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Ear Mesenchymal Stem Cells (EMSC) Can Differentiate Into Spontaneously Contracting Muscle Cells

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Abstract We have previously shown that cells isolated from the outer ears of adult mice are a source of mesenchymal stem cells that can be induced to differentiate into adipo-, osteo-, and chondrocytes. In this study, we demonstrate that ear mesenchymal stem cells (EMSC) express stromal cell-associated markers (CD44, CD73) and stem cell marker Sca-1 and can be differentiated into spontaneously contracting muscle cells. Treatment of cells with epidermal growth factor (EGF) change their morphology from fibroblast shapes into stick-like structures that show repeated spontaneous contractions. Under conditions that promote myogenic differentiation, EMSC expressed mRNA for myoD and ventricular specific myosin light chain (MLC-2v) and protein for connexin 43, sarcomeric α-actinin, myocyte enhancer factor 2c (MEF2c), myosin heavy chain (MyHC), myogenin, and sarco-endoplasmic reticulum Ca^{2+}ATPase (SERCA) 1. However, the cells were negative for Nkx2.5, GATA4, and ANP. Intracellular Ca^{2+} transients in spontaneously beating EMSC, visualized by Fluo-3AM, showed a frequency of Ca^{2+} oscillations ranging over 28–59/min (mean 41.17 ± SEM 1.54). We also demonstrated that small pieces of ear tissues (ear punches) collected from live mice provide sufficient numbers of EMSC to isolate, culture and differentiate them into myocytes. Due to the ease of acquiring an expanding repertoire of differentiated EMSC cell types by a noninvasive surgical procedure, we conclude that the ear may prove to be a potential source of autologous cells for regenerative medicine, as supported by the fact that ears are one of the best sources of cells for somatic cell nuclear transfer (SCNT). J. Cell. Biochem. 102: 122–135, 2007.

Key words: mesenchymal stem cells; differentiation; myocytes; cardiomyocytes

Adult stem cells inhabit a variety of tissues that range from bone marrow, muscle, and CNS through to adipose tissue, skin, umbilical cord, and other organs [Friedenstein et al., 1976; Gritti et al., 1996; Weiss et al., 1996; Erices et al., 2000; Asakura et al., 2001; Seale et al., 2001; Zuk et al., 2001; Jiang et al., 2002; Braun et al., 2003; Gimble and Guilak, 2003]. Since adult stem cells can differentiate into many cell types both in vitro and after in vivo transplantation, they carry a great promise in regenerative medicine, tissue engineering, and developmental studies. Potential therapeutic targets include the replacement of damaged myocardial muscle, bone, or cartilage.

Although injured tissues contain an endogenous population of stem cells that can theoretically contribute to the regenerative process, the functional recovery of the injured area appears to be hindered by the development of scar tissues as shown for injured skeletal muscle [Li and Huard, 2002; Orlic et al., 2002]. Similarly, it has been demonstrated that the adult heart contains a population of stem cells [Beltrami et al., 2003; Matsuura et al., 2004; Leri et al., 2005; Pfister et al., 2005] with the ability to differentiate into smooth muscle, myocytes, and endothelial cells that can potentially participate in its own myocardial repair [Urbanek et al., 2005; Yano et al., 2005]. However, the complex regulatory pathways that recruit endogenous stem cells to replace damaged cardiomyocytes or to secrete factors

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that can restrain scar formation are poorly understood [Matsuura et al., 2004; Urbanek et al., 2005].

An alternative approach has utilized adult stem cells from other differentiated tissues to reconstitute damaged muscles and dead myocardium. Hematopoietic stem cells, skeletal myoblasts, neonatal myocytes, embryonic, and mesenchymal stem cells have all been used, but each approach carries certain limitations [Murry et al., 1996; Ferrari and Mavilio, 2002; Orlic et al., 2002; Siminiak et al., 2003; Siminiak et al., 2004]. To differentiate embryonic stem cells or adult stem cells into the myogenic/cardiomyogenic lineage in vitro, a variety of inductive factors have been used including nitric oxide [Kanno et al., 2004], oxytocin [Paquin et al., 2002], and DMSO [Ridgeway et al., 2000]. While the most potential compound for induction of myogenic differentiation is 5-azacytidine [Makino et al., 1999; Matsuura et al., 2004; Xu et al., 2004], it is a potent DNA demethylating agent and its application in in vivo studies could be problematic due to oncogene activation [Sadikovic et al., 2004].

Our laboratory has shown that outer ears of mice contain a population of mesenchymal stem cells (EMSC). These cells possess the ability to differentiate into adipo-, osteo-, and chondrolineages in both primary cultures and clonally expanded cell lines [Gawronska-Kozak, 2004; Rim et al., 2005]. The present studies were undertaken to show that EMSC can differentiate into spontaneously contracting muscle cells and that small pieces of ear tissue (ear punches) collected from live mice, a minimally invasive procedure, provide sufficient numbers of EMSC to isolate, culture, and differentiate them into myocytes.

MATERIALS AND METHODS

Cell Culture

EMSC were isolated from the outer ears of C57BL/6J mice that had been sacrificed or from 4 mm ear punches obtained during the standard procedure used for marking live mice. Tissues were minced and digested with collagenase class I (2 mg/ml; Worthington Biochemical, Freehold, NJ) in a shaking bath for 1 h at 37°C. The tissue homogenate was filtered through a 70 μm cell strainer (Becton Dickinson Labware, NJ) and the cells were isolated by centrifugation at 360*g* for 5 min. Pelleted cells were resuspended for 1 min in a lysis buffer (Sigma Co. St. Louis, MO) and centrifuged at 360*g* for 5 min to remove erythrocyte contamination. Cells were plated in 35 mm Petri dishes ($P = 0$) in Dulbecco's Modified Eagle Medium (DMEM/F12; Life Technologies, New York, NY) supplemented with 15% of fetal bovine serum (FBS; Life Technologies) and antibiotics—complete medium. The nonadherent cell population was removed after 20 h and the adherent layer was washed with fresh media. Subconfluent primary cultures ($P = 0$) were trypsinized (0.05% trypsin/0.53 mM EDTA; Life Technologies), replated, and subcultured. All in vitro differentiation experiments were performed on cells from passage 1 and 2.

