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Generation of different fates from multipotent muscle stem cells

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SUMMARY

Although neuronal and mesenchymal stem cells exhibit multipotentiality, this property has not previously been demonstrated for muscle stem cells. We now show that muscle satellite cells of adult mice are able to differentiate into osteoblasts, adipocytes and myotubes. Undifferentiated muscle progenitor cells derived from a single satellite cell co-expressed multiple determination genes including those for MyoD and Runx2, which are specific for myogenic and osteogenic differentiation, respectively. Determination genes not relevant to the induced differentiation pathway were specifically downregulated in these cells. Similar multipotent progenitor cells were isolated from adult human muscle. Based on these observations, we propose a 'stock options' model for the generation of different fates from multipotent stem cells.

Key words: Stem cells, Mouse, Fate generation

INTRODUCTION

Tissue-specific stem cells (TS cells) have been detected in several tissues of adult mammals and are thought to be responsible for tissue regeneration after injury. Studies of neuronal stem cells and mesenchymal stem cells have suggested that multipotentiality is a common feature of mammalian TS cells (Bjornson et al., 1999; Galli et al., 2000; McKay, 1997; Prockop, 1997).

Skeletal muscle stem cells, also known as muscle satellite cells, are located adjacent to the plasma membrane of myofibers beneath the basement membrane. During muscle regeneration, satellite cells proliferate and then fuse together to form myotubes. Histopathological analysis has shown that muscle satellite cells differentiate into myotubes and myofibers exclusively (Saito et al., 1994), and there has been no evidence that these cells are able to differentiate into nonmuscle cells in vivo. However, both primary cultured mouse myoblasts and the immortalized mouse myoblastic cell line C2C12 differentiate into osteoblasts and adipocytes as well as myotubes under appropriate culture conditions (Chalaux et al., 1998; Fujii et al., 1999; Katagiri et al., 1994; Nishimura et al., 1998; Teboul et al., 1995; Yamamoto et al., 1997). Although these observations suggest that muscle satellite cells preserve multipotentiality, the source of the muscle-derived cells (so-called myoblasts) analyzed in these studies is unknown. Recently, multipotentiality of muscle satellite cells is also suggested by the analysis of multiclonal myoblasts derived from multiple satellite cells (Asakura et al., 2001). However, it has been unclear whether different fates are generated from a single satellite cell.

Specific cells within skeletal muscle exhibit apparent stem cell-like plasticity. Side population (SP) cells separated from dissociated muscle cells by fluorescence-activated cell sorting differentiate into muscle and hematopoietic cells (Gussoni et al., 1999; Jackson et al., 1999). However, SP cells constitute a distinct population from muscle satellite cells (Seale et al., 2000). Stem-like cells have also been isolated from primary cultured skeletal muscle cells and shown to exhibit multipotentiality (Lee et al., 2000a; Qu et al., 1998; Torrente et al., 2001). The origin of these cells is unknown, however, and their relation to muscle satellite cells is unclear.

Histopathological and molecular biological studies (Bischoff, 1986; Garry et al., 2000; Megeney et al., 1996; Saito et al., 1994; Seale et al., 2000) indicate that muscle satellite cells are largely responsible for postnatal muscle growth, regeneration and repair, even if SP cells or other stem-like muscle-derived cells also contribute to muscle regeneration. Clarification of the mechanisms of muscle regeneration will therefore require determination of the differentiation potential of muscle satellite cells and its regulation.

We have now characterized a clone of unmanipulated myogenic cells derived from a single mouse muscle satellite cell and revealed its multipotentiality in vitro. Furthermore, multipotent progenitor cells derived from muscle satellite cells

were shown to co-express multiple determination genes under growth conditions. On the basis of these observations, we propose a 'stock options' model for the lineage commitment of muscle satellite cells.

MATERIALS AND METHODS

Cell culture

Single fibers were isolated from the gastrocnemius muscles of an 8-week-old female transgenic mouse expressing GFP (Okabe et al., 1997) essentially as described previously (Bischoff, 1986; Rosenblatt et al., 1995), and were cultured for 6 days at 37°C under 10% CO_2 in 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, Cergr-Saint-Christophe, France) and glucose (4.5 mg ml^{-1}). Colonies derived from single satellite cells were isolated and replated on dishes coated with type I collagen (Sumilon, Tokyo, Japan). For low cell density culture, cells were isolated from gastrocnemius muscles of an 8-week-old female ICR mouse essentially as described (Pinset and Montarras, 1998). After culture for 6 days in pmGM, colonies derived from single satellite cells were pooled and cultured.

Normal human abdominal muscle tissue was obtained by biopsy from a 44-year-old woman with informed consent at the Kanagawa Cancer Center Research Institute. Human myogenic cells were isolated from a small piece of muscle according to the low cell density culture method.

For induction of myogenic differentiation, myogenic 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. Myogenic cells were cultured in pmDM supplemented with recombinant human BMP2 (250-500 ng ml^{-1}) (Strathman Biotech, Hamburg, Germany) to induce osteogenic differentiation. ALP activity in cells fixed with 4% paraformaldehyde was detected by incubation of the fixed cells for 20 minutes in a solution containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM $MgCl_2$, 0.01% naphthol AS-MX, and Fast Blue RR (0.5 mg ml^{-1}). To induce adipogenic differentiation, we cultured myogenic cells in DMEM supplemented with 10% FBS and 100 μM γ-linolenic acid (Sigma, St. Louis, MO) for up to 10 days. Formalin-fixed cells were stained with 0.3% oil red O (Sigma) in 60% isopropanol for 30 minutes at room temperature, and were photographed under epifluorescence conditions with a WIG filter. Nuclei of cells were visualized by staining with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) (0.5 μg/ml, Sigma).

