Differentiation (2001) 68:245–253

ORIGINAL ARTICLE

Atsushi Asakura · Motohiro Komaki
Michael A. Rudnicki

Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation

Accepted in revised form: 13 July 2001

Abstract Muscle satellite cells are believed to represent a committed stem cell population that is responsible for the postnatal growth and regeneration of skeletal muscle. However, the observation that cultured myoblasts differentiate into osteocytes or adipocytes following treatment with bone morphogenetic proteins (BMPs) or adipogenic inducers, respectively, suggests some degree of plasticity within the mesenchymal lineage. To further investigate this phenomenon, we explore the osteogenic and adipogenic potential of satellite cells isolated from adult mice. Our experiments clearly demonstrate that satellite cell-derived primary myoblasts, expressing myogenic markers such as MyoD, Myf5, Pax7 and desmin, differentiated only into osteocytes or adipocytes following treatment with BMPs or adipogenic inducers, respectively. However, satellite cells on isolated muscle fibers cultured in Matrigel readily differentiated into myocytes as well as osteogenic and adipogenic lineages, whereas primary myoblasts did not. Satellite cell-derived primary myoblasts isolated from mice lacking the myogenic transcription factor MyoD (*MyoD*-/-) differentiate into myocytes poorly *in vivo* and *in vitro* (Megeney et al., Genes Dev. 1996; Sabourin et. al, J. Cell Biol., 1999). Therefore, we tested whether *MyoD*-/- primary myoblasts display increased plasticity relative to wild type cells. Unexpectedly, the osteogenic or adipogenic differentiation potential of *MyoD*-/- primary myoblasts did not increase compared to wild-type cells. Taken together, these results strongly suggest that muscle satellite cells possess multipotential mesenchymal stem cell activity and are capable of forming osteocytes and adipocytes as well as myocytes.

Key words satellite cell · stem cell · MyoD · myogenesis · osteogenesis · adipogenesis · differentiation

A. Asakura · M. A. Rudnicki (✉)
Ottawa Health Research Institute
Molecular Medicine Program
501 Smyth Road
Ottawa, Ontario, K1H 8L6
Canada
e-mail: mrudnicki@ohri.ca
Tel: +1 613 739 6740
Fax: +1 613 737 8803

M. Komaki
CIHR in Periodontal Physiology
Faculty of Dentistry
University of Toronto
Toronto, Ontario, M5S 3E2
Canada

Introduction

During vertebrate embryogenesis, mesodermal progenitors give rise to distinct cell lineages, including skeletal myocytes, osteocytes, chondrocytes, and adipocytes, in response to distinct signals derived from surrounding tissues (Brand-Saberi et al., 1996). The existence of multipotential mesodermal progenitors in the embryo has been well studied using the C3H10T1/2 cell line derived from embryonic mesodermal cells. 10T1/2 cells readily differentiate into three distinct mesodermal cell lineages, skeletal myocytes, adipocytes, and chondrocytes following treatment with 5-azacytidine (Taylor and Jones, 1979). Treatment with bone morphogenetic proteins (BMPs) can induce osteogenic, chondrogenic, and adipogenic differentiation of 10T1/2 cells (Katagiri et al., 1990; Asahina et al., 1996). In addition, multipotential mesenchymal stem cells derived from bone marrow can differentiate into skeletal myocytes, adipocytes, osteocytes, and chondrocytes following treatment with various inducers as well as *in vivo* transplantation (Prockop, 1997; Pittenger et al., 1999; Liechty et al., 2000). There-

fore, these results suggest that there are common progenitors which give rise to mesenchymal progenies.

Satellite cells are believed to represent the committed myogenic stem cells of adult skeletal muscle. Satellite cells make up 2–5% of sublaminar nuclei and reside closely juxtaposed to skeletal muscle fibers beneath the basal lamina. Satellite cells are normally mitotically quiescent, but initiate proliferation when activated by growth factors induced by weight bearing or other trauma such as injury, to mediate the postnatal growth and regeneration of muscle. The myogenic precursor cells (MPCs), the progeny of activated satellite cells, undergo multiple rounds of cell division prior to their terminal differentiation to form multinucleated myotube (Grounds and Yablonka-Reuveni, 1993; Seale and Rudnicki, 2000). Satellite cells represent a unique population of committed myogenic progenitors that are distinct from their daughter myoblasts by both biochemical and biological criteria (Seale and Rudnicki, 2000).

The myogenic regulatory factors (MRFs), a group of skeletal-muscle specific basic helix-loop-helix (bHLH) transcription factors consisting of MyoD, Myf5, myogenin, and MRF4, play essential roles in satellite cell activation, proliferation, and differentiation (Grounds et al., 1992; Smith et al., 1994; Cornelison and Wold 1997; Seale and Rudnicki, 2000). Recently, we and other groups demonstrated that satellite cell-derived primary myoblasts isolated from adult mice lacking *MyoD* (*MyoD-/*) display accelerated growth rate and delayed terminal differentiation. Therefore, *MyoD-/-* primary myoblasts have been suggested to display characteristics that are more primitive than wildtype cells and may represent an intermediate stage between a stem cell and a myogenic precursor cell (Megeney et al., 1996; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999; Cornelison et al., 2000). We recently found that the paired-box transcription factor Pax7 is expressed in both quiescent and activated satellite cells. Homozygous *Pax7* gene knockout mice (*Pax7-/-*) that do not survive beyond two weeks after birth display complete lack of satellite cells in their muscles, suggesting the essential role of *Pax7* in satellite cell development (Seale et al., 2000).

Recent *in vitro* experiments demonstrate that cultured myogenic cells, which are believed to be already committed into muscle lineage, can differentiate into osteocytes or adipocytes by treatment with BMPs (Yamaguchi et al., 1991; Katagiri et al., 1994) or adipogenic inducers such as thiazolidinedione or fatty acids (Teboul et al., 1995), respectively. However, it remains unknown whether muscle satellite cells also are capable of differentiation into osteogenic and adipogenic lineages.

