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# Osteogenic Differentiation of Human Mesenchymal Stem Cells Cultured with Dexamethasone, Vitamin D3, Basic Fibroblast Growth Factor, and Bone Morphogenetic Protein-2

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## Abstract

*Purpose*: Human mesenchymal stem cells (hMSCs) are pursued for cell-based therapies of bone defects. Successful use of hMSCs will require them to be osteogenically differentiated before transplantation. This study was intended to determine the optimal combination(s) of supplements needed for inducing osteogenesis in hMSCs.

*Methods*: The hMSCs were cultured with combinations of β-glycerophosphate, dexamethasone (Dex), vitamin D3 (Vit-D3), basic fibroblast growth factor (bFGF), and bone morphogenetic protein-2 (BMP-2) to assess cell growth and osteogenesis. Osteogenic responses of the supplements were evaluated by alkaline phosphatase (ALP) activity, mineralization, and gene expression of ALP, Runx2, bone sialoprotein, and osteonectin. Adipogenesis was characterized based on Oil Red O staining, gene expression of peroxisome proliferator-activated receptor γ2, and adipocyte protein-2.

*Results*: Dex was found to be essential for mineralization of hMSCs. Cultures treated with Dex (100 nM), Vit-D3 (10/50 nM), and BMP-2 (500 ng/mL) demonstrated maximal calcification and up-regulation of ALP and bone sialoprotein expression. However, adipogenesis was up-regulated in parallel with osteogenesis in these cultures, as evident by the presence of lipid droplets and significant up-regulation of peroxisome proliferator-activated receptor γ2 and adipocyte protein-2 expression. An optimal condition was obtained at Dex (10 nM) and BMP-2 (500 ng/mL) for mineralization without increasing adipogenesis-related markers. The bFGF mitigated osteogenesis and enhanced adipogenesis. Vit-D3 appears essential for calcification only in the presence of bFGF.

*Conclusion*: Treatment of hMSCs with appropriate supplements at optimal doses results in robust osteogenic differentiation with minimal adipogenesis. These findings could be used in the cultivation of hMSCs for cell-based strategies for bone regeneration.

**Keywords:** human mesenchymal stem cells, osteogenesis, adipogenesis, bone tissue engineering, craniofacial defects

## INTRODUCTION

Bone deficiencies and defects due to congenital anomalies such as cleft palate are quite common in the clinical setting [1]. Despite significant variations in the nature of defects, autologous bone grafting is currently the frontline treatment for bone augmentation in a wide range of defects. However, there are numerous shortcomings to autologous bone grafting. In addition to donor site morbidity, other complications such as pain, wound infection, paresthesia, local tissue injury, and poor mobility, hamper the desired therapeutic outcomes [2]. To overcome the limitations related to bone harvesting and grafting, bone tissue engineering has been proposed as an alternative solution to prepare clinically useful bone grafts. In this approach, appropriate cells are cultivated in culture with biomaterials scaffolds and/or osteogenic supplements until a suitable bone graft is achieved. The tissue engineering approach, however, is often hampered by the need for large quantities

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of tissue-specific cells [3]. Human mesenchymal stem cells (hMSCs) from autologous bone marrow (i.e., bone marrow stromal cells) are the leading candidate for the source of cells in tissue engineering constructs, as they are readily available from the host, can be easily expanded in standard culture conditions, and have reliable osteogenic potential with no risk of immune rejection or tumorigenicity [4]. Successful use of hMSCs for augmentation of bone mass and repair requires these cells to be stimulated down the osteogenic pathway in vitro before transplantation. Among numerous agents used for inducing the osteogenic commitment of MSCs are bone morphogenetic proteins (BMPs), dexamethasone (Dex), basic fibroblast growth factor (bFGF), and vitamin D3 (Vit-D3).

The BMPs are multifunctional growth factors that are part of the transforming growth factor beta protein family. Among the BMPs, bone morphogenetic protein-2 (BMP-2) and BMP-7 are best known for their osteoinductive potential and are clinically used for bone repair and augmentation along with biomaterial implants. BMP-2 was proposed to require Dex to effectively induce osteogenic differentiation of rat MSCs [5]. Similarly, BMPs alone induced poor osteogenic commitment of hMSCs, but they improved Dex-induced osteogenesis of hMSCs, as measured by the alkaline phosphatase (ALP) induction and calcification in vitro [6,7]. Other studies also demonstrated that Dex in combination with ascorbic acid (AA) and β-glycerophosphate (GP) induced osteogenic differentiation of hMSCs based on enhanced ALP activity, expression of osteocalcin (OC) as well as in vitro calcification [8]. The growth factor bFGF is another protein that has been shown to augment the osteoinductive potential of BMP-2. bFGF is a prototypical mitogen that supports angiogenesis in vivo. Combinations of BMP-2 and bFGF demonstrated synergistic effects in osteogenic differentiation of MSCs in vitro and enhanced bone formation in vivo [9,10]. Subcutaneous implants supplemented with bFGF demonstrated enhanced ALP activity and calcification, since the presence of bFGF induced faster and stronger invasion of capillaries into implanted scaffolds, presumably resulting in an influx of osteoprogenitor cells from the enhanced vascular network [11]. It was also reported that bFGF and BMP-2 have a biphasic effect on osteoinduction; the stimulatory effect of bFGF was obtained at low bFGF doses, while bFGF exerts an inhibitory role in osteoinduction at high doses [12].

In addition to the protein growth factors, the active form of Vit-D3 has been shown to play an important role in skeletal homeostasis, as it displays anabolic effects on osteoblasts, resulting in increased bone formation [13]. In vitro studies demonstrated that treatment of hMSCs with Vit-D3 induced expression of both early and late stage osteogenic markers including ALP, bone sialoprotein (BSP), osteopontin, and OC [6,14]. Moreover, Vit-D3 improved Dex-induced osteogenic differentiation of human preosteoblasts and MSCs, resulting in increased ALP activity and matrix mineralization [6,14,15].

Taken together, we hypothesized that combining the optimal dose of BMP-2 and bFGF, along with Vit-D3 and Dex, will result in synergistic effects that may further augment osteogenic differentiation of hMSCs. To test this hypothesis, hMSCs were cultured with different concentrations of these osteoinductive reagents to explore the optimal combination(s) that will lead to robust osteogenesis in vitro. Our long-term aim was to determine the appropriate combination(s) of osteogenic supplements needed for developing a cell-based tissue engineering therapy for regeneration in bone defects. Toward this goal, this study took the first step by delineating the culture conditions that provided robust osteogenesis with minimal adipogenesis.

## MATERIALS AND METHODS

### Materials

Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine), Hank's Balanced Salt Solution (HBSS), and penicillin-streptomycin (10,000 U/mL–10,000 μg/mL solutions) were from Invitrogen (Grand Island, NY, USA). Master mix (2X) used for quantitative polymerase chain reaction (q-PCR) was developed by the Molecular Biology Service Unit in the Department of Biological Science at the University of Alberta (AB, Canada). The master mixture contained Tris (pH 8.3), KCl, $MgCl_2$, Glycerol, Tween 20, dimethyl sulfoxide, deoxynucleotide triphosphates, ROX, SYBR Green, and the antibody-inhibited Taq polymerase-Platinum Taq. Fetal bovine serum (FBS) was obtained from Atlanta Biologics (Lawrenceville, GA, USA). RNeasy kit was obtained from Qiagen (Valencia, CA, USA) and Agilent RNA 6000 Nano LabChip kit from Agilent Technologies (Santa Clara, CA, USA). Oligo$(dT)_{18}$ primer was obtained from Fermentas (Burlington, ON, Canada). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). CyQUANT cell proliferation kit and SYBR Green were from Molecular Probes (Portland, OR, USA). ALP substrate *p*-nitrophenol phosphate, 8-hydroxyquinoline, *o*-cresolphthalein, 2-amino-2-methyl-propan-1-ol (AMP), Dex, GP, AA, and Oil Red O stain were obtained from Sigma (St. Louis, MO, USA). Recombinant human bFGF was obtained from the Biological Resource Branch of NCI-Frederickton (Bethesda, MD, USA). Recombinant human BMP-2 was obtained from an E coli expression system, and its activity has been extensively reported in the literature [16,17]. The BMP-2 stock solution was reconstituted in $ddH_2O$.

