Donor Variation and Loss of Multipotency during In Vitro Expansion of Human Mesenchymal Stem Cells for Bone Tissue Engineering

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ABSTRACT: The use of multipotent human mesenchymal stem cells (hMSCs) for tissue engineering has been a subject of extensive research. The donor variation in growth, differentiation and in vivo bone forming ability of hMSCs is a bottleneck for standardization of therapeutic protocols. In this study, we isolated and characterized hMSCs from 19 independent donors, aged between 27 and 85 years, and investigated the extent of heterogeneity of the cells and the extent to which hMSCs can be expanded without loosing multipotency. Dexamethasone-induced ALP expression varied between 1.2- and 3.7-fold, but no correlation was found with age, gender, or source of isolation. The cells from donors with a higher percentage of ALP-positive cells in control and dexamethasone-induced groups showed more calcium deposition than cells with lower percentage of ALP positive cells. Despite the variability in osteogenic gene expression among the donors tested, *ALP*, *Collagen type 1*, *osteocalcin*, and *S100A4* showed similar trends during the course of osteogenic differentiation. In vitro expansion studies showed that hMSCs can be effectively expanded up to four passages (approximately 10–12 population doublings from a P0 culture) while retaining their multipotency. Our in vivo studies suggest a correlation between in vitro ALP expression and in vivo bone formation. In conclusion, irrespective of age, gender, and source of isolation, cells from all donors showed osteogenic potential. The variability in ALP expression appears to be a result of sampling method and cellular heterogeneity among the donor population. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:1029–1041, 2007

Keywords: human mesenchymal stem cells; bone tissue engineering

INTRODUCTION

The contemporary drawbacks of autograft-, allograft-, and xenograft-based bone regeneration methods such as an additional surgical procedure, infection, chronic pain, and donor dependency in successful healing[1–3] has generated large focus on the use of autologous cells for tissue engineering.[4–7] The method utilizes biodegradable materials, which provide an appropriate microenvironment to promote cell–material interaction, adhesion, and spreading. Current developments in biomaterial science allow the introduction of bioactive properties to ceramics and biodegradable polymers besides serving as carrier materials.[8] Furthermore, the identification of bone morphogenic protein[9] and other growth factors such as fibroblastic growth factor, epidermal growth factor, transforming growth factors, insulin-like growth factor 1, parathyroid hormone, vitamin D3, and the synthetic glucorticoid dexamethasone improved bone tissue engineering applications because they are known to induce osteogenic differentiation.[10–15]

The isolation of hMSCs and their extensive proliferation and ability to differentiate into osteogenic, adipogenic, chondrogenic, and myogenic lineages[16,17] has gained the attention of researchers to use hMSCs for potential clinical use. Multipotent cells have been isolated from many sources including adipose tissue, tibia, femur, lumbar spine, and trabecular bone.[18–20] Traditionally, hMSCs are isolated from an aspirate of bone marrow harvested from the iliac crest or acetabulum. The cells isolated from the latter source are multipotent in vitro and form bone in vivo.[16] Although hMSCs have superior proliferative capacity in vitro, it has been demonstrated that in vitro expanded hMSCs show a replicative senescence phenotype culminating in growth arrest and loss of multipotency.[21–23] Cellular senescence and growth arrest are known to occur when telomeres in one or more chromosomes reach a critical length.[24]

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Because no telomerase activity has been detected in mouse and human MSCs, they show limited proliferative capacity and multipotency in expanded cultures.[25,26] Retroviral transduction of telomerase into hMSCs extended the proliferative capacity up to 260 population doublings (PD) compared with 26 PD in the control cells while maintaining osteoblast markers, normal karyotype, and even enhanced in vivo bone formation.[27,28]

Bone tissue engineering could be improved by overexpression of genes such as *BMP2, BMP6, BMP9, Fra-1*, and *LIM mineralization protein-3* in MSCs to induce in vitro osteogenesis and in vivo bone formation.[29–34] However, ethical issues restrain the use of genetically modified cells for tissue engineering applications. Therefore, more focus has been put on the use of various osteoinductive stimuli, such as dexamethasone, BMP2, Vitamin D,[15] and statins[35] to enhance osteogenic differentiation of hMSCs in vitro.[36,37] We recently focused on the use of lithium,[38,39] Trichostatin A,[40] and cyclic AMP (manuscript in preparation) as potential compounds to stimulate various steps in the osteogenic process. Besides these developments in bone tissue engineering, the enormous donor variation in growth properties, osteogenic potential, and in vivo bone formation by hMSCs limits the standardization of therapeutic protocols.[41] Therefore, we investigated and characterized hMSCs from 19 independent donors to delineate the heterogeneity among the population and to determine how far the hMSCs can be effectively proliferated in vitro while retaining their differentiation abilities for tissue engineering applications.

MATERIALS AND METHODS

Isolation and Culture of hMSCs

Bone marrow aspirates (5–20 mL) were obtained from donors with written informed consent. The donors with known skeletal disease or drug history were excluded from the study. hMSCs were isolated and proliferated as described previously.[42] Briefly, aspirates were resuspended using 20-G needles, plated at a density of 5×10^5 cells/cm^2, and cultured in hMSC proliferation medium (PM) containing α-minimal essential medium (α-MEM, Life Technologies, Baltimore, MD), 10% fetal bovine serum (FBS, Cambrex, Walkersville, MD), 0.2 mM ascorbic acid (Asap, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), 10 μg/mL streptomycin (Life Technologies), and 1 ng/mL basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further subculturing or cryopreservation upon reaching near confluence. hMSC basic medium (BM) was composed of hMSC proliferation medium without bFGF, hMSC osteogenic medium (OM) was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (dex, Sigma, St. Louis, MO) and hMSC mineralization medium (MM) was composed of basic medium supplemented with 10^{-8} M dexamethasone and 0.01 M β-glycerophosphate (Sigma). For extensive proliferation of hMSCs, cells were seeded at 1000 cells/cm^2 and cultured in PM until they reached 80% confluence, then they were trypsinized and seeded again at 1000 cells/cm^2. Proliferation rate was calculated by counting the number of cells in triplicate before and after seeding. A fraction of cells from each passage was used for other biological assays.

