Mesenchymal Stem Cells from Adult Human Bone Marrow Differentiate into a Cardiomyocyte Phenotype *In Vitro*

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A method for isolating adult human bone marrow mesenchymal stem cells (MSCs) was established, and the ability of human MSCs to differentiate into cells with characteristics of cardiomyocytes *in vitro* was investigated. Selected MSC surface antigens were analyzed by flow cytometry. The MSCs at Passage 2 were treated with 5-azacytidine to investigate their differentiation into cardiomyocytes. Characteristics of the putative myogenic cells were determined by immunohistochemistry and transmission electron and confocal microscopies. The expression of myogenic specific genes was detected by reverse transcriptase-polymerase chain reaction (RT-PCR), real-time quantitative PCR, and DNA sequencing. The MSCs were spindle-shaped with irregular processes and were respectively positive for CD_{13}, CD_{29}, CD_{44}, CD_{71} and negative for CD_3, CD_{14}, CD_{15}, CD_{33}, CD_{34}, CD_{38}, CD_{45}, and HLA-DR. The myogenic cells differentiated from MSCs were positive for beta-myosin heavy chain (beta-MHC), desmin, and alpha-cardiac actin. When the myogenic cells were stimulated with low concentration of K^+ (5.0 m*M*), an increase in intracellular calcium fluorescence was observed. Myofilament-like structures were observed in electron micrographs of the differentiated myogenic cells. The mRNAs of beta-MHC, desmin, alpha-cardiac actin, and cardiac troponin T were highly expressed in the myogenic cells. These results indicate that 5-azacytidine can induce human MSCs to differentiate *in vitro* into cells with characteristics commonly attributed to cardiomyocytes. Cardiomyocytes cultured from bone marrow sources are potentially valuable for repairing injured myocardium. Exp Biol Med 229:623–631, 2004

Key words: mesenchymal stem cells; differentiation; cardiomyocytes

Introduction

Because adult cardiac muscle lacks the ability to regenerate damaged regions, postinfarction function is often seriously compromised and may lead to frank heart failure. Myocardial cell transplantation represents a potential therapy for treating heart failure and has generated significant interest in identifying cell types capable of restoring the injured myocardium (1, 2). Mesenchymal stem cells are bone marrow–derived cells that retain the ability to differentiate into various types of tissue cells and contribute to the regeneration of a variety of mesenchymal tissues including bone, cartilage, muscle, and adipose (1, 3, 4). Several research groups reported that 5-azacytidine induced murine or porcine mesenchymal stem cells (MSCs) to differentiate into cardiomyocytes *in vivo* or *in vitro* (5, 6).

Human MSCs have species-unrestricted immunomodulatory effects and the immunosuppressive properties (4) and have gained considerable attention due to their potential use for cell replacement therapy and tissue engineering. When human MSCs from adult bone marrow were transplanted directly into the adult murine myocardium, they differentiated into cardiomyocytes (2, 7). Another strategy is to differentiate adult bone marrow MSCs into cardiomyocytes *in vitro* prior to implantation. Because there was little information about the differentiation of adult human MSCs *in vitro*, the current study was conducted to investigate MSC differentiation into cardiomyocytes after treatment with 5-azacytidine.

Materials and Methods

Materials. Low-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum (HS) were purchased from Gibco (Grand Island, NY). Amphotericin, 5-azacytidine, Tyrode's balanced salt

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Table 1. Five Target Primers[a]

Target/control gene	Primer sequence (5'-3')	Amplicon size (bp)
Desmin	For: CCAACAAGAACAACGACG Rev:TGGTATGGACCTCAGAACC	408
Alpha-cardiac actin	For: GCCTTCCTCATTTAAAGCTC Rev: AACACCACTGCTCTAGCCACG	418
Beta-myosin heavy chain	For: GATCACCAACAACCCCTACG Rev: ATGCAGAGCTGCTCAAAGC	528
Cardiac troponin T	For: AGGCGCTGATTGAGGCTCAC Rev: ATAGATGCTCTGCCACAGC	416
GAPDH	For: CGGATTTGGTCGTATTGG Rev: TCAAAGGTGGAGGAGTGG	861

[a] *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; bp, base pairs.

solution, bFGF, trypsin-EDTA, mouse anti-human antibodies for desmin, beta-MHC, and alpha-cardiac actin were purchased from Sigma (St. Louis, MO). FITC-conjugated mouse anti-human antibodies (CD_3, CD_{14}, CD_{33}, CD_{34}, CD_{45}, CD_{71}, and HLA-DR), PE-conjugated mouse anti-human antibodies (CD_{13}, CD_{29}, CD_{38}, and CD_{44}), FITC-conjugated mouse IgG_1, and PE-conjugated mouse IgG_1 were purchased from Becton Dickinson (San Jose, CA). Trizol reagent and reverse transcriptase-polymerase chain reaction (RT-PCR) kit were purchased from Invitrogen (Carlsbad, CA). Primers were produced by Shanghai Bio-Engineering Company (Shanghai, China).