### Isolation and Culture of hMSCs

The bone marrow aspirates were isolated from three (15–48-year-old) patients undergoing routine

orthopedic surgical procedures under a protocol approved by the Institutional Health Research Ethics Board. The cells were cultured in a growth medium containing DMEM, 10% FBS, 100 U/mL penicillin, and 100 μg/mL of streptomycin supplemented with 5 ng/mL bFGF for a total of two passages according to a published procedure [18]. Upon confluence, the cells were split 1:3 using 0.05% trypsin/0.04% ethylenediaminetetraacetic acid. One day before the addition of osteogenic supplements, hMSCs at passages 3–6 were seeded in 24 well plates in a basic medium (BM: DMEM with 10% FBS, 50 mg/L AA, 100 U/mL penicillin, and 100 g/L of streptomycin). The cultures were incubated at 37°C with 5% $CO_2$.



### Osteogenic Treatment

Two series of experiments were conducted in this study. We first investigated the effect of different osteogenic supplements by exposing hMSCs to the BM-containing combinations of one concentration of the following supplements: 10 mM GP, 10 nM Dex, 10 ng/mL bFGF, and 1 μg/mL BMP-2. The control group was treated with BM only without any supplements. The hMSCs were then analyzed for cell proliferation (DNA assay) and differentiation (specific ALP activity) at Days 7 and 11. In a second experiment, the dose- and time-dependent changes in osteogenesis of hMSCs were investigated with the addition of different concentrations of Dex, bFGF, BMP-2, and Vit-D3. The effects of 36 possible treatments were investigated with all possible combination of Dex (10 and 100 nM), BMP-2 (0, 200, and 500 ng/mL), Vit-D3 (0, 10, and 50 nM), and bFGF (0 and 10 ng/mL), all in the presence of 10 mM GP. The control group was treated with BM alone without any supplements. After 15 and 25 days, the hMSCs were analyzed for total DNA content and specific ALP activity. Calcification (total $Ca^{2+}$ content) and adipogenesis (Oil Red O stain) were also assessed after 25 days of treatment. After 15 days, q-PCR was performed with a select group of treated hMSCs.

### Specific ALP Assay

The effects of the osteogenic supplements on ALP activity of hMSCs were measured as this enzyme is a critical predictor for mineralization [19]. Cultured hMSCs were washed with HBSS and lysed with an ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X100; pH 10.5). After 2 hr, 100 μL of cell lysates were added into 96 well plates, and an equal volume of 2 mg/mL ALP substrate *p*-nitrophenol phosphate solution was added to each well. The absorbance was periodically measured (once every 90 s) at 405 nm for 10 min. The ALP activity was normalized with the DNA content in each lysate to obtain the specific ALP activity (ALP/DNA) [20].

### DNA Assay

To quantify the total DNA content in the wells, the remaining cell lysates from the ALP assay were frozen at −20°C and measured at the end of the experiment to minimize differences. DNA content of hMSCs was analyzed using the CyQUANT DNA kit according to the manufacturer's instructions and measured with a fluorescent plate reader ($\lambda_{excitation}$ at 480 nm and $\lambda_{emission}$ at 527 nm). A DNA standard supplied with the kit was used to calculate the DNA concentrations in the cell lysates [20].

### Calcium Assay (Total $Ca^{2+}$ content)

The wells containing hMSCs lysate from the DNA assay were rinsed (×2) by HBSS and 0.5 mL of 0.5 M HCl was then added to dissolve the mineralized matrix overnight. On the following day, 20 μL of aliquots from each well were added to 50 μL of a solution containing 0.028 M 8-hydroxyquinoline and 0.5% (v/v) sulfuric acid, plus 0.5 mL of solution containing $3.7 \times 10^{-4}$ M *o*-cresolphthalein, and 1.5% (v/v) 2-amino-2-methylpropan-1-ol. The absorbance was quantified at 570 nm. A standard curve was developed using $Ca^{2+}$ standards obtained from Sigma and was used to convert the obtained absorbance values into $Ca^{2+}$ concentrations (in mg/dL) [20].

### Oil Red O Staining

After 25 days of treatment with the indicated supplements, hMSCs were fixed in 10% formalin for 1 hr, washed with 60% isopropanol, and left to dry completely. Cultured hMSCs were then stained with 0.21% (w/v) Oil Red O solution for 10 min, washed 4 times with $dH_2O$, and examined by microscopy.

### Comparison of Osteogenic and Adipogenic Potential of hMSCs

The extent of calcification was classified based on the obtained $Ca^{2+}$ content in cultures: (a) −: no calcification where $0 < [Ca^{2+}] < 3$ mg/dL; (b) −/+: poor calcification where $3 < [Ca^{2+}] < 8$ mg/dL; (c) +: moderate calcification where $8 < [Ca^{2+}] < 13$ mg/dL; and (d) ++: significant calcification where $[Ca^{2+}] > 13$ mg/dL. Adipogenesis was also classified with a similar semi-quantitative scale, based on the amount of positively stained lipid vacuoles for Oil Red O stain in the treated cultures: (a) −: no staining; (b) −/+: poor staining with only a few areas (<10%) of stain; (c) +: moderate areas (10–40%) of staining; and (d) ++: significant (>50%) areas of staining.

### Quantitative Polymerase Chain Reaction

The q-PCR was performed on hMSCs from three donors who were cultured for 15 days in BM and the three media formulations that gave the most osteogenesis (i.e., groups which resulted in the highest calcification in previous studies). The following were the study groups: Group 1: BM (control); Group 2: Dex

(10 nM) + BMP-2 (500 ng/mL); Group 3: Dex (100 nM) + Vit-D3 (10 nM) + BMP-2 (500 ng/mL); and Group 4: Dex (100 nM) + Vit-D3 (50 nM) + BMP-2 (500 ng/mL). After washing the monolayers with HBSS, RNA was extracted and purified with an RNeasy kit using QIAshredders and an RNase-free DNase set for on-column digestion of genomic DNA. RNA concentration was determined by a GE NanoVue spectrophotometer, and sufficient RNA quality was confirmed by an Agilent 2100 Bioanalyzer using an Agilent RNA 6000 Nano LabChip kit. All samples were deemed acceptable (RNA integrity number $\geq$ 6.9) with the exception of Group 4 of one of the three donors and was excluded from this study.

Each cDNA synthesis reaction was performed in 20 μL volume with 300 ng total RNA using M-MLV reverse transcriptase, as per the manufacturer's instructions. Moreover, a combination of 0.5 μL random primers and 0.5 μL oligos ($dT_{18}$) was also used to synthesize cDNA template.

The q-PCR was performed as SYBR Green assays using an Applied Biosystems (Streetsville, ON, Canada) 7500 Fast Real-time PCR System. Cycle conditions were set to 2 min of 95°C, followed by 40 cycles of 20 s at 95°C, 1 min at 60°C, and ending in a default dissociation step. Primers for the experiment were designed using Primer Express 3.0 (Applied Biosystems). All 10 μL q-PCR reactions consisted of 2.5 μL of cDNA template, 2.5 μL of 3.2 μM primers (combined concentration), and 5 μL of a proprietary 2X master mix (Tris, KCl, $MgCl_2$, Glycerol, Tween 20, dimethyl sulfoxide, deoxynucleotide triphosphates, ROX, SYBR Green, and the antibody inhibited Taq polymerase-Platinum Taq; pH 8.3).

Prior to the q-PCR experiment, stable expression of glyceraldehyde 3-phosphate dehydrogenase was confirmed for each group. All primer sets (Table 1) were validated with a four-sample 1/5 to 1/625 dilution series of a mixed cDNA sample composed of cDNA from each treatment; primer efficiency [$\Delta C_t$/log(dilution)] was found to be stable for each primer set for cDNA dilutions 1/25–1/625.

Each sample from the three donors was analyzed in triplicate for each target gene using a cDNA dilution of 1/60. Data were analyzed by the $\Delta\Delta C_t$ method using Group 1 (BM) of one of the donors as a calibrator and normalizing to glyceraldehyde 3-phosphate dehydrogenase. Hence data are reported as fold change compared to Group 1: BM (Figure 7).

### Statistical Analysis

All assays were performed in triplicate for each donor, for a total of three cell donors. The results were expressed as mean and standard deviation. Data were analyzed by a one-way analysis of variance using SPSS version 18.0 software package (SPSS, Chicago, IL, USA). Intergroup variations were analyzed using "Tukey HSD" testing. Statistical significance was determined by $p$-values $< 0.05$.