In this work, we demonstrate that satellite cell-derived primary myoblasts isolated from adult skeletal muscle differentiate into osteocytes or adipocytes following treatment with BMPs or adipogenic inducers, respectively. In addition, satellite cells isolated on muscle fibers more readily differentiate into osteocytes and adipocytes than primary myoblasts. The enhanced mesenchymal plasticity displayed by satellite cells therefore suggests that satellite cells represent a unique stem cell compartment with a mesenchymal repertoire of potential.

Methods

Transgenic mice

Myf5-nlacZ (Tajbakhsh et al., 1996a) transgenic mice were provided from Dr. Tajbakhsh. *MyoD-/-* mice (Rudnicki et al., 1992) were maintained by back crossing with Balb/c mice.

Cell culture

For isolation of satellite cell-derived primary myoblasts, hindlimb skeletal muscle derived from Balb/c, *MyoD-/-*, and heterozygous *Myf5-nlacZ* mice (two-month old) was digested with collagenase type B and dispase II (Roche Diagnostics). Primary myoblasts were cultured in HAM's F-10 medium supplemented with 20% FCS, 2.5 ng/ml basicFGF (R&D systems) on collagen-coated dishes, as described previously (Sabourin et al., 1999). Cultures were induced to muscle differentiation in Dulbecco's Modified Eagle's Medium (DMEM) with 5% horse serum and to osteogenic differentiation in DMEM supplemented with 5% horse serum and 100–400 ng/ml of human recombinant BMP7 (kindly provided by Dr. Sampath, Creative Biomolecules) or human recombinant BMP4 (R&D systems) for three to six days (Katagiri et al., 1994). Adipogenic differentiation of cultures was induced in DMEM supplemented with 5% horse serum with MDI-I cocktail; 0.5 mM methyl-isobutylxanthine (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich), 100 μM indomethacin (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich) followed by 10 μg/ml insulin, as described previously (Pittenger et al., 1999). The treatment cycle of MDI-I (two days) followed by Insulin (one day) was performed three times (total nine days). Single muscle fibers were isolated from hindlimb skeletal muscles prepared from Balb/c mice (two-month old) by digestion in 0.4% collagenase typeA (Roche Diagnostics), as described previously (Cornelison and Wold, 1997). Isolated muscle fibers were cultured in undiluted Matrigel (Beckton Dickinson) for 2 days followed by addition of DMEM supplemented with 10% FCS.

Histochemistry

Monoclonal anti-desmin (D33; Dako), monoclonal anti-myosin heavy chain (MF20; Developmental Studies Hybridoma Bank), monoclonal anti-Pax7 (Pax7; Developmental Studies Hybridoma Bank), monoclonal anti-myogenin (F5D; Developmental Studies Hybridoma Bank), monoclonal anti-MyoD (PharMingen), and polyclonal anti-Myf5 (sc-302, Santa Cruz Technology) were used for immunohistochemistry. Vectastain ABC kit (Vector) was used for detection of primary antibodies. For detection of osteoblastic cells, alkaline phosphatase (ALP) staining (Leukocyte Alkaline Phosphatase Assay Kit, Sigma-Aldrich) was used. Double staining was performed by X-gal-staining followed by ALP staining or by ALP-staining followed by immunostaining. LacZ expression was detected by X-gal staining overnight as described previously (Asakura et al., 1995). For detection of accumulated oil droplets, Oil Red-O (Sigma-Aldrich) staining was used followed by nuclear hematoxylin counter staining.

RT-PCR

Total RNA was reverse transcribed followed by 25–40 PCR cycles (RNA PCR Core Kit; Perkin-Elmer), using gene specific primer

pairs for β-actin (5′-CAC CCT GTG CTG CTC ACC GAG GCC-3′ and 5′-ACC GCT CGT TGC CAA TAG TGA TGA-3′), tissue non-specific alkaline phosphatase (TNAP) (5′-TTG AAA CTC CAA AAG CTC AAC ACCA-3′ and TCT CGT TAT CCGA GTA CCA GTC CC-3′), Runx2/CBFa1 (5′-ATG TTC ATT CGC CTC ACAA ACAA-3′ and 5′-CTG AGG CGG GAC ACC TAC TCT CAT-3′), osteocalcin (Fleet and Hock, 1994), osteopontin (Sodek et al., 1995), and PPARγ2 (Hansen et al., 1999).

Results

Previous work has demonstrated that proliferating primary myoblasts derived from satellite cells express myoblast markers such as MyoD, Myf5, desmin, but not terminal differentiation markers such as myogenin or myosin heavy chain (MHC) (Grounds et al., 1992; Smith et al., 1994; Cornelison and Wold, 1997; Sabourin et al., 1999). We re-examined expression of these muscle proteins in both proliferating and differentiating primary myoblasts isolated from two-month old adult mice. In addition, expression of Pax7 protein was examined in these cells since *Pax7* gene is expressed in both quiescent and activated satellite cells and plays an essential role in satellite cell development (Seale et al., 2000).

Immunohistochemical detection clearly revealed that almost all proliferating primary myoblasts expressed MyoD, Myf5 and Pax7 and desmin (Fig. 1). By contrast, few proliferating primary myoblasts expressed terminal differentiation markers such as myogenin and MHC (Fig. 1). Under low serum conditions, primary myoblasts underwent terminal differentiation and formed multinucleated myotubes that expressed myogenin, MHC, MyoD, and Myf5. By contrast, Pax7 expression was down-regulated in these cells (Fig. 1).

