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Autocrine Fibroblast Growth Factor 2 Increases the Multipotentiality of Human Adipose-Derived Mesenchymal Stem Cells

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ABSTRACT

Multipotent mesenchymal stem cells (MSCs), first identified in the bone marrow, have subsequently been found in many other tissues, including fat, cartilage, muscle, and bone. Adipose tissue has been identified as an alternative to bone marrow as a source for the isolation of MSCs, as it is neither limited in volume nor as invasive in the harvesting. This study compares the multipotentiality of bone marrow-derived mesenchymal stem cells (BMSCs) with that of adipose-derived mesenchymal stem cells (AMSCs) from 12 age- and sex-matched donors. Phenotypically, the cells are very similar, with only three surface markers, CD106, CD146, and HLA-ABC, differentially expressed in the BMSCs. Although colony-forming units-fibroblastic numbers in BMSCs were higher than in AMSCs, the expression of multiple stem cell-related genes, like that of fibroblast growth factor 2 (FGF2), the Wnt pathway effectors FRAT1 and frizzled 1, and other self-renewal markers, was greater in AMSCs. Furthermore, AMSCs displayed enhanced osteogenic and adipogenic potential, whereas BMSCs formed chondrocytes more readily than AMSCs. However, by removing the effects of proliferation from the experiment, AMSCs no longer out-performed BMSCs in their ability to undergo osteogenic and adipogenic differentiation. Inhibition of the FGF2/fibroblast growth factor receptor 1 signaling pathway demonstrated that FGF2 is required for the proliferation of both AMSCs and BMSCs, yet blocking FGF2 signaling had no direct effect on osteogenic differentiation. STEM CELLS *2008;26:1598–1608*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) are a rare cell type that were originally found in the bone marrow (bone marrow-derived mesenchymal stem cells [BMSCs]) during the 1960s [1]. Since then, populations of MSCs have been identified in many other tissues, including adipose tissue [2–4], umbilical cord blood [5–8], synovium [9], and muscle [10]. BMSCs have since become an attractive source of stem cells for tissue engineering applications and are currently in various phases of clinical trial [11–13]. Their potential applications are a direct result of their ability to differentiate into cells of the mesodermal lineage, including adipocytes, chondrocytes, osteoblasts, and myocytes [14–16]. However, their use has been somewhat limited because of their invasive isolation procedure and the decline of their proliferation and multilineage differentiation potential with age [17–19]. These issues have led to more thorough investigations for alternative sources of MSCs.

One of the interesting alternative sources of MSCs is adipose tissue harvested through liposuction, a much less invasive method than bone marrow aspiration. In addition, adipose tissue has the advantage of being generally available in much larger quantities than cord blood or bone marrow. Adipose-derived mesenchymal stem cells (AMSCs) are phenotypically similar to BMSCs and are also capable of multilineage differentiation in a manner resembling BMSCs [4, 5, 8]. However, direct comparison of multilineage differentiation capabilities of AMSCs and BMSCs has produced conflicting results [8, 20–23]. One key reason for the conflicting data may be that the ages of the donors often differ greatly when adipose and bone marrow are obtained from different donors [8, 22]. Age has a substantial effect on the ability of BMSCs to self-renew and differentiate [17–19]. Furthermore, the sex of the donor affects multipotentiality, particularly for BMSCs, because of factors such as menopause, corresponding hormonal imbalance, and the increasing incidence of osteoporosis (recently reviewed [24, 25]). Unfortunately, such considerations are often overlooked, contributing to inconsistent findings.

Here, we aimed to eliminate these confounding factors by comparing the multipotentiality of AMSCs and BMSCs from age- and sex-matched individuals. In this study, six BMSC and six AMSC donors were compared for their colony-forming ability, cell surface antigen expression, multilineage differenti-

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ation potential, and stem cell marker gene expression. BMSCs showed greater ability to form colonies and chondrocytes, whereas AMSC had higher proliferation rates and differentiated more readily into osteoblasts and adipocytes. This rapid growth appears to be linked to increased fibroblast growth factor 2 (FGF2) production, which positively influenced differentiation. When the growth effect was removed by seeding cells at confluence, BMSCs differentiated more readily into osteoblasts and adipocytes. Inhibition of fibroblast growth factor receptor 1 (FGFR1) signaling in these experiments had no direct effect on differentiation, demonstrating that FGF2 indirectly enhances osteogenic differentiation because of its proliferative effects.

MATERIALS AND METHODS

MSC Isolation and Cell Culture

Human MSCs were isolated from bone marrow mononuclear cells (BMNCs) obtained from six donors (four female [F], two male [M]; age range, 21–35 years; mean age, 26.83 years) (Cambrex, Walkersville, MD, http://www.cambrex.com), and six adipose samples (5 F, 1 M; age range, 21–33 years; mean age, 26.33 years) were obtained through vacuum pump-assisted liposuction (Hercules liposuction machine; Wells Johnson, Tucson, AZ, http://www.wellsgrp.com) from the abdominal regions of healthy adult donors after informed consent and the approval by the Institutional Review Board, National University Hospital, Singapore. BMNC fractions were seeded into 15-cm dishes in maintenance medium (Dulbecco's modified Eagle's medium [DMEM], 1g/l glucose, 10% fetal calf serum [FCS], 2 mM L-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin), and cells were allowed to adhere for 3 days before the first medium change. Lipoaspirates were processed using a modified protocol [4]. Adipose tissue was first washed three times with phosphate-buffered saline (PBS) and digested with 0.075% collagenase I (Gibco, Grand Island, NY, http://www.invitrogen.com) for 2 hours at 37°C with gentle, continuous shaking. Cells were pelleted at 500*g* and then plated on culture flasks. The cells were cultured in maintenance medium, with a medium change every 3–4 days, and routinely passaged upon 85% confluence using 0.125% trypsin. On replating, cells were seeded at 3,000 cells per cm^2. All cultures were maintained in a humidified incubator at 37°C with 5% CO_2. Only cells that were in passage 5 or 6 were used in these experiments.

Flow Cytometry

Cells were removed from culture using a nonenzymatic cell dissociation solution (CellStripper; Mediatech, Manassas, VA, http://www.cellgro.com) and washed once in PBS before counting. Cells (1×10^5) were then aliquoted into a 96-well plate, and cells were pelleted at 450*g* for 5 minutes. Prediluted antibody solutions in 2% FCS/PBS were subsequently added, and cells were incubated on ice for 20 minutes. Cells were then washed twice in 2% FCS/PBS before resuspension in 2% FCS/PBS and analyzed on a Guava PCA-96 benchtop flow cytometer (Guava Technologies, Hayward, CA, http://www.guavatechnologies.com). All samples were measured in triplicate for each cell line. All antibodies used are shown in supplemental online Table 1.

Colony-Forming Units-Fibroblastic Assay

Cells were plated at 80 cells per cm^2 in a 12-well plate and cultured for 12–14 days, with a single medium change after day 7. Cells were then fixed for 15 minutes in 100% methanol at room temperature before being stained with 0.1% Giemsa stain in methanol for 10 minutes. Wells were washed with water and allowed to air dry. Colonies were counted only when they were greater than 50 cells in size and were not in contact with another colony.

DNA Quantification

DNA was quantified using the Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) according to the manufacturer's instructions and read at 495/515 nm using a Victor3 1420 Multilabel plate reader (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com).

Multilineage Differentiation

MSCs for adipocyte and osteoblast differentiation were removed from culture as described above and then washed once in maintenance medium before being seeded into either chamber slides for immunohistochemistry, 12-well plates for histological stains, or 6-well plates for protein and RNA isolation.

