells were cultured in pmDM supplemented with 0.1% DMSO (a–c) or 10 μM H89 (d–f) for 6 h. Lamellipodium formation (arrowheads in a, b, and c) was severely inhibited by H89 (d). Accumulation of F-actin (b and c) on the leading edge of lamellipodia was not observed in lamellipodia that were formed in H89-treated cells (arrowheads in d, e, and f). Squares in (a) and (d) represent the areas that were magnified in (b) and (c), and (e) and (f), respectively. Images in (a), (b), (d) and (e) were obtained by phase-contrast microscopy, and those in (c) and (f) were obtained by epifluorescence microscopy. Scale bars: 50 μm in a and d; 25 μm in b, c, e, and f.

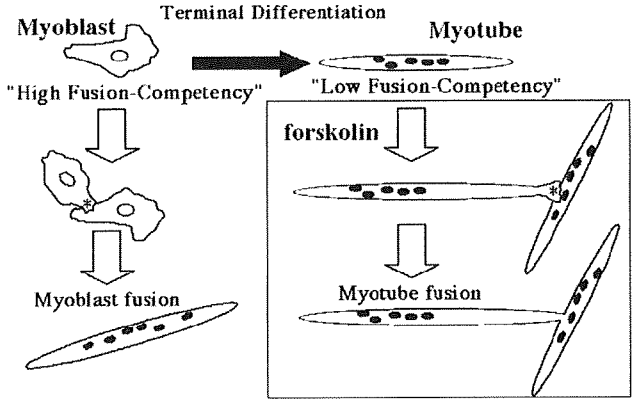

Fig. 9 – Role of localized PKA on myogenic cell fusion. Differentiating myoblasts have the capacity of cell fusion whereas myotubes tend to lose it during terminal muscle differentiation. Myoblast fusion requires the PKA activity localized in lamellipodia. Ability of cell fusion is induced by forskolin through elevating activity of PKA localized in lamellipodia of myotubes. Asterisks represent PKA-localized lamellipodia. A square indicates a process of forskolin-induced myotube–myotube fusion.

expression of MyHC (Fig. 8A, right panel) and myogenin (data not shown).

To determine whether the PKA pathway is involved in lamellipodium formation by mononucleated progenitor cells, Ric10 cells were cultured for 6 h in pmDM supplemented with DMSO (vehicle) or pmDM supplemented with H89. Ric10 cells frequently protruded lamellipodia in control cultures (Figs. 8Ba, b, and c). In contrast, H89 prevented lamellipodium formation, resulting in marked morphological changes in Ric10 cells (Fig. 8Bd). F-actin was accumulated in the leading edge of lamellipodia in mononucleated progenitor cells (Figs. 8Bb and c), as shown in forskolin-stimulated myotubes (Fig. 4B). However, F-actin was not accumulated on the leading edge of lamellipodia in H89-treated Ric10 cells even if they were formed (Figs. 8Be and f). These data suggest that localized PKA activity is required for the lamellipodium formation that is prerequisite for cell fusion of both mononucleated myoblasts and small myotubes (Fig. 9).

Discussion

In the present study, we describe the cell fusion of terminally differentiated myotubes, which has not been shown previously either in vivo or in vitro. Multinucleated myotube fusion results in large, branched myotubes, which we designated "myosheet". Time-lapse recording strongly suggests that branched myotubes are formed by cell fusion of a myotube with another myotube but not with a myoblast. Branched myotubes are usually found in cultured myogenic cells under differentiation-inducing conditions. In addition, branched myofibers are rare in healthy muscle but can be found in regenerated muscle. These observations indicate that terminally differentiated multinucleated myotubes still have the capacity to fuse with each other under appropriate conditions both in vitro and in vivo. The myotube–myotube fusion shown here was not an unusual event and did not depend on a specific cell line or the culture conditions used in our experiments.

We show here that the cAMP–PKA pathway localized in lamellipodia plays a pivotal role in myotube fusion, which is a multistep process. The enhancement of lamellipodium formation by localized PKA activity demonstrates that PKA regulates the early steps of myotube fusion, such as myotube–myotube contact and recognition. Furthermore, the localized PKA might also be involved in the specification of fusion-competent areas of the plasma membrane because only the leading edge of lamellipodia seems to acquire fusion competency.

The role of cAMP in myogenesis has not been established because inconsistent results were reported in previous papers. Membrane-permeable cAMP analogs or compounds that stimulate adenylate cyclase inhibited the expression of muscle-specific genes in the cell line BC3H1 [18,19]. BC3H1 cells are non-fusing cells derived from a mouse intracranial tumor [20], and their expression of muscle-specific genes is rapidly reversible by altering the level of serum or growth factors in the culture medium [21]. BC3H1 cells have been used as a unique model system to reveal the mechanisms by which the expression of muscle-specific genes is regulated. However, they are not true myogenic cells that have the ability to differentiate irreversibly into myofibers. Therefore, we should note that the effects of cAMP in BC3H1 cells are not always the same as in true myogenic cells derived from muscle satellite cells.

Dibutyryl cAMP inhibits the expression of the muscle-specific transcription factor myogenin [22] and terminal muscle differentiation in the mouse myoblastic cell line C2C12 and rat myoblastic cell line L6 [23]. However, the effective concentration of dbcAMP was extremely high (1–3 mM) in those studies. Thus, these results might represent a spurious effect of dbcAMP rather than a true effect of intracellular cAMP because butyrate inhibits myogenesis by interfering with the transcriptional activity of MyoD and myogenin [24]. Cyclic AMP also inhibits the myogenesis induced by vasopressin or insulin-like growth factor I (IGF-I) in L6-C5, a rat myoblastic cell clone selected for its ability to undergo myogenesis when stimulated by these peptides [25–27]. Thus, the inhibition seems to be a specific event in the vasopressin- or IGF-I-induced myogenesis of certain myogenic cell lines. In contrast, the present study demonstrates that intracellular cAMP does not inhibit the expression of muscle-specific genes and cell fusion in both established and primary cultured mouse myogenic cells derived from muscle satellite cells. Similar results have been shown in C2C12 cells [28] and primary cultured chicken myoblasts [29]. Therefore, we should note that an appropriate dose of a cAMP-elevating reagent does not inhibit the expression of muscle-specific genes in primary cultured myogenic cells that are derived from muscle satellite cells.

