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Biochemical and Biophysical Research Communications 318 (2004) 119–124
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Isolation and enrichment of skeletal muscle progenitor cells from mouse bone marrow

Satyakam Bhagavati* and Weimin Xu

Department of Neurology, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, USA

Received 25 March 2004

Abstract

There is great interest in the therapeutic potential of non-hematopoietic stem cells obtained from bone marrow called mesenchymal stem cells (MSCs). Rare myogenic progenitor cells in MSC cultures have been shown to convert into skeletal muscle cells in vitro and also in vivo after transplantation of bone marrow into mice. To be clinically useful, however, isolation and expansion of myogenic progenitor cells is important to improve the efficacy of cell transplantation in generating normal skeletal muscle cells. We introduced into MSCs obtained from mouse bone marrow, a plasmid vector in which an antibiotic (Zeocin) resistance gene is driven by MyoD and Myf5 enhancer elements, which are selectively active in skeletal muscle progenitor cells. Myogenic precursor cells were then isolated by antibiotic selection, expanded in culture, and shown to differentiate appropriately into multinucleate myotubes in vitro. Our results show that using a genetic selection strategy, an enriched population of myogenic progenitor cells, which will be useful for cell transplantation therapies, can be isolated from MSCs.

Keywords: Mesenchymal stem cell; Myogenic precursors; Skeletal muscle cells

Bone marrow contains hematopoietic stem cells which provide a continuous source of progenitors for all hematopoietic cells. Several reports have shown that bone marrow also contains stem-like cells for non-hematopoietic mesenchymal tissues, called mesenchymal stem cells (MSCs) [1–3]. MSCs are progenitors of fat, cartilage, and bone [1–3] and in addition have been experimentally induced to undergo unorthodox differentiation, occasionally forming neural [4] and skeletal muscle cells [5]. Much interest has been directed at the possibility of using MSCs for cell transplantation therapies to repair disease and damaged tissues, such as skeletal muscle. However, in vitro differentiation of MSCs into skeletal muscle cells is inconsistent and infrequent [2]. This matches in vivo data, where only very limited engraftment of transplanted bone marrow derived muscle progenitors into mouse skeletal muscle has been described [6,13,14].

A reliable and consistent method for the isolation of skeletal muscle progenitor cells from bone marrow MSCs is important to permit expansion of these cells ex vivo for eventual use for cell transplantation therapies to repair diseased or damaged skeletal muscle. We report here the isolation of myogenic progenitor cells on the basis of the expression of Myf5 and MyoD. Myf5 and MyoD (along with myogenin and MRF4) are basic helix–loop–helix transcription factors required both for the commitment of mesodermal progenitors to myoblasts and subsequent differentiation of committed myoblasts to contractile myotubes [7,8]. Myf5 transcripts appear prior to those of the other three myogenic factor genes in newly formed somites of mouse embryos, followed by the expression of MyoD [7]. Transcriptional enhancer elements have been identified which regulate the embryonic expression of Myf5 and MyoD genes. Myf5 expression in the somite epaxial muscle progenitor cells in the mouse embryo has been shown to be activated by a 651 bp transcriptional enhancer [9], and muscle specific expression of MyoD in mouse embryos has been shown to be activated by a 258 bp enhancer [10].

We isolated continuously growing MSC cultures on the basis of their enhanced adhesiveness to plastic in tissue culture plates [2], and confirmed, as previously

* Corresponding author. Fax: 1-718-270-3840.
E-mail address: satyakamb@hotmail.com (S. Bhagavati).

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doi:10.1016/j.bbrc.2004.03.192

described [1–3], the ability of these MSCs to differentiate into fat, cartilage or bone. Next, to isolate myogenic progenitor cells we generated stably transfected MSC clones, using a vector containing the Myf5 and MyoD enhancers described above, driving the expression of a Zeocin resistance gene. Zeocin selection resulted in the isolation of several clones which on expansion were found to have an enriched population of myogenic precursor cells and could be differentiated into multinucleate myotubes. Thus, using this genetic selection strategy, myogenic progenitor cells can be isolated from MSC cultures and expanded and differentiated into skeletal muscle cells in vitro.