For osteogenesis, cells were seeded at 3,000 cells per cm^2 in maintenance medium in 6-well and 12-well plates and chamber slides and incubated as described above for 24 hours before being changed to osteogenic medium (maintenance medium, 10 nM dexamethasone, 25 μg/ml ascorbic acid, and 10 mM β-glycerophosphate) or fresh maintenance medium. Cells were then maintained for up to 28 days, with a medium change every 3–4 days. After 14 days, cells in the chamber slides were fixed in 4% paraformaldehyde (PFA) and stored at 4°C in PBS for immunocytochemistry. After 14 and 28 days the cells were stained with alizarin red S for calcium and by the von Kossa method for calcium phosphate. RNA was extracted using the Nucleospin RNA extraction kit according to the manufacturer's instructions (Macherey-Nagel, Bethlehem, PA, http://www.mn-net.com), and protein samples were extracted as described below.

For adipogenesis, cells were seeded at 18,000 cells per cm^2 in maintenance medium and incubated as described above for 2 days. Medium was removed, and cells were washed once in PBS before the addition of adipogenic maintenance medium (DMEM, 4.5 g/l glucose, 10% FCS, L-glutamine, and penicillin and streptomycin) or adipogenic medium (adipogenic maintenance medium with 10 μg/ml insulin, 115 μg/ml methyl-isobutylxanthine, 1 μM dexamethasone, and 20 μM indomethacin). Cells were then maintained for up to 28 days, with a medium change every 3–4 days. After 14 and 28 days, the cells were stained with oil red O to stain the lipid droplets. RNA and protein were extracted as for the osteoblasts.

For chondrogenesis, cells were counted and resuspended at 5×10^5 cells per milliliter in chondrogenic medium (DMEM with Cambrex chondrogenic single aliquots) with or without 10 ng/ml TGFβ3 (Cambrex), and then 500-μl aliquots were put into 15-ml tubes before centrifugation at 150*g* at room temperature for 10 minutes and incubation at 37°C for 2 days. After 2 days, the tubes contained loose round pellets. Pellets were maintained for 21 days, with a medium change every 3–4 days, before RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) or cell pellets were fixed in 4% PFA and embedded for cryosectioning. Serial sections were made before slides were stored at −80°C for immunohistochemistry. When osteogenic and adipogenic differentiation was investigated under confluent conditions, cells were seeded at 30,000 cells per cm^2 and allowed to reach confluence before switching to the relevant differentiation medium and cultured as described above.

Immunohistochemistry

Chondrocyte pellet slides were removed from −80°C and put at 37°C with a drop of PBS on the section for 30 minutes prior to fixation in 4% PFA for 30 minutes at room temperature. Slides were washed with PBS three times for 5 minutes before incubation with 0.3% H_2O_2 in water for 20 minutes. Slides were then washed three times as described above and blocked in 5% goat serum, 0.1% Triton X-100 in PBS for 1 hour. Slides were subsequently incubated with the relevant primary antibody or isotype control at 4°C overnight in a humidified chamber. All secondary antibodies were obtained from the ABC kits (Vectastain, Vector Labs, Burlingame, CA, http://www.vectorlabs.com) and used according to the manufacturer's instructions. Slides were developed using the DAB kit (Dako, Glostrup, Denmark, http://www.dako.com) according to the manufacturer's instructions. Cells grown in chamber slides under osteogenic were removed from culture, washed in PBS, and immediately fixed in 4% PFA before being stained as described above. All antibodies used are shown in supplemental online Table 1.

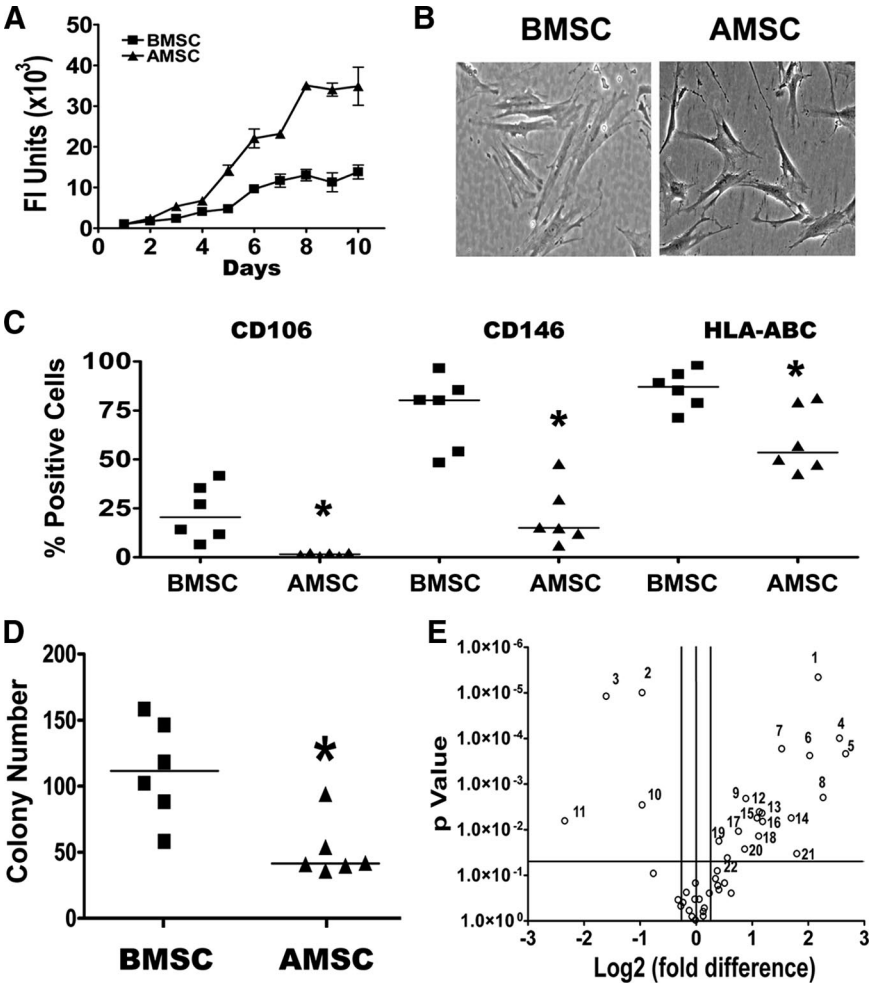

Figure 1. Phenotypic and genotypic analysis of AMSCs and BMSCs. Cells were grown to passage 5 and then analyzed for their growth rate, cell surface markers, CFU-F ability, and stem cell marker expression. **(A):** Growth rate analysis of AMSCs and BMSCs, as measured by DNA content. Graph shows the mean ± SEM. **(B):** Phase-contrast pictures demonstrating the difference in morphology between AMSCs and BMSCs when initially plated for the cell growth assays. **(C):** Differentially expressed surface markers in AMSCs and BMSCs. **(D):** CFU-F colonies formed in BMSCs and AMSCs. **(E):** Volcano plot showing the differential expression of stem cell-related genes. Circles on the right represent genes overexpressed by AMSCs, and those on the left represent genes overexpressed by BMSCs. Numbered circles in the top left or top right quadrants were significantly overexpressed by one of the two groups and are identified in Table 1. For all assays, $n = 6$ for BMSCs and AMSCs. *, $p < .02$. Abbreviations: AMSC, adipose-derived mesenchymal stem cell; BMSC, bone marrow-derived mesenchymal stem cell; CFU-F, colony-forming units-fibroblastic; FI, fluorescence intensity; HLA-ABC, human leukocyte antigen ABC.