We and others have demonstrated that cAMP enhances myogenic cell fusion [29–31]. Intracellular cAMP levels rise upon the onset of cell fusion and then decline after fusion in myogenic cell culture [30,32,33]. The nuclear cAMP–PKA pathway was previously assumed to be involved in the regulation of transcriptional activity of the MyoD family

[22,23] or the expression of cAMP phosphodiesterase during myogenesis [34]. In addition, the present study suggests that the localized activity of PKA in lamellipodia is an important facet of a cell-to-cell contact/recognition step of muscle cell fusion.

Compared with the enhancement of myotube fusion, the effect of forskolin on myoblast fusion was not so clear. However, H89 or StHt31S inhibited myoblast fusion severely. Therefore, while localized PKA activity is also required for myoblast fusion, intracellular cAMP levels should not limit the PKA activity because cAMP is markedly produced in myoblasts upon the onset of fusion. In contrast, intracellular cAMP levels might limit PKA activity in myotubes because cAMP levels decline after myoblast fusion [30,32,33]. Here we propose that intracellular cAMP modulates the competence for cell fusion in myogenic cells. The present study reveals a new aspect of the cAMP–PKA pathway in terminal skeletal muscle differentiation.

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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.yexcr.2007.10.006.

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Osteogenic properties of human myogenic progenitor cells

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ABSTRACT

Here, we identified human myogenic progenitor cells coexpressing Pax7, a marker of muscle satellite cells and bone-specific alkaline phosphatase, a marker of osteoblasts, in regenerating muscle. To determine whether human myogenic progenitor cells are able to act as osteoprogenitor cells, we cultured both primary and immortalized progenitor cells derived from the healthy muscle of a nondystrophic woman. The undifferentiated myogenic progenitors spontaneously expressed two osteoblast-specific proteins, bone-specific alkaline phosphatase and Runx2, and were able to undergo terminal osteogenic differentiation without exposure to an exogenous inductive agent such as bone morphogenetic proteins. They also expressed the muscle lineage-specific proteins Pax7 and MyoD, and lost their osteogenic characteristics in association with terminal muscle differentiation. Both myoblastic and osteoblastic properties are thus simultaneously expressed in the human myogenic cell lineage prior to commitment to muscle differentiation. In addition, C3 transferase, a specific inhibitor of Rho GTPase, blocked myogenic but not osteogenic differentiation of human myogenic progenitor cells. These data suggest that human myogenic progenitor cells retain the capacity to act as osteoprogenitor cells that form ectopic bone spontaneously, and that Rho signaling is involved in a critical switch between myogenesis and osteogenesis in the human myogenic cell lineage.

1. Introduction

Ectopic ossification in skeletal muscle is characteristic of certain muscle diseases in humans (Kocyigit et al., 2001; Mahboubi et al., 2001). Bone marrow stromal cells are thought to be the primary source of osteoprogenitor cells in the body. Although intravenously transplanted mouse bone marrow cells settle in skeletal muscles (Brazelton et al., 2003; Ferrari

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et al., 1998; LaBarge and Blau, 2002), these cells have not been shown to migrate to muscle during ectopic ossification. Previous studies have suggested that osteoprogenitor cells reside in skeletal muscle, although their source has remained unknown (Bosch et al., 2000; Levy et al., 2001). Recent studies, however, suggest that vascular smooth muscle cells are candidates for osteoprogenitors in human skeletal muscle (Hegyi et al., 2003; Levy et al., 2001).

Muscle satellite cells are skeletal muscle stem cells that are activated to proliferate and then fuse with each other to form myofibers during muscle regeneration. Histopathologic and molecular biological studies indicate that progenitor cells derived from muscle satellite cells differentiate exclusively into myotubes and myofibers in vivo. However, our clonal analysis demonstrated that mouse muscle satellite cells actually preserve the ability to undergo osteogenic terminal differentiation in vitro in a bone morphogenetic protein (BMP) stimulation-dependent manner (Wada et al., 2002). Our previous results also suggest the possibility that failure of the restriction of phenotypic plasticity triggers ectopic ossification in muscle satellite cells (Wada et al., 2002).

A previous study referred to bone marrow stromal cells, which form bone spontaneously, as "determined osteoprogenitor cells" and to osteoprogenitor cells from extraskeletal sources, which require inductive agents to express osteogenic phenotypes, as "inducible osteoprogenitor cells" (Friedenstein and Kuralesova, 1971). In mice, descendants of muscle satellite cell undergo osteogenic differentiation only when exposed to BMPs in vitro (Asakura et al., 2001; Hashimoto et al., 2004; Wada et al., 2002). In the absence of BMPs, these cells preferentially undergo myogenic terminal differentiation in vitro. However, it remains to be determined whether osteogenic differentiation of human myogenic cells depends on BMP stimulation, although our previous study demonstrated that these cells are able to undergo osteogenic terminal differentiation in vitro (Wada et al., 2002). In addition, the involvement of BMPs in ectopic bone formation in skeletal muscle has not been shown in human disorders. Therefore, it is likely that ectopic bone formation in human skeletal muscle can be triggered in a BMP-independent manner, although "determined osteoprogenitor cells" have not been identified in human skeletal muscle.

We here show that muscle progenitor cells spontaneously expressed both a marker of muscle satellite cells and a marker of osteoblasts in regenerating human muscle. We then cultured both primary and immortalized human myogenic progenitor cells to determine whether they can undergo osteogenic differentiation without exogenous BMPs. We found that exogenous BMP stimulation is not necessary to induce ossification in both primary and immortalized human myogenic progenitor cells. Therefore, we propose the hypothesis that human myogenic progenitor cells preserve the ability to act as "determined osteoprogenitor cells" and undergo osteogenic terminal differentiation when myogenic differentiation is impaired in human muscle diseases. In addition, the immortalized human myogenic progenitor cell lines used have a multipotentiality that enables us to analyze the nature of human myogenic progenitor cells in detail, as previously done in mouse and rat myoblastic cell lines. They will also provide a model system for genetic modification and transplantation of human myogenic cells.

