Fibroblast Growth Factor-2 Enhances Proliferation and Delays Loss of Chondrogenic Potential in Human Adult Bone-Marrow-Derived Mesenchymal Stem Cells

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We compared human mesenchymal stem cells (hMSCs), expanded long term with and without fibroblast growth factor (FGF) supplementation, with respect to proliferation, and the ability to undergo chondrogenesis *in vitro*. hMSCs expanded in FGF-supplemented medium proliferated more rapidly than the control cells. Aggregates of FGF-treated cells exhibited chondrogenic differentiation at all passages tested although, in some preparations, differentiation was diminished after seventh passage. Aggregates made with control cells differentiated along the chondrogenic lineage after first passage but exhibited only marginal differentiation after fourth and failed to form cartilage after seventh passage. Microarray analysis of gene expression identified 334 transcripts differentially expressed in fourth passage control cells that had reduced chondrogenic potential, compared with the fourth passage FGF-treated cells that retained this capacity, and 243 transcripts that were differentially expressed when comparing them to the first passage control cells that were also capable of differentiating into chondrocytes. The intersection of these analyses yielded 49 transcripts differentially expressed in cells that exhibited chondrogenic differentiation *in vitro* compared with the cells that did not. Among these, angiopoietin 1, secreted frizzled-related protein 1, and six transmembrane epithelial antigen of the prostate 1 appear to be of higher relevance. These preliminary data must now be validated to verify whether different gene expression profiles translate into functional differences. In summary, these findings suggest that the chondrogenic potential of hMSCs is vulnerable to cell expansion and that care should be exercised when expanding these cells for cartilage tissue engineering applications. Supplementation with FGF-2 allows reaching target cell numbers more rapidly and extends the level of expansion within which these cells are useful for tissue-engineered cartilage repair.

Introduction

TISSUE ENGINEERING IS the process of creating functional living replicas of tissues through the use of cells, scaffolds, and biomolecules. Tissue engineering, and its application to regenerative medicine, stands to benefit greatly from progress in the isolation, expansion, and characterization of multipotent stem cells from adult tissues.

Stem cells are defined as cells with the ability to renew themselves through cell division and, under certain conditions, differentiate and acquire specialized phenotypes.^{1,2} Mesenchymal stem cells (MSCs) are derived from the stroma of bone marrow. Human MSC (hMSC) preparations have significant, although highly variable and not unlimited, proliferative potential.³ In some cases, MSCs can be expanded through 30–40 population doublings (10^9 – 10^{12} :1 expansion),

whereas other preparations stop proliferating after 4–5 population doublings (50:1 expansion).^{4–6} This different behavior may be because of multiple factors, such as the protocol used to obtain the bone marrow,^{4–7} the low frequency of MSCs in marrow,⁸ and the age and condition of the donor.^{5,9}

Interestingly, the loss of differentiation potential is not generalized. It has been reported that hMSCs maintain their osteogenic potential through extensive subcultivation.^{4,5} Further, in some cases, late-passage hMSCs spontaneously differentiate into osteoblasts.^{10,11} However, as cells approach senescence they lose their ability to differentiate into adipocytes.⁵ The ability to differentiate into chondrocytes is lost in earlier passages.¹²

Self-renewal and pluripotentiality, the two key properties that characterize a stem cell, are progressively lost in MSC preparations as cultured MSCs senesce *in vitro*.^{5,10–12} By and

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large, cell-based tissue engineering therapies require large numbers of cells that make extensive *in vitro* subcultivation an absolute requirement. This loss of proliferation and differentiation potential are critical hurdles that must be overcome if MSC-based tissue engineering and regenerative medicine are going to make the transition from the laboratory bench to the clinic.

Recent reports have demonstrated that hMSCs cultured in the presence of fibroblast growth factor 2 (FGF-2) can undergo more population doublings than control cells before they lose their proliferative capacity.^{13–17} Our long-term goal is to identify the cellular mechanisms involved in the regulation of the chondrogenic potential of hMSCs. We have demonstrated that gene expression analysis by microarray can aid in the identification of the molecular mechanisms¹⁸ underlying the aspects of these phenomena, through generation of association networks that link differentially expressed genes with the cellular processes in which they participate. The fact that FGF-2 treatment has the potential to delay the loss of chondrogenic potential can be exploited to help us identify the mechanisms that regulate this potential.

Materials and Methods

Isolation of hMSCs

Six hMSC preparations were used in this study. These cells were derived from bone marrow aspirated from the iliac crest of normal healthy human donors at the Hematopoietic Stem Cell Core Facility at Case Western Reserve University after informed consent was obtained under the terms of an Internal Review Board–approved protocol.

The procedures for establishing human bone-marrow-derived MSC cultures followed previously published methods.^{19,20} Briefly, bone marrow aspirates were washed with control medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM-LG) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) from a selected lot (lot # ACQ 23291; Hyclone, Logan, UT)²⁰ (DMEM-LG + 10% FBS) and subjected to a preformed Percoll (Sigma Chemical, St. Louis, MO) density gradient to isolate mononucleated cells. Serum lot selection is a standard procedure performed before purchasing a new shipment of serum; all these experiments were conducted with serum from a single lot. The mononucleated cells were washed with control medium and seeded at a density of 1.8×10^5 cells/cm² in control medium to establish primary cultures of human bone-marrow-derived MSCs. All cell culture was done at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Establishment of study groups

From the first medium change (day 4), some of the plates were assigned to receive control medium consisting of DMEM-LG + 10% FBS, and the rest of the plates received DMEM-LG + 10% FBS supplemented with 10 ng/mL of FGF-2 (Biological Resources Branch of the National Cancer Institute, Bethesda, MD). This dose was chosen based on the previous studies.¹⁸ Cultures were fed twice a week.

