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Hedgehog Signaling and Osteoblast Gene Expression are Regulated by Purmorphamine in Human Mesenchymal Stem Cells

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ABSTRACT

Several biological events are controlled by Hedgehog (Hh) signaling, including osteoblast phenotype development. This study aimed at evaluating the gene expression profile of human mesenchymal stem cells (hMSCs) treated with the Hh agonist, purmorphamine, focusing on Hh signaling and osteoblast differentiation. hMSCs from bone marrow were cultured in non-osteogenic medium with or without purmorphamine (2 μM) for periods of up to 14 days. Purmorphamine up-regulated gene expression of the mediators of Hh pathway, *SMO*, *PTCH1*, *GLI1*, and *GLI2*. The activation of Hh pathway by purmorphamine increased the expression of several genes (e.g., *RUNX2* and *BMPs*) related to osteogenesis. Our results indicated that purmorphamine triggers Hh signaling pathway in hMSCs, inducing an increase in the expression of a set of genes involved in the osteoblast differentiation program. Thus, we conclude that Hh is a crucial pathway in the commitment of undifferentiated cells to the osteoblast lineage. J. Cell. Biochem. 113: 204–208, 2012.

KEY WORDS: GENE EXPRESSION; HEDGEHOG; MESENCHYMAL STEM CELL; OSTEOBLAST; PURMORPHAMINE

Mesenchymal stem cells (MSCs) are non-hematopoietic, plastic adherent progenitor cells isolated from the bone marrow, which are capable of differentiating into the osteogenic, chondrogenic, and adipogenic lineages both in vitro and in vivo [Prockop, 1997]. The investigation of small molecules to modulate MSCs differentiation could lead the discovery of new therapeutic agents to treat a plethora of pathologies, including bone-related diseases like osteoporosis.

Purmorphamine, a 2,6,9-trisubstituted purine, is a Hedgehog (Hh) agonist that directly target Smoothened (SMO) transmembrane protein [Sinha and Chen, 2006]. In vertebrates, the activation of Hh pathway is regulated by three proteins, which are Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) [Pathi et al., 2001]. In the absence of a ligand, SMO is inhibited by Patched 1 (PTCH1) and consequently is unable to transduce the signal. Binding ligand to PTCH1 reverses the repression of SMO and leads the activation of a signaling cascade by translocation of the transcriptional factor GLI2 to the nucleus [Murone et al., 1999; Briscoe, 2006].

Activation of Hh signaling by purmorphamine promotes the transcription of various genes, including *Gli1*, *Ptch*, and alkaline phosphatase (*Alp*) [Wu et al., 2004; Plaisant et al., 2009]. Purmorphamine increased ALP activity in both mouse embryonic mesoderm fibroblast C3H10T1/2 and pre-osteoblast MC3T3-E1 cell lines and up-regulated *Runx2* expression in C3H10T1/2 cells [Ding et al., 2002; Wu et al., 2002; Wu et al., 2004]. It has been shown that purmorphamine increased ALP activity and bone-like formation in osteoblasts differentiated from human mesenchymal stem cells (hMSCs) [Beloti et al., 2005]. In contrast, by activating Hh signaling, purmorphamine inhibited osteoblast differentiation in human multipotent adipose-derived stem cells (hMASCs) and MSCs from bone marrow [Plaisant et al., 2009].

Outcomes from the literature are inconclusive and for that reason, up to now, how Hh pathway modulates osteoblast differentiation

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from hMSCs remains a challenging question. Therefore, our study was designed to investigate the expression of genes related to Hh pathway and osteoblast phenotype development in hMSCs cultured in presence of the Hh agonist, purmorphamine.

MATERIALS AND METHODS

PURMORPHAMINE

Purmorphamine [2-(1-naphthoxy)-6-(4-morpholinoanilino)-9-cyclohexylpurin] (Calbiochem, Gibbstown, NJ) was reconstituted in dimethylsulfoxide (Sigma, St. Louis, MO) to make a 4.8 mM stock solution and then diluted with culture medium to 2 μM.