For induction of bone matrix formation in vitro, Mouse myogenic cells were cultured in pmDM supplemented with BMP-2 and 10 mM β-glycerophosphate for up to 8 days. Paraformaldehyde-fixed cells were stained with the calcium-staining dye Alizarin Red S (0.01%, Sigma) for 30 minutes.

Immunofluorescence, immunocytochemical and immunoblot analyses

For immunofluorescence or immunocytochemical analysis, paraformaldehyde-fixed cells were incubated for 12 to 36 hours at 4°C with a mouse monoclonal antibody to Runx2 (Zhang et al., 2000), a mouse monoclonal antibody to MyoD (Novocastra, Newcastle, UK), rabbit antibodies to rat myogenin (Hashimoto et al., 1994), a mouse monoclonal antibody to MHC (Bader et al., 1982), a mouse monoclonal antibody to Pax7 (Ericson et al., 1996) (DSHB, Iwoa City, IA), goat antibodies to mouse osteocalcin (Biomedical Tech, Stoughton, MA), rabbit antibodies to nestin (Arimatsu et al., 1999), rabbit antibodies to desmin (Progen, Heiderberg, Germany) or rabbit antibodies to GFP (Medical and Biological Laboratory, Nagoya, Japan) in the presence of 0.1% saponin (Sigma). Biotinylated or Cy3-labeled antibodies to mouse or rabbit immunoglobulin G as well as fluorescein isothiocyanate (FITC)-labeled antibodies to goat immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME) were used as secondary antibodies. The biotinylated antibodies were detected with streptavidin-conjugated horseradish peroxidase or FITC. The antibody to Runx2 was detected by biotinylated antibodies to mouse immunoglobulin G and a TSA Direct kit (New England Nuclear, Boston, MA). Cell nuclei were stained with DAPI.

Immunoblot analysis and corresponding sample preparation were performed as described (Hashimoto and Ogashiwa, 1997; Hashimoto et al., 1995). Immune complexes were detected by colorimetry with a BCIP/NBT detection kit (Nakarai, Kyoto, Japan) or with the use of chemiluminescence reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured myogenic cells with TRIzol (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 Your Prime cDNA synthesis kit (Amersham Pharmacia Biotech) and random hexamers as primers. Targeted genes were amplified by PCR with the following primers (sense and antisense, respectively) and conditions: 5′-AGGACACGACTGCTTT-CTTC-3′ and 5′-GCACCGCAGTAGAGAAGTGT-3′ (25 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds) for the mouse *Myod1* (encoding MyoD) gene; 5′-TGAGATTTGTGGGCCGGAGC-3′ and 5′-GGGACACCTACTCT-CATACTGG-3′ (25 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds) for the mouse *Runx2* gene; 5′-TTGCTGAACGTGAAGCCCATCGAGG-3′ and 5′-GTCCTTGTA-GATCTCCTGGAGCAG-3′ (30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 s) for the mouse *Pparg* (encoding PPARγ) gene; 5′-TGACGGAGCAGGAACAGCAG-3′ and 5′-GACGAAGGCGAGTGAGAATC-3′ (25 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds) for the mouse muscle creatine kinase gene; 5′-TTCATGTCCAAGCAGGAGGG-CAA-3′ and 5′-ACCGTAGATGCGTTTGTAGGCGGT-3′ (27 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 s) for the mouse osteocalcin gene; 5′-GCATGGACTGTGGTCATGAG-3′ and 5′-CCATCACCATCTTCCAGGAG-3′ (22 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds) for the mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene; 5′-GTCTTACCCCTCCTACCTGA-3′ and 5′-TGCCTGGCTCTTCTT-ACTGA-3′ (30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 30 seconds) for the human *RUNX2* gene; and 5′-GCATGGACTGTGGTCATGAG-3′ and 5′-CCATCACCATCTTCC-AGGAG-3′ (30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds) for the human *GAPDH* gene.

Cell transfection and inducible expression of myogenin

The ecdysone-inducible expression system (Invitrogen, San Diego, CA) was modified for the present study. The inducible expression plasmid pISE was constructed by introducing an internal ribosome entry site (Hashimoto and Ogashiwa, 1997) and the GFP cDNA into the pIND(SP1) vector. The rat myogenin cDNA (Wright et al., 1989) was then subcloned into pISE to generate the myogenin expression plasmid pISEmgn. MMCs (5×10^4 cells in a 35 mm dish) were transfected with 0.5 μg of pISEmgn (or pISE) and 1.5 μg of pVgRXR in the presence of 9 μl of FuGENE6 transfection reagent (Roche Diagnostic, Mannheim, Germany) as described (Hashimoto and Ogashiwa, 1997; Hashimoto et al., 1995; Hashimoto et al., 1994). Expression of both myogenin and GFP was induced simultaneously by ponasterone A in pISEmgn-transfected cells cultured in pmDM supplemented with BMP2 (250 ng ml^{-1}).

RESULTS

Multipotentiality of muscle satellite cells

We isolated clones of mouse myogenic cells (MMCs) from a single muscle satellite cell by the single-fiber culture method (see Materials and Methods). Prior to the culture, satellite cells on isolated myofibers expressed CD34 (data not shown). One of the isolated clones, GB1T, was characterized further in this study. To compare the properties of the monoclonal MMCs with those of other MMCs, we also prepared multiclonal MMCs by the low cell density culture method.