Isolation of Human MSCs. Human MSCs were isolated as described previously for rat bone marrow (8), with added modifications. Bone marrow from six healthy volunteers was aspirated from the posterior iliac crest and collected with added heparin (6000 U). Mononuclear cells were isolated by centrifugation through 1.073 g/ml Ficoll at 1100 *g* for 30 mins. The cells were rinsed twice with PBS and seeded at 1 to $2 \times 10^5/cm^2$ in complete medium (low-glucose DMEM, 10% FBS, 5% HS, 100 U/ml penicillin and streptomycin) at 37°C in a humid 5% CO_2 air atmosphere. Three days later, nonadherent cells (hematopoietic cells) were removed by replacing the medium. After 10 days in culture, adherent cells formed homogenous colonies. The adherent cells were resuspended after trypsin treatment and re-plated at a density of $8000/cm^2$ (approximately 1:3). The medium was changed every 3 days, and MSCs from Passage 2 were used for the differentiation studies.

Surface Antigens of Human MSCs. Human MSCs at Passage 2 were treated with 0.25% trypsin-EDTA, harvested, and washed twice with PBS. The cells were incubated on ice with labeled mouse anti-human antibodies for CD_{14}, CD_{15}, CD_{34}, CD_{71}, HLA-DR (FITC-conjugated), CD_3, CD_{13}, CD_{29}, CD_{33}, CD_{38}, CD_{44} (PE-conjugated), and CD_{45} (Percp-conjugated). Control groups were incubated with FITC- and PE-conjugated antibodies against mouse IgG_1. The labeled cells were analyzed by flow cytometry.

Myogenic Differentiation *In Vitro*. Human MSCs of the second passage were re-suspended after trypsin treatment and washed twice with Tyrode's balanced salt solution (Sigma). The cells were re-suspended in complete medium and seeded into 35-mm dishes at a density of 1×10^4 cells/dish. Twenty-four hours after seeding, the medium was changed to complete medium containing 5-azacytidine (10 μmol/l), bFGF (10 μg/l), and amphotericin (0.25 mg/l). After incubating for another 24 hrs, the cells were washed twice with Tyrode's balanced salt solution and the medium was changed to complete medium without 5-azacytidine and amphotericin. The medium was changed twice a week thereafter until the experiment was terminated 2 weeks after the drug treatment. After completing the protocol, aliquots of the cells were prepared for immunohistochemistry, transmission electron microscopy, confocal laser scanning microscopy, RT-PCR, and real-time quantitative RT-PCR.

Immunohistochemistry. The myogenic cells that differentiated from the MSCs adherent to chamber slides were fixed for 10 mins with methanol at –20°C. After washing three times with PBS, the cells were incubated at 4°C overnight with the primary antibodies directed against beta-MHC epitopes, alpha-cardiac actin, or desmin, respectively. The incubation with secondary-antibody was at 37°C for 30 mins. The reaction with the diaminobenzidine (DAB) reagent was 5–10 mins. The cells were mounted for microscopic examination with neutral gum. Cells with brown granular DAB reaction product in the cytoplasm were considered positive for the protein in question.

Transmission Electron Microscopy. Cells were washed three times with PBS (pH 7.4), fixed with PBS containing 2.5% glutaraldehyde for 2 hrs and embedded in epoxy resin. Ultra-thin sections were cut horizontally to the growing surface. The sections were double-stained in uranyl acetate and lead citrate prior to inspection in the transmission electron microscope.

Confocal Laser Scanning Microscopy. Cells induced with 5-azacytidine were plated on cover slips (0.5 cm × 0.5 cm) and were incubated with Fluo-3 and 5.0 m*M* KCl. The cellular morphology and Ca^{2+} transients were observed under confocal laser scanning microscope.

RT-PCR. Total RNA was extracted with Trizol reagent from both untreated MSCs and myogenic cells differentiated from MSCs. For RT-PCR, cDNA was

synthesized in a 20-μl reaction volume containing 4 μg of total RNA and SuperScript II RT, according to the instructions of the manufacturer. The endogenous "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was also quantified to normalize differences in the added RNA and efficiency of reverse transcription. The thermal profile for PCR was 94°C for 2 mins, followed by 35 cycles of 30 secs at 94°C, with 1 min annealing intervals (60°C for desmin, alpha-cardiac actin, and beta-MHC and 58°C for cardiac troponin T) followed by 1 min extension at 72°C. An additional 10-min incubation at 72°C was included after completion of the last cycle. The PCR products were size-fractioned by electrophoresis on 2% agarose gels. The five specific primers used are illustrated in Table 1.

Real-Time Quantitative PCR. Real-time quantitative PCR was conducted with SYBR Green. Reaction mixtures contained 0.2 μl of 100 × SYBR Green I (Biowhittaker Molecular Application, BMA), 1 μl cDNA , 5 pmol of each primer, 2 m*M* $MgCl_2$, 200 n*M* dNTP, and 1 unit of Taq DNA polymerase in a final volume of 25 μl. The temperature profiles were the same as listed above for RT-PCR. At the end of each cycle, the SYBR Green was measured spectroflurometrically.

Melting curve analyses were conducted after completion of the cycling process with the aid of a temperature ramp (from 45°C to 95°C at 0.5°C/2 sec) and continuous fluorescence monitoring. All reactions were independently repeated twice in duplicate to ensure the reproducibility of the results. Data were viewed and analyzed by using the Rotor-Gene Real-Time Analysis Software (Rotor-Gene 2000, CR, Sydney, Australia). Amplification plots and the corresponding dissociation curves were examined for each sample. External controls were constructed, consisting of cDNA plasmid standards (9) to obtain standardized quantitative results.

DNA Sequencing. The PCR products for human desmin and cardiac troponin T were inserted with the aid of DNA ligase into pMD18-T plasmid vectors. The plasmids were expanded in *Escherichia coli*, harvested, and the subsequent DNA sequencing of the PCR products was performed on a CEQ 2000XL system (Foster City, CA).

