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Characterization of Mesenchymal Stem Cells Isolated From Murine Bone Marrow by Negative Selection

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Abstract Mesenchymal stem cells (MSCs) are typically enriched from bone marrow via isolation of the plastic adherent, fibroblastoid cell fraction. However, plastic adherent cultures elaborated from murine bone marrow are an admixture of fibroblastoid and hematopoietic cell types. Here we report a reliable method based on immunodepletion to fractionate fibroblastoid cells from hematopoietic cells within plastic adherent murine marrow cultures. The immunodepleted cells expressed the antigens Sca-1, CD29, CD44, CD81, CD106, and the stem cell marker nucleostemin (NST) but not CD11b, CD31, CD34, CD45, CD48, CD90, CD117, CD135, or the transcription factor Oct-4. They were also capable of differentiating into adipocytes, chondrocytes, and osteoblasts in vitro as well as osteoblasts/osteocytes in vivo. Therefore, immunodepletion yields a cell population devoid of hematopoietic and endothelial cells that is phenotypically and functionally equivalent to MSCs. The immunodepleted cells exhibited a population doubling time of approximately 5–7 days in culture. Poor growth was due to the dramatic down regulation of many genes involved in cell proliferation and cell cycle progression as a result of immunodepletion. Exposure of immunodepleted cells to fibroblast growth factor 2 (FGF2) but not insulin-like growth factor (IGF), murine stem cell factor, or leukemia inhibitory factor (LIF) significantly increased their growth rate. Moreover, 82% of the transcripts down regulated by immunodepletion remain unaltered in the presence of FGF2. Exposure to the later also reversibly inhibited the ability of the immunodepleted cells to differentiate into adipocytes, chondrocytes, and osteoblasts in vitro. Therefore, FGF2 appears to function as a mitogen and self-maintenance factor for murine MSCs enriched from bone marrow by negative selection. J. Cell. Biochem. 89: 1235–1249, 2003. © 2003 Wiley-Liss, Inc.

Key words: mesenchymal stem cells; marrow stromal cells; FGF2

Mesenchymal stem cells (MSCs) from various species can be enriched from whole bone marrow by isolation of fibroblastoid cells via their preferential attachment to tissue culture plastic [Friedenstein et al., 1970; Ashton et al., 1984; Thomson et al., 1993; Hurwitz et al., 1997; Pittenger et al., 1999]. Although this fractionation method yields a phenotypically and functionally heterogeneous fibroblastoid cell population [Kuznetsov et al., 1997; Phinney et al., 1999; Tremain et al., 2001] its use remains widespread due to the lack of specific antigens for direct isolation of MSCs from bone marrow. In contrast, plastic adherent cultures elaborated from the bone marrow of rodents contain both fibroblastoid and hematopoietic cell types. Hematopoietic cells constitute a large percentage of plastic adherent murine marrow cultures due to their ability to adhere directly to tissue culture plastic as well as bind to fibroblastoid [stromal] cells via engagement with adhesion molecules, cytokine receptors, and extra cellular matrix proteins [Kerk et al., 1985; Bearpark and Gordon, 1989; Simmons et al., 1992; Deryugina and Muller-Sieburg, 1993]. Moreover, hematopoietic cells persist in these cultures even after serial passage due to

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the ability of stromal cells to support granulopoiesis and B cell lymphopoiesis even in the absence of exogenous growth factors and cytokines [Witte et al., 1987; Phinney et al., 1999a].

Several methods have been reported to separate fibroblastoid and hematopoietic cells in plastic adherent murine bone marrow cultures. Wang and Wolf [1990] demonstrated that when murine bone marrow cells are plated at very low density, fibroblastoid colonies arise that are devoid of hematopoietic cell types. However, the method is impractical as a purification scheme because it yielded only 27 fibroblastoid colonies of 5 or more cells from a total of 200 culture dishes. Modderman et al. [1994] reported that repeated exposure of plastic adherent murine bone marrow cultures to the cytotoxin potassium thiocyanate selectively poisoned macrophages and other hematopoietic cell types, producing an enriched population of fibroblastoid cells. However, the authors failed to determine if the later contained a pool of progenitors capable of multi-lineage mesenchymal cell differentiation. Van Vlasselaer et al. [1994] described a method to isolate mesenchymal progenitors via two-color cell sorting using anti-Sca-1 antibodies and wheat germ agglutinin. This approach yielded a cell population devoid of hematopoietic cell markers that was capable of forming bone nodules in vitro. However, the sorted cell population exhibited reduced clonogenicity and osteogenic potential as compared to unsorted cells, indicating that sheer forces generated during the sort adversely affected survival of the progenitor cells. No assessment was made of the multi-potentiality of the sorted cell population, as well. Finally, although the mouse monoclonal antibody Stro-1 has been used to isolate stromal cell precursors from human bone marrow [Simmons and Torok-Storb, 1991], it is not useful for fractionating mouse bone marrow cultures.

In the absence of a reliable purification scheme most laboratories continue to employ the method of plastic adherence to isolate murine MSCs, despite its limitations. Accordingly, the engraftment or therapeutic potential of murine MSCs has been evaluated in vivo by administering the plastic adherent cell fraction of bone marrow to experimental animals [Pereira et al., 1998; Kotton et al., 2001; Jin et al., 2002]. Since this population includes hematopoietic cells that possess an appreciable engraftment potential in vivo the aforementioned studies are confounded in that they do not provide a direct measure of the contribution made by MSCs to the experimental outcome. The lack of a reliable method to isolate MSCs from murine bone marrow also prohibits the use of genetically altered and mutant mouse strains to study basic aspects of their biology.

Previously, we used antibodies against CD11b to isolate fibroblastoid cells with adipogenic and chondrogenic potential from plastic adherent mouse marrow cultures [Kopen et al., 1999]. Here, we describe an improved protocol that yields a cell population characteristic of MSCs based on expression of various stem cell specific antigens and the ability to differentiate into adipocytes, chondrocytes, and osteoblasts in vitro as well as osteoblasts/osteocytes in vivo. We also show that fibroblast growth factor 2 (FGF2) is mitogenic for the cells and reversibly inhibits their ability to undergo cellular differentiation. Collectively, these studies describe a reliable method to isolate and culture expand in an undifferentiated state murine MSCs and as such will facilitate further research into the biology and therapeutic potential of these adult stem cells.