2. Results

2.1. Osteogenic properties of a human myogenic cell lineage in vivo

The transcription factor Pax7 is expressed exclusively in muscle satellite cells in mouse skeletal muscle (Seale et al., 2000). To determine whether Pax7 is a specific marker for human muscle satellite cells, we probed cryosections prepared from normal back muscle with antibodies to this protein. Pax7-positive nuclei were detected exclusively between the basement membrane and the sarcolemma, which were recognized by antibodies to laminin and dystrophin, respectively (Fig. 1A), suggesting that Pax7 is indeed specifically expressed in human muscle satellite cells (Reimann et al., 2004). Quiescent muscle satellite cells expressing Pax7 did not express bone-specific alkaline phosphatase (ALP), an early marker of osteogenic differentiation. In contrast, ALP was detected histochemically in the small regenerating myofibers present in humans with skeletal muscle diseases including Duchenne muscular dystrophy (DMD) (Fig. 1B–D) (Nonaka et al., 1981). To determine whether human myogenic progenitor cells express osteogenic markers during muscle regeneration, we examined the expression of ALP in muscle of individuals with DMD. In DMD muscles, muscle regeneration is spontaneously triggered and myogenic progenitor cells are present, although ectopic bone has not been found. Immunofluorescence analysis revealed that the ALP activity in small myofibers of a 5-month-old boy with DMD was attributable to bone-specific ALP located at the plasma membrane (Fig. 1C and D). Probing cryosections with antibodies to Pax7 and bone ALP also revealed several mononucleated cells coexpressing Pax7 and ALP in the regenerating muscle of a 4-month-old boy with DMD (Fig. 1B). One or two double-positive cells were found in each cryosection ($\sim$20 mm^2) of muscles from these two boys with DMD. These results thus indicate that myogenic progenitor cells with both myoblastic and osteoblastic properties are present in regenerating human skeletal muscles in vivo. Pax7 expression suggests that these cells might be descendants of muscle satellite cells. The results suggest that human myogenic progenitors are probable candidates for osteoprogenitor cells.

2.2. Coexpression of myoblast- and osteoblast-specific proteins in primary human myogenic progenitor cells in vitro

We previously isolated a primary human myogenic progenitor cell clone, Hu5, from the healthy subcutaneous muscle of a nondystrophic woman (Fig. 2A). To determine whether human muscle progenitor cells are able to act as osteoprogenitor cells, we characterized Hu5 cells. Expression of Pax7 was detected in the majority of Hu5 cells (more than 80%) in early passages (less than 6 passages), but not during successive passages (Fig. 2B). Further, Hu5 cells differentiated spontaneously into prominent myotubes in vitro on achieving confluence (Fig. 2C). They also underwent terminal osteogenic

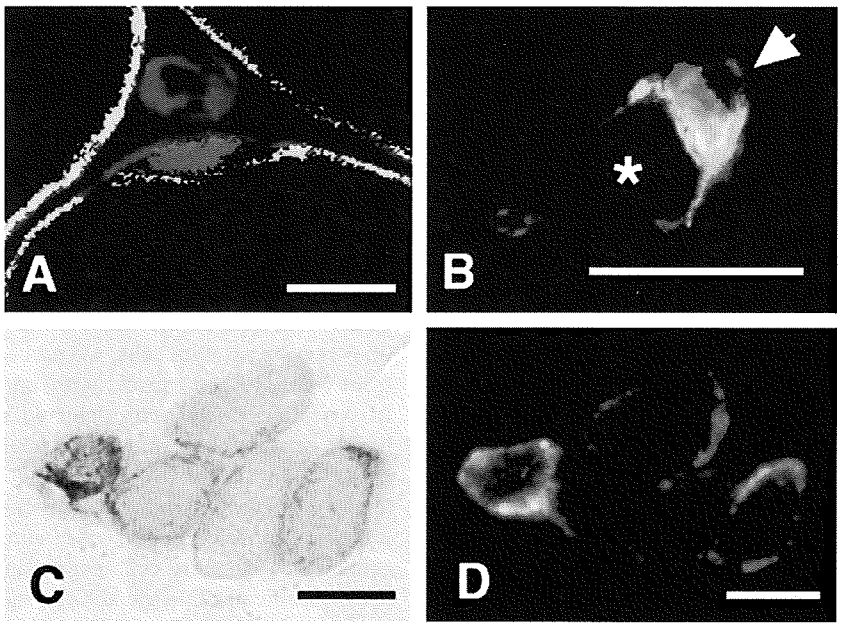

Fig. 1 – Coexpression of myogenic- and osteogenic-specific markers in cells present in skeletal muscle of individuals with DMD. (A) Confocal immunofluorescence analysis of a skeletal muscle section from a nondystrophic 62-year-old woman using antibodies to Pax7, laminin, and dystrophin. A muscle satellite cell expressing Pax7 (red) was detected between the basement membrane containing laminin (blue) and the sarcolemma containing dystrophin (green). (B) Cryosections of the biceps brachii muscle of a 4-month-old boy with DMD were subjected to immunofluorescence analysis with antibodies to Pax7 (red) and to bone-specific ALP (green). The arrow indicates a cell expressing both Pax7 and ALP that was located adjacent to a small, newly regenerated myofiber expressing ALP (asterisk). (C) Cryosections of the biceps brachii muscle of a 5-month-old boy with DMD were subjected to staining of ALP activity with Fast Blue RR. ALP activity was restricted to small, newly regenerated myofibers. (D) Immunofluorescence analysis revealed the presence of bone-specific ALP at the plasma membrane of the same myofibers shown in (C). Scale bars: 10 μm (A) and 20 μm (B–D).

differentiation accompanied by calcification in the presence of both BMP2 and βGP, whereas myogenic differentiation is prevented (Hashimoto et al., 2006). In addition, a phosphate donor, βGP, alone induced calcification in Hu5 cells without BMP2 stimulation (Fig. 2D). Unexpectedly, ALP was detected histochemically in all unstimulated Hu5 cells (Fig. 2E) whereas primary cultured mouse myogenic cells express bone ALP only when stimulated with BMP2 (Wada et al., 2002). The observation is consistent with result that Hu5 cells underwent osteogenic terminal differentiation without BMP2 stimulation. Double staining revealed that more than 80% of undifferentiated Hu5 cells coexpressed Pax7 and bone ALP in the absence of BMP2 (Fig. 2F) in a similar manner to mononucleated myogenic cells in vivo (Fig. 1E). In addition, Hu5 cells also express determination genes, including those for MyoD and Runx2, which are specific for myogenic and osteogenic differentiation, respectively (Table 2). The Hu5 cell, which is a myogenic progenitor cell clone derived from the healthy muscle, thus exhibits both myoblast- and osteoblast-specific properties. The expression of both myogenic- and osteogenic-specific proteins was also demonstrated in six independent clones of myogenic progenitor cells isolated from the subcutaneous muscle of another woman (see Fig. S1 online). We found no clones that retain only myoblast- or osteoblast-specific properties under our culture conditions. The results suggest that human myogenic progenitor cells coexpress myoblast- and osteoblast-specific proteins during in vitro culture.