Flow Cytometry Analysis

Phenotypic characterization of undifferentiated EMSC was performed by flow cytometry. Cells isolated from outer ears of C57BL/6J mice during three independent experiments (n = 8 animals per experiment) were cultured and cryopreserved at concentrations of $1.0–2.0 \times 10^6$/ml in DMEM/F12 media containing 20% FBS and 10% dimethylsulfoxide. Two days before analysis, cells were rapidly thawed in a 37°C water bath, resuspended in 10 ml medium and centrifuged. Supernatant containing dimethylsulfoxide was discarded and cells were plated in 100 mm petri dishes in DMEM/F12 media containing 10% FBS. On the day of analysis, unattached cells in cultures were washed out with PBS and adherent cells were harvested by trypsinization. Cells were centrifuged, washed three times in PBS with 0.5% BSA, resuspended at a concentration of 4×10^6/ml and then incubated in blocking buffer (containing 25 μg/ml of IgG) for 10 min on ice. A total of 25 μl of cell suspension (1×10^5 cells) was aliquoted per tube per antibody and incubated with 10 μl (1 μg/10 μl) of phycoerythrin (PE) conjugated antibodies (anti-CD 90, -CD 73, -Sca-1, -CD 117, -CD 44, -CD4 or -CD 45; BD Pharmingen, San Diego). Incubations were performed on ice and protected from light for 40 min. Following labeling, cells were washed in PBS with 0.5% BSA, centrifuged and fixed in 300 μl of cold Cytofix™ buffer (BD Biosciences Pharmingen). The assay was performed using a flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed with a Macintosh G3 workstation, which contains graphics software packages used for data acquisition and

analysis. The total number of cells counted for each sample was 10,000.

Myogenic Differentiation

Experiment I. Subconfluent EMSC from passage 0 were trypsinized, resuspended in complete medium and seeded in six-well plates at the density of 1.5×10^5 cells/well. To test for myogenic potential, EMSC were seeded on gelatin (porcine gelatin, Sigma) coated, collagen (Becton Dickinson Labware) coated or uncoated wells. Forty-eight hours after plating (at approximately 90% confluency), the complete medium was removed and cells were washed with HBSS. From this point, cells were treated with one of three types of myogenic differentiation media. Media I contained HAM's F10 (Life Technologies), 20% FBS (Life Technologies) and 2.5 ng/ml bFGF (Promega); media II consisted of DMEM/F12, 0.1% ITS, 5% FBS and 0.2 μg/ml epidermal growth factor (EGF) (Life Technologies); media III contained DMEM/F12, 10% FBS, 5% horse serum, 50 μM hydrocortisone and 0.1 μM dexamethasone. Cells were monitored microscopically every day for the appearance of spontaneous contraction. The cultures were terminated on Day 7 of differentiation and cells were collected for RT-PCR assays. The experiment was repeated several times using different groups of C57BL/6J mice as a source of cells. Half of the experiments were performed on the EMSC isolated from ear punches of C57BL/6J mice.

Experiment II. Experiment I showed that Media II (containing EGF) was the most potent for stimulating EMSC into spontaneously contracting myocytes. We next determined a time course for the differentiation of EMSC into myocytes. EMSC were seeded in six-well plates and were stimulated with media II for 2, 4, or 7 days. The experiment was terminated at Day 7 and cells were collected for protein extraction.

C2C12 Cell Line

C2C12 cells, a myogenic cell line derived from mouse muscle satellite cells, were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in growth medium (DMEM with 4 mM L-glutamine, 4.5 g/L glucose, 10% FBS) and induced to form myotubes in differentiation media (growth medium with 5% horse serum in place of fetal bovine serum).

Analysis by Reverse Transcription-PCR

Total RNA was extracted from differentiated and undifferentiated EMSC using Trizol Reagent as recommended by the supplier (Life Technologies). Heart and muscle tissues collected from C57BL/J6 mice were first homogenized and then RNA was extracted by Trizol Reagent. First-strand cDNA was synthesized from 1 μg of total RNA primed with oligo dT (Life Technologies) using Moloney mouse leukemia virus reverse transcriptase (200 U/assay) as described by the supplier (Life Technologies) in the presence of 1 μl of RNAse inhibitor, 0.1 M dithiothreitol and 0.5 mM of each dNTP. Oligonucleotides were purchased from One Trick Pony Oligos Division (Ransom Hill Bioscience, CA). Amplification was performed on cDNA using sets of primers for GATA4, Nkx2.5, atrial specific myosin light chain (MLC-2a), ventricular specific myosin light chain (MLC-2v), ANF, and myoD as described by Planat-Benard et al. [2004].

Western Blot Analysis

Total cell lysates were prepared by adding 150 μl of RIPA buffer containing proteinase inhibitor cocktails (Sigma) and phosphatase inhibitor cocktail I and II (Sigma). Total protein (50 μg) was separated on 12% SDS–polyacrylamide gels and transferred onto a PVDF membrane (Millipore). The blots were then incubated with antibodies against desmin (BD Biosciences), connexin 43 (BD Transduction Laboratories), myogenin (Developmental Hybridoma Bank), sarcomeric α-actinin (Sigma), myosin heavy chain (MyHC; Upstate Cell Signaling Solution), myocyte enhancer factor 2c (MEF 2c; Cell Signaling Technology), sarco-endoplasmic reticulum Ca2+ ATPase 1 (Sarco-endoplasmic reticulum Ca^{2+}ATPase (SERCA) 1; Affinity BioReagents), or sarco-endoplasmic reticulum Ca2+ ATPase 2 (SERCA 2; Affinity BioReagents) according to the manufacturers' protocols. Bands were visualized using the Odyssey imaging system (LI-COR Bioscience) with fluorescent (IRDye800^{TM} or Cy5.5) labeled secondary antibodies according to the manufacturers' protocol.