MATERIALS AND METHODS

Isolation of Murine MSCs

MSCs were isolated as previously described [Kopen et al., 1999; Phinney et al., 1999a]. Briefly, whole bone marrow obtained from the long bones of 4 week-old FVB/N mice was suspended in α-MEM media containing L-glutamine but no ribonucleosides or deoxyribonucleosides (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal calf sera (Lot # F0091, Atlanta Biologicals, Norcross, GA), penicillin (100 U/ml), and streptomycin (100 U/ml). Cells (1.4×10^6 cells/cm^2) were cultured at 37°C with 5% CO_2 in a humidified chamber for 72 h and then the non-adherent cells were removed by aspiration. Cells were cultured an additional 5–7 days with a single media change and then harvested by gentle scraping after a 5 min incubation in 0.25% trypsin with 1 mM EDTA. In some cases cells were incubated for 48 h prior to harvest with 5 μM 5-bromo-2-deoxyuridine (BrdU). The cell pellet was dispersed by gentle agitation, resuspended in 20 ml of HBBS, and filtered through a 70 μm filter (Falcon, Franklin Lakes, NJ) to remove cell clumps. Cells were suspended in HBSS at a maximum density of

40×10^6 cells/ml and incubated on a rotator for 1 h at 4°C prior to immunodepletion. Antibodies used for immunodepletion were bound to streptavidin-conjugated M-280 Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions. MSCs (up to 40×10^6 cells) were incubated successively on a rotator at 4°C for approximately 50 min with magnetic beads (5 beads/cell) conjugated to anti-CD11b, anti-CD34, and anti-CD45 antibodies (10 μg/mg beads). The immunodepleted cells were diluted in 5 ml of HBSS, counted, plated at 8,600 cells/cm^2, and cultured 7 days in media with or without FGF2 (20 ng/ml) prior to differentiation. Alternatively, immunodepleted cells (40,000) were plated into 6-well plates and cultured with media changes every 2 days in the presence of various growth factors, which were added daily, to monitor effects on cell proliferation.

Fluorescence-Activated Cell Sorting (FACS)

Aliquots (2.5×10^5) of immunodepleted cells were suspended in 50 μl of wash buffer (0.1% sodium azide, 1.0% BSA in PBS) containing a rat anti-mouse CD16/CD32 antibody (Fc Block; PharMingen, Franklin Lakes, NJ) at a concentration of 1 μg/1×10^6 cells and incubated for 3–5 min at 4°C in the dark. Wash buffer (50 μl) containing 5 μg of the appropriate primary antibody (PharMingen) was added and the cells incubated for an additional 20 min. Cells were washed twice with 200 μl of wash buffer and where necessary were incubated for 20 min in wash buffer (100 μl) containing 5 μg of a fluorochrome-conjugated secondary antibody (PharMingen). The extent of cell labeling was evaluated using a Beckman Coulter Model Epics XL (Beckman Coulter, Fullerton, CA). Isotype controls were run in parallel using the same concentration of each antibody tested.

RNA Isolation, PCR, and DNA Microarray Analysis

Total RNA was isolated from immunodepleted cells or the murine embryonic stem (ES) cell line D3 (ATCC) using the RNAeasy Kit (Qiagen, Valencia, CA). Total RNA from murine brain was obtained from Clonetech, Palo Alto, CA. All RNA samples were treated with DNAase I during purification. Total RNA was converted to cDNA using the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 100 μl in 1× Taq polymerase buffer (Sigma, St. Louis, MO) with 10 μM dNTPs, 2.5 U Taq DNA polymerase, and 100 pmoles of the following primer sets. Osteocalcin (OC) (accession number NM_031368), forward primer (bp 4–22) 5′-CAGACAAGTCCCACACAGCAGC-3′ and reverse primer (bp 69–87) 5′-AGAGCAGCCAAAGCCGAGC-3′; osteopontin (OP) (accession number AF515708), forward primer (bp 259–285) 5′-GATGATGACGATGATGATGACGATGGA-3′ and reverse primer (bp 728–752) 5′-AGGCTGGCTTTGGAACTTGCTTGAC-3′; bone sialoprotein (BSP) (accession number L20232), forward primer (bp 698–718) 5′-CAAGCGTCACTGAAGCAGGTG-3′ and reverse primer (bp 971–994) 5′-CATGCCCCTTGTAGTAGCTGTATT-3′; adipsin (accession number NM_013459), forward primer (bp 265–286) 5′-ACTCCCTGTCCGCCCCTGAACC-3′ and reverse primer (bp 674–697) 5′-CGAGAGCCCCACGTAACCACACCT-3′; collagen 18(α1) (accession number NM_009929), forward primer (bp 330–353) 5′-TTCCGGGACTTTTCGCTGCTGTTT-3′ and reverse primer (bp 780–806) 5′-TGCCCGGTCTTCATCATCATCTTCTTC-3′; FGF2 (accession number NM_00800006), forward primer (bp 12–29) 5′-CGGCATCACCTCGCTTCC-3′ and reverse primer (bp 408–429) 5′-CTTCTGTCCAGGTCCCGTTTTG-3′; NST (accession number AY181025), forward primer (bp 829–847) 5′-GGGAAAAGCAGTGTCATTA-3′ and reverse primer (bp 408–429) 5′-GGGATGGCAATAGTAACC-3′; Oct-4 (accession number X52437), forward primer (bp 119–139) 5′-TGTCCGCCCGCATACGAGTTC-3′ and reverse primer (bp 1231–1248) 5′-CAGGGGCCGCAGCTTACACAT-3′; Nestin (accession number NM_016701), forward primer (bp 4679–4699) 5′-TCAAGGGGAGGCCAGGAAGGA-3′ and reverse primer (bp 5103–5123) 5′-CTGCAGCCCCACTCAAGCCATC-3′; GAPDH (accession number W64025), forward primer (bp 58–79) 5′-CAAGATGGTGAAGGTCGGTGTG-3′ and reverse primer (bp 501–523) 5′-GGGGTAAGCAGTTGTGTCAGGAT-3′. A total of 500 ng of input cDNA was used for each PCR reaction, which were amplified for 25–32 cycles 94°C for 30 s, 55–65°C for 45 s, and 72°C for 90 s with an initial heat activation step at 94°C for 4 min and a final extension step at 72°C for 10 min.

Experimental procedures for GeneChip microarray were performed according to the

Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Biotinylated cRNA was hybridized to the Affymetrix MG-U74Av2 array, which contains approximately 6,000 murine genes. The expression data were analyzed using Affymetrix MicroArray Suite v5.0. Signal intensities of all probe sets were scaled to the target value of 2,500. Microarray analyzes were performed once with a single preparation of RNA from each cell population.

Differentiation of Immunodepleted Cells In Vitro

Immunodepleted cells cultured for 7 days with or without FGF2 (20 ng/ml) were induced to differentiate into chondrocytes using the method described by MacKay et al. [1998] or were plated into 6-well plates (Falcon) at a density of 10,415 cells/cm^2 and exposed to osteogenic stimuli for 21 days as previously described [Phinney et al., 1999a]. Alternatively, approximately 2.5×10^5 immunodepleted cells were cultured in micromass for 5 weeks as described above except that the concentration of dexamethasone used was 10 nM and it was removed from the media after 3 weeks. Adipogenic differentiation was induced as previously described except that immunodepleted cells were initially plated at approximately 50% confluence prior to the onset of differentiation [Kopen et al., 1999]. Accumulation of lipid vacuoles was demonstrated by fixing cells in ice cold methanol for 2 min and then staining cells with Oil Red O and toluidine blue.

Osteogenic Differentiation of Immunodepleted Cells In Vivo

A single fracture was induced in the tibia of anesthetized female mice by releasing a weight bearing blunt-edged (2 mm thick) steel blade (approximately 200 g) through a predetermined distance, which supplies sufficient force to displace the tibia a distance of approximately 0.5 of its diameter. The displacement induces a closed fracture by three point bending. Approximately 5 min after induction of the fracture approximately 50,000 BrdU-labeled, male, immunodepleted cells were injected into the resulting hematoma using a 10 µl Hamilton syringe and a 33-gauge needle. The fracture was immobilized using an external splint to encourage proper healing and minimize discomfort. Mice were sacrificed 3 weeks after fracture induction and the fracture callus examined histologically for the presence of engrafted donor cells.