Image Analysis

Bioquant Image Analysis software (Bioquant Image Analysis Corp., Nashville, TN, http://www.bioquant.com) was used to quantify the average density of stained wells. Photographs of the 12-well plates and specimen slides were taken with the same light intensity and exposure to keep consistency within samples. Using the image analysis software, the digital images were loaded into the field of view, and a consistent region of interest was placed over each well/specimen. The average density of the wells was recorded for each sample and is reported in units of grayscale. The density of the negative stains was subtracted from the recorded density for each sample and then normalized to DNA content. In these measurements, a darker stain correlates with minimal light transmission, giving a higher density value.

SDS-Polyacrylamide Gel Electrophoresis and Blotting

Cells were lysed, protein concentrations were quantified, and 15 mg was resolved by SDS-polyacrylamide gel electrophoresis as previously described [26]. All antibodies used are shown in supplemental online Table 1.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using an RNA Isolation Nucleospin1 RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. RNA (250 ng) was reverse transcribed using the SuperScript III and random hexamers kit (Invitrogen). Oligonucleotides were designed using Primer Express1 software, version 2.0 (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) and synthesized by ProLigo (Sigma-Aldrich, Singapore, http://www.sigmaaldrich.com) or were purchased from Applied Biosystems. The specific sequences are outlined in supplemental online Table 2. Polymerase chain reaction (PCR) products were sequenced by the Institute of Molecular and Cell Biology Sequencing Facility (Singapore). Real-time PCR was performed as previously described [27].

PCR Array

The human stem cell RT^2 Profiler PCR Array (Genomax Technologies, Singapore, http://www.genomax.com.sg) was used to look at the expression of stem cell markers in the two groups of MSCs following the manufacturer's instructions. For analysis, the BMSC group was selected as the control group and AMSCs as the test group. All fold change data are thus represented as a fold change from the bone group. Anything above 1 is considered an upregulation of gene expression in AMSCs, and any value less than 1 indicates a downregulation of gene expression in AMSCs compared with BMSCs. A fold change of greater than 1.2 with a p value of $<.05$ was considered to be significant.

FGF2 Enzyme-Linked Immunosorbent Assay

Cells were plated at 3,000 cells per cm^2 and cultured in maintenance medium for up to 10 days. At days 4, 7, and 10, medium- and matrix-bound proteins were removed, and FGF2 levels were measured using an FGF2 Quantikine enzyme-linked immunosorbent assay (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). Removal of matrix-bound proteins was as described previously [28].

Table 1. Stem cell self-renewal and maintenance gene expression in bone marrow-derived mesenchymal stem cells (BMSCs) and adipose-derived mesenchymal stem cells

Significantly different expression				No significantly different expression[a]		
Number	Symbol	Log_2 (FC)	*p* value	Symbol	Log_2 (FC)	*p* value
1	*BMP2*	2.18	4.6×10^{-6}	*ABCG2*	−0.23	4.0×10^{-1}
2[b]	*NOTCH2*	−0.96	1.0×10^{-5}	*ADAR*	−0.01	9.7×10^{-1}
3[b]	*CXCL12*	−1.60	1.2×10^{-5}	*APC*	−0.07	8.1×10^{-1}
4	*ALDH1A1*	2.56	1.0×10^{-4}	*AXIN1*	0.51	1.5×10^{-1}
5	*IGF1*	2.67	2.2×10^{-4}	*BMP1*	−0.32	3.5×10^{-1}
6	*FGF2*	2.03	2.4×10^{-4}	*BTRC*	−0.12	6.0×10^{-1}
7	*ALDH2*	1.53	1.7×10^{-4}	*CCNA2*	0.39	1.7×10^{-1}
8	*CCND2*	2.27	2.0×10^{-3}	*CCND1*	0.13	6.3×10^{-1}
9	*FZD1*	0.89	2.1×10^{-3}	*CCNE1*	0.13	7.9×10^{-1}
10[b]	*GJA1*	−0.96	2.9×10^{-3}	*CDC2*	0.63	2.5×10^{-1}
11[b]	*JAG1*	−2.34	6.5×10^{-3}	*EP300*	0.24	2.5×10^{-1}
12	*GCN5L2*	1.13	4.1×10^{-3}	*FGF1*	−0.76	9.2×10^{-2}
13	*MYST1*	1.18	4.4×10^{-3}	*FGFR1*	0.15	5.3×10^{-1}
14	*FRAT1*	1.70	5.6×10^{-3}	*GJB1*	0.06	3.4×10^{-1}
15	*HSPA9B*	1.09	5.6×10^{-3}	*IPF1*	−0.01	1.5×10^{-1}
16	*HDAC2*	1.19	6.7×10^{-3}	*ISL1*	−0.02	3.4×10^{-1}
17	*MYC*	0.76	1.1×10^{-2}	*MYST2*	0.35	1.2×10^{-1}
18	*DTX2*	1.12	1.4×10^{-2}	*NOTCH1*	0.41	2.1×10^{-1}
19	*PPARD*	0.41	1.8×10^{-2}	*NUMB*	0.38	8.1×10^{-2}
20	*DVL1*	0.87	2.7×10^{-2}	*PARD6A*	−0.27	4.8×10^{-1}
21	*GJB2*	1.80	3.4×10^{-2}	*RB1*	−0.17	2.4×10^{-1}
22	*CDC42*	0.56	4.2×10^{-2}			

[a]Columns under "No significantly different expression" indicate genes that are not significantly differently expressed in either population.
[b]Genes that are overexpressed in the BMSCs. Numbers indicate the dot that coincides with a particular gene in Figure 2A.

SU5402 Studies

Cells were seeded at 3,000 or 30,000 cells per cm^2 and allowed to adhere overnight. For proliferation and signaling under normal growth conditions, cells were cultured for 6 days in the presence of dimethyl sulfoxide (DMSO) or SU5402 (Calbiochem, San Diego, http://www.emdbiosciences.com) at 25, 50, or 100 μM before being washed twice in PBS and lysis in 100 μl of 2 × SDS buffer (Sigma-Aldrich). FGF2 effects on signaling were observed by culturing cells for up to 2 days under serum-free conditions in the presence of DMSO or SU5402 before the addition of 2.5 ng/ml FGF2 or an equivalent volume of PBS for 5 minutes. Cells were washed and lysed as described above. Proteins were resolved as described above. Osteogenic differentiation studies were carried out as described above, but in the presence of 25 μM SU5402.

Statistical Analyses

Standard analyses were carried out using the nonparametric Mann-Whitney *t* test. Significant differences were considered those with a *p* value of $<.05$. For growth curves, a two-way analysis of variance was performed with Bonferroni's post-test analysis.

RESULTS

Phenotypic Comparison of AMSCs and BMSCs

DNA was collected daily from cultured cells and quantified as a measure of cell growth. Upon plating of cells at 3,000 cells per cm^2, the AMSCs grew more quickly than the BMSCs (Fig. 1A). Both cell groups had equivalent growth up to 4 days in culture, followed by a significant change in the growth of the AMSCs until confluence at day 8. Surprisingly, both preparations reached confluence at the same time, although the BMSCs had threefold less DNA, indicating the presence of fewer cells. Comparison of the morphology of the cells revealed differences between the two donor groups (Fig. 1B; supplemental online Fig. 1). The AMSCs appeared to have a more compact, spindle-like morphology, whereas the BMSCs appeared larger and more spread out.