Materials and methods

Isolation and culture of mouse MSCs

Two 6 weeks old Balb/c mice were killed and both femurs and tibia were flushed with BMC medium, centrifuged at 4000 rpm for 4 min, resuspended in BMC medium [11] (60% DMEM-LG, 40% MCDB-201, 1× insulin–transferrin–selenium, 1× lenolenic acid/bovine serum albumin, 10^{-9} M dexamethasone, and 10^{-4} M ascorbic acid 2-phosphate) (Gibco-BRL, NY and Sigma, St. Louis, MO) with 2% fetal calf serum (GIBCO, NY) and with 10 ng/ml of epidermal growth factor (EGF), 10 ng/ml of platelet derived growth factor (PDGF-BB), and 1000 U/ml of leukemia inhibitory factor (LIF) (Chemicon, CA), and plated onto fibronectin coated tissue culture plates. Supernatant was discarded after 4 h and again the next day and cultures were maintained at 2×10^3 cells/cm^2 by sub-culturing every 2–3 days at a 1:2 dilution. A continuous growing culture was obtained after 2–3 weeks. MSCs have been cultured for over 40 population doublings using this method. Cell morphology was observed and pictures were taken after 4 weeks in culture under phase contrast using an inverted microscope.

Vector construction

We constructed a vector carrying the Myf5 and MyoD enhancer elements driving the expression of the Zeocin resistance gene (PZMDMYF) (Fig. 2A). A 258 bp core enhancer element from the MyoD gene and a 651 bp Myf5 enhancer element were amplified by PCR from mouse genomic DNA. The 651 bp Myf5 enhancer element was inserted into the *Ssp*I site of pZeoSV plasmid (Invitrogen) and the 258 bp MyoD enhancer was inserted into the *Acl*I site of the same plasmid. The MyoD enhancer element has been shown to force skeletal muscle expression of MyoD on heterologous promoters. The Myf5 and MyoD muscle enhancer elements were placed upstream of the CMV promoter to drive expression of the Zeocin resistance gene.

Confirmation of selective efficacy of PZMDMYF plasmid in skeletal muscle cells

Plasmid PZMDMYF was linearized and electroporated into two muscle cell lines, C2C12 (mouse muscle) and RD-1 (rhabdomyosarcoma) and two non-muscle cell lines, HepG2 (hepatic cell line) and normal mouse fibroblasts. Two days after electroporation, Zeocin 250 μg/ml selection was started and stably transfected clones were obtained after 2–3 weeks.

Selection and isolation of muscle progenitor cells in MSC cultures

The vector PZMDMYF constructed as described above (Fig. 2A) was linearized with *Apa*I, electroporated into bone marrow MSC cultures, and 2 days later subjected to Zeocin 250 μg/ml selection for 2–4 weeks to select for muscle progenitor cells. Four of seven surviving Zeocin resistant clones (MSC-PZ) were expanded in proliferation medium (BMC medium, 2% FCS, EGF, PDGF-BB, and LIF) and checked for differentiation into skeletal muscle cells in differentiation medium (BMC medium, 2% FCS, and 2% horse serum) after 1–2 weeks. All four clones were also grown in the following culture conditions to determine if the number of skeletal muscle cells detected could be increased: (1) BMC medium, 2% FCS, and sonic hedgehog (SHH) (200 ng/ml), (2) BMC medium, 2% FCS, insulin-like growth factor-1 (IGF-I) (40 ng/ml), transforming growth factor β-I (2 ng/ml), and basic fibroblast growth factor(bFGF1) (30 ng/ml).

