

Msx1 regulates proliferation and differentiation of mouse dental mesenchymal cells in culture

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The homeobox, msh-like 1 (MSX1) protein is essential for cell proliferation and differentiation. Tooth germ development of *Msx1* knockout mouse is arrested at the bud stage, impeding an understanding of its role beyond this stage of tooth development. The aims of this study were to investigate the potential role of MSX1 in the regulation of proliferation and differentiation of dental mesenchymal cells in culture, and to preliminarily explore its underlying mechanism of action. Tooth germs were isolated from embryonic day (E)15.5 mice. The mesenchyme was separated and digested into a single-cell suspension, and then cultured in vitro. Isolated dental mesenchymal cells were transfected with MSX1 small interfering RNA, and the effects on cell proliferation, cell cycle distribution, and the expression of bone morphogenetic protein 2 (*Bmp2*) and bone morphogenetic protein 4 (*Bmp4*) were studied. We also compared the expression levels of alkaline phosphatase (*Alp*), type I collagen (*Col1A*), osteocalcin (*Ocn*), runt-related transcription factor 2 (*Runx2*), dentin sialophosphoprotein (*Dspp*) and dentin matrix protein 1 (*Dmp1*), and mineralized nodule formation, between control and MSX1 siRNA-transfected groups after the induction of odontoblast differentiation. Knockdown of *Msx1* expression was associated with decreased cell proliferation, prolonged time in the S phase of the cell cycle, enhanced odontoblast differentiation, and elevated *Bmp2* and *Bmp4* expression. We conclude that MSX1 may promote proliferation and prevent the differentiation of dental mesenchymal cells by the inhibition of *Bmp2* and *Bmp4* expression.

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The tooth germ is viewed as an excellent model for studying the sequential and complex regulatory mechanisms of early organogenesis (1, 2). Tooth germ development is regulated at the spatial and temporal levels, and each stage has different regulatory systems (3). Classical epithelial and mesenchymal tissue reorganization experiments have revealed that before the bud stage, the potential for tooth formation resides in the prospective dental epithelium (4), and after the bud stage this potential shifts to the dental mesenchyme (5).

Msx genes are homeobox genes that encode transcription factors which play an important role during vertebrate organogenesis (6). Homeobox, msh-like 1 (*Msx1*) and homeobox, msh-like 2 (*Msx2*) are known to be expressed in tooth germs during tooth development (7). Specifically, *Msx1* is strongly expressed in the dental mesenchyme and is excluded from the dental epithelium throughout all stages of odontogenesis, with its expression reaching a peak in the dental mesenchyme during the cap stage and progressively declining during the bell stage before odontoblast differentiation (8). It has been reported that MSX1 is essential for

mediating craniofacial bone and tooth development and that homozygous *Msx1* mutations lead to tooth agenesis in mice (9). *Msx2* is first expressed in the dental mesenchyme at the bud stage, and then expression shifts to the dental epithelium in the molar region (10). As tooth-formation potential shifts to the dental mesenchyme after the bud stage, MSX1 may play a critical role in the regulation of tooth germ development after this stage.

The MSX1 protein functions as a transcriptional regulator that controls cellular proliferation and differentiation during embryonic development (11–13). It has been proposed that MSX1 expression is required to maintain multiple cells, including dental mesenchymal cells, in a proliferative and undifferentiated state (13–15). The role of MSX1 in regulating tooth germ development has primarily been demonstrated in *Msx1* knockout mice, in which tooth development is arrested at the bud stage (16). Previous research has shown that during the bud stage, bone morphogenetic protein 2 (*Bmp2*) and bone morphogenetic protein 4 (*Bmp4*) are downstream targets of MSX1 in the dental epithelium

and mesenchyme, respectively (16–19). Interestingly, *Bmp2* expression shifts to the dental mesenchyme at the cap stage (20). It has been demonstrated that BMP4 is an important effector of MSX1 during tooth morphogenesis, and that MSX1 is essential for odontoblast and dental pulp survival (21). Recently, Jia *et al.* (22) reported that the synergistic action of BMP4 and MSX1 activates mesenchymal odontoblast differentiation potential during maxillary molar morphogenesis and sequential tooth formation; however, the regulatory roles of MSX1 in stages beyond the bud stage are still unclear.

The fact that *Msx1* is strongly expressed in the dental mesenchyme, and that tooth-formation potential shifts from the epithelium to the dental mesenchyme after the bud stage, led us to hypothesize that MSX1 may play an important role in the later stages of tooth development. Previous studies have demonstrated that MSX1 regulates the proliferation and differentiation of multiple cell types (13–15), and thus MSX1 might participate in the regulation of tooth-germ development through regulating dental mesenchymal cell proliferation and odontoblast differentiation. Although traditional gene-knockout techniques are important for analysing gene function during organ development, they are expensive and time consuming. Additionally, in the case of tooth development, it is difficult to analyse the potential roles of MSX1 beyond the bud stage because tooth germ development is arrested at this stage (16). Recently, small interfering RNA (siRNA) has been successfully used to knock down target gene expression to study gene function in mammalian cells (23, 24). Thus, by using siRNA to selectively knock down *Msx1* expression in dental mesenchymal cells *in vitro*, we attempted to elucidate whether MSX1 could regulate the proliferation dental mesenchymal cells and their differentiation into odontoblasts.