Statistics. All data were tested for Gaussian distribution, and statistical analyses were performed by paired *t* tests when applicable. Spearman's correlation coefficients were used to evaluate correlative relationships. Variables were described by mean ± standard deviation (SD). Statistical analysis was performed using the SAS v6.12 software (SAS Institute Inc., Cary, NC).

Results

The Characteristics of Human MSCs and Differentiated Myogenic Cells. After 3 days in primary culture, MSCs adhered to the plastic surface, presenting a small population of single cells. The cells were spindle-

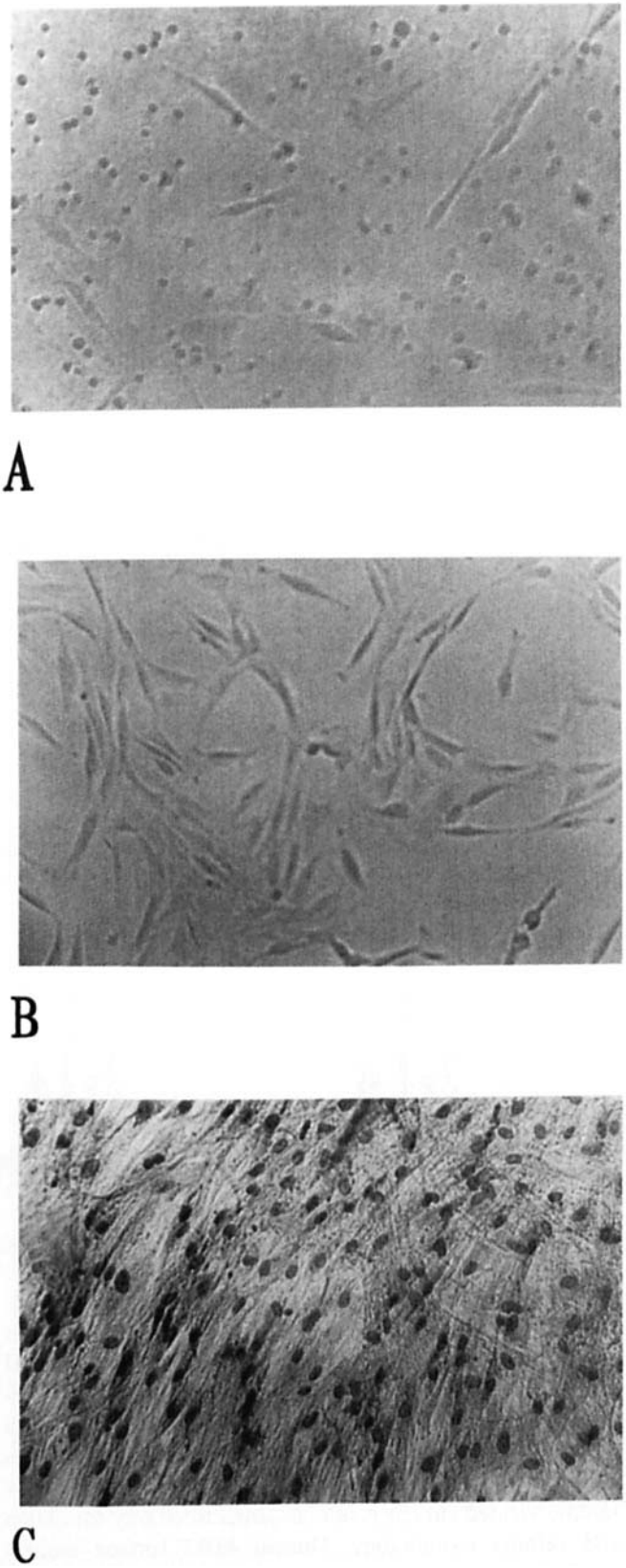


Figure 1. (A) mesenchymal stem cells (MSCs) from human bone marrow cultured for 3 days (magnification: ×100). (B) MSCs from human bone marrow cultured for 7 days (magnification: ×100). (C) MSCs stained by Giemsa cultured for 10 days (magnification: ×100).