We next analyzed a panel of 21 surface markers for their expression on donor MSCs (summarized in supplemental online Table 3). All staining for hematopoietic markers was negative, and levels for nonhematopoietic stains were similar except for three: only CD106 (20% and 1.5%), CD146 (71% and 20%), and human leukocyte antigen ABC (HLA-ABC) (84% and 59%) showed significant differences, with BMSCs having greater expression than AMSCs (Fig. 1C). The BMSCs also have a corresponding increase in their colony-forming units-fibroblastic (CFU-F) ability, with BMSCs demonstrating a two-fold greater ability to form colonies compared with AMSCs (111.6 ± 37.1, compared with 51.2 ± 21.8), suggesting that our BMSC preparations contained more stem cells than the AMSC preparations (Fig. 1D).

Stem Cell Marker Expression Is Increased in AMSCs

To determine the expression of stem cell markers in the two cell populations, we carried out a quantitative stem cell PCR array. Of the 84 genes, 58 were expressed in one or both of the cell samples, and of these, 31 were differentially expressed. Significantly, 26 of the genes were upregulated in the AMSCs and only 5 in the BMSCs (supplemental online Table 4). Of the 22 stem cell marker or maintenance genes differentially overexpressed, 18 were in the AMSCs, including the important positive regulators of the Wnt pathway FRAT1 and the Wnt receptor Frizzled 1; the cell cycle regulators FGF2, *Myc*, CDC42, and cyclin D2; and the self-renewal markers Myst1 and HSPA9b (Fig. 1E; Table 1). These findings suggest that AMSCs may actually contain more stem cells than BMSCs, in contradiction to the CFU-F results. To investigate this apparent discordance, multilineage differentiation assays were conducted.

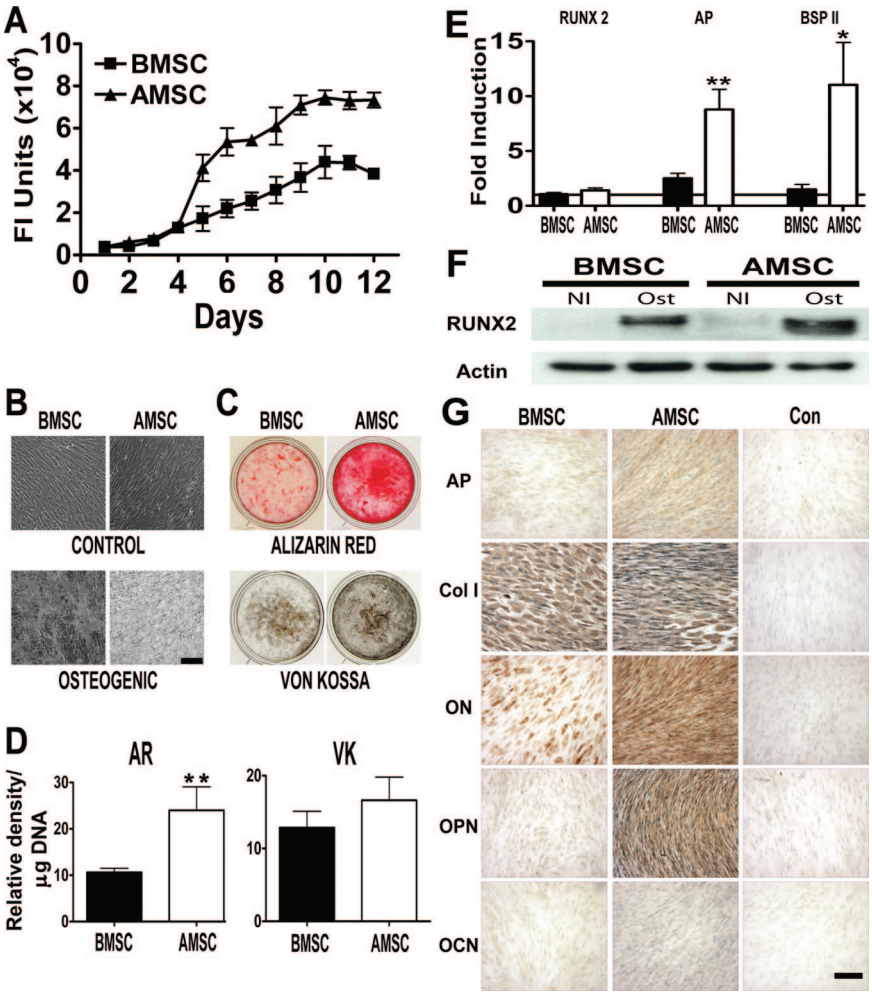

Figure 2. Osteogenic differentiation is enhanced in AMSCs. To compare the osteogenic potential of the cells from each source, a differentiation assay lasting 14 and 28 days was performed. **(A):** Growth was analyzed during osteogenic differentiation as previously described. **(B):** Phase-contrast pictures of cells from both source cultured under normal or osteogenic conditions. **(C, D):** Representative VK and AR staining **(C)** and their quantification using image analysis **(D)**. Scale bar = 20 μm. **(E):** Quantitative real-time polymerase chain reaction analysis of osteogenic markers at day 14 of culture. Graphs are represented as FI compared with their respective Con (black line on graphs). **(F):** Western blot of RUNX2 protein levels at day 14. **(G):** Cells were stained with antibodies specific for osteogenic proteins or their relevant isotype Con and visualized using DAB. Scale bar = 20 μm. All graphs show the mean ± SEM. *, $p < .02$; **, $p < .005$. Abbreviations: AMSC, adipose-derived mesenchymal stem cell; AP, alkaline phosphatase; AR, alizarin red; BMSC, bone marrow-derived mesenchymal stem cell; BSP II, bone sialoprotein II; Col I, collagen I; Con, control; FI, fluorescence intensity; NI, non-induced; OCN, osteocalcin; ON, osteonectin; OPN, osteopontin; Ost, osteogenic medium; VK, von Kossa.

Osteogenic Differentiation

Cells were grown in osteogenic medium for up to 28 days to test their ability to differentiate into osteoblasts. Growth rates were assayed over the first 12 days, and although both sets of cells reach confluence at the same time (days 9–10), there were approximately twice as many cells in the AMSC samples, as measured by DNA content (Fig. 2A), similar to the growth in maintenance medium. There were also differences in cell morphology (supplemental online Fig. 1). After 28 days of differentiation, the AMSCs had secreted much more matrix than the BMSCs in osteogenic medium; the cell layer was completely obscured in the AMSC cultures yet still visible in the BMSCs (Fig. 2B). Cells were stained with alizarin red or with the von Kossa procedure after a total of 14 or 28 days of differentiation. After a total of 14 days, there was no significant staining observed (data not shown), but after 28 days, significant differences in staining between BMSC and AMSC osteogenic cultures could be seen (Fig. 2C, 2D), with AMSCs exhibiting increased staining densities compared with BMSCs even after normalization for DNA content (normalized staining densities for alizarin red of 23.98 and 10.6, and for von Kossa of 16.62 and 12.86, respectively).

