ARTICLE
Journal of Cellular Biochemistry 114:2718–2728 (2013)

Journal of Cellular Biochemistry

Exogenous Polyamines Promote Osteogenic Differentiation by Reciprocally Regulating Osteogenic and Adipogenic Gene Expression

Mon-Juan Lee,[1]* Yuhsin Chen,[1,2] Yuan-Pin Huang,[3] Yi-Chiang Hsu,[4] Lan-Hsin Chiang,[1] Tzu-Yu Chen,[1] and Gwo-Jaw Wang[5,6,7,8]

[1]*Department of Bioscience Technology, Chang Jung Christian University, Tainan, Taiwan*

[2]*Taichung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Taichung, Taiwan*

[3]*Department of Cosmetics and Fashion Styling, Cheng Shiu University, Kaohsiung, Taiwan*

[4]*Graduate Institute of Medical Sciences, Chang Jung Christian University, Tainan, Taiwan*

[5]*Orthopaedic Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan*

[6]*Department of Orthopaedics, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*

[7]*Department of Orthopaedics, National Cheng Kung University Hospital, Tainan, Taiwan*

[8]*Department of Orthopedic Surgery, University of Virginia, Charlottesville, Virginia*

ABSTRACT

Polyamines are naturally occurring organic polycations that are ubiquitous in all organisms, and are essential for cell proliferation and differentiation. Although polyamines are involved in various cellular processes, their roles in stem cell differentiation are relatively unexplored. In this study, we found that exogenous polyamines, putrescine, spermidine, and spermine, promoted osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) without inducing cell death or apoptosis. Alkaline phosphatase (ALP) activity and the mRNA level of osteogenic genes, including Runx2, ALP, osteopontin, and osteocalcin, were up-regulated by exogenous polyamines. When hBMSCs were cultured at high cell density favoring adipocyte formation, exogenous polyamines resulted in down-regulation of adipogenic genes such as PPARγ, aP2, and adipsin. Extracellular matrix mineralization, a marker for osteoblast maturation, was enhanced in the presence of exogenous polyamines, while lipid accumulation, an indication of adipogenic differentiation, was attenuated. Exogenous polyamines increased the mRNA expression of polyamine-modulated factor 1 (PMF-1) and its downstream effector, spermidine/spermine N^1-acetyltransferase (SSAT), while that of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, was suppressed. These results lead to possible connections between polyamine metabolism and osteogenic differentiation pathways. To summarize, this study provides evidence for the involvement of polyamines in osteogenic differentiation of hBMSCs, and is the first to demonstrate that osteogenic and adipogenic differentiation are reciprocally regulated by exogenous polyamines. J. Cell. Biochem. 114: 2718–2728, 2013.

KEY WORDS: POLYAMINES; OSTEOGENIC DIFFERENTIATION; ADIPOGENIC DIFFERENTIATION; MESENCHYMAL STEM CELLS

Development of new pharmaceuticals or therapeutic alternatives to stimulate bone formation is critical in the treatment of skeletal diseases. Currently, the only drug that stimulates bone formation is parathyroid hormone (PTH), which is approved by the U. S. Food and Drug Administration (FDA) to treat severe osteoporosis in both men and women [Neer et al., 2001]. However, it was shown in

The authors confirm that there is no conflict of interest.

Grant sponsor: National Science Council, Taiwan; Grant number: NSC 101-2314-B-309-001-MY3; Grant sponsor: Academic Research Funds of Chang Jung Christian University, Tainan, Taiwan; Grant sponsor: Taichung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Taichung, Taiwan.

*Correspondence to: Mon-Juan Lee, Department of Bioscience Technology, Chang Jung Christian University, No. 1, Changda Rd., Gueiren District, Tainan 71101, Taiwan. E-mail: mjlee@mail.cjcu.edu.tw

Manuscript Received: 22 November 2012; Manuscript Accepted: 17 June 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 24 June 2013

DOI 10.1002/jcb.24620 • © 2013 Wiley Periodicals, Inc.

animal studies that PTH exhibits the risk of inducing the formation of osteosarcoma [Kuijpers et al., 2002]. Although bisphosphonates, the most widely prescribed osteoporosis medications, are effective in suppressing the growth of osteoclasts, its bone-forming capability has not been proven clinically.

Polyamines are naturally occurring organic polycations derived from amino acids. These molecules of low molecular weight are ubiquitous in all organisms, and are involved in various biological processes such as proliferation, differentiation, and apoptosis [Lux et al., 1980; Heby, 1981; Pendeville et al., 2001; Pignatti et al., 2004]. They are associated with the growth and development of several tissue types, including embryonic development, and may participate in tissue repair by facilitating tissue remodeling [Babal et al., 2000; Lopez-Garcia et al., 2008]. Compared to PTH, the low molecular-weight polyamines have the advantage of lower cost of production, and are tightly regulated by their biosynthetic and catabolic pathways. In addition, the various polyamine analogs developed as anticancer drugs may provide the basis for screening polyamine-derived compounds with bone-stimulating activity [Wolff et al., 2003; Dredge et al., 2009].

Polyamines have been implicated in the growth and development of bone and cartilage [Matsui-Yuasa et al., 1985; Vittur et al., 1986]. Polyamines are abundant in the ossifying area of epiphyseal cartilage of calf scapulas, and alkaline phosphatase (ALP) activity, a marker for osteogenic differentiation, is enhanced in the presence of polyamines [Vittur et al., 1986]. In rabbit costal chondrocytes, the activity of the polyamine catabolic enzyme, spermidine/spermine N^1-acetyltransferase (SSAT) is found to be regulated by PTH [Matsui-Yuasa et al., 1985]. The polyamine spermine has been shown to promote osteogenic differentiation of goat adipose tissue-derived mesenchymal stem cells (ADSCs) [Tjabringa et al., 2006, 2008]. Up-regulation of Runx2 and osteopontin, markers for early- and late-stage osteogenic differentiation, respectively, as well as ALP activity, is observed in goat ADSCs after a short-term stimulation with spermine [Tjabringa et al., 2008]. In addition, spermine is found to inhibit both nitric oxide (NO) production and COX2 gene expression, both of which are involved in the mechanical adaptation of bone during mechanical loading [Tjabringa et al., 2006]. Nevertheless, the role of polyamines and their metabolic pathways in relation to osteogenesis remains poorly understood. Besides, for the purpose of drug development, the long-term effect of a bone-stimulating agent and its efficacy in promoting terminal differentiation of stem cells have to be considered.

In this study, we hypothesize that due to functional and structural similarities, putrescine (PUT) and spermidine (SPD), in addition to spermine (SPM), may also be involved in bone formation. Physiologically, polyamines compensate for each other's loss to maintain their homeostasis. Inhibition of spermidine synthase results in an increase in spermine to compensate for the reduced spermidine level in rat tissue [Shirahata et al., 1993]. We therefore assume that exogenous putrescine, spermidine, and spermine may function in stem cell differentiation by activating similar signal transduction pathways. This report aims at determining the efficacy and specificity of polyamines in promoting bone formation. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were induced to differentiate in the presence of putrescine, spermidine, or spermine. The effect of polyamines on the terminal differentiation and gene expression of hBMSCs was evaluated, and the correlation of polyamine signal transduction to osteogenic differentiation pathways was discussed.