Previous work demonstrated that the C2C12 muscle cell line and primary myoblasts isolated from new born mice differentiate into osteogenic or adipogenic cells following treatment with BMPs (bone morphogenetic proteins) or adipogenic inducers, respectively (Katagiri et al., 1994; Teboul et al., 1995). However, it remains unknown whether muscle satellite cells, the adult stem cells of skeletal muscle, also are capable of differentiation into non-muscle lineages. To investigate whether muscle satellite cells are committed or multipotential stem cells, we first used BMPs to examine the osteogenic differentiation capacity of satellite cell-derived primary myoblasts. Staining for alkaline phosphatase (ALP), one of the early markers for osteogenesis, clearly showed that treatment with BMP7 could induce osteogenic differentiation of primary myoblasts in a dose dependent manner, while BMP7 suppressed formation of MHC-expressing myotubes (Fig. 2A). RT-PCR experiments clearly revealed that osteogenic marker mRNAs for tissue-non-specific alkaline phosphatase and osteocalcin were up-regulated in primary myoblast culture following treatment with BMP7 for six days (Fig. 2B). By contrast,

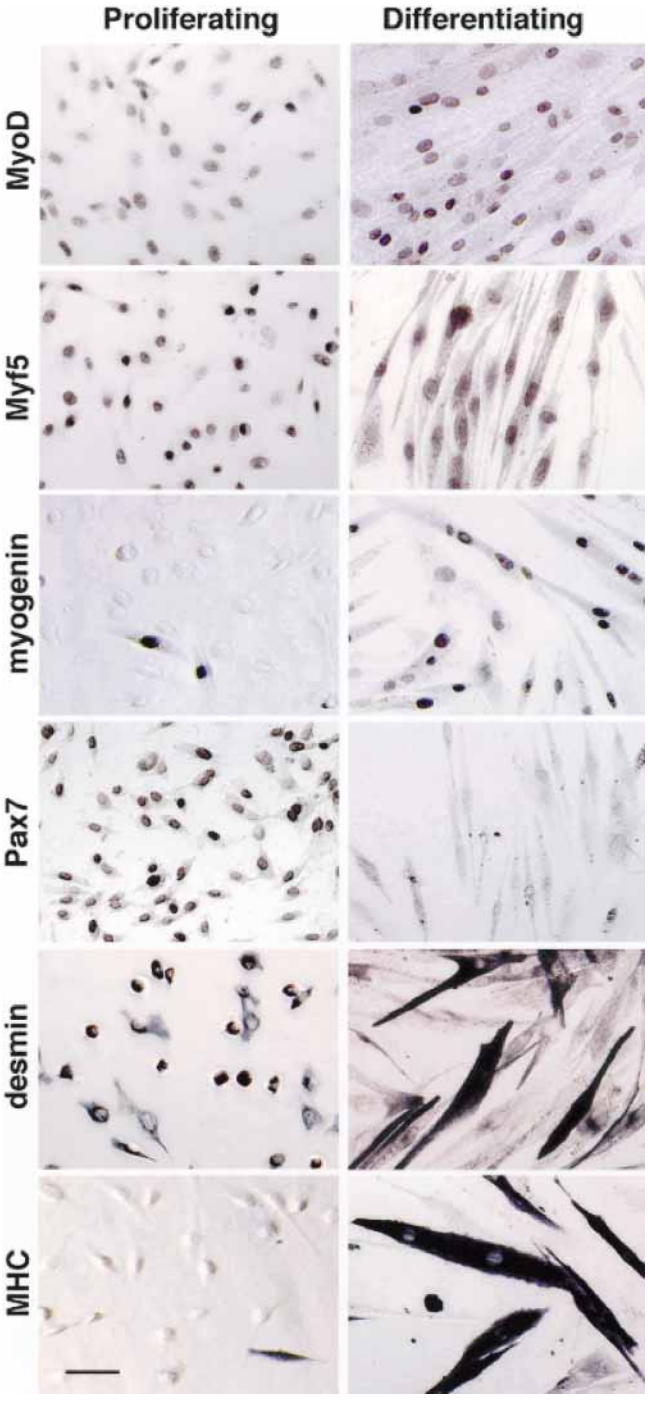

Fig. 1 Muscle marker expression in primary myoblasts derived from adult mice. Proliferating primary myoblasts expressed myoblast markers, MyoD, Myf5, and Pax7 in nucleus, and desmin in cytoplasm. By contrast, muscle differentiation markers, myogenin in nucleus and myosin heavy chain (MHC) in cytoplasm were rarely detected in these cells. In differentiation medium for three days, primary myoblasts initiated terminal differentiation to form multinucleated myotubes. MyoD, Myf5, myogenin, desmin, and MHC were expressed in the differentiating cells. By contrast, Pax7 expression was down-regulated in these cells. *Scale bar*, 50 μm.