Material and methods

Animals

Pregnant ICR mice were purchased from the Department of Laboratory Animal Science of Peking University Health Science Centre. All animals were handled in accordance with Institutional and National guidelines for the care and use of laboratory animals. The protocol was approved by Peking University Animal Ethics Committee (LA2012-58).

Cell culture

Dental mesenchymal cells were isolated and cultured at embryonic day 15.5 (E15.5), when the tooth germ of fetal mouse is at the cap stage, and dental mesenchyme, which contains dental papilla and dental follicle, is undifferentiated. A stereo microscope (Olympus, Tokyo, Japan) was used to dissect tooth germs from embryos. At E15.5, the mandibular second molar at the distal part of the mandibular first molar has entered into the bud stage, and is easily identifiable under a stereo microscope. The mandibular second molar and surrounding tissues were carefully

removed using a fine needle to avoid tissue contamination (Supporting Fig. S1). For each experiment, dental mesenchymal cells were isolated from 25–30 mandibular first molar germs and cultured *in vitro*. In brief, the isolated tooth germs were incubated in 1.5 mg ml⁻¹ of dispase II (Roche, Mannheim, Germany) for 20 min at 37°C to separate the epithelium and mesenchyme. The isolated mesenchyme was then digested with 0.25% trypsin (Gibco-Invitrogen, Carlsbad, CA, USA) at 37°C for 10 min, and dissociated into a single-cell suspension by gentle pipetting. Approximately 1 × 10⁶ cells were plated in a 60-mm dish and cultured in high-glucose Dulbecco's modified Eagle's minimum essential medium (DMEM), supplemented with 15% fetal bovine serum (FBS; Gibco), at 37°C and 5% CO₂. Cells passaged two or three times were used for our siRNA transfection study described below.

Msx1 gene knockdown in dental mesenchymal cells

To determine the efficiency of our siRNA transfection, red fluorescent control siRNA (BLOCK-iT Alexa Fluor Red Fluorescent Control; Invitrogen) was transfected into dental mesenchymal cells using Lipofectamine RNAiMAX (Invitrogen). The nucleotide sequences of the *Msx1* siRNA were as follows: 5'-UCU UCU GGC AGC UUG AGG AGU CCU C-3' (sense) and 5'-GAG GAC UCC UCA AGC UGC CAG AAG A-3' (antisense). Stealth RNAi siRNA Negative Control Med GC (Invitrogen) was used as a scrambled siRNA control, and cells transfected with this scrambled siRNA were used as the experimental control group.

Dental mesenchymal cells were transfected using RNA interference (RNAi) duplex–Lipofectamine RNAiMAX complexes according to the manufacturer's protocol. In brief, RNA interference (RNAi) duplexes (24-well plate, 30 pmol per well; six-well plate, 150 pmol per well; 60-mm dish, 300 pmol) were diluted in Opti-MEM I Medium (24-well plate, 100 µl per well; six-well, 500 µl per well; 60-mm dish, 1,000 µl) and then gently mixed in a RNase-free centrifuge tube. Lipofectamine RNAiMAX (24-well plate, 1.5 µl per well; six-well plate, 7.5 µl per well; 60-mm dish, 15 µl) was then added to the centrifuge tube containing the diluted RNAi molecules, mixed gently and incubated for 20 min at room temperature. Finally, the RNAi duplex–Lipofectamine RNAiMAX complexes were added to the cell suspension and incubated at 37°C in a CO₂ incubator.

RNA isolation, RT, and real-time PCR

Cells were harvested and total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Isolated RNA was purified by removing genomic DNA with a DNase I, RNase-free kit (Fermentas, Glen Burnie, MD, USA). One microgram of total RNA from each group (control and *Msx1* siRNAs) was used for synthesis of cDNA using the AMV Reverse Transcriptase kit (Fermentas), according to the manufacturer's protocol. Semiquantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) with SYBR Green (Roche). All samples were run in triplicate in 96-well plates, with each well containing 1.0 µl of cDNA diluted 1 in 20 to give a total reaction volume of 20 µl. Reactions were performed at 50°C for 2 min and then at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The real-time PCR primers are presented in Table 1. For data analysis, the levels of target

gene expression in samples relative to the level of expression in the control samples were calculated using the comparative cycle threshold method ($\Delta\Delta CT$). The expression levels of target gene expression were normalized to the expression of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Western blotting

The levels of expression of MSX1, BMP2, and BMP4 proteins were measured by western blotting. Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Appligen, Beijing, China), according to the manufacturer's instructions. Protein levels were calculated using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Beijing, China). Equal amounts of protein samples were separated by electrophoresis through a 12% SDS polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Blots were blocked with 5% skim milk, followed by incubation with the following primary antibodies: rabbit anti-BMP2, rabbit anti-BMP4 (both Abcam, Hong Kong, China), mouse anti-MSX1 (Abcam), and mouse anti-GAPDH (Abmart, Shanghai, China). Blots were then incubated with goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Origene, Beijing, China) and visualized by enhanced chemiluminescence (Appligen).