Subculturing

To keep their growth at an exponential rate and prevent spontaneous differentiation or loss of differentiation poten-

tial, hMSCs had to be subcultured before the cells became confluent.^{19,21} Typically, they were passaged when the cultures were 80–90% confluent. Primary cultures were usually subcultured around 14 ± 3 days. Subsequently, the cells were subcultured approximately every 7 ± 2 days. Plates assigned to the different study groups (control or FGF treated) were subcultured at the same time, which resulted in different levels of confluence in the treatment groups as a result of the previously reported differences in cell proliferation and cell size.¹⁸ Cells were subcultured by trypsinization, counted, and reseeded at a density of 4.5×10^3 cells/cm².

Assessment of cell expansion

Trypsinized cell suspensions were counted manually in triplicate using a Neubauer hemocytometer. Population doublings were calculated as the base 2 logarithm of the number of cells obtained at the end of a given passage divided by the number of cells seeded:

$$\text{Population doublings} = \log_2 = \left(\frac{\text{cells at end of passage}}{\text{cells seeded}} \right).$$

For primary cultures we used the number of colonies formed in primary culture as the denominator, assuming that one colony is derived from one MSC:

$$\text{Population doublings} = \log_2 = \left(\frac{\text{cells at end of primary culture}}{\text{number of colonies}} \right).$$

Population doubling times for each passage and treatment group were calculated by dividing the number of population doublings in that passage by the duration of the passage time in days.

Chondrogenic assay

At the end of first, fourth, and seventh passages, the cells were resuspended in chondrogenic medium at 1.25×10^6 cells/mL. Chondrogenic medium is high-glucose DMEM (Invitrogen) supplemented with 1% ITS + Premix (BD Biosciences, Franklin Lakes, NJ), 100 μ M ascorbate-2-phosphate (Wako, Richmond, VA), 10^{-7} M dexamethasone (Sigma Chemical), and 10 ng/mL transforming growth factor- β 1 (Peprotech, Rocky Hill, NJ). This is the same chondrogenic differentiation medium formulation used in all our^{18,22–25} and other's^{12,26} previous studies. This medium is serum free and contains no FGF. Two hundred microliter aliquots containing 2.5×10^5 cells were placed in polypropylene 96-well plates (Phenix, Hayward, CA), centrifuged at 500 g, and placed in the incubator at 37°C in a humidified atmosphere of 95% air and 5% CO₂.^{24,27–29} After 12–16 h, the cells coalesced and formed a free-floating aggregate. The chondrogenic medium was changed three times per week. On days 7, 14, and 21, cell aggregates were harvested. Replicate aggregates from each group were processed for histologic and immunohistologic evaluation, or for proteoglycan and DNA quantification.

DNA assay

For the chondrogenic aggregate cultures, cell numbers were determined indirectly by measuring the DNA content. Aggregates were digested with papain (Sigma Chemical).³⁰

The papain-digested extract was combined with 0.1 N NaOH, incubated at room temperature for 20 min, and neutralized with 0.1 N HCl in 5 M NaCl, 100 mM NaH₂PO₄, and this mixture combined with 1 mL of 0.7 µg/mL Hoechst 33258 (Sigma Chemical) in water. Fluorescence was read in a GENios Pro microplate reader (Tecan, Durham, NC; $\lambda_{\text{Ex}} = 340 \text{ nm}$, $\lambda_{\text{Em}} = 465 \text{ nm}$) and compared with that of a certified Calf Thymus DNA standard (Amersham, Piscataway, NJ).

Glycosaminoglycan quantification

Aggregates were papain digested as described above. A nitrocellulose membrane (0.45 µm pore size) was placed into a dot-blot apparatus. A 250-µL aliquot of 0.02% Safranin O (Sigma Chemical) in 50 mM sodium acetate (pH 4.8) was pipetted into each well, and 25-µL aliquots of the papain-digested extracts were then pipetted into the wells. Vacuum was applied until the samples filtered through. The wells were rinsed with distilled water, and the filter was removed from the apparatus and allowed to air dry. The individual dots were cut out, transferred to microcentrifuge tubes, and eluted for 20 min in 10% cetylpyridinium chloride (Sigma Chemical) at 37°C. The absorbance of these extracts was read at 536 nm and compared with that of the chondroitin sulfate standards (Seikagaku America, East Falmouth, MA). Proteoglycan content was normalized to DNA content of the chondrogenic cultures.³¹

Collagen immunohistochemistry

To further evaluate chondrogenesis in the aggregates, adjacent sections were stained for collagen types I, II, and X, using anti-Collagen Type I (Clone Col-1; Sigma Chemical, Cat# C2456), anti-Collagen Type II (Developmental Studies Hybridoma Bank, Iowa City, IA; Cat# II-II6B3), and anti-Collagen Type X (from Dr. Gary J. Gibson, Breech Research Laboratory, Bone & Joint Center, Henry Ford Hospital & Medical Centers, Detroit, MI) antibodies, respectively. Sections were deparaffinized in xylene, digested with pronase for 15 min, and then blocked with 5% bovine serum albumin (BSA) in PBS for 30 min. Either primary antibodies or control immunoglobulin G were applied in 1% BSA in PBS for 1 h. Secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Chemicon, Temecula, CA). In some cases, 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. Wet mounts were immediately documented with a 10× objective, using a fluorescence microscope and a SPOT-RT digital camera. The sections were evaluated qualitatively for the presence (or absence) and location of the immunoreactivity.