To determine the multipotentiality of unmanipulated muscle satellite cells, we cultured GB1T under various conditions. Immunofluorescence analysis revealed that GB1T expressed MyoD, Pax7, desmin and nestin (Fig. 1A-D), all proteins that are known to be expressed in muscle lineage cells. GB1T therefore appeared to preserve the myogenic phenotype during in vitro culture. GB1T cells differentiated into myotubes when cultured in the differentiation medium pmDM (Fig. 1F), although they continued to proliferate as undifferentiated cells in the growth medium pmGM (Fig. 1E). Furthermore, GB1T differentiated into immature osteoblasts expressing alkaline phosphatase (ALP), an early marker of osteogenic differentiation, when cultured for 2-4 days in pmDM supplemented with bone morphogenetic protein 2 (BMP2) (Fig. 1G); ALP activity was not detected in GB1T cultured in the absence of BMP2. GB1T also differentiated into adipocytes, containing numerous lipid droplets in the cytoplasm, when cultured in the presence of 100 μM γ-linolenic acid for 6 days (Fig. 1H). These results indicate that unmanipulated satellite cells derived from adult skeletal muscle preserve multipotentiality.

The multiclonal MMCs also differentiated into myotubes, osteoblasts and adipocytes under appropriate culture conditions (data not shown). We did not detect any differences in morphology, differentiation potential or gene expression patterns between the monoclonal and multiclonal MMCs analyzed in the present study. A sufficient number of multiclonal MMCs was obtained easily at early passages, whereas the number of monoclonal MMCs at early passages was not enough for multiple experiments. The results shown for subsequent experiments were therefore obtained with the multiclonal MMCs (unless indicated otherwise), although similar results were obtained with the monoclonal cells.

Co-expression of multiple lineage determination genes in undifferentiated MMCs

The expression of master genes essential for myogenesis, osteogenesis, and adipogenesis was examined in undifferentiated MMCs by RT-PCR analysis. Undifferentiated MMCs expressed the genes for MyoD, a muscle-determining factor; Runx2, a transcription factor essential for osteogenesis (Komori et al., 1997; Otto et al., 1997); and PPARγ, a nuclear receptor essential for adipogenesis (Barak et al., 1999; Kubota et al., 1999; Rosen et al., 1999) (Fig. 2A).

We next focused on determination of the myogenic and osteogenic lineages in MMCs. The expression of master and differentiation marker genes for osteogenesis was examined under conditions specific for myogenic or osteogenic differentiation. Whereas the expression of *Runx2* in MMCs was markedly inhibited under myogenic differentiation conditions, it was sustained during osteogenesis induced by BMP-2 (Fig. 2B). The expression of myogenic and osteogenic differentiation marker genes encoding muscle creatine kinase and osteocalcin, respectively, was induced under the respective culture conditions.

To determine whether a *Runx2*-expressing subpopulation of MMCs is lost from the culture under myogenic differentiation conditions, we examined the expression of Runx2 protein in undifferentiated MMCs by immunofluorescence analysis. Runx2 was present in the nuclei of all MMCs (Fig. 2C,D). The

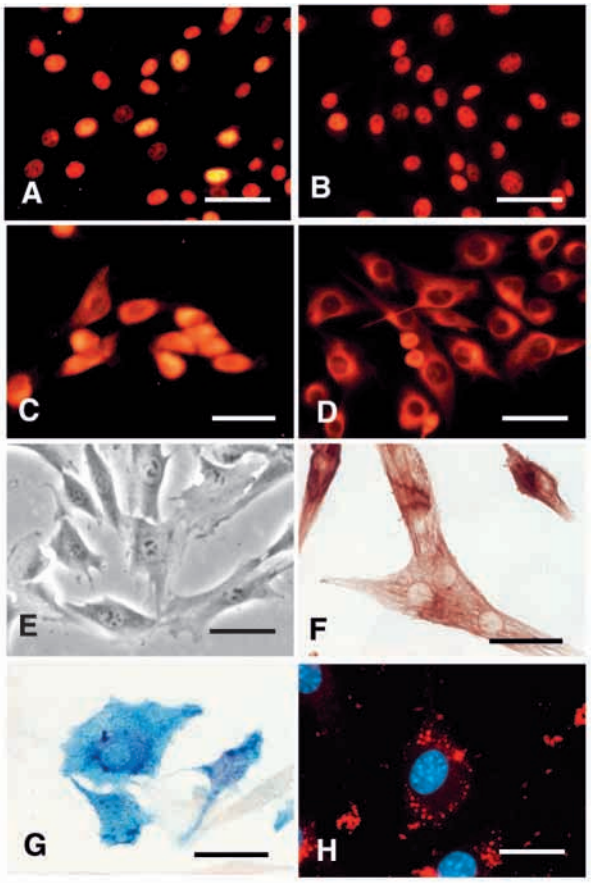

Fig. 1. Multipotentiality of MMCs derived from muscle satellite cells. (A-D) Expression of myogenic lineage markers in a clone (GB1T) of primary cultured MMCs was determined by immunofluorescence analysis with antibodies to MyoD (A), to Pax7 (B), to desmin (C) or to nestin (D), as well as Cy3-conjugated secondary antibodies. (E) Undifferentiated MMCs resembled fibroblasts when cultured in pmGM. (F) MMCs differentiated into myotubes expressing sarcomeric myosin heavy chain (immunostained with a horseradish peroxidase reaction product) after culture for 4 days in pmDM. (G) MMCs differentiated into immature osteoblasts expressing ALP (activity detected by staining with Fast Blue RR) when cultured in pmDM supplemented with BMP2 (250-500 ng ml^{-1}) for 4 days. (H) MMCs differentiated into adipocytes containing many lipid droplets in their cytoplasm (as revealed by staining with oil red O; nuclei were detected by staining with DAPI) when cultured for 6 days in DMEM supplemented with 10% FBS and 100 μM γ-linolenic acid. Images in A-D,H were obtained by epifluorescence microscopy, that in E was obtained by phase-contrast microscopy, and those in F,G were obtained by bright-field microscopy. Scale bars: 50 μm.