2.3. *Preservation of dual lineage-specific properties in immortalized human myogenic progenitor cells*

In contrast to primary cultured mouse myogenic cells, the primary myogenic progenitor cell clone Hu5 ceased proliferation and underwent replicative senescence after 10–12 passages. Hu5 cells also lost differentiation potential along with cellular senescence, making it difficult to characterize Hu5 cells undergoing replicative senescence. Therefore, to facilitate analysis of the myogenic and osteogenic properties of Hu5 cells at the molecular level, we used the cells that were immortalized in vitro by introduction of the reverse transcriptase component of human telomerase (hTert) and human papillomavirus (HPV)-16 E7 (Hashimoto et al., 2006).

One of the Hu5-derived clones retaining myogenic differentiation potential, E18, was subjected to further analyses, although similar results were obtained with the other eight independent clones derived from individual Hu5 cells. The muscle lineage markers desmin, nestin, and a master gene for myogenesis, *MyoD*, were expressed in both parent Hu5 and immortalized E18 cells (Table 2).

In addition, Runx2 (Cbfa1), an essential transcription factor for osteogenesis (Komori et al., 1997; Otto et al., 1997),

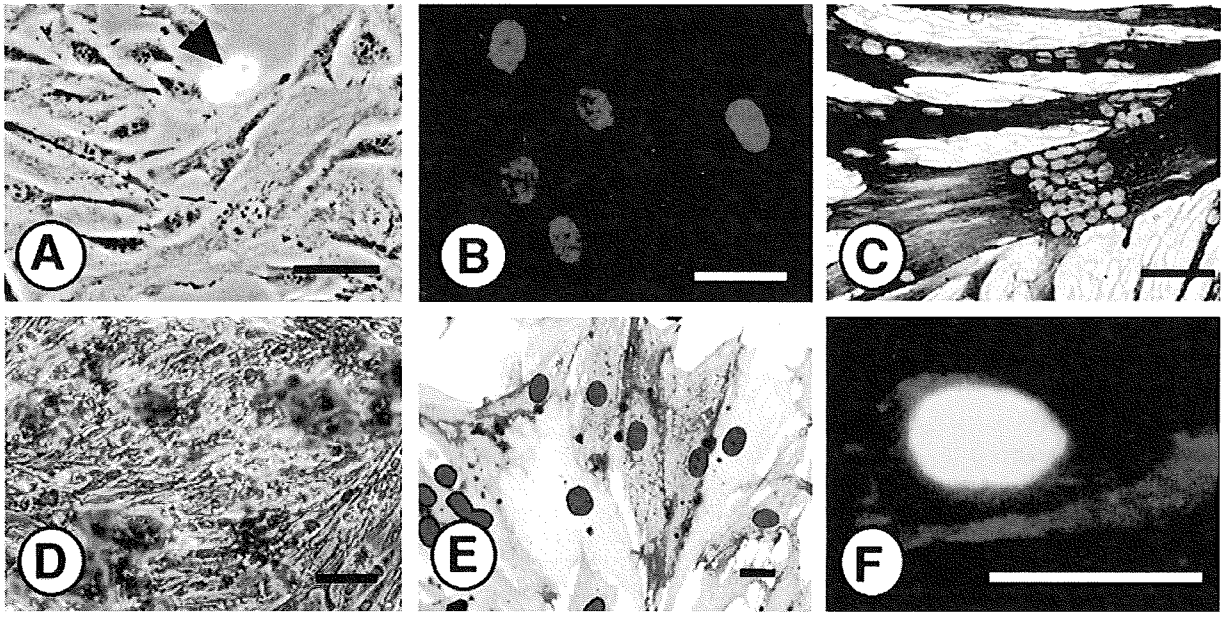

Fig. 2 – Multipotentiality of parental primary human myogenic cells. (A) The primary human myogenic cell clone Hu5 was grown in pmGM and examined by phase-contrast microscopy. The arrowhead indicates dividing cells. (B) Undifferentiated Hu5 cells (passage 6) were subjected to immunofluorescence analysis 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	CTCGCCACTGCCTTTATCTT			
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).

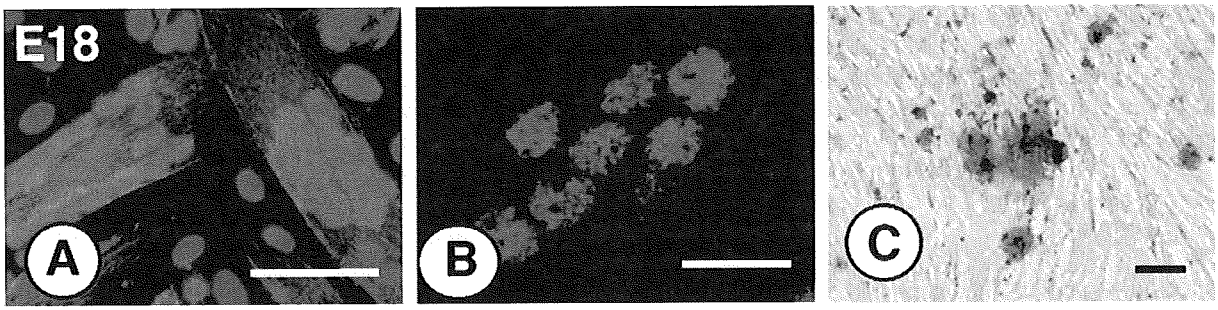

Fig. 3 – Multipotentiality of an immortalized human myogenic progenitor cell clone E18. (A and B) The Hu5-derived clone E18 differentiated into myotubes after culture for 6 days in pmDM. The cells were then subjected to immunofluorescence analysis with antibodies (red) to MHC in (A) or MyoD in (B). Nuclei 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.