METHODS

CELL CULTURE

Human BMSCs ($CD14^-$, $CD45^-$, $CD29^+$, $CD44^+$, $CD90^+$, and $CD105^+$) isolated from red marrow of a single normal donor were purchased from Cellular Engineering Technologies (CET), Inc. (Coralville, IA). The cells were cultured in CET Human Mesenchymal Stem Cell Expansion Media (CET, Inc.) supplemented with Invitrogen MSC-Qualified Fetal Bovine Serum (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Human BMSCs between passages 3 and 5 were used in this study. Cells were seeded at 10^4 cells/well in 24-well plates for cell viability assay, alizarin red S staining, oil red O staining and ALP activity assay, at 5×10^4 cells/well in 6-well plates for total RNA extraction, and at 8×10^4 cells/well in 10-cm dishes for cell cycle and apoptosis analysis. For osteogenic differentiation-related studies, hBMSCs were treated 2 days after seeding. For adipogenic differentiation-related analysis, cells were cultured for another 6 days after seeding to reach 100% confluence before treatment. Under the treatment of exogenous polyamines, cell culture, and differentiation media were supplemented with 1 mM aminoguanidine to inhibit bovine serum amine oxidase.

CELL VIABILITY ASSAY

Human BMSCs were treated with osteogenic induction medium (OIM), which consists of DMEM supplemented with 10^{-7} M dexamethasone, 10 mM β-glycerolphosphate, 50 μM L-ascorbate 2-phosphate, and 1 mM aminoguanidine, or with various concentrations of polyamines in OIM for 2 or 14 days. Cell viability was analyzed by WST-1 assay using Quick Cell Proliferation Colorimetric Assay Kit (BioVision, Milpitas, CA) and quantitated spectrophotometrically by the optical density at 450 nm, with a reference wavelength of 650 nm.

CELL CYCLE ANALYSIS

Human BMSCs were treated with OIM or with exogenous polyamines in OIM for 7 days. Cells were harvested with 0.05% trypsin–EDTA (Life Technologies) and resuspended in cold PBS, followed by incubation in ice-cold 70% ethanol on ice for at least 8 h. Cells were then pelleted and resuspended in 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase in PBS. After 30 min of incubation in the dark, the cells were analyzed by a FACSCalibur flow cytometer using the CellQuest software program (Becton Dickson, San Jose, CA). At least 10^4 cells were analyzed for each sample. The percentages of cells in sub-G1, G0/G1, S, and G2/M phases were calculated using the WinMDI 2.8 program [Trotter, 2000].

APOPTOSIS ANALYSIS

Human BMSCs were treated with OIM or with exogenous polyamines in OIM for 7 days. Cells were harvested with 0.05% trypsin–EDTA

(Life Technologies), washed twice with cold PBS, and resuspended in 1× Annexin V Binding Buffer (BD Pharmingen, Becton Dickson). A 100 μl cell suspension was mixed with 100 μg/ml of PI and 5 μl of FITC Annexin V (BD Pharmingen, Becton Dickson), and was incubated in the dark for 15 min at room temperature. The suspension was then mixed with another 400 μl of 1× Annexin V Binding Buffer, and analyzed by a FACSCalibur flow cytometer.

OSTEOGENIC DIFFERENTIATION AND ALIZARIN RED S STAINING

Osteogenic differentiation assay was performed as previously described [Chen et al., 2012]. Human BMSCs were treated with various concentrations of polyamines prepared in OIM for 14 days. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS at room temperature for 15 min, followed by washing with PBS and ddH_2O. The level of extracellular matrix calcification was determined by staining hBMSCs with 0.2% alizarin red S (Sigma-Aldrich) in ddH_2O (pH 6.4) for 20 min at room temperature. After washing three times with ddH_2O, the 24-well plate was air-dried at room temperature. To quantify the level of matrix mineralization, cell-bound alizarin red S was dissolved in 10% cetylpyridinium chloride and shaken at room temperature for 10 min. The concentration of alizarin red S in each sample was determined by measuring optical density at 570 nm and interpolating on a standard curve produced by various concentrations of alizarin red S standards. The level of matrix mineralization was determined by normalizing alizarin red S concentration (μM) to the total amount of DNA (μg), which was determined by Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) according to the manufacturer's instructions.

ALKALINE PHOSPHATASE ACTIVITY ASSAY

The ALP activity of hBMSCs was determined by staining the cells with Pierce 1-Step NBT/BCIP Solution (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Human BMSCs were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS and ddH_2O, and incubated with the NBT/BCIP solution for another 15 min. After washing three times with ddH_2O, the 24-well plate was air-dried at room temperature. The resulting black-purple stain was solubilized by dimethyl sulfoxide (DMSO). After centrifugation at 12,000 rpm for 10 min to remove insoluble precipitates, the level of ALP activity was determined by the optical density of solubilized ALP stain at 570 nm.

ADIPOGENIC DIFFERENTIATION AND OIL RED O STAINING

Human BMSCs were induced to differentiate adipogenically using the Chemicon Mesenchymal Stem Cell Adipogenesis kit (Millipore, Billerica, MA). Cells were maintained in DMEM until reaching confluence, followed by treatment with PUT, SPD, or SPM prepared in adipogenic induction medium (AIM), which consists of DMEM supplemented with 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), 10 μg insulin/ml, 100 μM indomethacin, and 1 mM aminoguanidine. Cells were cultured alternately in polyamine-containing AIM or adipogenesis maintenance medium (DMEM containing 10 μg/ml insulin and 1 mM aminoguanidine) for another 3 weeks according to the manufacturer's instructions. The amount of lipid droplets accumulated in hBMSCs was determined by oil red O staining. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, followed by washing with PBS and ddH_2O. Oil red O staining solution (0.36% in 60% isopropanol) was then added and reacted with hBMSCs for 50 min at room temperature. After washing three times using ddH_2O, the accumulated lipid droplets were observed with phase-contrast microscopy. Cell-bound oil red O was extracted with 500 μl isopropanol per well and its intensity was determined by the optical density at 540 nm.

REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from hBMSCs by the Trizol® Reagent (Life Technologies), and reverse transcription was carried out using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. The cDNA was diluted to a final concentration of ~1 ng/μl and reacted with gene-specific primer pairs and Applied Biosystems SYBR® Green PCR Master Mix (Life Technologies) according to the manufacturer's protocol. The primer sequences for Runx2 (NM_004348), liver/bone/kidney-type ALP (NM_000478), osteopontin (NM_000582), osteocalcin (NM_199173), PPARγ (NM_015869), aP2 (NM_001442), adipsin (NM_001928), ODC (NM_002539), PMF-1 (NM_007221), and SSAT (NM_002970) were designed by Origene (Rockville, MD). Primer specificity was confirmed by Primer-BLAST developed at NCBI, and primer PCR efficiency was validated to be close to 100%. Genes of interest were detected and amplified by Applied Biosystems 7300 Real-Time PCR System (Life Technologies) with the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min, followed by melting curve analysis. Amplicons were visualized by electrophoresis on a 1.4% agarose gel to ensure the presence of a single product. The mRNA level of each gene was analyzed by the Applied Biosystems Sequence Detection Software V1.2 (Life Technologies) and normalized to that of GAPDH. Relative gene expression was calculated by the comparative Ct ($2^{-\Delta\Delta ct}$) method [Livak and Schmittgen, 2001] and expressed as fold changes (x-fold) relative to the control.

STATISTICAL ANALYSIS

Statistical analysis was performed on data from at least three independent experiments using hBMSCs from the same donor. Significant difference relative to the control was tested using one-way ANOVA followed by post hoc tests. Levels of significance of $P < 0.05$ and 0.01 were accepted as significant and highly significant, respectively.