## RESULTS

### Initial Response of hMSCs to Osteogenic Supplements

The DNA and specific ALP activity of hMSCs were investigated in short time culture (Days 7 and 11) in BM (control) and medium supplemented with bFGF, BMP-2, and bFGF/BMP-2 combination. The summary of the DNA analysis is provided in Figure 1A (Day 7) and 1B (Day 11). At Day 7, hMSCs treated in the absence of growth factors did not show any differences in DNA content with different media, indicating no effect of GP, Dex, and GP+Dex combinations on cell proliferation. In the presence of bFGF, the combination of BM+GP+Dex significantly enhanced DNA content as compared with control cultures (BM only) and cultures treated with BM+GP ($p < 0.05$), while BM+Dex demonstrated higher DNA amount as compared with BM only ($p < 0.05$). The hMSCs treated with BMP-2 did not show a significant variation in DNA content among the treatment groups. In the presence of the bFGF/BMP-2 combination, the hMSCs cultured in BM+Dex and BM+GP+Dex demonstrated higher DNA content as compared with hMSCs cultured in BM alone and BM+GP ($p < 0.05$).

On Day 11, there was no effect for bFGF, BMP-2, or bFGF/BMP-2 groups in terms of total DNA content for hMSCs cultured in BM (Figure 1B). On the other hand, BMP-2 treated hMSCs gave increased DNA content when cultured in BM+Dex and BM+GP+Dex as compared with cells treated with BM+GP ($p < 0.05$), but not as compared with BM alone ($p > 0.05$).

The summary of the specific ALP activity is provided in Figure 2A (Day 7) and 2B (Day 11). At Day 7 in the absence of growth factors, hMSCs cultured with

Table 1. Sequence of the forward and reverse primers used for the q-PCR

| Target gene (NCBI Ref. #) | Forward primer (5′ to 3′) | Reverse primer (5′ to 3′) |
|---|---|---|
| GAPDH NM_002046.3 | ACCAGGTGGTCTCCTCTGACTTC | GTGGTCGTTGAGGGCAATG |
| PPARγ NM_015869.4 | AGACATTCAAGACAACCTGCTACAA | GGAGCAGCTTGGCAAACAG |
| BSP NM_004967.3 | AAGCTCCAGCCTGGGATGA | TATTGCACCTTCCTGAGTTGAACT |
| aP2 NM_001442.2 | CATAAAGAGAAAACGAGAGGATGATAAA | CCCTTGGCTTATGCTCTCTCA |
| ON NM_003118.2 | TCCGTACGGCAGCCACTAC | GCATGGCTCTCAAGCACTTG |
| Runx2 NM_004348.3 | TCAGCCCAGAACTGAGAAACTC | TTATCACAGATGGTCCCTAATGGT |
| ALP NM_000478.4 | AGAACCCCAAAGGCTTCTTC | CTTGGCTTTTCCTTCATGGT |

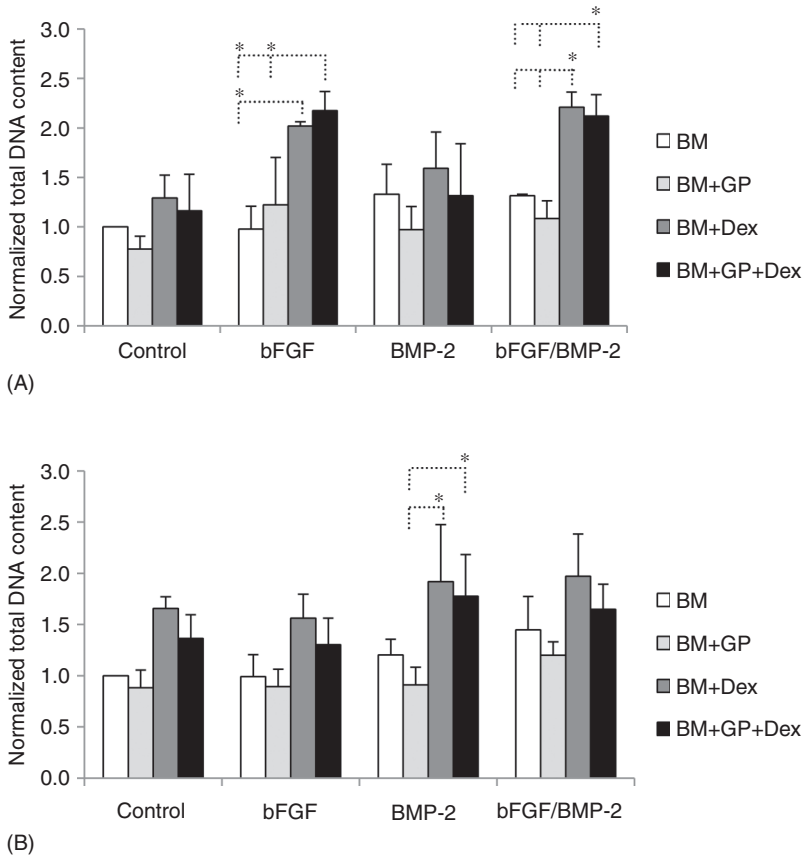



Figure 1. Effect of different osteogenic supplements (GP "10 mM", Dex "10 nM", bFGF "10 ng/mL", and BMP-2 "1 μg/mL") on total DNA content of the hMSCs. The analysis was conducted on Day 7 (A) and Day 11 (B). Data presented are the summary from three cultures of hMSCs derived from three different donors. Due to significant variations in the DNA amounts of different donors, all samples were normalized with the control treatment of individual donors (i.e., hMSCs treated with BM alone; indicated to be equivalent to 1.0). *Indicates the significantly different groups at $p < 0.05$.



BM+Dex and BM+GP+Dex gave significantly elevated ALP activity as compared to the cells cultured in BM alone and BM+GP ($p < 0.001$). A similar effect was also evident in the presence of bFGF, BMP-2, and bFGF/BMP-2 combination; cells cultured in BM+Dex and BM+GP+Dex showed higher ALP activity as compared to cells cultured in BM or BM+GP ($p < 0.05$). However, the specific ALP responses obtained from the BM+Dex- and BM+GP+Dex-cultured hMSCs were generally attenuated in the presence of growth factors ($p < 0.005$), compared with the cells treated with no growth factors. Addition of GP failed to enhance ALP activity under all conditions, and Dex addition was essential for such a response.

The specific ALP activity was generally elevated in all cultures on Day 11 (Figure 2B). In the absence of growth factors, or the presence of bFGF and BMP-2 alone, the ALP activity was again elevated in cells cultured in BM+Dex or BM+GP+Dex, as compared with BM and BM+GP cultured hMSCs ($p = 0.005$). Among the cells treated with the bFGF/BMP-2 combination, hMSCs cultured in BM+Dex gave higher ALP activity as compared with BM and BM+GP cultures ($p = 0.005$). Addition of bFGF alone or in combination with BMP-2 significantly decreased the ALP activity obtained in BM+Dex and BM+GP+Dex media, as compared with similar cultures in the absence of growth factors ($p < 0.05$).

### Dose-Dependent Response of hMSCs to Dex, BMP-2, Vit-D3, and bFGF

Longer term osteogenesis of hMSCs was then investigated by culturing the cells in BM containing GP (10 mM) and in the presence of various concentrations of supplements. The GP was added to media since this supplement is known to be essential for in vitro mineralization. The control group was treated with BM without any supplements. As before, the DNA content and specific ALP activity were determined in addition to in vitro calcification.