Identification of skeletal muscle cells in Zeocin selected MSC-PZ cultures

RT-PCR. RNA was isolated from MSC-PZ clones, DNase treated, and cDNA synthesized. PCR was performed using the following primers (expected PCR product size), myf5, 5′-GCTGAGGGAACAGGTGGAGA-3′, 5′-GGAGTGATCATCGGGAGAGAG (379 bp); myogenin, 5′-CCAGCGAGGGAATTTAGCTGACTC-3′, 5′-CCCTGCCTGTTCCCGGTATCATCA-3′ (444 bp); desmin, 5′-TCTACGAGGAGGAGATGCGC-3′, 5′-GGACCTGCTGTTCCTGAAGC-3′ (317 bp); and myoD, 5′-TCTTCCCAACTGTCCTTTCGA-3′, 5′-GATTTCCAACACCTGACTCGC-3′ (570 bp).

Immunocytochemistry. Continuously growing mouse marrow MSC cultures were analyzed with the following antibodies: anti-mouse H-2K^k (MHC Class I), rat anti-mouse CD45, rat anti-mouse CD44, rat anti-mouse Ly-6G, and rat anti-mouse TER-119 (all from BD Pharmingen, CA). Proliferating or differentiated Zeocin resistant MSC-PZ clones were analyzed with the following antibodies: rabbit polyclonal anti-myf5 (Santa Cruz, CA), monoclonal anti-desmin (Sigma, MO). FITC or Cy3 conjugated secondary anti-mouse or anti-rat or anti-rabbit IgG was obtained from Jackson Immunoresearch, PA or Sigma, MO. Cells were visualized by fluoroscopy and the percentage of Myf5 positive cells in all four MSC-PZ clones was calculated by counting positively stained cells in 20 randomly chosen 20× fields in four separate cultures for each clone.

Results

Culture of undifferentiated mouse bone marrow derived MSC cultures

Mesenchymal stem cells (MSCs) from mouse bone marrow were isolated based on the enhanced adherence of MSCs to plastic in tissue culture plates [2]. After 2–3 weeks in culture a continuously growing population of cells was obtained. Cells were small and spindle shaped with a doubling time of 18–24 h and were grown continuously for 40 population doublings with no changes in morphology (Fig. 1A). Reverse transcription polymerase chain reaction (RT-PCR) of MSC cultures showed the presence of two transcription factors important in maintaining the undifferentiated state in embryonic stem cells, Oct-4 and Rex-1 (Fig. 1B). These factors have also been shown to be expressed in murine marrow derived multipotent adult progenitor cells [11]. The cultured MSCs were CD44, CD45, major histocompatibility complex (MHC) class I, TER-119, and