RESULTS

EFFECT OF EXOGENOUS POLYAMINES ON CELL VIABILITY

Cell viability assay (WST-1 assay) was performed to determine the maximum tolerated concentration of exogenous polyamines for hBMSCs. When treated with various concentrations of polyamines in the presence of OIM for 2 days, the viability of hBMSCs was slightly increased at 100 μM PUT and 10 μM SPD. However, the number of viable cells was significantly decreased at 100 μM SPD and 100 μM SPM (Fig. 1A). Prolonged incubation of hBMSCs with polyamines resulted in significant decrease in cell viability compared to the control at all polyamine concentrations tested, as well as in the

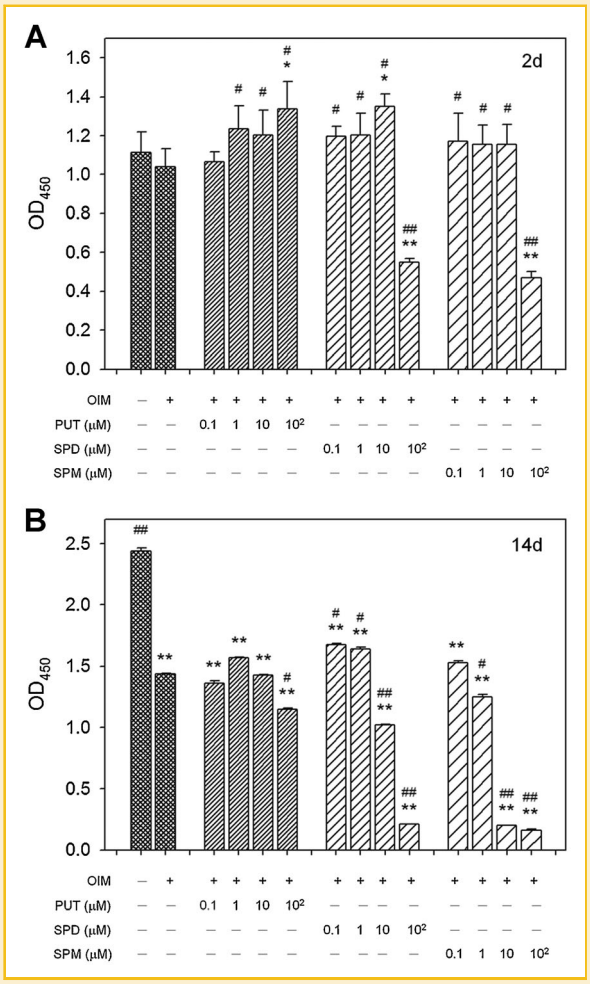

Fig. 1. Effect of exogenous polyamines on the viability of hBMSCs. Human BMSCs were treated with osteogenic induction medium (OIM) or with various concentrations of putrescine (PUT), spermidine (SPD), or spermine (SPM) in OIM for (A) 2 or (B) 14 days. Cell viability was determined by WST-1 assay and expressed as optical density at 450 nm (OD_{450}). Error bars represent standard deviations ($n \geq 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the control (DMEM); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to OIM.

presence of OIM (Fig. 1B). Less than 50% of hBMSCs were viable after incubating with 10–100 μM SPD or SPM for 14 days (Fig. 1B).

EFFECT OF EXOGENOUS POLYAMINES ON CELL CYCLE AND APOPTOSIS

Human BMSCs were treated with 1 μM PUT, 1 μM SPD, or 1 μM SPM in OIM for 7 days, and subjected to cell cycle analysis to examine whether the reduced cell viability observed in Figure 1B was correlated with cell death. As shown in Figure 2A, the cell cycle profile of hBMSCs revealed that a large subset of cells resided in the growth arrest state (G0/G1 phase) and G2/M phase. There were no dead (sub-G1) cells in untreated hBMSCs or those treated with OIM or exogenous polyamines, indicating that the decrease in cell number caused by OIM or exogenous polyamines (Fig. 1B) was not attributed to cell death. Further detection of apoptotic cells with annexin V also indicated that OIM or exogenous polyamines did not induce apoptosis at the concentrations tested (Fig. 2B).

EFFECT OF EXOGENOUS POLYAMINES ON MATRIX MINERALIZATION

The effect of polyamines on matrix mineralization, one of the markers for osteoblast maturation, was determined by the amount of calcium deposition, which can be detected by alizarin red S through the formation of the bright-red calcium–alizarin red S complex. Calcium deposition in hBMSCs usually requires 3 weeks of culture in OIM to reach an abundant level. In the absence of OIM, exogenous polyamines were unable to induce matrix mineralization within 3 weeks (data not shown). Therefore, hBMSCs were treated with exogenous polyamines in the presence of OIM so that their effect on terminal differentiation could be seen within the limited life span of hBMSCs in vitro. As expected, after being cultured for 14 days in OIM, calcium–alizarin red S complex was barely observed in hBMSCs (Fig. 3A). However, when hBMSCs were treated with OIM supplemented with various concentrations of polyamines, matrix mineralization was drastically enhanced, and was most significant at the highest tolerated concentrations of exogenous polyamines (Fig. 3A). When hBMSCs were incubated in OIM in the presence of 100 μM PUT, 1 μM SPD, or 1 μM SPM, the amount of cell-bound alizarin red S (μM alizarin red S/μg DNA) was increased to 2.27-, 1.91-, or 1.96-fold, respectively, of those cultured in OIM (Fig. 3B). The enhancement in matrix mineralization suggests that exogenous PUT, SPD, and SPM are capable of accelerating the osteogenic differentiation of hBMSCs.

EFFECT OF EXOGENOUS POLYAMINES ON OSTEOGENIC GENE EXPRESSION

We next studied the effect of exogenous polyamines on the mRNA level of several osteogenic genes, including Runx2 and ALP, which belong to the early-onset genes responsible for osteogenic differentiation, as well as osteopontin and osteocalcin, which are late-onset genes related to extracellular matrix mineralization. The mRNA level of Runx2 and ALP was significantly elevated when hBMSCs were cultured in the presence of OIM for 7 days, and exogenous polyamines further enhanced the expression of both genes (Fig. 4A,B). Up-regulation of osteopontin and osteocalcin by OIM was less significant compared to Runx2 and ALP, but in the presence of exogenous polyamines, the mRNA level of osteopontin and osteocalcin was significantly higher than that of OIM (Fig. 4C,D). These results suggest that exogenous polyamines may direct hBMSCs toward the osteoblast lineage by up-regulating both early- and late-onset genes related to osteogenesis.

EFFECT OF EXOGENOUS POLYAMINES ON ALKALINE PHOSPHATASE ACTIVITY

Up-regulation of ALP activity is an indication of early-stage osteogenesis. Treatment of hBMSCs for 7 and 14 days with 1–100 μM PUT, 0.01–1 μM SPD, or 0.01–1 μM SPM prepared in OIM resulted in significant increase in ALP activity compared to OIM alone (Fig. 5). Optical density of ALP stain in the presence of 100 μM PUT, 1 μM SPD, or 1 μM SPM was 1.47-, 1.79-, or 1.77-fold, respectively, that of OIM at day 7 (Fig. 5B), consistent with the up-regulation of ALP mRNA expression by exogenous polyamines (Fig. 4B).