Histology and Immunocytochemistry

Mice subjected to fracture induction were perfused with 10 ml PBS followed by 10 ml 4% buffered paraformaldehyde using a 27-gauge butterfly catheter. The fractured and contralateral tibias were dissected and placed back in fixative for 6 h at 4°C on a rotator, decalcified in cold 5% formic acid for 1.5 h at 4°C, processed and embedded in paraffin, and sectioned at 4 µm onto positively charged slides. Slides were incubated in 3% H_2O_2 for 15 min to quench endogenous peroxidase activity and then stained with an antibody against BrdU (Dako, Carpinteria, CA) as previously described [Kopen et al., 1999]. The extent of antibody staining was visualized by incubating the slides with horseradish peroxidase-conjugated streptavidin at a dilution of 1:100 for 30 min at room temperature and then with AEC (Dako) for 30 min at room temperature in the dark.

Osteogenic micromass pellets were fixed for 15 min in 4% buffered paraformaldehyde and then transferred to a 7% solution of sucrose prepared in PBS, and incubated at 4°C on a rotator for several hours. The pellets were then embedded in glycol methacrylate (Technovit 8100) as described by the manufacturer (Energy Beam Sciences, Agawam, MA). To demonstrate mineral deposition sections (1.5 µm) were stained with Alizarin Red S. Immunohistochemistry was carried out using a goat anti-mouse type I collagen antibody (Hybridoma Bank, University of Iowa, clone CIICI) and visualized using the chromagen substrate AEC (Dako). Chondrogenic micromass pellets were fixed in zinc formalin and embedded in paraffin as described above. To demonstrate glycosaminoglycan deposition, sections were stained with 1% toluidene blue for several seconds, rinsed in tap water, dehydrated, and mounted.

Image Analysis

Fluorescent micrographs were obtained using a Leica RX-DMV upright fluorescent microscope attached to a digital camera (Cooke Sensicam High Performance) and rendered using Slidebook® software (Intelligent Imaging Innovations, Denver, CO). Bright field images were obtained using a Nikon E800 upright fluorescent microscope equipped with a 35-mm camera (Nikon). Processed photos were digitized using

an AGFA Duoscan HiD scanner and Photoshop 5.0 software (Adobe).

RESULTS

Isolation of Murine MSCs by Negative Selection

We and others have demonstrated that plastic adherent cultures elaborated from murine bone marrow are an admixture of fibroblastoid and hematopoietic cell types, the later of which persist in the cultures even after serial passage [Xu et al., 1983; Witte et al., 1987; Phinney et al., 1999a]. Accordingly, we developed a fractionation scheme wherein plastic adherent cells are cultured for 8–10 days to maximize the yield of fibroblastic cells but minimize their differentiation [Phinney et al., 1999a] and then subjected to three rounds of immunodepletion using antibodies against CD11b, CD34, and CD45. This procedure removed essentially all hematopoietic lineages from the plastic adherent cell fraction (Fig. 1). The average cell yield after immunodepletion was $23 \pm 8\%$ of the total number of plastic adherent marrow cells (Table I). The yield from the BALB/C strain was $6.8 \pm 3.3\%$, which is significantly less than that obtained from the FVB/N strain ($P \leq 0.0008$). FACS analysis revealed that the immunodepleted cells uniformly expressed CD9, CD29, and CD81, expressed variable levels of Sca1, CD44, and CD106 but did not express CD11b, CD31, CD34, CD45, CD48, CD90, CD117, and CD135 (Figs. 1 and 2). The cells also expressed the stem cell markers NST [Tsai and McKay, 2002] and nestin but not Oct-4, a transcription factor highly expressed in ES cells [Nichols et al., 1998] (Fig. 3). Based on their surface antigen profile and differentiation potential (see below) the immunodepleted cells are characteristic of MSCs.

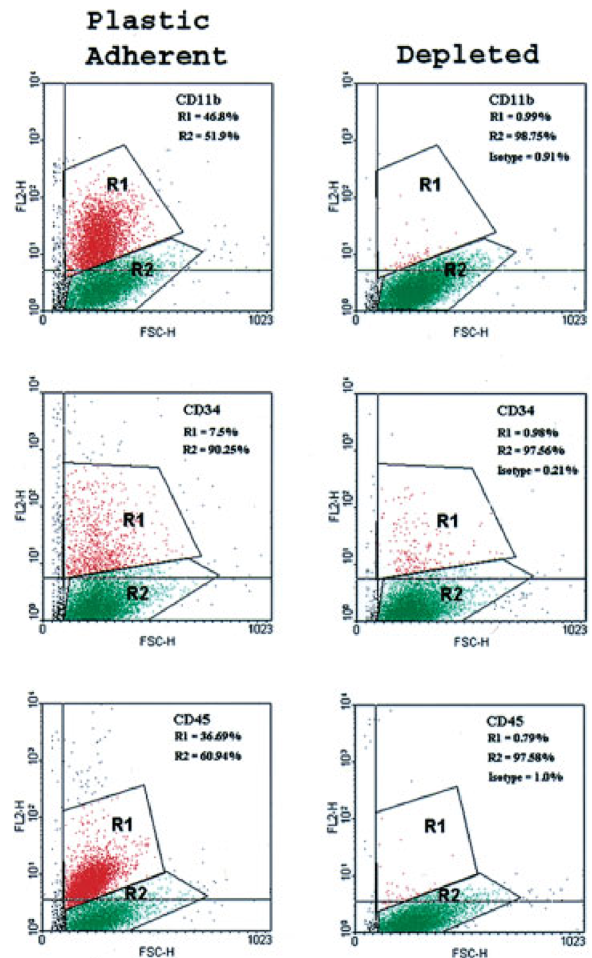

Fig. 1. FACS analysis of plastic adherent murine marrow cells prior to and after immunodepletion. Plastic adherent cells elaborated from murine bone marrow were harvested after 8 days of culture and analyzed by FACS to determine the percentage of CD11b, CD34, and CD45 expressing cells. The same plastic adherent population was then subjected to three rounds of immunodepletion using antibodies against CD11b, CD34, and CD45 and re-analyzed by FACS. R1, percent of total cells that express respective antibody; R2, percent of total cells that do not express the respective antibody.

