## **Research Article**

# Role of MSX1 in Osteogenic Differentiation of Human Dental Pulp Stem Cells

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Msh homeobox 1 (MSX1) encodes a transcription factor implicated in embryonic development of limbs and craniofacial tissues including bone and teeth. Although MSX1 regulates osteoblast differentiation in the cranial bone of young animal, little is known about the contribution of MSX1 to the osteogenic potential of human cells. In the present study, we investigate the role of MSX1 in osteogenesis-induction medium, runt-related transcription factor-2 (*RUNX2*), bone morphogenetic protein-2 (*BMP2*), alkaline phosphatase (*ALPL*), and osteocalcin (*OCN*) mRNA levels, as well as alkaline phosphatase activity, increased on days 4–12, and thereafter the matrix was calcified on day 14. However, knockdown of *MSX1* with small interfering RNA abolished the induction of the osteoblast-related gene expression, alkaline phosphatase activity, and calcification. Interestingly, DNA microarray and PCR analyses revealed that *MSX1* knockdown induced the sterol regulatory element-binding protein 2 (*SREBP2*) transcriptional factor and its downstream target genes in the cholesterol synthesis pathway. Inhibition of cholesterol synthesis enhances osteoblast differentiation of various mesenchymal cells. Thus, MSX1 may downregulate the cholesterol synthesis-related genes to ensure osteoblast differentiation of human dental pulp stem cells.

#### 1. Introduction

Msh homeobox 1 (MSX1) is a homeobox transcriptional factor involved in limb-pattern formation and craniofacial development and specifically in odontogenesis. Mouse *Msx1* mutations cause craniofacial malformation and tooth agenesis [1]. *Msx1*-knockout mice show arrested tooth development at the bud stage and embryonic lethal defects [2]. Msx1 is expressed at high levels in craniofacial skeletal

cells during early postnatal development [3], and transgenic mice expressing Msx1 under the control of the alpha (I) collagen promoter exhibit increased osteoblast number, cell proliferation, and apoptosis [4], suggesting Msx1 may have a role in craniofacial bone modeling. MSX1 is also expressed at high levels in the dental mesenchyme at the cap and bell stages [5] and may be a suppressor for cell differentiation that maintains mesenchymal cells in a proliferative state to ensure robust craniofacial and tooth development [6].

In addition, MSX1 is an upstream and downstream regulator for the bone morphogenetic protein BMP2/BMP4 signaling pathway [7, 8]. Mutations in human *MSX1* also cause cleft lip/palate and tooth agenesis [9, 10]. However, the role of MSX1 in human craniofacial and tooth development has not been fully understood.

Dental pulp stromal cells isolated from whole pulp tissue can differentiate into osteoblasts, odontoblasts, endothelial cells, nerve cells, and adipocytes in vitro. Some of these cells identified by several cell surface antigens are referred to as dental pulp stem cells (DPSCs) [11, 12]. DPSCs may play a role in dentinogenesis/osteogenesis in both developing and injured teeth. Furthermore, these cells are a promising source of cell-based regenerative therapies for dental, skeletal, vascular, and neuronal diseases [13, 14]. Human DPSCs (hDPSCs) have not been fully characterized at the molecular level, but a previous reported showed that MSX1 is expressed at higher levels in hDPSCs than in bone marrow-derived mesenchymal stem cells and fibroblasts [15]. MSX1 may participate in the control of primary or secondary dentin formation and reparative dentin or osteodentin/bone formation in injured pulp tissue, in addition to the physiological role such as the maintenance of dental pulp stem/progenitor cells in healthy teeth. In the present study, we explored the role of MSX1 in pulpal mesenchymal cells using human DPSCs in culture.

Statins are a class of drugs that function as specific inhibitors of 3-hydoroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol synthesis. Numerous studies have shown that statins exert bone anabolic effects in osteoblasts and osteogenic precursor cells [16, 17]. Simvastatin enhances alveolar bone remodeling in the tooth extraction socket [18], enhances bone fracture healing [19], and reduces alveolar bone loss and tooth mobility in chronic periodontitis [20]. In addition, simvastatin enhances odontoblast/osteoblast differentiation of DPSCs and mesenchymal stem cells isolated from other tissues [17, 21, 22]. These studies indicate a close relationship between cholesterol synthesis and osteoblast differentiation.

Here, we demonstrated the role of MSX1 in osteoblast differentiation and cholesterol synthesis in hDPSCs using small interfering RNA (siRNA) against *MSX1*. DNA microarray analyses revealed that knockdown of *MSX1* in hDPSCs undergoing osteogenic differentiation abolished the expression of various osteoblast-related genes but enhanced the expression of cholesterol synthesis-related genes. Our results suggest that MSX1 enhances osteoblast differentiation and calcification in hDPSCs through repression of cholesterol synthesis genes and induction of osteoblast-related genes.

#### 2. Material and Methods

2.1. Human DPSCs. Extracted healthy deciduous teeth were collected from 6–12-year-old children following protocols approved by the ethical authorities at Hiroshima University (permit number: D88-2). Written informed consent was obtained from the subject or subject's parent. Pulp tissue specimens from deciduous teeth were minced and digested with 3 mg/mL collagenase type I (Life Technologies,

Carlsbad, CA, USA) and 4 mg/mL dispase (Roche Diagnostics, Mannheim, Germany) in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) for 1h at 37°C. Single cell suspension was obtained by passing cells through a 70  $\mu$ m cell strainer (CORNING, Corning, NY, USA). The cells were incubated in DMEM supplemented with 20% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 1% penicillin-streptomycin (Life Technologies) at 37°C in 95% air and 5% CO<sub>2</sub> [23]. Forming colonies were separated by incubation with Accutase (Funakoshi Co., Ltd., Tokyo, Japan), and isolated cells were transferred to passage cultures with DMEM supplemented by 10% FBS and 1% penicillinstreptomycin. The culture medium was changed every 2 days. Cells at passages 3–9 were used in subsequent experiments.

2.2. FACS Analysis. Cells were harvested with Accutase and fixed in 4% paraformaldehyde. Cells were centrifuged at 1,500 ×g for 5 min and resuspended at 5 ×  $10^6$  cells/mL in PBS containing 0.5% bovine serum albumin (BSA). Aliquots containing 10<sup>5</sup> cells were incubated with individual phycoerythrin- (PE-) conjugated antibodies or isotype control PE-conjugated IgG $\kappa$  for 30 min at room temperature and then washed in PBS supplemented with 3% FBS. Samples were analyzed using a FACS Aria flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data were analyzed using CELLQUEST software (Becton Dickinson). The following monoclonal antibodies were used: PE-conjugated antibodies against CD73 (mouse IgG1k; Biolegend, San Diego, CA, USA), CD90 (mouse IgG1k; Biolegend), CD105 (mouse IgG1 $\kappa$ ; Biolegend), and CD166 (mouse IgG1 $\kappa$ ; Santa Cruz Biotechnology, Texas, USA). PE-conjugated isotype control mouse IgG1 $\kappa$  (Biolegend) was used as the control.

2.3. MSX1 Knockdown. MSX1 siRNA oligonucleotides (s8999 and s224066) were purchased from Life Technologies. The sequences are 5'-GCAUUUAGAUCUACACUCUtt-3' (sense) and 5'-AGAGUGUAGAUCUAAAUGCta-3' (antisense) for s8999 and 5'-GCAAGA AAAGCGCAGAGAAtt-3' (sense) and 5'-UUCUCUGCGCUUUUCUUGCct-3' (antisense) for s224066. Silencer select negative control #1 siRNA (Life Technologies) was used as the control.