Table 1
Real-time PCR primer sequences

Gene symbol	Primer sequence
<i>Gapdh</i>	Forward 5'-AATGGATTGGACGCATTGGT-3' Reverse 5'-TTTGCACCTGGTACGTGTTGAT-3'
<i>CcnA</i>	Forward 5'-TGGATGGCAGTTTGAATCACC-3' Reverse 5'-CCCTAAGGTACGTGTGAATGTC-3'
<i>Cdk2</i>	Forward 5'-CCTGCTTATCAATGCAGAGGG-3' Reverse 5'-TGCGGGTACCATTTTCAGC-3'
<i>Alp</i>	Forward 5'-CCAACTCTTTGTGCCAGAGA-3' Reverse 5'-GGCTACATTGGTGTGAGCTTTT-3'
<i>Col1A</i>	Forward 5'-GGAGGGCAATAAGGTAGTGAA-3' Reverse 5'-CCATAGATGCGTTTGTAGGC-3'
<i>Ocn</i>	Forward 5'-GGAGGGCAATAAGGTAGTGAA-3' Reverse 5'-CCATAGATGCGTTTGTAGGC-3'
<i>Dspp</i>	Forward 5'-ATTCCGGTTCCCCAGTTAGTA-3' Reverse 5'-CTGTTGCTAGTGGTGCTGTT-3'
<i>Dmp1</i>	Forward 5'-CATTCTCCTTGTGTTCCCTTTGGG-3' Reverse 5'-TGTGGTCACTATTTGCCTGTG-3'
<i>Runx2</i>	Forward 5'-AGAGTCAGATTACAGATCCAGG-3' Reverse 5'-AGGAGGGGTAAGACTGGTCATA-3'
<i>Msx1</i>	Forward 5'-TGCTGCTATGACTTCTTTGCC-3' Reverse 5'-GCTTCCTGTGATCGGCCAT-3'
<i>Bmp2</i>	Forward 5'-TGGAAGTGGCCATTAGAGG-3' Reverse 5'-CTGTGTTTCATCTTGGTGCAAAG-3'
<i>Bmp4</i>	Forward 5'-GTATCTGGTCTCCGTCCCTG-3' Reverse 5'-GTCGCTCCGAATGGCACTA-3'

Alp, alkaline phosphatase; *Bmp2*, bone morphogenetic protein 2; *Bmp4*, bone morphogenetic protein 4; *CcnA*, cyclin A; *Cdk2*, cyclin-dependent kinase 2; *Col1A*, type I collagen; *Dmp1*, dentin matrix protein 1; *Dspp*, dentin sialophosphoprotein; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Msx1*, homeobox, msh-like 1; *Ocn*, osteocalcin; *Runx2*, runt-related transcription factor 2.

Cell-proliferation assays

A Cell-Light EdU Imaging Detection kit (Ribo-Bio, Guangzhou, China) was used to measure cell proliferation, according to the manufacturer's instructions. Cells were incubated with 5-ethynyl-2'-deoxyuridine (EdU) 24, 48, and 72 h after transfection, in a 24-well plate for 2 h then fixed with 4% paraformaldehyde at room temperature for 30 min. Next, 0.2% Triton X-100 was added and incubated for 10 min; the cells were then incubated with Apollo 550 fluorescent azide reaction buffer for 30 min and stained with 5 mg ml⁻¹ of Hoechst (Ribo-Bio) for 30 min at room temperature. Images were taken and analysed using High Content Imaging Pathway 855 software (BD, Franklin Lakes, NJ, USA). The percentage of EdU-positive cells was calculated as a fraction of the total number of Hoechst-stained cells.

Cell-cycle assay

Our cell-cycle distribution assay was performed by flow cytometry (FACS CantoII; BD). Briefly, after transfection for 24, 48, and 72 h, cells were harvested and fixed in 70% ethanol overnight at 4°C, centrifuged, and the supernatant was removed. The cells were then washed with PBS and incubated with RNase A, DNase, and protease-free solutions (Fermentas) for 30 min to remove RNA. Next, the cells were stained with propidium iodide (50 mg ml⁻¹), and flow cytometry was used to detect the cell-cycle phase of control and *Msx1* siRNA-transfected cells, according to the manufacturer's instructions. To examine the cell cycle in more detail, real-time PCR was used to detect the expression of the S-phase-related genes, cyclin A (*CcnA*) and cyclin-dependent kinase 2 (*Cdk2*) (see Table 1 for primer sequences).