CULTURE OF hMSCs

hMSCs were obtained from bone marrow of two donors (one female: 13-year old and one male: 15-year old) following the research protocols approved by the Committee of Ethics in Research from the University of Sao Paulo. Cells were grown in α-MEM (Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 50 μg/ml gentamicin (Gibco Life Technologies), and 0.3 μg/ml fungizone (Gibco Life Technologies). Subconfluent cells in primary culture were harvested after treatment with 1 mM EDTA (Gibco Life Technologies) and 0.25% trypsin (Gibco Life Technologies) and subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ) at a density of 2×10^4/well in presence of vehicle (dimethylsulfoxide) or 2 μM of purmorphamine. During the culture period, of up to 14 days, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and the medium was changed every 3 days.

TOTAL RNA EXTRACTION

At 7 and 14 days, total RNA was extracted using the SV Total RNA Isolation Kit (Promega Corporation, Madison, WI) according to the manufacturer's recommendations. RNA samples were eluted from the columns in 25 μl of RNase-free water and stored at −80°C until use. The concentration and purity was determined by optical density at a wavelength of 260, 280, and 230 nm using GeneQuant® spectrophotometer (GE Healthcare, Buckinghamshire, UK).

GENE EXPRESSION OF Hh PATHWAY MEDIATORS

Gene expression was evaluated at 7 and 14 days using primer sequences designed at the Primer-BLAST, which are presented in Table I. Total RNA from samples was reversely transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Assays were performed in a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). SybrGreen PCR MasterMix (Invitrogen, Carlsbad, CA), specific primers (Invitrogen), and 12.5 ng of cDNA were used in each reaction. The standard PCR conditions were 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 s) and 60°C (1 min), followed by the standard denaturation curve. The amount of mRNA was normalized using β-actin as a housekeeping gene and calibrated by the cells exposed to the vehicle for 7 days or 14 days and data were calculated using the ΔΔCT method [Livak and Schmittgen, 2001].

TABLE I. Primer Sequences and Product Size (bp) for Real-Time PCR

Target	Acession number	Sense and anti-sense sequences	bp
β-actin	NM_001101.3	ACGGGGTCACCCACACTGTGC CCGCTCGTTGCCAATAGTGATGA	495
SMO	NM_005631.3	CGGCAAGCTCGTGCTCTGGT GGGTGGCCTGGCAGAGGGTA	143
PTCH1	NM_001083602.1	CGCTCCGAGCAGGGGTTGAC AGGCCCACAACCAAGAACTTGCC	147
GLI1	NM_005269.2	TGCACCGAGGGCCCACTCTT AGGGAGCTGGGTGAGGTGCG	134
GLI2	NM_005270.4	GCCTGGACCTGCAGCGGATG GAAGGCTGGGCTGAGGGCAC	131

OSTEOBLAST GENE EXPRESSION PROFILING

To evaluate expression of genes related to osteoblast phenotype development, hMSCs were exposed to purmorphamine or vehicle for 7 and 14 days. First strand synthesis was performed with 1 μg of total RNA using the RT^2 PCR Array First Strand Kit (Superarray Bioscience Corporation, Frederick, MD). Samples were then screened to identify the expression of 84 osteoblast genes (Osteogenesis PCR Array, Cat # PAHS-026) by means of the RT^2 Profiler PCR Array System (Superarray Bioscience Corporation). Changes in the SYBR Green/ROX fluorescence ratios were detected on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Five housekeeping genes (β-actin, B2M, HPRT1, RPL13A, and GAPDH) were used to normalization. Results were calibrated by the cells exposed to vehicle for 7 and 14 days and analyzed by RT^2 Profiler PCR Array Data Analysis Template v3.2. Data were calculated using the ΔΔCT method [Livak and Schmittgen, 2001].

STATISTICAL ANALYSES

All experiments were carried out in triplicates ($n = 3$), data were compared by student's *t*-test and the level of significance was established at $P \leq 0.05$. The Benjamini–Hochberg multiple test correction were used to correct all data.

RESULTS

GENE EXPRESSION OF Hh PATHWAY MEDIATORS

Modulation of Hh signaling by purmorphamine in hMSCs was evaluated at 7 and 14 days through the gene expression of the membrane receptors *SMO* and *PTCH1*, and the transcriptional factors *GLI1* and *GLI2* (Fig. 1). Gene expression of *SMO* was up-regulated at 7 days ($P \leq 0.05$) and down-regulated at 14 days ($P \leq 0.05$) by purmorphamine. *PTCH1* expression was increased by purmorphamine at 7 days ($P \leq 0.05$) and not affected at 14 days ($P \geq 0.05$). Purmorphamine up-regulated the expression of *GLI1* and *GLI2* at 7 ($P \leq 0.05$) and 14 days ($P \leq 0.05$).