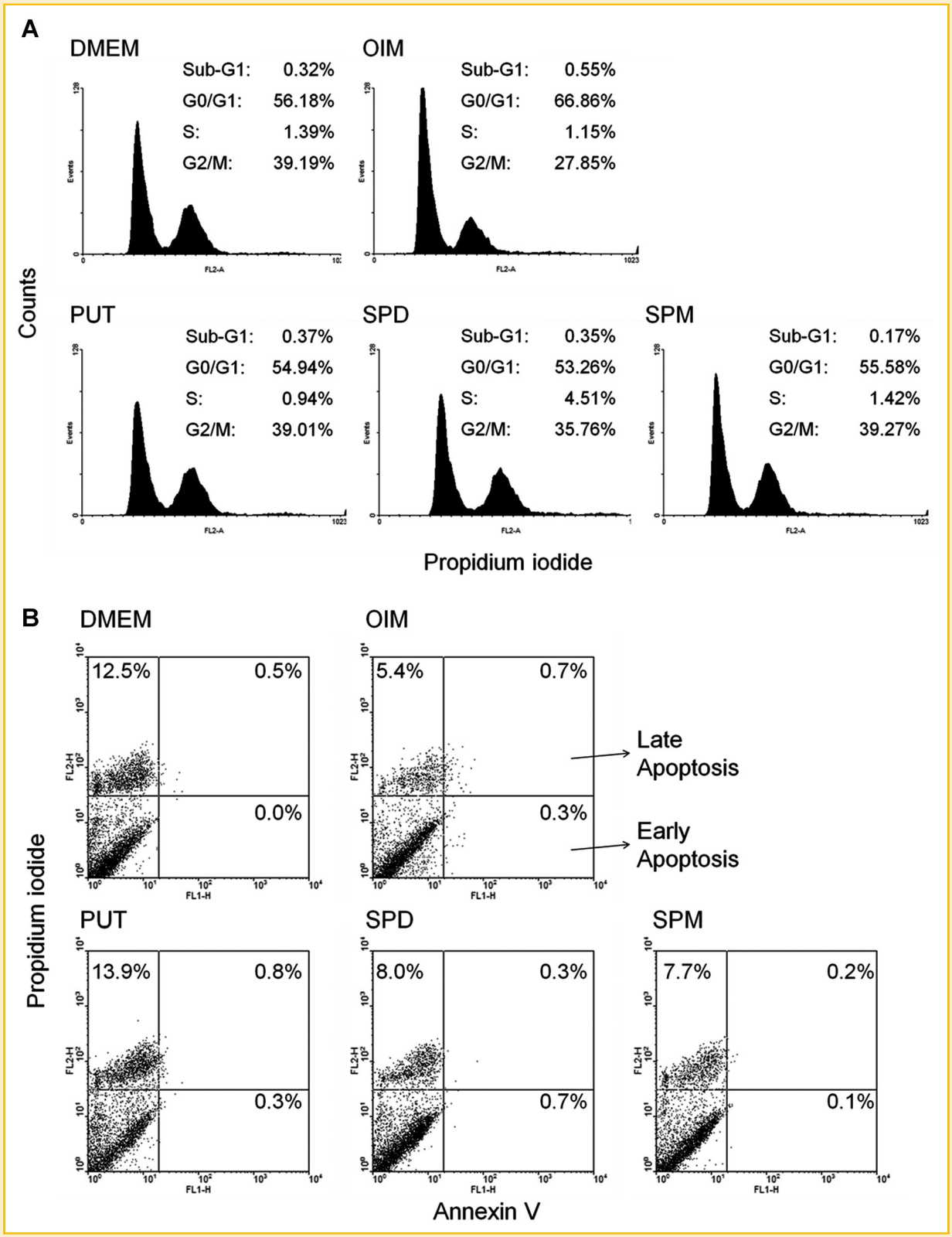

Fig. 2. Effect of exogenous polyamines on cell cycle and apoptosis of hBMSCs. Human BMSCs were treated with osteogenic induction medium (OIM), 1 μM putrescine (PUT), 1 μM spermidine (SPD), or 1 μM spermine (SPM) in OIM for 7 days and then subjected to (A) cell cycle analysis, in which the number of cells in sub-G1, G0/G1, S, and G2/M phases was estimated by propidium iodide (PI) staining and calculated using the WinMDI 2.8 program [Trotter, 2000], or (B) staining with FITC annexin V and PI to determine the fraction of apoptotic cells.

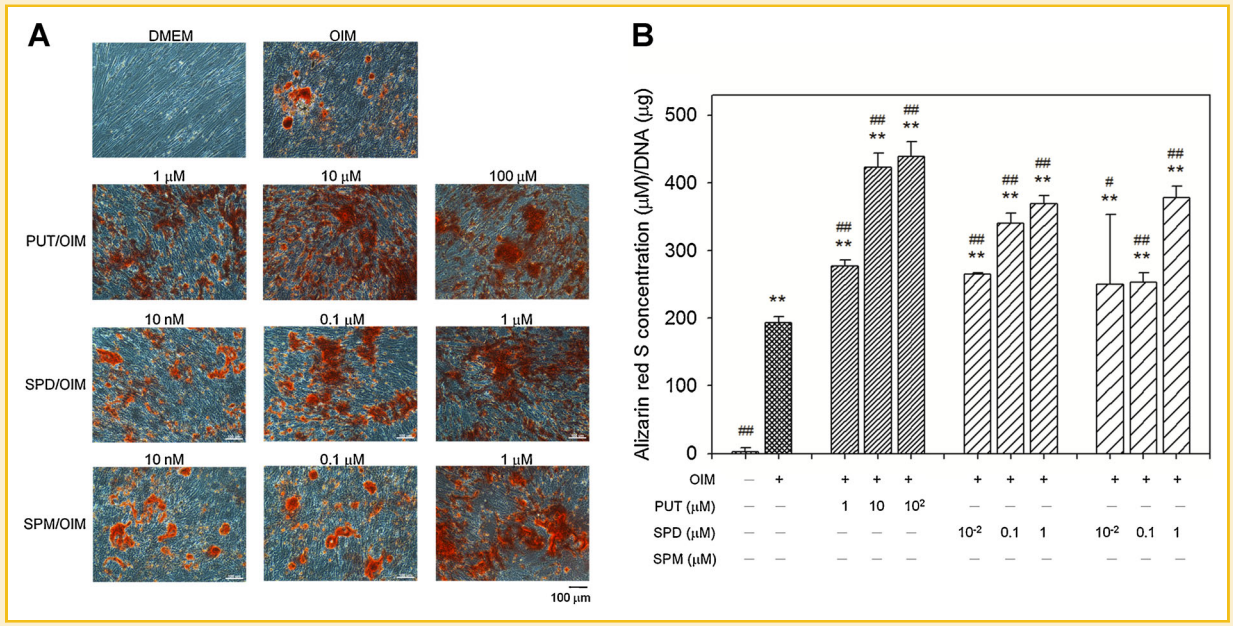

Fig. 3. Effect of exogenous polyamines on matrix mineralization of hBMSCs. Human BMSCs were treated with various concentrations of putrescine (PUT), spermidine (SPD), or spermine (SPM) in combination with osteogenic induction medium (OIM) for 14 days. A: The cells were stained with alizarin red S and observed with phase-contrast microscopy. B: The concentration of cell-bound alizarin red S was quantitated spectrophotometrically at OD_{570} and normalized to the amount of DNA (μg). Error bars represent standard deviations ($n \geq 3$). $^{**}P < 0.01$, compared to the control (DMEM); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to OIM.

EFFECT OF EXOGENOUS POLYAMINES ON ADIPOGENIC GENE EXPRESSION

Because there exists a reciprocal relationship between the osteogenic and adipogenic differentiation pathways [Gimble and Nuttall, 2012; Huang et al., 2012], whether exogenous polyamines suppress adipogenesis to promote osteogenic differentiation of hBMSCs was examined. Human BMSCs were cultured at high cell densities that favor adipogenesis, and treated with 1 μM PUT, 1 μM SPD, or 1 μM SPM in the presence of adipogenesis-inducing reagents for 7 days to examine the effect of individual polyamines on the expression of adipogenic genes. When cultured in AIM, a significant increase in the mRNA levels of PPARγ, aP2, and adipsin was observed in hBMSCs compared to the control (Fig. 6). Addition of exogenous polyamines drastically reduced the expression of PPARγ, aP2, and adipsin under adipogenic conditions, suggesting that exogenous polyamines were capable of suppressing adipogenic gene expression at the transcriptional level (Fig. 6).