ALP Analysis by Flow Cytometry

hMSCs were seeded at 5000 cells/cm^2 and allowed to attach for 10 to 15 h in BM, then cells were incubated with 10^{-8} M dexamethasone for the denoted time periods. Each experiment was performed in triplicate with a negative control (cells grown in BM) and a positive control (cells grown in OM) and one or more experimental conditions. At the end of culture period, the cells were trypsinized and incubated for 30 min in block buffer [PBS with 5% bovine serum albumin, BSA (Sigma) and 0.05% NaN2], then incubated with primary antibody (anti-ALP, B4-78 [Developmental Studies Hybridoma Bank, University of Iowa]) diluted in wash buffer (PBS with 1% BSA and 0.05% NaN2) for 30 min or with isotype control antibodies. Cells were then washed three times with wash buffer and incubated with secondary antibody (goat antimouse IgG PE, DAKO) for 30 min. Cells were washed three times and suspended in 250 μL wash buffer with 10 μL Viaprobe (Pharmingen, Uppsala, Sweden) for live/dead cell staining, and only live cells were used for further analysis. ALP expression levels were analyzed on a FACS Caliber (Becton Dickinson Immuno cytometry systems, Fullerton, CA). The percentage ALP positive cells were calculated compared to untreated cells and expressed as relative ALP expression compared to respective controls. The data was analyzed using Student's *t*-test ($p < 0.05$).

Mineralization and Calcium Deposition

For mineralization, hMSCs were seeded in MM at 5000 cells/cm^2 in T25 culture flasks and cultured for 30 days with cells cultured in BM as negative control ($n = 4$). The total calcium deposition was assayed using a calcium assay kit (Sigma diagnostics; 587A) according to manufacturer's protocol. Briefly, the culture medium was aspirated, washed twice with calcium and magnesium free PBS (Life Technologies) and incubated overnight with 0.5 N HCl on an orbital shaker at room temperature. The supernatant was collected for direct

measurement or stored at −20°C. The calcium content was measured at 620 nm (Bio-tek Instruments, Burlington, VT) and expressed as μg calcium/flask ($n = 3$). The data was analyzed using Student's t-test at $p < 0.05$. To visualize the mineralized area one flask from each group was stained with von Kossa staining.

Adipogenic Assay

To study the adipogenic differentiation ability of expanded hMSCs, cells from each passages were seeded in adipogenic medium [Dulbecco's minimal essential medium (DMEM; Life Technologies), 10% FBS, 0.5 mM isobutylmethylxanthine (Sigma), 1 mM dexamethasone, 10 mM insulin (Sigma), 200 mM indomethacin (Sigma)] at 5000 cells/cm^2 in triplicate and grown for 21 days. Medium was refreshed twice a week and lipid formation was visualized with Oil red O (Sigma) staining. Briefly, the cells were fixed overnight in formol [3.7% formalin plus $CaCl_2$ $2H_2O$ (1 g/100 mL)], rinsed with water, incubated for 5 min in 60% isopropanol, and stained for 5 min in freshly filtered Oil red O solution [stock: 500 mg of Oil red O (Sigma), 99 mL of isopropanol, 1 mL of water; stain: 42 mL of stock plus 28 mL of water]. At least three images were taken at different locations of the flasks at same magnification and the number of adipocytes were counted in those three images and statistically analyzed using Student t-test ($p < 0.05$).

Gene Expression Analysis by qPCR

The effect of dexamethasone on expression of osteogenic marker genes was analyzed by seeding hMSCs at 5000 cells/cm^2 in T75 flasks in BM and OM for 1, 2, 4, 10, and 21 days. Total RNA was isolated using an Rneasy mini kit (Qiagen, Chatsworth, CA) and on column DNase treated with 10 U RNase free DNase I (Gibco, Rockville, MD) at 37°C for 30 min. DNAse was inactivated at 72°C for 15 min. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Two micrograms of RNA were used for first-strand cDNA synthesis using Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One microliter of 100 × diluted cDNA was used for collagen type 1 (*COL1*) and 18s rRNA amplification and 1 μL of undiluted cDNA was used for other genes. PCR was performed on a Light Cycler real-time PCR machine (Roche, Indianapolis, IN) using SYBR green I master mix (Invitrogen). Data were analyzed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to exclude background fluorescence. Expression of osteogenic marker genes are calculated relative to 18s rRNA levels by the comparative ΔCT method[43] and the statistical significance was found using Student's t-test at $p < 0.05$. The primers used in the study are listed in Table 1.

In Vivo Bone Formation

To evaluate the donor variation on in vivo bone formation by hMSCs, we seeded 200,000 hMSCs in BM onto 2–3 mm biphasic calcium phosphate particles (BCP, three particles per condition) prepared and sintered at 1150°C as described previously.[44] The cells were cultured for a further 7 days on the BCP particles. Six immune-deficient male mice (Hsd-cpb:NMRI-nu, Harlan) were anesthetized by intramuscular injection of 0.05 mL of anesthetics (1.75 mL ketamine 100 μg/mL, 1.5 mL xylazine 20 mg/mL, and 0.5 mL atropine 0.5 mg/mL). Four subcutaneous pockets were made and each pocket was implanted with three particles. The incisions were closed using a vicryl 5-0 suture. After 6 weeks the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck, Rahway, NJ) in 0.14 M cacodylic acid (Fluka, Haupphage, NY) buffer pH 7.3, dehydrated, and embedded in methyl methacrylate (Sigma) for sectioning. Approximately