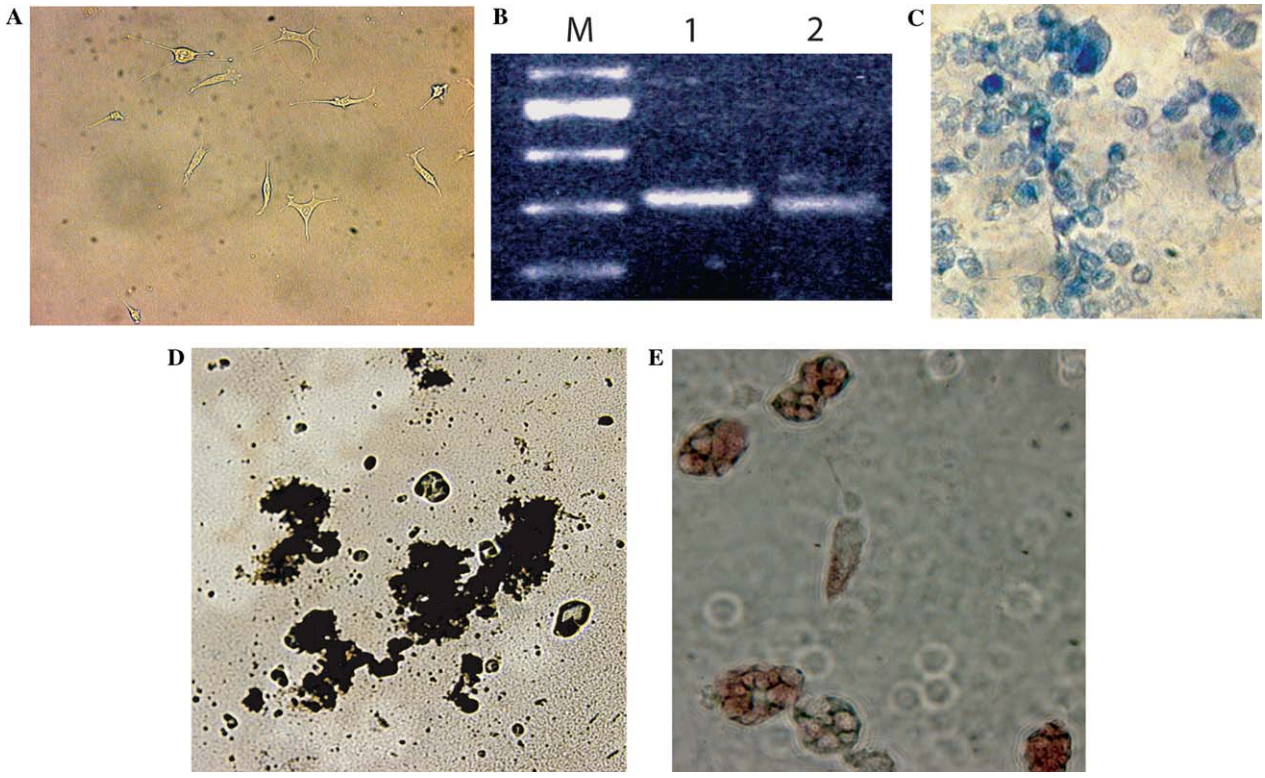

Fig. 1. (A,B) Bone marrow derived mesenchymal stem cell cultures (MSCs). (A) Morphology of typical MSCs after 3–4 weeks in culture. Cells are small and spindle or stellate in shape. (B) RT-PCR analysis of MSCs shows expression of lane 1: Oct-4 and lane 2: Rex-1. (C–E) Differentiation of MSCs into cells of mesenchymal lineage: (C) cartilage formation shown by toluidine blue staining, (D) Von Kossa staining of calcium phosphate crystals shows bone formation, and (E) lipid laden cells stained with oil-red-O.

Ly-6G negative, indicating the absence of hematopoietic cells or their precursors in the MSC cultures. We confirmed that the MSCs have the potential to develop into fat, cartilage, and bone (Figs. 1C–E) as described previously [1–3].

Confirmation that plasmid PZMDMYF is selectively active in skeletal muscle cells

Plasmid PZMDMYF (Fig. 2A) was electroporated into two muscle cell lines, C2C12 and RD-1 and two non-muscle cell lines, HepG2 (hepatic cell line) and normal mouse fibroblasts. Under Zeocin 250 μg/ml selection, rapid death was seen in fibroblasts and HepG2 cells with no surviving cells after 2–3 weeks. In contrast, numerous (18–20) resistant colonies were seen growing vigorously under Zeocin selection in the muscle cell lines C2C12 and RD-1. Thus, the MyoD and Myf5 enhancers drive expression of the Zeocin resistance gene selectively in skeletal muscle cells.