Immunocytochemistry

EMSC were grown and maintained in chamber slides (Nalge Nunc Int.) in DMEM/F12

medium containing 15% FBS. When the cells reached 90% confluency, media were changed to differentiation medium II (containing EGF). After 7 days of incubation, cells were fixed in 4% paraformaldehyde for 20 min, washed in PBS and treated with 3% H_2O_2 in methanol (Sigma) for 20 min to block endogenous peroxidases. The processed slides were then incubated overnight (4°C) with primary antibodies against SERCA 1 (1:200; Affinity BioReagents), SERCA 2 (1:200; Affinity BioReagents) or sarcomeric α-actinin (1:500; Sigma). Antibody binding was detected with the ABC complex (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA). Peroxidase activity was revealed using 3.3′-diaminobenzidine (Sigma) as a substrate. Two types of controls were performed: (a) the primary antibody was omitted during the immunostaining procedure; (b) the primary antibody was substituted with nonspecific IgG during the procedure. Slides were counterstained with hematoxylin and cells were visualized with a Zeiss microscope (Axioskop 40) and photographed with a Kodak digital camera (DC290 Zoom).

Measurement of Intracellular Free Calcium Concentration

EMSC were seeded at a density of 1.5×10^5 cells/dish in 35 mm dishes in complete medium. The medium was changed 48 h later to differentiation medium II (containing EGF) and incubated for an additional 4 days. After visual inspection for the presence of spontaneously beating cells under phase-contrast microscopy, cells were washed three times with HBSS solution (1.26 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 5.3 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 138 mM NaCl, 0.338 mM NaH_2PO_4, and 5.5 mM dextrose; Life Technologies) and loaded with 10 μM of Fluo-3 acetoxymethyl ester (Fluo-3-AM; Molecular Probes, Carlsbad, CA) for 30 min at room temperature in the dark. Culture dishes were then washed three times in HBSS and mounted onto the microscope stage (Nikon). Fluo-3 fluorescence was measured in these cells using an excitation filter of 495 nm on the selection illuminator (Illuminator D6), an emitter of 520 nm, and a low light camera (Photometrics) interfaced with the microscope and controlled by Metamorph software as described previously [Hermann et al., 2005].

RESULTS

Immunophenotype of EMSC

Flow cytometric analysis was used to characterize EMSC by demonstrating the presence or absence of selective cell surface molecular phenotypes on cells isolated from outer ears of C57BL/6J mice. Cells were examined for the expression of stem cell markers (Sca-1, CD117), hematopoietic markers (CD45, CD4), and stromal cell markers (CD44, CD73, CD90). Undifferentiated EMSC were positive for stem cell marker Sca-1 (82.77 ± 7.8) but negative for CD117 (2.48 ± 1.2) (Fig. 1). Simultaneously, cells were negative for hematopoietic markers CD45 (4.47 ± 1.3) and CD4 (3.23 ± 1.04) (Fig. 1). The stromal associate marker CD44 was expressed by $96.95\% \pm 2.2$ of cells, whereas $65.74\% \pm 11.1$ of the cells were positive for CD73 and only $5.5\% \pm 1.5$ for CD90 (Fig. 1).

Myogenic Potential of EMSC

To establish EMSC potentiality for differentiation into the myogenic lineage and to extend their usefulness, the present study was performed on cells isolated from ear punches obtained with standard procedures used for marking mice (Fig. 2) and/or from the outer ears of C57BL/6J mice (Figs. 3–6). This model allows one to conduct simultaneously in vivo and in vitro studies without requiring the sacrifice of animals.

Experiment I. To determine the optimal conditions for differentiating EMSC into myocytes, two approaches were undertaken. First, we tested three different types of differentiation media: medium I (supplemented with bFGF), medium II (supplemented with EGF), and medium III (supplemented with dexamethasone and hydrocortisone) and second, the optimal type of adherent surface conditions for myogenic differentiation using gelatin coated, collagen type IV coated or uncoated dishes was evaluated. Each media type (I, II, and III) was evaluated on each seeding surface (gelatin, collagen, and uncoated).

Morphology. Cells were monitored daily by phase contrast microscopy. During the first 48 h, when the cells were maintained in complete medium, they showed a typical fibroblast-like phenotype, a morphology that was retained under control, unstimulated conditions during the entire experiment. After 48 h, when the media was replaced with one of the three