Multi-Lineage Differentiation of Immunodepleted Murine MSCs (IDmMSCs)

IDmMSCs exposed to the PPAR-γ agonist ETYA in the presence of insulin and rabbit serum underwent adipogenic differentiation as evidenced by accumulation of large lipid vesicles in their cytoplasm that stained strongly with Oil Red O (Fig. 4A). Notably, the number of cells exhibiting adipogenic differentiation varied from 20% to 50% between experiments and appeared to be adversely affected by high initial plating density. Alternatively, when cultured in micromass in the presence of TGF-β3 the cells adopted a morphology characteristic of chondrocytes and secreted an extra cellular matrix rich in glycosaminoglycans, as evidenced by staining with toluidine blue (Fig. 4B). Finally, when cultured in media supplemented with dexamethasone, ascorbic acid, and β-glycerolphosphate the cells expressed high levels of alkaline phosphatase (data not shown) and deposited an extra cellular matrix that stained strongly with the calcium binding dye Alizarin Red S (Fig 4C). Concurrently, levels of OC, OP, and BSP mRNAs also showed significant increases between 10 and 21 days of culture,

TABLE I. Yield of IDmMSCs From Murine Bone Marrow

Mouse strain	Total marrow cells/ animal ($\times 10^9$)	Plastic adherent cells/animal ($\times 10^6$)	IDmMSCs/animal ($\times 10^6$)	Yield of viable IDmMSCs (%)
FVB/N	0.041 ± 0.011	0.46 ± 0.31	0.1 ± 0.053	23 ± 8.0
Balb/C	0.05 ± 0.018	0.42 ± 0.19	0.033 ± 0.012	6.8 ± 3.3

The number of total bone marrow cells, plastic adherent cells, and IDmMSCs obtained per animal from FVB/N and BALB/C inbred strains were calculated. Yields of IDmMSCs were calculated as a percentage of the total number of plastic adherent cells. Differences in the number of IDmMSCs per animal ($P \leq 0.002$) and yield of viable IDmMSCs ($P \leq 0.0008$) between FVB/N and BALB/C strains was statistically significant as determined using a two-tailed Student's t-test.

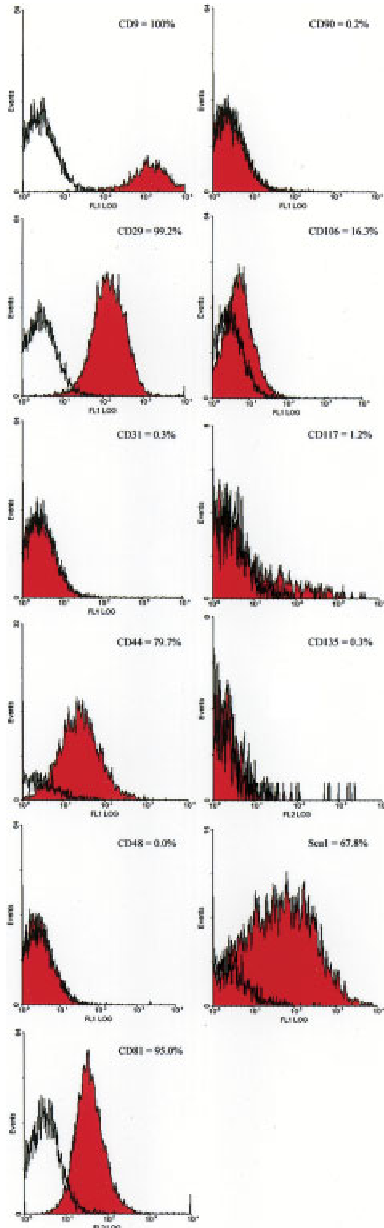

Fig. 2. Phenotype of IDmMSCs. Histograms showing the relative staining intensity of IDmMSCs for various cell surface antigens analyzed by FACS. Numbers indicate the percentage of cells in the population whose staining intensity (red) with the respective antibody was greater than that compared to the isotype control (black).

demonstrating the differentiation of cells to an osteogenic fate (Fig. 5). Osteogenic differentiation was also evident when IDmMSCs were cultured in micromass as described above except that dexamethasone was added at a concentration of 10^{-8} M and was removed after 3 weeks. Under these conditions the cells displayed a morphology characteristic of mineralizing osteoblasts as evidenced by their round morphology and localization within lacunae (Fig. 4F). The extra cellular matrix of the micromass also stained strongly with antibodies against type I collagen (Fig. 4D) and with Alizarin Red S (Fig. 4E). However, osteogenic differentiation by micromass culture was unpredictable in that some pellets contained bone, others were a mixture of bone and cartilage, and some consisted of only fibrous tissue.

To determine whether IDmMSCs differentiated into mesenchymal cells fate in vivo, we assayed their osteogenic capacity using an induced fracture model we developed in mice. A single fracture was created in a mouse's tibia by three point bending and immediately thereafter

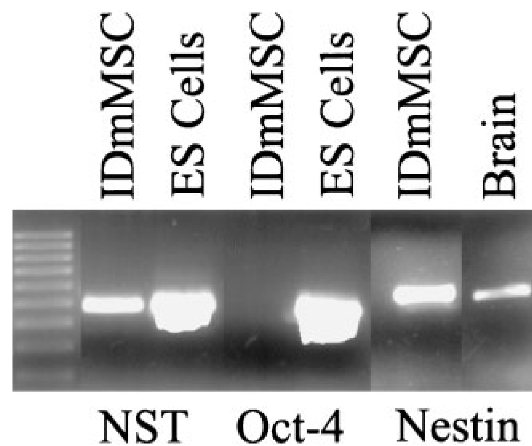

Fig. 3. RT-PCR analysis of stem cell marker gene expression. Expressed transcripts corresponding to nucleostemin (NST), Oct-4, and nestin were assayed by RT-PCR in IDmMSCs, the mouse embryonic stem (ES) cell line D3 (ES cells) and total RNA derived from mouse brain (Brain). The amplified products shown were cloned and sequenced to confirm their identity.

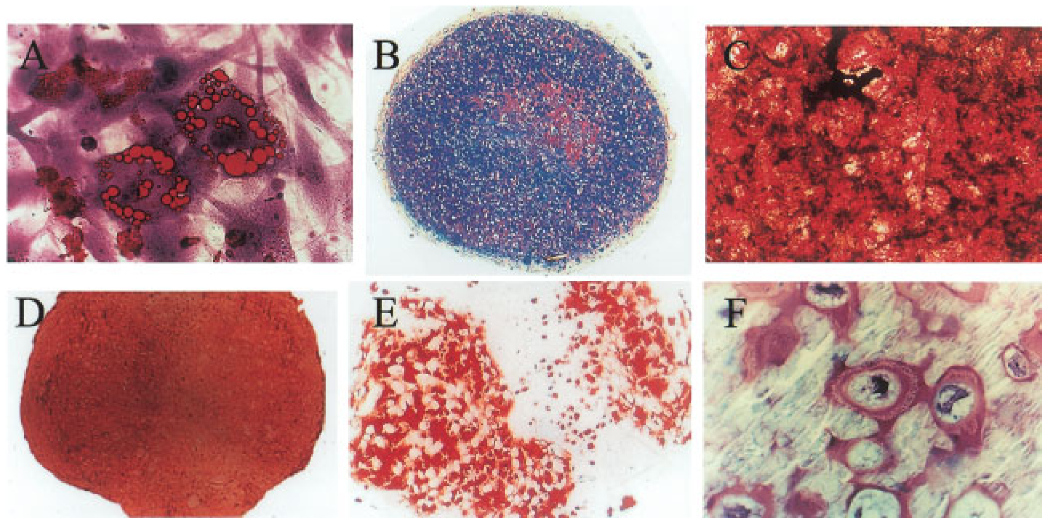

Fig. 4. Multi-lineage mesenchymal cell differentiation of IDmMSCs in vitro. **A**: Phase-contrast image of IDmMSCs cultured in adipogenic media and then fixed and stained with Oil Red O and toluidine blue. Note the accumulation of numerous Oil Red O stained lipid vesicles within the cells. **B**: Histological section of a micromass pellet stained with toluidine blue and examined under bright field illumination. The uniform, intense blue staining reveals the rich glycosaminoglycan content of the pellet, indicative of robust chondrogenic differentiation. **C**: Bright field image of IDmMSCs cultured in osteogenic media and stained with Alizarin Red S. The intense red staining indicates deposition by the cells of a calcified extra cellular matrix. **D**: Histological section of an osteogenic micromass pellet stained with an antibody against type I collagen. **E**: Osteogenic micromass pellet stained with Alizarin Red S showing calcified extra cellular matrix. **F**: Higher magnification of (E) showing osteoblast-like cells within the micromass pellet.