Isolation of skeletal muscle progenitor cells from MSC cultures

We isolated myogenic precursor cells in our MSC cultures based on their expression of Myf5 and MyoD. In MSC cultures stably transfected with the PZMDMYF vector, the Myf5 and MyoD enhancers drive expression of the Zeocin resistance gene. After Zeocin selection for 2–4 weeks, seven Zeocin resistant colonies were obtained and four clones were expanded and analyzed further. RT-PCR analysis of MSC-PZ clones expanded in the presence of EGF, PDGF-BB, and LIF showed the expression of multiple muscle specific genes, Myf5, MyoD, myogenin, and desmin (Fig. 2B). MSC-PZ clones growing in proliferation medium were also analyzed using anti-myf5 antibody. Nuclear expression of Myf5 transcription factor was seen in $35 \pm 3\%$, $39 \pm 5\%$, $38 \pm 2\%$, and $46 \pm 4\%$ of cells (Fig. 2C) in the clones examined. Myf5 negative non-muscle cells had the morphology of undifferentiated MSCs. Analysis of MSC-PZ cultures maintained in proliferation medium showed cytoplasmic expression of desmin in mononuclear myoblasts (Fig. 3A) and after 1 week in differentiation medium, in multinucleate myotubes (Figs. 3B and C). The morphology of the myotubes was similar to that of mouse muscle cells in culture. Occasionally waves of visible contractions of the multinucleated myotubes were seen. Varying culture conditions by using different growth factor combinations did not result in more efficient muscle cell isolation.

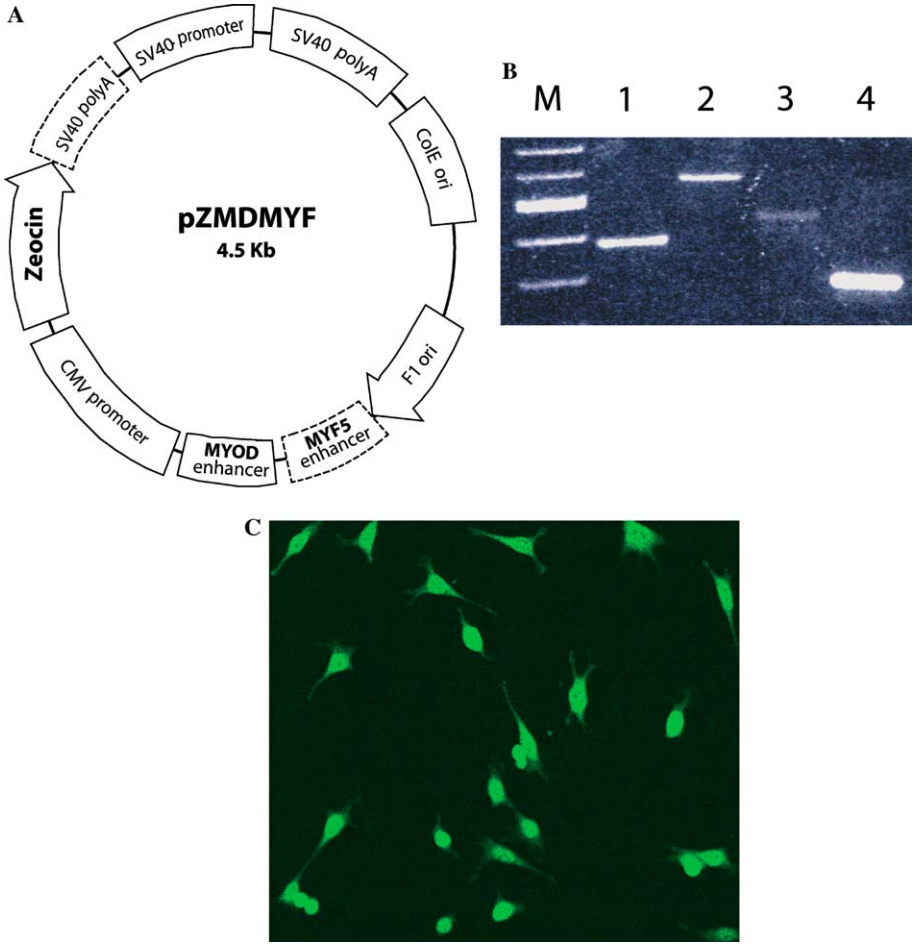

Fig. 2. (A) Vector PZMDMYF. Two hundred and fifty-eight base pairs of MyoD enhancer and a 651 bp Myf5 enhancer placed upstream of a CMV promoter driving the expression of a Zeocin resistance gene in plasmid pZeoSV (Invitrogen, CA). (B) RT-PCR analysis of MSC-PZ cultures shows expression of muscle specific genes in: lane 1, Myf5 (379 bp); lane 2, MyoD (570 bp); lane 3, myogenin (444 bp); and lane 4, desmin (317 bp). (C) MSC-PZ cultures obtained after 3 weeks Zeocin selection in proliferation medium show nuclear expression of Myf5 protein.