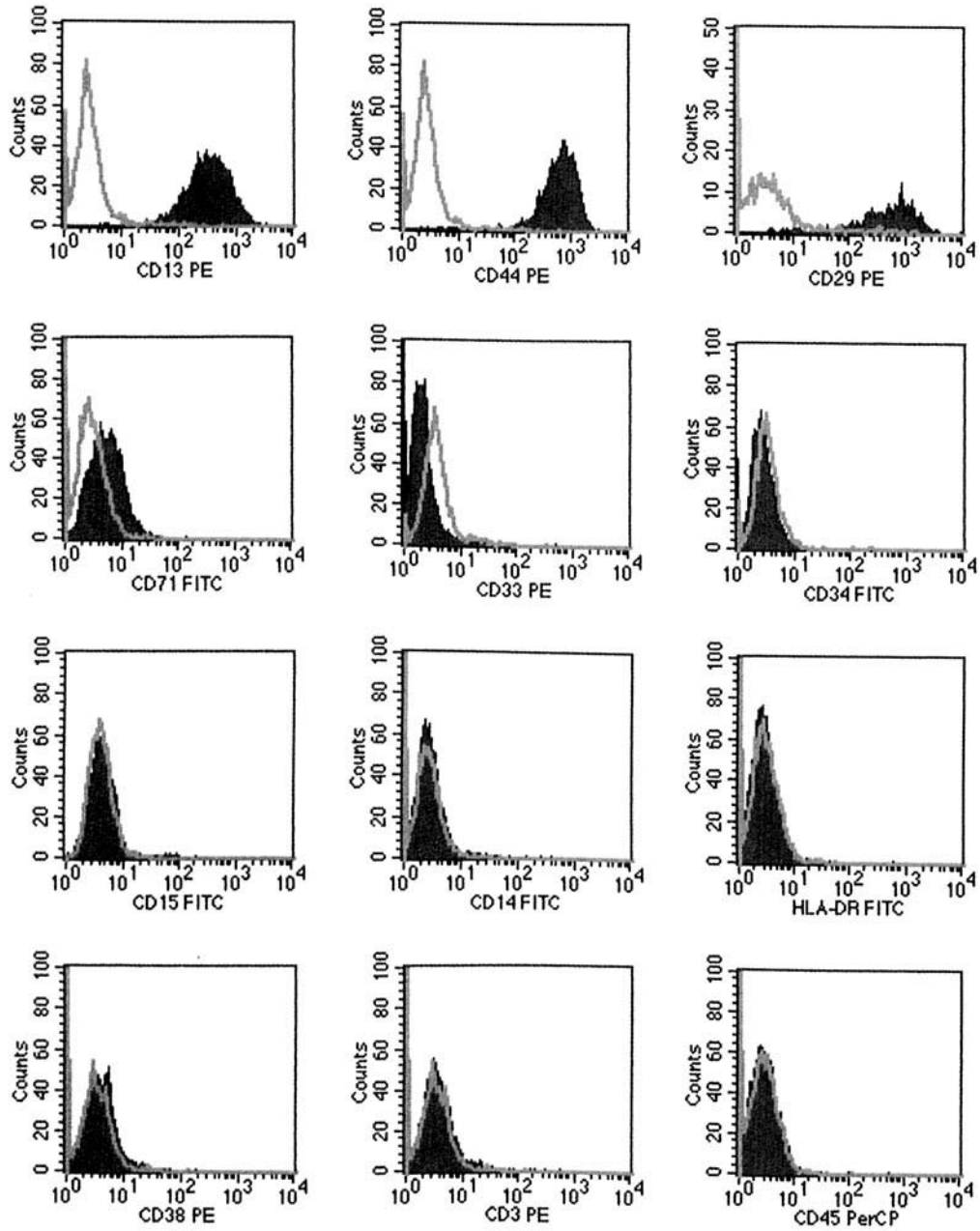
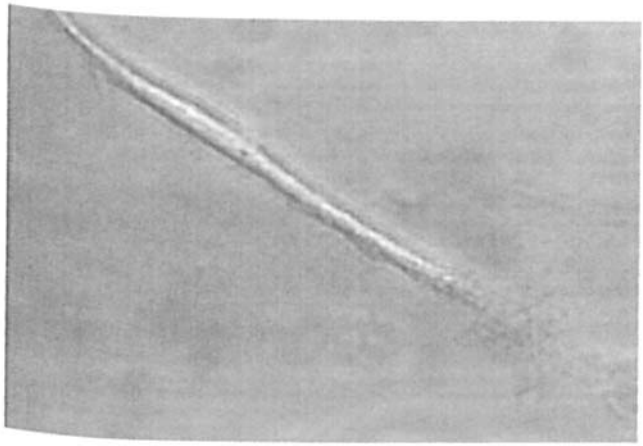


Figure 2. Surface antigens of human mesenchymal stem cells (MSCs) detected by flow cytometry (FCM). Human MSCs were positive for CD_{13}, CD_{29}, CD_{44}, CD_{71}, and negative for CD_3, CD_{14}, CD_{15}, CD_{33}, CD_{34}, CD_{38}, CD_{45}, and HLA-DR.

shaped with one nucleus (Fig. 1A and C). Seven to 10 days after initial plating, the cells looked like long spindle-shaped fibroblastic cells and began to form colonies. After re-plating, the cells were polygonal or spindle-shaped, with long processes (Fig. 1). Human MSC isolation experiments were conducted on six healthy volunteers. Each marrow sample yielded similar results in terms of colony formation and cellular morphology. Human MSC surface antigen profiles obtained by flow cytometry (Fig. 2) were positive for CD_{13}, CD_{29}, CD_{44}, CD_{71}, and negative for CD_3, CD_{14}, CD_{15}, CD_{33}, CD_{34}, CD_{38}, CD_{45}, and HLA-DR.

The morphological differentiation from MSCs to myogenic-like cells evolved gradually after 5-azacytidine induction. During exposure to 5-azacytidine, some adherent cells died, whereas the surviving cells began to proliferate and differentiate. One week later, approximately 30% of all of the remaining adherent cells had enlarged and had assumed ball-like or stick-like morphologies. Within 2–3 weeks, the cells connected with adjoining cells and formed myotube-like structures. The cells looked like long spindle-shaped fibroblastic cells when observed under confocal laser scanning microscope (Fig. 3).