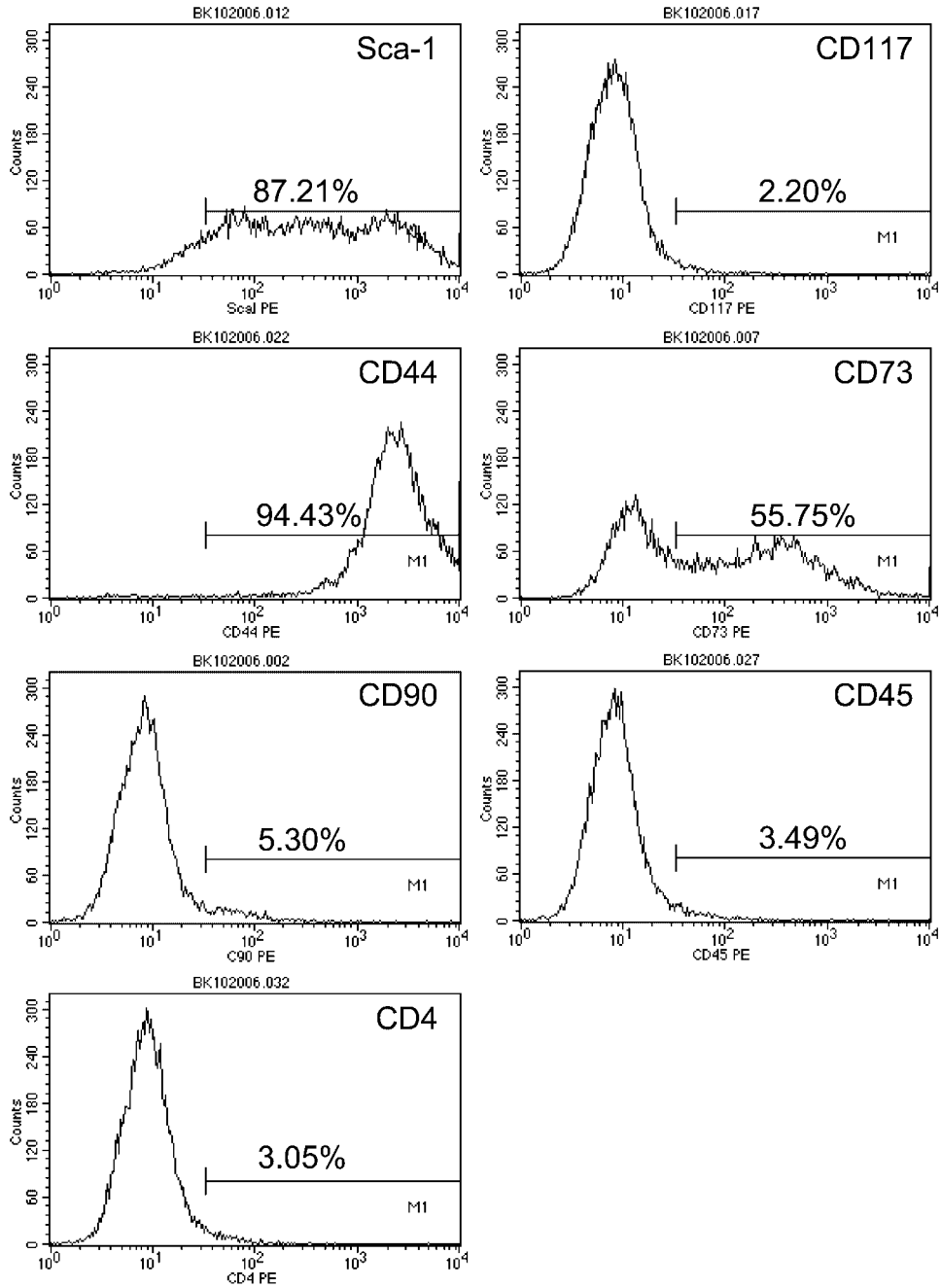

Fig. 1. A representative flow cytomeric analysis of EMSC at passage 1. Cells were labeled with phycoerythrine-conjugated antibodies against CD45, CD4, CD44, Sca-1, CD117, CD90, or CD73. The percentage of cells staining positive is indicated on each panel. The data represent the mean of three experiments (n = 3). Each experiment consisted of a pool of cells collected from eight animals.

differentiation media, gradual changes in cells morphology were observed. On Day 3, EMSC cultured in medium I (bFGF) became elongated and spindle-shaped with parallel orientations. This morphological pattern was most prominent on Day 7 (Fig. 2A). The morphology of EMSC cultured in medium II (EGF) differed dramatically (Fig. 2C,D). Two different populations of the cells were observed; the first consisted of epithelioid-shaped cells densely arranged and closely attached to the bottom surface and the second was composed of cells with stick-like shapes [Makino et al., 1999] which were lying on the top of the first layer