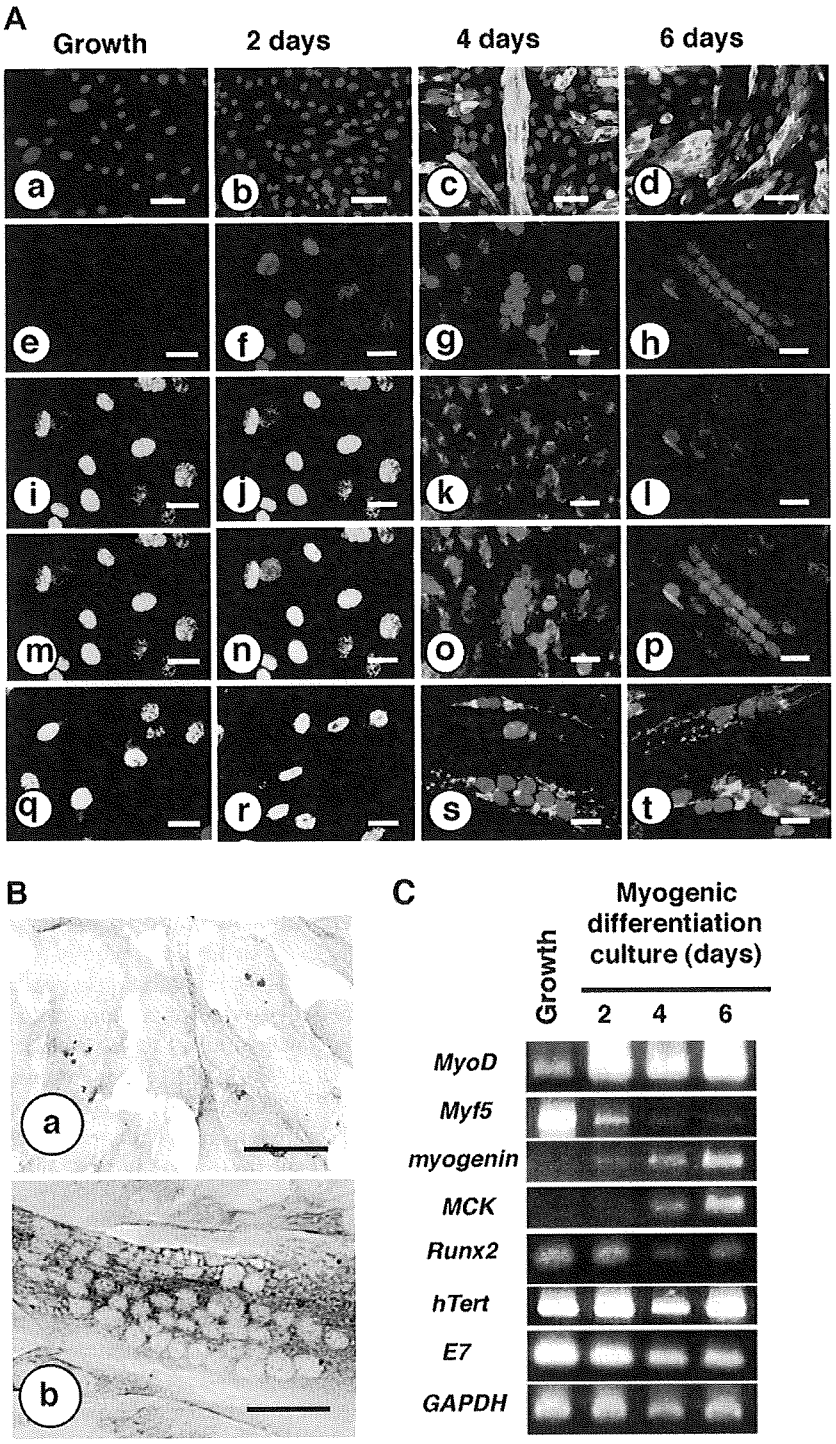

Fig. 4 – Myogenic differentiation of the immortalized human myogenic progenitor cell clone E18. (A) Immortalized Hu5-derived clone E18 cells were cultured under growth conditions or in pmDM for 2, 4, or 6 days (to induce myogenic terminal differentiation), as indicated. Expression of myogenic- or osteogenic-specific 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

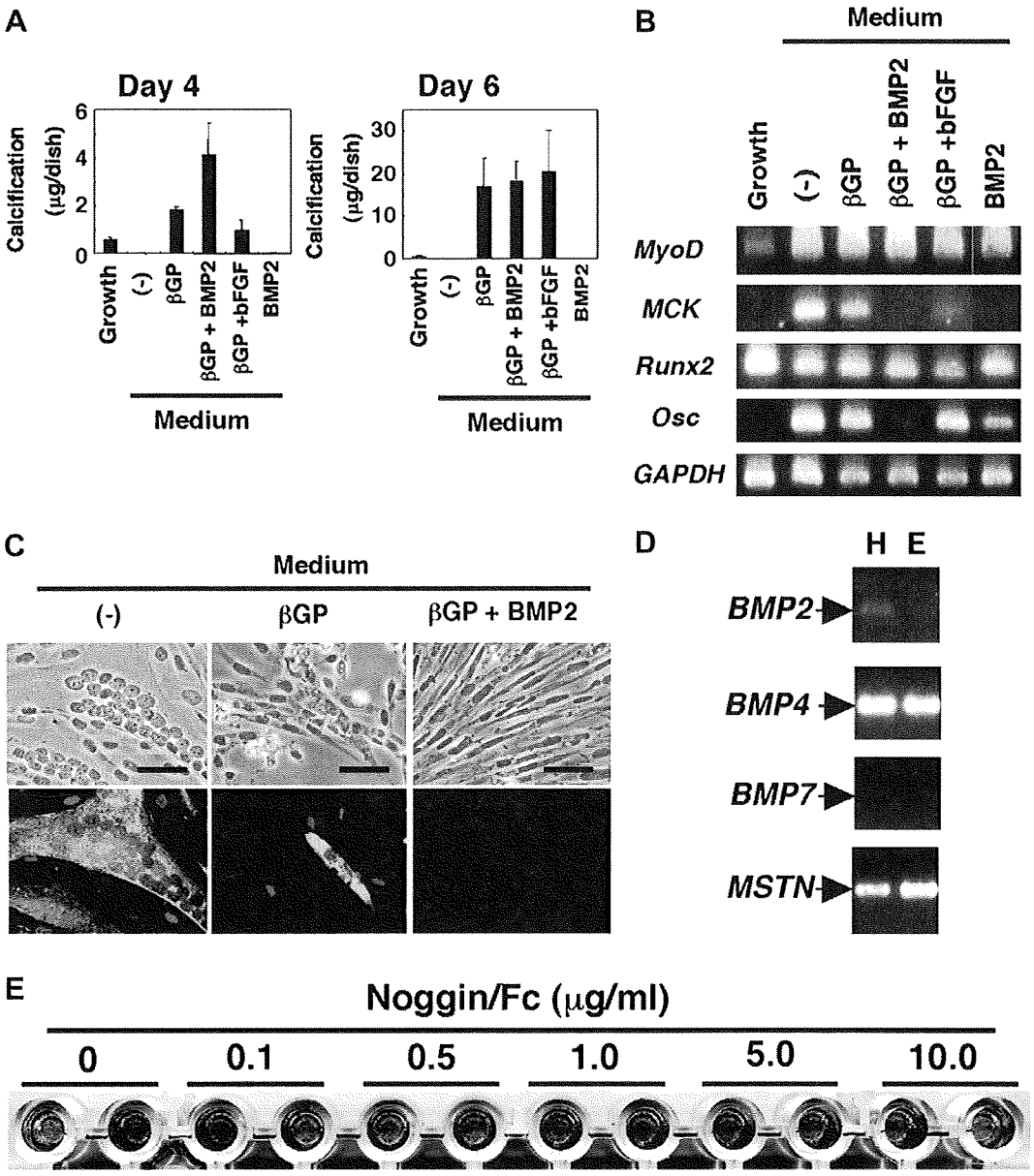

Fig. 5 – Osteogenic terminal differentiation of an immortalized human myogenic progenitor cell clone E18. (A) E18 cells were cultured for 4 or 6 days in growth medium or serum-containing medium supplemented (or not) with either βGP (10 mM) alone, βGP plus BMP2 (500 ng/ml), βGP plus bFGF (10 ng/ml), or 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,