#### *DNA content*

The DNA content of the treatment groups was generally lower on Day 15 (Figure 3A) than that of the control BM group without supplements. The DNA content of hMSCs treated with 10 nM Dex+0 nM

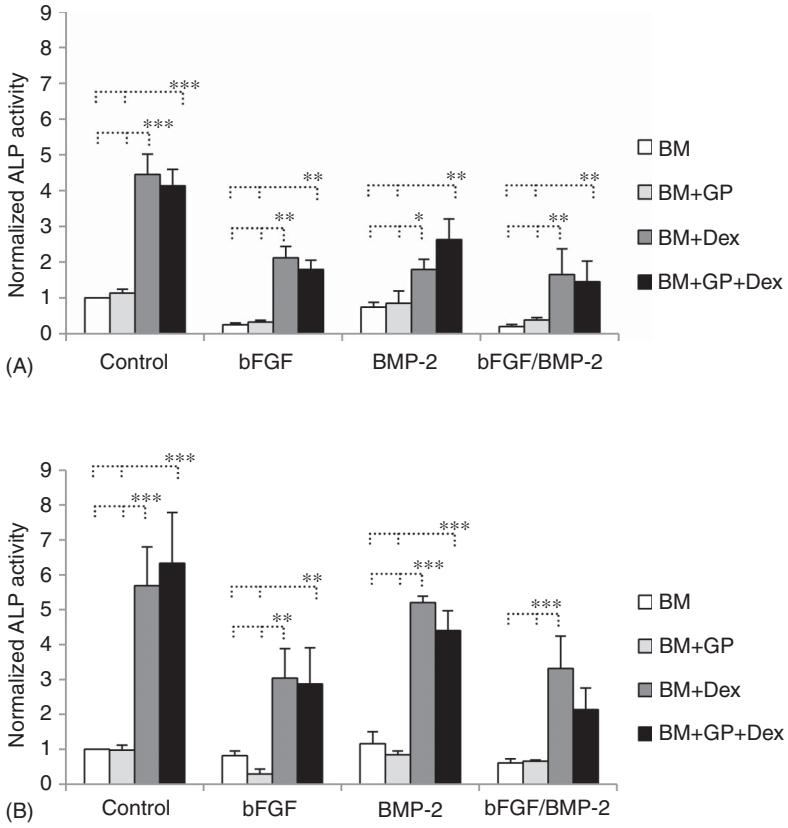



Figure 2. Effect of different osteogenic supplements (GP "10 mM", Dex "10 nM", bFGF "10 ng/mL", and BMP-2 "1 μg/mL") on specific ALP activity of hMSCs. The analysis was conducted on Day 7 (A) and Day 11 (B). Data presented are the summary from three cultures of hMSCs derived from three different donors. Due to significant variations in the ALP activity of different donors, all samples were normalized with the control treatment of individual donors (i.e., hMSCs treated with BM alone).
***, **, and *indicate the significantly different groups at $p < 0.001$, $p < 0.005$, and $p < 0.05$, respectively, as compared to cultures treated with BM and BM+GP.

Vit-D3+0 ng/mL BMP-2 was significantly higher than the hMSCs treated with 10 nM Dex+50 nM Vit-D3+0 ng/mL BMP-2 ($p < 0.005$). Similarly, cultures treated with 10 nM Dex+0 nM Vit-D3+500 ng/mL BMP-2 demonstrated higher DNA content as compared with similar cultures treated with 10 nM ($p < 0.05$) and 50 nM ($p < 0.001$) Vit-D3. On the other hand, the bFGF significantly increased the total DNA content of hMSCs treated with 100 nM Dex+0/50 nM Vit-D3+500 ng/mL BMP-2, as compared with similar treatments without bFGF ($p < 0.001$). Increasing Dex and BMP-2 concentrations did not show any detrimental effects on the DNA content of hBMC cultures.

In longer cultures (Day 25), there was no significant effect of increasing Dex or Vit-D3 on the DNA content of the treated hMSCs (Figure 3B). Addition of bFGF, however, increased DNA content of cultures treated with 10/100 nM Dex+0 nM Vit-D-3+500 ng/mL BMP-2 as compared with similar cultures treated in the absence of bFGF ($p < 0.05$).

### *Specific ALP activity*

On Day 15, the specific ALP activities of hMSCs were generally higher without bFGF treatment (Figure 4A). In the absence of bFGF, Vit-D3 (50 nM) had a stimulatory role in specific ALP activity, which was considerably increased by treatments with 100 nM Dex+0 ng/mL BMP-2, 100 nM Dex+200 ng/mL BMP-2, and 10 nM Dex+500 ng/mL BMP-2 ($p < 0.05$) as compared to control cultures. The specific ALP activity was also stimulated in cultures treated with 50 nM Vit-D3+100 nM Dex+200 ng/mL BMP-2 as compared with cells treated similarly but without Vit-D3 ($p < 0.05$). In the presence of bFGF, cells treated with 100 nM Dex+50 nM Vit-D3+200 ng/mL BMP-2 displayed reduced specific ALP activity ($p < 0.005$) as compared with similar cultures treated in the absence of bFGF. Although there was a general trend of increasing specific ALP activity with increasing Vit-D3 concentration in the presence of bFGF, there were no significant differences among the groups on Day 15. Increasing Dex and BMP-2 concentrations did not appear to change the specific ALP activity of treated hMSCs in this time frame.

On Day 25, there was a general reduction in the specific ALP activity among the groups (compare scales in Figure 4A and B). Vit-D3 again appeared to increase specific ALP activity, as evident by the increased ALP

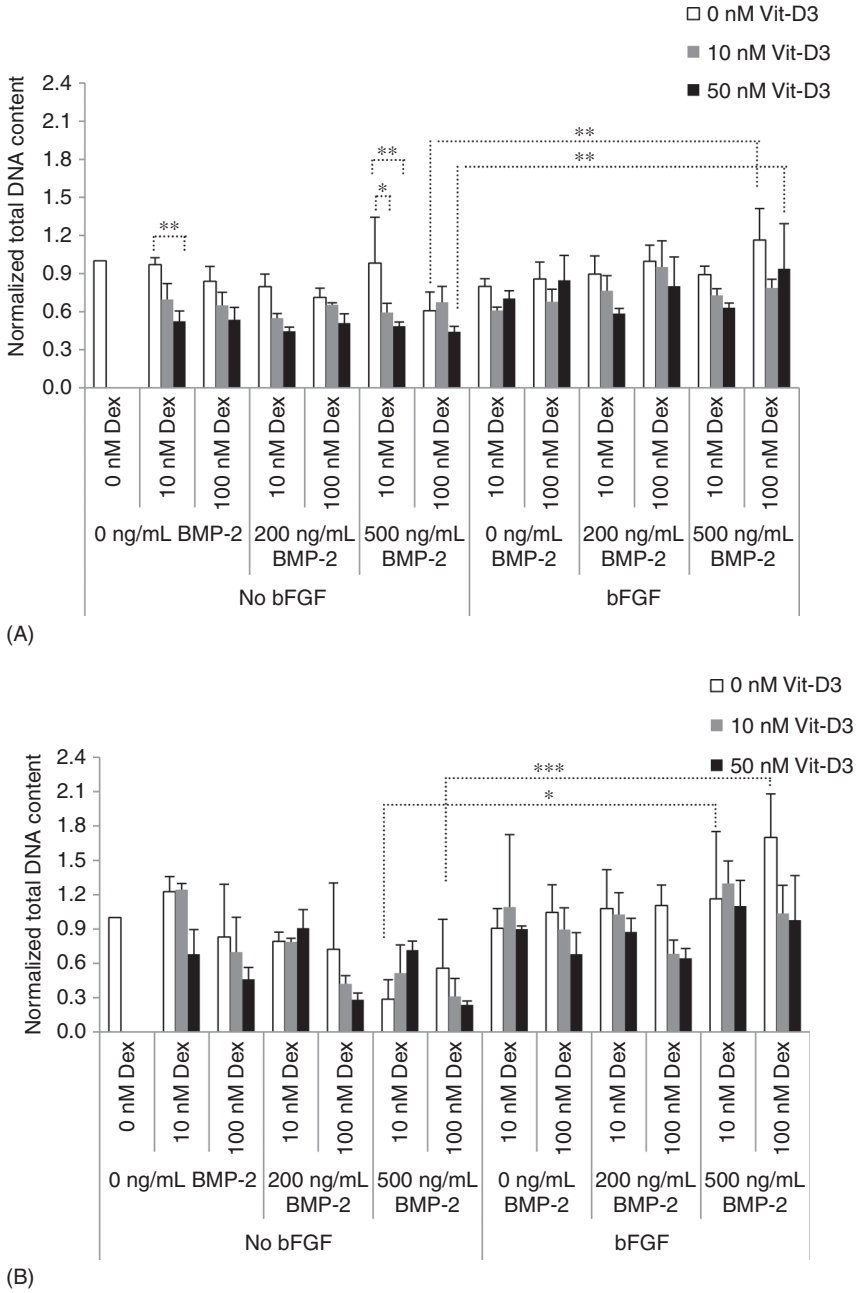



Figure 3. Effect of different osteogenic treatments on total DNA content of the hMSCs. The analysis was conducted on Day 15 (A) and Day 25 (B). The data is a summary from three cultures of hMSCs derived from three different donors. Due to significant variations in the DNA amounts of different donors, all samples were normalized with the control treatment of individual donors (i.e., hMSCs treated with BM alone).
***, **, and *indicate the significantly different groups at $p < 0.001$, $p < 0.005$, and $p < 0.05$, respectively.