Human DPSCs were seeded at  $5 \times 10^4$  cells/well in 24multiwell plates coated with type I collagen with 0.5 mL DMEM supplemented with 10% FBS. After 24 h, siRNA was transfected into cells with Lipofectamine 2000 (Life Technologies) and cells were incubated for an additional 48 h.

2.4. Osteogenic Differentiation of hDPSCs and Alizarin Red Staining. After the cultures became confluent, hDPSCs were incubated with 0.5 mL of DMEM supplemented with 10% FBS, 10 mM  $\beta$ -glycerophosphate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), 50 µg/mL ascorbic acid 2-phosphate (Sigma), 2 mM L-glutamine (Sigma), 100 nM dexamethasone (Sigma), and 1% penicillin/streptomycin (osteogenesisinduction medium) as described [15]. For evaluation of calcification, cells incubated with osteogenesis-induction medium for 14 days were fixed at room temperature in 95% ethanol for

Gene	Primer $(5' \rightarrow 3')$	Probe
MCV1 (conco)	F: CTCGTCAAAGCCGAGAGC	Dacha Universal Droba # 7
WIGHT (Selise)	R: CGGTTCGTCTTGTGTTTGC	Roche Oniversal Probe # 7
MSV1 (anticonco)	F: GCCAGCCCTCTTAGAAACAG	Pocha Universal Drobe # 50
WISKI (antiselise)	R: AATAAAGCAGCCCCTCGTTC	Roche Oniversal Probe # 50
RUNIX2	F: CAGTGACACCATGTCAGCAA	Roche Universal Probe # 66
RUNAL	R: GCTCACGTCGCTCATTTTG	Koche Oniversai 1100e # 00
RMP2	F: CGGACTGCGGTCTCCTAA	Roche Universal Probe # 49
Divit 2	R: GGAAGCAGCAACGCTAGAAG	
OSY	F: CAGCAGCTAAACTTGGAAGGA	Roche Universal Drobe # 76
001	R: TGCTTTCGCTTGTCTGAGTC	Koche Oniversai i 1000 # 70
OCN	F: GCCTCCTGAAAGCCGATGT	5'-CCAACTCGTCACAGTCCGGATTGAGCT-3'
OCIV	R: AAGAGACCCAGGCGCTACCT	5-comercereneredent render-5
AIPI	F: TCACTCTCCGAGATGGTGGT	Roche Universal Probe # 12
	R: GTGCCCGTGGTCAATTCT	Roche Oniversul 1100e # 12
SOX9	F: GTACCCGCACTTGCACAAC	Roche Universal Probe # 61
0011)	R: TCTCGCTCTCGTTCAGAAGTC	
PPARv	F: GACAGGAAAGACAACAGACAAATC	Roche Universal Probe # 7
ΠΠΑγ	R: GGGGTGATGTGTTTGAACTTG	
SRFBP2	F: GCCCTGGAAGTGACAGAGAG	Roche Universal Probe # 21
SICEDI Z	R: TGCTTTCCCAGGGAGTGA	
HMGCS1	F: TCTGTCTACTGCAAAAAGATCCAT	Roche Universal Probe # 59
11110001	R: TGAAGCCAAAATCATTCAAGG	
HMGCR	F: GTTCGGTGGCCTCTAGTGAG	Roche Universal Probe # 65
million	R: GCATTCGAAAAAGTCTTGACAAC	
FDPS	F: GGCCACTCCAGAACAGTACC	Roche Universal Probe # 75
1210	R: CCTCATATAGCGCCTTCACC	
CYP51A1	F: TGCAGATTTGGATGGAGGTT	Roche Universal Probe # 64
	R: CCTTGATTTCCCGATGAGC	
DHCR7	F: GCCATGGTCAAGGGCTAC	Roche Universal Probe # 60
DIION/	R: TTGTAAAAGAAATTGCCTGTGAAT	

TABLE 1: Primer and probe sequences used for RT-qPCR.

MSX1: msh homeobox 1; RUNX2: runt-related transcription factor-2; BMP2: bone morphogenetic protein-2; OSX: osterix; OCN: osteocalcin; ALPL: alkaline phosphatase liver type; SOX9: SRY- (sex determining region Y-) box 9; PPARy: peroxisome proliferator activated receptor gamma; SREBP2: sterol regulatory element-binding protein 2; HMGCS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCR: HMG-CoA reductase; FDPS: farnesyl diphosphate synthase; CYP51A1: Cytochrome P450 Family 51 Subfamily A Polypeptide 1; DHCR7: 7-dehydrocholesterol reductase; F: forward; R: reverse.

10 min and stained with 1% alizarin red S for 30 min. The cellmatrix layers were washed 6 times with sterile water.

2.5. Alkaline Phosphatase Activity. Human DPSCs were washed twice with saline and homogenized ultrasonically with 1% NP-40 in saline. Alkaline phosphatase activity was determined using Lab Assay ALP (Wako, Osaka, Japan). DNA concentration was determined with the Quant- $iT^{TM}$  PicoGreen dsDNA Assay Kit (Life Technologies) to calculate alkaline phosphatase activity/µg DNA.

2.6. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was isolated and cDNA was synthesized as described [15]. The cDNA samples were amplified using Universal PCR Master Mix (Life Technologies) with primers (Table 1) and TaqMan probes were purchased from Roche Diagnostics (Basel, Switzerland). GAPDH primers/probe set was used for normalization. After amplification of DNA, expression levels were determined with the ABI prism 7900 HT sequence detection system (Life Technologies).

2.7. DNA Microarray. After the induction of osteogenic differentiation for 4 days, total RNA was isolated from MSX1-knockdown and control hDPSCs using TRIzol (Life Technologies, Japan) and an RNeasy Mini Kit (Qiagen, Chatsworth, CA). DNA microarray analysis was performed using the SurePrint G3 Human GE  $8 \times 60 \text{ K}$  v2 Microarray (Agilent Technologies, Santa Clara, CA, USA). Raw data were standardized by the global median normalization method using GeneSpring (Silicon Genetics, Redwood City, CA, USA). The raw data were deposited in the Gene Expression Omnibus database (GSE69992).

2.8. Statistical Analysis. Results are expressed as mean  $\pm$  SD. Differences between two groups were analyzed by two-way





FIGURE 1: (a) A microscope image of cultured hDPSCs isolated from human primary teeth with no induction. (b) Positive expression of several cell surface antigens for mesenchymal stem cells, including CD73, CD90, CD105, and CD166, in hDPSCs. Similar cell surface antigen expression pattern was obtained with hDPSCs isolated from different donors (data not shown).

ANOVA with Tukey's *post hoc* test for multiple comparisons. In all analyses, P < 0.05 indicated statistically significant differences between values.

#### 3. Results

3.1. Mesenchymal Stem Cell Markers Expressed in Cultured hDPSCs. Human DPSCs from postnatal human primary

teeth were used to explore the functional role of MSX1. These cells exhibited a fibroblastic shape (Figure 1(a)) and showed expression of mesenchymal stem cell surface markers CD73 (>90%), CD90 (>90%), CD105 (>10%), and CD166 (>30%) (Figure 1(b)) as expected from previous studies [24].