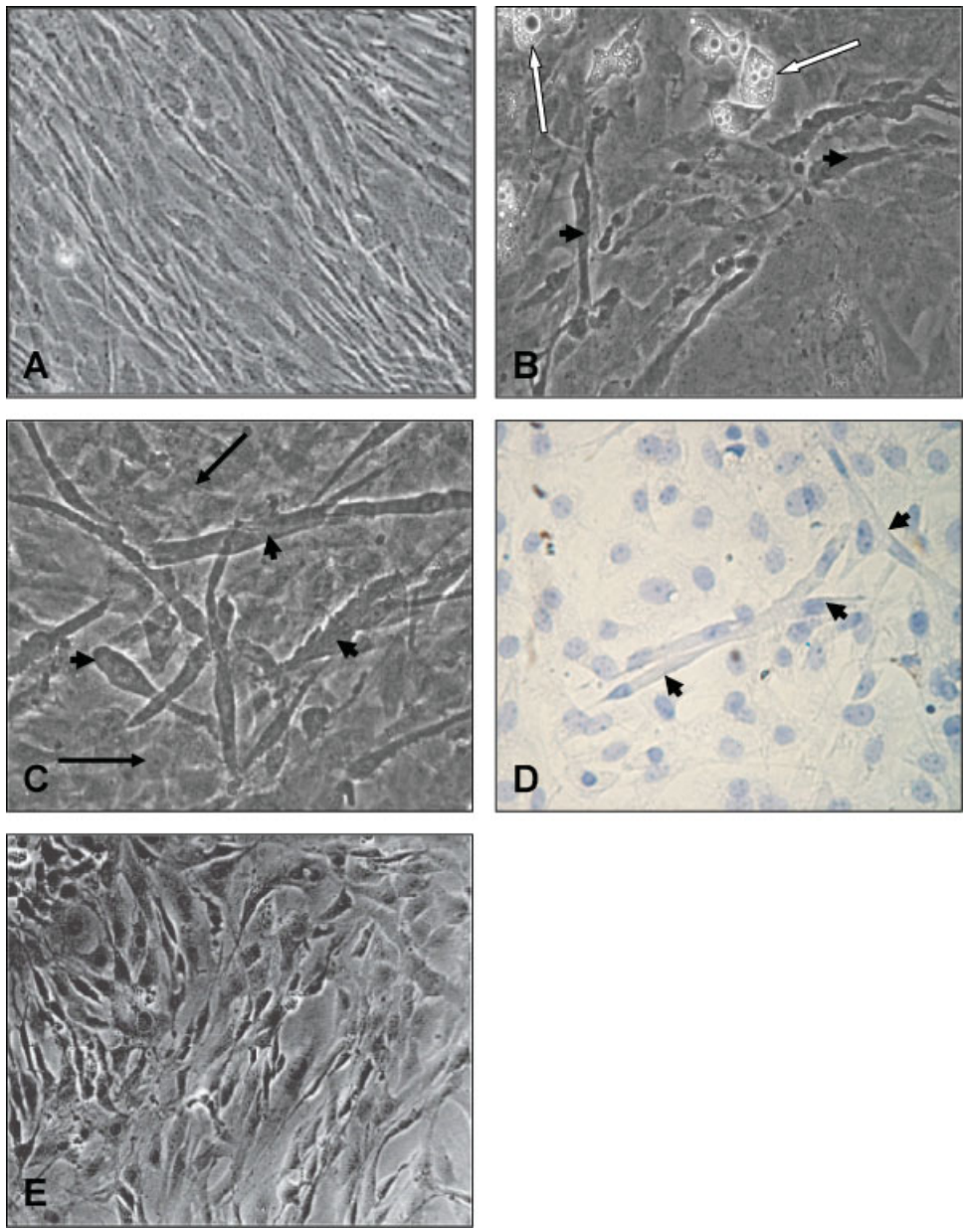

Fig. 2. EMSC isolated from the ear punches of C57BL/6J mice differentiate into myogenic lineage. Phase-contrast (**A**–**C**) and light-microscope (**D**) photographs of EMSC after 7 days of treatment. A: medium I with bFGF, (B) medium III with dexamethasone, (C,D) medium II with EGF. **E**: shows the morphology of control unstimulated EMSC. White arrows—adipocytes; black arrows—bottom-layered cells; arrowheads—myocytes. D: cells were fixed in 4% paraformaldehyde and stained with hematoxylin to visualize nuclei.

(Fig. 2C,D). On Day 3, the stick-like cells were characterized by repeated spontaneous contraction and relaxation in the absence of exogenous stimuli (supplemental online video 1). The contractile activity of the differentiated EMSC occurred in a random fashion without a synchronous pattern between distinct foci. Spontaneously beating cells were anchored to the bottom-layer of cells and exerted a motive force on them during the contractile phase (supplemental online video 1). Most of the stick-like cells were mononuclear, and some binuclear; however, multinucleated cells were never observed (Figs. 2D,5C). EMSC cultured in medium III displayed a mixture of the cells with different phenotypes. The most frequently

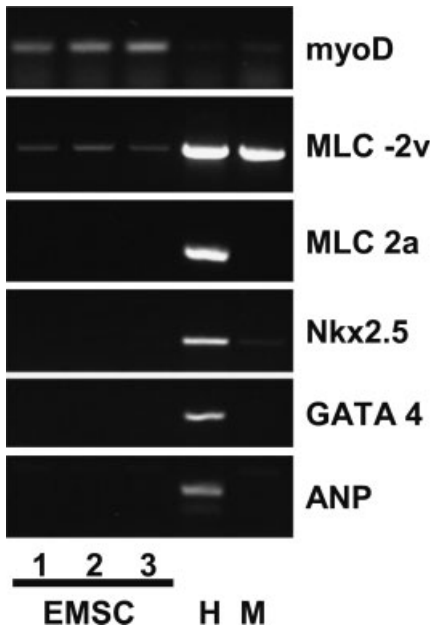

Fig. 3. Expression of myogenic-specific markers in EMSC under different culture conditions. RNA samples from EMSC cultured in (1) medium I (containing bFGF), (2) medium II (containing EGF), (3) medium III (containing dexamethasone) were analyzed by RT-PCR. Molecular markers included myoD, MLC-2v, MLC2a, Nkx2.5, GATA 4, and ANP. (H) heart and (M) skeletal muscles of C57BL/6J mice.

observed cells possessed prominent lipid droplets, indicating adipocyte differentiation (Fig. 2B, arrows). Most of the cells were epithelioid-shaped and few had myocyte-like characteristics; however, the morphology of myocyte-like cells differed from those cultured in medium II by being smaller, shorter and always mononucleated. The spontaneous beating potentiality was observed sporadically (Fig. 2B). Thus, on the basis of microscopic examined cultures, media II (with EGF) showed the greatest potentiality of differentiation of EMSC into myogenic lineage. No differences in EMSC morphology in regards to myogenic differentiation were observed among cells plated on gelatin, collagen, or uncoated dishes within the particular stimulation media (data not shown).

Gene expression. The molecular characterization of the myogenic potentiality of EMSC was initially determined by RT-PCR assays on RNA extracted from differently stimulated cultures (Fig. 3). Total RNA obtained from heart tissues and skeletal muscles of C57BL/J6 mice were used as controls. Irrespective of the type of myogenic stimulation, differentiated EMSC showed no expression of cardiomyocyte-specific genes including ANP, GATA4, and Nkx2.5 (Fig. 3). On the contrary, EMSC under all stimulating culture conditions showed strong MyoD expression. Interestingly, the EMSC under all three differentiation conditions expressed myosin light chain-2 ventricular form (MLC-2v), but not the atrial form (MLC-2a).

Experiment II. Next, we determined the length of incubation in EGF containing medium necessary for EMSC to acquire their optimal myogenic phenotype. EMSC collected from C57BL/6J mice were seeded at a density of 1.5×10^5 cells/dish in six-well uncoated plates.

Subconfluent cultures were stimulated with differentiation medium II for various times: 2, 4, or 7 days. Afterward, cultures were rinsed twice with serum-free medium, changed to the DMEM/F12 medium with 5% FBS and maintained for up to 7 days to the end of experiment. Control cultures, without myogenic stimulation, were maintained in DMEM/F12 medium containing 5% FBS. On Day 7, cells were harvested and Western Blot analyses were performed. Protein extracted from differentiated C2C12 cells and protein from heart and muscle tissues of C57BL/6J mice were used as controls.