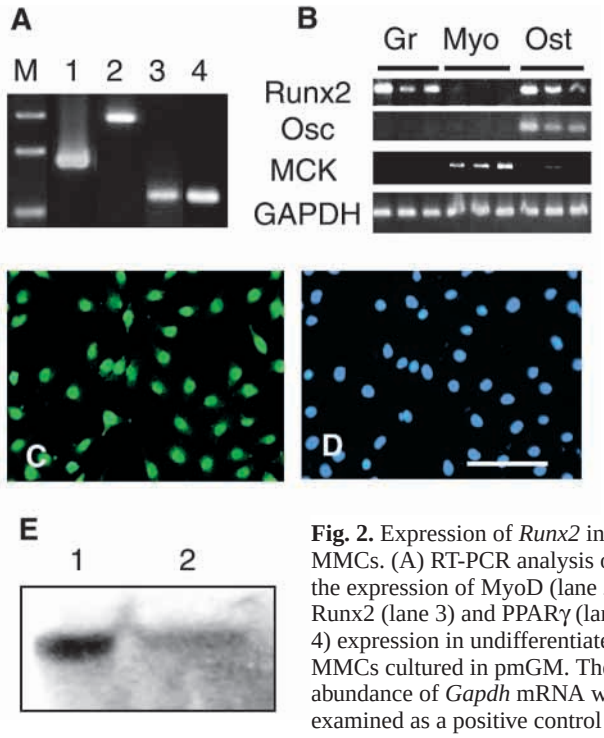

Fig. 2. Expression of *Runx2* in MMCs. (A) RT-PCR analysis of the expression of MyoD (lane 2), Runx2 (lane 3) and PPARγ (lane 4) expression in undifferentiated MMCs cultured in pmGM. The abundance of *Gapdh* mRNA was examined as a positive control (lane 1). DNA size markers in lane M represent 200, 300 and 400 bp. (B) RT-PCR analysis of the expression of *Runx2*, osteocalcin (Osc) and muscle creatine kinase (MCK) in undifferentiated growing (Gr), myogenically differentiated (Myo) and osteogenically differentiated (Ost) MMCs. The abundance of *Gapdh* mRNA was again analyzed as a positive control. Three independent samples were analyzed for each experimental condition. (C) Immunofluorescence analysis of Runx2 expression in undifferentiated MMCs. (D) Nuclei in the same field as that shown in C were visualized by DAPI staining. (E) Immunoblot analysis of Runx2 expression in total lysates (20 μg of protein) of undifferentiated MMCs (lane 1) and C2C12 cells (lane 2). Scale bar: 100 μm.

downregulation of *Runx2* expression in MMCs under myogenic differentiation conditions was therefore not due to the selective elimination of a *Runx2*-expressing subpopulation. Immunoblot analysis revealed that the abundance of Runx2 in undifferentiated MMCs was markedly greater than that in C2C12 cells (Fig. 2E). A low level of *Runx2* expression in C2C12 cells was also observed previously (Katagiri et al., 1994; Lee et al., 2000b; Zhang et al., 2000).

Critical period for induction of osteogenic differentiation by BMP-2

The downregulation of *Runx2* expression during myogenesis suggested that the osteogenic differentiation potential of MMCs may change. To detect any alteration in the response of MMCs in myogenic differentiation culture to BMP2 as well as to determine the reversibility of BMP2-induced osteogenesis, we exposed MMCs to BMP2 for various periods during culture under myogenic differentiation conditions (Fig. 3A). BMP2 induced marked ALP expression in MMCs that were exposed to this factor for 0-12, 12-24 or 12-48 hours of a 48-hour culture period (Fig. 3B). Exposure to BMP2 within a critical period thus induced osteogenic differentiation even after withdrawal of BMP2. The osteogenesis triggered by BMP2 during this critical period thus appears to be irreversible. By contrast, MMCs lost the potential to undergo osteogenic differentiation after culture for 24 hours under myogenic differentiation conditions.