Table 1. Primer Sequences Used for qPCR Studies

Gene	Sequence	Product Length (bp)
18s rRNA	F-5′cggctaccacatccaaggaa3′ R-5′gctggaattaccgcggct3′	187
Collagen 1	F-5′agggccaagacgaagacatc3′ R-5′agatcacgtcatcgcacaaca3′	138
Osteopontin (OP)	F-5′ccaagtaagtccaacgaaag3′ R-5′ggtgatgtcctcgtctgta3′	348
Osteonectin (ON)	F-5′actggctcaagaacgtcctg3′ R-5′gagagaatccggtactgtgg3′	438
Osteocalcin (OC)	F-5′ggcagcgaggtagtgaagag3′ R-5′gatgtggtcagccaactcgt3′	138
S100A4	F-5′agcttcttggggaaaaggac3′ R-5′ccccaaccacatcaagagg3′	200
Alkaline phosphotase (ALP)	F-5′gacccttgacccccacaat3′ R-5′gctcgtactgcatgtcccct3′	70

10 μm-thick, undecalcified sections were processed on a histological diamond saw (Leica saw microtome cutting system). The sections were stained with basic fuchsin and methelyine blue. Basic fuchsin stains newly formed bone pink and methylene blue stains the remaining fibrous tissue blue. The calcium phosphate ceramic material remains unstained and appears black in the sections. At least five sections were made from each sample and scanned using Minolta Dimage Scan. The bone formation is expressed as percentage bone areas considering the total available pore area for new bone growth as 100%.

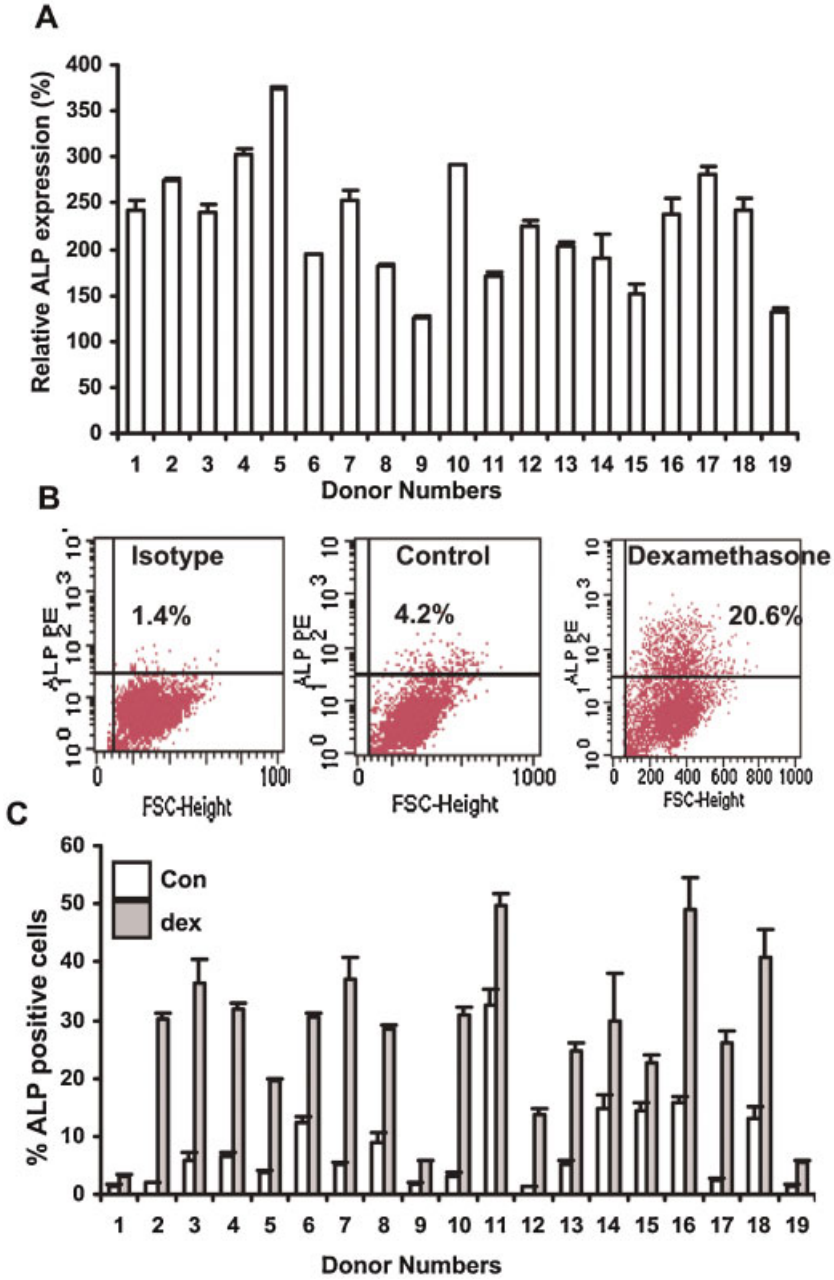

Figure 1. Donor variation in ALP expression. (A) Effect dexamethasone on ALP induction by hMSCs from 19 donors. ALP expression was analyzed by flowcytometry and expressed as percentage relative to untreated cells of the same donor. (B) A representative dot plot used to calculate the ALP positive cell fraction in control and dexamethasone-treated groups. (C) Percentage of ALP positive cells in untreated (Con) and dexamethasone-treated (dex) cell populations. Error bars represent the standard deviation. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