Western Blot analysis. We analyzed expression of two groups of protein connected with (1) cardiomyocyte differentiation: connexin 43, desmin, sarcomeric α-actinin, MEF 2c, and SERCA 2 and (2) skeletal muscle myogenic differentiation: MyHC, myogenin, and SERCA 1 (Fig. 4). Differentiated EMSC expressed sarcomeric α-actinin and MEF 2c with no expression in cells cultured in medium without EGF. The protein levels were highest in EMSC differentiated for 7 days with very faint expression after 2 days of differentiation. Both proteins were also present in C2C12 cells and heart tissue extracts. Connexin 43 expression was observed in EMSC after 7 days of differentiation. The connexin 43 specific band, with a molecular weight of about 43 kDa, corresponds to that found in heart tissues. Additionally, a weak positive reaction was observed in C2C12 cells. Anti-desmin and anti-SERCA 2 antibodies showed no reactions with EMSC protein extracts, but showed positive staining in heart tissues (desmin and SERCA 2) and C2C12 cells (desmin). In contrast to the lack of SERCA 2 expression in studied cells, SERCA 1 antibody revealed the presence of a strong characteristic

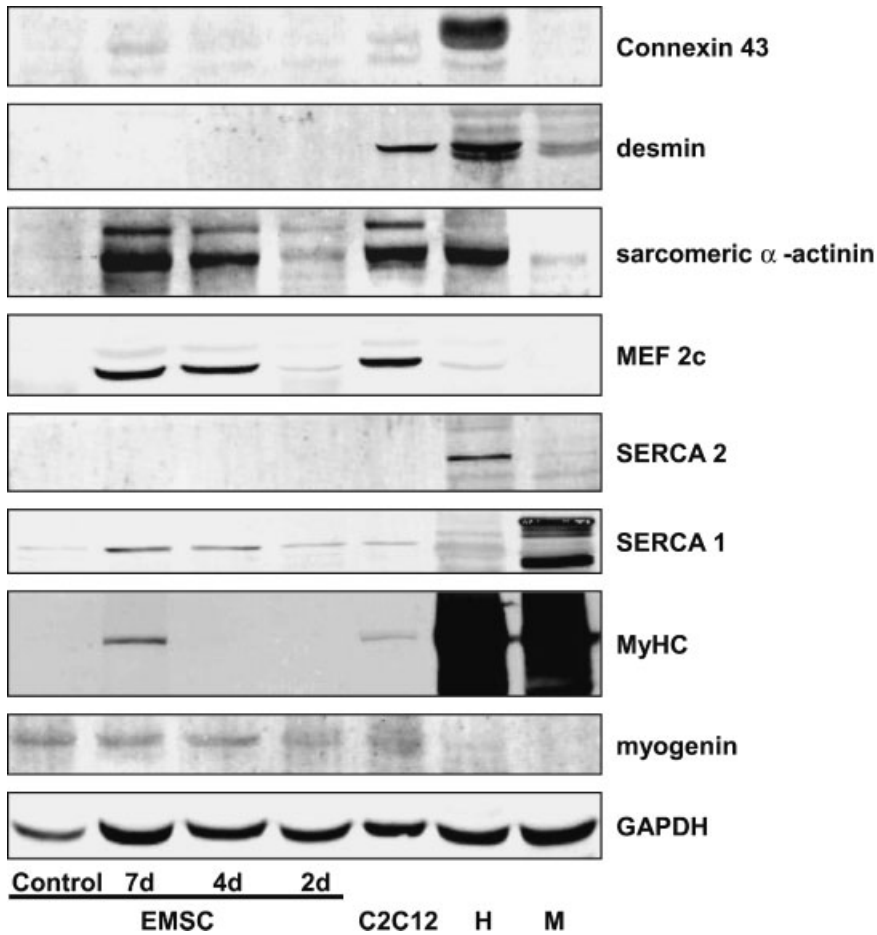

Fig. 4. Western Blot analysis of connexin 43, desmin, sarcomeric α-actinin, myocyte enhancer factor 2c (MEF 2c), myosin heavy chain (MyHC), myogenin, SERCA 1, and SERCA 2. EMSC were induced for 2, 4, or 7 days in myogenic medium II (EGF) or in control medium. C2C12—cell line, (H) heart and (M) skeletal muscles of C57BL/6J mice.

100 kDa band in EMSC that reached a maximum after 7 days of differentiation. A very faint band was also observed in control EMSC. Strong MyHC expression, typified by a 220 kDa band, was observed only in EMSC that had been in differentiation media for 7 days. Finally, myogenin was present in all EMSC samples independent of culture conditions (differentiated or control) and in protein extracts from C2C12 cells.

Immunocytochemistry. RT-PCR and Western Blot data are based on total RNA and protein (respectively) content collected from the entire population of EMSC differentiated into myocytes. Morphological analysis of cells after 7 days of differentiation revealed the existence of two populations of cells: bottom-layered epithelioid-shaped cells and stick-like, spontaneously contracting cells on the top. To demonstrate whether individual cells acquire the expression of proteins specific to muscle cells, we performed immunocytochemical staining for sarcomeric α-actinin and SERCA 1 and SERCA 2. EMSC stimulated for 7 days with EGF showed positive staining for the presence of SERCA 1 (Fig. 5A) and were negative for SERCA 2 (Fig. 5B). The deposits of positive staining for SERCA 1 were distributed within the cytoplasm of spontaneously contracting cells (Fig. 5A). The contracting cells also stained positively with anti-sarcomeric-α-actinin antibody (Fig. 5C).

$[Ca^{2+}]$ Oscillation. The most impressive feature of myocyte expression in EMSC is their ability to spontaneously contract (supplemental online video 1). It has been shown that in