OSTEOBLAST GENE EXPRESSION PROFILING

At 7 and 14 days, we examined whether modulation of Hh signaling by purmorphamine affects the expression of genes involved in osteogenesis. Among the 84 evaluated genes, 27 genes were up-regulated and 9 were down-regulated by purmorphamine at 7 days. At 14 days, the number of up-regulated genes increased to 40, while

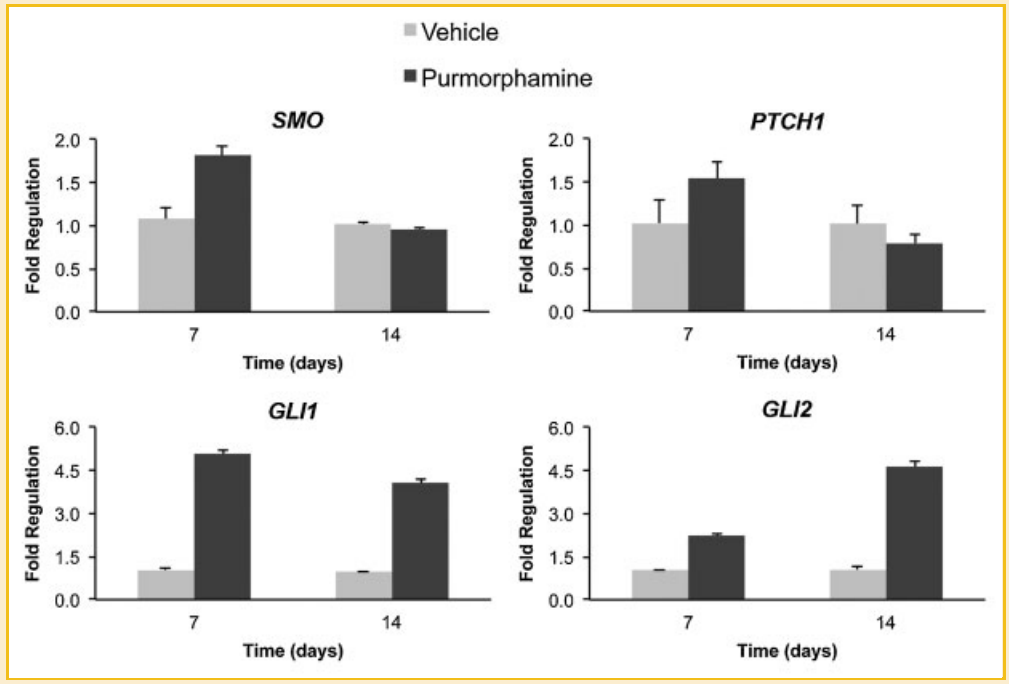

Fig. 1. Modulation of Hh signaling by purmorphamine in hMSCs at 7 and 14 days. Gene expression of *SMO* was higher at 7 days ($P \leq 0.05$) and lower at 14 days ($P \leq 0.05$) in presence of purmorphamine. Purmorphamine up-regulated *PTCH1* expression at 7 days ($P \leq 0.05$) and *GLI1* and *GLI2* at 7 ($P \leq 0.05$) and 14 days ($P \leq 0.05$). Data are presented as mean ± standard deviation.

12 genes were down-regulated. Despite a slight reduction at 7 days, Hh pathway induced a significant increased in the expression of the key transcriptional factors of both osteogenesis (*RUNX2*) and chondrogenesis (*SOX9*). The gene expression of a class of relevant proteins for osteoblast differentiation was up-regulated by Hh pathway activation, specially *BMP2* and *BMP4*, *SMAD1*, *SMAD2*, and *SMAD4* and *TGF-β2*. In general, Hh also increased the gene expression of growth factors (e.g., *FGFs*, *IGFs*, *PDGF*, and *VEGFs*) and integrin receptors that enable cells to respond to signals from extracellular matrix, which play important roles in cell growth, differentiation, and survival. Despite the up-regulation of the majority of genes related to osteogenesis, some bone matrix proteins, namely collagen type I, α1, *ALP*, and osteocalcin were down-regulated by Hh signaling activation. For a detailed view of osteoblast gene regulation, see Table II.