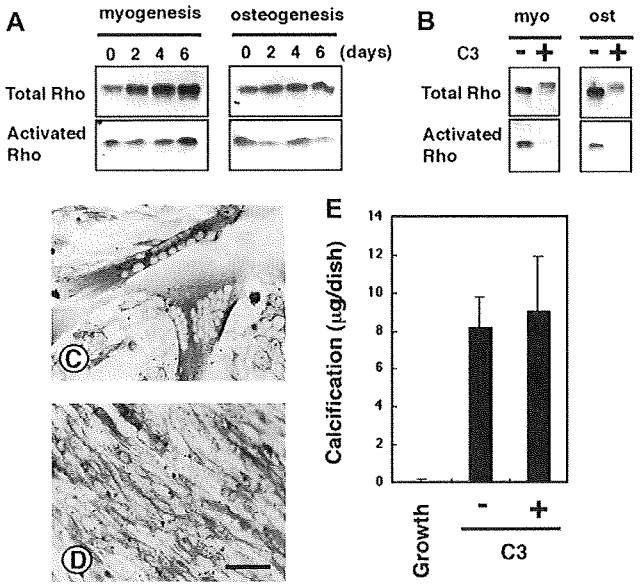

Fig. 6 – Involvement of Rho signaling in the regulation of myogenensis but not osteogenesis of human myogenic progenitor cell E18. (A) E18 cells were cultured for up to 6 days in pmDM (left panels) or medium containing 10% FBS and 10 mM βGP (osteogenesis-inducing medium) (right panels). Cellular proteins 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 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.

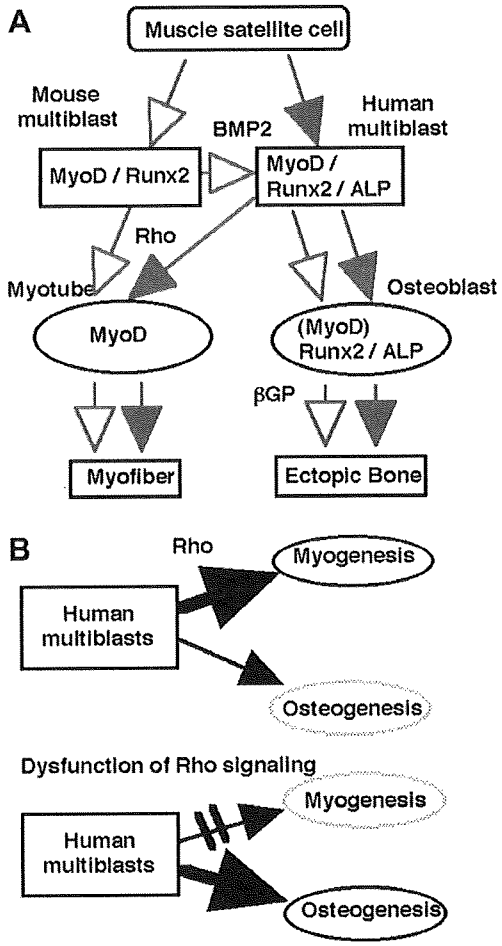

Fig. 7 – Differentiation pathways of human myogenic progenitor cells (multiblasts). (A) Similar but distinct myogenic (red arrows) and osteogenic (blue arrows) differentiation pathways of human (filled arrows) and mouse (open arrows) myogenic cells. Human myogenic progenitor cells (multiblasts) express 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).

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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Cardiovascular, Pulmonary and Renal Pathology

Lipid Droplet-Associated Proteins Protect Renal Tubular Cells from Fatty Acid-Induced Apoptosis

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Proteinuria is a major cause of tubulointerstitial kidney damage, and free fatty acids bound to albumin are thought to play an important role in its pathogenesis. However, the mechanism whereby proteinuria causes tubulointerstitial damage to the kidney is unclear. Using primary human renal proximal tubular cells, we observed that albumin replete with fatty acids (rBSA) and defatted albumin (dBSA) complexed with linoleic acid (LA) induced significantly more apoptosis than did defatted albumin alone. Oxidative stress was partially involved in apoptotic induction by LA/dBSA but not by rBSA. Administration of fatty acid-bound BSA increased the number of lipid droplets (LDs) and the LD-associated proteins, adipocyte differentiation-related protein and TIP47. LDs are organelles that store esterified fatty acids, and the LD-associated proteins are presumed to facilitate LD formation. Knockdown of adipocyte differentiation-related protein or TIP47 by RNA interference enhanced induction of apoptosis by both rBSA and LA/dBSA. Apoptotic induction was observed similarly when either rBSA or LA/dBSA was applied to only the apical surfaces of polarized LLC-PK1 cells. The present results suggest that LDs and LD-associated proteins have protective effects against apoptosis induced by fatty acid-bound albumin by sequestering free fatty acids. Therapeutic manipulation of these LD-associated proteins could aid in the amelioration of nephritic diseases. ***(Am J Pathol 2008, 173:1286–1294; DOI: 10.2353/ajpath.2008.080137)***

Functional impairment of the kidney during glomerulonephritis correlates better with the degree of tubulointerstitial atrophy than glomerular alteration.[1] Many studies have shown that proteinuria is a major cause of damage in the renal tubules and interstitium.[2–4] Albumin is the predominant protein in the urine of nephritic patients. *In vitro* studies have shown that albumin affects intracellular signaling pathways in proximal tubular epithelial cells,[5,6] induces them to generate various chemoattractants and extracellular matrices,[7,8] and changes the balance between cell proliferation and cell death.[9,10]

The mechanism whereby proteinuria causes tubulointerstitial damage is unclear. Although many studies have concluded that albumin itself is important for the development of the pathological changes, other studies have inferred that free fatty acids (FFAs) bound to albumin play critical roles.[9–11] In mice, FFA-bound albumin caused more severe tubulointerstitial damage, including cortical apoptosis, than albumin depleted of FFA.[12,13] Also, in cultured proximal tubular cells (PTCs), FFA-bound albumin induced apoptosis by activating peroxisome proliferator activated receptor (PPAR)-γ.[11] These results suggest that FFAs are involved in the pathogenesis of tubulointerstitial damage.