Induction of odontoblast differentiation

Our analysis of odontoblast differentiation induction included real-time PCR, an alkaline phosphatase (ALP) activity assay, and Alizarin Red staining and quantification. In order to induce odontoblast differentiation, 24 h after transfection, the regular culture medium was changed to odontoblast differentiation medium (high-glucose DMEM supplemented with 10% FBS, 10 nmol l⁻¹ dexamethasone, 10 mmol l⁻¹ β-glycerophosphate, and 50 mg ml⁻¹ L-ascorbic acid phosphate), and the medium was changed every 3 d thereafter.

Real-time PCR

Seven days after the induction of odontoblast differentiation, cells from both groups were harvested for total RNA isolation, cDNA synthesis, and real-time PCR. The levels of mRNA expressed by the following genes were studied: *Alp*, type I collagen (*Col1A*), osteocalcin (*Ocn*), runt-related transcription factor 2 (*Runx2*), dentin sialophosphoprotein (*Dspp*), and dentin matrix protein 1 (*Dmp1*) (see Table 1 for primer sequences).

Alkaline phosphatase

Seven days after induction of odontoblast differentiation, total protein was extracted and ALP activity was measured using an ALP detection kit (Nanjing Jiancheng, Nanjing, Jiangsu, China), according to the manufacturer's

instructions. In brief, a 100- μ l aliquot of freshly prepared p-nitrophenyl-phosphate solution (p-Nitrophenyl Phosphate, Disodium Salt; Nanjing Jiancheng) was added to 200 μ l of protein mixed with Tris/glycin/Triton buffer and incubated for 30 min at 37°C. The optical density of p-nitrophenol at 405 nm was measured by spectrophotometry, and calculated ALP activity was expressed as units (U) per milligram of protein.

Alizarin Red staining

Fourteen days after the induction of odontoblast differentiation in six-well plates, cells of both groups were fixed with 4% paraformaldehyde and incubated at room temperature for 15 min, rinsed three times with PBS, and stained with Alizarin Red solution at room temperature for 20 min. Finally, the cells were rinsed three times with PBS and digital images were collected. To quantify Alizarin Red staining, 10% acetic acid was added to each well and incubated for 30 min. Next, the cells and acetic acid were vortexed for 30 s and heated to 85°C for 10 min. After cooling, the slurry was centrifuged at 20,000 *g* for 15 min, and the supernatant was collected. The supernatant was then transferred to a 96-well plate, and the absorbance was measured at 405 nm by spectrophotometry.

Statistical analysis

Statistical analysis was performed using SPSS software, version 15.0 (SPSS, Chicago, IL, USA). A *P* value of <0.05 was considered to indicate a statistically significant difference between experimental groups. Continuously distributed data were expressed as mean \pm SD and analysed by one-way ANOVA or the paired *t*-test. A chi-square analy-

sis was performed to compare percentages. All experiments were repeated at least three times.

Results

RNAi transfection and knockdown efficiency

The transfection efficiency was assessed by red fluorescence 24 h after transfection with the scrambled siRNA. The highest transfection efficiency (~90%) of dental mesenchymal cells grown in a 24-well plate was achieved by transfection with 30 pmol of scrambled siRNA and 1.5 μ l of RNAiMAX (Fig. 1A). To determine the time point at which the knockdown of *Msx1* was highest, the mRNA and protein expression levels of *MSX1* were assessed 24, 48, and 72 h after transfection with *Msx1* siRNA. The mRNA knockdown efficiency was highest at 24 h (Fig. 1B), and the protein knockdown efficiency was highest at 48 h (Fig. 1C).

Knockdown of *Msx1* inhibits dental mesenchymal cell proliferation

After transfection with *Msx1* siRNA, cell proliferation was assayed at 24, 48, and 72 h. We found that at each time point the number of proliferating cells observed in the *Msx1* siRNA group was lower compared with the number of proliferating cells in the control group (Fig. 2A). Quantitative analysis of cell proliferation confirmed that the rate of cell proliferation was signifi-