3.2. MSX1 Knockdown Abolishes Osteogenic Differentiation of hDPSCs. Human DPSCs were transfected with two



FIGURE 2: Effects of MSX1 knockdown on calcification of hDPSCs. hDPSCs were transfected with either MSX1 siRNA (s8999 or s224066) or control siRNA and incubated for 2 days in growth medium before the cultures became confluent. Thereafter, the cultures were exposed to osteogenesis-induction medium. (a) The calcified matrix was stained with alizarin red on day 14. (b) MSX1 mRNA level was quantified by RT-qPCR at 48 h after transfection. Values are averages ±SD for three cultures. \*\* P < 0.01.

different siRNAs for *MSX1* or control siRNA and then exposed to osteogenesis-induction medium. Both siRNA oligonucleotides targeting *MSX1* (s8999 and s224066) abolished *MSX1* mRNA expression at 48 h and subsequent matrix calcification on day 14 (Figure 2). We selected *MSX1* siRNA (s8999) for subsequent studies.

Next, we examined the effect of MSX1 knockdown on alkaline phosphatase activity and the expression of osteoblast-related genes in hDPSCs after the onset of osteogenesis (Figure 3). In hDPSCs transfected with control siRNA, alkaline phosphatase activity and RUNX2, BMP2, osterix (OSX), osteocalcin (OCN; also known as BGLAP), and alkaline phosphatase liver type (ALPL) mRNA levels increased on days 4-12 after the onset of differentiation. MSX1 mRNA levels also increased on days 4-12. However, MSX1 knockdown abolished the induction of alkaline phosphatase activity (Figure 3(a)) and the increases in ALPL, RUNX2, BMP2, OCN, and MSX1 mRNA levels, although it further increased OSX mRNA levels (Figure 3(b)). It should be noted that the incubation with MSX1 siRNA abolished MSX1 expression at least until day 12 after the onset of osteogenic differentiation.

Next, we examined whether MSX1 knockdown might influence the expression of other master genes including a master regulator of chondrogenesis SOX9 and a master regulator of adipogenesis  $PPAR\gamma$  (Figure 4). In control hDPSCs, no significant changes in the expressions of SOX9 and  $PPAR\gamma$  were observed after the exposure to osteogenesis-induction medium. Under these conditions, MSX1 knockdown increased the expression of  $PPAR\gamma$  on days 4–8, although it had little effect on the expression level of SOX9.

3.3. MSX1 Knockdown Downregulated and Upregulated a Variety of Genes. To characterize the effects of MSX1 knockdown on osteogenic differentiation, we performed DNA microarray analyses on day 4 after exposure to osteogenesisinduction medium. MSX1 knockdown decreased and increased mRNA levels of 2923 and 3480 genes, respectively, which were selected with cut-off values of >1.5-fold change and *t*-test P < 0.05. Tables 2 and 3 show lists of down-regulated and upregulated genes in *MSX1*-knockdown hDPSCs, respectively.

To understand MSX1 actions in hDPSCs differentiating into osteoblasts, we performed a gene-set approach using the 2923 downregulated and 3480 upregulated genes. The WikiPathways analysis showed that the *MSX1* knockdown downregulated various genes involved in focal adhesion, endochondral ossification, integrin-mediated cell adhesion, matrix metalloproteinases, calcium regulation, and insulin signaling (Table 4), whereas it upregulated genes involved in sterol regulatory element-binding protein (SREBP) signaling, cholesterol biosynthesis, adipogenesis, and fatty acid biosynthesis (Table 5). These findings revealed that MSX1 regulates various cellular processes in hDPSCs differentiating into osteoblasts.

3.4. MSX1 Knockdown Upregulates Cholesterol Synthesis-Related Genes. In MSX1-knockdown hDPSCs, "SREBP signaling" and "cholesterol biosynthesis" were the top 1st and 3rd upregulated gene sets, respectively (Table 5). The SREBP2 master transcriptional factor regulates the expression of all genes encoding enzymes in cholesterol synthesis pathway [25]. Because cholesterol synthesis is closely linked with osteoblast differentiation [17], we examined the effect of MSX1 knockdown on the expression of these genes. DNA microarray analyses showed that all cholesterol synthesisrelated genes, including SREBP2, were significantly upregulated by MSX1 knockdown on day 4 (Figure 5(a)). Quantitative RT-PCR analyses confirmed that MSX1 knockdown increased SREBP2, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), HMG-CoA reductase (HMGCR), farnesyl diphosphate synthase (FDPS), Cytochrome P450 Family 51 Subfamily A Polypeptide 1 (CYP51A1), and 7-dehydrocholesterol reductase (DHCR7) mRNA levels (Figure 5(b)).



FIGURE 3: Effects of *MSX1* knockdown on the expression of osteogenic markers in hDPSCs. (a) Alkaline phosphatase activity in cultures was determined on days 0–12 after exposure to osteogenesis-induction medium. (b) The mRNA levels of osteoblast-related genes, including *RUNX2*, *BMP2*, *OSX*, *OCN*, and *ALPL*, along with the *MSX1* mRNA level were quantified by RT-qPCR on the indicated days. Values are averages  $\pm$ SD for three cultures. \**P* < 0.05; \*\**P* < 0.01.

Gene symbol	Gene name	Gene ID	Fold change
Cllorf96	Chromosome 11 open reading frame 96	NM_001145033	92.53
JAM2	Junctional adhesion molecule 2	NM_021219	70.90
MLC1	Megalencephalic leukoencephalopathy with subcortical cysts 1	NM_015166	65.56
NPPC	Natriuretic peptide C	NM_024409	54.40
CPXM1	Carboxypeptidase X (M14 family), member 1	NM_019609	51.08
EFCC1	EF-hand and coiled-coil domain containing 1	NM_024768	44.56
SFRP4	Secreted frizzled-related protein 4	NM_003014	43.96
JAM2	Junctional adhesion molecule 2	NM_021219	38.31
LOC200772	Uncharacterized LOC200772	NR_033841	34.41
S100A8	S100 calcium binding protein A8	NM_002964	31.72
JAM2	Junctional adhesion molecule 2	NM_001270408	31.42
CPXM1	Carboxypeptidase X (M14 family), member 1	NM_019609	29.44
SFRP4	Secreted frizzled-related protein 4	NM_003014	28.11
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	NM_000222	27.48
CLCA2	Chloride channel accessory 2	NM_006536	24.88
LINC00473	Long intergenic nonprotein coding RNA 473	NR_026860	24.34
ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2.8-sialyltransferase 4	NM_005668	24.16
TEX29	Testis expressed 29	NM 152324	23.52
PTGDR2	Prostaglandin D2 receptor 2	NM 004778	23.31
CXCL14	Chemokine (C-X-C motif) ligand 14	NM 004887	22.63
HS6ST2	Henoran sulfate 6 O sulfatransferase 2	NM 001077188	22.60
1100012	Carbohydrate (N-acetylgalactosamine 4-sulfate 6-0) Sulfotransferase	11112001077100	22.01
CHST15	15	NM_015892	21.84
PIANP	PILR alpha associated neural protein	NM_153685	21.67
SNAP25	Synaptosomal-associated protein, 25 kDa	NM_003081	21.55
CBLN2	Cerebellin 2 precursor	NM_182511	21.30
FRAS1	Fraser syndrome 1	NM_025074	21.24
SECTM1	Secreted and transmembrane 1	NM_003004	20.76
NFE2	Nuclear factor, erythroid 2	NM_006163	20.46
GABBR2	Gamma-aminobutyric acid (GABA) B receptor, 2	NM_005458	19.22
MSX1	Msh homeobox 1	NM_002448	19.08
SCARA5	Scavenger receptor class A, member 5 (putative)	NM_173833	18.79
PRSS35	Protease, serine, 35	NM_153362	18.60
WNT2B	Wingless-type MMTV integration site family, member 2B	NM_004185	17.95
BMP2	Bone morphogenetic protein-2	NM_001200	17.63
NDRG4	NDRG family member 4	NM_022910	17.39
CRTAM	Cytotoxic and regulatory T cell molecule	NM_019604	16.80
RASL12	RAS-like, family 12	NM_016563	15.91
THBD	Thrombomodulin	NM_000361	15.26
CHST1	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	NM_003654	14.90
DIO3OS	DIO3 opposite strand/antisense RNA (head to head)	NR_002770	14.81
CCR1	Chemokine (C-C motif) receptor 1	NM_001295	14.81
TMEM35	Transmembrane protein 35	NM_021637	14.79
HAS1	Hvaluronan synthase 1	NM 001523	14.68
SCN1B	Sodium channel voltage-gated type I beta subunit	NM 199037	14 34
ADAMTS17	ADAM metallopentidase with thrombospondin type 1 motif 17	NM 139057	14.12
CALNETIC	UDP-N-acetyl-alpha-D-galactosamine:polypeptide		12.04
GALN115	N-acetylgalactosaminyltransferase 15	NM_054110	13.86
RHOH	Ras homolog family member H	NM_004310	13.80