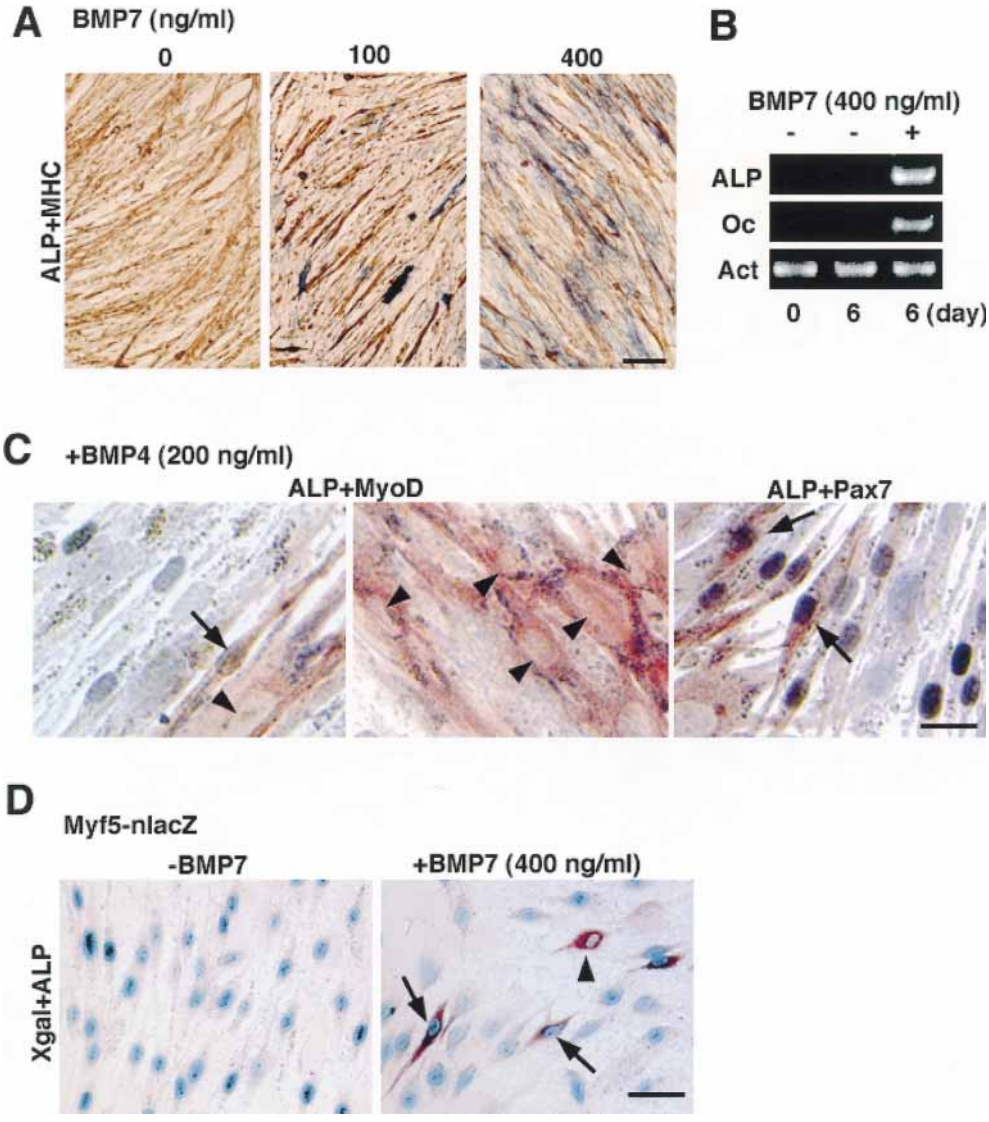

Fig. 2 Osteogenic differentiation of primary myoblasts by treatment with BMPs. **A** Osteogenic differentiation of primary myoblasts was induced by treatment with BMP7 for six days. Alkaline phosphatase (ALP)-staining (*blue color*) and anti-MHC antibodies (*brown color*) were used simultaneously for detection of osteocytes and myocytes, respectively. **B** RT-PCR experiment clearly revealed that osteogenic marker mRNAs for tissue non-specific alkaline phosphatase (ALP) and osteocalcin (Oc) were up-regulated in primary myoblast cultures following treatment with BMP7 for six days. β-actin (Act) was used as an internal control. **C** Some of the ALP-positive osteocytes (*red color*) co-expressed MyoD or Pax7 (*black color in nucleus*) following treatment with BMP4 (200 ng/ml) for three days (*arrows*), while most of the ALP-positive osteocytes were negative for MyoD (*arrowheads*). **D** Primary myoblasts isolated from *Myf5-nlacZ* heterozygous mice expressed lacZ in their nucleus (*blue color*). Some of the ALP-positive cells (*red color*) induced by treatment with BMP7 (400 ng/ml) for three days co-expressed lacZ (*arrows*), **C**). *Arrowhead*, ALP-positive Myf5-nlacZ-negative cells. *Scale bars*, **A** 100 μm, **C** 20 μm, **D** 50 μm.

without BMP-treatment, primary myoblasts never formed ALP-positive osteogenic cells but exclusively differentiated into MHC expressing myotubes (Fig. 2A). These results indicate that satellite cell-derived primary myoblasts retain an ability to differentiate into osteocytes.

Treatment with BMPs suppresses transcriptional activity of myogenic HLH proteins including MyoD and down-regulates MyoD gene expression in C2C12 cells (Katagiri et al., 1994, 1997). Therefore, one possibility is that, prior to osteogenic differentiation, treatment with BMPs may first force primary myoblasts to de-differentiate into multipotential mesenchymal progenitors that do not express any lineage-specific markers. To test this possibility, expression of muscle-specific transcription factors, MyoD and Pax7, were examined following treatment with BMP4. Significantly, a few ALP-weak positive osteocytes derived from primary myoblasts co-expressed MyoD or Pax7 in their nucleus, while the majority of ALP-strong positive osteocytes did not express these transcription factors (Fig. 2C). Next, heterozygous *Myf5-nlslacZ* knock-in mice were used for isolation of primary myoblasts. The expression of Myf5-nlslacZ in the transgenic mice recapitulates the endogenous *Myf5* gene expression during embryogenesis and adult skeletal muscle regeneration (Tajbakhsh et al, 1996a; Cooper et al., 1999; Beauchamp et al., 2000 and data not shown). Proliferating cells and differentiating myotubes expressed nuclear lacZ (Fig. 2D). The ALP-positive osteocytes induced by treatment with BMP7 for three days consisted of lacZ-positive and -negative cells. Taken together, these results suggest that primary myoblasts differentiate directly to osteogenic cells rather than de-differentiating to form a common progenitor for myogenic and osteogenic lineages.

Previous experiments demonstrated that C2C12 myoblasts and primary myoblasts isolated from new born mice can differentiate into the adipogenic lineage follow-

ing treatment with adipogenic inducers such as fatty acids or thiazolidinediones (Teboul et al., 1995), or by blocking the Wnt-signaling pathway (Ross et al., 2000). Therefore, we examined whether primary myoblasts isolated from adult mice also have an ability to undergo adipogenic differentiation. We used MDI-I (methyl-isobutylxanthine, dexamethasone, indomethacin and insulin) cocktail as an osteogenic inducer, which efficiently induces adipogenesis of mesenchymal stem cells isolated from bone marrow (Pittenger et al., 1999). Treatment with MDI-I for nine days clearly induced adipogenic differentiation of primary myoblasts judged by cytoplasmic accumulation of oil droplets stained with Oil-Red O, while without this treatment, primary myoblasts never underwent adipogenic differentiation (Fig. 3C). These results indicate that satellite cell-derived primary myoblasts also possess an ability to give rise to adipocytes.