approximately 50,000 IDmMSCs labeled with the nucleotide analogue BrdU were injected into the resulting hematoma. Approximately 3 weeks after fracture induction donor derived IDmMSCs were evident within newly formed compact bone at the site of fracture and appeared morphologically indistinguishable from host osteocytes. In contrast, donor-derived cells were not detected distal to the site of the fracture or in the contralateral tibia (Fig. 6). Genomic DNA prepared from tibias was also analyzed by real-time PCR using primers and a probe specific for sequences within the mouse Y chromosome [McBride et al., 2003]. Y chromo-

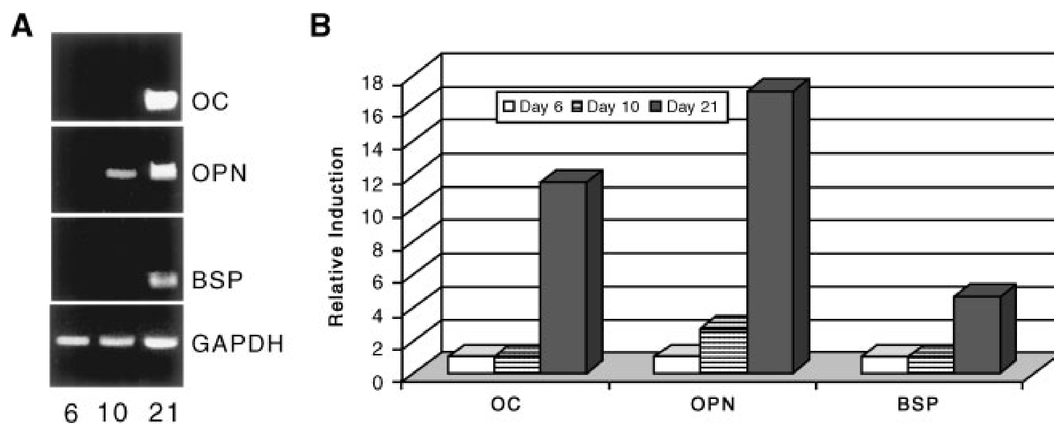

Fig. 5. RT-PCR analysis of bone-specific gene expression in IDmMSCs. **A**: Expression levels of transcripts corresponding to osteocalcin (OC), osteopontin (OP), and bone sialoprotein (BSP) were measured by semi-quantitative PCR in IDmMSCs exposed to osteo-inductive media (OIM) for 6, 10, and 21 days. **B**: Histogram of data in (A) showing relative induction levels of bone-specific transcripts in IDmMSCs. Expression levels of OC, OP, and BSP were quantified by measuring the fluorescent intensity of each respective band in the gel and then normalizing it to that of GAPDH for each time point.

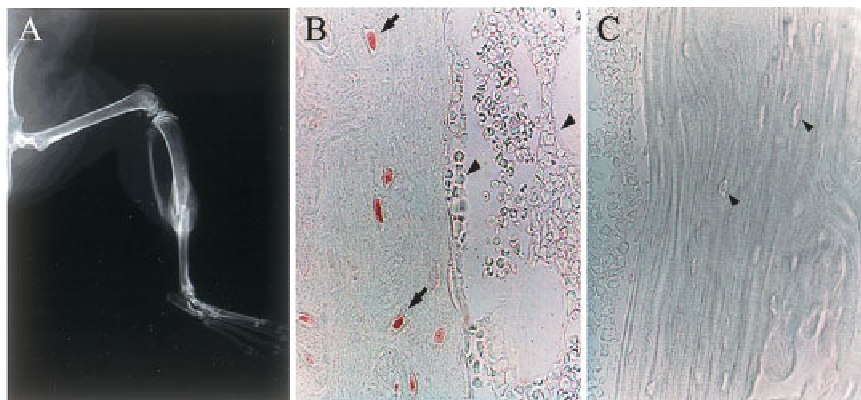

Fig. 6. Osteogenic differentiation of IDmMSCs in vivo. **A**: X-ray of ossified callus 3 weeks after fracture induction by three point bending. **B**: Histological section of the fracture callus in (A) stained with an anti-BrdU antibody reveals donor-derived MSCs within newly formed compact bone at the site of the fracture (arrows), but no donor-derived cells are evident within bone marrow (arrowheads). **C**: Histological section of the contralateral tibia stained with anti-BrdU antibodies as in (B) reveals only host-derived osteocytes (arrowheads).

some specific sequences were detected in DNA samples prepared from fractured tibias of female transplant recipients but not in that of control animals, confirming the engrafted cells were of donor origin (data not shown). Therefore, IDmMSCs were able to engraft at sites of induced fracture, integrate into newly formed bone, and adopt a morphology indistinguishable from host osteocytes. Presently, we have not explored whether the engrafted cells affect the rate of healing or mechanical strength of the fractured tibia.

Growth Kinetics of IDmMSCs

Although plastic adherent marrow cells from murine bone marrow exhibit robust growth in vitro [Phinney et al., 1999a], IDmMSCs doubled in number only after approximately 5–7 days in culture (Fig. 7A). Plating the cells at densities ranging from 3 to 5,000 cells/cm^2 did not increase their growth rate (data not shown). Therefore, we compared the transcript profiles of freshly prepared (FD) IDmMSCs to those that were cultured for 7 days (7D) in vitro after immunodepletion. The expression levels of 71 transcripts were altered 5-fold or greater in 7D versus FD cells, and 65 (92%) of these were down regulated (Fig. 7B). The latter included genes involved in cell proliferation and cell cycle progression, consistent with the poor growth of IDmMSCs (Table II). Other transcripts down regulated included procollagens XV and XVIII, the protease inhibitor Spi2, and TIE1. In contrast, only six transcripts were up regulated by 5-fold or greater in 7D versus FD cells, including FGF2 (Table II). Continuous exposure to FGF2 (20 ng/ml) immediately following immunodepletion induced a statistically significant, 4.5-fold increase in the growth rate of IDmMSCs as compared to untreated cells ($P \leq 0.01$) (Fig. 7A). This effect of FGF2 showed a biphasic dose response in that 5 or 100 ng/ml had no effect on growth but 20 ng/ml significantly stimulated growth. In contrast, exposure of IDmMSCs to insulin-like growth factor (IGF), stem cell factor (SCF), or leukemia inhibitory factor (LIF) produced a measurable increase in growth rate but it was not statistically significant ($P \geq 0.05$) as compared to untreated cells. IDmMSCs exhibited reduced growth rates at passage 2 regardless of whether or not they were exposed to exogenously added mitogens.