 TABLE 2: The list of downregulated genes in MSX1-knockdown cells (top 50).

Gene name				Gene ID	Fold change
G protein-coupled receptor 68				NM_003485	13.63
Tachykinin 3				NM_013251	13.37
MicroRNA 1247				AF469204	13.13
T T T T T T T T T T T T T T		10 PPARy 8 - 6 - 4 - 0 0 Control sil	** T T 4 (I RNA 7	** 	
1 siRNA $ \Box^{INS} $		MSX1 siR	NA <sup>**</sup>		
	Gene name G protein-coupled receptor 68 Tachykinin 3 MicroRNA 1247	Gene name G protein-coupled receptor 68 Tachykinin 3 MicroRNA 1247	Gene name G protein-coupled receptor 68 Tachykinin 3 MicroRNA 1247	Gene name G protein-coupled receptor 68 Tachykinin 3 MicroRNA 1247 MicroRNA 1247 M	Gene name Gene name Gene ID NM_003485 NM_013251 MicroRNA 1247 AF469204 M_003485 NM_013251 AF469204 M_003485 NM_003485 NM_013251 AF469204 M_003485 N

TABLE 2: Continued.

FIGURE 4: Effects of *MSX1* knockdown on the expression of the master genes of chondrogenesis and adipogenesis in hDPSCs. The mRNA levels of *SOX9* and *PPARy* in hDPSCs transfected with MSX1 siRNA or control siRNA were quantified by RT-qPCR on the indicated days. Values are averages  $\pm$ SD for 3 cultures. \*\* *P* < 0.01; NS: not significant.

#### 4. Discussion

Previous studies showed that mouse MSX1 was implicated in craniofacial bone development [1, 2, 4]. In mouse embryos, MSX1 suppresses precocious differentiation and calcification in dental mesenchymal cells and maintains these cells in a proliferative state to ensure subsequent craniofacial and tooth development [6, 26]. High levels of osteoblast number, cell proliferation, and apoptosis in MSX1 transgenic mice suggest that MSX1 modulates mouse craniofacial bone modeling [4]. However, the role of MSX1 in human cells remains poorly understood. In the present study, we demonstrated that MSX1 plays an essential role in osteogenic differentiation of hDPSCs.

In human DPSC cultures, *MSX1* knockdown resulted in suppressed expression of *RUNX2*, *ALPL*, *BMP2*, and *OCN*. These results demonstrate that MSX1 modulated the major signaling/transcriptional pathways regulating hard tissue differentiation to enhance osteogenic potential of hDPSCs. However, *MSX1* knockdown unexpectedly increased the mRNA level of *OSX*, another transcriptional factor involved in osteoblast maturation. This indicates MSX1 does not activate the entire osteogenesis program, perhaps because MSX1 cooperates with other transcription factors to fully control osteogenesis. *MSX1* knockdown enhanced *PPARy* expression under the osteogenesis-induced condition, suggesting that MSX1 negatively regulates adipogenic differentiation. MSX1 may direct hDPSCs into the osteoblast lineage by preventing them from differentiating into the adipogenic lineage. *MSX1*  knockdown also resulted in downregulation of various genes involved in focal adhesion, integrin-mediated cell adhesion, matrix metalloproteinases, calcium regulation, insulin signaling, and other processes. The extensive effect of *MSX1* knockdown on the entire gene expression profile emphasizes a crucial role of MSX1 in hDPSCs undergoing differentiation into osteoblasts.

Bidirectional transcription of the Msx1 gene has been previously reported [27-29]. In embryonic and newborn mice, sense and antisense Msx1 transcripts are differently expressed during development. In 705IC5 mouse odontoblasts, overexpression of Msx1 antisense RNA decreased the expression of Msx1 sense transcript, whereas overexpression of Msx1 sense RNA increased Msx1 antisense transcript. Thus, expression of mouse Msx1 is controlled by the balance of the two transcripts. In our experiments, however, MSX1 antisense transcript was not detected during osteogenic differentiation of hDPSCs irrespective of siRNA knockdown of MSX1 (data not shown). The presence of MSX1 antisense transcript in humans has so far been reported only in the embryo. Therefore, Msx1 antisense RNA does not seem to be involved in the MSX1 expression in hDPSCs. Under these conditions, the expression of Msx1 sense transcripts was markedly depressed in hDPSCs after treatment with MSX1 siRNA, indicating that the knockdown experiments worked appropriately regardless of the presence or absence of the *Msx1* antisense transcript.

MSX2, a paralog of MSX1, has been shown to enhance osteogenic differentiation of various mesenchymal cells,