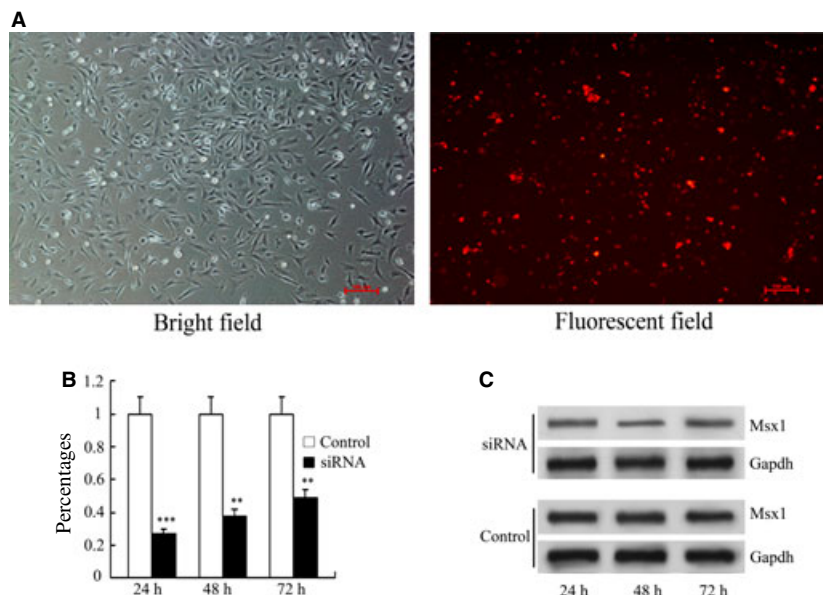


Fig. 1. Transfection efficiency of small interfering RNA (siRNA) and homeobox, msh-like 1 (*Msx1*) knockdown efficiency at various time points. (A) Representative photographs of transfected dental mesenchymal cells: (a) bright field microscopy image; (b) fluorescence microscopy image (scale bar for both images = 100 μ m). Quantification of *Msx1* knockdown at the mRNA (B) and protein (C) levels is also shown 24, 48, and 72 h after transfection with control and *Msx1* siRNAs. The knockdown efficiency at 24, 48, and 72 h was 27, 38, and 49%, respectively, at the mRNA level. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ***P* < 0.01; ****P* < 0.001.

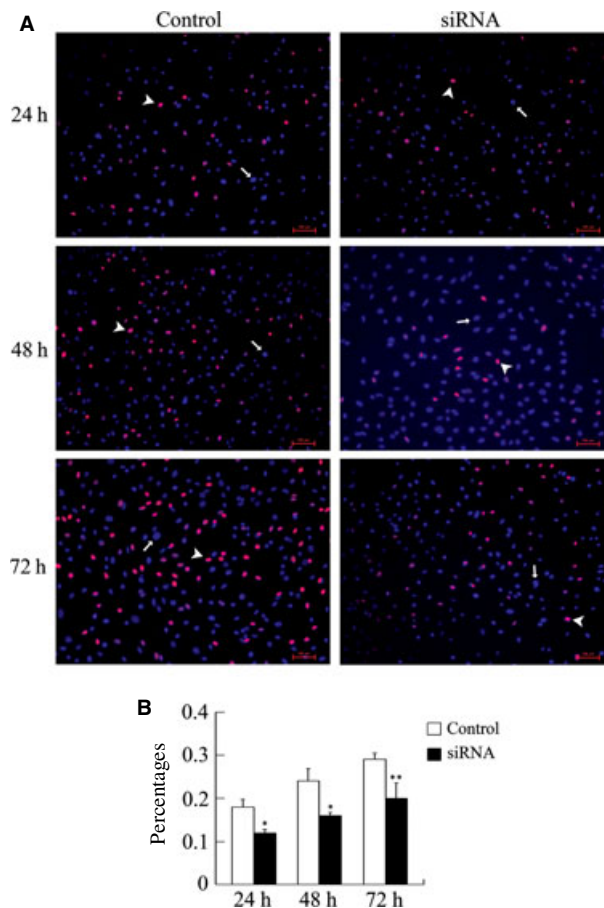


Fig. 2. Effect of homeobox, msh-like 1 (*Msx1*) gene knockdown on dental mesenchymal cell proliferation. (A) Representative photographs of control and *Msx1* small interfering RNA (siRNA)-transfected cells, 24, 48, and 72 h after transfection, are shown following incorporation of 5-ethynyl-2'-deoxyuridine (EdU) to assess cell proliferation. Arrowheads, EdU-positive cells; arrows, Hoescht-stained cells; scale bars = 100 μ m. (B) Quantification of the rate of cell proliferation in control and *Msx1* siRNA-transfected cells are plotted 24, 48, and 72 h after transfection. The rate of cell proliferation in the control group at 24, 48, and 72 h was 18, 24, and 29%, respectively, while the rate in the *Msx1* siRNA group at each time point was 12, 16, and 20%, respectively. The rate of cell proliferation of the *Msx1* siRNA group at each time point was significantly lower than that of the control group. * $P < 0.05$; ** $P < 0.01$ (chi-square test).

cantly lower at each time point in the *Msx1* siRNA group (Fig. 2B). These results indicate that MSX1 might promote dental mesenchymal cell proliferation under physiological conditions.

Knockdown of *Msx1* prolongs the S phase of the cell cycle in dental mesenchymal cells

The effect of *Msx1* knockdown on the cell cycle of dental mesenchymal cells was also studied. Analysis by flow cytometry demonstrated that the *Msx1* siRNA group exhibited a significantly higher proportion of cells in the S phase and a lower proportion of cells in

the G1 phase, compared with the control group at 24, 48, and 72 h after transfection (Fig. 3A). There was no significant difference in the number of cells in the G2 phase between the two groups at each time point. Furthermore, the mRNA expression levels of the S-phase-related genes, *CcnA* and *Cdk2*, were significantly reduced in the *Msx1* siRNA group compared with the control group at 24, 48, and 72 h after transfection (Fig. 3B). Taken together, these results indicate that MSX1 may participate in regulating cell cycle progression of dental mesenchymal cells, and that *Msx1* knockdown leads to S-phase arrest.