Immunohistochemistry. About 80% of the resulting myogenic cells differentiated from MSCs were positive for desmin (Fig. 4A), beta-myosin heavy chain (Fig. 4B),



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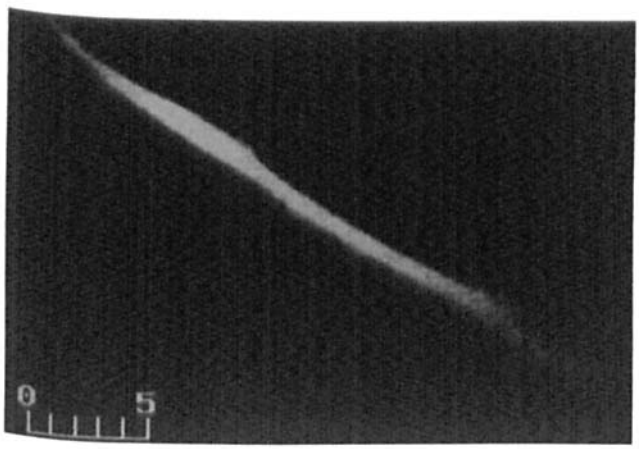
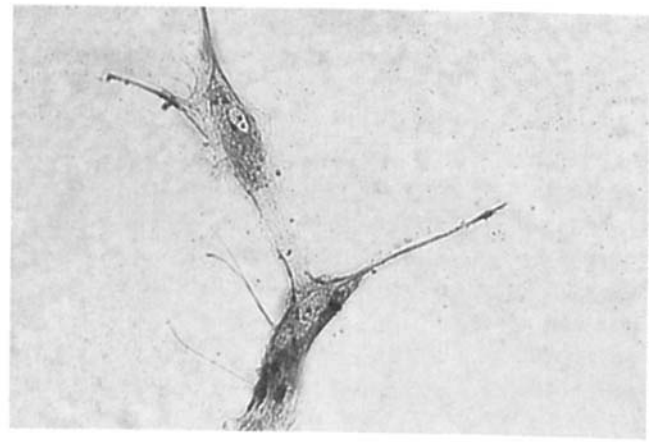


B

Figure 3. (A) Myogenic cell differentiated from mesenchymal stem cells (MSCs) treated with 5-azacytidine for 24 hrs and cultured for 2 weeks, viewed under confocal laser scanning microscope (magnification: ×400). (B) Myogenic cell differentiated from MSCs treated with 5-azacytidine for 24 hrs and cultured for 2 weeks, viewed under confocal laser scanning microscope (stained with Fluo-3; magnification: ×400; the scale bar represents 5 μm). The figure represents two experiments.

and alpha-cardiac actin (Fig. 4C). The uninduced cells were negative for these specified cardiomyocyte markers.

Myofilaments and Ca^{2+} Flux. Figure 5 includes representative transmission electron micrographs for control (A) and 5-azacytidine–treated cells (B). Numerous myofilments were evident in the cytoplasm of the myogenic cells and were absent in the untreated, uninduced cells. The myogenic cells displayed both spontaneous rhythmic Ca^{2+} fluxes (Fig. 6A) and KCl (5.0 m*M*)-induced Ca^{2+} fluxes (Fig. 6B) when observed under confocal laser scanning microscope. The KCl-stimulated Ca^{2+} fluxes peaked rapidly and gradually declined thereafter (Fig. 6B).



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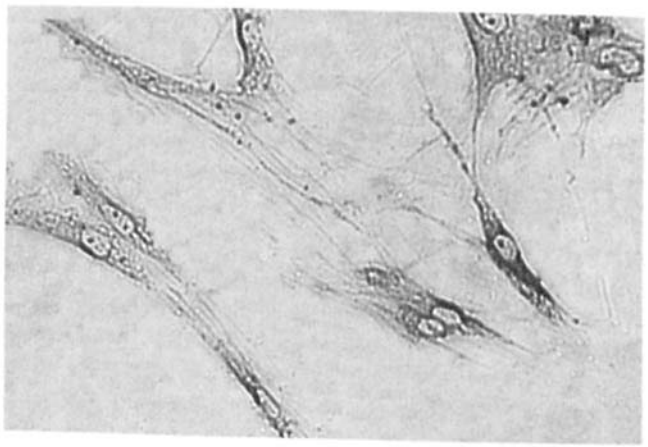

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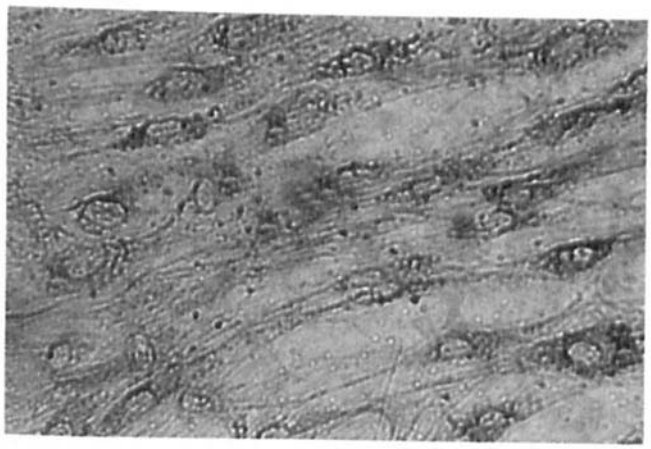
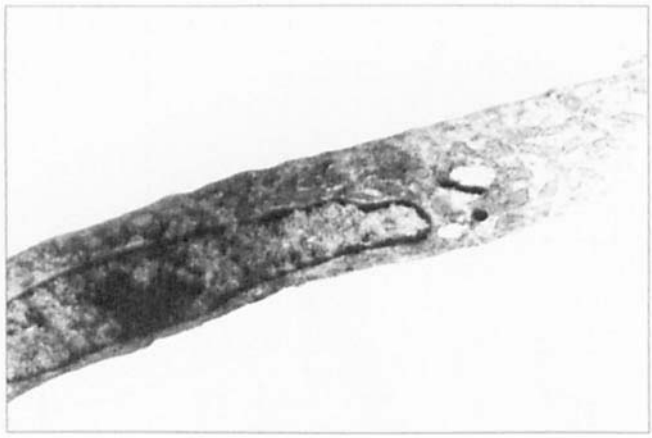