Gene expression profiling of hMSCs

To attempt to determine the molecular basis of the effect of FGF-2 on hMSCs, total RNA was Trizol extracted from three independent hMSC preparations at the end of first, fourth, and seventh passages. DNA microarray was performed by the Case Western Reserve University (CWRU) Gene Expression Array Core Facility, using methods that have been described previously.^{32–37} Briefly, total RNA was cleaned-up using Qiagen (Valencia, CA) spin columns (only RNA samples with 260/280 nm absorbance ratios between 1.9 and 2.1 were carried further). Five to eight micrograms of total RNA

was converted into double-stranded cDNA using Superscript II reverse transcriptase (Gibco BRL, Rockville, MD) and an oligo-dT primer with a T7 RNA polymerase promoter linked to its 5' end. cDNA was cleaned and used in an *in vitro* transcription (IVT) reaction to generate adequate amplified, biotin-labeled cRNA (50–80 µg). cRNA was then fragmented and 15 µg added to 300 µL hybridization cocktail (100 mM 2-(N-morpholino)ethanesulfonic acid (MES), 1 M NaCl, 20 mM ethylenediaminetetraacetic acid, 0.01% Tween 20; 0.1 mg/mL Herring Sperm DNA, 0.5 mg/mL acetylated BSA, and Affymetrix IVT controls, as per manufacturer). Four IVT products of bacterial genes were added at final concentrations ranging from 1.5 to 100 pM. Spiked transcripts served as hybridization controls and were always monitored as part of the quality control regimen. Fragmented biotin-labeled cRNA was added, and the final volume was adjusted to 300 µL with molecular grade water. Samples were hybridized onto the Affymetrix (Santa Clara, CA) H-U133A 2.0 human microarrays, which represent ~18,400 full-length genes and expressed sequence tag clusters from UniGene build 133 for 16 h at 45°C. The manufacturer's standard posthybridization wash, double-stain, and scanning protocols use an Affymetrix GeneChip Fluidics Station 400 and a Hewlett Packard Gene Array scanner.

Raw data from microarray scans were initially normalized and analyzed with Affymetrix Microarray Suite v.5.0. Pairwise comparisons were made between samples from the different culture conditions. Transcripts were defined as differentially regulated if they met the following criteria: (i) consistent increase/decrease call in all replicates of a specific condition, based on the Wilcoxon's signed rank test ($p < 0.05$) and (ii) absolute value of the average fold difference ≥ 2.0 (Tables 2 and 3).

Affymetrix-provided transcript annotations were replaced with official gene nomenclature using National Center for Biotechnology Information (NCBI) databases, and gene functions were assigned based on gene ontology and other data in NCBI LocusLink (www.ncbi.nlm.nih.gov/LocusLink/).

Statistical analyses

The statistical significance of the differences in population doubling times was determined by paired *t*-test. Analyses of the biochemical measurements were performed by two-factor repeated measurements analysis of variance and paired *t*-tests; differences were considered significant for p -values < 0.05 .

Results

Proliferation

FGF-treated hMSCs exhibited higher proliferation rates than those expanded in control conditions. The population doubling time for FGF-treated hMSCs was always shorter than that of the cells expanded under control conditions at any passage ($p < 0.01$). This is shown in Figure 1 where the slope of the growth curves approximate the proliferation rates of the treatment groups. Plates assigned to the different study groups (control or FGF treated) were subcultured at the same time, which resulted in different levels of confluence in the treatment groups as a result of the previously reported

FIG. 1. FGF-treated cells proliferate faster than control cells. Cumulative population doublings of hMSCs expanded in control conditions (white) or in the presence of FGF-2 (black). Mean and standard deviation from six individual hMSC preparations. The average population doubling times are estimated as the slope of a linear regression fitted to the data from each treatment group. At every passage ($p < 0.01$), the population doubling time was shorter for hMSCs expanded in the presence of FGF-2 than for those grown under control conditions. FGF-2, fibroblast growth factor 2; hMSCs, human mesenchymal stem cells.

differences in cell proliferation and cell size.¹⁸ In no case were control cultures more confluent than their FGF-treated counterparts at the time of subcultivation.

Chondrogenic potential

The chondrogenic potential of hMSCs was affected by both time in culture and culture conditions (Table 1). FGF-treated hMSCs always formed aggregates that were larger than the ones made with the matching cells expanded in control conditions after 7, 14, and 21 days of chondrogenic culture.

At the end of the first passage, histology (Fig. 2) revealed a more developed and more homogenous cartilaginous matrix in aggregates made with FGF-treated hMSCs (Fig. 2E–H) compared with those made with cells expanded in control conditions (Fig. 2A–D).

Aggregates made with fourth passage FGF-treated hMSCs (Fig. 3E–H) presented an overall appearance very similar to

that of the aggregates made with first-passage cells although they were, in most cases, slightly smaller. Aggregates made with fourth passage cells expanded in control conditions (Fig. 3A–D) demonstrated little or no chondrogenic differentiation.

In all of the cell preparations tested, aggregates made with seventh passage FGF-treated cells were smaller than those made with first or fourth passage FGF-treated cells. In some of the preparations, the aggregates exhibited robust chondrogenic differentiation (identified as “++” in Table 1), whereas some other preparations (identified as “+” or “±” in Table 1) exhibited weaker or limited chondrogenic differentiation (Fig. 4E–H). None of cells expanded under control conditions exhibited signs of chondrogenic differentiation at the end of the seventh passage (identified as “–” in Table 1) (Fig. 4A–D).

Analysis of the DNA content of the aggregates demonstrated no differences in the amounts of DNA per aggregate between the treatment groups ($p = 0.39$) but DNA content decreased as a function of time in culture ($p < 0.01$). In contrast, analysis of the glycosaminoglycan (GAG) content revealed that, within any given passage, aggregates made with FGF-treated cells contained more GAG, both per aggregate and normalized to DNA content ($p < 0.05$) (Fig. 5). GAG content diminished as a function of time in culture in both treatment groups but much more rapidly in the cells expanded in control conditions. In aggregates made with control cells, the GAG content per DNA dropped significantly ($p < 0.01$) between first and fourth passage. It remained at the same low level in fourth and seventh passage ($p = 0.26$), while in aggregates made with FGF-treated cells, a similar decrease did not take place until passage 7 (first to fourth passage $p = 0.11$; fourth to seventh passage $p < 0.01$). It should be noted that these passage numbers correspond to different population doublings; in all cases the control group had a lower number of population doublings. The fact that, despite this, the differences are in favor of the FGF-treated cells strengthens the case for the use of this supplement.