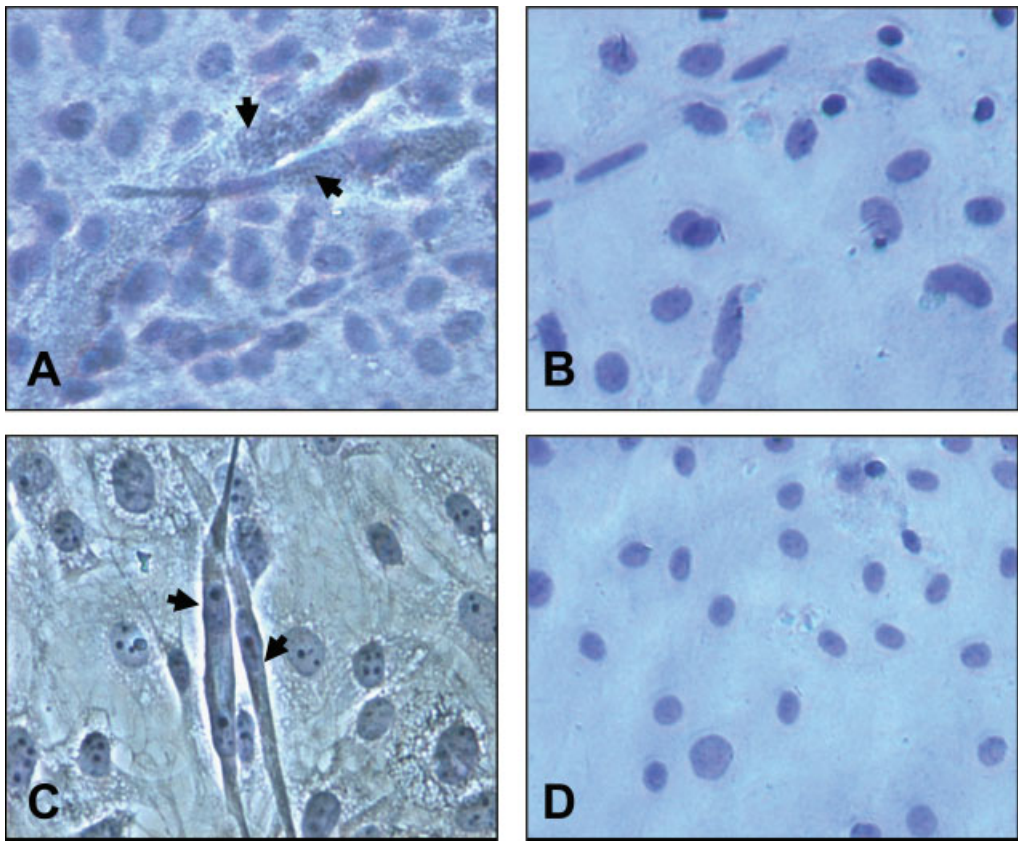

Fig. 5. Immunostaining of EMSC-derived myocytes. **A**: immunodetection of SERCA 1 positive cells (arrowheads), (**B**) negative staining with SERCA 2 antibodies, (**C**) positive staining with anti-sarcomeric-α-actinin (arrowheads), (**D**) control of immunostaining procedure in which nonspecific IgG was substituted for the primary antibody show no staining.

spontaneously beating cells, the occurrence of action potentials coincide with the rapid uptake of Ca^{2+} [Sartiani et al., 2002; Blatter et al., 2003]. It suggests that differentiated EMSC may have functional Ca^{2+} transduction channels. To ascertain that the spontaneous beating of cells depends on intracellular levels of Ca^{2+}, we analyzed [Ca^{2+}] transients in differentiated

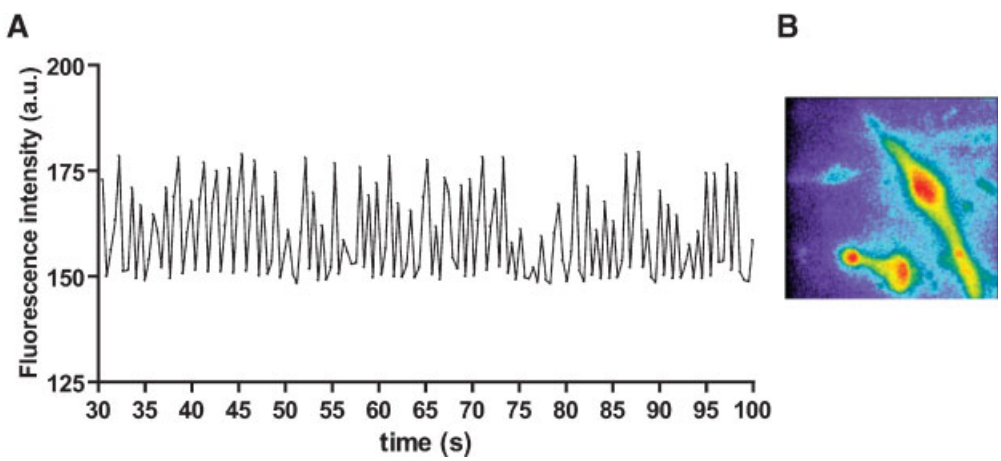

Fig. 6. **A**: A typical continuous recording of [Ca^{2+}] transients in beating cells derived from EMSC as determined by Fluo-3AM; (**B**) color image of [Ca^{2+}] transients in differentiated EMSC (supplemental online video 3).

EMSC (supplemental online videos 2 and 3). Figure 6 shows the rhythmic variation in fluorescence intensity of Fluo-3-AM-loaded cells measured in a spontaneously beating region of differentiated EMSC. The frequency of Ca^{2+} oscillations ranged over 28–59/min (mean 41.17 ± SEM 1.54). The amplitudes of the Ca^{2+} signals varied from 2 to 98 with a mean of 19.75 (±SEM 3.73). Spontaneous beating of neighboring cells was observed as independent events resulting in nonsynchronous calcium transients.

DISCUSSION

The results in this paper have extended the range of pluripotency of EMSC to include the myogenic cell lineage, in addition to their capacity to differentiate into chondro-, adipo-, and osteolineage as established earlier [Gawronska-Kozak, 2004; Rim et al., 2005]. The stem cell character of the undifferentiated EMSC is principally reflected in the fact that 83% of EMSC express the stem cell marker Sca-1.

Sca-1 has been commonly used to characterize a mesenchymal stem cell population in bone marrow [Goodell et al., 1996; Parmar et al., 2003; Peister et al., 2004], in skeletal and cardiac muscles [Asakura et al., 2001; Matsuura et al., 2004; Pfister et al., 2005] and adipose-derived cells [Zheng et al., 2006]. Moreover, it has been demonstrated that among cardiac side population cells, the greatest potential for cardiomyogenic differentiation is restricted to Sca-1 positive cells [Matsuura et al., 2004; Pfister et al., 2005]. Additionally, we showed that EMSC are positive for CD44 and CD73 markers that were previously reported to characterize populations of human and rat bone marrow-derived and adipose tissue-derived mesenchymal stem cells [Pittenger et al., 1999; Mitchell et al., 2006; Yoshimura et al., 2007]. However, EMSC express neither the hematopoietic stem cell marker CD117, nor hematopoietic cell markers CD45 and CD4, strongly suggesting that EMSC belong to a mesenchymal stem cell population residing in ear tissue and that they are not derived from circulating hematopoietic cells.