EFFECT OF EXOGENOUS POLYAMINES ON LIPID ACCUMULATION

Human BMSCs were cultured at high cell densities that favor adipogenesis, and treated with various concentrations of polyamines under adipogenic induction conditions for 21 days. Lipid accumulation, an indication of adipocyte formation, was then quantified by oil red O staining. It was found that the amount of lipid droplets formed in hBMSCs after adipogenic induction for 21 days was 6.7-fold that of the control. Treatment with 1–100 μM PUT, 0.01–1 μM SPD, or 0.01–1 μM SPM in the presence of adipogenesis-inducing reagents attenuated lipid accumulation (Fig. 7). The amount of lipid droplets reduced to 0.66-, 0.65-, or 0.54-fold of AIM in the presence of 100 μM PUT, 1 μM SPD, or 1 μM SPM, respectively (Fig. 7B). It is therefore suggested that exogenous polyamines were effective in suppressing adipogenic terminal differentiation while promoting osteogenic differentiation.

EFFECT OF EXOGENOUS POLYAMINES ON GENES RELATED TO POLYAMINE METABOLISM

Ornithine decarboxylase (ODC) is the enzyme responsible for the rate-limiting step of polyamine biosynthesis [Yuan et al., 2001; Palanimurugan et al., 2004; Kurian et al., 2011; Ramot et al., 2011]. Besides, polyamines activate the transcription of the polyamine catabolic enzyme, SSAT, through polyamine-modulated factor 1 (PMF-1), a transcriptional cofactor regulated by polyamines [Wang et al., 1999]. The effect of exogenous polyamines on the mRNA level of ODC, PMF-1, and SSAT was therefore examined under osteogenesis-inducing conditions in order to correlate polyamine metabolic pathways with osteogenic differentiation. The mRNA expression of ODC and PMF-1 were unaffected compared to the control when hBMSCs were cultured in OIM (Fig. 8A,B). However, supplementing OIM with exogenous polyamines resulted in down-regulation of ODC, and up-regulation of PMF-1. On the other hand, the mRNA level of SSAT was increased in the presence of OIM, and exogenous polyamines further enhanced its expression (Fig. 8C). These results indicate that intracellular polyamine metabolism may be regulated by exogenous polyamines to stimulate osteogenic differentiation of hBMSCs.

DISCUSSION

This study demonstrated that exogenous polyamines reduced the viability of hBMSCs during osteogenic differentiation (Fig. 1), but did

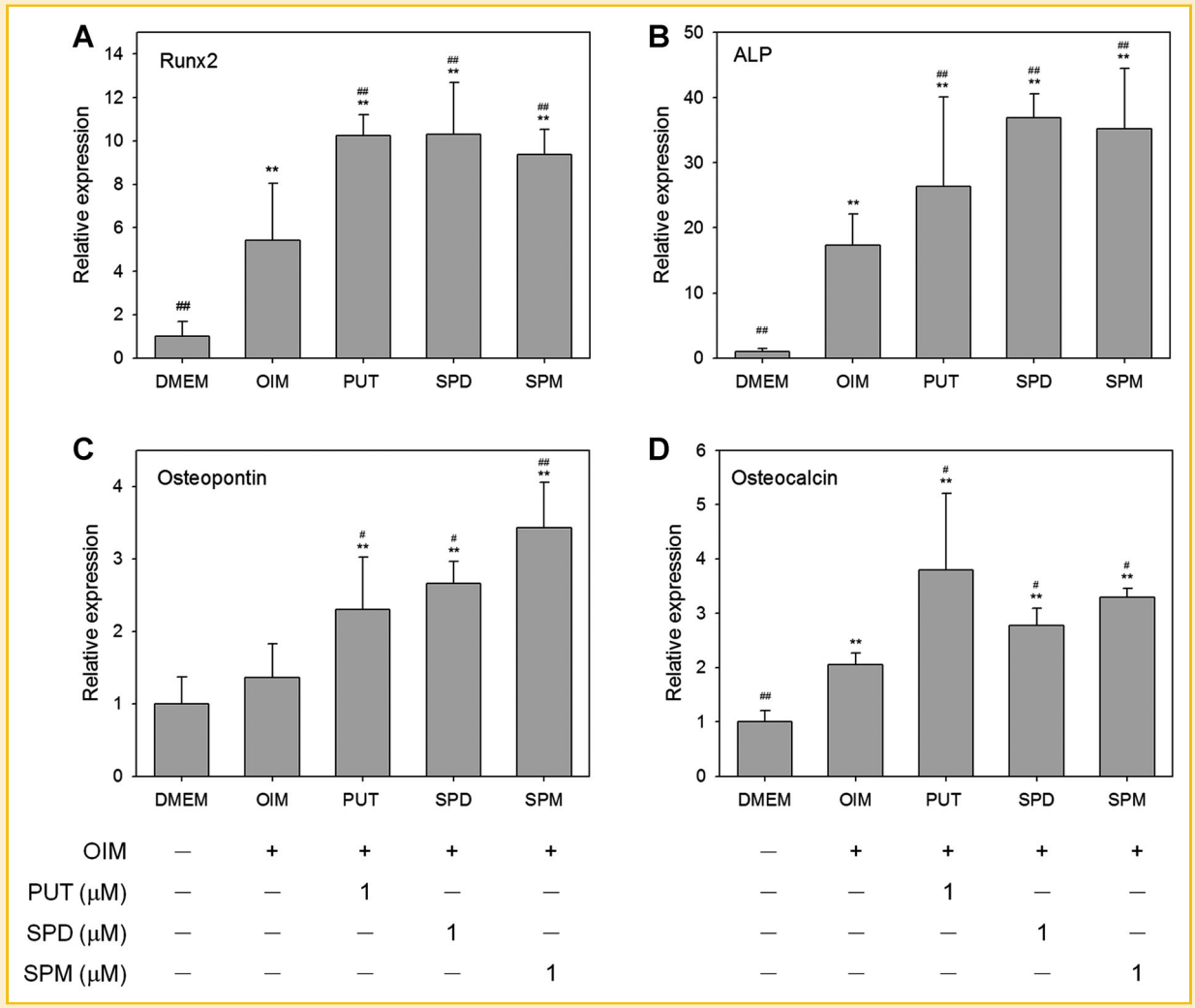

Fig. 4. Effect of exogenous polyamines on the osteogenic gene expression of hBMSCs. Human BMSCs were treated with osteogenic induction medium (OIM), 1 μM putrescine (PUT), 1 μM spermidine (SPD), or 1 μM spermine (SPM) in OIM for 7 days. Relative gene expression of (A) Runx2, (B) ALP, (C) osteopontin, and (D) osteocalcin was determined by real-time PCR. Error bars represent standard deviations ($n \geq 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the control (DMEM); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to OIM.

not result in cell death or apoptosis (Fig. 2). Exogenous polyamines accelerated matrix mineralization of hBMSCs (Fig. 3) by up-regulating osteogenic genes such as Runx2, ALP, osteopontin and osteocalcin (Fig. 4), and ALP activity (Fig. 5). In the meantime, exogenous polyamines suppressed adipogenic gene expression and lipid accumulation (Figs. 6 and 7), indicating that exogenous PUT, SPD, and SPM reciprocally regulate osteogenic and adipogenic differentiation pathways to direct hBMSCs toward osteoblast maturation.

In addition to promoting osteogenic differentiation as discussed in this study and by Tjabringa et al. [2008], SPM (0.5–1 mM) is found to induce the generation of contractile muscle fibers in mouse embryonic stem (ES) cells [Sasaki et al., 2008], while SPD (0.1–1 μM) is reported to promote hair growth from hair follicle epithelial stem cells [Ramot et al., 2011]. Although our data demonstrated that adipogenic gene expression in hBMSCs is suppressed by exogenous polyamines (Fig. 6), other researchers suggest that SPD is indispensable for adipocyte formation in 3T3-L1 fibroblasts, and polyamine metabolism is involved in adipogenesis [Vuohelainen et al., 2010; Ishii et al., 2012]. Moreover, there is also evidence pointing to the involvement of SPD and SPM (1–10 μM) in the terminal differentiation of osteoarthritic chondrocytes [Facchini et al., 2012]. Consequently, the function of exogenous polyamines in cell differentiation is diverse and depends on the various stem cell and committed cell types.