MLXPL         MLX interacting protein-like         NM. 02931         74.63           CRUEP         Cytokine receptor-like factor 1         NM. 007702         69.84           MTPA2         Microtubule associated protein 2         NM. 002702         69.84           MAP2         Microtubule associated protein 2         NM. 00274         62.64           PRODH         Proline dehydrogenase (oxidase) 1         NM. 0018355         30.84           ERCH2         Glutamate-rich 2         XM.000714892         29.67           DMD         Dystrophin         NM. 00001805         30.84           ERCH2         Glutamate-rich 2         XM.00011402         21.89           DMD         Dystrophin         NM.00000401         21.88           PLN4         Perilipin 4         NM.0000326         21.22           SAA1         Serum amyloid A2         NM.4000326         21.22           SAA2         Serum amyloid A2         NM.400374         20.98           PARD6B         Par-6 family cell polarity regulator beta         NM.400374         20.98           PARD6B         Par-6 family cell polarity regulator beta         NM.400326         11.52           VLSP33         Ubiquitin specific pertidase 53         NM.400350         16.47 <t< th=""><th>Gene symbol</th><th>Gene name</th><th>Gene ID</th><th>Fold change</th></t<>	Gene symbol	Gene name	Gene ID	Fold change
CRLF1Cytokine receptor-like factor 1NN.0047073.44ATP1A2ATPace, Nat/K + transporting, alpha 2 polypeptideNM.00070260.44ATP1A2Microtubule associated polypeptideNM.0027462.64PRODMProline delydrogenase (oxidas) 1NM.00171489229.67JONRD3LON peptidase N-terminal domain and ring finger 3NM.000171489229.67DMDDystrophinNM.000171427.91SAISerom anyloid A1NM.000031426.65CLDN20Claudin 20NM.000014621.92DMDDystrophinNM.000031426.65CLDN20Claudin 20NM.000031420.64RLF1Retinaldelyde binding potein 1NM.00027420.92SAA2Serom anyloid A221.2223.62SAA2Serom anyloid A221.2223.62CNA3Anoctamin 3NM.00037420.98PARD68Par-6 family cell polarity regulator betaNM.03025420.92SAA2Serom anyloid A216.3517.41LOC284501Uncharacterized LOC284501XE.11082816.35USP33Ubiquitin specific peptidase 53NM.019050316.47CLGNCalmeginNM.01945216.35KLHDC78Kelch domain containing 78NM.10284314.44PSG9Pregnarcy specific beta-lybcoprotein 9NM.0027412.32COLA44Colagen, rype IV, alpha 4NA100748213.33COLA44Colagen, rype IV, alpha 4NA100748212.72	MLXIPL	MLX interacting protein-like	NM_032951	74.63
ATP1A2         ATPase, Na+/K + transporting, alpha 2 polypeptide         NN 000702         69.84           MAP2         Microtubule-associated protein 2         NM 00237         62.64           PRODFI         Proline d/bydrogenase (oxidac) 1         NM 00335         50.32           LONRP3         LON peptidase N-terminal domain and ring finger 3         NM 0001714892         29.47           DMD         Dystrophin         NM 000101         27.91           SAAI         Serum anyloid A1         NM 000101346         24.19           DMD         Dystrophin         NM 000001346         24.19           DMD         Dystrophin         NM 000001346         24.19           DMD         Dystrophin         NM 000001346         24.19           DMD         Dystrophin         NM 0000026         21.22           SAAI         Serum anyloid A2         NM 000026         21.22           SAA2         Serum anyloid A2         NM 000026         21.22           SAA2         Serum anyloid A2         NM 000036         6.32           USQUITA         Retaladehyde binding protein 1         NM 000374         20.88           SAA2         Serum anyloid A2         NM 00103         6.52           USAGUITA         Anotarini 3	CRLF1	Cytokine receptor-like factor 1	NM_004750	73.44
MAP2         Microtubule-associated protein 2         NM. 002374         62.64           PRODH         Proline dehydrogenase (oxidase) 1         NM. 002375         50.32           LON Peytidase N-terminal domain and ring finger 3         NM.00031855         30.84           ERICH2         Glutamate rich 2         XM.000714892         29.67           DMD         Dystrophin         NM.000031         26.65           CLDN20         Claudin 20         NM.000031         26.65           CLDN20         Claudin 20         NM.0000314         21.88           PLIN4         Pertipin 4         NM.000080400         21.64           RLBP1         Retinaldehyde binding protein 1         NM.0003754         20.98           PARD6B         Par-6 family cell polarity regulator beta         NM.032521         20.62           ANO3         Anoctarnin 3         NM.03744         20.88           RCN16         Pata-family cell polarity regulator beta         NM.03744         12.62           LOC284561         Uncharacterized LOC284561         XR.110828         16.52           USP33         Ubiquitin specific peptidase 53         NM.019050         16.15           PLCEL-AS1         PLCEL antisense RNA 1         NR.038433         14.44           PSO <td>ATP1A2</td> <td>ATPase, Na+/K+ transporting, alpha 2 polypeptide</td> <td>NM_000702</td> <td>69.84</td>	ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	NM_000702	69.84
PRODHProline dehydrogenase (oxidase) 1NML 01633550.32LONRP3LON peptidase N-terminal domain and ring finger 3NML 0001385530.34ENCH2Glutamate-rich 2XML 00017489229.67DMDDystrophinNNL 004003126.65CLDN20Claudin 20NML 00003126.65CLDN20Claudin 20NML 00003121.84DMDDystrophinNNL 00400121.84DMDPostrophinNML 00032621.22SAA2Serum amyloid A2NML 00032621.22SAA2Serum amyloid A2NML 00032621.52SAA2Serum amyloid A2NML 00032621.52SAA2Serum amyloid A2NML 00032621.52SAA2Serum amyloid A2NML 00032621.52LOC24561Uncharacterized LOC24561NML 00432117.61LOC24561Uncharacterized LOC24561NML 00432616.52USP33Ubiquitin specific peptidase 53NML 00436215.55LUC24561Uncharacterized LOC24561NML 00436215.66LUC24561ClauneginNML 00436215.65PLCELASPregnacy specific bert-legicoprotein 9NML 0043714.23PLGELASPregnacy specific bert-legicoprotein 9NML 0043915.65PLCELASPregnacy specific bert-legicoprotein 9NML 0043915.65PLCELASPregnacy specific bert-legicoprotein 9NML 0043915.65PLCELASPregnacy specific bert-legicoprotein 9NML 0043915.65 <td>MAP2</td> <td>Microtubule-associated protein 2</td> <td>NM_002374</td> <td>62.64</td>	MAP2	Microtubule-associated protein 2	NM_002374	62.64
LONRF3LON peptidase N-terminal domain and ring finger 3NM.0003185530.84ERICH2Glutamate-rich 2XM.00171489229.67DMDDystrophinNM.00003126.65CLDN20Clandin 20NM.000031424.91DMDDystrophinNM.0010314624.19DMDDystrophinNM.0010314624.19DMDDystrophinNM.0010314621.64RIBP1Retinaldchyde binding protein 1NM.0010346021.64SAA2Serua mayloid A2NM.03075420.98SAA2Serua mayloid A2NM.03075420.98ANO3Anotamin 3NM.03075420.98ANO3Anotamin 3NM.03075420.98ANO3Anotamin 3NM.03075420.98CNIfePotastimi inwardly rectifying channel, subfamily J, member 16NM.7074117.41LOC284561Uphquitin specific peptidase 53NM.0905016.15USP33Ubiquitin specific peptidase 53NM.0905016.15ICEI-ANIPCE1 antisense RNA 1NM.3343314.44PSGPPregnancy specific beta-1-glycoprotein 9NM.00274143.33CNHADAlkyin repeat domain 1(cardiac muscle)NM.01439113.58PDEAPlosphodiesterase 6A, CGMP-specific, rod, alphaNM.0009212.98REST2Bestrophin 2Scific and, alphaNM.0009212.98LICELASInosito Heakisphopshipa kinase 3NM.001661912.85LICELASInosito Heakisphopshipa kinase 4NM.00092	PRODH	Proline dehydrogenase (oxidase) 1	NM_016335	50.32
ERICH2         Glutamate-rich 2         XM.001714892         29.67           DMD         Dystrophin         NM.001001         2791           SAA1         Serum amyloid A1         NM.00103146         24.19           DMD         Dystrophin         NM.0010346         24.19           DMD         Dystrophin         NM.001080400         21.64           RLBP1         Retinaldehyde binding protein 1         NM.001080400         21.64           RADD8         Pars 6 family cell polarity regulator beta         NM.03526         21.22           SAA2         Serum amyloid A2         NM.0303754         20.98           RADD6         Pars 6 family cell polarity regulator beta         NM.031418         17.58           KCN16         Potassium inwardity rectifying channel, subfamily J. member 16         NM.70741         17.41           LOC284561         Uncharacterized LOC284561         NM.019050         16.15           LCE1 ASI         PLCE1 antisense RNA1         NR.033969         15.15           PLCE1 ASI         PLCE1 antisense RNA1         NR.033969         15.15           KLHDC7B         Kelch domain (cardiac muscle)         NM.401491         13.35           PDF6A         Phosphodiesterase 6A, GMP-specific, rod, alpha         NM.00092         12.98	LONRF3	LON peptidase N-terminal domain and ring finger 3	NM_001031855	30.84