To confirm these results, osteogenic markers were analyzed at day 14 by quantitative real-time polymerase chain reaction (qPCR), immunohistochemistry, and Western blotting. Under osteogenic conditions, qPCR revealed that AMSCs had greater inductions of alkaline phosphatase (8.8-fold, compared with 2.5-fold) and of bone sialoprotein II (11-fold, compared with 1.5-fold) compared with basal levels (Fig. 2E). These are early markers of proliferation and osteogenesis. Interestingly, despite the apparently poor induction of RUNX2 mRNA, its protein levels were significantly increased above those of controls in osteogenic cultures (Fig. 2F). There was also appreciably more RUNX2 in the AMSCs than in the BMSCs. Immunocytochemical staining for alkaline phosphatase confirmed the qPCR results, as staining was much more intense in the AMSCs, even though another early marker of osteogenesis, collagen I, was similarly expressed in the two cell populations. The intensities of staining for osteonectin, osteopontin, and osteocalcin, later markers of osteogenesis, were also greater in the AMSCs (Fig. 2G). Overall, these findings demonstrate that the AMSCs exhibit stronger indications of osteoblastic differentiation than the BMSCs.

Adipogenic Differentiation

For adipocyte differentiation, the cells are plated at a much higher density and consequently the growth phase is much reduced. The AMSCs reach an initial plateau phase of growth at day 3, whereas the BMSCs are confluent at day 5, yet, in contrast to the lower plating densities, there appear to be similar numbers of cells at confluence (Fig. 3A compared with Figs. 1A, 2A). The morphology of cells following 28 days of differentiation revealed the extensive formation of lipid droplets in the cell layers (Fig. 3B, top panel). Again, very little positive staining was observed at day 14 with oil red O (data not shown), but at day 28, AMSCs have a normalized staining density of

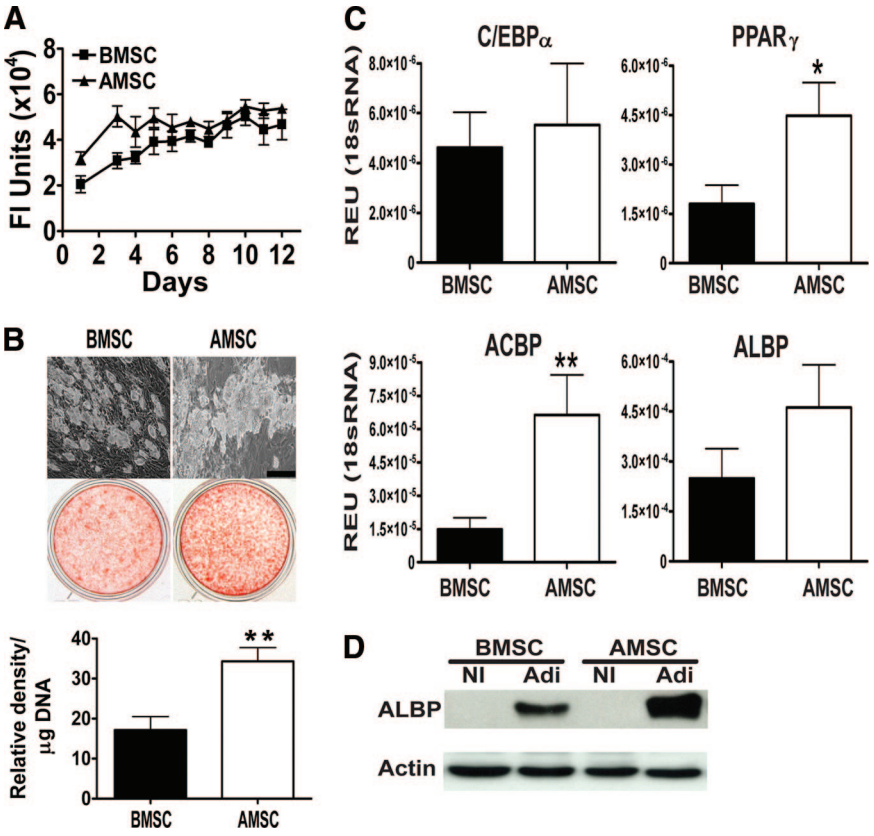

Figure 3. Adipogenic differentiation is enhanced in AMSCs. To compare the adipogenic potential of the cells from each source, a differentiation assay lasting 28 days was performed. **(A):** Growth was analyzed as described previously. **(B):** Phase-contrast pictures and oil red O staining for oil droplets quantified as described above. Scale bar = 20 μm. **(C):** Quantitative real-time polymerase chain reaction analysis was performed for the adipogenic markers ACBP, ALBP, C/EBPα, and PPARγ in AMSCs and BMSCs. Graphs are represented as relative expression units compared with 18S rRNA. **(D):** Western blot for ALBP shows the differential expression in AMSCs and BMSCs. All graphs show the mean ± SEM. *, $p < .05$; **, $p < .01$. Abbreviations: ACBP, acyl-coA-binding protein; Adi, adipogenic medium; ALBP, adipocyte lipid-binding protein; AMSC, adipose-derived mesenchymal stem cell; BMSC, bone marrow-derived mesenchymal stem cell; C/EBPα, CCAAT/enhancer-binding protein; FI, fluorescence intensity; NI, non-induced; PPARγ, peroxisome proliferator-activated receptor γ; REU, relative expression units.

34.30, compared with 17.16 for BMSCs (Fig. 3B), showing that AMSCs differentiate into adipocytes more readily than BMSCs. Analysis by qPCR demonstrated that AMSCs upregulate both the critical transcription factor peroxisome proliferator-activated receptor γ (PPARγ) and one of the adipogenesis proteins under its control, acyl-CoA binding protein [29], significantly more than BMSCs (4-fold and 2.5-fold upregulation in AMSCs over BMSCs, respectively) during differentiation (Fig. 3C). The adipocyte lipid-binding protein, which is also under the control of PPARγ [29], showed a similar trend, although the results did not reach statistical significance at the transcription level, yet AMSCs expressed a significantly greater amount of the protein than the BMSCs (Fig. 3D). There was, however, no appreciable difference in the induction of another critical adipogenic-related transcription factor, C/EBPα. These results show that AMSCs differentiate into adipocytes more readily than BMSCs.

Chondrogenic Differentiation

The final differentiation assay evaluated the ability of MSCs to form chondrocytes. Cells were grown in pellet cultures for 21 days before analysis by immunohistochemistry and qPCR. Histological staining demonstrated the notably different morphology of the AMSC pellets as compared with the BMSC pellets (Fig. 4A). Both pellets stained positively for Alcian Blue, but the AMSC chondrocyte pellets developed fibrous, loosely packed outer layers, with the more densely packed inner core containing large hypertrophic chondrocytes (arrows). In contrast, the BMSCs demonstrated a more uniform distribution of cells throughout the whole pellet, with a very thin fibrous outer layer as visualized by H&E staining. Interestingly, the loosely packed outer layers showed the most intense stain for proteoglycans, with the central regions having a lighter, more uniform stain, particularly in the BMSCs. The size of the chondrocyte pellets was also vastly increased in the AMSCs (average size of 2.3 mm, compared with 1.49 mm for BMSCs) despite the addition of the same number of cells into the initial pellets (supplemental online Fig. 2).

Further analysis by qPCR of genes associated with chondrogenesis demonstrated that three of the four markers were upregulated in BMSCs (Fig. 4B). Two early markers, collagen II and SOX-9, were upregulated (12-fold and 5-fold, respectively) compared with AMSCs, and this was also reflected in the immunohistochemistry for these two proteins (Fig. 4C). The expression of aggrecan, the major proteoglycan associated with chondrogenesis, was also upregulated, but to a lesser extent (only 1.5-fold), and this was again confirmed at the protein level (Fig. 4C). Both cell groups showed a large upregulation of collagen X above control cultures, although BMSCs had greater induction at this time point (18-fold, compared with 13-fold increases above control levels). For all stains, the AMSC pellets have strong staining in the outer fibrous cover and in the inner core, whereas the BMSCs have a more uniform stain. Together, these results show that both BMSCs and AMSCs can form chondrocytes but that BMSCs have greater chondrogenic potential than AMSCs.