Knockdown of *Msx1* promotes odontoblast differentiation of dental mesenchymal cells

The effect of *Msx1* knockdown on the differentiation of dental mesenchymal cells into odontoblasts was studied next. The results of our real-time PCR analysis revealed that the expression levels of the early and late odontoblast differentiation markers *Alp* and *Ocn* were significantly increased in the *Msx1* siRNA group, as was the expression of *Coll1*, which is the main component of the dentin collagenous protein framework. Additionally, the expression levels of markers essential for dentin mineralization – *Runx2*, *Dspp*, and *Dmp1* – were significantly increased compared with the control group (Fig. 4A). In agreement with our gene-expression data, *Alp* activity was significantly higher in the *Msx1* siRNA group compared with the control group (data not shown). Small, round, Alizarin Red-positive mineralized nodules were observed 14 d after the induction of differentiation. There appeared to be more pronounced mineralization in the *Msx1* siRNA group (Fig. 4B), which was verified by our quantitative analysis of Alizarin Red staining (data not shown). Based on these results, we speculate that MSX1 might inhibit the differentiation of dental mesenchymal cells into odontoblasts under physiological conditions.

Knockdown of *Msx1* promotes *Bmp2* and *Bmp4* expression in dental mesenchymal cells

In order to gain insight into the mechanistic action of *Msx1* knockdown, we assessed the gene and protein expression levels of BMP2 and BMP4 at 24, 48, and 72 h after transfection with scrambled and *Msx1* siRNAs. Our results show that knockdown of *Msx1* expression was accompanied by a significant increase in the levels of *Bmp2* and *Bmp4* mRNA (Fig. 5A) and protein (Fig. 5B) expressed. Based on these results, we speculate that MSX1 inhibits *Bmp2* and *Bmp4* expression, at both mRNA and protein levels, in dental mesenchymal cells.

Discussion

It has previously been reported that MSX1 regulates cell proliferation during embryogenesis (12, 25, 26) and that its action, through multiple signalling pathways,

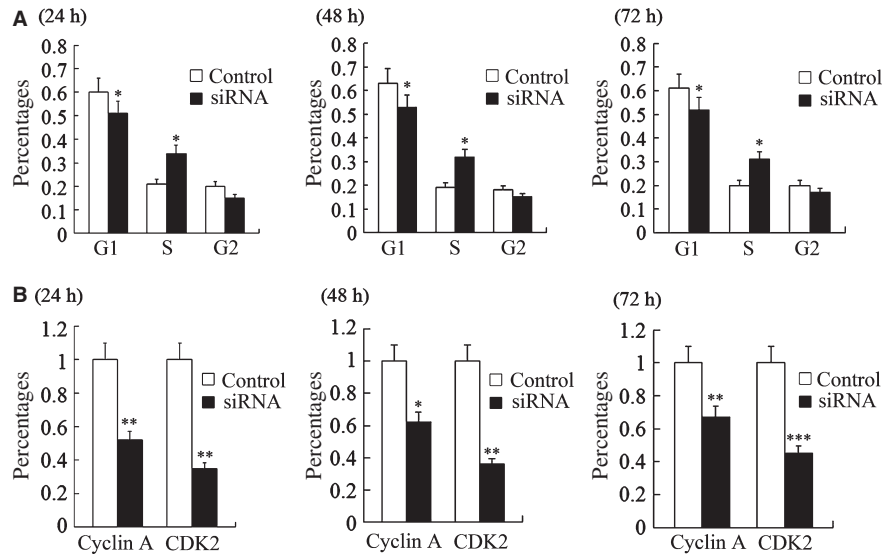


Fig. 3. Effect of homeobox, msh-like 1 (*Msx1*) knockdown on progression of the cell cycle in dental mesenchymal cells. (A) Analysis of the cell cycle in control and *Msx1* small interfering RNA (siRNA)-transfected cells, 24, 48, and 72 h after transfection, showing that cells with decreased *Msx1* expression have a prolonged S phase and spend less time in the G1 phase. (B) Real-time PCR analysis showed downregulated expression of the S-phase-related genes, cyclin A (*CcnA*) and cyclin-dependent kinase 2 (*Cdk2*), in *Msx1* siRNA-transfected cells compared with control cells 24, 48, and 72 h after transfection. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

promotes the proliferation of dental mesenchymal cells, thus influencing tooth size (27). It has also been reported that MSX1 regulates the cell cycle and pro-

motes the proliferation of neural crest cells during odontogenesis by downregulating CDK inhibitor p19^{INK4d} activity (15). In this study, we found that MSX1 knockdown inhibited the proliferation of dental mesenchymal cells, implying that MSX1 promotes the proliferation of dental mesenchymal cells under physiological conditions.