Myogenin, a member of the MyoD family of proteins, is essential for the terminal differentiation of skeletal muscle (Hasty et al., 1993; Nabeshima et al., 1993). Immunoblot analysis revealed that myogenin expression was induced after culture of MMCs for 12 hours under myogenic differentiation conditions, and that the expression of sarcomeric myosin heavy chain (MHC), a marker of terminal muscle differentiation, was apparent after 24 hours (Fig. 3C). The ability of MMCs to respond to BMP2 was thus lost at the same time that

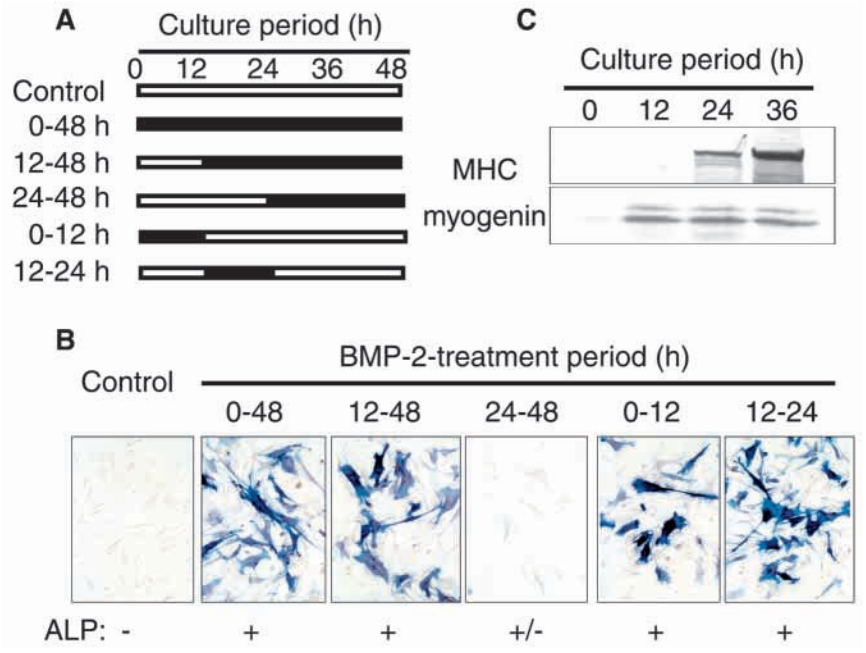

Fig. 3. Critical period for BMP2-induced osteogenesis. (A,B) Sensitivity of MMCs to BMP2 under myogenic differentiation conditions. MMCs were cultured in the absence (white bars) or presence (black bars) of BMP2 for various intervals up to 48 hours (A). Numbers on the left represent the period of exposure to BMP2. At the end of the 48-hour culture, the cells were stained for ALP activity (B); images were obtained by bright-field microscopy. The level of ALP activity in each culture is shown as +, +/– or – below the images. (C) Induction of myogenesis-specific proteins in MMCs under myogenic differentiation conditions. Cells were cultured in pmDM for the indicated times, after which total lysates (20 μg of protein) were subjected to immunoblot analysis with antibodies to MHC and to myogenin.

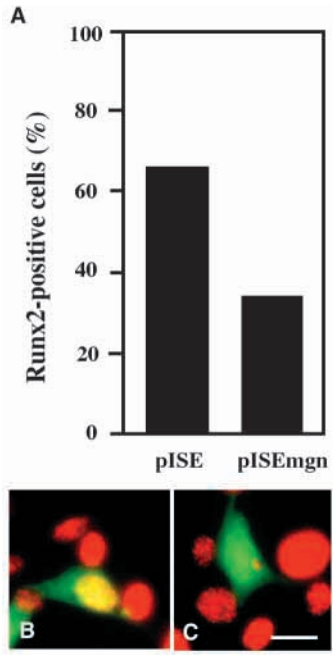

Fig. 4. Suppression of BMP2-induced osteogenesis by myogenin. MMCs were transfected with the myogenin expression plasmid pISEmgn or the control vector pISE. (A) Expression of myogenin was induced by ponasterone A simultaneously with the onset of stimulation with BMP2 for 30 hours. The expression of Runx2 was determined by immunofluorescence analysis in 132 or 177 GFP-expressing cells transfected with pISE or pISEmgn, respectively (A). MMCs transfected with pISE (B) or pISEmgn (C) were probed with antibodies to GFP and to Runx2, and immune complexes were detected by FITC or Cy3 fluorescence, respectively. Runx2 (red) was present in nuclei, whereas GFP (green) was localized to both nuclei and cytoplasm. Intrinsic fluorescence of GFP was severely decreased after the immunostaining procedure. Scale bar: 20 μm.

irreversible terminal differentiation into skeletal muscle cells was apparent by the expression of MHC.

Inhibition of osteogenic differentiation by myogenin

The loss of osteogenic differentiation potential in MMCs during myogenesis suggested that a myogenesis-specific factor (or factors) is responsible for the downregulation of *Runx2* expression. A possible candidate for the suppressor of osteogenesis was myogenin, given that MMCs lost the ability to undergo osteogenic differentiation after the induction of myogenin expression. To determine whether myogenin contributes to the downregulation of Runx2 expression, we transfected MMCs with a bicistronic expression plasmid (pISEmgn) that encodes both myogenin and green fluorescent protein (GFP). Effects of myogenin on the growth properties of MMCs were avoided by inducing the expression of myogenin with ponasterone A under osteogenic differentiation conditions. Immunofluorescence analysis revealed that Runx2 was expressed in 66% of GFP-positive MMCs transfected with the control vector pISE, indicating that Runx2 expression was inhibited slightly by transfection itself (Fig. 4A,B). By contrast, Runx2 was detected in no more than 32% of GFP-expressing MMCs transfected with pISEmgn (Fig. 4A,C). In addition, myogenin was detected in only ~50% of GFP-positive MMCs transfected with pISEmgn, probably because myogenin is more labile than is GFP. These results thus indicated that forced expression of myogenin resulted in marked inhibition of Runx2 expression in MMCs even under osteogenic differentiation conditions.