DMDDystrophinNM.00401027.91SAAISerum anyloid A1NM.00100134624.19DMDDystrophinNM.00100134624.19DMDDystrophinNM.00100134624.18PLN4Perilipin 4NM.00032621.22SAA2Serum anyloid A2NM.00375420.98PARD6BPar-6 family cell polarity regulator betaNM.03075420.98PARD6BPar-6 family cell polarity regulator betaNM.03075420.98PARD6BPar-6 family cell polarity regulator betaNM.0314817.84KCNJ6Potassium inwardly rectifying channel, subfamily J, member 16NM.17074117.41LOC284561Ucharacterized LOC284561XR.10082016.57USP33Ubiquitin specific peptidase 53NM.01905016.47CLGNCalmeginNM.00456216.35USP33Ubiquitin specific peptidase 53NM.01905016.15PLCE1-ASIPLCE1-ASINM.0396915.15PLCE1-ASIPLCE1-ASINM.01905015.37KLHDC7BKelch domain containing 7BNM.03041013.38PSG9Prograncy specific beta-1-glycoprotein 9NM.01843314.44PSG9Prograncy specific beta-1-glycoprotein 9NM.00040113.58PDE6APhosphodisetrase 6A, CGMP-specific, rod, alphaNM.00040112.32ANKRD1Ankyrin repeat domain 1 (cardiac muscle)NM.01661912.85BEST2Bearophin 2NM.01661912.8512.52DNA2Prograncy	ERICH2	Glutamate-rich 2	XM_001714892	29.67
SAAI         Serum amyloid A1         NM.000331         26.65           CLDN20         Claudin 20         NM.00001346         24.19           DMD         Dystrophin         NN.0000326         21.22           RLBP1         Retinaldehyde binding protein 1         NM.000326         21.22           SAA2         Serum amyloid A2         NN.03074         20.98           PARD6B         Par-6 family cell polarity regulator beta         NM.03248         17.57           ANO3         Anoctamin 3         MJ.030418         17.58           ICC284561         Uncharacterized LOC284561         NL.10828         16.52           USP53         Ubiquitin specific peptidase 53         NM.019050         16.47           CLGN         Calmegin         NM.04362         16.35           USP53         Ubiquitin specific peptidase 53         NM.019050         16.15           PLCE1 Attisense RNA 1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NM.18433         14.44           PSG9         Pregnancy specific beta-lykoprotein 9         NM.0017449         13.23           COL4A4         Collagen, type IV, alpha 4         NL000092         12.85           PLACS         Plosophin 2         12.7	DMD	Dystrophin	NM_004010	27.91
CLDN20         Claudin 20         NML.001001346         24.19           DMD         Dystrophin         NML.00108140         21.83           PLIN4         Perilpin 4         NML.00108140         21.64           RLBP1         Retinaldehyde binding protein 1         NML.00108140         21.64           RLBP1         Retinaldehyde binding protein 1         NML.00108140         21.22           SAA2         Serum amyloid A2         NML.001074         20.98           PARD6B         Par-6 family cell polarity regulator beta         NML.0031418         17.54           ANO3         Anoctamin 3         NML.0010741         17.41           LOC248561         Uncharacterized LOC284561         NML.001050         16.47           CLGN         Calmegin         NML.002362         16.35           USP33         Ubiquitin specific peptidase 53         NML.009050         16.15           KLHDC7B         Kelch domain containing 7B         NML.038433         14.44           PSG9         Prognancy specific beta-1-glycoprotein 9         NML.00784         14.33           ERICH2         Glutamate-rich 2         XM.00114892         13.56           DPE6A         Plosphodiesterase 6A, GMP-specific, rod, alpha         NM.006401         13.23	SAA1	Serum amyloid A1	NM_000331	26.65
DMDDystrophinNM.00402121.88PLN4Perlipin 4NM.00008040021.64PLN4Perlipin 4NM.00008040021.64SLBP1Retinaldehyde binding protein 1NM.00075420.98PARD68Par-6 family cell polarity regulator betaNM.030252119.56ANO3Anoctamin 3NM.03141817.58KCN16Portassim inwardly rectifying channel, subfamily J, member 16NM.17074117.41LOC284561Uncharacterized LOC284561KR.11082816.52USP33Ubiquitin specific peptidase 53NM.01905016.47CLGNCalmeginNM.13843314.41PCE1-ASIPLCE1 antisense RNA 1NR.0396915.15PLCE1-ASIPLCE1 antisense RNA 1NR.0396915.35PLCE1-ASIPergnancy specific beta-1-glycoprotein 9NM.00278414.23PDE6APhosphodiesterase 6A, cGMP-specific, rod, alphaNM.00040013.23PDE6APhosphodiesterase 6A, cGMP-specific, rod, alphaNM.00064013.23PLACSPLACSPLACS12.7217.64PLACSPostenybin 2NM.01668112.85BEST2Destrophin 2NM.00571112.22PNAH2Orsonecoid 2NM.0060810.51PCK83Inoistio hexakisphosphate kinase 3NM.00541112.22PNAH2Orgenecoid 2NM.0060810.51PCK84Inoistio hexakisphosphate kinase 3NM.00641013.58PCG00COl200 moleculeNM.0006810.51 <td>CLDN20</td> <td>Claudin 20</td> <td>NM_001001346</td> <td>24.19</td>	CLDN20	Claudin 20	NM_001001346	24.19
PLIN4         Perifyn 4         NML.001080-000         21.64           RLBP1         Retinaldehyde binding protein 1         NML.000326         21.22           SAA2         Serum amyloid A2         NML.030754         20.98           PARD6B         Par-6 family cell polarity regulator beta         NML.032211         19.56           AN03         Anoctamin 3         NML.03178         17.87           KCN116         Potassium inwardly rectifying channel, subfamily J, member 16         NML.0010828         16.52           US253         Ubiquitin specific peptidase 53         NML.019050         16.47           CLGN         Calmegin         NML.00362         16.35           PLCE1-ASI         PLCE1 antiense RNA1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NML.0017482         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NML.0017482         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NML.001401         13.23           PDE6A         Phosphodiesterase 6A, CGMP-specific, rod, alpha         NML.004070         12.98           PLAC8         Placenta-specific 8         NML.001061         12.35           DST21         Bestrophin 2         NML.00	DMD	Dystrophin	NM_004021	21.88
RLBP1         Retinal dehyde binding protein 1         NM.000326         21.22           SAA2         Serum amyloid A2         NM.030754         20.98           PARD6B         Par 6 family cell polarity regulator beta         NM.032521         19.56           AN03         Anoctamin 3         NM.031418         17.578           KCN116         Potassium inwardly rectifying channel, subfamily J, member 16         NM.107741         17.41           LOC284561         XR.110828         16.52         15.35           USP33         Ubiquitin specific peptidase 53         NM.019050         16.47           CLGN         Calmegin         NM.03869         15.15           PLCE1-ASI         PLCE1 antisense RNA 1         NR.033969         15.15           PLCE1-ASI         Columanic containing 7B         NM.104302         13.66           ANKRD1         Ankyrin repeat domain (cardiare muscle)         NM.000714892         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiare muscle)         NM.010401         13.23           COL4A4         Collagen, type IV, alpha 4         NM.000714892         12.92           PDE6A         Phosphodiesterase 6A, CGMP-specific, rod, alpha         NM.007762         12.72           IP6K3         Inositol hexakisphosphate kinase 3 <td>PLIN4</td> <td>Perilipin 4</td> <td>NM_001080400</td> <td>21.64</td>	PLIN4	Perilipin 4	NM_001080400	21.64
SAA2         Serum anyloid A2         NM.030754         20,98           PARD68         Par-6 family cell polarity regulator beta         NM.032521         19:56           ANO3         Anoctamin 3         NM.0310741         17:41           LOC284561         Uncharacterized LOC284561         XR.110828         16:52           USP33         Ubiquitin specific peptidase 53         NM.09050         16:47           LGEN         Calmegin         NM.09050         16:45           VLPD7B         Kelch domain containing 7B         NM.09050         16:15           PLCE1 antisense RNA 1         NR.033969         15:15           SPG9         Pregnancy specific beta-l-glycoprotein 9         NM.0002784         14:23           ERICH2         Glutamate-rich 2         XM.001714892         13:56           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NM.002784         14:23           COL4A4         Collagen, type IV, alpha 4         NM.00092         12:98           PLACS         Placenta-specific 8         NM.016169         12:85           BEST2         Bestrophin 2         NM.020877         12:07           INHB8         Inhibitin, beta B         NM.020136         16:15           INHB2         Inhibitin, beta B<	RLBP1	Retinaldehvde binding protein 1	NM_000326	21.22