C

Figure 4. Myogenic cells differentiated from mesenchymal stem cells (MSCs) treated with 5-azacytidine for 24 hrs and cultured for 2 weeks were positive for (A) desmin (magnification: ×200), (B) beta-MHC (magnification: ×200), and (C) alpha-cardiac actin (magnification: ×200). The figure represents three experiments.



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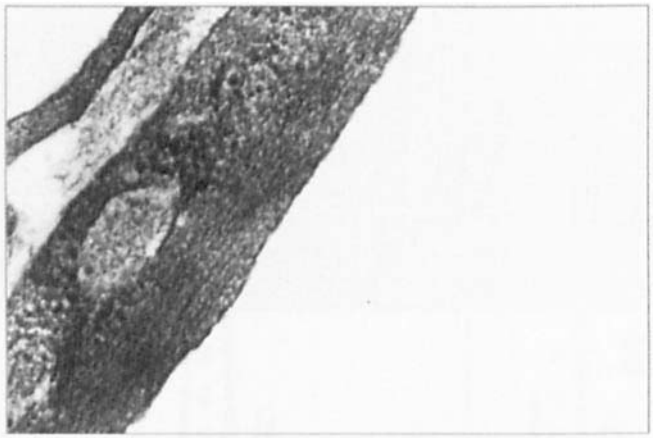

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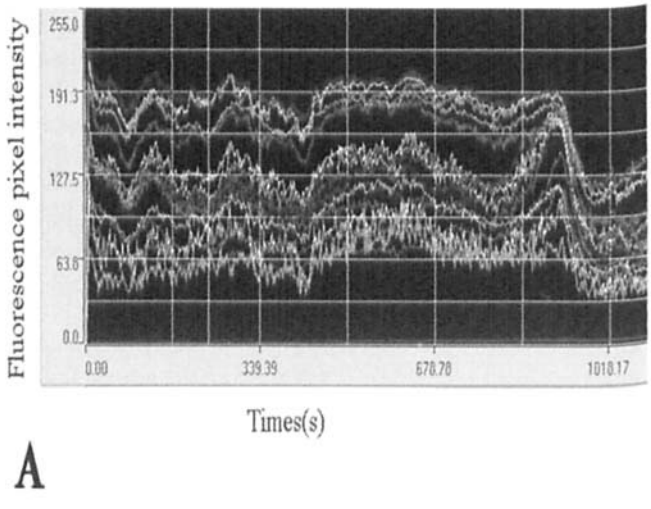
Figure 5. Transmission electron micrographs of (A) control mesenchymal stem cells (MSCs) (magnification: ×4800) without visible myofilaments in the cytoplasm and (B) myogenic cell (magnification: ×9280) differentiated from MSCs treated with 5-azacytidine for 24 hrs and cultured for 2 weeks with many myofilments in the cytoplasm. The figure represents three experiments.



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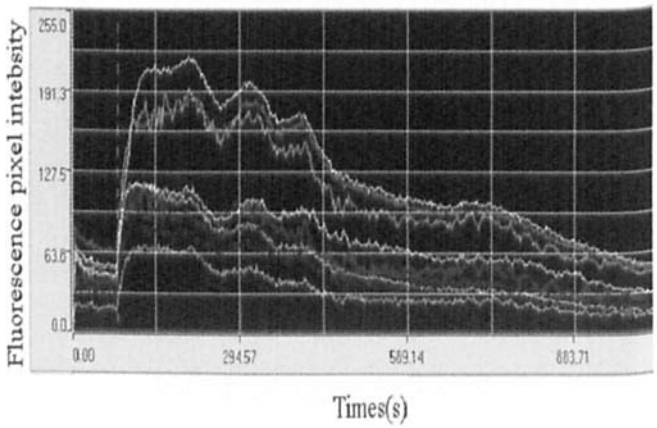

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Figure 6. (A) Rhythmic Ca^{2+} fluctuations of myogenic cell differentiated from mesenchymal stem cells (MSCs) treated with 5-azacytidine for 24 hrs and cultured for 2 weeks, recorded by confocal laser scanning microscopy. (B) Ca^{2+} flux in myogenic cells differentiated from MSCs treated with 5-azacytidine for 24 hrs and cultured for 2 weeks, recorded by confocal laser microscopy during stimulation with 5.0 m*M* KCL. The figure represents two experiments.

Cardiomyocyte-Associated Gene Expression. RT-PCR of myognic cell extracts produced four bands on agarose gels (Fig. 7A and B), consistent respectively with desmin (408bp), beta-MHC (528bp), alpha-cardiac actin (418bp), and cardiac troponin T (416bp). In control groups, the expression of these same genes was barely detectable for desmin and beta-MHC and undetectable for alpha-cardiac actin and troponin T. The real-time quantitative PCR confirmed that gene expression of alpha-cardiac actin, desmin, beta-MHC, and cardiac troponin T was higher in experimental groups than untreated control groups ($P < 0.05$; Fig. 8). Each amplicon had a unique melting temperature (T_m). DNA sequencing results confirmed that the human cardiac troponin T and desmin genes amplified by PCR were identical to their respective reference sequences provided by GenBank (X74819; NM 001927).