DISCUSSION

To date, there is some controversy regarding the effects of Hh signaling on osteoblast differentiation as contrasting outcomes from studies carried out with different cell lines are noticed. Here, we have shown that purmorphamine activates Hh signaling in hMSCs. Besides, Hh pathway activation increased the expression of a panel of genes related to osteoblast phenotype development in hMSCs.

It has been demonstrated that purmorphamine activates Hh signaling pathway, inducing osteogenesis in the rodent cell line C3H10T1/2 [Wu et al., 2004; Sinha and Chen, 2006]. In addition, the Hh signaling is triggered by purmorphamine in hMASCs and hMSCs as indicated by *GLI1* up-regulation [Plaisant et al., 2009]. Our results confirm that purmorphamine activates Hh signaling pathway in hMSCs, as observed by up-regulation of gene expression of Hh pathway mediators, *SMO*, *PTCH1*, at 7 days, and *GLI1* and *GLI2* at 7 and 14 days. Thus, purmorphamine is a useful Hh pathway agonist in human and rodent cell lines.

Hh signaling activation up-regulated several genes involved in osteoblast differentiation, including *RUNX2*, *BMP* superfamily, *SMADs*, growth factors, and integrin receptors (for details, see Table II). As extensively mentioned, RUNX2 is the key transcriptional factor to initiate bone formation [Komori et al., 1997; Lian and Stein, 2003]. Some members of BMP superfamily like BMP2 and BMP4, which were increased by Hh here, are important factors that regulate osteoblast differentiation through SMAD (SMAD1, SMAD2, and SMAD4) pathway activation, all of them also up-regulated by Hh signaling [Hughes et al., 1995; Yamaguchi et al., 2000; Canalis et al., 2003]. *SMAD3*, an inhibitor of osteoblast differentiation, was down-regulated by Hh activation, suggesting that in addition to induce osteogenesis, Hh may also disrupt cell processes which impair this event [Kaji et al., 2006].

Growth factors (e.g., *FGFs*, *IGFs*, *PDGF*, and *VEGFs*) and integrin receptors play important roles in cell growth, differentiation, and survival [Bikle, 2008; Ng et al., 2008; Canalis, 2009]. Here, we have showed that Hh signaling increased gene expression of these growth factors and receptors. Some bone matrix proteins as collagen type I, α1, *ALP*, and osteocalcin were down-regulated by Hh signaling activation, which may affect extracellular matrix mineralization. In contrast with our results, as a consequence of Hh pathway activation in hMASCs and hMSCs, it was observed that gene expression of *RUNX2*, osteopontin, osteoprotegerin, and osteonectin were inhibited [Plaisant et al., 2009]. Such discrepancy

TABLE II. Effect of Hg Pathway Activation on Osteoblast Gene Expression Profiling in hMCS at 7 and 14 Days