PARD6B         Par-6 family cell polarity regulator beta         NML.032521         19.56           ANO3         Anoctamin 3         NML.031418         17.58           KCN116         Potassium inwardly rectifying channel, subfamily J, member 16         NML.10828         16.52           UC284561         Ucharacterized LOC284561         KL.10828         16.52           USP53         Ubiquitin specific peptidase 53         NM.019050         16.47           CLGN         Calmegin         NM.04362         16.35           USP53         Ubiquitin specific peptidase 53         NM.019050         16.15           PLCE1-AS1         PLCE1 antisense RNA 1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NM.002784         14.23           ERICH2         Glutamate-rich 2         XM.001714892         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NM.002784         13.23           COLA4         Collagen, type IV, alpha 4         NM.000092         12.98           PLAC8         Placenta-specific 8         NM.01619         12.85           BEST2         Bestrophin 2         NM.00619         12.85           IPKA3         Inositol hexakisphosphate kinase 3         NM.002797         10	SAA2	Serum amyloid A2	NM_030754	20.98
ANO3         Anoctamin 3         NML.031418         17.58           KCNJ16         Potassium inwardly rectifying channel, subfamily J, member 16         NML.170741         17.41           LOC284561         Uncharacterized LOC284561         XR.110828         16.52           USP53         Ubiquitin specific peptidase 53         NM.019050         16.47           CLGN         Calmegin         NM.004362         16.35           USP53         Ubiquitin specific peptidase 53         NM.019050         16.15           PLCELASI         PLCEI antisense RNA 1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NM.1084333         14.44           PSG9         Pregnancy specific beta-1-glycoprotein 9         NM.002784         14.23           ERICH2         Glutamate-rich 2         XM.001714892         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NM.004400         13.23           COL4A4         Collagen, type IV, alpha 4         NM.0000410         13.23           COL4A4         Collagen, type IV, alpha 4         NM.000872         12.07           INKB         Inositol hexakisphosphate kinase 3         NM.0020877         12.07           INALB         Inhibin, beta B         NM.0020877 <td>PARD6B</td> <td>Par-6 family cell polarity regulator beta</td> <td>NM_032521</td> <td>19.56</td>	PARD6B	Par-6 family cell polarity regulator beta	NM_032521	19.56
KCNJ16         Portassium inwardly rectifying channel, subfamily J, member 16         NM.170741         17.41           LOC284561         Uncharacterized LOC284561         XR.110828         16.52           USP33         Ubiquitin specific peptidase 53         NM.019050         16.47           CLGN         Calmegin         NM.004362         16.35           USP33         Ubiquitin specific peptidase 53         NM.019050         16.15           PLCE1-AS1         PLCE1 antisense RNA 1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NM.108433         14.44           PSG9         Pregnacy specific beta-1-glycoprotein 9         NM.002784         14.23           ERICH2         Glutamate-rich 2         XM.001714892         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NM.014391         13.58           PDE6A         Phosphodiesterase 6A, cGMP-specific, rod, alpha         NM.000400         12.85           BEST2         Bestrophin 2         NM.017682         12.72           IPAG8         Inositol hexakisphosphate kinase 3         NM.020877         12.07           INHB         Inibitin, beta B         NM.002193         11.63           LOC100506544         Uncharacterized LOC100506544 <td>ANO3</td> <td>Anoctamin 3</td> <td>NM_031418</td> <td>17.58</td>	ANO3	Anoctamin 3	NM_031418	17.58
LOC284561         Uncharacterized LOC284561         XR_110828         16.52           USP53         Ubiquitin specific peptidase 53         NM_019050         16.47           CLGN         Calmegin         NM_004362         16.35           USP53         Ubiquitin specific peptidase 53         NM_019050         16.15           PLCE1-AS1         PLCE1 antisense RNA 1         NR_033969         15.15           KLHDC7B         Kelch domain containing 7B         NM_108433         14.44           PSG9         Pregnancy specific beta-1-glycoprotein 9         NM_002784         14.23           ERICH2         Glutamate-rich 2         XM_001714892         13.68           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NM_00014191         13.58           PDE6A         Phosphodiesterase 6A, GMP-specific, rod, alpha         NM_000092         12.98           PLAC8         Placenta-specific 8         NM_0006092         12.85           BEST2         Bestrophin 2         NM_0017682         12.72           DNAH2         Dynein, axonemal, heavy chain 2         NM_0020877         12.07           NHHB8         Inhibin, beta B         NM_0020877         12.07           NME125         Transmembrane protein 125         NM_104626         10.57 <td>KCNJ16</td> <td>Potassium inwardly rectifying channel, subfamily I, member 16</td> <td>NM_170741</td> <td>17.41</td>	KCNJ16	Potassium inwardly rectifying channel, subfamily I, member 16	NM_170741	17.41
USP53         Ukiquitin specific peptidaes 53         NM.019050         16.47           CLGN         Calmegin         NM.019050         16.35           USP53         Ukiquitin specific peptidaes 53         NM.019050         16.15           PLCE1-ASI         PLCE1 antisense RNA 1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NM.012784         14.23           ERICH2         Glutamate-rich 2         XM.001714892         13.66           ANKRD1         Ankyrin repeat domain (cardiac muscle)         NM.014391         13.58           PDE6A         Phosphodiesterase 6A, cGMP-specific, rod, alpha         NM.000092         12.98           PLAC8         Placenta-specific 8         NM.0107682         12.72           PA6A         Placenta-specific 78         NM.0107682         12.72           PLAC8         Placenta-specific 78         NM.0107682         12.72           IPA6X3         Inositol hexakisphosphate kinase 3         NM.017682         12.72           IPA6X3         Inositol hexakisphosphate kinase 3         NM.020877         10.9           TMEM125         Transmembrane protein 125         NM.140266         10.57           ORM2         Orosomucoid 2         NM.00103493         9.85 </td <td>LOC284561</td> <td>Uncharacterized LOC284561</td> <td>XR_110828</td> <td>16.52</td>	LOC284561	Uncharacterized LOC284561	XR_110828	16.52
CLONCompetinis period of the peri	USP53	Ubiquitin specific pentidase 53	NM_019050	16.47
Charley Construction         NML 019050         16.15           USP53         Ubiquitin specific peptidase 53         NML 019050         16.15           PLCE1 ASI         PLCE1 antisense RNA 1         NR.033969         15.15           KLHDC7B         Kelch domain containing 7B         NML 002784         14.23           PSG9         Pregnancy specific beta-1-glycoprotein 9         NML 002784         14.23           ERICH2         Glutamate-rich 2         XM.001714892         13.66           ANKRD1         Ankyrin repeat domain 1 (cardiac muscle)         NM.014391         13.58           PDE6A         Phosphodiesterase 6A, cGMP-specific, rod, alpha         NML000092         12.98           PLAC8         Placenta-specific 8         NML.01619         12.85           BEST2         Bestrophin 2         NM.016282         12.72           IP6K3         Inositol hexakisphosphate kinase 3         NML020877         12.07           INHBB         Inhibin, beta B         NML002193         11.63           LOC100506544         Uncharacterized LOC100506544         AK057177         10.9           TMEMD25         Transmembrane protein 125         NM.144626         10.57           ORM2         Orosomucoid 2         NM.0000608         10.51	CLGN	Calmegin	NM 004362	16.35
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KCNB1         Potassium voltage-gated channel_Shab-related subfamily_member 1         NM 004975         8 97	GATA3	GATA binding protein 3	NM 001002295	9.03
10003000000000000000000000000000000000	KCNB1	Potassium voltage-gated channel. Shab-related subfamily member 1	NM_004975	8.97