Concomitant with its effects on cell growth, FGF2 also significantly altered the transcript profile of IDmMSCs. Out of the 65 transcripts that were down regulated by 5-fold or greater in 7D versus FD cells, expression levels of 53 (82%) of these transcripts were essentially unaltered (≤2-fold difference) if the cells were continuously cultured for 7D in FGF2 (7D + FGF) immediately after immunodepletion (Table II). For example, procollagens XV and XVIII were down regulated 40.9-fold and 99-fold in 7D cells but only 2.7-fold and 1.4-fold in 7D + FGF cells as compared to FD cells, respectively. Concurrently, FGF2 exposure induced expression of 34

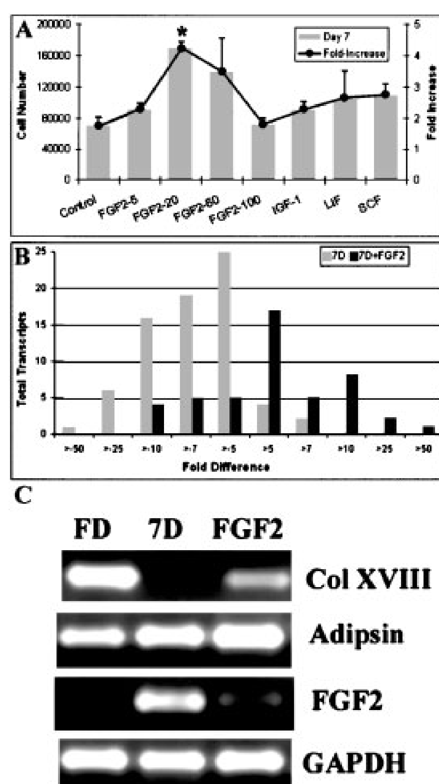

Fig. 7. Effect of FGF2 on growth rate and transcript profile of IDmMSCs. **A**: Aliquots (40,000) of freshly isolated IDmMSCs were cultured for 7 days in media alone (control) or media supplemented with 5, 20, 50, or 100 ng/ml FGF2, 20 ng/ml IGF, 1,000 U/ml LIF, or 20 ng/ml SCF. After 7 seven days the total number of cells was quantified by counting. The results are expressed as the mean cell number from a single experiment run in triplicate and the bars represent the sample standard deviation (*$P \leq 0.01$, Student's *t*-test). Fold increase was determined by dividing the total cell number at 7 days for each treatment group by the number of cells initially plated. **B**: Number of unique transcripts whose expression levels were altered by 5-fold or greater in IDmMSCs cultured for 7 days without (7D) or with FGF2 (7D + FGF2) as compared to cells that were freshly immunodepleted (FD). Graph shows total number of transcripts and their fold-difference between the respective samples as determined using microarray analysis. **C**: Expressed transcripts corresponding to procollagen (Col) XVIII, adipsin, and FGF2 were assayed by semi-quantitative RT-PCR in populations of IDmMSCs as described in (B).

transcripts in IDmMSCs (Fig. 7B, Table III). These included transcripts expressed specifically in adipose tissue or involved in lipid metabolism such as adipsin, stearoyl-coenzyme A desaturase 1, 11beta-hydroxysteroid dehydrogenase, apolipoprotein C1, and adipoQ. FGF2 also induced expression of BMP2 and Dmp-1, gene products that regulate osteogenic differentiation and deposition of extra cellular matrix proteins, respectively. Quantitative differences in the expression levels of mRNAs for adipsin, procollagen XVIII, and FGF2 in FD, 7D, and 7D + FGF populations were validated by semi-quantitative PCR (Fig. 7C).

TABLE II. Comparison of Transcript Levels Between IDmMSC Populations

	Fold difference	
Transcript	7D vs. FD	7D + FGF vs. FD
Procollagen type XVIII, alpha 1	−99.7	−2.7
Spi2 protease inhibitor	−48.9	−6.2
Procollagen type XV	−40.9	−1.4
TIE1	−26.5	−4.1
Thymidine kinase	−20.1	−2.3
IGFBP3	−18.7	1.5
GRO1 oncogene	−18.2	−12.9
Uridine phosphorylase	−17.4	−3.1
Proliferation associated gene 1	−13.4	−2.6
c-fos	−8.9	1.1
Polo-like kinase homolog	−6.6	1.0
Early growth response 1	−6.3	−1.0
DNA topoisomerase II alpha	−5.3	−1.4
Sulfonylurea receptor 2	−5.1	−2.2
Small inducible cytokine A2	−5.1	−1.2
Cell surface antigen AA4	−5.1	−2.5
BMP4	3.1	1.3
Stem cell growth factor	3.4	1.1
cDNA clone	5.5	2.3
Osteomodulin	5.5	1.0
FGF2	5.7	5.0
CAMK1-beta 2	5.9	1.4
Inteferon-induced protein	7.0	6.3
cDNA clone	7.8	17.5

DNA microarrays were used to compare the fold-difference in transcript abundance between MSCs that were freshly immunodepleted (FD) to those that were cultured for 7 days in media alone (7D) or media supplemented with FGF2 (7D + FGF) following immunodepletion. The table lists transcripts whose expression levels were altered by 5-fold or greater in 7D versus FD cells. The table also compares the fold-difference in these transcripts between 7D + FGF and FD cells, illustrating how exposure to FGF2 affects transcription in IDmMSCs. Positive values indicate fold increase in expression, and negative values indicate the fold decrease in expression between experimental samples.

FGF2 Reversibly Inhibits Differentiation of IDmMSCs

Although IDmMSCs readily differentiated into adipocytes, chondrocytes, and osteoblasts in vitro, exposure of cells to FGF2 inhibited their cellular differentiation. IDmMSCs cultured in FGF2 for 7 days prior to transfer to micromass cultures showed a complete lack of chondrogenic differentiation, even though FGF2 was not present during the 6 week time course of differentiation (Fig. 8A). Similarly, transient exposure to FGF2 inhibited adipogenic differentiation on IDmMSCs approximately 10-fold ($P \leq 0.01$) (Fig. 8B). FGF2 also significantly reduced the extent of osteogenic differentiation as evidenced by a visible reduction in the extent of Alizarin Red S stain bound

TABLE III. Transcripts Induced in IDmMSCs by FGF2

Transcript	Fold difference	
	7D + FGF vs. FD	7D vs. FD
Adipsin	110.9	1.8
Coagulation factor V	34.5	1.3
MRP8 calcium binding protein	28.6	−1.7
PAF acetylhydrolase	24.2	1.3
Angiogenin-3 precursor	21.3	−1.6
cDNA clone	17.5	7.8
Dmp-1 gene	16.3	1.1
Matrix metalloproteinase 3	15.5	−4.2
Glycerolphosphate dehydrogenase 1	12	2.1
cDNA clone	11.5	1.2
Adipocyte-specific mRNA	10.7	1.1
Rab3D	10.6	1.9
Stearoyl-coenzyme A desaturase 1	9.4	−1.7
Cytochrome P450	9.0	1.3
cDNA clone	7.5	1.1
ALDR (adrenoleukodystrophy protein)	7.2	1.2
Aldehyde dehydrogenase 3 (aldh3)	7.0	−1.7
Retinal oxidase/aldehyde oxidase	6.9	1.9
11beta-hydroxysteroid dehydrogenase	6.7	−1.7
Apolipoprotein C1	6.5	1.5
BMP2	6.5	−1.9
Inteferon-induced protein	6.3	5.5
Long chain fatty acyl CoA synthetase	6.2	1.3
MCP-8	6.2	−2.2
CD24a	5.9	1.2
cDNA clone	5.4	2.8
cDNA clone	5.4	2.2
Monocarboxylate transporter 1	5.3	−1.5
AdipoQ	5.3	−1.8
Plasma selenoprotein P (SELP)	5.3	2.1
Type 2 deiodinase	5.3	3.4
cDNA clone	5.1	3.5
Carboxylesterase	5.1	4.2