The MSX1 protein is known to regulate the cell cycle during embryogenesis by upregulating expression of cyclin D1; thus, by preventing exit from the cell cycle, MSX1 inhibits the terminal differentiation of progenitor cells (28). Forced expression of MSX1 in the human ovarian cancer cell line, OVCAR3, markedly increases the duration of the G1 phase, thus suppressing cell proliferation (29). Furthermore, expression of cyclin A is significantly reduced in the tooth germs of *Msx1* knockout mice, indicating S-phase arrest of dental mesenchymal cells (15). In this study, we demonstrated that *Msx1* knockdown significantly prolonged the S phase of dental mesenchymal cells and reduced the expression of the S-phase-related genes, *CcnA* and *Cdk2*. Thus, *Msx1* knockdown-decreased cell proliferation is associated with S-phase arrest.

It is generally believed that *Msx1* is expressed in multiple undifferentiated tissue types and inhibits cell differentiation. When tooth germs enter differentiation, *Msx1* expression decreases (8). In the myogenic lineage, *Msx1* is expressed in myogenic precursors during development but is not expressed in differentiated myotubes (30), and forced expression of MSX1 inhibits osteogenic and myogenic differentiation (28). In this study, we demonstrated that downregulated *Msx1* expression could promote the expression of *Alp*, *Col1A1*, *Ocn*, *Runx2*, *Dspp*, and *Dmp1*, and increase ALP activity and mineralized nodule formation. Through modulat-

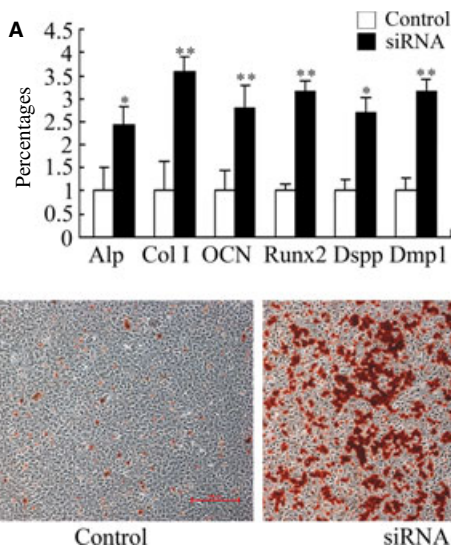


Fig. 4. Odontoblast differentiation potential of dental mesenchymal cells was increased by downregulation of homeobox, msh-like 1 (MSX1). (A) The mRNA expression levels of alkaline phosphatase (*Alp*), type I collagen (*Col1A*), osteocalcin (*Ocn*), runt-related transcription factor 2 (*Runx2*), dentin sialophosphoprotein (*Dspp*), and dentin matrix protein 1 (*Dmp1*) were measured by real-time PCR in control and *Msx1* small interfering RNA (siRNA) groups, 7 d after *Msx1* knockdown, revealing a consistent increase in all genes studied. (B) Representative images of Alizarin Red-stained cells, 14 d after *Msx1* knockdown, show increased mineralization in *Msx1* siRNA-transfected cells. Scale bars = 500 μ m; * $P < 0.05$; ** $P < 0.01$.

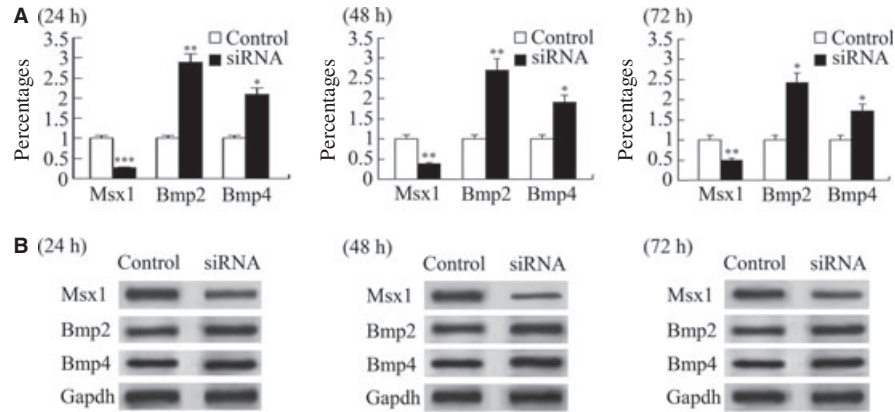


Fig. 5. Bone morphogenetic protein 2 (BMP2) and bone morphogenetic protein 4 (BMP4) expression levels in dental mesenchymal cells were modulated by homeobox, msh-like 1 (*Msx1*) knockdown. The effects of *Msx1* knockdown on expression of *Bmp2* and *Bmp4* mRNA (A) and BMP2 and BMP4 protein (B) were assessed 24, 48, and 72 h after *Msx1* knockdown. Decreased expression of *Msx1* mRNA and MSX1 protein was counteracted by increased expression of *Bmp2* and *Bmp4*. ** $P < 0.01$; *** $P < 0.001$.

ing the expression of *Alp*, *Col1A1*, *Ocn*, *Runx2*, *Dspp*, and *Dmp1*, MSX1 could inhibit the differentiation of dental mesenchymal cells into odontoblasts. Mineralized nodule formation represents the potential of dental mesenchymal cells to differentiate into odontoblasts. The more pronounced mineralized nodule formation observed after knockdown of *Msx1* expression suggests that MSX1 may inhibit the odontoblast differentiation potential of dental mesenchymal cells.