Studies on polyamine transport have shown that polyamines are not uniformly distributed in mammalian cells, and endogenous and exogenous polyamines are sequestered in different cellular compartments, indicating that exogenous polyamines may exert their effect through pathways distinct from their endogenous counterparts [Poulin et al., 2006]. It is therefore of particular interest to elucidate the mechanism underlying the osteogenic potential of exogenous polyamines. Because ODC activation is an indication of cell proliferation in many cell types, including mesenchymal stem cells

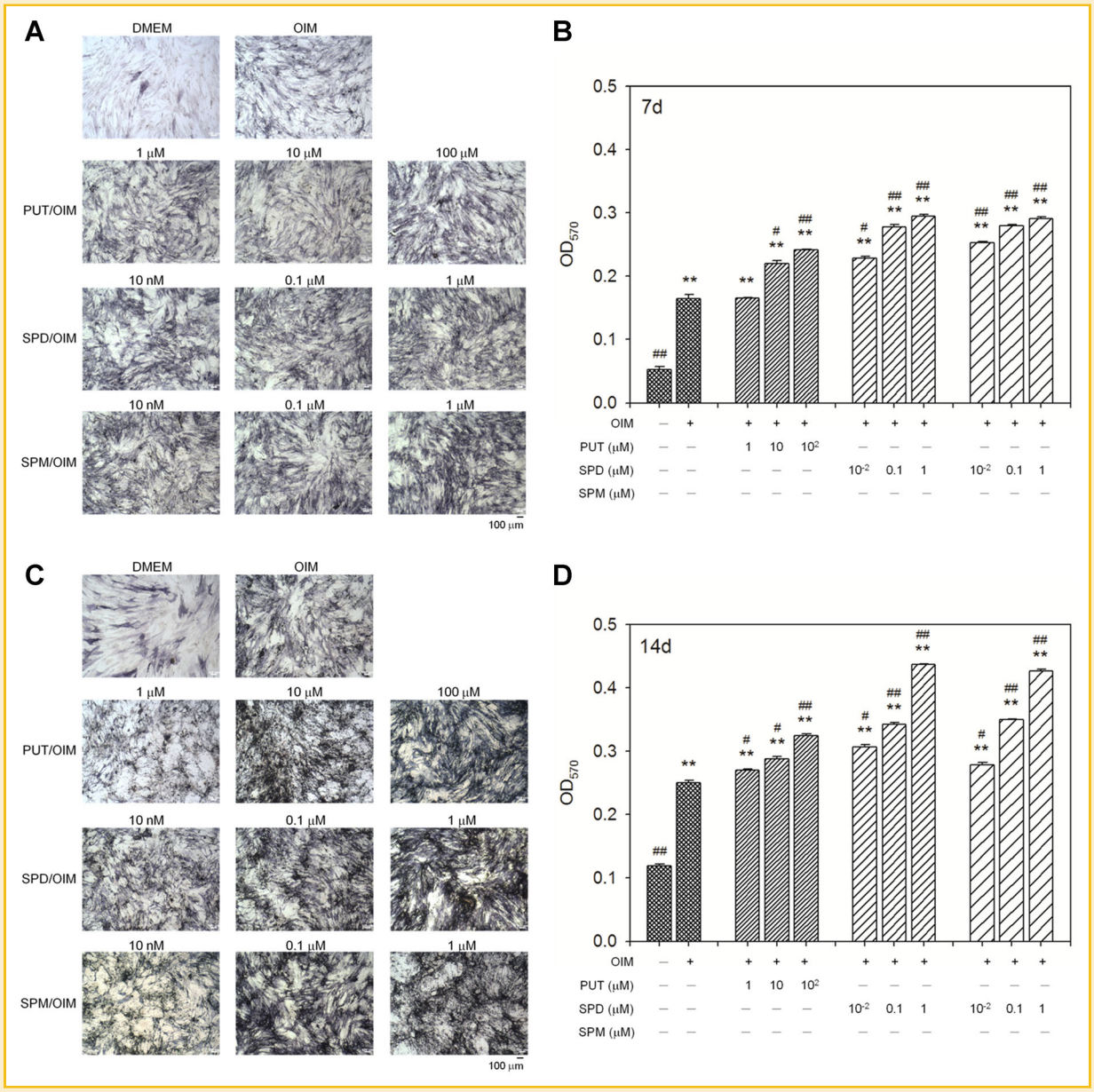

Fig. 5. Effect of exogenous polyamines on the alkaline phosphatase (ALP) activity of hBMSCs. Human BMSCs were treated with exogenous polyamines in OIM for (A,B) 7 or (C,D) 14 days, followed by cell staining with Pierce 1-Step NBT/BCIP Solution (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were observed with phase-contrast microscopy, and the level of alkaline phosphatase activity was determined by the optical density of solubilized ALP stain at 570 nm. Error bars represent standard deviations ($n \geq 3$). $^{**}P < 0.01$, compared to the control (DMEM); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to OIM.

[Nemoto et al., 2002; Curran et al., 2005; Lee et al., 2011], it is reasoned that the reduced cell viability in the presence of exogenous polyamines (Fig. 1) may result from suppression of the expression of ODC mRNA (Fig. 8A). In addition, a possible connection between ODC suppression (Fig. 8A) and osteogenic differentiation may be attributed to up-regulation of ALP (Fig. 4B), because polyamines regulate Sp1, the major positive regulator of ODC transcription that is in turn antagonized by Sp3, the transcription factor that activates ALP [Kumar and Butler, 1997; Yusa et al., 2000; Huang et al., 2006]. Suppression of ODC by exogenous polyamines may therefore be associated with growth inhibition and activation of ALP. On the other hand, up-regulation of PMF-1, and hence SSAT may be necessary to maintain intracellular polyamine homeostasis in the presence of exogenous polyamines (Fig. 8B,C). Interestingly, SSAT expression can be induced by OIM alone without an increase in the mRNA level of PMF-1 (Fig. 8B,C), suggesting that other factors in the osteogenic differentiation pathway may regulate SSAT, and that polyamine catabolism may be associated with osteogenesis.

Because polyamines and their metabolic pathways are ubiquitous in almost all cell types, it may be challenging to target exogenous polyamines directly to specific cells or tissues. In addition, exogenous polyamines may be catabolized by polyamine oxidase or SSAT to

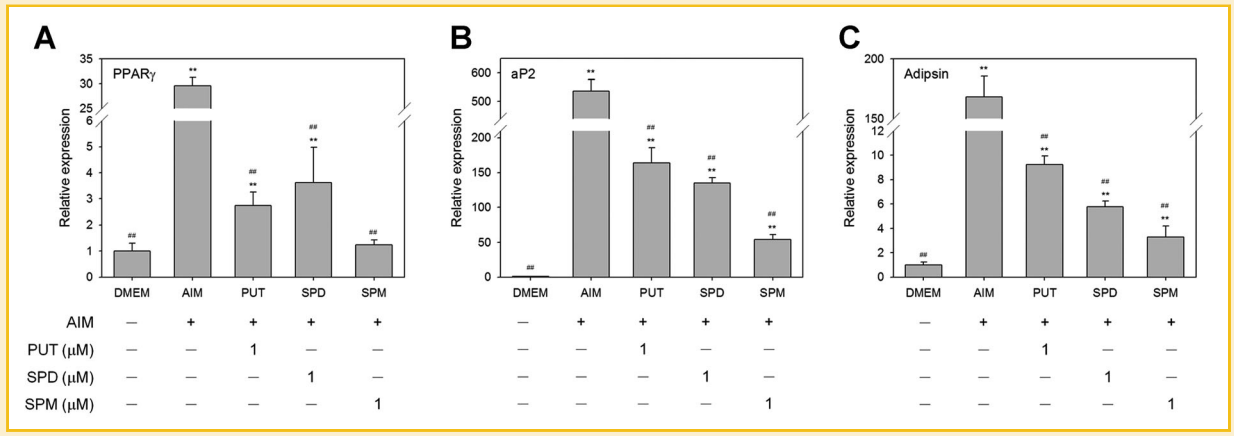

Fig. 6. Effect of exogenous polyamines on the adipogenic gene expression of hBMSCs. Human BMSCs were cultured at high cell densities that favor adipogenesis, and treated with adipogenic induction medium (AIM), 1 μM putrescine (PUT), 1 μM spermidine (SPD), or 1 μM spermine (SPM) in AIM for 7 days. Relative gene expression of (A) PPARγ, (B) aP2, and (C) adipsin was determined by real-time PCR. Error bars represent standard deviations ($n \geq 3$). $^{**}P < 0.01$, compared to the control (DMEM); $^{\#\#}P < 0.01$, compared to AIM.