We previously demonstrated that primary myoblasts isolated from adult mice lacking the *MyoD* gene (*MyoD*-/-) display an accelerated growth rate and delayed terminal differentiation, as compared to wildtype cells. Therefore, *MyoD*-/- primary myoblasts have been suggested to display characteristics that are more primitive than wildtype cells and may represent an intermediate stage between a stem cell and a myogenic precursor cell (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999; Cornelison et al., 2000). Proliferating *MyoD*-/- primary myoblasts expressed Myf5 and Pax7, but not MyoD (Fig. 3A). Therefore, one possibility is that *MyoD*-/- primary myoblasts may have a greater potential to differentiate into osteocytes and adipocytes compared to wildtype cells since MyoD has been shown to play a crucial role in muscle differentiation of satellite cells. To test this possibility, osteogenic or adipogenic differentiation potential of *MyoD*-/- primary myoblasts was examined by treatment with BMP4 or MDI-I, respectively. Unexpectedly, osteogenic or adipogenic differentiation potential of *MyoD*-/- primary myoblasts did not increase compared to that of wild-type cells (Fig. 3B, C). Therefore, MyoD expression appears not be sufficient to suppress either osteogenic or adipogenic differentiation of satellite cell-derived primary myoblasts.

To further investigate the hypothesis that muscle satellite cells are multipotential stem cells, single muscle fibers were isolated from adult skeletal muscle. Because satellite cells on the freshly isolated muscle fibers do not express any myogenic HLH proteins, including MyoD or Myf5, these cells may maintain more intact features as stem cells than do primary myoblasts in culture (Cornelison and Wold, 1997). Single muscle fibers derived from adult mice were cultured in Matrigel for up to several weeks. Matrigel is a solubilized basement membrane matrix that supports the growth and differentiation of many different cell types but does not contain any strong osteogenic or adipogenic inducers. Under these culture conditions, single muscle fibers survived at least one week to a maximum of one month. Satellite cells initiated their proliferation on the fibers (day 1) and migrated into the Matrigel (day 3) (Cornelison and Wold, 1997). Migrated satellite cells predominantly formed myoblast and multi-nuclei myotube clusters. However, by day 4 satellite cells on the muscle fibers (Fig. 4A) and migrated cells by day 10 initiated the accumulation of

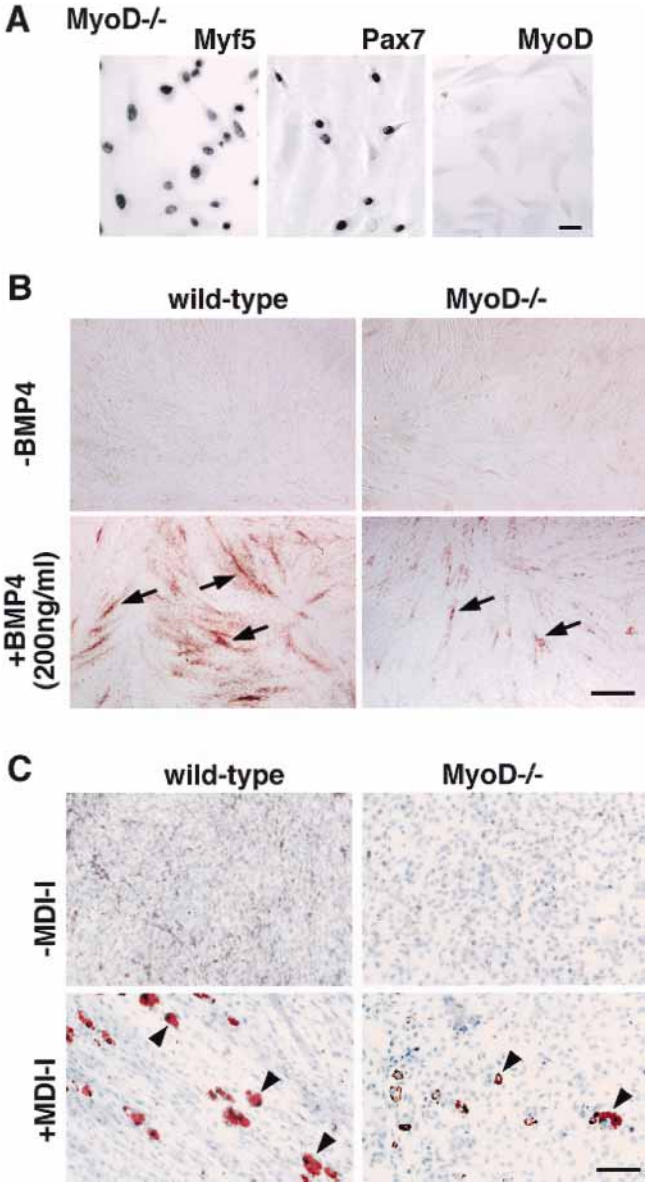

Fig. 3 Osteogenic and adipogenic differentiation potential of *MyoD*-/- primary myoblasts. **A** Proliferating primary myoblasts derived from *MyoD*-/- adult mice expressed myoblast markers, Myf5 and Pax7 in nucleus, but not MyoD. Wild-type and *MyoD*-/- primary myoblasts were treated with BMP4 (200 ng/ml) for three days (**B**) or treated with adipogenic cocktail (MDI-I) for nine days (**C**). BALP-staining showed BMP4-induced osteogenic differentiation of wild-type and *MyoD*-/- primary myoblasts. **C** Oil-Red O-staining showed adipogenic differentiation of wild-type and *MyoD*-/- primary myoblasts. Hematoxylin was used for counter staining nuclei. *Arrows*, ALP-positive osteocytes; *Arrowheads*, Oil Red O-positive adipocytes. *Scale bars*, **A** 20 μm, **B** 200 μm, **C** 100 μm.