activity in 100 nM Dex+50 nM Vit-D3+0 ng/mL BMP-2, 100 nM Dex+10 nM Vit-D3+200 ng/mL BMP-2, 100 nM Dex+50 nM Vit-D3+200 ng/mL BMP-2, 10 nM Dex+50 nM Vit-D3+500 ng/mL BMP-2, and 100 nM Dex+50 nM Vit-D3+500 ng/mL BMP-2 groups as compared with the control group ($p < 0.05$ in all cases). The specific ALP activity was also stimulated in cultures treated with 50 nM Vit-D3+100 nM Dex+0 ng/mL BMP-2 and 50 nM Vit-D3+100 nM Dex+200 ng/mL BMP-2 as compared with the cells treated similarly but without Vit-D3 ($p < 0.05$). Increasing Dex concentration from 10 to 100 nM in cultures treated with 50 nM Vit-D3 and 200 ng/mL BMP-2 significantly increased the ALP activity ($p < 0.05$). Addition of bFGF to 100 nM Dex+50 nM Vit-D3+200 ng/mL BMP-2 decreased the ALP activity in treated cells as compared with the cells exposed to similar conditions but without bFGF ($p < 0.05$). There

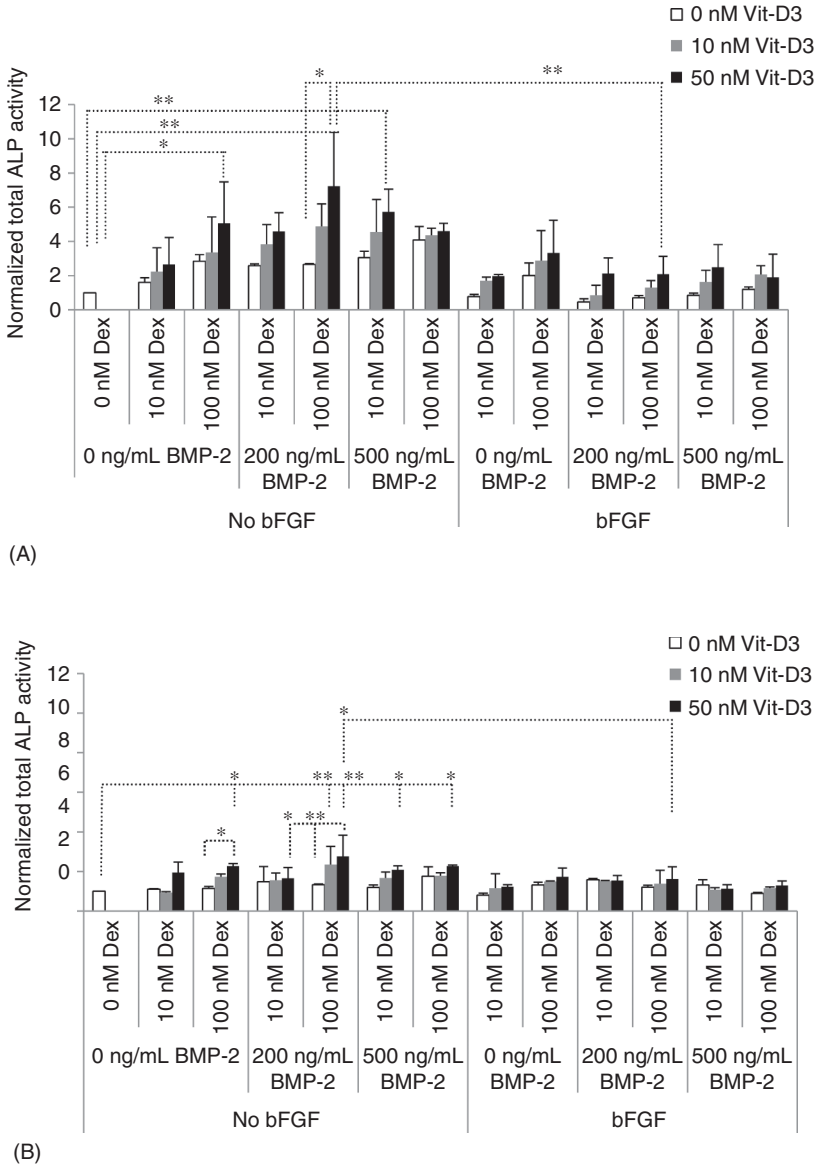



Figure 4. Effect of different osteogenic treatments on specific ALP activity of the hMSCs. The analysis was conducted on Day 15 (A) and Day 25 (B). Data presented are the summary from three cultures of hMSCs derived from three different donors. Due to significant variations in the DNA amounts of different donors, all samples were normalized with the control treatment of individual donors (i.e., hMSCs treated with BM alone).
** and *indicate the significantly different groups at $p < 0.005$ and $p < 0.05$, respectively.



was no effect of increasing BMP-2 concentration (from 0 to 500 ng/mL) on specific ALP activity of hMSCs at this time point.

### *Calcification*

There was a lack of calcification for hMSCs on Day 15 (not shown), but calcification was evident on Day 25 (Figure 5). Cells cultured in BM without any supplements did not yield any calcified deposits over the 25 days, indicating a lack of dystrophic calcification under our experimental conditions. In the absence of any growth factors, calcification was evident with increasing Dex concentration from 10 to 100 nM ($p < 0.05$). In the absence of bFGF, increasing Dex concentration from 10 to 100 nM also enhanced calcification of hMSCs treated with Vit-D3 (10 or 50 nM) and BMP-2 (200 and 500 ng/mL) ($p < 0.01$ in all cases). In the presence of bFGF, increasing Dex concentration from 10 to 100 nM also enhanced calcification only in hMSCs treated with Vit-D3 (10 or 50 nM) and BMP-2 (0 and 500 ng/mL) ($p < 0.01$ in all cases).

The effect of Vit-D3 on the calcification of hMSCs was dependent on other supplements. In the absence of bFGF, a detrimental effect of Vit-D3 (50 nM) was seen for cultures treated with 10 nM Dex and 200 or 500 ng/mL BMP-2 ($p < 0.05$) as compared with similar cultures treated without Vit-D3. In the presence of bFGF, only hMSCs treated with 100 nM Dex and, 10 and 50 nM Vit-D3 showed significant calcification irrespective of the BMP-2 concentration ($p < 0.01$).

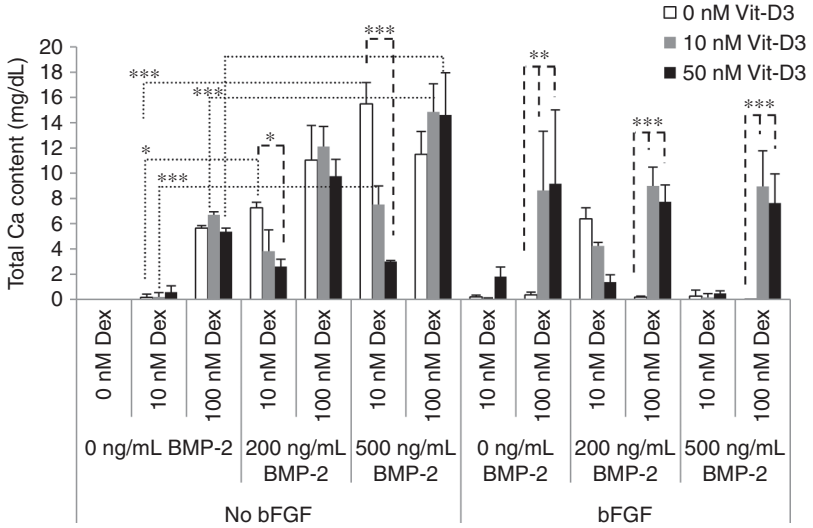



Figure 5. Effect of different osteogenic supplement combinations on calcification of hMSCs on Day 25. Each bar represents the mean + SD from three donors and no normalization was used in this analysis. Lines indicate significant changes in calcification due to Vit-D3 (dashed line) and BMP-2 (dotted line).
***$p < 0.005$, **$p < 0.01$, and *$p < 0.05$.