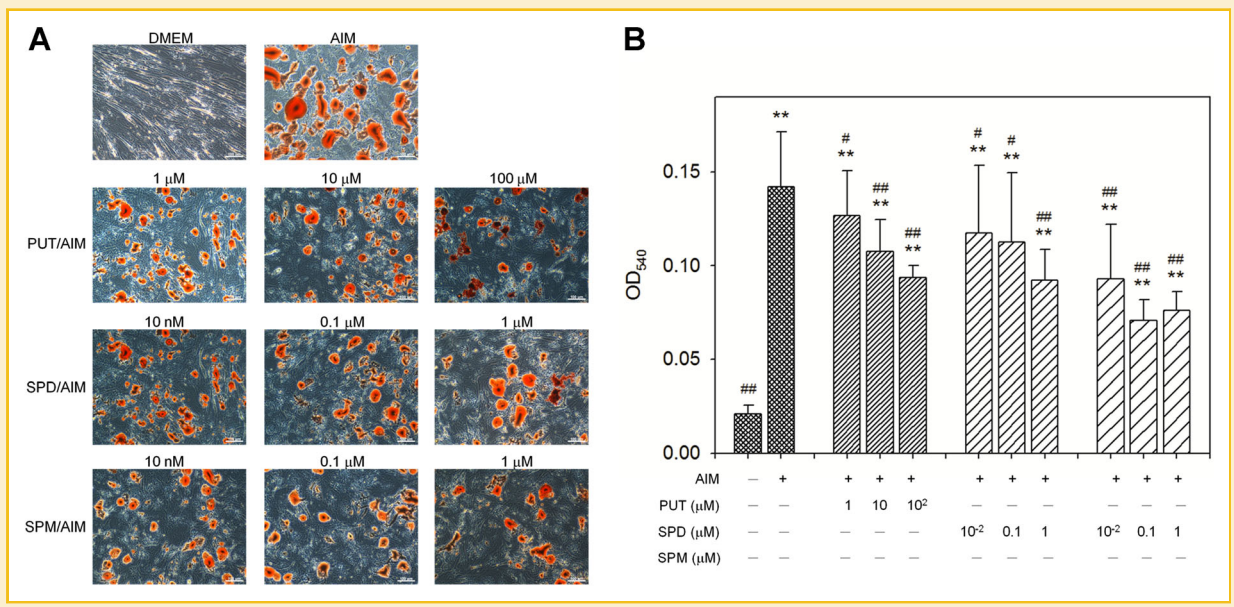

Fig. 7. Effect of exogenous polyamines on lipid accumulation of hBMSCs. Human BMSCs were cultured at high cell densities that favor adipogenesis, and treated with various concentrations of putrescine (PUT), spermidine (SPD), or spermine (SPM) under adipogenic conditions for 21 days. A: Cells were stained with oil red O and observed with phase-contrast microscopy. B: Cell-bound oil red O was extracted with isopropanol and its intensity was determined colorimetrically at OD_{540}. Error bars represent standard deviations ($n \geq 3$). $^{**}P < 0.01$, compared to the control (DMEM); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to AIM.

regulate intracellular polyamine levels, rendering it difficult to accumulate enough concentration of exogenous polyamines to induce osteogenic differentiation under physiological conditions. Nevertheless, the correlation between polyamine metabolism and the differentiation fate of hBMSCs established in this study provides a new direction for the development of novel bone-stimulating drugs, which may exert their osteogenic activity by interfering with intracellular polyamine homeostasis.

In conclusion, we have proved in this study that exogenous PUT, SPD, and SPM were capable of inducing osteogenic differentiation in hBMSCs by reciprocally regulating osteogenic and adipogenic gene expression and terminal differentiation. The osteogenic activity of exogenous polyamines may be associated with suppression of ODC and activation of ALP, resulting in the onset of osteogenic differentiation. Further investigations are necessary to elucidate the crosstalk between pathways of polyamine metabolism and osteogenesis. Studies on polyamines as novel osteogenic inducers not only help to explore the mechanism underlying their osteogenic activity, but also provide the basis for the development of polyamine-derived new drugs that stimulate bone formation.

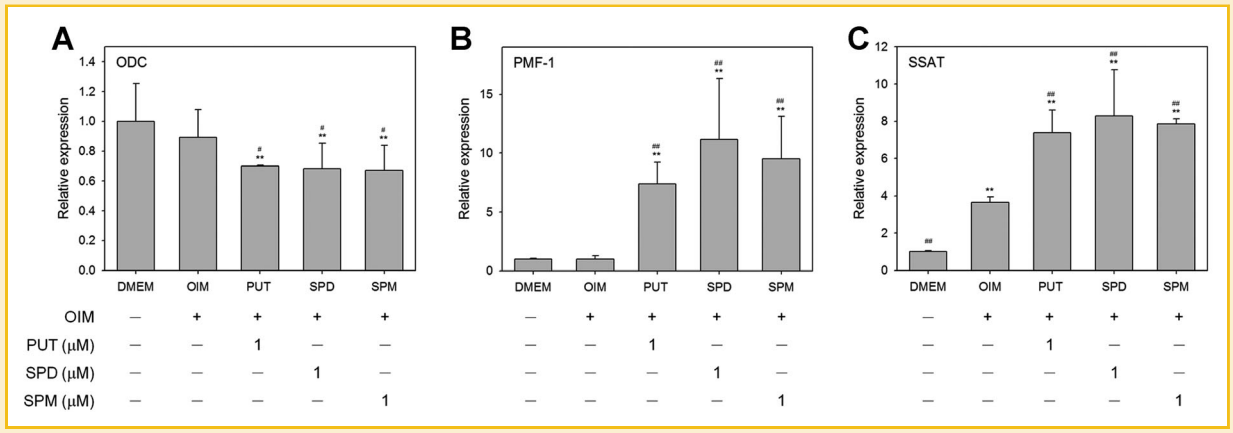

Fig. 8. Effect of exogenous polyamines on the expression of polyamine-regulated genes in hBMSCs. Human BMSCs were treated with osteogenic induction medium (OIM), 1 μM putrescine (PUT), 1 μM spermidine (SPD), or 1 μM spermine (SPM) in OIM for 7 days. Relative gene expression of (A) ODC, (B) PMF-1, and (C) SSAT was determined by real-time PCR. Error bars represent standard deviations ($n \geq 3$). $^{**}P < 0.01$, compared to the control (DMEM); $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to OIM.

ACKNOWLEDGMENTS

This study is supported by the National Science Council, Taiwan (NSC101-2314-B-309-001-MY3), the Academic Research Funds of Chang Jung Christian University, Tainan, Taiwan, and Taichung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan, Taichung, Taiwan.

REFERENCES

Babal P, Manuel SM, Olson JW, Gillespie MN. 2000. Cellular disposition of transported polyamines in hypoxic rat lung and pulmonary arteries. Am J Physiol Lung Cell Mol Physiol 278:L610–L617.