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RESULTS

Donor Variation in Response to Dexamethasone

In vitro differentiation of hMSCs is characterized by change in the morphology of the cells and expression of the early osteogenic marker gene alkaline phosphatase (ALP). We investigated the donor variability in response to a well-know osteogenic inducer, dexamethasone. As shown in Figure 1A, the induction of ALP relative to untreated hMSCs of 19 donors varied between 1.3- and 3.8-fold, indicating a vast variation in response to an osteogenic signal. Because we noticed a donor variation in ALP induction we calculated the percentage of ALP positive cells by gating for ALP positive cell fraction in control and dexamethasone-treated cells (Fig. 1B). The percentage of ALP positive cells in the untreated group varied between 1 and 33%, indicating varying amounts of ALP-positive cells in the initial culture. Upon dexamethasone treatment, the percentage of ALP positive cells varied between 3 and 50%, with an average of 27% (Fig. 1C). We continuously monitored ALP expression from D12 of P2 cells over a period of 15 days, and we observed induction kinetics reaching peak expression between 5 and 10 days, dropping back to the basal level there after (Fig. 2A). This phenomenon of decrease in dexamethasone-induced ALP expression after 7 days is consistently observed in our earlier studies. Further, no statistical correlation was observed when ALP index (ratio of ALP-positive cells in dexamethasone-treated and control group) was plotted against age, gender, or source of isolation (Fig. 2B–D). In addition, P2 cells from donors 16, 17, and 18 were tested for their ability to mineralize in vitro. As shown by von Kossa staining (Fig. 3A), the cells from donors 16 and 18 showed in vitro mineralization; however, calcium quantification showed no significant difference between the donors. But donor 17 did not show any calcium deposition (Fig. 3B), again emphasizing the donor variability. To further understand this variability we compared the percentage of ALP positive cells in untreated and dexamethasone-treated cells from these donors to in vitro mineralization ability. The high number of ALP-positive cells in D16 and D18 correlated to their in vitro mineralization potential. In contrast, the absence of mineralization in

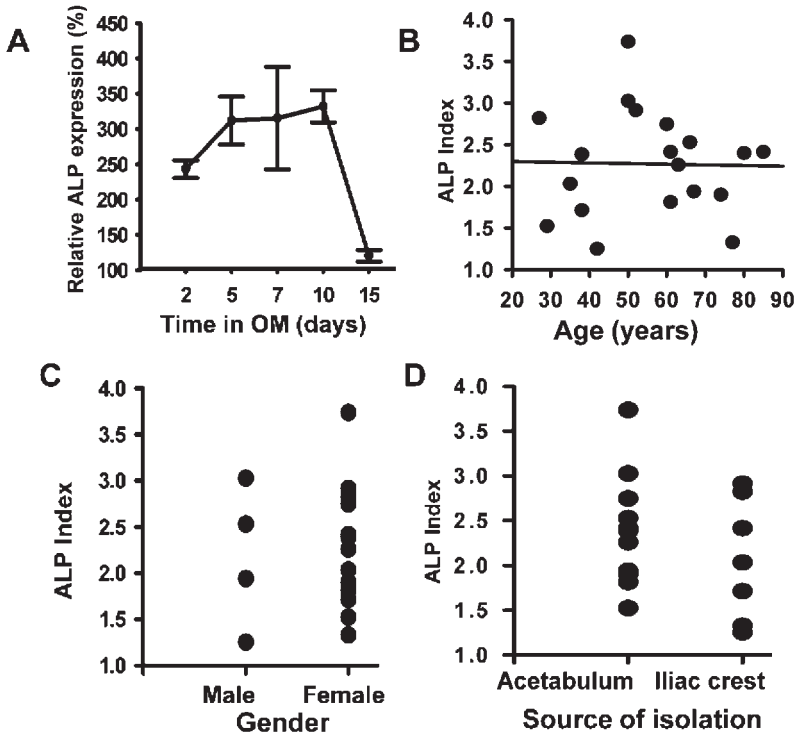

Figure 2. Correlation between ALP and age, gender, or source of isolation. (A) ALP induction profile of P2 cells from D12 over a period of 15 days in osteogenic medium (OM). Error bars represent the standard deviation. (B–D) Correlation of ALP index (ratio of ALP positive cells in dexamethasone-treated and control group) with donor age ($r^2 = 0.0005$), gender ($r^2 = 0.0523$), and source of isolation ($r^2 = 0.00056$).

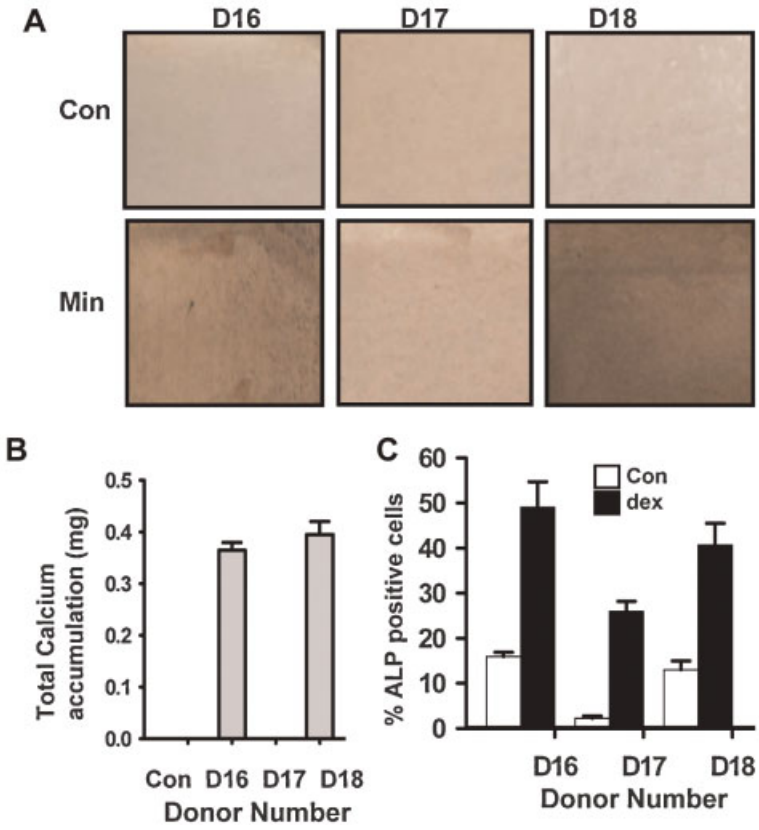

Figure 3. Donor variation in in vitro mineralization potential. (A) von Kossa staining of hMSCs from D16, D17, and D18 grown in basic medium (con) and mineralization medium (min) for 30 days. Note the black staining in dex-treated cells of D16 and D18, indicating mineralization. (B) Calcium accumulation by hMSCs from D16, 17, and 18 in MM after 30 days. No detectable calcium was measured in control-treated cells (Con). (C) Percentage ALP-positive cells in untreated (white bars) and dexamethasone treated (black bars) cells from D16, D17, and D18. Error bars represents standard deviation ($p < 0.05$).