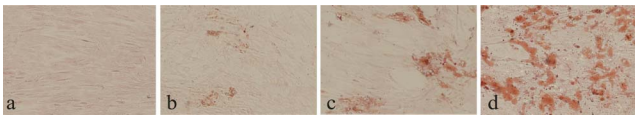
In the absence of bFGF, BMP-2 was stimulatory for calcification; for example, increasing BMP-2 concentration from 0 to 500 ng/mL in cultures with 10 nM Dex+0 or 10 nM Vit-D3, and 100 nM Dex+10 or 50 nM Vit-D3 showed a BMP-2 dose-dependent increase in calcification ($p < 0.05$). Addition of bFGF resulted in significant reduction in mineralization in all cultures treated with the highest BMP-2 concentration (500 ng/mL) except one culture (10 nM Dex+50 nM Vit-D3) as compared to similar cultures without bFGF ($p < 0.05$ in all cases). Cultures treated with 100 nM Dex+0 nM Vit-D3+200 ng/mL BMP-2 and in the presence of 10 ng/mL bFGF demonstrated inhibition of calcification as compared to cultures exposed to the same treatment but without bFGF ($p < 0.005$).

### Comparison of Osteogenic and Adipogenic Response of hMSCs

Following treatment of hMSCs with different supplements, some cultures gave lipid droplet-like deposits in cells, which were positively stained with Oil Red O, whereas hMSCs grown in BM did not produce such results. A comparison between calcification (osteogenesis) and Oil Red O staining (adipogenesis) in treated hMSCs was then pursued to investigate the best supplement combination for enhanced osteogenesis without adipogenesis. Adipogenesis was characterized based on Oil Red O staining and classified into "No", "Poor", "Moderate", and "High" based on the amount of positively stained lipid vacuoles (Figure 6), as well as specific changes in molecular markers (Figure 7). Calcification from Figure 5 was used as a measure of osteogenesis for comparison purposes and summarized in Figure 6.

Based on Oil Red O staining, Dex appeared to be most influential in adipogenesis. At Day 15, in the absence of bFGF, 10 nM Dex did not demonstrate any adipogenesis in any culture, but the adipogenesis was evident as the concentration of the Dex was increased to 100 nM in all groups, except the 0 nM Vit-D3+0 ng/mL BMP-2 and 0 nM Vit-D3+500 ng/mL BMP-2

(A)




(B)

| | | | Adipogenesis (D15) | | Adipogenesis (D25) | | Osteogeneis (D25) | |
|---|---|---|---|---|---|---|---|---|
| | | | 10 nM Dex | 100 nM Dex | 10 nM Dex | 100 nM Dex | 10 nM Dex | 100 nM Dex |
| No b-FGF | 0 ng/mL BMP-2 | 0 nM Vit D3 | – | – | – | – | – | –/+ |
| | | 10 nM Vit D3 | – | –/+ | – | –/+ | – | –/+ |
| | | 50 nM Vit D3 | – | + | – | + | – | –/+ |
| | 200 ng/mL BMP-2 | 0 nM Vit D3 | – | –/+ | – | + | –/+ | + |
| | | 10 nM Vit D3 | – | –/+ | – | + | –/+ | + |
| | | 50 nM Vit D3 | – | –/+ | –/+ | + | – | + |
| | 500 ng/mL BMP-2 | 0 nM Vit D3 | – | – | – | –/+ | ++ | + |
| | | 10 nM Vit D3 | – | –/+ | – | –/+ | –/+ | ++ |
| | | 50 nM Vit D3 | – | + | –/+ | + | – | ++ |
| b-FGF | 0 ng/mL BMP-2 | 0 nM Vit D3 | – | – | – | – | – | – |
| | | 10 nM Vit D3 | – | – | – | –/+ | – | + |
| | | 50 nM Vit D3 | – | – | – | –/+ | – | + |
| | 200 ng/mL BMP-2 | 0 nM Vit D3 | – | –/+ | – | ++ | –/+ | – |
| | | 10 nM Vit D3 | –/+ | + | –/+ | –/+ | –/+ | + |
| | | 50 nM Vit D3 | –/+ | + | –/+ | –/+ | – | –/+ |
| | 500 ng/mL BMP-2 | 0 nM Vit D3 | –/+ | –/+ | –/+ | ++ | – | – |
| | | 10 nM Vit D3 | –/+ | + | –/+ | + | – | + |
| | | 50 nM Vit D3 | –/+ | ± | –/+ | + | – | –/+ |

Figure 6. Adipogenic differentiation in hMSCs based on Oil Red O staining. Adipogenesis was classified based on the amount of positively stained lipid droplets in treated cultures. (A) Typical spectrum of adipogenesis seen in cultures (a) –: no staining; (b) –/+: poor staining with only a few areas (<10%) of Oil Red O stain; (c) +: moderate areas (10–40%) stained with Oil Red O Stain; and (d) ++: significant (>50%) areas of Oil Red O stain. (B) Summary of adipogenesis in hMSCs after 15 and 25 days of treatment with different osteogenic supplements. Calcification from Figure 5 was summarized and was used as a measure of osteogenesis for comparison purposes and summarized in this figure as follow: –: no calcification ($Ca^{2+}$= 0–3 mg/dL); –/+: poor calcification ($Ca^{2+}$= 3–8 mg/dL); +: moderate calcification ($Ca^{2+}$= 8–13 mg/dL); and ++: significant calcification ($Ca^{2+}$> 13 mg/dL).

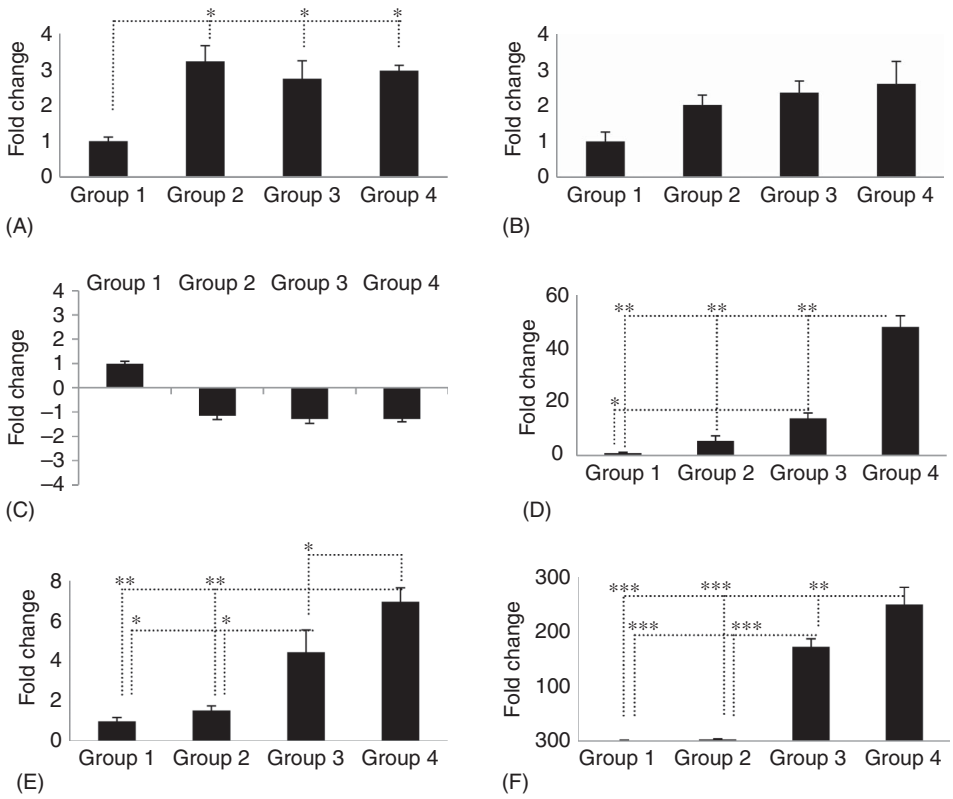



Figure 7. Quantitative analysis of osteogenic and adipogenic gene markers at Day 15. The following were the specific groups: Group 1: BM (control); Group 2: Dex (10 nM) and BMP-2 (500 ng/mL); Group 3: Dex (100 nM), Vit-D3 (10 nM), and BMP-2 (500 ng/mL); and Group 4: Dex (100 nM), Vit-D3 (50 nM), and BMP-2 (500 ng/mL). (A) ALP, (B) Runx2, (C) ON, (D) BSP, (E) PPARγ2, and (F) aP2. Data represent mean + SD from 3 donors.
***$p = 0.000$, **$p < 0.001$, and *$p < 0.05$.

groups. Stronger adipogenesis was seen in the presence of bFGF; 10 nM Dex gave some adipogenesis in all groups treated in the presence of BMP-2 (200 and 500 ng/mL) irrespective of Vit-D3 concentration (except 0 nM Vit-D3+200 ng/mL BMP-2). All 100 nM Dex groups treated in the presence of BMP-2 (200 and 500 ng/mL) demonstrated adipogenesis, which increased in the presence of Vit-D3 irrespective of its concentration (10 or 50 nM).