As previously reported,¹⁸ even successful chondrogenic aggregates made with cells expanded under control conditions (Fig. 2) presented an outer layer of flattened cells embedded in a fibrous extracellular matrix (ECM) that did not contain type II collagen (Fig. 2C) but did contain type I collagen (Fig. 2B), as determined by immunostaining at 3 weeks. In contrast, aggregates made with FGF-treated cells did not exhibit this fibrous outer layer, and type II collagen immunoreactivity was present throughout the aggregate (Fig. 2G).

TABLE 1. CHONDROGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELL PREPARATIONS

		ND1045	ND1047	ND1120	ND1183	ND1192	ND1251
Control	P1	+	+	+	+	+	+
	P4	±	–	±	±	–	±
	P7	–	–	–	–	–	–
FGF-2	P1	++	++	++	++	++	++
	P4	++	++	++	++	+	++
	P7	++	++	+	+	±	+

Preparations ND1183, ND1192, and ND1251 were used for analyses of gene expression. –, no chondrogenic differentiation; ±, chondrogenic differentiation was observed in some areas of the aggregate but not throughout; +, chondrogenic differentiation was observed throughout the aggregate; ++, chondrogenic differentiation was observed throughout the aggregate and GAG content was higher than 35 μ g of GAG per aggregate.

FGF-2, fibroblast growth factor 2; GAG, glycosaminoglycan.

FIG. 2. Aggregates made with first passage FGF-treated cells are larger than those made with first passage control cells. Photomicrographs of aggregates made with first passage hMSCs expanded in control (A–D) conditions or in the presence of FGF-2 (E–H) after 21 days in chondrogenic culture. (A, E) Toluidine blue staining; (B, F) Immunostaining for Type I collagen; (C, G) immunostaining for Type II collagen; (D, H) immunostaining for Type X collagen. All the panels are shown at the same magnification. Color images available online at www.liebertonline.com/ten.

FIG. 3. Aggregates made with fourth passage FGF-treated exhibit more robust and complete chondrogenic differentiation than aggregates made with fourth passage control cells. Photomicrographs of aggregates made with fourth passage hMSCs expanded in control (A–D) conditions or in the presence of FGF-2 (E–H) after 21 days in chondrogenic culture. (A, E) Toluidine blue staining; (B, F) immunostaining for Type I collagen; (C, G) immunostaining for Type II collagen; (D, H) immunostaining for Type X collagen. All the panels are shown at the same magnification. Color images available online at www.liebertonline.com/ten.

FIG. 4. Aggregates made with FGF-treated cells differentiate into chondrocytes but those made with seventh passage control cells do not. Photomicrographs of aggregates made with seventh passage hMSCs expanded in control (A–D) conditions or in the presence of FGF-2 (E–H) after 21 days in chondrogenic culture. (A, E) Toluidine blue staining; (B, F) immunostaining for Type I collagen; (C, G) immunostaining for Type II collagen; (D, H) immunostaining for Type X collagen. All the panels are shown at the same magnification. Color images available online at www.liebertonline.com/ten.

Type X collagen immunoreactivity was present throughout the ECM of aggregates made with FGF-treated cells except for a peripheral area of approximately six cell layers (Fig. 2H). Type X collagen immunoreactivity was also present throughout the matrix of aggregates made with cells expanded under control conditions (Fig. 2D).

Gene expression

Pair-wise comparisons between cells from different passages within the same treatment group (Table 2) or different treatment groups within the same passage (Table 3) were made for each cell preparation to determine the effect of time in culture and culture conditions on gene expression, respectively. In both treatment groups, the highest number of differentially expressed genes was detected in the comparison of first to seventh passage. In control cells, the comparison of fourth to seventh passage identified six transcripts that were differentially expressed in all three preparations, whereas in the FGF-treated cells, the fourth passage cells had fewer differentially expressed transcripts when compared with the first passage cells (Table 2). When comparing cells within the same passage, the number of differentially expressed transcripts as a result of the different conditions was similar for all the three passages tested (Table 3).

We further analyzed the transcripts identified as differentially expressed in the comparison of control cells at the first and fourth passages and the comparison of the control and FGF-treated fourth passage cells. These are comparisons between chondrogenic and nonchondrogenic cells (Table 2).

The intersection of these two groups of transcripts yielded 46 genes differentially expressed in chondrogenic versus nonchondrogenic cells (Table 4). Almost 20% of these differentially expressed genes are involved in intracellular signaling; approximately 15% relate to immune responses and 12% to epidermis development. Half of the signaling-related genes were upregulated and the other half downregulated in cells with chondrogenic potential, while all of those related to immune responses were upregulated and all of the genes related to epidermis development were downregulated.

Discussion

Cell-based therapies require large numbers of cells with the necessary characteristics for successful implantation, engraftment, and function. For cartilage tissue engineering, for example, approximately $8\text{--}10 \times 10^7$ cells/mL of tissue are required as starting material.³⁸ Table 5 shows the projected cell requirements for a variety of repair diameters and thicknesses, assuming disc-shaped defects. The current state-of-the-art in hMSC technology does not consistently achieve this degree of proliferation without partial or total loss of chondrogenic potential. This loss^{3,5,10,12} limits the applicability of hMSC-based therapies.

As we have reported previously, exposure of hMSCs to FGF-2 during expansion increases the cell yield and enhances their chondrogenic potential. By first passage, the FGF-treated cells average three population doublings more than the control cells: The FGF-treated cells are smaller than their control counterparts, allowing more cells per cm^2 at the same

FIG. 5. Aggregates made with FGF-treated cells have higher GAG content than those made with control cells. GAG content normalized to DNA content in aggregates made with hMSCs expanded in control conditions (white) or in the presence of FGF-2 (black) after 3 weeks in chondrogenic culture. The numbers are represented as percentage of the GAG per DNA content of aggregates made with first passage FGF-treated cells. Mean and standard deviation from six independent experiments performed with six individual hMSC preparations. Differences are significant between control and FGF groups ($p < 0.01$ for first and fourth passage and $p < 0.05$ for seventh passage). Within control cells, differences are significant between first and fourth passages ($p < 0.01$) and first and seventh passages ($p < 0.01$) but not between fourth and seventh passages ($p = 0.135$). For FGF-treated cells, differences are significant between first and seventh passages ($p < 0.01$) and fourth and seventh passages ($p < 0.01$) but not between first and fourth passages ($p = 0.06$). GAG, glycosaminoglycan.