The morphological phenotype, the expression of muscle-related genes and proteins together with spontaneous contractility by EGF-stimulated EMSC provide strong evidence of myogenic differentiation. However, the evidence (see Figs. 3–5) indicates that the cultures did not display a definitive "cardiocyte" or "skeletal myocyte" phenotype. Rather, the cultures contained a heterogeneous mixture of cells with a partially differentiated myogenic phenotype. Differentiated EMSC express myogenic regulatory transcription factors (MRFs), such as myoD and myogenin, which commit/differentiate cells to skeletal muscles [Pownall et al., 2002; Buckingham et al., 2003; Hadchouel et al., 2003]. The expression of myogenin, as estimated by Western Blot analysis, was present not only in differentiated EMSC, but also in control/unstimulated cells as well. On the contrary, MEF2c, a transcriptional factor belonging to a second group of factors involved in myogenic differentiation—MEF2 [Ma et al., 2005], was expressed only in EMSC differentiated for 4–7 days. Although the expression of MEF2c has been implicated as an essential transcriptional factor in cardiac development [Jamali et al., 2001; Zang et al., 2004; Phan et al., 2005], other studies have shown its involvement in skeletal muscle maturation [Ridgeway et al., 2000; Dodou et al., 2003; Wilson et al., 2004]. We did not detect the expression of transcriptional factors which are considered to be essential for heart development including Nkx2.5, GATA 4, and ANF [Zang et al., 2004]. Nevertheless, connexin 43, a protein known to participate in the formation of gap junctions in the heart, and the ventricular isoform of myosin light chain (MLC-2v), which is expressed in heart tissue, were both present in differentiated EMSC [Makino et al., 1999; Gassanov et al., 2004; Matsuura et al., 2004].

The multi-step process of muscle differentiation involves expression of muscle-specific genes and proteins followed by morphological changes, including the formation of multinucleated myotubes and eventually the contractile apparatus [Sabourin and Rudnicki, 2000]. SERCA is an important component of the contraction mechanism in the skeletal and cardiac muscle because it mediates muscle relaxation by stimulating reuptake of cytosolic Ca^{2+} into the sarcolemic reticulum. There are two isoforms: SERCA 1, the fast (glycolytic) muscle isoform expressed in skeletal muscle, and SERCA 2a, the slow (oxidative) muscle isoform expressed in cardiac muscle [Yao et al., 1998; Simonides et al., 2001]. Our Western Blot

and immunocytochemical data showed the expression of SERCA 1 protein in differentiated EMSC and in skeletal muscle tissues and the lack of SERCA 2a in EMSC that is normally expressed in heart samples. Differentiated EMSC, expressing SERCA 1 in their contractile apparatus, displayed features of spontaneous, nonstimulated contractile activity, which was observed after only 3 days of differentiation. The frequency of calcium transients in differentiated EMSC varied from 28–59/min and are similar to that recorded for mouse fetal cardiomyocytes using the FDSS6000 (instrumentation used for high throughput screening to detect calcium oscillation; www.usa.hamamatsu.com), rat cardiomyocytes [Pfister et al., 2005; Uhlen et al., 2006], and embryonic stem cells differentiated into cardiomyocytes [Kehat et al., 2001; Mummery et al., 2003]. On the contrary, C2C12 myotubes showed occasional spontaneous calcium transients varying from one to tens of beats, but only when they were co-cultured on a feeder layer of dermal fibroblasts [Cooper et al., 2004]. EMSC appear to have the ability to create a supportive environment simultaneously that enable myocytes to acquire spontaneous contractility. This ability to contract distinguishes EMSC from other adult stem cells with apparent myogenic potentiality [Makino et al., 1999; Mizuno et al., 2002; Gaustad et al., 2004; Shim et al., 2004]. It is possible that EGF stimulated EMSC differentiation in a manner similar to that observed with the embryonal carcinoma P19 cell line, where the presence of dimethyl sulfoxide resulted in the development of both cardiac and skeletal muscle [Ridgeway et al., 2000].

Our findings underscore the relative facility with which a population of cells in the ear is able to express the functional characteristics of a stem cell. These findings may be relevant to ongoing somatic cell nuclear transfer (SCNT) studies [Kato et al., 2000; Rideout et al., 2001; Cho et al., 2004]. Here, the literature has noted that among all adult cells tested, ear cells (outer ears) appear to be one of the most commonly used donor cells for giving live clonogenic progeny following nuclear transfer, especially in livestock species [Kato et al., 2000; Cho et al., 2004; Jang et al., 2004; Silvestre et al., 2004; Jang et al., 2005]. It has been shown that cloning from ES cells is more effective than that from somatic, terminally differentiated cells. Moreover, Hochedlinger and Jaenisch [2002] have posited that a majority of the surviving clones, in which cells from somatic tissues were used as donors, are derived from the nuclei of rare somatic stem cells present in adult tissues rather than from the nuclei of differentiated cells. Accordingly, our studies raise the possibility that success in animal reproductive cloning, in which ear cells were used as a source for the donor cell, may in fact be attributed to the presence of pluripotent mesenchymal stem cells. Though EMSC have not been identified in other species to date, it is possible that enrichment of EMSC from ear punch biopsies could provide a means to improve the overall efficiency of animal reproductive cloning.

In conclusion, these studies, demonstrating that EMSC isolated from the outer ears or ear punches of mice are able to differentiate into spontaneously beating myocytes, have extended our earlier experiments showing that EMSC can differentiate into adipocytes, osteocytes, and chondrocytes. Furthermore, because of the ease of acquiring an expanding repertoire of differentiated cell types by a non-invasive surgical procedure, EMSC may provide a potentially useful source of autologous cells for repairing damaged tissues and may be a useful source of donor cells for SCNT.

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