At Day 25, in the absence of bFGF, 10 nM Dex gave some adipogenesis with 200 and 500 ng/mL BMP-2 with 50 nM Vit-D3, but the level of adipogenesis was increased as the Dex concentration was increased to 100 nM. Stronger adipogenesis was seen with the addition of bFGF; almost all 100 nM Dex groups (except 0 nM Vit-D3+0 ng/mL BMP-2) and 10 nM Dex groups treated with BMP-2 (except 0 nM Vit-D3+200 ng/mL BMP-2) gave adipogenesis. The strongest adipogenesis was seen in the presence of bFGF (10 ng/mL) plus 100 nM Dex+0 nM Vit-D3+200 /500 ng/mL BMP-2. In bFGF-treated groups, Vit-D3 addition had a negative effect on the adipogenesis activity of hMSCs in some cases (in the presence of BMP-2), but a stimulatory effect in others (in the absence of BMP-2).

The q-PCR results for the expression levels of osteogenic and adipogenic markers are summarized in Figure 7. Only cells cultured in BM (control) and the three most osteogenic media were assessed for osteogenic and adipogenic gene expression. The ALP expression level was increased 2.7–3.0-fold ($p < 0.05$) in hMSCs exposed to osteogenic treatments compared to control cultures (Figure 7A). The specific ALP activity (as measured by colorimetric assay on Day 15) and ALP mRNA levels (as measured by q-PCR on Day 15) gave comparable responses in hMSCs under these treatments. The expression of Runx-2 in all osteogenic groups was 2.0–2.6-fold higher as compared to the cultures in BM (Figure 7B). Osteonectin (ON) was slightly decreased in osteogenic media as compared to control; however, this difference was not significant (Figure 7C). BSP expression was pronouncedly increased in Groups 3 and 4 by approximately 14-fold ($p < 0.05$ as compared to control) and approximately 48-fold ($p < 0.001$ as compared to all groups), respectively (Figure 7D). The adipogenic genes peroxisome proliferator-activated receptor γ2 (PPARγ2) and adipocyte protein-2 (aP2) were significantly upregulated in groups 3 and 4, but not in group 2 (Figure 7E). The PPARγ2 expression was 4.4-fold increased in Group 3 ($p < 0.05$ as compared to all groups) and approximately 7-fold in Group 4 ($p < 0.05$ as compared to all groups). Moreover, the expression of aP2 was increased by approximately

172-fold in Group 3 ($p < 0.000$ as compared to groups 1 and 2) and by approximately 250-fold in Group 4 ($p < 0.001$ as compared to all groups) (Figure 7F).

## DISCUSSION

In tissue engineering-based cellular therapies, one will rely on osteogenic cells cultivated in designer scaffolds to create a viable bone tissue in a bony defect site. It is desirable to use hMSCs and induce them into osteogenic pathway during in vitro culture before implantation. Therefore, the aim of this study was to investigate the effects of Dex, BMP-2, Vit-D3, and bFGF on hMSC osteogenesis in vitro to determine their potential for developing a cell-based therapy. Although there is extensive literature on in vitro osteogenic differentiation of hMSCs, there were no studies that simultaneously investigated osteogenesis and adipogenesis with a wide range of concentrations and combinations of different supplements (16 treatments in initial experiments and 37 treatments in subsequent experiments presented in our study). Most of the previous reports on differentiation of hMSCs focused on either osteogenic or adipogenic differentiation of hMSCs exposed, respectively, to osteogenesis or adipogenesis inducing media. We avoided this approach and evaluated the osteogenic and adipogenic differentiation concurrently in hMSC cultures to provide a complete picture in clarifying the role of the supplements Dex, Vit-D3, bFGF, and BMP-2. To our best knowledge, most reports focused on osteogenic differentiation and lacked adipogenesis data in hMSC cultures exposed to osteogenic media containing BMP-2, Vit-D3, or bFGF, which might explain some of the reported deleterious effects of these agents on osteogenic differentiation in hMSCs [21–23]. Jaiswal et al. [8] extensively studied the osteogenic effects of Dex at 1–1000 nM, GP at 1–10 mM, and AA at 0.01 to 4 mM on hMSCs and did not observe any adipogenesis with different doses of Dex (1–1000 nM) based on Oil Red O staining, but they did not study the associated adipogenic gene expression at the mRNA level. Moreover, Piek et al. [24] extensively studied osteogenic differentiation induced by Dex (100 nM), BMP-2 (250 ng/mL), and Vit-D3 (10 nM) and identified the role of the proto-oncogene c-*myc* as a regulator of osteogenesis; however, they did not explore the effects of these supplements on the adipogenic differentiation of hMSCs. On the other hand, our study presented balanced osteogenesis and adipogenesis data for several combinations of supplements and presented optimal conditions for osteogenesis with minimal induction of adipogenesis based on ALP activity, calcification, and expression of specific osteogenic and adipogenic markers. Our study reported similar responses in hMSCs derived from the three different donors, so that our findings could be generally applicable to a wider population, although the latter extrapolation will require further investigation with additional cell sources.

The studies performed here initially relied on DNA content and ALP activity to investigate the response of hMSCs to the supplements. The total DNA content was used as a measure of cell mass and to detect any detrimental effects of osteogenic supplements on cell proliferation. ALP is considered to be an early marker for osteoblastic differentiation that becomes upregulated in vitro within 2 weeks of osteogenesis [25]. It promotes mineralization through hydrolysis of pyrophosphate and ATP (an inhibitor of mineralization), and it is essential for phosphate production at local sites needed for the hydroxyapatite crystallization [19]. The specific ALP activity (as measured by a colorimetric assay on Day 15) and ALP mRNA levels (as measured by q-PCR on Day 15) demonstrated comparable responses in hMSCs, suggesting that the ALP activity colorimetrically measured throughout this study could be linked to gene expression. Our results demonstrated that GP alone did not affect cell viability (i.e., DNA content) and failed to promote ALP activity at any time point, but the addition of Dex was essential for the desired ALP response. Dex exposure additionally induced cellular proliferation of hMSCs in early culture (Days 7 and 11). Our data were similar to the study by Jaiswal et al. [8], who demonstrated a significant increase in ALP activity and mineralization in hMSCs exposed to osteogenic media containing 10 nM Dex, unlike cells grown with GP (10 mM) alone.

We expected the prototypical morphogen BMP-2 with its ability to induce de novo bone to impart significant osteogenesis in hMSCs. On the contrary, Jorgensen et al. [6] reported that BMP-2 alone did not affect ALP activity and poorly induced in vitro calcification by hMSCs. We made a similar observation in this study where, based on ALP activity as a measure of osteogenic differentiation, the BMP-2 effect was enhanced when hMSCs were additionally exposed to BM+Dex or BM+GP+Dex combinations, so that these supplements might be needed for a strong BMP-2 effect in culture. The bFGF, on the other hand, acted to increase cellular mass under numerous culture conditions in this study, while reversing osteogenesis. This was consistent with the literature on the mitogenic effects of bFGF on MSCs [26,27] and anti-osteogenic effects mediated by bFGF in human and rat MSCs [22,27].

We subsequently investigated dose-dependent responses of the cultured hMSCs to the supplements. Unlike the early time points, Dex did not change cellular proliferation or ALP activity of hMSCs at late time points (Days 15 and 25), but it enhanced mineralization in a dose-dependent manner, as noted earlier [8]. However, increased mineralization of hMSCs at higher Dex concentrations also resulted in significant appearance of Oil Red O-stained cells. Increased adipogenesis was confirmed under select conditions, based on elevated expression of the adipogenic markers PPARγ2 and aP2. PPARγ2 is an early stage marker [28] and aP2 is a

late stage marker of adipogenic differentiation [29]. The expression of aP2 is limited to adipocytes in vitro and in vivo, and an adipose-specific enhancer component was identified in the 5′ flanking region of the gene [30]. In contrast, Jaiswal et al. [8] did not observe any adipogenesis in hMSCs treated with different doses of Dex (1-1000 nM) based on Oil Red O staining, but they did not study the associated adipogenic gene expression at the mRNA level. This might be due to different isolation protocol and/or variability in the cell source. Having adipogenesis in the induced cells is not desirable, since it might reduce the osteogenic cell pool at the transplant site, and it is imperative to minimize this activity for cultures destined for clinical application.