D17 correlated to a low ALP expression level (Fig. 3C).

Expression Profile of Osteogenic Markers

We further investigated the donor heterogeneity by studying the expression profile of other osteogenic markers such as collagen type 1 (*Col-1*), noncollagenous proteins of the extra cellular matrix such as osteopontin (*OP*), osteocalcin (*OC*), and osteonectin (*ON*) from P2 cells of D12, D11, D16, and D18 during a time course of osteogenic differentiation. Among the genes studied, *ALP, COL-1*, and *ON* increased progressively during osteogenic differentiation and declined at later stages (Fig. 4A). *OC* expression was stable in the beginning of the culture period and decreased later. Expression of calcium binding protein S100A4, a negative regulator of mineralization persisted in early phase of osteogenic differentiation and declined before the onset of mineralization.

In Vitro Senescence of hMSCs

Cell-based bone tissue engineering needs enormous amounts of multipotent cells for successful clinical application that requires in vitro expansion of the isolated cells. hMSCs isolated from human trabecular bone show a typical phenomenon of cellular senescence including morphological change, decreased proliferation, and declined ALP and Col-1 expression.[22] To document how far the isolated hMSCs can be in vitro proliferated while retaining their differentiation abilities, we serially passaged hMSCs from D11 up to nine passages. First, we observed a morphological change from thin and spindle-shaped in early passage cells to large, flattened, and irregularly shaped in late passage cells (Fig. 5A). Growth

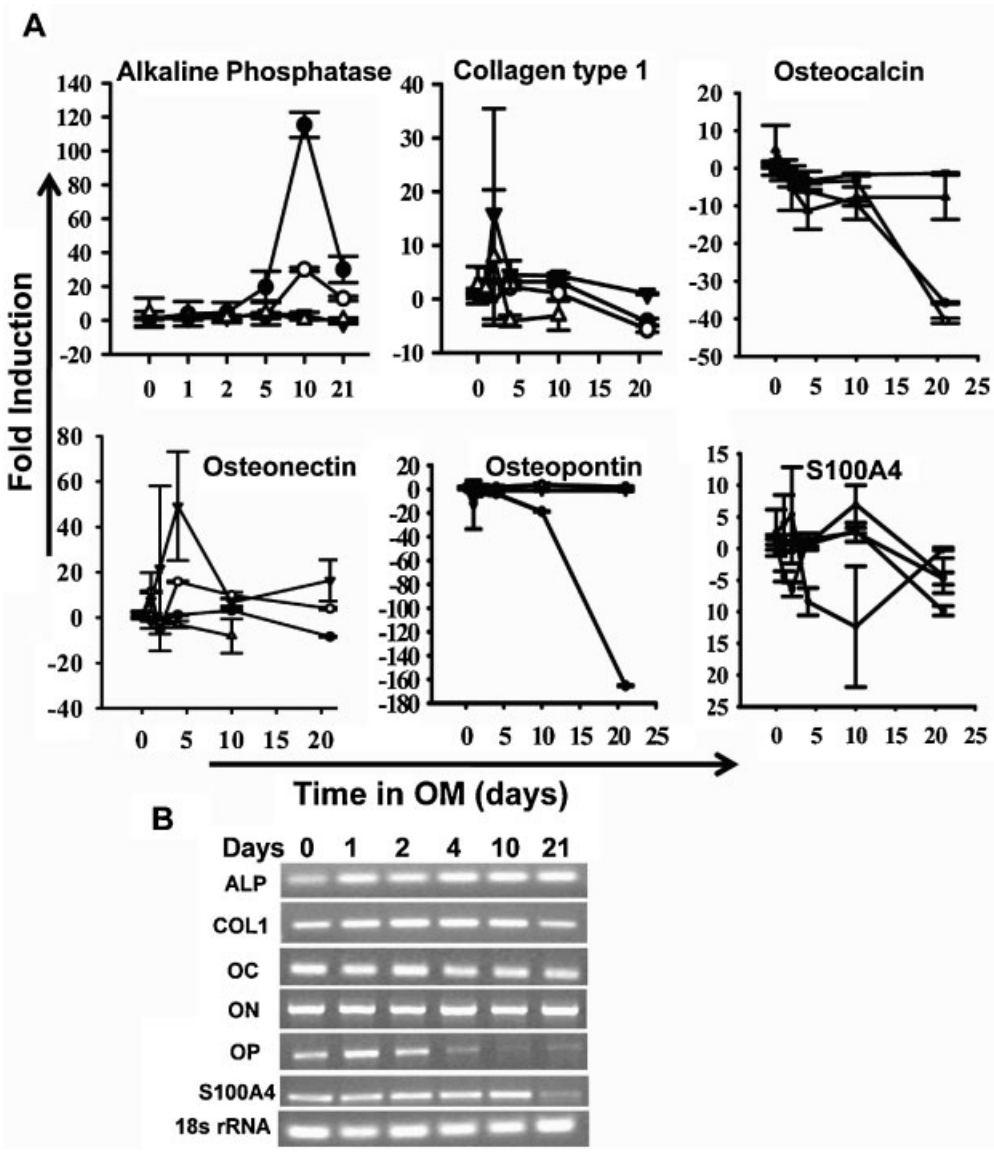

Figure 4. Osteogenic gene expression in dexamethasone-induced hMSCs. (A) Gene expression profiles osteogenic markers in osteogenic medium (OM). Expression is indicated as fold induction compared to cells grown in BM and normalized to 18s rRNA. ALP, alkaline phosphatase; col1, collagen type 1; OP, Osteopontin; ON, Osteonectin; OC, Osteocalcin; S100A4, calcium binding protein S100A4. Error bars represent standard deviation. (B) Agarose gel of the qPCR samples from a donor in A.

kinetic analysis showed a rapid increase in proliferation in P2 and P3, followed by a phase of slow growth (Fig. 5B). To our surprise, some P6 cells regained proliferative potential and formed independent colonies. We could culture those cells for another three passages and then the cells absolutely stopped dividing and died in P9. To confirm this we serially passaged cells from two other independent donors and observed the same phenomenon (data not shown). We are further investigating this unique pattern of hMSC growth in detail by serially passaging hMSCs from a number donors and studying gene expression profile of serially passaged cells by microarray in parallel with their ability to form bone in vivo.