DNA microarrays were used to compare the transcript profile of MSCs that were freshly immunodepleted to those that were cultured for 7 days in media supplemented with FGF2 (7D + FGF) following immunodepletion. The table lists those transcripts that were induced by exposure of IDmMSCs to FGF2 (7D + FGF vs. FD). The table also compares the fold-difference in these transcripts between 7D + FGF and FD cells, illustrating how exposure to FGF2 affects transcription in IDmMSCs. Positive values indicate fold increase in expression, and negative values indicate the fold decrease in expression between experimental samples.

to the extra cellular matrix and a 3.5-fold decrease in its calcium content as compared to 7D cells ($P \leq 0.005$) (Fig. 8C,D). To determine whether this inhibitory effect of FGF2 was reversible, we cultured IDmMSCs in FGF2 for 7 days and then withdrew it from the media for 2, 5, or 7 days before the cells were induced to differentiate. The osteogenic capacity of the cells increased as a function of FGF2 withdrawal time such that after 5 or 7 days there was no significant difference in osteogenic differentiation, as measured by calcium deposition, between untreated IDmMSCs and those exposed to FGF2 (Fig. 9A,B). A similar result was seen for adipogenic differentiation, but recovery of chondrogenic differentiation did not occur until FGF2 was withdrawn from the culture media for several weeks. The later is likely due to the fact that FGF2 down regulates expression of various adhesion molecules that promote cellular aggregation and are necessary to promote chondrogenic differentiation (data not shown).

DISCUSSION

Currently there is great interest in using MSCs as therapeutic agents to treat a broad spectrum of diseases, including spinal cord injury [Hofstetter et al., 2002], stroke [Chen et al., 2001], myocardial infarction [Shake et al., 2002; Toma et al., 2002], and pulmonary fibrosis [Kotton et al., 2001]. To better exploit genetically altered mouse strains, which provide effective experimental models to study both the biology and evaluate the therapeutic potential of stem cells, we developed a scheme to isolate MSCs from murine bone marrow using immunodepletion. This approach entails the limited expansion ex vivo of plastic adherent marrow

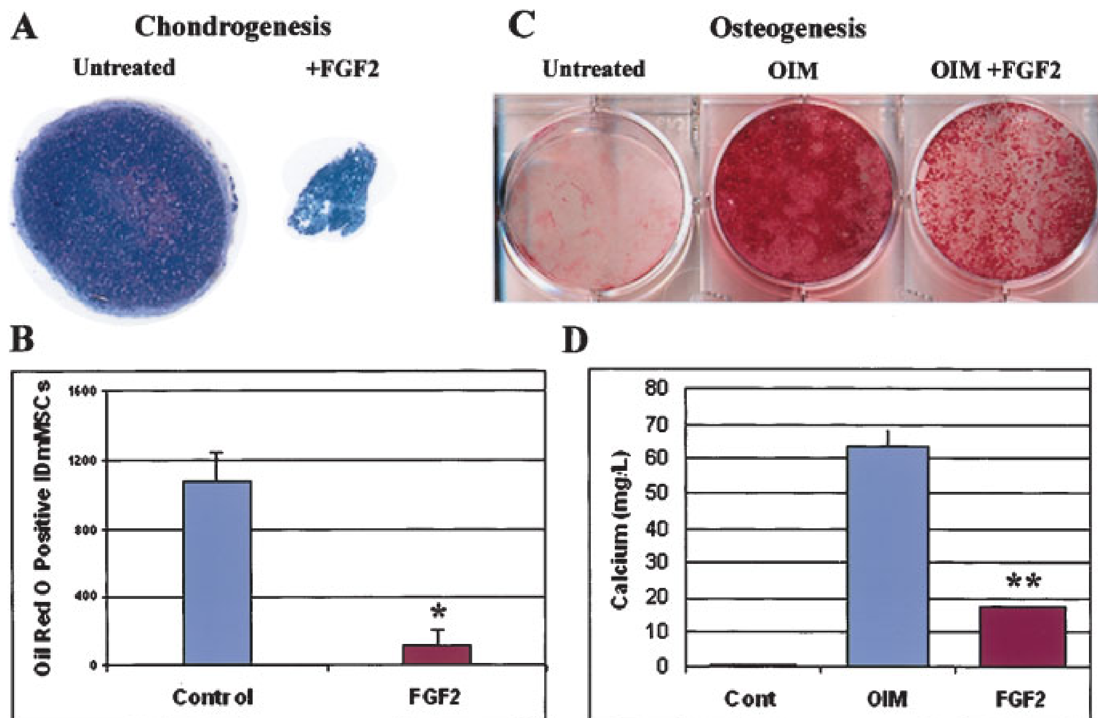

Fig. 8. FGF2 inhibits cellular differentiation of IDmMSCs. IDmMSCs were cultured for 7 days in media alone or in media containing FGF2 and then subjected to in vitro differentiation. **A**: Histological sections of chondrogenic pellets stained with toluidine blue that were generated by culturing IDmMSCs under micromass conditions for 6 weeks. **B**: Total number of Oil Red O positive IDmMSCs after exposure of cells to adipogenic differentiation for 7 days. Numbers are expressed as the mean value determined by counting eight representative microscopic fields and the error bars represent the sample standard deviation (*$P \leq 0.01$, Student's *t*-test). **C**: Extent of Alizarin Red S staining of the extra cellular matrix in untreated IDmMSCs or cells exposed to OIM for 21 days. **D**: Quantification of the total amount of calcium contained in the extra cellular matrix deposited by cells in (C). Values represent the mean of a single experiment done in triplicate and the bars represent the sample standard deviation (**$P \leq 0.005$, Student's *t*-test).

cells followed by their separation from contaminating hematopoietic lineages using anti-CD11b, anti-CD34, and anti-CD45 antibodies conjugated to paramagnetic beads. The immunodepleted cell population expressed Sca-1 but not CD117 (c-kit), CD135 (Flt-3), and CD31, antigens typically associated with hematopoietic and endothelial cells [Jackson et al., 2001]. They also expressed common MSC markers, including CD9, CD29, CD44, CD81, and CD106, as well as the stem cell-specific marker NST [Tsai and McKay, 2002]. Moreover, the cells differentiated into adipocytes, chondrocytes, and osteoblasts in vitro as well as osteocytes in vivo. IDmMSCs have also been shown to engraft in the CNS and differentiate into astrocytes [Kopen et al., 1999; McBride et al., 2003] as well as engraft in lung where they adopt an epithelial-like morphology and co-purify with type II epithelial cells [Ortiz et al., 2003]. Although we acknowledge that classification as a stem cell requires demonstrating self-renewal and multi-lineage differentiation at the level of a single cell, the lack of suitable assays to evaluate the ability of MSCs to serially reconstitute an ablated stem cell compartment in vivo represents a formidable obstacle toward this end. Accordingly, cells that exhibit multi-lineage mesenchymal differentiation in vitro, which may include bona fide stem cells and different classes of progenitors, are all promiscuously referred to as MSCs in the literature. Clearly, based on their morphology, phenotype, and differentiation potential in vitro and in vivo our immunodepleted cells are analogous to MSC populations described from human and rat bone marrow.