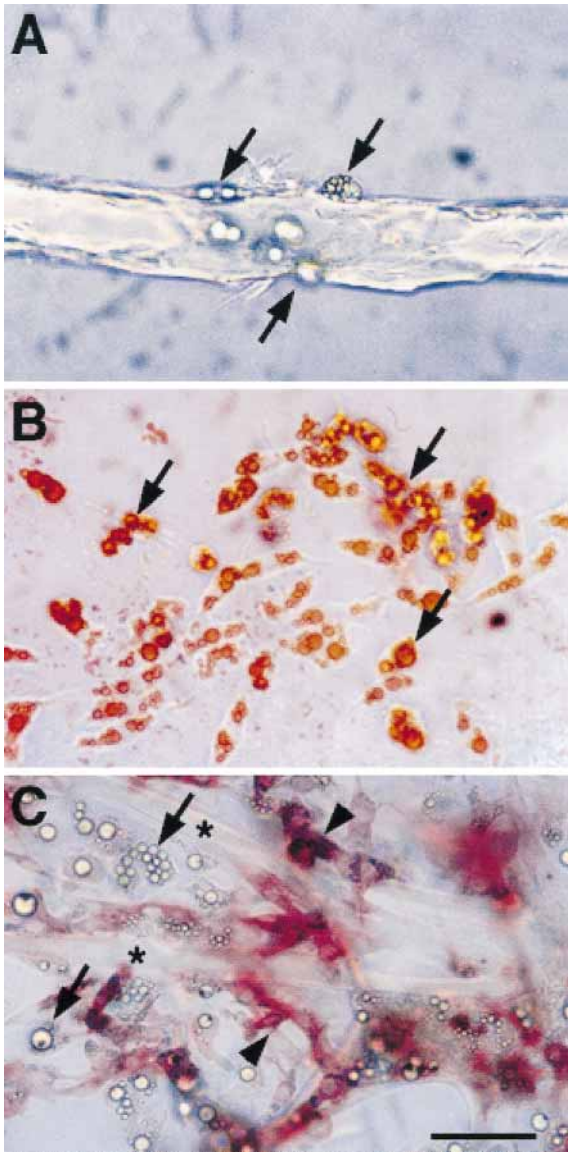

Fig. 4 Satellite cells on muscle fibers gave rise to adipocytes and osteocytes in Matrigel culture. Single muscle fibers derived from adult mice were cultured in Matrigel for four days (**A**) and ten days (**B, C**). Satellite cell-derived adipocytes containing oil droplets in their cytoplasm were detected on muscle fibers (**A**). Migrated satellite cells differentiated into Oil-Red O-positive adipocytes (**B**). ALP-staining showed that osteocytes were also detected between adipocytes and myotubes (**C**). *Arrows*, adipocytes; *Arrowheads*, osteocytes; *Asterisks*, myotubes. *Scale bar*, 100 μm.

oil droplet in their cytoplasm confirmed by Oil Red O staining (Fig. 4B). In addition, cells strongly positive for ALP were observed surrounding the myotubes (Fig. 4C). RT-PCR experiments confirmed that both osteogenic (osteopontin and osteocalcin) and adipogenic (PPARγ2) markers were expressed in the single muscle cultures in Matrigel (data not shown). These result suggest that intact satellite cells have an intrinsic capacity to differentiate into myocytes, adipocytes, and osteocytes.

Discussion

Previous work has demonstrated that myogenic cells such as C2C12 myoblasts, primary myoblasts isolated from new born mice, or clonal myogenic cells isolated from adult skeletal muscle display osteogenic (Katagiri et al., 1994; Lee, J.Y. et al., 2000) or adipogenic (Teboul et al., 1995) differentiation capability following treatment with BMPs or adipogenic inducers, respectively. In addition, blocking the Wnt signaling pathway also induces adipogenic differentiation of C2C12 cells (Ross et al., 2000). However, it remained unclear whether muscle satellite cells, adult stem cells for skeletal muscle, possess similar differentiation capabilities. In this work, we clearly demonstrate that muscle satellite cells can give rise to osteocytes and adipocytes as well as skeletal myocytes.

Proliferating satellite cell-derived primary myoblasts expressed several myoblast markers, such as MyoD, Myf5, Pax7, and desmin, indicating that primary myoblasts are already committed to the myogenic cell lineage (Grounds et al., 1992; Smith et al., 1994; Cornelison and Wold, 1997; Seale et al., 2000; and this work). However, treatment with BMPs or adipogenic inducers stimulated osteogenic or adipogenic differentiation of the primary myoblasts, respectively. During osteogenic differentiation of primary myoblasts, muscle-specific transcription factors, such as MyoD, Myf5, and Pax7, were transiently co-expressed with ALP, an early osteogenic marker. Therefore, osteogenic differentiation of primary myoblasts seems to be direct conversion from the myogenic to the osteogenic lineage, rather than mediated through a common progenitor. Direct cell lineage conversion between differentiated cell types is termed "transdifferentiation" (Eguchi and Kodama, 1993). For example, smooth to skeletal muscle transdifferentiation occurs during esophagus development, in which smooth muscle cells directly differentiate into skeletal muscle (Kablar et al., 2000).

However, proliferating C2C12 cells (Lee, K.S. et al., 2000) or primary myoblasts (data not shown) also express low levels of the osteogenic transcription factor Runx2/CBFa1 (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997), suggesting that they retain some inherent capacity for osteogenic differentiation. Moreover, satellite cells on muscle fibers readily differentiated into both osteocytes and adipocytes in Matrigel which does not contain strong osteogenic or adipogenic inducers. By contrast, primary myoblasts strictly formed differentiated myocytes in this culture. Therefore, satellite cells on muscle fibers exhibit an enhanced capacity to differentiate into osteocytes and adipocytes, compared to cultured primary myoblasts. MyoD or Myf5 expression is undetectable in satellite cells on freshly isolated muscle fibers (Cornelison and Wold, 1997). However, MyoD and Myf5 are highly expressed in primary myoblasts.