We also studied the differentiation potential of serially passaged hMSCs by inducing them into osteogenic and adipogenic lineages. ALP expression analyses showed that dexamethasone induced ALP expression in early passages but

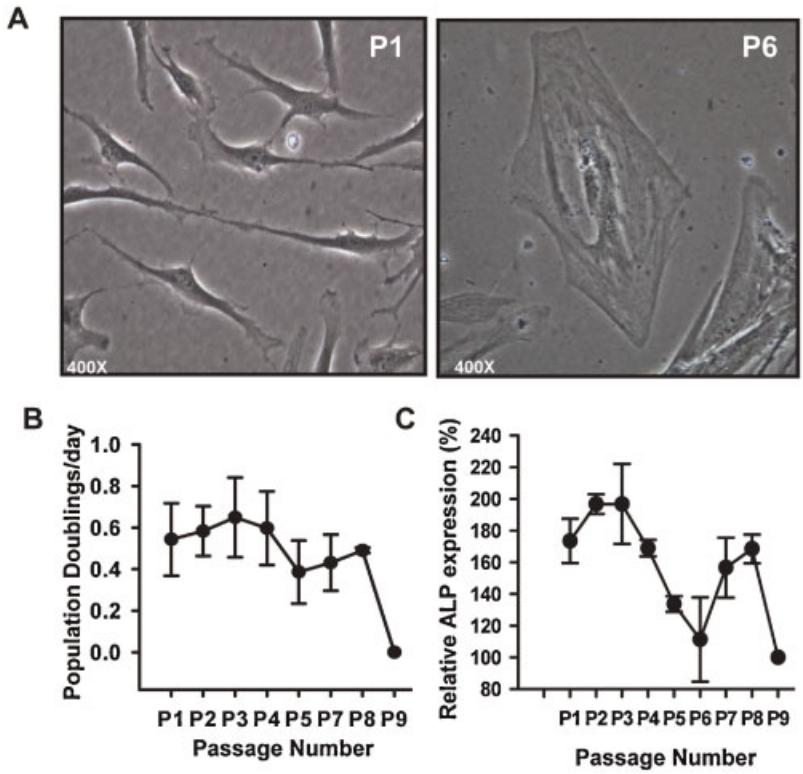

Figure 5. In vitro senescence of hMSCs. (A) Images showing a change in morphology from thin and spindle shaped in passage 1 to large and flattened in passage 6. (B) Growth kinetics of serially passaged hMSCs expressed as population doubling per day. (C) Relative ALP expression in serially passaged hMSCs compared to the controls of the same passage cells. Error bars represent the standard deviation.

failed to do so in P6. However, the recovered colonies from P6 showed a slight ALP induction in P7 and P8 (Fig. 5C). In P9, the hMSCs completely lost responsiveness to dexamethasone. No further analyses could be performed; thereafter, due to cessation of cell growth and cell death. In vitro mineralization studies showed that the cells were able to mineralize in vitro up to P3, and no mineralization was observed in P4 and later passages. The calcium deposition by P2 cells was significantly higher than P1 cells. This could be possibly due to the fact that we used cryopreserved cells of P0 and the cells may have had a lag phase of growth. Further, we observed a significant decrease in the calcium accumulation in P3 and cells from P4, and later passages failed to mineralize as determined by their inability to accumulate calcium (Fig. 6A). In contrast, the cells were able to differentiate into adipogenic lineage up to P5. Although no significant differences were observed between P2 and P3 cells, there was a significant drop in adipogenic differentiation ability of P4 and P5 cells compared with P2 and P3 (Fig. 6B).

In Vivo Bone Formation

We further tested whether the large variation between individual donors in ALP expression and in vitro mineralization would be reflected on in vivo bone formation by hMSCs. We implanted hMSCs from D16, 17, 18, and 19 in immune-deficient mice for 6 weeks. The samples were explanted and stained as explained in Materials and Methods. We observed bone formation by cells from donor 16, 17, and 18, but no bone was formed by donor 19 (Fig. 7). The comparative analyses of ALP expression by these donors with in vivo bone formation suggest a correlation between in vitro ALP expression and in vivo bone formation. Although D17 did not mineralize in vitro and had lower percentage of ALP positive cells in control and dexamethasone-induced groups, we observed more bone formation compared to D16.