Induction of osteogenesis prior to downregulation of MyoD expression

Our results indicated that either the myogenic or the osteogenic differentiation pathway was selected by MMCs under the corresponding differentiation-inducing conditions. BMP2 has previously been shown to suppress the expression of MyoD in C2C12 cells (Katagiri et al., 1997; Katagiri et al., 1994). However, it was unclear whether the downregulation of MyoD expression is required for induction of osteogenesis in MMCs. We therefore examined MyoD abundance in MMCs during BMP2-induced osteogenesis. MyoD expression was maintained and ALP was not expressed during myogenesis (Fig. 5A). By contrast, MMCs lost MyoD protein and began to express ALP during exposure to BMP2 (Fig. 5B,C). After culture with BMP2 for 2 days, 40-50% of MMCs expressed MyoD, whereas they all expressed ALP (Fig. 5B); after 4 days, however, only 20-30% of MMCs expressed MyoD, whereas, again, they all expressed ALP (Fig. 5C). The expression of MyoD was thus downregulated after the induction of ALP expression. Downregulation of MyoD expression is therefore not required for the osteogenic differentiation of MMCs.

Bone matrix formation by myogenic cells

Our results suggested that muscle satellite cells are candidate osteoprogenitor cells. To determine whether myogenic cells derived from muscle satellite cells are able to undergo terminal osteogenic differentiation, we examined the ability of BMP-2-stimulated MMCs to form bone matrix under various culture conditions. Calcium deposition was not detected in either growing or myogenic differentiation cultures of MMCs. Slightly flattened MMCs became round and formed nodules after 6 days of culture in the presence of BMP2 (Fig. 6A). The nodules resembled those formed by mineralizing bone marrow stromal cells (Maniatopoulos et al., 1988). However, no calcium deposition was detected with the calcium-staining dye Alizarin Red S in BMP2-stimulated MMC cultures. Culture of MMCs in pmDM containing both BMP2 and β-glycerophosphate, the latter of which is used as an in vitro source of phosphate ions (Maniatopoulos et al., 1988), resulted in the formation of nodules, the central regions of which were opaque (Fig. 6B). More than 20 opaque nodules were formed in the center of 35 mm culture dishes after culture of cells for 6 days in medium containing both BMP2 and β-glycerophosphate. The opaque regions of the nodules stained prominently with Alizarin Red S (Fig. 6C,D). Moreover, brief incubation with the chelating agents EGTA or EDTA resulted

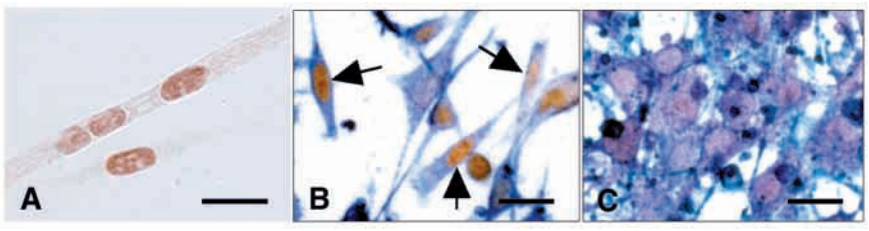

Fig. 5. Expression of MyoD and ALP in MMCs during myogenic and osteogenic differentiation. MMCs were cultured in pmDM for 6 days to induce myogenic differentiation (A) or in the presence of BMP2 for 2 (B) or 4 (C) days to induce osteogenic differentiation. The cells were then subjected to staining for ALP activity (blue) and immunostaining for MyoD (brown). Arrows in B indicate cells expressing both MyoD and ALP. Scale bars: 20 μm.