To identify downstream targets of MSX1 that are involved in regulating the differentiation of dental mesenchymal cells into odontoblasts, we assessed the expression of the putative MSX1 target genes, *Bmp2* and *Bmp4*. Our results show that knockdown of *Msx1* expression significantly promotes the expression of BMP2 and BMP4. Bone morphogenetic protein 2 is a crucial regulator of odontoblast differentiation that has been shown to stimulate odontoblast differentiation in vitro as well as dentin formation in vivo (31). Furthermore, BMP4 is known to play an important role in dentin formation and odontoblast differentiation by stimulating ALP activity and the synthesis of collagen, OCN, and RUNX2 (32, 33). Thus, MSX1 may regulate odontoblast differentiation through the BMP2 and BMP4 signalling pathways. However, JIA *et al.* (22) reported that conditional knockout of *Bmp4* expression caused mandibular molar development arrest at the bud stage but that the development of maxillary teeth was normal. This study indicates that increased *Bmp4* expression is not necessary for odontoblast differentiation, at least in the maxillary molars. Therefore, we speculate that developmental regulation of odontoblast differentiation may be different between the mandibular and maxillary molars; however, further research will be required to identify these different pathways.

Previous studies in *Msx1* knockout mice showed that expression of *Bmp2* and *Bmp4* were suppressed at the bud stage (16, 17); however, in our study we found that *Bmp2* and *Bmp4* expression levels were up-

regulated after *Msx1* knockdown. Possible explanations for this inconsistency are as follows. (i) *Msx1* expression was knocked down but not totally suppressed, and therefore residual *Msx1* expression may stimulate the transcription of *Bmp2* and *Bmp4*. (ii) During molar development, MSX1 physically cooperates with other proteins, such as paired box 9 (PAX9) and BarH-like homeobox 1 (BARX1), to form a multiprotein complex (34, 35). Specifically, MSX1 interacts with PAX9 post-transcription (34), and the interaction between MSX1 and BARX1 could upregulate *Bmp4* expression (35). Hence, in this study, a decrease of MSX1 would impede physical protein interactions that may lead to different protein arrangements affecting *Bmp2* and *Bmp4* expression. (iii) The transcriptional repressor function of MSX1 is critical for inhibiting cell differentiation in embryogenesis (36), whereas BMP2 and BMP4 promote cell differentiation during development (37, 38). (iv) Dental mesenchymal cells of the cap stage are undifferentiated (39), and the maximal expression of MSX1 during the cap stage suppresses the expression of *Bmp2* and *Bmp4* to maintain the undifferentiated state of the cap stage. Finally, (v) regulation of tooth germ development, like that of other tissues, is spatially and temporally specific. In this regard, ISHII *et al.* (40) demonstrated that *Msx1* knockout embryos showed elevated expression of *Bmp4* in the cranial mesenchyme, including a migrating neural crest of the first and second pharyngeal arch, which was attributed to specific tissues and stages. Furthermore, ROYBAL *et al.* (41) found ectopic bone islands derived from the neural crest between the reduced frontal bones in *Msx1* mutant embryos showing elevated expression of *Bmp2*, which is involved in ectopic bone formation. These authors proposed that *Msx1* plays a dual role in calvarial development and that this is required for the differentiation and proliferation of osteogenic cells within rudiments, and MSX1 is further required to suppress osteogenic gene expression upon rudiment growth. In summary, resid-

ual *Msx1* expression and the physical cooperation of MSX1 with other proteins, the opposite roles of MSX1 and BMP2/BMP4 in cell differentiation, and the specific stages of tooth germ development may explain how MSX1 inhibits the expression of *Bmp2* and *Bmp4* in the dental mesenchyme at the cap stage.

In conclusion, we propose that MSX1 promotes dental mesenchyme proliferation, regulates the cell cycle of dental mesenchymal cells, and prevents odontoblast differentiation via inhibition of *Bmp2* and *Bmp4* expression at the cap stage during tooth development. The MSX1 protein may play a dual role during early tooth development, being required for initiation of the tooth germ during the bud stage and then for the proliferation and the prevention of odontoblast differentiation. In general, the main functions of MSX1 during embryonic development may be the promotion of cell proliferation and the inhibition of odontoblast differentiation in a stage- and tissue-specific manner.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Separation of mandibular first molar and mandibular second molar under a stereo microscope.