level of confluence.¹⁸ In addition, the FGF-treated hMSCs exhibit enhanced chondrogenic potential. After four passages, the FGF-treated cells have doubled eight times more than the control (a 256-fold increase in yield). The degree of differentiation, reflective of the interindividual variability among cell preparations, was variable. Nonetheless, they retain a chondrogenic potential which is very diminished or lost entirely in the control cells. After seven passages, no control cells from any of the cell preparations tested retained any chondrogenic potential, whereas some of the FGF-treated cells did. This is consistent with our previous findings of stimulatory effects of FGF-2 on the proliferation and differentiation of bone-marrow-derived progenitors¹⁸ and are confirmed by other groups.^{13,15–17} However, in these reports,

TABLE 2. NUMBER OF DIFFERENTIALLY EXPRESSED TRANSCRIPTS AS A FUNCTION OF TIME IN CULTURE

		ND1183	ND1192	ND1251	Common	
Control	P1 vs. P4	535	468	419	40	} 37
	P1 vs. P7	846	1026	589	139	
	P4 vs. P7	206	393	252	6	
FGF-2	P1 vs. P4	666	640	520	33	} 56
	P1 vs. P7	1453	1260	1623	249	
	P4 vs. P7	405	643	651	82	

TABLE 3. NUMBER OF DIFFERENTIALLY EXPRESSED TRANSCRIPTS AS A FUNCTION OF CULTURE CONDITIONS

		ND1183	ND1192	ND1251	Common	
P1	Control vs. FGF-2	1315	889	1604	329	} 118
P4	Control vs. FGF-2	1079	893	1420	330	
P7	Control vs. FGF-2	1293	861	1734	387	

bone-marrow-derived progenitor cells exhibited differentiation potential only when expanded in the presence of FGF-2 or cultured at low density.¹⁶ In our hands, early-passage hMSCs expanded under control conditions do exhibit chondrogenic potential, which is enhanced in the FGF-treated hMSCs. These differences might be explained by our rigorous screening and testing of serum lots to select those that support the growth of hMSCs and maintain their multipotentiality. This is an important and documented element of our technology,²⁰ as the use of suboptimal serum results in the rapid loss of multipotentiality and slow expansion of hMSCs. Subtle differences in the protocols (seeding density of primary and subsequent cultures, or formulation of the base media) may also play a role in the lack of differentiation potential reported by others for cells grown without FGF.^{13,15,16} Analysis of DNA and GAG content suggests that the larger size of the aggregates from FGF-treated cells is because of the increased matrix production, and not because of the presence of more cells in the aggregates. Histologic analyses of the pellets revealed qualitative differences between the treatment groups beyond the difference in the size of the pellets.

Clearly, if hMSCs are to be of value to clinical tissue engineering, it will be critical to understand how their proliferation and differentiation potentials are regulated or by what mechanism FGF-2 supplementation enhances their proliferative and chondrogenic potentials. In this study, we take a first step toward this goal and present gene expression signatures of hMSCs with enhanced chondrogenic potential. As in most microarray studies, our dataset presents some sample-to-sample variability³⁹; the genes identified here as differentially expressed met very stringent selection criteria, increasing our confidence in the data. The analysis revealed differences due to both time in culture and culture conditions. The effects of time in culture in control cells were rapid, with most of the expression changes occurring during the initial passages, and the cells then settling into a fairly stable phenotype with only very few changes occurring later on. Many of these changes might be part of the adaptation of the cells to the monolayer culture conditions. But, in conjunction with the rapid sequential loss of differentiation potentials, this suggests that, under ordinary culture conditions, chondrogenic potential and, perhaps, true “stemness” is a very transient property of hMSCs.

Unsurprisingly, given the mitogenic effects of the treatment, cell signaling, metabolism, and cell-cycle-related genes figure prominently in the list of genes differentially expressed in the FGF-treated cells compared with their control counterparts. We also observed robust changes in the ECM-related gene expression, which trend toward reduced expression of