Differentiation at Confluence

We next investigated whether the enhanced ability of AMSCs to differentiate into osteoblasts and adipocytes was due to their increased growth rate. Cells were seeded at 30,000 cells per cm^2 and allowed to reach confluence before they were switched to osteogenic or adipogenic medium. After 14 and 28 days, RNA was extracted or cells were stained with alizarin red, von Kossa, or oil red O. Under these conditions BMSCs have significantly higher alizarin red and von Kossa staining than the AMSCs after normalization to DNA content at days 14 and 28 (Fig. 5A, 5B). This was also reflected in gene expression analysis, with significantly upregulated osteopontin and osteocalcin at day 28

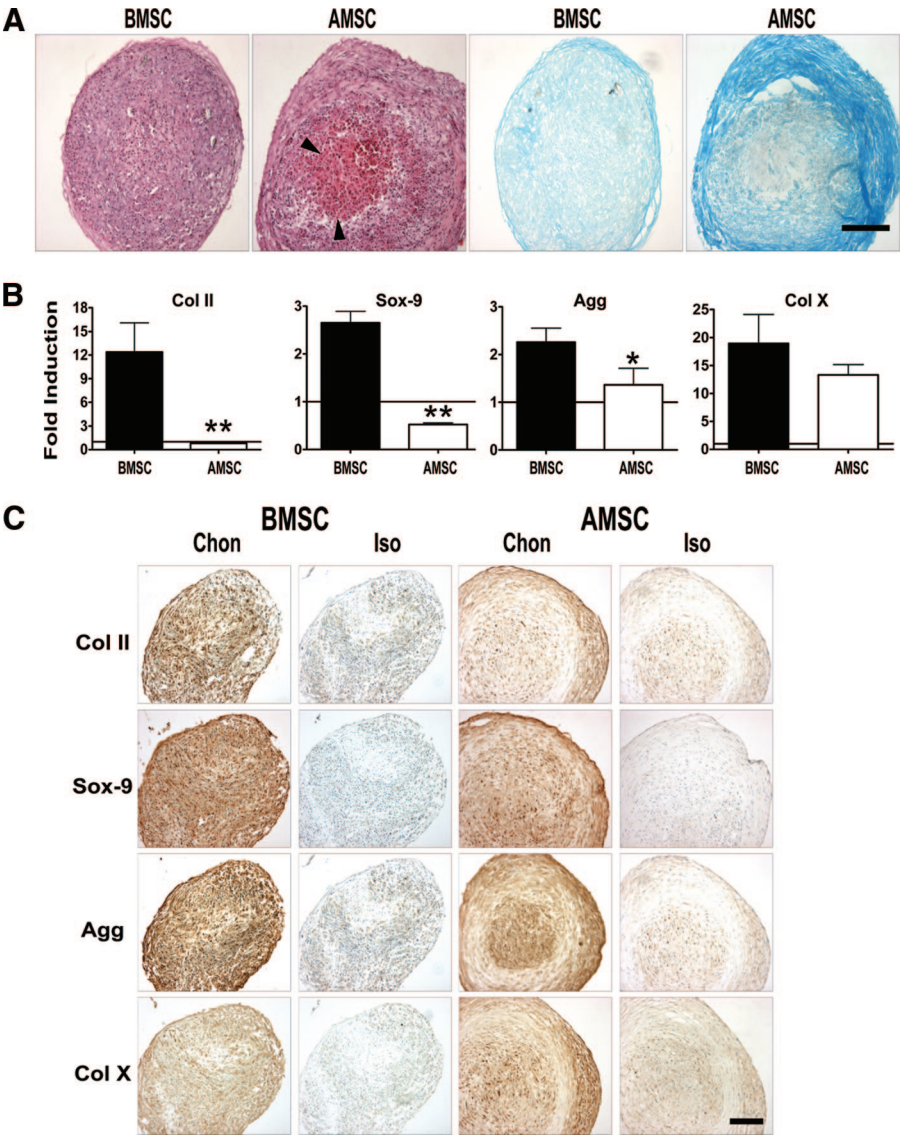

Figure 4. BMSCs form Chons more readily than AMSCs. To compare the chondrogenic potential of the cells from each source, we performed a differentiation assay lasting 21 days. **(A):** Sample H&E and Alcian Blue staining show the distinctly different morphologies of AMSC- and BMSC-derived Chons. Scale bar = 400 μm. **(B):** Quantitative real-time polymerase chain reaction (qPCR) of the chondrogenic markers shows that BMSCs have higher expression of the earlier developmental genes Col II, SOX-9, and Agg. Col X expression was not significantly different between the two populations of cells. **(C):** Immunohistochemical analysis of the same early and late markers largely confirms the qPCR results. Cells were stained with primary antibodies for chondrogenic markers or their relevant isotype controls and visualized using DAB. All graphs show the mean ± SEM. *, $p < .05$; **, $p < .01$. Abbreviations: Agg, aggrecan; AMSC, adipose-derived mesenchymal stem cell; BMSC, bone marrow-derived mesenchymal stem cell; Chon, chondrocyte; Col II, collagen II; Col X, collagen X; Iso, isotype.

(Fig. 5C). For adipogenesis, the trend was the same as for osteogenesis, although none of the differences between the two groups were statistically significant (Fig. 5A–5C). This suggests that AMSCs exhibit enhanced differentiation ability only because of their increased proliferation rate.

FGF2/FGFR1 Signaling Is Not Required for Osteogenic Differentiation

To determine why AMSCs exhibited an increased proliferation rate, we chose to analyze the FGF2 and Wnt pathways further, as these are involved with MSC proliferation and are overexpressed in AMSCs (Table 1). We found that over the course of the growth phase of AMSCs and BMSCs, AMSCs secreted significantly more FGF2 than BMSCs at each day of sampling (Fig. 6A) and also had increased β-catenin activity, as measured by a luciferase reporter assay (supplemental online Fig. 4). Since FGF2 is known to increase BMSC proliferation and is thought to be important for osteogenic differentiation, we investigated the effect of the FGFR1 inhibitor SU5402 on both these processes. We found that SU5402 significantly reduced signaling through FGFR1, as demonstrated by reductions in AKT and ERK phosphorylation (Fig. 6B). Furthermore, the presence of SU5402 inhibits the activation of these pathways when they are induced by the addition of exogenous FGF2 to serum-starved cells (Fig. 6C). Proliferation of AMSCs was inhibited at all SU5402 concentrations after 6 days compared with the carrier control (Fig. 6D), although significant cell death was seen at the two higher concentrations (data not shown). Under conventional osteogenic conditions, differentiation was completely abrogated after 28 days in the presence of 25 μM SU5402 (Fig. 6D, inset). Therefore, to study the effect of the