FFAs are potentially harmful to cellular functions, but cells readily esterify them to form triacylglycerol and cholesterol esters. The esters are then stored in lipid droplets (LDs), consisting of a globular mass of lipid esters surrounded by a phospholipid monolayer.[14,15] In fact, administration of FFAs increases the number and size of LDs in many kinds of cells in culture, including PTCs.[16] Recent studies revealed that a number of proteins are associated with LDs, and that their expression is increased on FFA loading. Furthermore, engagement of LDs in intracellular lipid trafficking, lipid metabolism, sig-

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nal transduction, and other cellular functions has been suggested.[15,17–19]

In view of the novel functions attributed to LDs and LD-associated proteins, we aimed to study whether their manipulation could modify the effect of FFAs on PTCs. For this purpose, we used primary human PTCs and two PTC lines and confirmed that albumin replete with FFAs as well as defatted albumin complexed with long-chain FFAs induced a higher degree of apoptosis than defatted albumin alone. Using this experimental system, we found that reduction of LD-associated proteins, ie, adipose differentiation-related protein (ADRP) and TIP47, by RNA interference increased apoptosis induced by FFA-bound albumins. The result suggests that manipulation of LD-associated proteins could be a potential target of therapeutic intervention for nephrotic diseases.

Materials and Methods

Reagents

Rabbit anti-human TIP47 antibody was raised as described.[20] Mouse anti-ADRP antibody (Progen; Richlands BC, Queensland, Australia), and secondary antibodies (Jackson ImmunoResearch, West Grove, PA; Invitrogen, Carlsbad, CA; Pierce Chemical, Rockford, IL) were obtained commercially. Bovine serum albumin replete with FFAs (rBSA, catalog no. A9306; Sigma Chemical, St. Louis, MO; and endotoxin <0.1 ng/mg, catalog no. 013-15104; Wako Pure Chemical, Osaka, Japan), and essentially FFA-free BSA (dBSA, catalog no. 017-15141; Wako) were used. Oleic acids (OA, Sigma), linoleic acid (LA, Sigma), and docosahexaenoic acid (DHA, Sigma) were vigorously mixed with dBSA in phosphate-buffered saline at a molar ratio of 6:1,[21] filter-sterilized, and added to culture media at the final fatty acid concentration of 400 μmol/L (OA, LA) or 100 μmol/L (DHA). This concentration of FFA was used because the molar ratio of FFA to albumin could reach up to 8.59,[22] and the albumin concentration in the proximal tubular lumen could be as high as 2.9 mg/ml, or 43 μmol/L.[23] In the current experimental condition, the BSA concentration was 4.4 mg/ml when the fatty acid was used at 400 μmol/L. Vitamin E and desferrioxamine were purchased from Sigma. The FFA content of the rBSA preparation was analyzed by gas chromatography by Mitsubishi Kagaku BCL Inc. (Tokyo, Japan).

Cell Culture

Human primary PTCs (RPTECs; Cambrex, Walkersville, MD), showing characteristics of PTCs *in vivo*, were cultivated in renal epithelial cell basal medium (REBM, Cambrex) supplemented with REGM SingleQuots (0.5 μl/ml hydrocortisone, 10 pg/ml hEGF, 0.5 μg/ml epinephrine, 6.5 pg/ml triiodothyronine, 10 μg/ml transferrin, 5 μg/ml insulin, 50 μg/ml gentamicin, 50 pg/ml amphotericin B, and 0.5% fetal bovine serum). HK-2 cells, an immortalized human PTC line, were obtained from American Type Culture Collection (Rockville, MD) and were cultured in K1 medium. The K1 medium contained Ham's F-12/Dulbecco's modified Eagle's medium (1:1, Sigma), 12.5 mmol/L HEPES, 10% heat-inactivated fetal bovine serum (JRH Biosciences Inc., Lenexa, KS), and select hormones as described.[24] LLC-PK1 cells, a pig PTC line, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan), and grown in 199 medium (AppiChem, Darmstadt, Germany) supplemented with 10% fetal bovine serum. LLC-PK1 cells were grown in Transwell chambers (Corning, Corning, NY), and used for experiments 3 days after reaching confluence. All of the cells were kept at 37°C in 5% CO_2/95% air.

Immunofluorescence Microscopy and Data Analysis

Cells were fixed with 3% formaldehyde and 0.05% glutaraldehyde for 30 minutes, permeabilized with 0.01% digitonin for 30 minutes, and treated with 3% BSA before immunolabeling for ADRP and TIP47. LDs were stained with BODIPY493/503 (Invitrogen). Images were obtained with an Axiovert fluorescence microscope equipped with Apotome (Carl Zeiss, Oberkochen, Germany) and analyzed with Image J software (National Institutes of Health, Bethesda, MD) as described.[18] Contrast and brightness of micrographs were adjusted by Adobe Photoshop 7.0 for data presentation.

RNA Interference and cDNA Transfection

For RNA interference (RNAi), siGENOME duplexes (Dharmacon Inc., Lafayette, CO) were used to knock down the expression of TIP47 and ADRP. A control RNA duplex, siControl nontargeting siRNA, was also obtained from Dharmacon. RNAi was conducted by electroporation using the Gene Pulser 2 system (Bio-Rad, Hercules, CA).[25] RPTECs (1.5×10^6) were electroporated (600 V, exponential decay 300 ms, 1 pulse) with 10 μg of siRNA in 400 μl of siPORT siRNA electroporation buffer (Ambion, Austin, TX) using 2-mm electroporation cuvettes. Cells were analyzed 3 days after RNAi.

Human ADRP and TIP47 cDNAs were cloned by polymerase chain reaction and inserted into the pcDNA3.1 vector (Invitrogen). The plasmid vectors were introduced into the cells by Lipofectamine2000 (Invitrogen), and the cells were analyzed 3 days later. The transfection protocol was optimized by using pEGFP-C1 vector (Clontech, Mountain View, CA), and the efficiency was estimated as ~60%.