TABLE 4. DIFFERENTIALLY EXPRESSED TRANSCRIPTS IN CELLS WITH DIFFERENT CHONDROGENIC POTENTIAL

(A) Genes downregulated in cells with chondrogenic potential			
<i>Fold change control, P4 vs. P1</i>	<i>Fold change P4, control vs. FGF</i>	<i>Gene symbol</i>	<i>Gene title</i>
-14.33	-7.56	MBP	Myelin basic protein
-9.33	-4.71	KRT18	Keratin 18
-6.33	-36.69	FLG	Filaggrin
-4.67	-106.25	KRT14	Keratin 14
-4.00	-65.29	KRT34	Keratin 34
-3.50	-4.52	COMP	Cartilage oligomeric matrix protein
-3.50	-5.57	FAM129A	Family with sequence similarity 129, member A
-3.00	-4.49	PSG1	Pregnancy-specific beta-1-glycoprotein 1
-3.00	-8.06	FAM38B	Family with sequence similarity 38, member B
-3.00	-3.19	TRPC4	Transient receptor potential cation channel, subfamily C, 4
-2.67	-4.46	BST1	Bone marrow stromal cell antigen 1
-2.67	-32.57	KRT16	Keratin 16
-2.67	-3.26	DEPDC6	DEP domain containing 6
-2.67	-4.22	C21orf7	Chromosome 21 open reading frame 7
-2.50	-3.48	ANKRD1	Ankyrin repeat domain 1
-2.50	-3.33	KRT17	Keratin 17
-2.50	-4.42	KIAA1199	KIAA1199
-2.50	-3.57	HSPB8	Heat shock 22 kDa protein 8
-2.33	-2.31	ABLIM1	Actin-binding LIM protein 1
-2.33	-7.56	PSG5	Pregnancy-specific beta-1-glycoprotein 5
-2.33	-3.30	PSG4	Pregnancy-specific beta-1-glycoprotein 4
-2.33	-3.05	PSG6	Pregnancy-specific beta-1-glycoprotein 6
-2.00	-3.17	BAMBI	BMP and activin membrane-bound inhibitor homolog
-2.00	-2.66	COBLL1	COBL-like 1
-2.00	-4.52	PSG9	Pregnancy-specific beta-1-glycoprotein 9
-2.00	-4.07	MFAP5	Microfibrillar-associated protein 5
(B) Genes upregulated in cells with chondrogenic potential			
33.50	218.42	HLA-DRA	Major histocompatibility complex, class II, DR alpha
12.50	47.85	COLEC12	Collectin subfamily member 12
10.50	129.40	HLA-DRB1/3	Major histocompatibility complex, class II, DR beta 1/beta 3
6.50	22.25	SFRP1	Secreted frizzled-related protein 1
5.00	17.59	SEPP1	Selenoprotein P, plasma, 1
5.00	22.95	HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1
4.50	35.67	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1
4.00	8.71	CH25H	Cholesterol 25-hydroxylase
3.50	9.03	PDGFRL	Platelet-derived growth factor receptor-like
3.33	8.12	IFI44L	Interferon-induced protein 44-like
3.00	2.43	GAS1	Growth arrest-specific 1
3.00	3.72	MDK	Midkine
3.00	9.41	DIRAS3	DIRAS family, GTP-binding RAS-like 3
2.50	2.83	MARCKSL1	MARCKS-like 1
2.50	2.20	FUCA1	Fucosidase, alpha-L-1, tissue
2.50	4.71	STEAP1	Six transmembrane epithelial antigen of the prostate 1
2.50	3.74	ANGPT1	Angiopoietin 1
2.50	3.22	IFI44	Interferon-induced protein 44
2.50	3.63	NLGN4X	Neurologin 4, X-linked
2.00	7.05	SLC1A3	Solute carrier family 1 member 3

differentiation-related ECM molecules and enhanced expression of ECM catabolism-related genes. The pervasiveness of this pattern suggests that it is more than merely related to the enhanced cell division, but rather represents a fundamental shift in the phenotype of the cells. This is supported by the fact that merely delivering a mitogenic stimulus via platelet-derived growth factor-beta polypeptide (PDGF-BB) had a similar effect on cell division, but failed to improve the maintenance of chondrogenic potential (data not shown). In

future experiments, we plan to use PDGF-BB-stimulated cells in the expression array experiments to tighten the focus on nonmitogenic FGF-2 effects.

Our previous gene expression studies in first passage cells¹⁸ suggested that FGF-2 might be mediated through mitogen-activated protein kinase (MAPK) and Wnt signaling pathways.⁴⁰⁻⁴⁴ Here, we focused on the differences between hMSC preparations that can successfully differentiate into chondrocytes and those that cannot. Some differences were

TABLE 5. PROJECTED CELL REQUIREMENTS FOR DIFFERENT-SIZED REPAIRS ASSUMING AN INITIAL SEEDING DENSITY OF 100×10^6 CELLS/ML

Diameter (cm)	Thickness (cm)		
	0.3	0.4	0.5
1.0	2.36×10^7	3.14×10^7	3.93×10^7
2.0	9.42×10^7	1.26×10^8	1.57×10^8
3.0	2.12×10^8	2.83×10^8	3.53×10^8
4.0	3.77×10^8	5.03×10^8	6.28×10^8
5.0	5.89×10^8	7.85×10^8	9.82×10^8

consistent with those reported previously between the first passage control and FGF-treated cells.¹⁸ We found differences in Wnt signaling between chondrogenic and nonchondrogenic cells with upregulation of secreted frizzled-related protein 1 and downregulation of pregnancy-specific beta-1-glycoprotein 1 in chondrogenic cells. The effects on MAPK signaling were not through elements of the feedback loop as previously reported,¹⁸ but rather through the upstream regulators of MAPK signaling such as angiopoietin 1 and midkine, which are upregulated in cells with chondrogenic potential. These transcripts may have a role not only in the maintenance of the chondrogenic potential but also in the overall physiology of hMSCs; angiopoietin 1 has been implicated in the paracrine tissue repair activity displayed by MSCs^{45–47} and as an antiapoptotic factor⁴⁸; secreted frizzled-related protein 1 has also been reported to play a role in the tissue repair activity of MSCs^{49,50} and in controlling osteogenic differentiation.^{51–53} Additionally, a recent report indicates that six transmembrane epithelial antigen of the prostate 1, also upregulated in cells with chondrogenic potential, may be a marker for MSCs.⁵⁴ Our data here must now be validated at the level of protein expression and signaling pathway activation to verify whether the different gene expression profiles detected translate into functional differences.

In summary, the chondrogenic potential of MSCs is vulnerable to cell expansion; the expanded cells may exhibit weak or no chondrogenic potential, rendering them suboptimal or useless for the intended application. Supplementation of the growth medium with FGF-2 not only allows investigators to reach target cell numbers quicker but also extends the level of expansion within which these cells are still useful for tissue-engineered cartilage repair. The FGF-treated cells remain chondrogenic after 30 population doublings, whereas control cells are no longer chondrogenic after approximately 20 population doublings, that is over 1000-fold difference in the number of cells. Taken together, our results indicate that we must be extremely careful when these cells are extensively expanded to achieve a target cell number for cartilage tissue engineering applications in particular, but perhaps also when used in other applications.

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Disclosure Statement

No competing financial interests exist.