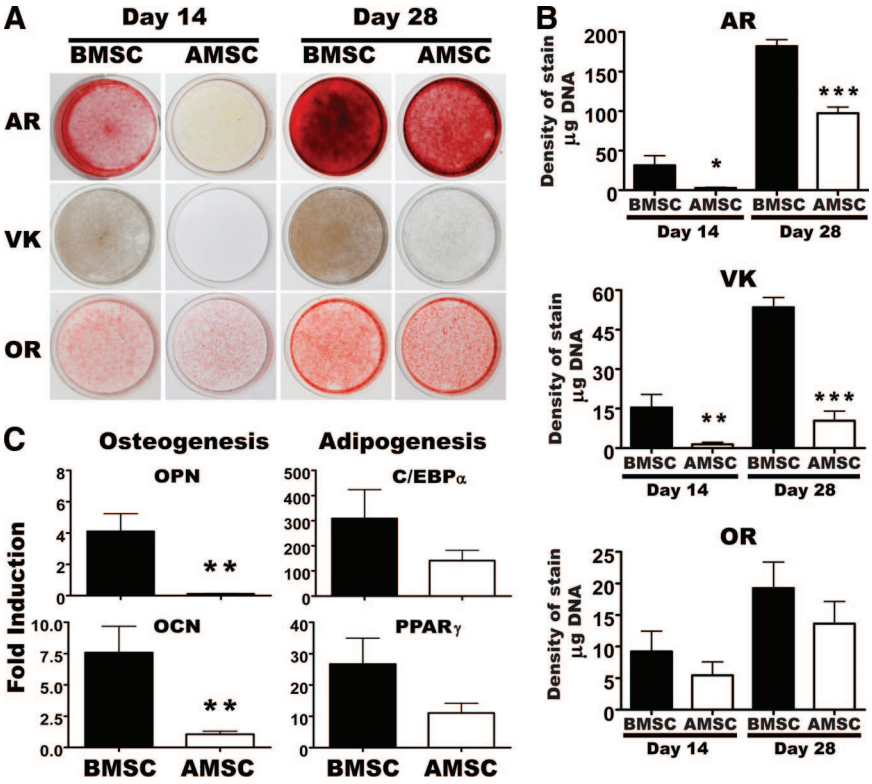

Figure 5. Differentiating cells at confluence reverses osteogenic and adipogenic potential. Cells were plated at 30,000 cells per cm^2 and allowed to reach confluence before the addition of osteogenic or adipogenic induction medium and analysis after 14 and 28 days. **(A):** Cells were stained histologically for AR, VK, and OR and then quantified relative to DNA content in **(B)**. **(C):** Quantitative real-time polymerase chain reaction analysis of day 28 cultures for the osteogenic genes OPN and OCN and the adipogenesis genes C/EBPα and PPARγ1. All graphs show the mean ± SEM. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: AMSC, adipose-derived mesenchymal stem cell; AR, alizarin red; BMSC, bone marrow-derived mesenchymal stem cell; C/EBPα, CCAAT/enhancer-binding protein; OCN, osteocalcin; OPN, osteopontin; OR, oil red O; PPARγ, peroxisome proliferator-activated receptor γ; VK, von Kossa.

inhibitor on osteogenesis, cells were seeded at 30,000 cells per cm^2 as described above and grown for up to 28 days in normal osteogenic medium alone or with DMSO or 25 μM SU5402. At days 14 and 28, there were no significant differences in alizarin red and von Kossa staining among the three culture conditions, even when the density of stain was normalized to DNA content (Fig. 6E, 6F). This suggests that FGF2/FGFR1 signaling is dispensable for osteogenic differentiation and is only required for the initial proliferation of the cells.

DISCUSSION

In this study we compared the phenotype and multilineage differentiation capability of BMSCs and AMSCs from 12 age- and sex-matched donors. Reports of the effect of age on BMSC life span and differentiation potential are contradictory, with some data suggesting that age accelerates senescence and some suggesting that it has no effect [17–19, 30–34]. Sex is also likely to play a role in MSC self-renewal and differentiation potential, as women over 60 will have gone through menopause and its associated bone homeostasis dysfunction and osteoporosis [24, 25]. When comparisons of AMSCs and BMSCs have been made, there has been very little attention given to either the age or sex of the donors [8, 21–23]. Obviously the ideal protocol is to harvest MSC populations from different tissues within the same donor [9, 35, 36], yet this is not always possible. To limit the effects of these factors, in this study we have used age- and sex-matched individuals, so that any differences can be attributed to the difference in the cell source. Another consideration is the ethnicity of the donors from whom clinical material was obtained. Since ethnicity can have a large impact on obesity and osteoporosis, it is possible that stem cell behavior may be affected. We were unable to guarantee that our donors were ethnically matched because of patient confidentiality, and this is a limitation of our study. Although we cannot rule out an effect of ethnicity, the bone marrow and adipose tissue was derived from young donors (mean ages, 26.8 and 26.3 years, respectively), so the impact of osteoporosis and obesity on ethnic grounds was likely to be minimized.

Phenotypically the cells were very similar, with only CD106, CD146, and HLA-ABC showing differential (increased) expression in BMSCs. Both populations were capable of forming CFU-Fs, although this ability was enhanced in the BMSCs (Fig. 1). The differences in CD106 and HLA-ABC expression have been described previously [4, 5] and are particularly interesting as CD106 correlates with increased CFU-F ability in BMSCs [37], possibly explaining the differences seen in our MSC cultures. The HLA-ABC differences are also interesting as MSCs have been demonstrated to be nonimmunogenic and immunosuppressive in vitro and in vivo [38–40], and as AMSCs express less HLA-ABC than BMSCs, they may have more potential in transplantation.

Analysis of AMSC and BMSC multilineage differentiation demonstrated that both cell groups were able to differentiate into adipocytes, chondrocytes, and osteoblasts. AMSCs appeared to differentiate more readily into osteoblasts and adipocytes, whereas BMSCs showed more chondrogenic potential. Under normal adipogenic and osteogenic differentiation conditions, AMSCs proliferate faster than BMSCs, resulting in greater cell numbers at confluence under osteogenic conditions and reaching confluence faster in adipogenic conditions. This may be why there was enhanced differentiation of AMSCs into adipocytes and osteoblasts, as they have more time to differentiate during adipogenesis, and the vastly greater cell numbers during osteogenesis allows for greater production of matrix and faster differentiation into osteoblasts.

From the qPCR data, it appears that BMSCs form chondrocytes more readily than AMSCs, reflecting the notably different staining patterns for chondrogenic markers in the pellets. In the AMSCs, the pellets were not uniform, with many regions not expressing any of the markers, whereas the BMSC pellets had