Symbol	Description	Fold regulation 7 days	Fold regulation 14 days
Bone matrix proteins			
ALPL	Alkaline phosphatase, liver/bone/kidney	–	−1.62
BGLAP	Osteocalcin	–	−1.42
BGN	Biglycan	–	–
BMP superfamily			
BMP2	Bone morphogenetic protein 2	2.99	–
BMP3	Bone morphogenetic protein 3	5.58	–
BMP4	Bone morphogenetic protein 4	1.30	2.52
BMP5	Bone morphogenetic protein 5	–	–
BMP6	Bone morphogenetic protein 6	–	1.64
GDF10	Growth differentiation factor 10	–	6.54
TGFB1	Transforming growth factor, beta 1	−1.13	1.68
TGFB2	Transforming growth factor, beta 2	1.48	4.29
TGFB3	Transforming growth factor, beta 3	–	−3.15
Receptors			
CD36	CD36 molecule (thrombospondin receptor)	3.10	4.70
CDH11	Cadherin 11, type 2, OB-cadherin	–	3.72
EGFR	Epidermal growth factor receptor	1.13	3.32
FGFR1	Fibroblast growth factor receptor 1	1.40	–
FGFR2	Fibroblast growth factor receptor 2	–	−1.58
FLT1	Fms-related tyrosine kinase 1	–	–
ICAM1	Intercellular adhesion molecule 1 (CD54)	−1.18	7.03
SCARB1	Scavenger receptor class B, member 1	–	2.15
TGFBR1	Transforming growth factor, beta receptor I	2.56	3.22
TGFBR2	Transforming growth factor, beta receptor II	–	–
VCAM1	Vascular cell adhesion molecule 1	1.50	−1.59
VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor	1.36	–
IGF1R	Insulin-like growth factor 1 receptor	1.70	1.46
PHEX	Phosphate regulating endopeptidase homolog, X-linked	2.52	–
Growth factors			
EGF	Epidermal growth factor (beta-urogastrone)	–	2.36
FGF1	Fibroblast growth factor 1 (acidic)	1.23	3.84
FGF2	Fibroblast growth factor 2 (basic)	3.30	3.79
IGF1	Insulin-like growth factor 1 (somatomedin C)	3.20	1.75
IGF2	Insulin-like growth factor 2 (somatomedin A)	1.85	–
PDGFA	Platelet-derived growth factor alpha	–	2.78
VEGFA	Vascular endothelial growth factor A	1.19	−1.55
VEGFB	Vascular endothelial growth factor B	–	3.90
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	–	5.13
CSF3	Colony stimulating factor 3 (granulocyte)	–	–
Integrin receptors			
ITGA1	Integrin, alpha 1	2.41	5.00
ITGA2	Integrin, alpha 2	2.38	3.15
ITGA3	Integrin, alpha 3	−1.29	1.88
ITGB1	Integrin, beta 1	–	4.06
Collagen			
COL10A1	Collagen, type X, alpha 1	–	–
COL11A1	Collagen, type XI, alpha 1	1.63	1.49
COL12A1	Collagen, type XII, alpha 1	1.20	1.28
COL14A1	Collagen, type XIV, alpha 1	−1.24	1.58
COL15A1	Collagen, type XV, alpha 1	–	2.27
COL1A1	Collagen, type I, alpha 1	1.26	−5.62
COL1A2	Collagen, type I, alpha 2	–	–
COL2A1	Collagen, type II, alpha 1	–	–
COL3A1	Collagen, type III, alpha 1	–	−1.41
COL4A3	Collagen, type IV, alpha 3	–	2.86
COL5A1	Collagen, type V, alpha 1	−1.15	2.63
Cartilage-related genes			
COMP	Cartilage oligomeric matrix protein	–	2.49
SOX9	SRY (sex determining region Y)-box 9	−1.28	3.79
Metalloproteinases			
BMP1	Bone morphogenetic protein 1	–	2.23
MINPP1	Multiple inositol polyphosphate histidine phosphatase, 1	1.45	2.80
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	–	–
MMP2	Matrix metallopeptidase 2	–	1.27
MMP8	Matrix metallopeptidase 8	–	–
MMP9	Matrix metallopeptidase 9	–	–
Transcription factors			
MSX1	Msh homeobox 1	–	
NFKB1	Nuclear factor of kappa in B-cells 1 (p105)	1.35	−4.43
RUNX2	Runt-related transcription factor 2	−1.06	1.96
SMAD1	SMAD family member 1	−1.11	1.56
SMAD2	SMAD family member 2	3.03	1.18
SMAD3	SMAD family member 3	1.18	−6.67
SMAD4	SMAD family member 4	–	1.53

(*Continued*)

TABLE II. (*Continued*)

Symbol	Description	Fold regulation 7 days	14 days
TWIST1	Twist homolog 1	–	–
Other genes			
CTSK	Cathepsin K	–	−1.64
FN1	Fibronectin 1	−1.14	–
SERPINH1	Heat shock protein 47	–	–
STATH	Statherin	–	–
TUFT1	Tuftelin 1	1.32	3.19
TFIP11	Tuftelin interacting protein 11	–	2.31
ANX5	Annexin A5	–	−1.15

Data were expressed as fold regulation relative to cells grown in presence of vehicle at 7 and 14 days, respectively. All depicted values are statistically significant at $P \leq 0.05$.
"–" indicates lack of effect relative to vehicle.

could be related to distinct osteoblast differentiation status of cultures used in both studies, since we cultivated hMSCs under non-osteogenic condition and they used medium supplemented with ascorbic acid, glycerophosphate and dexamethasone (osteogenic medium). In this context, it is possible to suggest that the effect of activation of Hh signaling on osteoblast differentiation of hMSCs is closely related to the culture conditions and the stage of osteoblast maturation.

In conclusion, the present study indicates that purmorphamine triggers Hh signaling pathway in hMSCs cultured in non-osteogenic environment, and such activation up-regulates the expression of a set of genes involved in the osteoblast differentiation program. Thus, we postulate that Hh is a key pathway to drive undifferentiated cells to the osteoblast lineage.

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