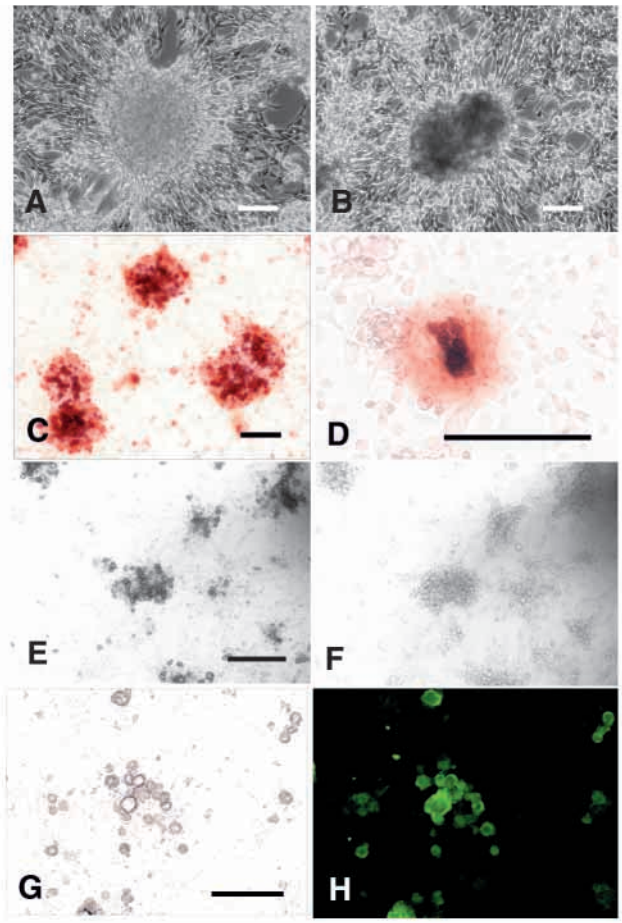

Fig. 6. Bone matrix formation by MMCs. MMCs were cultured for 6 days in pmDM supplemented with either BMP2 alone (A) or both BMP2 and 10 mM β-glycerophosphate (B-H). Transparent nodules were formed by the cells cultured with BMP2 alone (A). By contrast, many opaque nodules were detected in the presence of β-glycerophosphate (B). Nodules formed by multiclonal (C) and monoclonal (D) MMCs stained with Alizarin Red S. Incubation of fixed cells with 200 mM EGTA for 5 minutes resulted in the removal of the opaque material; the same field of a culture before and after EGTA treatment is shown in E and F, respectively. (G,H) Osteocalcin was detected in the opaque matrix by immunofluorescence analysis with antibodies to osteocalcin and FITC-conjugated secondary antibodies. (H) The same field is shown in G. Scale bars: 100 μm. Images in A and B were obtained by phase-contrast microscopy, whereas those in C-G were obtained by bright-field microscopy.

in complete removal of the opaque material (Fig. 6E,F), after which none of the nodules stained with Alizarin Red S (data not shown). The opaque material was thus probably composed of deposited calcium. The opaque material appeared to accumulate in the extracellular matrix around cells in nodules (Fig. 6E,G). In addition, osteocalcin (Gla protein), a marker of late osteogenic differentiation that is present in bone matrix (Maniatopoulos et al., 1988), was shown to colocalize with the opaque material (Fig. 6G,H). The opaque material formed by the MMCs was thus bone matrix. Ascorbic acid and dexamethasone, which enhance bone formation by bone marrow stromal cells (Maniatopoulos et al., 1988), did not affect mineralization in BMP2-stimulated MMC cultures. In contrast to MMCs, C2C12 cells formed neither bone matrix nor nodules under the conditions shown to induce bone formation by MMCs, although they did express ALP in response to BMP2 stimulation (data not shown). These results thus indicated that BMP2 induces osteogenic terminal differentiation in MMCs, as well as the expression of osteogenesis marker genes.

Multipotentiality of human myogenic cells

Our results suggested that muscle satellite cells might be a source of autologous osteogenic cells for bone repair in patients. We therefore also determined whether human myogenic cells (HMCs) exhibit multipotentiality in vitro. One of the isolated HMC clones, Hu5, was selected for further study. All of the HMCs expressed the muscle lineage markers MyoD (Fig. 7A) and Pax7 (Fig. 7B). HMCs differentiated into myotubes when cultured in pmDM (Fig. 7C). These cells therefore appeared to retain the myogenic phenotype during in vitro culture. Calcium deposition was not observed in either growing or myogenic differentiation cultures of Hu5 (Fig. 7C). BMP2 prevented myogenesis of HMCs (Fig. 7D). Furthermore, Alizarin Red S staining revealed mineralization of opaque nodules formed by BMP2-stimulated Hu5 cells (Fig. 7D). The nodules formed by HMCs were fewer in number and smaller in size than were those formed by MMCs, and neither β-glycerophosphate nor dexamethasone enhanced

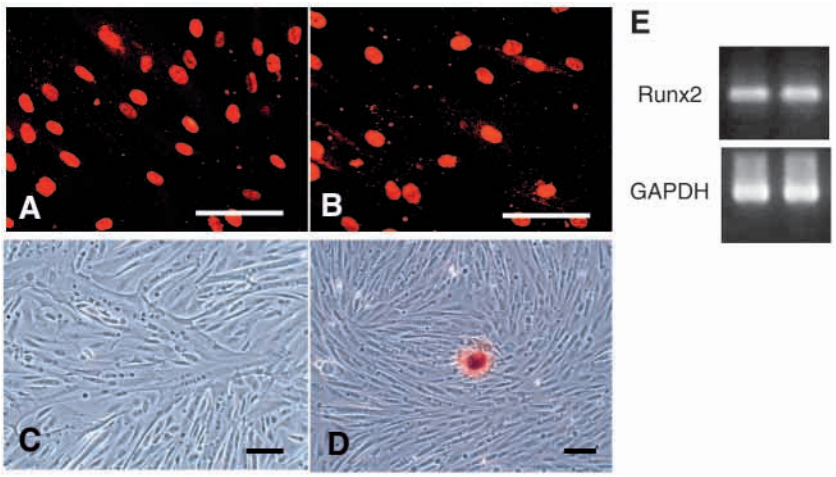

Fig. 7. Multipotentiality of HMCs. (A,B) Undifferentiated growing HMCs were subjected to immunofluorescence analysis with antibodies to MyoD (A) or to Pax7 (B) and with Cy3-conjugated secondary antibodies. Both MyoD and Pax7 were present in the nuclei of all HMCs. (C,D) HMCs were cultured in pmDM for 6 days in the absence (C) or presence (D) of BMP2, and were then stained with Alizarin Red S for the detection of calcium deposition and examined by phase-contrast microscopy. Prominent myotubes developed in the culture shown in C. By contrast, myogenic differentiation was inhibited and calcium deposition was induced in the culture shown in D. Scale bars: 100 μm in A-D. (E) RT-PCR analysis of *Runx2* expression in undifferentiated HMCs. Results are shown for two independent samples.