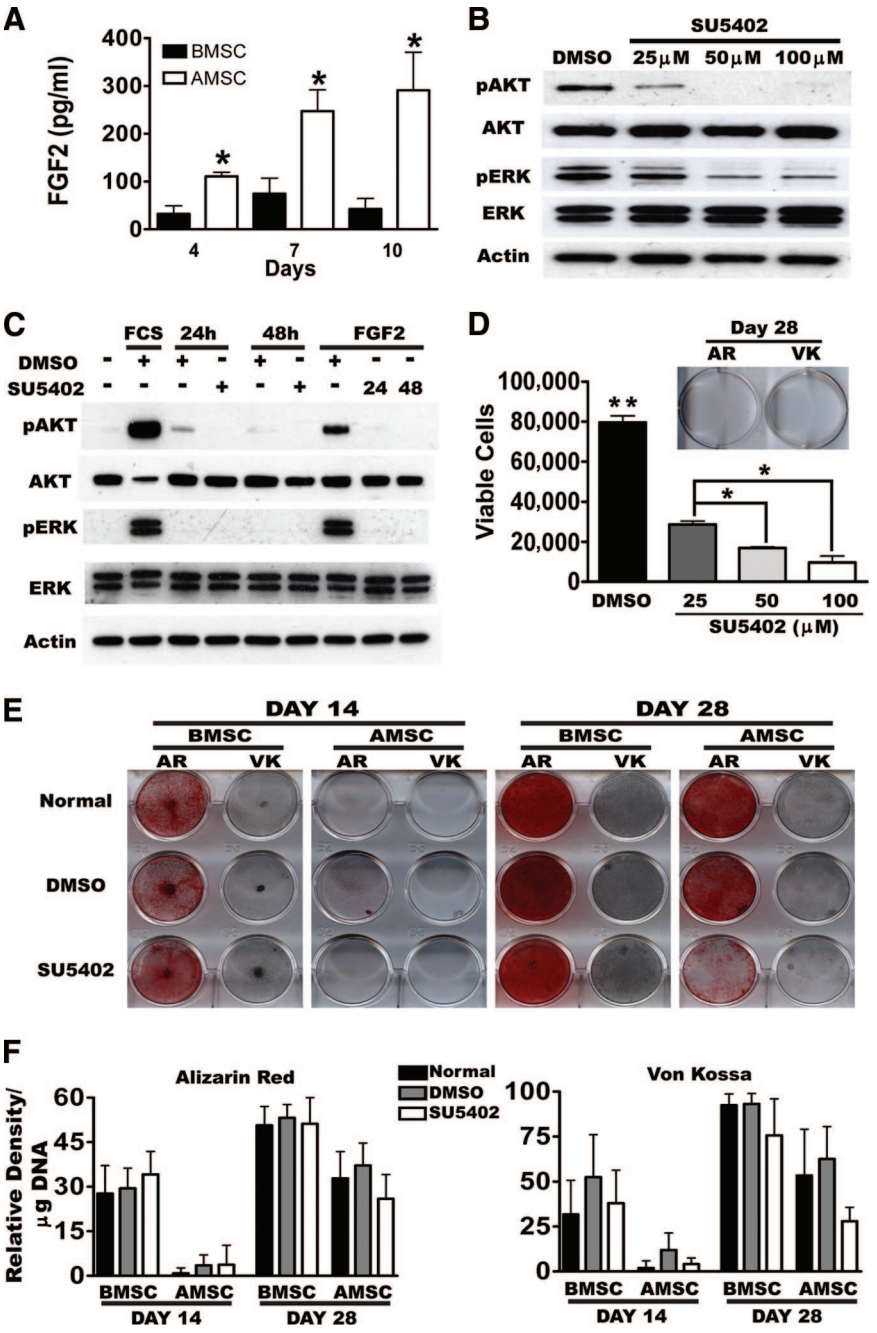

Figure 6. FGF2 positively influences osteogenic differentiation via an indirect mechanism. **(A):** To determine the causes of the increased AMSC proliferation, we carried out an FGF2 enzyme-linked immunosorbent assay on matrix-bound FGF2 released by washing the cell layer in 2 M NaCl [28]. **(B):** The effect of SU5402 on FGFR1 signaling in AMSCs culture in maintenance medium after 6 days. **(C):** Inhibition of FGF2 signaling in serum-starved cells exposed to SU5402. **(D):** The effect on AMSC proliferation after 6 days and osteogenic differentiation after 28 days (inset) in the presence of SU5402. **(E, F):** The effect of SU5402 on osteogenic differentiation of cells after confluence. Abbreviations: AMSC, adipose-derived mesenchymal stem cell; AR, alizarin red; BMSC, bone marrow-derived mesenchymal stem cell; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FGF2, fibroblast growth factor 2; h, hours; VK, von Kossa.

almost uniform expression for all markers. Within the AMSC cultures, the qPCR data only reflect average RNA levels from a pool of cells that may vary in differentiation status, complicating such an analysis of chondrogenic genes, whereas BMSCs may have more uniformity in their differentiation, giving rise to higher expression levels overall. Alternatively, we cannot rule out the possibility that the AMSCs are actually further along this differentiation pathway compared with the BMSCs and that the time point for optimal chondrogenic gene upregulation of AMSCs has been missed. The staining intensity of the late-stage marker collagen X in the AMSC pellet is greater than in the BMSC pellet, although, again, the staining is more uniform in the latter. In addition, collagen X is associated with the development of hypertrophic chondrocytes [41], and the central core of the AMSC pellets, containing the hypertrophic chondrocytes, does stain strongly for collagen X. Supporting this concept, Zuk et al. demonstrated that collagen II and aggrecan gene expression was absent after 10 days of culture, whereas collagen X began expression at day 14 [4]. This may explain why there was very little collagen II and aggrecan mRNA in the AMSC pellets at the day 21 time point.

Of note also is that the enhanced AMSC differentiation capability occurs only when an initial proliferation phase is involved in the process. When proliferation was removed from the process, such as during chondrogenesis, or osteogenic and adipogenic differentiation when cells were seeded at high density, BMSCs in fact outperform AMSC cultures. This suggests that the apparent enhanced multipotentiality of AMSCs was dependent on their increased proliferation rate. Mechanisms underlying this appear to be FGF2-related, as AMSCs produced increased amounts of FGF2 that was found bound into the matrix and not in the conditioned medium (data not shown),

confirming previous findings [28]. Addition of exogenous FGF2 has been demonstrated to accelerate growth and increase the life span of BMSCs in culture (T. Helledie, C. Dombrowski, I. Lee et al. manuscript submitted for publication) [42–44], which is further reflected here with the upregulation of a number of cell cycle control genes and self-renewal markers in our AMSCs. Moreover, this exogenous FGF2 also modifies their morphology, making cells smaller and more spindle-shaped [45], as also seen here. It is likely that the increased production of FGF2 by AMSCs helps to drive their proliferation, as culturing the cells with the FGFR1 inhibitor SU5402 inhibits proliferation and signaling in a manner identical to the drug's effects in BMSCs (manuscript in preparation). We also show that, in addition to the inhibition of proliferation, inhibition of FGF2 signaling in confluent cultures during osteogenic differentiation does not enhance or inhibit differentiation. However, when proliferation is required in the differentiation experiment, the addition of SU5402 completely abrogates differentiation. These findings have implications for the role of FGF2 in the osteogenic process. The presence of FGF2 is generally thought to be advantageous for bone formation, both in vitro and in vivo. The results here suggest that FGF2 is only indirectly required for mineralization, probably because it speeds up the process of cell growth, thus stimulating cells to reach confluence more quickly, or for there to be more cells when confluence is reached. This is consistent with previous findings from our group demonstrating that stimulating rat osteoblasts with a single dose of FGF2 increased their proliferation, and consequently their mineralization, compared with control cultures [26].

We also found that, in addition to FGF2, Wnt pathway genes had greater expression levels in AMSCs. The overexpression of genes involved in the Wnt pathway in AMSCs, BMSCs, and cord blood-derived mesenchymal stem cells compared with HS68 fibroblasts has been described previously [5], suggesting that Wnt signaling may be important for MSC maintenance regardless of source. The differential effects of FGF2 and Wnt on BMSC and AMSC self-renewal are therefore of interest in light of the results in this study.

Conclusion

We have found that AMSCs have a multilineage capacity that is at least equal to that of age- and sex-matched BMSCs. We have shown that the proliferation rate of MSCs can greatly enhance their multilineage differentiation potential. AMSCs displayed an increased proliferation rate, as a result of increased endogenous FGF2 production, and corresponding enhanced differentiation. The relative ease of access and the potential numbers of MSCs that can be isolated from adipose tissue compared with bone marrow offer significant advantages for the use of these cells in MSC transplantations.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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