Discussion

Mesenchymal stem cells have been isolated from adult bone marrow (10–12). These cells can be expanded *ex vivo* and induced, either *in vitro or in vivo*, to terminally differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, myotubes, neural cells, and hematopoietic-

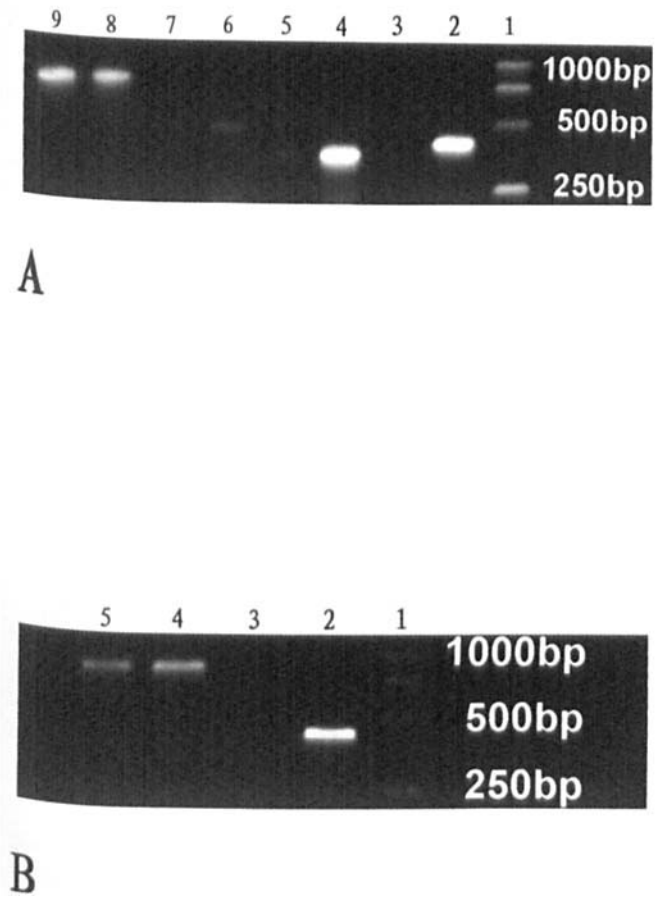

Figure 7. (A) Control mesenchymal stem cells (MSCs) versus myogenic cells differentiated from MSCs expressed the mRNAs for desmin, beta-myosin heavy chain (beta-MHC), and alpha-cardiac actin as detected by RT-PCR (Lane 1, marker; Lane 2, alpha-cardiac actin; Lane 3, control for alpha-cardiac actin; Lane 4, desmin; Lane 5, control for desmin; Lane 6, beta-MHC; Lane 7, control for beta-MHC; Lane 8, glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*]; Lane 9, control for *GAPDH*). (B) Control MSCs versus myogenic cells differentiated from MSCs expressed mRNA for cardiac troponin T (Lane 1, marker; Lane 2, cardiac troponin T; Lane 3, control for cardiac troponin T; Lane 4, *GAPDH*; Lane 5, control for *GAPDH*). The figure represents three experiments.

supporting stroma. The pluripotent character of these cells makes them an attractive therapeutic tool. The percentage of MSCs in adult bone marrow cells is very small, as only approximately 0.001%–0.01% of cells isolated from the Ficoll (1.073 g/ml) density interface are adherent cells. Therefore, it is very important to isolate and expand human MSCs *in vitro*. The method reported here was modified from that described by Wakitani (8) for isolating the cells from adult bone marrow in rats. The primary deviation from that method was use of 1.073 g/ml Ficoll, which was empirically determined to raise the efficiency of isolating MSCs from adult human bone marrow. The cells obtained from all six volunteers had similar growth characteristics in culture and all expressed the same specified surface antigens. From Passage 2 to Passage 6, the cellular karyotype was normal; the cell cycles were similar (data not shown). These results indicate that adult human MSCs have strong self-renewal ability and genetic stability *in vitro*.

Several research groups reported that MSCs were able to proliferate and potentially differentiate *in vitro* (10, 13, 14). The data presented above demonstrate that the ability of human MSCs to proliferate remains strong between Passage 2 and Passage 6 but gradually declines in later passages (data not shown). Therefore, the second-passage human MSCs were selected to investigate whether these cells would differentiate into cardiomyocytes *in vitro* when they were treated with 5-azacytidine. The morphological studies showed that the cells appeared spindle-shaped and gradually increased in size during culture. After 2–3 weeks in culture, myotube-like structures were formed. The changes of morphology may be associated with expression of proteins maintaining cytoskeleton (5, 6). The appearance of cytoplasmic myofilaments in transmission electron micrographs provided an important cardiomyocyte marker and was consistent with prior reports by others (8, 10, 15). Therefore, myogenic cells differentiated from adult bone marrow MSCs and cardiomyocytes had similar morphological and ultrastructural characteristics.