Discussion

Bone marrow derived MSCs have generated great interest as a potential source of multipotent progenitor cells which may eventually be useful for cell transplantation in humans for the repair of diseased or damaged tissues [11,12]. MSCs have been shown to consistently generate fat, cartilage, and bone and rarely skeletal muscle and neural cells, in vitro [1–5]. To be useful for cell transplantation therapies for muscle diseases such as dystrophies, however, a pure or enriched population of myogenic precursor cells is necessary. In addition, myogenic progenitor cells could be expanded ex vivo and transplanted to introduce exogenous genes for the expression of therapeutic hormones or coagulation factors. However, in vitro derivation of skeletal muscle cells by chemical treatment is inconsistent and infrequent and transplantation of bone marrow into mice results in very limited engraftment of skeletal muscle [2,14]. New strategies are therefore needed to enrich for myogenic progenitor cells from bone marrow.

In one strategy used to isolate cells of interest from a heterogeneous cell population, plasmid vectors active only in specific subsets of cells have been used to isolate and expand specific cell populations [15,16]. Examples of this method include studies in which embryonic stem cell clones were obtained in which the β-galactosidase or green fluorescent protein gene was expressed selectively as reporter genes in cardiac muscle cells [15,16]. We report here the use of a similar strategy to isolate and enrich skeletal muscle cells from mouse bone marrow derived MSC cultures. MSC cultures were stably transfected with a plasmid engineered to contain Myf5 and MyoD enhancer elements. These transcriptional enhancers drive the expression of an antibiotic (Zeocin) resistance gene in the infrequent myogenic progenitor cells in MSC cultures and not in cells of other lineages in MSCs. Myogenic precursors obtained under positive Zeocin selection were shown to express the transcription factor Myf5, which is one of the earliest genes to be expressed during mammalian embryonic myogenesis and were shown to differentiate into desmin positive