DISCUSSION

Easy isolation, rapid in vitro expansion, and multipotency of hMSCs attributes to the attrac-

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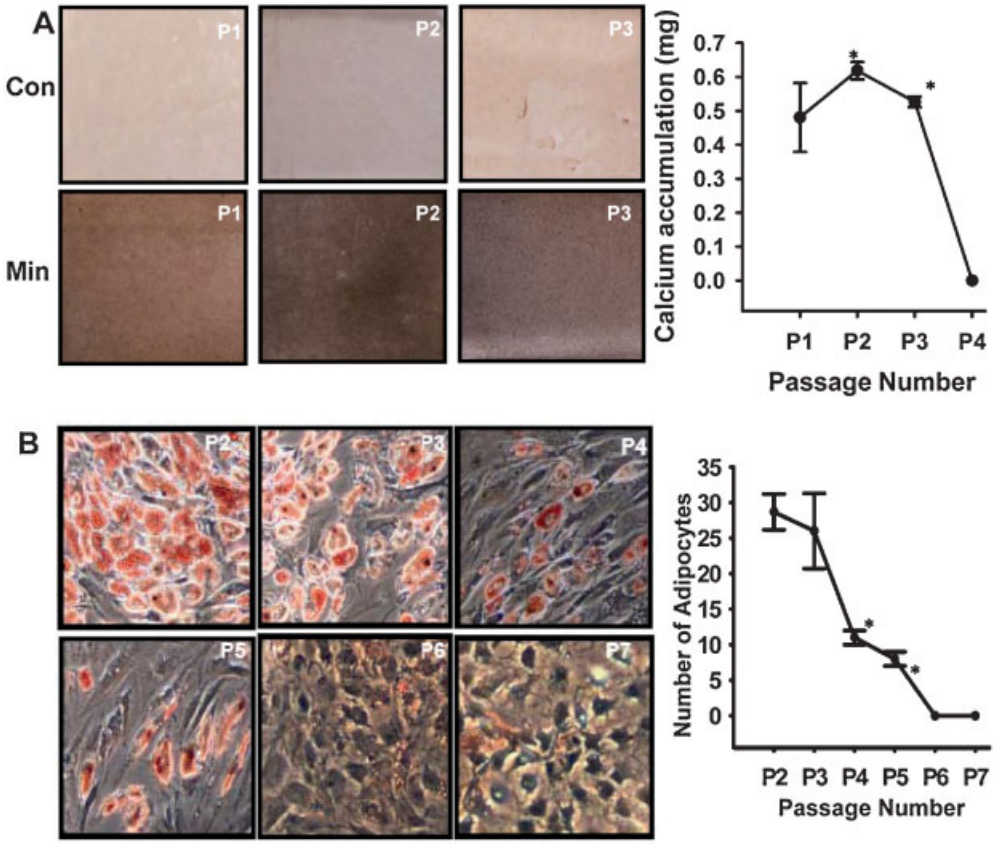

Figure 6. Osteogenic and adipogenic potential of serially passaged hMSCs. (A) von Kossa staining (left upper panel) and quantification of calcium deposition (right upper panel) in serially passaged hMSCs. (B) Adipogenic differentiation was visualized by staining with Oil red O (left lower panel) and the number of adipocytes were quantified. (left lower panel). Error bars represent the standard deviation.

tiveness as a candidate for regenerative medicine and tissue engineering. Some clinical trials have shown that hMSCs loaded onto different carrier materials produced clinically relevant amounts of bone.[45] Successful bone tissue engineering using hMSCs mainly depends on the quality of the cells, ability to proliferate, differentiate in vitro, and to form bone in vivo.

In this report, we isolated and characterized hMSCs from 19 independent donors. First, we analyzed ALP expression by all the donors because it is known to be a pre-osteogenic marker upregulated during osteoblast differentiation and represents the percentage of committed osteoprogenitor cells.[46] The cells from different donors exhibited a vast difference in ALP induction by dexamethasone. The percentage ALP-positive cells in untreated and dexamethasone-treated cells showed marked differences among the 19 donors tested. The presence of higher percentage of ALP-positive cells in the initial culture did not always resulted in high ALP induction. This suggests that the fold induction in ALP expression by dexamethasone is independent of initial number of ALP-positive cells that may have been influenced by various unknown factors. ALP analyses further suggest no statistical correlation with age, gender, or source of isolation, although hMSCs from 10 donors out of 19 belong to an age group over 60 year and the sample size in male and female group are inadequate, which limits the evident conclusions. Further, we deliberately selected donors over 40 years of age and above because they represent major targets for bone tissue engineering. This could be possibly due to the considerable variation in the composition of the initial aspirate and population of committed progenitors, which will effect ALP expression in vitro. Another possible explanation is that differential sampling methods by different physicians may result in varying heterogeneity in the final cell composition as reported previously.[41,47] However, we observed no