The influence of Vit-D3 on osteogenesis was previously investigated with hMSCs [21]; Vit-D3 (10 nM) markedly inhibited cellular proliferation, enhanced ALP activity, and reduced mineralization in Dex (10 nM) treated hMSCs. Under consideration, increasing Vit-D3 concentration from 0 to 50 nM in the presence of Dex resulted in reduced DNA content and mineralization in cultures treated in the absence of bFGF, which is in accordance with others' observations. Reduced mineralization might be due to reduced cell proliferation induced by increasing Vit-D3 concentration. The addition of Vit-D3 was stimulatory for adipogenesis in hMSCs used in our study, in line with observations in the rat calvarial cells cultured with similar concentrations of the supplements [31], where adipogenesis was increased in a dose-dependent manner for Vit-D3 (from 0.1 to 100 nM) and Dex (from 1 to 100 nM). A synergistic effect for Vit-D3 and Dex on adipogenesis was evident in hMSCs (this study) and rat calvarial osteoblasts [31]. Interestingly, addition of Vit-D3 (10 or 50 nM) plus Dex (100 nM) significantly enhanced calcification of hMSCs after 25 days only in the presence of 10 ng/mL bFGF, irrespective of the BMP-2 concentration. It is likely that the Vit-D3 and Dex inhibited bFGF-induced adipogenesis and supported osteogenesis in hMSCs as evidenced by reduction in lipid formation concurrent with a significant increase in mineralization at this time point.

The BMP-2 gave dose-dependent mineralization in hMSCs, consistent with other studies on the activity of this morphogenetic protein on rat and hMSCs [23,27]. Conversely, the highest BMP-2 concentration (500 ng/mL) reduced cell mass of cultured hMSCs at Day 25, which was significantly improved by co-treatment with bFGF. It is likely that cell mass (as measured by the DNA content) might have been reduced due to enhanced extracellular mineralization caused by BMP-2. In parallel with osteogenesis, BMP-2 also gave enhanced adipogenesis in some cases, for example, in combination with Dex (100 nM) and bFGF (10 ng/mL). Previous studies reported negative effects of Dex on osteogenesis as a result of preferential adipogenic differentiation in rat calvarial cells (based on Oil Red O staining), in cells treated with 10 nM Dex and 100 ng/mL BMP-2 [32]. BMP-7 (100 ng/mL) also gave elevated expression of adipocyte-specific genes aP2, adiponectin, and lipoprotein lipase in hMSCs in osteogenic media (10 mM GP, 50 μg/mL AA, and 100 nM Dex) [33]. BMP-7 (50–200 ng/mL) was also capable of inducing adipogenesis in hMSCs cultured in conditions favoring chondrogenic differentiation in the absence of transforming growth factor-β3 [34]. Therefore, there seems to be consistent data in the literature that the presence of BMPs might stimulate adipogenesis under "osteogenic" culture conditions.

Combinations of BMP-2 and bFGF demonstrated synergistic osteogenic effects during differentiation of hMSCs and bone formation in vivo [35]. The BMP-2/bFGF co-treatment used in our experiment was intended to investigate this synergistic action, but no such synergistic effects were evident on osteogenic responses of hMSCs. In fact, bFGF treatment consistently deteriorated BMP-stimulated osteogenic differentiation in hMSCs, in line with previous studies on hMSCs [22]. However, the latter studies did not investigate adipogenesis. The stronger adipogenesis seen in hMSCs cultured in the presence of bFGF in this study might explain the reduction in osteogenesis in treated hMSCs. It is likely that bFGF increased the population of other cell lineages as they share the same multipotent precursors in the bone marrow [36]. Conversely, Akita et al. [35] demonstrated significant enhancement of ALP activity following treatment of hMSCs with bFGF (2.5 ng/mL) and BMP-2 (50 ng/mL) for 4 days after 6 days of osteogenic treatment with Dex (100 nM), AA (0.05 mM), and GP (10 mM). Sequential addition of the media supplements and the lower concentration of BMP-2 and bFGF used in Akita's study might lead to increased ALP activity and presumably osteogenesis, unlike our results.

Osteogenesis in our strongly mineralizing cultures was also confirmed based on specific changes at the mRNA levels of ALP, BSP, ON, and Runx-2. Runx-2 is expressed in preosteoblasts, immature osteoblasts, and early mature osteoblasts [37]. ON is involved with the onset of crystal nucleation [38]. BSP indicates a late phase of osteoblast differentiation and an initial phase of mineralization [39]. Our study reported considerable enhancement of in vitro calcification in cultures enriched with the highest concentration of BMP-2 and Dex (i.e., cultures treated with 100 nM Dex+10 nM Vit-D3+500 ng/mL BMP-2, and 100 nM Dex+50 nM Vit-D3+500 ng/mL BMP-2) more than the lower concentrations. This was confirmed with increased expression of ALP, Runx-2, and BSP as compared untreated control cells. Unlike other markers, ON expression was not significantly changed in hMSCs and we note a similar observation in a previous independent study [18]. Adipogenesis in these cultures paralleled osteogenesis, given by significant up-regulation of the adipogenic markers PPARγ2 and aP2. Our results are consistent with a previous report [33], which indicated enhanced

expression of osteogenic markers (ALP, Runx-2, osteopontin, and OC) as well as adipogenic markers (aP2, adiponectin, and lipoprotein lipase) in hMSCs exposed to similar osteogenic media (10 mM GP, 100 nM Dex, but with 100 ng/mL BMP-7). A critical issue is to identify culture conditions that optimize osteogenesis with no or minimal adipogenesis. In our hands, this condition was attained at 10 nM Dex, 500 ng/mL BMP-2, and without Vit-D3 and bFGF. It must be stated that this conclusion is based on addition of media supplements to hMSCs simultaneously. It is likely that sequential addition of the media supplements might alter this picture and lead to different results. This was considered beyond the scope of the current study. We can envision using conditions that do not support osteogenesis initially (e.g., culture in BM + bFGF supplementation) following by exposure to osteogenic supplements (e.g., Dex and BMP-2) when sufficient cell expansion occurs.

The concept of reconstructing craniofacial defects with MSCs from bone marrow was successfully validated in different animal models [40,41], with osteogenically induced cells yielding better bone induction in animal models [42]. However, the use of hMSCs for bone regeneration in humans is rare. Gimbel et al. [43] implanted collagen scaffolds seeded with bone marrow aspirates into human cleft defects and reduced morbidity compared to autologous grafts, but they did not report any quantitative measurement of bone formation at defects. Behnia et al. [44] implanted hMSCs combined with a demineralized bone mineral/calcium sulphate scaffold to obtain <50% bone fill. Both studies used hMSCs with no osteogenic conditioning. Hibi et al. [45], on the other hand, used osteogenically induced hMSCs to repair an alveolar cleft in a 9-year-old patient, which resulted in approximately 79% bone fill after 9 months post-operatively with successful eruption of lateral incisor and canine. The conditioning was attempted with platelet-rich plasma, whose osteogenic effects are difficult to dissect due to its various constituents. No attempts have been made to optimize the osteogenic conditioning of the cells using purified supplements (such as the ones used in this study) before transplantation. Using purified reagents might be a better approach since it can provide better control over the potency and reproducibility of cellular differentiation. The outcome of cell-based therapies for bone regeneration could be accordingly optimized with such an approach, potentially providing a superior alternative for autologous grafts. Our studies delineated the conditions for phenotypic differentiation of hMSCs and it will be important to explore in vivo potential of phenotypically differentiated hMSC for translation into clinics.

In conclusion, Dex was found to be most essential for osteogenesis of hMSCs in vitro, but high concentrations of Dex (100 nM) also enhanced adipogenesis of hMSCs. Vit-D3 appeared to be essential for calcification only in the presence of bFGF. But in the absence of bFGF, increasing Vit-D3 in culture (e.g., from 0 to 50 nM) did not have any additive effect on mineralization and, in fact, increased adipogenesis in some cases. BMP-2 demonstrated a dose-dependent increase in mineralization as its concentration increased from 0 to 500 ng/mL, but its effect was more pronounced in the presence of Dex. Although bFGF (10 ng/mL) was beneficial in enhancing DNA content in some cases, it deteriorated osteogenic and enhanced adipogenic features of the cultured hMSCs. Our results indicated that under appropriate priming with the optimal Dex and BMP-2 concentrations, hMSCs could be stimulated for osteogenesis with minimal adipogenesis. These studies provide a framework for obtaining optimal osteogenesis with hMSCs and this will be indispensable for clinical tissue engineering efforts that will rely on conditioned cells to induce a viable bone tissue in desired repair sites.

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