 TABLE 3: The list of upregulated genes in MSX1-knockdown cells (top 50).

TABLE 3:	Continued.
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Gene symbol	Gene name	Gene ID	Fold change
OCA2	Oculocutaneous albinism II	NM_000275	8.90
PDZRN4	PDZ domain containing ring finger 4	NM_013377	8.86

TABLE 4: The WikiPathways analysis selected gene sets significantly downregulated in MSX1-knockdown cells.

Ranking	Pathway	P value	Gene counts (gene number of pathway)
1	Focal adhesion	4.20E - 09	36 (188)
2	IL-4 signaling pathway	2.55E - 05	13 (55)
3	Endochondral ossification	6.27E - 05	14 (64)
4	Muscle cell tarbase	7.95E - 05	41 (336)
5	Integrin-mediated cell adhesion	8.22E - 05	18 (99)
6	Matrix metalloproteinases	9.72E - 05	9 (31)
7	Regulation of toll-like receptor signaling pathway	1.12E - 04	22 (150)
8	Calcium regulation in the cardiac cell	1.30E - 04	23 (149)
9	MicroRNAs in cardiomyocyte hypertrophy	3.35E - 04	15 (105)
10	Insulin signaling	4.14E - 04	23 (161)

TABLE 5: The WikiPathways analysis selected gene sets significantly upregulated in MSX1 knockdown cells.

Ranking	Pathway	P value	Gene counts (gene number of pathway)
1	SREBP signalling	< 1.00E - 44	24 (50)
2	Lymphocyte tarbase	< 1.00E - 44	78 (420)
3	Cholesterol biosynthesis	1.86E - 17	16 (17)
4	Muscle cell tarbase	1.78E - 10	65 (336)
5	Epithelium tarbase	9.47E - 10	52 (278)
6	Folate metabolism	1.47E - 09	22 (68)
7	Adipogenesis	3.19E - 08	30 (131)
8	Leukocyte tarbase	1.47E - 07	28 (128)
9	Fatty acid biosynthesis	1.73E - 06	10 (22)
10	SREBF and miR33 in cholesterol and lipid homeostasis	8.09E - 06	8 (18)

including C3H10T1/2 cells and aorta myofibroblasts [30–32]. MSX1 and MSX2 activate aortic adventitial osteoprogenitors via overlapping yet distinct mechanisms [33]. MSX2, unlike MSX1, enhances *OSX* expression without an increase in *RUNX2* expression in aortic myofibroblasts [30], suggesting distinct actions of MSX1 and MSX2 in osteoblast differentiation.

The molecular mechanism by which MSX1 activates the differentiation program remains unclear. MSX1 regulates transcriptional activity of target genes either by directly binding to the specific DNA MSX1-binding motif (C/GTAATTG) or through interactions with other transcriptional regulators. Interestingly, MSX1 binds to various transcriptional regulators, including Sp1, Sp3, Dlx3, Dlx5, PAX3, PAX9, BarH-like homeobox 1/BARX1, and PIAS1 [34–37]. Depending on the partner in the complex, MSX1 activates or represses transcription in the MSX1-interacting network of transcription factors [26, 38]. Moreover, MSX1 modifies chromatin structure near target genes by histone methylation [35, 39]. The interactions of MSX1 with various transcriptional regulators may account for the extensive changes in the expression levels of many genes (~6400) by *MSX1* knockdown.

Statins, drugs for hyperlipidemia, enhance osteogenic differentiation of various mesenchymal cells, including osteoblast precursor cells, mesenchymal stem cells, and DPSCs, by inhibiting the synthesis of farnesyl pyrophosphate, decreasing cellular cholesterol, and activating the Ras-PI3K-Akt/MAPK signaling pathway, thereby increasing the expression of BMP2 and RUNX2 [17], although the underlying mechanisms are still controversial. Statins also suppress osteoclast function and enhance mandibular bone formation in vivo [40]. Interestingly, a previous study showed that simvastatin induces odontoblast differentiation of hDPSCs in vitro and in vivo [22]. However, no studies have shown the involvement of transcription factor(s) in the control of cholesterol synthesis during osteoblast differentiation. Here we found for the first time that MSX1 suppresses the entire cholesterol synthesis pathway in osteoblast differentiating hDPSCs by repressing SREBP2 and other related genes. This suppression of cholesterol synthesis may facilitate osteoblast



FIGURE 5: Effects of *MSX1* knockdown on the expression of cholesterol synthesis-related genes, which are direct targets for SREBP2, in hDPSCs. (a) The cholesterol biosynthesis pathway is shown and the bold frames indicate target genes for the master transcriptional factor SREBP2. Microarray analysis indicates all genes involved in cholesterol synthesis are upregulated by *MSX1* knockdown. The numbers in brackets represent the fold changes in gene expression in *MSX1*-knockdown cells as compared with the control cells. (b) The mRNA levels of genes relevant to cholesterol synthesis, including *SREBP2*, *HMGCS1*, *HMGCR*, *FDPS*, *CYP51A1*, and *DHCR7*, were quantified by RT-qPCR analysis. Values are averages  $\pm$ SD for three cultures. \*\* *P* < 0.001; \*\*\* *P* < 0.001.

differentiation. It is also interesting to note that various mutations in the cholesterol synthesis pathway, including 7-dehydrocholesterol reductase (*DHCR7*), cause craniofacial anomalies including cleft palate, suggesting the role of cholesterol synthesis in craniofacial development [41].

In conclusion, here we revealed for the first time that MSX1 is indispensable for osteoblast-like differentiation and calcification in hDPSCs derived from deciduous teeth. Furthermore, MSX1 was found to modulate a wide variety of genes, including cholesterol synthesis-related genes, during osteogenic differentiation of hDPSCs. We have not examined the effects of MSX1 siRNA in definitive teeth, although MSX1 may also function as a positive regulator of osteogenesis in definitive teeth as MSX1 mRNA levels are high in both definitive and deciduous teeth [15]. Our findings will provide new insights into the role of MSX1 in development and repair of teeth and may be useful in DPSC-based regenerative therapy.

#### **Competing Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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