Chen HT, Lee MJ, Chen CH, Chuang SC, Chang LF, Ho ML, Hung SH, Fu YC, Wang YH, Wang HI, Wang GJ, Kang L, Chang JK. 2012. Proliferation and differentiation potential of human adipose-derived mesenchymal stem cells isolated from elderly patients with osteoporotic fractures. J Cell Mol Med 16:582–593.

Curran JM, Chen R, Hunt JA. 2005. Controlling the phenotype and function of mesenchymal stem cells in vitro by adhesion to silane-modified clean glass surfaces. Biomaterials 26:7057–7067.

Dredge K, Kink JA, Johnson RM, Bytheway I, Marton LJ. 2009. The polyamine analog PG11047 potentiates the antitumor activity of cisplatin and bevacizumab in preclinical models of lung and prostate cancer. Cancer Chemother Pharmacol 65:191–195.

Facchini A, Borzi RM, Olivotto E, Platano D, Pagani S, Cetrullo S, Flamigni F. 2012. Role of polyamines in hypertrophy and terminal differentiation of osteoarthritic chondrocytes. Amino Acids 42:667–678.

Gimble JM, Nuttall ME. 2012. The relationship between adipose tissue and bone metabolism. Clin Biochem 45:874–879.

Heby O. 1981. Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19:1–20.

Huang Y, Keen JC, Pledgie A, Marton LJ, Zhu T, Sukumar S, Park BH, Blair B, Brenner K, Casero RA, Jr., Davidson NE. 2006. Polyamine analogues down-regulate estrogen receptor alpha expression in human breast cancer cells. J Biol Chem 281:19055–19063.

Huang S, Wang S, Bian C, Yang Z, Zhou H, Zeng Y, Li H, Han Q, Zhao RC. 2012. Upregulation of miR-22 promotes osteogenic differentiation and inhibits adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells by repressing HDAC6 protein expression. Stem Cells Dev 21:2531–2540.

Ishii I, Ikeguchi Y, Mano H, Wada M, Pegg AE, Shirahata A. 2012. Polyamine metabolism is involved in adipogenesis of 3T3-L1 cells. Amino Acids 42:619–626.

Kuijpers G, Schneider B, Stadel B, Colman E. 2002. Recombinant human parathyroid hormone. Preclinical data on rat osteosarcoma were not dismissed. BMJ 324:1218; author reply 1218.

Kumar AP, Butler AP. 1997. Transcription factor Sp3 antagonizes activation of the ornithine decarboxylase promoter by Sp1. Nucleic Acids Res 25:2012–2019.

Kurian L, Palanimurugan R, Godderz D, Dohmen RJ. 2011. Polyamine sensing by nascent ornithine decarboxylase antizyme stimulates decoding of its mRNA. Nature 477:490–494.

Lee NK, Skinner JP, Zajac JD, MacLean HE. 2011. Ornithine decarboxylase is upregulated by the androgen receptor in skeletal muscle and regulates myoblast proliferation. Am J Physiol Endocrinol Metab 301:E172–E179.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408.

Lopez-Garcia C, Lopez-Contreras AJ, Cremades A, Castells MT, Marin F, Schreiber F, Penafiel R. 2008. Molecular and morphological changes in placenta and embryo development associated with the inhibition of polyamine synthesis during midpregnancy in mice. Endocrinology 149:5012–5023.

Lux GD, Marton LJ, Baylin SB. 1980. Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. Science 210:195–198.

Matsui-Yuasa I, Otani S, Morisawa S, Takigawa M, Enomoto M, Suzuki F. 1985. Induction of spermidine/spermine N1-acetyltransferase by parathyroid hormone in rabbit costal chondrocytes in culture. J Biochem 97:387–390.

Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. 2001. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med 344:1434–1441.

Nemoto T, Hori H, Yoshimoto M, Seyama Y, Kubota S. 2002. Overexpression of ornithine decarboxylase enhances endothelial proliferation by suppressing endostatin expression. Blood 99:1478–1481.

Palanimurugan R, Scheel H, Hofmann K, Dohmen RJ. 2004. Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme. EMBO J 23:4857–4867.

Pendeville H, Carpino N, Marine JC, Takahashi Y, Muller M, Martial JA, Cleveland JL. 2001. The ornithine decarboxylase gene is essential for cell survival during early murine development. Mol Cell Biol 21:6549–6558.

Pignatti C, Tantini B, Stefanelli C, Flamigni F. 2004. Signal transduction pathways linking polyamines to apoptosis. Amino Acids 27:359–365.

Poulin R, Soulet D, Gagnon B, Rivest S, Audette M. 2006. Evidence for a multistep model for eukaryotic polyamine transport. In: Wang JY, Casero RA, editors. Polyamine cell signaling: Physiology, pharmacology, and cancer research. Totowa, NJ: Humana Press, Inc. pp 415–432.

Ramot Y, Tiede S, Biro T, Abu Bakar MH, Sugawara K, Philpott MP, Harrison W, Pietila M, Paus R. 2011. Spermidine promotes human hair growth and is a novel modulator of human epithelial stem cell functions. PLoS ONE 6:e22564.

Sasaki T, Matsuoka H, Saito M. 2008. Generation of a multi-layer muscle fiber sheet from mouse ES cells by the spermine action at specific timing and concentration. Differentiation 76:1023–1030.

Shirahata A, Takahashi N, Beppu T, Hosoda H, Samejima K. 1993. Effects of inhibitors of spermidine synthase and spermine synthase on polyamine synthesis in rat tissues. Biochem Pharmacol 45:1897–1903.

Tjabringa GS, Vezeridis PS, Zandieh-Doulabi B, Helder MN, Wuisman PI, Klein-Nulend J. 2006. Polyamines modulate nitric oxide production and COX-2 gene expression in response to mechanical loading in human adipose tissue-derived mesenchymal stem cells. Stem Cells 24:2262–2269.

Tjabringa GS, Zandieh-Doulabi B, Helder MN, Knippenberg M, Wuisman PI, Klein-Nulend J. 2008. The polymine spermine regulates osteogenic differentiation in adipose stem cells. J Cell Mol Med 12:1710–1717.

Trotter J. 2000. WinMDI 2.8 software. Windows multiple document interface flow cytometry application.

Vittur F, Lunazzi G, Moro L, Stagni N, de Bernard B, Moretti M, Stanta G, Bacciottini F, Orlandini G, Reali N, Casti A. 1986. A possible role for polyamines in cartilage in the mechanism of calcification. Biochim Biophys Acta 881:38–45.

Vuohelainen S, Pirinen E, Cerrada-Gimenez M, Keinanen TA, Uimari A, Pietila M, Khomutov AR, Janne J, Alhonen L. 2010. Spermidine is indispensable in differentiation of 3T3-L1 fibroblasts to adipocytes. J Cell Mol Med 14:1683–1692.

Wang Y, Devereux W, Stewart TM, Casero RA, Jr. 1999. Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine N(1)-acetyltransferase gene. J Biol Chem 274:22095–22101.

Wolff AC, Armstrong DK, Fetting JH, Carducci MK, Riley CD, Bender JF, Casero RA, Jr., Davidson NE. 2003. A Phase II study of the polyamine analog N1,N11-diethylnorspermine (DENSpm) daily for five days every 21 days in patients with previously treated metastatic breast cancer. Clin Cancer Res 9: 5922–5928.

Yuan Q, Ray RM, Viar MJ, Johnson LR. 2001. Polyamine regulation of ornithine decarboxylase and its antizyme in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 280:G130–G138.

Yusa N, Watanabe K, Yoshida S, Shirafuji N, Shimomura S, Tani K, Asano S, Sato N. 2000. Transcription factor Sp3 activates the liver/bone/kidney-type alkaline phosphatase promoter in hematopoietic cells. J Leukoc Biol 68: 772–777.