Confocal laser scanning microscopy was used to investigate the functions of the induced myogenic cells differentiated from the MSCs. These induced myogenic cells had rhythmic Ca^{2+} fluctuations and rapid increases in Ca^{2+} flux when stimulated with 5.0 m*M* KCL. Thus, these newly differentiated cells displayed functional characteristics similar to cardiomyocytes and skeletal muscle cells.

To confirm the specificity of the differentiation for myocytes, the cells were stained with Sudan black, and clear yellow droplets stained brown to black were observed in the cytoplasm of some cells (data not shown). These presumptive adipocytes usually appeared in clusters, which suggested that they were derived from common precursors. Other cells such as chondrocytes or osteoblasts were not observed, which suggested that the conditions were not optimal for their differentiation. Thus, 5-azacytidine can promote the differentiation of adult human MSCs into a cardiomyocyte-like phenotype, but the process is less than completely specific, as adipocyte-like cells were also observed.

Desmin and cardiac troponin T, known to be early markers of myogenic differentiation, are important structures of muscle tissue and play a role in contraction of muscle cells. Other markers include alpha-cardiac actin and beta-MHC. In this study, three proteins, desmin, alpha-cardiac actin, and beta-MHC, were found in the cytoplasm of the cells induced with 5-azacytidine (Fig. 4). The RT-PCR results showed that mRNAs of beta-MHC and desmin were highly expressed in the myogenic cells differentiated from the MSCs (Fig. 7A and B). The cells also specifically expressed mRNAs of alpha-cardiac actin and cardiac troponin T. The real-time PCR also confirmed the above results. Notably, the gene expression of alpha-cardiac actin and troponin T increased in experimental groups, and those

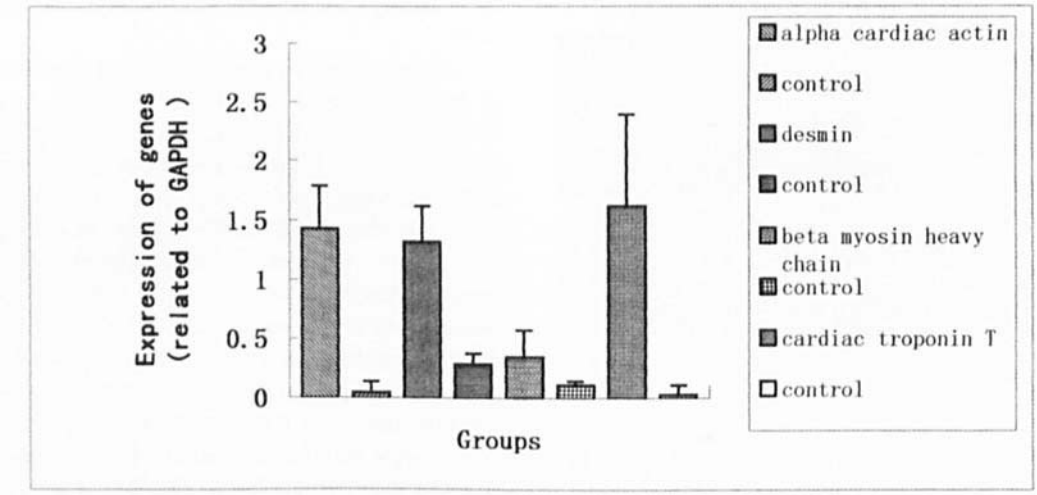

Figure 8. The mRNA expression of alpha cardiac actin, beta-myosin heavy chain, desmin, and cardiac troponin T ($n = 6$). All values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and expressed as mean and standard deviation. Each measure of gene expression in the experimental group was significantly higher than its respective measure in the control group ($P < 0.05$).

of beta-MHC and desmin were higher than that in the control groups ($P < 0.05$; Fig. 8). The exact identity of PCR products was confirmed by sequences analysis. However, the mechanism by which 5-azacytidine increased the expression of these specific markers is unclear. The expression may be associated with activation of the myogenic gene, MyoD, secondary to hypomethylation of selected cytosines involved in activating phenotype-specific genes (16, 17).

Bone marrow cells were recently reported as able to migrate into skeletal and cardiac muscle and then differentiate into the skeletal and cardiac muscle cells. This suggests that such a process may normally contribute to tissue maintenance or regeneration (15, 18–22). Other reports have demonstrated that rat and mouse bone marrow cells have the ability to regenerate infarcted myocardium (11, 23, 24). Contrary to this and earlier reports (5, 6, 8, 10), Liu Y *et al.* (25) reported that rat MSCs could not be expanded and induced to differentiate into cardiomyocytes by 5-azacytidine treatment. In this regard, the methods for isolation and culture conditions may be very important. Repeated experiments with adult human MSCs suggested that DMEM containing 10% FBS and 5% HS was optimal for the expansion of MSCs and the expression of the myogenic properties. The bFGF was included in the culture medium until the analysis experiments were performed because of earlier reports that bFGF could increase the expression of the myogenic phenotype and promote the formation of myotubes (8). Thus, there may be a synergistic action between bFGF and 5-azacytidine in the process of the differentiation. The modifications described in this report produced an efficient differentiation into a cardiomyocyte-phenotype.

Our results indicate that mesenchymal stem cells derived from adult human bone marrow can differentiate into cells with cardiomyocyte-phenotypes *in vitro*. The prevailing evidence suggests that bone marrow stem cells can regenerate myogenic cells in cardiac tissue (1, 7, 26). These advances raise the prospect that damaged cardiac tissues might be repaired by administered adult human bone marrow MSCs.

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