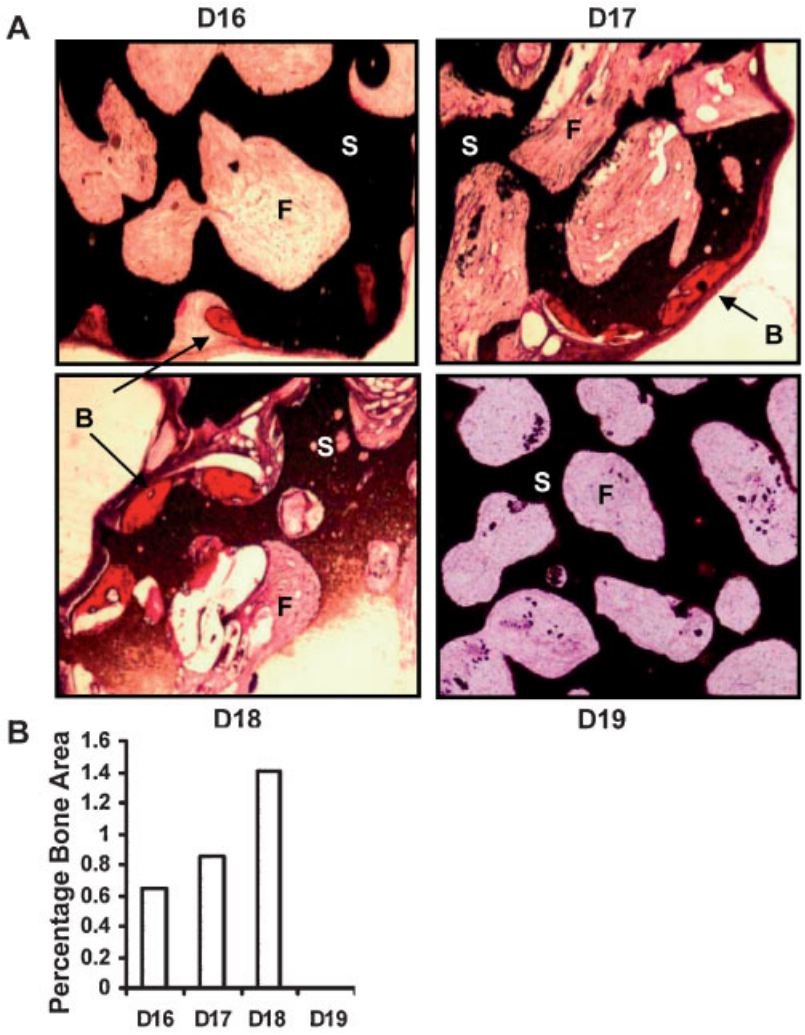

Figure 7. Donor variation in in vivo bone formation. (A) Representative histological sections of bone formation in immune-deficient mice by cells from D16, D17, D18, and D19 after 6 weeks of implantation. S, Scaffold material; F, fibrous tissue; B, basic fuchsin-stained newly formed bone. (B) Bone histromorphometric analyses of the donors from A.

statistical correlation between the ALP index and different physicians who harvested bone marrow (data not shown). Our data suggest that the variability in ALP expression reflects on the functional difference in osteogenic differentiation of hMSCs. The hMSCs with higher percentage of basal and dexamethasone-induced ALP-positive cells showed higher in vitro mineralization and calcium deposition (Fig. 2). However, this explanation may not rule out the differences in the physiological status and clinical history of the patients, which would account for in vitro variations. Collectively, our data demonstrate that, first, irrespective of age, gender, and source of isolation of hMSCs, cells from all donors responded to an osteogenic signal by dexamethasone. Second, hMSCs isolated from donors by different sampling methods might cause the variation in the heterogeneity of the cell population, which would affect in vitro cell behavior.

Gene expression analyses of osteogenic markers in hMSCs further demonstrated variation in gene expression levels but the expression profiles of *ALP, COL-1*, *OC*, and *S100A4* followed the same trend. For instance, ALP showed peak expression on day 10, but the fold induction varied between 3-fold and 115-fold, indicating the ability of hMSCs from various donors to respond to an osteogenic signal. This variation in hMSC gene expression is consistent with the studies from other groups.[48] Efforts have been made to minimize the heterogeneity of hMSCs using monoclonal antibodies to unique cell surface antigens such as SH2, cluster designation (CD)-antibodies specific for hMSCs,

PCR with known cell surface, extracellular matrix, and soluble proteins.[49–51] However, the individual clones isolated using these markers still exhibited a differential capacity to form new bone in vivo.[52]

We investigated whether ALP expression could be used as an indicator of bone-forming capacity. Seeding hMSCs onto porous BCP particles and subcutaneous implantation in immune-deficient mice for 6 weeks showed bone formation by three donors out of four. D19, the donor that yielded no bone, had the lowest relative ALP expression of all four donors investigated. Interestingly, D18 had the highest percentage of ALP-positive cells, and from this donor we observed the most robust bone formation in vivo. This is in line with previous work from our lab demonstrating a correlation between the fold upregulation of ALP by dexamethasone treatment in vitro and bone formation in vivo.[53] These results further substantiate the idea that the bone-forming capacity of an hMSC culture can be predicted by analyzing its gene expression profile. Because the resolution of ALP analysis is not sufficient to determine the bone-forming capacity of an MSC culture in advance, our current research is focused on identifying new diagnostic markers, which predict the bone-forming capacity of hMSCs. We approach this by isolating hMSCs from over 80 donors, analyzing their gene expression profiles and in parallel assessing their in vitro differentiation abilities and bone-forming capacity in vivo.

Proliferation of hMSCs is accompanied by loss of multipotency and cellular senescence. This limits the extent to which cells can be expanded. Consistent with previous studies, we observed a typical change in morphology from thin spindle shaped fibroblastic to large, flattened, and irregularly shaped during in vitro expansion of hMSCs. Furthermore, we show that the serially passaged hMSCs exhibited progressive loss of replicative potential and multipotency. Interestingly, we noticed that some cells in P5 regained growth and formed independent colonies, which was also observed in two other donors. To our knowledge, this is the first observation of this phenomenon in hMSCs. We are currently investigating whether the recovery of proliferative capacity of hMSCs in P6 resembles the escape from senescence during immortalization. We acknowledge the fact that in true immortalization, the cells divide indefinitely, whereas in hMSCs, cells loose proliferative potential after a few extra cell division cycles. We observed peak growth, *ALP* expression, calcium accumulation, and adipogenic differentiation in P2 and P3 cells, and this could be due to the fact that we used frozen cells of P0, which showed lag phase in growth and differentiation.[54] From our studies we conclude that hMSCs can be expanded in vitro up to P3 (which approximates to 10–12 population doublings starting from a P0 culture) while retaining multipotency for effective use in tissue engineering applications.

Despite the fact that hMSCs reproducibly form bone when implanted in animal models, the bone typically does not bridge the whole implant.[55,56] To optimize bone tissue engineering, various labs invest in optimizing the proliferation[57] and differentiation of hMSCs in vitro.[12,38,39,58] By better understanding of molecular pathways such as MAPK pathway,[59] Rho kinase,[60] Wnt,[39] Notch,[61] and receptor tyrosine kinases[12] we could improve bone tissue engineering. We recently discovered that PKA activation in hMSCs during in vitro expansion substantially enhances in vivo bone formation (manuscript in preparation).

In conclusion, use of hMSCs for cell and tissue engineering applications depends on the ability to in vitro expand while retaining their multipotency. Large variation among the donors in composition of cells (progenitor to committed osteoblast), growth, and response to osteogenic signals may limit these applications. Careful analyses of multiple samples from the same donor and use of genetic knowledge to enhance in vitro and in vivo osteogenic differentiation may improve tissue engineering applications.

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