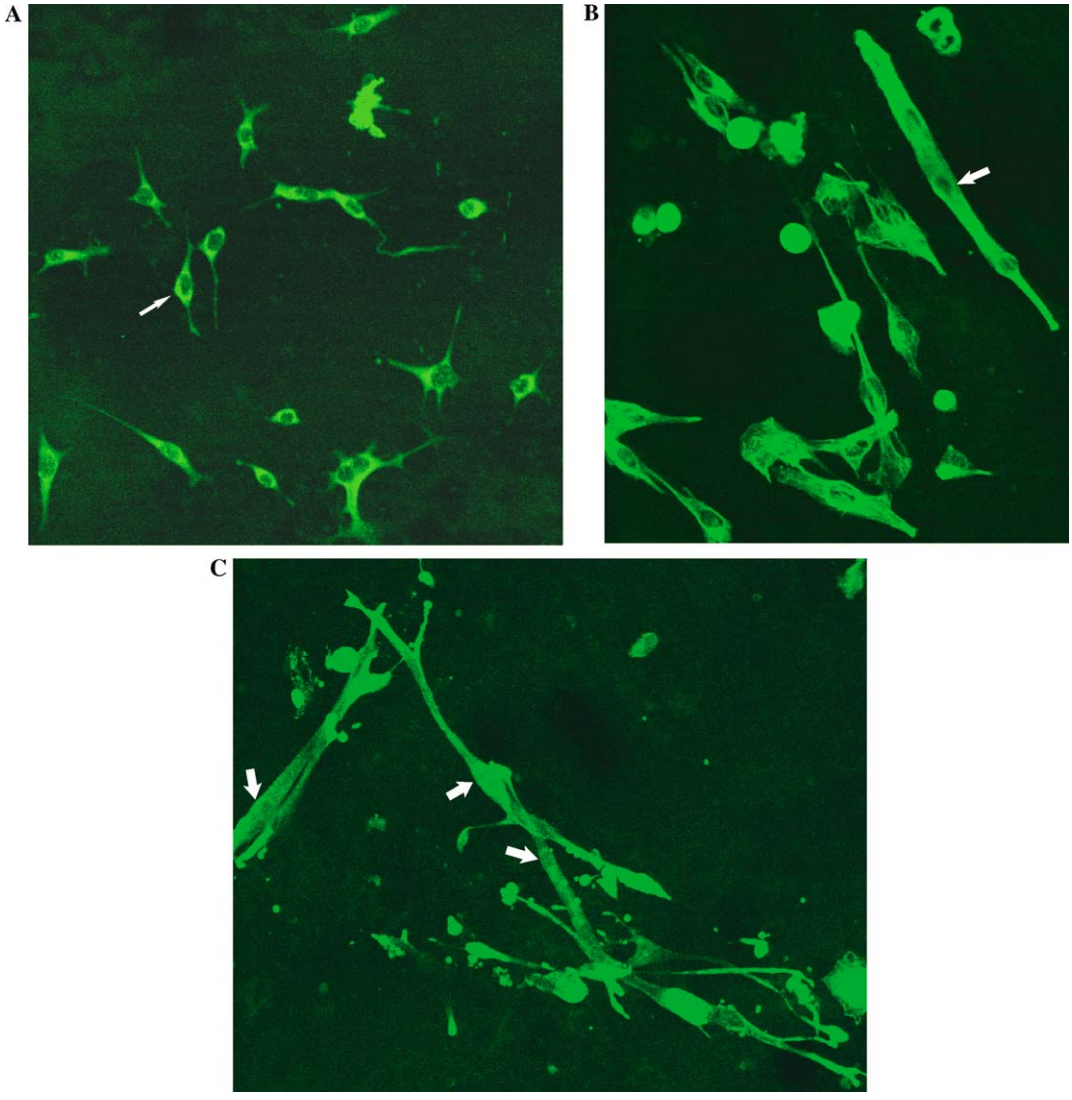

Fig. 3. Desmin expression in MSC-PZ cultures grown in: (A) proliferation medium (arrow shows representative mononuclear myoblast with cytoplasmic expression of desmin) and (B,C) in differentiation medium showing desmin expression in multinucleate myotubes (arrows).

multinucleate myotubes. However, expansion of Zeocin resistant clones always resulted in a mixture of muscle and non-muscle cells and a pure population of muscle cells was not obtained. The continued presence of non-muscle cells even after prolonged Zeocin selection implies that stem and progenitor cells are in a dynamic state, constantly changing in response to the microenvironment and cell density, and can maintain the myogenic phenotype only under the effect of specific growth factor signals which are yet undefined. In spite of this limitation, the level of enrichment we obtain using this technique (35–46%) is significant as the skeletal muscle differentiation pathway is rarely successfully obtained from bone marrow MSCs. Future improvements, when growth factors which selectively promote myogenic development and differentiation are identified, should result in improvements in this technique.

Successful development of stem cell based therapies for human diseases requires purifying the desired cell lineage from mixed cell populations and demonstrating that the cells are capable of normal differentiation. The results provided here show that, using a genetic selection strategy, a population of cells enriched in vitro for myogenic precursor cells can be obtained from bone marrow derived MSCs, expanded in culture, and differentiated normally. The transplantation of such bone marrow derived muscle progenitor cell populations will have an advantage over myoblast transplantation for the treatment of muscular dystrophies, where the systemic delivery of normal skeletal muscle cells throughout the body is crucial. Enriched preparations of myogenic precursor cells, obtained as described here and then expanded and purified further by removing non-myogenic cells, should prove useful for cell

transplantation therapies in muscular dystrophies or for the introduction of exogenous therapeutic genes. Similar strategies may also prove useful in obtaining myogenic precursor cells from embryonic stem cells.

Acknowledgment

This work is supported by a grant from the NIH to S.B.

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