Taken together, these observations therefore suggest that satellite cells undergo osteogenic and adipogenic differentiation from a plastic progenitor rather than transdifferentiation of a committed myogenic cell.

Experiments using gene knock-out mice demonstrated that myogenic progenitors lacking expression of MyoD and Myf5 differentiate into non-muscle cell lineages such as cartilage or dermis during embryogenesis (Tajbakhsh, 1996b; Kablar et al., 1999). Primary myoblasts lacking *MyoD* but expressing Myf5 did not exhibit increased osteogenic or adipogenic differentiation capabilities, compared to wild-type cells. Therefore, MyoD expression alone may not suppress either osteogenic or adipogenic differentiation of primary myoblasts, while MyoD has been shown to be required for myogenic differentiation of satellite cells (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999; Cornelison et al., 2000). Since Myf5 expression is up-regulated in *MyoD-/-* primary satellite cells compared to wild-type cells (Sabourin et al., 1999), it is possible to hypothesize that Myf5 expression may partially suppress non-myogenic differentiation of *MyoD-/-* primary satellite cells. In support of this, satellite cells on freshly isolated muscle fibers, in which expression of MyoD or Myf5 is undetectable (Cornelison and Wold, 1997), readily undergo both adipogenic and osteogenic differentiation. Interestingly, ectopic expression of Msx1, a homeobox type transcription factor, in C2C12 cells suppresses expression of muscle-specific bHLH genes and induces de-differentiation of multinucleated myotubes. The de-differentiated myotube-derived mononuclear cells as well as Msx1-induced myoblasts also exhibit an enhanced capacity to differentiate into osteocytes and adipocytes, compared to their parent cells (Odelberg et al., 2000). Since Msx1 expression is detectable in satellite cells on freshly isolated muscle fibers but down-regulated in activated satellite cells in culture (Cornelison et al., 2000), Msx1 may play a crucial role in the differentiation plasticity of satellite cells.

Several *in vivo* observations have suggested the existence of mesenchymal progenitors within skeletal muscles. For example, expansion of adipose tissue within skeletal muscles occurs in response to denervation (Dulor et al., 1998) and in some muscle diseases including muscular dystrophy (Lin et al., 1969) and mitochondrial myopathy (DiMauro et al., 1980). Similarly, a replacement of muscle mass by adipose is observed in compound mutant mice (*MyoD-/-:Myf5-/-*) (Rudnicki et al., 1993). Moreover, transplantation of BMP into adult skeletal muscles induces ectopic osteogenesis within these muscles (Urist and Strates, 1971). Similarly, BMP2/4 have been implicated in ectopic ossification of muscles in a human disease, Fibrodysplasia Ossificans Progressiva (FOP) (Shafritz et al., 1996).

Recent work has revealed a novel stem cell population in skeletal muscle called muscle-derived stem cells, isolated by fluorescence activated cell sorting (FACS) of side population (SP) cells, on the basis of Hoechst dye exclusion. Muscle-derived stem cells exhibit the capacity to reconstitute the entire hematopoietic repertoire and to participate in the formation of new myofibers following tail vein injection into lethally irradiated mice (Gussoni et al., 1999; Jackson et al., 1999). However, muscle SP cells are present in *Pax7*-deficient muscle that entirely lacks satellite cells (Seale et al., 2000), indicating that muscle-derived stem cells represent a distinct cell population from satellite cells. It remains unknown whether muscle-derived stem cells are also capable of differentiation into osteogenic and adipogenic lineages. Further experimentation will elucidate whether satellite cells or muscle-derived stem cells are the origin of those adipose cells and osteocytes *in vivo*.

In conclusion, we present direct evidence that muscle satellite cells display multipotential mesenchymal stem cell activity and are capable of forming osteocytes and adipocytes as well as myocytes.

Acknowledgements We thank Shahragim Tajbakhsh for providing *Myf5-nlacZ* strain of mice. We thank Sela J. Cheifetz for providing primers. We also thank Patrick Seale for critical reading of the manuscript. This work was supported by grants to M.A.R. from the Muscular Dystrophy Association, the National Institutes of Health, the Canadian Institutes of Health Research, and the Canada Research Chair Program. A.A. is supported by Development Grant from the Muscular Dystrophy Association. M.A.R. holds the Canada Research Chair in Molecular Genetics.

References

Asahina, I., Sampath, T.K. and Hauschka, P.V. (1996) Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp Cell Res 222:38–47.

Asakura, A., Lyons, G.E. and Tapscott, S.J. (1995) The regulation of MyoD gene expression: conserved elements mediate expression in embryonic axial muscle. Dev Biol 171:386–398.

Beauchamp, J.R., Heslop, L., Yu, D.S.W., Tajbakhsh, S., Kelly, R.G., Wernig, A., Buckingham, M.E., Partridge, T.A. and Zammit, P.S. (2000) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151:1221–1234.

Brand-Saberi, B., Wilting, J., Ebensperger, C. and Christ, B. (1996) The formation of somite compartments in the avian embryo. Int J Dev Biol 40:411–420.

Cooper, R.N., Tajbakhsh, S., Mouly, V., Cossu, G., Buckingham, M. and Butler-Browne, G.S. (1999) *In vivo* satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112:2895–2901.

Cornelison, D.D., Olwin, B.B., Rudnicki, M.A. and Wold, B.J. (2000) MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. Dev Biol 224: 122–137.

Cornelison, D.D. and Wold, B.J. (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191:270–283.

DiMauro, S., Mendell, J.R., Sahenk, Z., Bachman, D., Scarpa, A., Scofield, R.M. and Reiner, C. (1980) Fatal infantile mitochondrial myopathy and renal dysfunction due to cytochrome-c-oxidase deficiency. Neurology 30: 795–804.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L. and Karsenty, G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Dulor, J.P., Cambon, B., Vigneron, P., Reyne, Y., Nougues, J., Casteilla, L. and Bacou, F. (1998) Expression of specific white adipose tissue genes in denervation-induced skeletal muscle fatty degeneration. FEBS Lett 439:89–92.