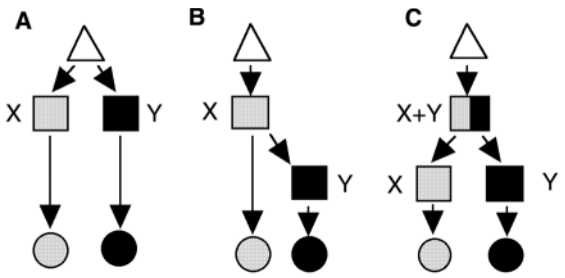

Fig. 8. Models for the generation of different fates from multipotent stem cells. The divergence model (A), trans-determination model (B), and stock options model (C) are shown. Triangles, squares and circles represent tissue stem cells, progenitor cells and differentiated cells, respectively. X and Y represent lineage-specific determination genes.

mineralization by BMP2-stimulated HMCs. RT-PCR analysis also revealed that, as in undifferentiated MMCs, Runx2 was expressed in undifferentiated HMCs (Fig. 7E). These data thus indicate that HMCs are multipotent and retain the ability to undergo osteogenic terminal differentiation.

DISCUSSION

Although neuronal and mesenchymal stem cells exhibit multipotentiality, the multipotentiality of muscle satellite cells has not previously been demonstrated either in vitro or in vivo. By clonal analysis of MMCs derived from single muscle satellite cells, we have now demonstrated the multipotentiality of these latter cells. Our results and those of previous studies on neuronal and mesenchymal stem cells suggest that multipotentiality is a common feature of TS cells. However, the biological significance of the multipotentiality of TS cells remains unclear, given that their differentiation potential is not fully exploited during tissue regeneration in vivo.

Ectopic bone formation within skeletal muscle has been described. Osteoprogenitor cells have thus been thought to reside in skeletal muscle, although their source has remained unknown (Bosch et al., 2000). We have now shown that MMCs form bone in vitro in the presence of β-glycerophosphate. These cells are therefore able to undergo terminal osteogenic differentiation. Our results suggest that the descendants of muscle satellite cells are responsible for ectopic ossification of skeletal muscle, including that caused by genetic deficiency (Feldman et al., 2000).

Bone marrow stromal cells are considered a source of autologous osteogenic cells for bone repair. However, the collection of these cells entails substantial risk to the individual. Furthermore, freshly isolated bone marrow stromal cells are limited in number and are difficult to culture continuously in order to provide sufficient cells for therapy. Our demonstration of the multipotentiality of HMCs and MMCs suggests that myogenic satellite cells as well as previously described muscle-derived stem-like cells might serve as an alternative source of autologous osteogenic cells for the repair of bone defects.

Undifferentiated myogenic cells were shown to express multiple differentiation-determining genes that are essential for the commitment to distinct cell lineages. The co-expression of these determination genes was apparent in independently isolated MMCs as well as HMCs. The expression of *Runx2* was thus not induced artifactually in myogenic cells during culture. Furthermore, undifferentiated MMCs continued to express several myogenic lineage markers. These observations thus indicate that *Runx2*-expressing MMCs preserve the myogenic phenotype during in vitro culture. Muscle satellite cells were previously thought to be already committed to the myogenic lineage. Our results, however, suggest that the fate of muscle satellite cells is not committed even in adult skeletal muscle. The myogenic commitment of muscle satellite cells during muscle regeneration probably involves both the maintenance of *Myod1* expression and the repression of nonmyogenic determination genes including *Runx2*. The present study suggests that myogenin suppresses osteogenesis by inhibiting *Runx2* expression during myogenesis.

In contrast to myogenic differentiation, commitment of muscle satellite cells to the osteogenic lineage was accompanied by the suppression of myogenic determination genes, including those for MyoD and myogenin. The observation that *Runx2* is already expressed in undifferentiated myogenic cells before BMP2-induced osteogenesis suggests that osteogenic differentiation of muscle satellite cells might not be trans-determined. Given that they also express genes essential for the commitment to nonmyogenic lineages and that they preserve multipotentiality, it might be more accurate to refer to these multipotent progenitor cells as ‘multiblasts’ (multiple tissue blasts) rather than as myoblasts.

Myogenesis of C2C12 cells is thought to be inhibited by suppression of the expression of MyoD and myogenin genes by both Runx2 and Smad proteins, which act downstream of the BMP2 receptor (Fujii et al., 1999; Lee et al., 2000b; Nishimura et al., 1998). However, we have now shown that the induction of ALP preceded the down-regulation of MyoD expression during osteogenesis of MMCs. The downregulation of MyoD expression is thus not a prerequisite for osteogenic differentiation. MMCs are able to undergo osteogenesis directly; they do not first have to undergo dedifferentiation as has been proposed for the trans-differentiation of iris epithelial cells of adult newts into lens epithelial cells (Yamada and McDevitt, 1984).

An understanding of the mechanisms of tissue regeneration requires characterization of the mechanisms by which different cell fates are generated from multipotent stem cells. According to the ‘divergence’ model, a lineage determination gene, such as *Myod1* or *Runx2*, is induced in uncommitted multipotent stem cells and converts them to the corresponding committed monopotent progenitor cells (Fig. 8A). In the ‘trans-determination’ model, the determination gene expressed initially (‘X’ in Fig. 8B) is downregulated and another determination gene (‘Y’ in Fig. 8B) is induced in stem cell-derived progenitor cells. In addition to these models, we now propose an alternative that we refer to as the ‘stock options’ model for the generation of different fates from multipotent stem cells. According to this model, stem cells are induced to express multiple determination genes and are converted to multipotent progenitor cells (multiblasts). Depending on the differentiation-inducing signal, the progenitor cells select an option for the terminal differentiation pathway (Fig. 8C). During muscle regeneration, muscle satellite cells would thus be committed to the myogenic lineage as a result of the process proposed in the stock options model.

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