References

1. Lagasse, E., Shizuru, J.A., Uchida, N., Tsukamoto, A., and Weissman, I.L. Toward regenerative medicine. *Immunity* **14**, 425, 2001.
2. Smith, A.G. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* **17**, 435, 2001.
3. Minguell, J.J., Erices, A., and Conget, P. Mesenchymal stem cells. *Exp Biol Med* **226**, 507, 2001.
4. Bruder, S.P., Jaiswal, N., and Haynesworth, S.E. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* **64**, 278, 1997.
5. Digirolamo, C.M., Stokes, D., Colter, D., Phinney, D.G., Class, R., and Prockop, D.J. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* **107**, 275, 1999.
6. Phinney, D.G., Kopen, G., Righter, W., Webster, S., Tremain, N., and Prockop, D.J. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J Cell Biochem* **75**, 424, 1999.
7. Blazsek, I., Delmas Marsalet, B., Legras, S., Marion, S., Machover, D., and Misset, J.L. Large scale recovery and characterization of stromal cell-associated primitive haemopoietic progenitor cells from filter-retained human bone marrow. *Bone Marrow Transplant* **23**, 647, 1999.
8. Koc, O.N., Peters, C., Aubourg, P., Raghavan, S., Dyhouse, S., DeGasperis, R., Kolodny, E.H., Yoseph, Y.B., Gerson, S.L., Lazarus, H.M., Caplan, A.I., Watkins, P.A., and Krivit, W. Bone marrow-derived mesenchymal stem cells remain host-derived despite successful hematopoietic engraftment after allogeneic transplantation in patients with lysosomal and peroxisomal storage diseases. *Exp Hematol* **27**, 1675, 1999.
9. Galotto, M., Berisso, G., Delfino, L., Podesta, M., Ottaggio, L., Dallorso, S., Dufour, C., Ferrara, G.B., Abbondandolo, A., Dini, G., Bacigalupo, A., Cancedda, R., and Quarto, R. Stromal damage as consequence of high-dose chemo/radiotherapy in bone marrow transplant recipients. *Exp Hematol* **27**, 1460, 1999.
10. Conget, P.A., and Minguell, J.J. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* **181**, 67, 1999.
11. Banfi, A., Bianchi, G., Notaro, R., Luzzatto, L., Cancedda, R., and Quarto, R. Replicative aging and gene expression in long-term cultures of human bone marrow stromal cells. *Tissue Eng* **8**, 901, 2002.
12. Pittenger, M.F., Mbalaviele, G., Black, M., Mosca, J.D., and Marshak, D.R. Mesenchymal stem cells. In: Koller, M.R., Palsson, B.O., and Masters, J.R.W., eds. *Primary Mesenchymal Cells*. Boston: Kluwer Academic Publishers, 2001, pp. 189–207.
13. Martin, I., Muraglia, A., Campanile, G., Cancedda, R., and Quarto, R. Fibroblast growth factor-2 supports *ex vivo*

- expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology* **138**, 4456, 1997.
14. Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quarto, R. Proliferation kinetics and differentiation potential of *ex vivo* expanded human bone marrow stromal cells: implications for their use in cell therapy. *Exp Hematol* **28**, 707, 2000.
 15. Mastrogiacomo, M., Cancedda, R., and Quarto, R. Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells. *Osteoarthritis Cartilage* **9 Suppl A**, S36, 2001.
 16. Bianchi, G., Banfi, A., Mastrogiacomo, M., Notaro, R., Luzzatto, L., Cancedda, R., and Quarto, R. *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* **287**, 98, 2003.
 17. Tsutsumi, S., Shimazu, A., Miyazaki, K., Pan, H., Koike, C., Yoshida, E., Takagishi, K., and Kato, Y. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun* **288**, 413, 2001.
 18. Solchaga, L.A., Penick, K., Porter, J.D., Goldberg, V.M., Caplan, A.I., and Welter, J.F. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* **203**, 398, 2005.
 19. Haynesworth, S.E., Goshima, J., Goldberg, V.M., and Caplan, A.I. Characterization of cells with osteogenic potential from human bone marrow. *Bone* **13**, 81, 1992.
 20. Lennon, D.P., Haynesworth, S.E., Bruder, S.P., Jaiswal, N., and Caplan, A.I. Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation. *In Vitro Cell Dev Biol* **32**, 602, 1996.
 21. Lennon, D.P., and Caplan, A.I. Isolation of human marrow-derived mesenchymal stem cells. *Exp Hematol* **34**, 1604, 2006.
 22. Solchaga, L.A., Welter, J.F., Lennon, D.P., and Caplan, A.I. Generation of Pluripotent Stem Cells and Their Differentiation to the Chondrocytic Phenotype. Totowa, NJ: The Humana Press, 2004.
 23. Hanada, K., Solchaga, L.A., Caplan, A.I., Hering, T.M., Goldberg, V.M., Yoo, J.U., and Johnstone, B. BMP-2 induction and TGF-beta 1 modulation of rat periosteal cell chondrogenesis. *J Cell Biochem* **81**, 284, 2001.
 24. Penick, K.J., Solchaga, L.A., and Welter, J.F. High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. *Biotechniques* **39**, 687, 2005.
 25. Welter, J.F., Solchaga, L.A., and Penick, K.J. Simplification of aggregate culture of human mesenchymal stem cells as a chondrogenic screening assay. *Biotechniques* **42**, 732, 2007.
 26. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Graig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
 27. Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., and Yoo, J.U. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* **238**, 265, 1998.
 28. Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* **4**, 415, 1998.
 29. Yoo, J.U., Barthel, T.S., Nishimura, K., Solchaga, L., Caplan, A.I., Goldberg, V.M., and Johnstone, B. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am* **80**, 1745, 1998.
 30. Ponticello, M.S., Schinagl, R.M., Kadiyala, S., and Barry, F.P. Gelatin-based resorbable sponge as a carrier matrix for human mesenchymal stem cells in cartilage regeneration therapy. *J Biomed Mater Res* **52**, 246, 2000.
 31. Carrino, D.A., Arias, J.L., and Caplan, A.I. A spectrophotometric modification of a sensitive densitometric Safranin O assay for glycosaminoglycans. *Biochem Int* **24**, 485, 1991.
 32. Khanna, S., Merriam, A.P., Gong, B., Leahy, P., and Porter, J.D. Comprehensive expression profiling by muscle tissue class and identification of the molecular niche of extraocular muscle. *FASEB J* **17**, 1370, 2003.
 33. Porter, J.D., Khanna, S., Kaminski, H.J., Rao, J.S., Merriam, A.P., Richmonds, C.R., Leahy, P., Li, J., and Andrade, F.H. Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proc Natl Acad Sci USA* **98**, 12062, 2001.
 34. Porter, J.D., Khanna, S., Kaminski, H.J., Rao, J.S., Merriam, A.P., Richmonds, C.R., Leahy, P., Li, J., Guo, W., and Andrade, F.H. A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum Mol Genet* **11**, 263, 2002.
 35. Porter, J.D., Leahy, P., Khanna, S., Cheng, G., and Veigl, M.L. Applicability and practice of genome-wide expression profiling: insights from studies of skeletal muscle diversity and disease mechanisms. In: Appasani, K., ed. *Perspectives in Gene Expression*. Westboro, MA: Eaton Publishing, 2003, pp. 221–244.
 36. Porter, J.D., Merriam, A.P., Khanna, S., Andrade, F.H., Richmonds, C.R., Leahy, P., Cheng, G., Karathanasis, P., Zhou, X., Kusner, L.L., Adams, M.E., Willem, M., Mayer, U., and Kaminski, H.J. Constitutive properties, not molecular adaptations, mediate extraocular muscle sparing in dystrophic mdx mice. *FASEB J* **17**, 893, 2003.
 37. Porter, J.D., Merriam, A.P., Leahy, P., Gong, B., and Khanna, S. Dissection of temporal gene expression signatures of affected and spared muscle groups in dystrophin-deficient (mdx) mice. *Hum Mol Genet* **12**, 1813, 2003.
 38. Solchaga, L.A., Tognana, E., Penick, K., Baskaran, H., Goldberg, V.M., Caplan, A.I., and Welter, J.F. A rapid seeding technique for the assembly of large cell/scaffold composite constructs. *Tissue Eng* **12**, 1851, 2006.
 39. Abeyta, M.J., Clark, A.T., Rodriguez, R.T., Bodnar, M.S., Pera, R.A., and Firpo, M.T. Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum Mol Genet* **13**, 601, 2004.
 40. Stanton, L.A., Underhill, T.M., and Beier, F. MAP kinases in chondrocyte differentiation. *Dev Biol* **263**, 165, 2003.
 41. Seghatolleslami, M.R., Roman-Blas, J.A., Rainville, A.M., Modaressi, R., Danielson, K.G., and Tuan, R.S. Progression of chondrogenesis in C3H10T1/2 cells is associated with prolonged and tight regulation of ERK1/2. *J Cell Biochem* **88**, 1129, 2003.
 42. Hoffman, L.M., Weston, A.D., and Underhill, T.M. Molecular mechanisms regulating chondroblast differentiation. *J Bone Joint Surg Am* **85A Suppl 2**, 124, 2003.
 43. Tufan, A.C., Daumer, K.M., and Tuan, R.S. Frizzled-7 and limb mesenchymal chondrogenesis: effect of misexpression and involvement of N-cadherin. *Dev Dyn* **223**, 241, 2002.
 44. Tuli, R., Tuli, S., Nandi, S., Huang, X., Manner, P.A., Hozack, W.J., Danielson, K.G., Hall, D.J., and Tuan, R.S. Transforming growth factor-beta-mediated chondrogenesis of human

- mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. *J Biol Chem* **278**, 41227, 2003.
45. Shyu, K.G., Wang, B.W., Hung, H.F., Chang, C.C., and Shih, D.T. Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. *J Biomed Sci* **13**, 47, 2006.
 46. Wu, Y., Chen, L., Scott, P.G., and Tredget, E.E. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* **25**, 2648, 2007.
 47. Chen, L., Tredget, E.E., Wu, P.Y., and Wu, Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* **3**, e1886, 2008.
 48. Liu, X.B., Jiang, J., Gui, C., Hu, X.Y., Xiang, M.X., and Wang, J.A. Angiopoietin-1 protects mesenchymal stem cells against serum deprivation and hypoxia-induced apoptosis through the PI3K/Akt pathway. *Acta Pharmacol Sin* **29**, 815, 2008.
 49. Mirotsov, M., Zhang, Z., Deb, A., Zhang, L., Gnecci, M., Noiseux, N., Mu, H., Pachori, A., and Dzau, V. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci USA* **104**, 1643, 2007.
 50. Dufourcq, P., Descamps, B., Tojais, N.F., Leroux, L., Oses, P., Daret, D., Moreau, C., Lamaziere, J.M., Couffignal, T., and Duplaa, C. Secreted frizzled-related protein-1 enhances mesenchymal stem cell function in angiogenesis and contributes to neovessel maturation. *Stem Cells* **26**, 2991, 2008.
 51. Bodine, P.V., Billiard, J., Moran, R.A., Ponce-de-Leon, H., McLarney, S., Mangine, A., Scrimo, M.J., Bahy, R.A., Stauffer, B., Green, J., Stein, G.S., Lian, J.B., and Komm, B.S. The Wnt antagonist secreted frizzled-related protein-1 controls osteoblast and osteocyte apoptosis. *J Cell Biochem* **96**, 1212, 2005.
 52. Cho, S.W., Yang, J.Y., Sun, H.J., Jung, J.Y., Her, S.J., Cho, H.Y., Choi, H.J., Kim, S.W., Kim, S.Y., and Shin, C.S. Wnt inhibitory factor (WIF)-1 inhibits osteoblastic differentiation in mouse embryonic mesenchymal cells. *Bone* **44**, 1069, 2009.
 53. Gaur, T., Lengner, C.J., Hovhannisyan, H., Bhat, R.A., Bodine, P.V., Komm, B.S., Javed, A., van Wijnen, A.J., Stein, J.L., Stein, G.S., and Lian, J.B. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* **280**, 33132, 2005.
 54. Vaghjiani, R.J., Talma, S., and Murphy, C.L. Six-transmembrane epithelial antigen of the prostate (STEAP1 and STEAP2)-differentially expressed by murine and human mesenchymal stem cells. *Tissue Eng Part A* **15**, 2073, 2009.

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