Western Blotting

Total cell lysates were prepared in a sodium dodecyl sulfate-sample buffer, an equal amount of protein (30 μg) was electrophoresed in 15% acrylamide gels, and the protein was transferred to nitrocellulose membranes. The blots were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The reaction was detected with the SuperSignal West dura extended duration substrate (Pierce).

Apoptosis and Cell Viability Assays and Thiobarbituric Acid Reactive Substance (TBARS) Assay

DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) using an ApoAlert DNA fragmentation kit (Clontech). Nuclei were counterstained with 4,6-diamidino-2-phenylindole. More than five areas, each containing 30 to 170 cells, were randomly selected for each sample under identical microscopic settings. The ratio of positive cells was counted and given in percentages.

Activated caspases were detected by the CaspACE FITC-VAD-FMK *in situ* marker (Promega, Madison, WI) that was added directly to living cells and incubated for 20 minutes. The cells were then fixed and observed with a microscope. For flow cytometric analysis, cells were detached from the substrate by a trypsin-ethylenediaminetetraacetic acid solution after fixation and all of the cells were subjected to analysis in a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ). Fluorescence was excited at 488 nm and measured at 530 nm. For each experiment, control samples obtained from cells cultured in the complete medium containing 10% FCS were analyzed along with the experimental groups, and a threshold was set so that 1% of the control cells were classified as apoptotic. Cell viability was determined with the Cell Counting Kit 8 (Wako). The amount of TBARS including lipid hydroperoxides, which increase as a result of oxidative stress, was measured by the TBARS assay kit (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

Where appropriate, experimental data were analyzed by unpaired *t*-tests, assuming unequal variance. A P value of <0.05 was considered statistically significant.

Results

FFA-Bound Albumin Induces Apoptosis in Cultured PTCs

We first tested whether FFAs induce apoptosis of cultured human PTCs (RPTECs). Because albumin itself could induce apoptosis, we compared the effect of FFA-free BSA versus the same concentration of FFA-bound BSA using two different combinations: first, dBSA that was depleted of FFAs was compared with the same concentration (4.4 mg/ml) of dBSA prebound with linoleic acid (18:2) or oleic acid (18:1) (LA/dBSA, OA/BSA; FFA concentration, 400 μmol/L); second, dBSA and rBSA at 30 mg/ml were compared. Gas chromatographic analysis revealed that the medium with 30 mg/ml of rBSA contained ~380 μmol/L FFAs, among which 56.6% (w/w) were saturated, ie, stearate, palmitate, and myristate, and the rest were unsaturated, ie, linoleate and oleate. An albumin concentration of 30 mg/ml is higher than that is found in the normal proximal tubule lumen *in vivo*, but was necessary to reproduce the FFA concentration seen in the nephrotic renal tubule.[11] The ratio of apoptotic cells was examined by TUNEL staining (Figure 1, A and B) and fluorescence microscopic analysis of the caspase marker (FITC-VAD-FMK) (Figure 1, C and D). Both assays showed that LA/dBSA and rBSA induced a significant increase in apoptosis compared to dBSA at the same

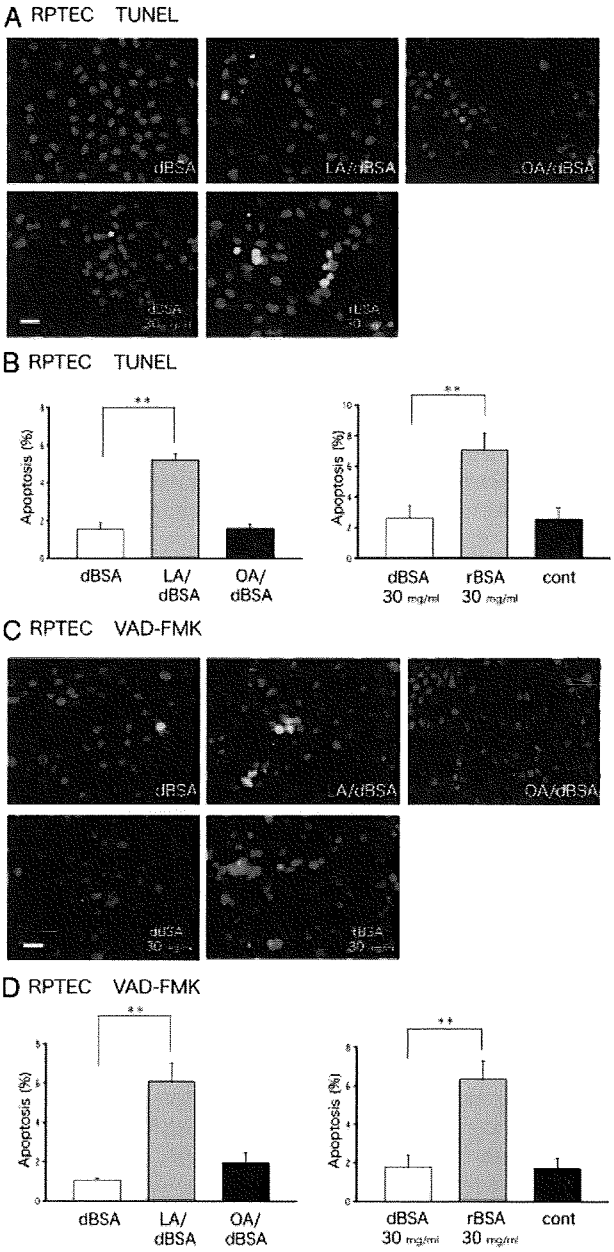

Figure 1. Fatty acids bound to BSA increase apoptosis in human PTCs (RPTECs). Human PTCs, cultured in medium containing 0.5% FCS, were treated with five different combinations of BSA and FFAs for 24 hours: i) dBSA (4.4 mg/ml); ii) 400 μmol/L LA/dBSA (BSA concentration, 4.4 mg/ml); iii) 400 μmol/L OA/BSA (BSA concentration, 4.4 mg/ml); iv) dBSA (30 mg/ml); and v) rBSA (30 mg/ml; FFA concentration, 380 μmol/L). **A** and **B:** TUNEL assay; **C** and **D:** FITC-VAD-FMK assay. **A** and **C:** Photographs show representative areas. **B** and **D:** The number of positive cells were counted in eight random areas in three independent experiments and averaged (mean ± SD, $^{**}P < 0.01$). In controls, no protein was added to the culture medium. Both assays showed that LA/dBSA and rBSA significantly increased apoptosis compared to dBSA at the same concentration. Scale bars = 10 μm.