Eguchi, G. and Kodama, R. (1993) Transdifferentiation. Curr Opin Cell Biol 5:1023–1028.

Fleet, J.C. and Hock, J.M. (1994) Identification of osteocalcin mRNA in nonosteoid tissue of rats and humans by reverse transcription-polymerase chain reaction. J Bone Miner Res 9:1565–1573.

Grounds, M.D., Garrett, K.L., Lai, M.C., Wright, W.E. and Beilharz, M.W. (1992) Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. Cell Tissue Res 267:99–104.

Grounds, M.D. and Yablonka-Reuveni, Z. (1993) Molecular and cell biology of skeletal muscle regeneration. Mol Cell Biol Hum Dis Ser 3:210–256.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M. and Mulligan, R.C. (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394.

Hansen, J.B., Petersen, R.K., Larsen, B.M., Bartkova, J., Alsner, J. and Kristiansen, K. (1999) Activation of peroxisome proliferator-activated receptor gamma bypasses the function of the retinoblastoma protein in adipocyte differentiation. J Biol Chem 274:2386–2393.

Jackson, K.A., Mi, T. and Goodell, M.A. (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci USA 96:14482–14486.

Kablar, B., Krastel, K., Ying, C., Tapscott, S.J., Goldhamer, D.J. and Rudnicki, M.A. (1999) Myogenic determination occurs independently in somites and limb buds. Dev Biol 206:219–231.

Kablar, B., Tajbakhsh, S. and Rudnicki, M.A. (2000) Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factor-dependent. Development 127:1627–1639.

Katagiri, T., Akiyama, S., Namiki, M., Komaki, M., Yamaguchi, A., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A. and Suda, T. (1997) Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. Exp Cell Res 230:342–351.

Katagiri, T., Yamaguchi, A., Ikeda, T., Yoshiki, S., Wozney, J.M., Rosen, V., Wang, E.A., Tanaka, H., Omura, S. and Suda, T. (1990) The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172:295–299.

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A. and Suda, T. (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. and Kishimoto, T. (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Lee, J.Y., Qu-Petersen, Z., Cao, B., Kimura, S., Jankowski, R., Cummins, J., Usas, A., Gates, C., Robbins, P., Wernig, A. and Huard, J. (2000) Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol 150:1085–1100.

Lee, K.S., Kim, H.J., Li, Q.L., Chi, X.Z., Ueta, C., Komori, T., Wozney, J.M., Kim, E.G., Choi, J.Y., Ryoo, H.M. and Bae, S.C. (2000) Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2 and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783–8792.

Liechty, K.W., MacKenzie, T.C., Shaaban, A.F., Radu, A., Moseley, A.M., Deans, R., Marshak, D.R. and Flake, A.W. (2000) Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med 6:1282–1286.

Lin, C.H., Hudson, A.J. and Strickland, K.P. (1969) Fatty acid metabolism in dystrophic muscle in vitro. Life Sci 8:21–26.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E. and Rudnicki, M.A. (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev 10:1173–1183.

Odelberg, S.J., Kollhoff, A. and Keating, M.T. (2000) Dedifferentiation of mammalian myotubes induced by msx1. Cell 103:1099–1109.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B. and Owen, M.J. (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. and Marshak, D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Prockop, D.J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74.

Ross, S.E., Hemati, N., Longo, K.A., Bennett, C.N., Lucas, P.C., Erickson, R.L. and MacDougald, O.A. (2000) Inhibition of adipogenesis by Wnt signaling. Science 289:950–953.

Rudnicki, M.A., Braun, T., Hinuma, S. and Jaenisch, R. (1992) Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71:383–390.

Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H. and Jaenisch, R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75:1351–1359.

Sabourin, L.A., Girgis-Gabardo, A., Seale, P., Asakura, A. and Rudnicki, M.A. (1999) Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol 144:631–643.

Seale, P. and Rudnicki, M.A. (2000) A new look at the origin, function, and "Stem-Cell" status of muscle satellite cells. Dev Biol 218:115–124.

Seale, P., Sabourin, L.A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M.A. (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102:777–786.

Shafritz, A.B., Shore, E.M., Gannon, F.H., Zasloff, M.A., Taub, R., Muenke, M. and Kaplan, F.S. (1996) Overexpression of an osteogenic morphogen in fibrodysplasia ossificans progressiva. N Engl J Med 335:555–561.

Smith, C.K., 2nd, Janney, M.J. and Allen, R.E. (1994) Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol 159:379–385.

Sodek, J., Chen, J., Nagata, T., Kasugai, S., Todescan, R., Jr., Li, I.W. and Kim, R.H. (1995) Regulation of osteopontin expression in osteoblasts. Ann NY Acad Sci 760:223–241.

Tajbakhsh, S., Bober, E., Babinet, C., Pournin, S., Arnold, H. and Buckingham, M. (1996a) Gene targeting the myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fibres as well as early embryonic muscle. Dev Dyn 206:291–300.

Tajbakhsh, S., Rocancourt, D. and Buckingham, M. (1996b) Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf-5 null mice. Nature 384:266–270.

Taylor, S.M. and Jones, P.A. (1979) Multiple new phenotypes in-

duced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.

Teboul, L., Gaillard, D., Staccini, L., Inadera, H., Amri, E.Z. and Grimaldi, P.A. (1995) Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. J Biol Chem 270:28183–28187.

Urist, M.R. and Strates, B.S. (1971) Bone morphogenetic protein. J Dent Res 50:1392–1406.

Yablonka-Reuveni, Z., Rudnicki, M.A., Rivera, A.J., Primig, M., Anderson, J.E. and Natanson, P. (1999) The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev Biol 210:440–455.

Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J.M., Rosen, V., Wang, E.A., Kahn, A.J., Suda, T. and Yoshiki, S. (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. J Cell Biol 113:681–687.