The one distinctive characteristic of IDmMSCs is their lack of cell proliferation in vitro. In contrast, human and rat MSCs can be propagated extensively in vitro [Lennon et al., 1995; Colter et al., 2000]. Several recent

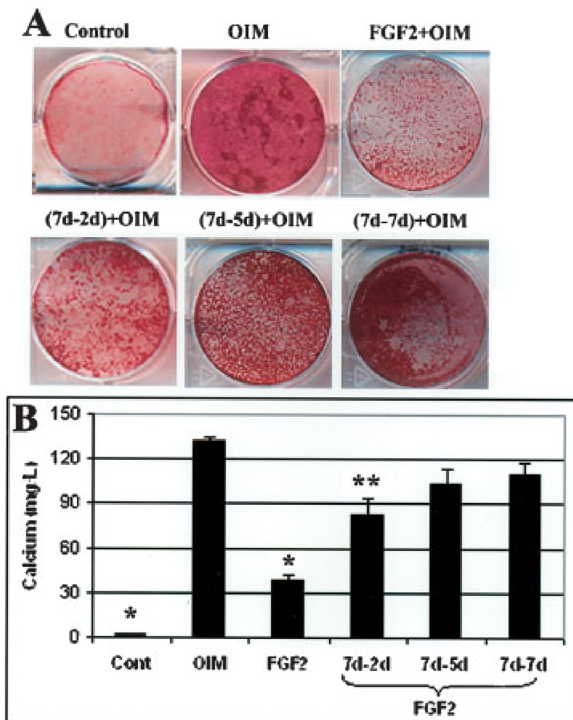

Fig. 9. FGF2 withdrawal restores IDmMSCs ability to undergo osteogenic differentiation. **A**: IDmMSCs were culture for 7 days in media alone or media containing FGF2. Cells were then cultured an additional 21 days in normal media (control) or OIM and then stained with Alizarin Red S. Alternatively, cells exposed to FGF2 for 7 days were then cultured 2 (7d–2d), 5 (7d–5d), or 7 (7d–7d) days in media without FGF2 prior to exposure to OIM for 21 days. **B**: Histogram showing calcium content of the extra cellular matrix from populations in (A) ($^*P \leq 0.001$; $^{**}P \leq 0.01$; Student's *t*-test).

reports have documented that human MSCs loose multi-potency with continued passage [DiGirolamo et al., 1999; Mauraglia et al., 2000; Pittenger et al., 2001] indicating that factors that support long-term maintenance of the bona fide stem cells are unknown. Our data suggests that growth of murine MSCs are dependent on factors that are eliminated during immunodepletion, and as such the likely source of these factors is the hematopoietic cell fraction. These findings are consistent with previous studies showing that growth of fibroblastoid colonies from murine marrow requires the presence of hematopoietic or lymphoid cells [Friedenstein et al., 1976]. Additionally, Kuznetsov et al. [1997a] showed that formation of stromal fibroblast colonies from mouse bone marrow was inhibited by neutralizing antibodies to PDGF, EGF, and TGF-β. Our results demonstrate that FGF2 stimulates the growth of IDmMSCs, affording a 4.5-fold expansion of the cells in 1 week. Consistent with these findings, FGF2 was previously shown to be mitogenic for human and mouse marrow stromal cells [Pri-Chen et al., 1998; Walsh et al., 2000; Zhang et al., 2002], human, rabbit, and canine MSCs [van den Bos et al., 1997; Tsutsumi et al., 2001], human calvarial osteoblasts [Debiais et al., 1998], and rat osteo-progenitor cells [Tanaka et al., 1999]. Importantly, IDmMSCs cultured in FGF2 fail to sustain a high growth rate after serial passage, indicating that the cells also require other mitogenic factors. Our immunodepletion scheme provides a useful experimental system to evaluate these factors systematically.

FGF2 also reversibly inhibited the ability of IDmMSCs to undergo cellular differentiation. Previous studies have shown that FGF2 may induce or inhibit this process depending upon the experimental system [Pitaru et al., 1993; Hanada et al., 1997; Pri-Chen et al., 1998; Enomoto-Iwamoto et al., 2000; Nakajima et al., 2001]. More recent studies suggest that this paradoxical affect is due to differentiation stage-specific effects of FGF2, stimulating growth and inhibiting differentiation of uncommitted progenitors but accelerating the maturation of committed precursors [Martin et al., 1997; Debiais et al., 1998; Walsh et al., 2000]. Accordingly, the inability of FGF2 to completely inhibit osteogenic differentiation of IDmMSCs likely reflects the existence of multi-potential progenitors and osteogenic precursors within the population. Differentiation of the later into mature osteoblasts would also explain up regulation of BMP2 and DMP1 seen by microarray analysis. The ability of FGF2 to inhibit osteogenic differentiation is attributed to down regulation of core binding factor alpha 1 (cbfa1) and up regulation of TWIST, transcriptional regulators that are requisite for and negatively regulate osteoblastic differentiation, respectively [Lee et al., 1999; Tsuji and Noda, 2001]. Analysis of the IDmMSC transcriptome by serial analysis of gene expression indicated the cells express both cbfa1 and TWIST (D.G. Phinney, unpublished results).

The growth characteristic and phenotype of IDmMSCs clearly distinguishes them from mesenchymal adult progenitor cells (MAPCs) recently described by Jiang et al. [2002]. MAPCs are isolated from bone marrow cells cultured 3–4 weeks on plastic dishes in media containing LIF, EGF, and PDGF. The $CD45^-$/$Ter119^-$

population is then selected, replated at low density, and cultured for several more weeks. MAPCs are characterized as the small percentage of cells (1%) that yield continuously growing cultures under these conditions. MAPCs are CD44 and c-kit negative and express the LIF receptor, Oct-4, and high levels of SSEA-1. In contrast, IDmMSCs are CD44 positive and do not express Oct-4, SSEA-1, or the LIF receptor. Another distinction between IDmMSCs and MAPCs is that the later cells cannot be established from bone marrow directly, or bone marrow cultured for 3–4 weeks in the absence of LIF, EGF, and PDGF. The appearance of MAPCs only after expansion under selective conditions, together with their apparent lack of replicative senescence, indicates they are characteristic of immortalized cell lines. Therefore, MAPCs and IDmMSCs are phenotypically and functionally distinct.

In summary, these studies describe a reliable method to isolate MSCs from murine bone marrow, describe their phenotypic and functional characteristics, and demonstrate that FGF2 has both mitogenic affects and reversibly inhibits differentiation of the cells. Therefore, immunodepletion coupled with exposure to FGF2 provides a means to expand ex vivo in an undifferentiated state MSCs elaborated from murine bone marrow.

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