Bcl11b regulates enamel matrix protein expression and dental epithelial cell differentiation during rat tooth development

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Abstract. Amelogenesis, beginning with thickened epithelial aggregation and ending with highly mineralized enamel formation, is a process mediated by a complex signaling network that involves several molecules, including growth and transcription factors. During early tooth development, the transcription factor B-cell CLL/lymphoma 11B (*Bcl11b*) participates in dental epithelial cell proliferation and differentiation. However, whether it affects the postnatal regulation of enamel matrix protein expression and ameloblast differentiation remains unclear. To clarify the role of *Bcl11b* in enamel development, the present study initially detected the protein expression levels of Bcl11b during tooth development using immunohistochemistry, from the embryonic lamina stage to the postnatal period, and demonstrated that *Bcl11b* is predominantly restricted to cervical loop epithelial cells at the cap and bell stages, whereas expression is reduced in ameloblasts. Notably, the expression pattern of *Bcl11b* during tooth development differed between rats and mice. Knockdown of *Bcl11b* by specific small interfering RNA attenuated the expression of enamel-associated genes, including amelogenin, X-linked (*Amelx*), ameloblastin (*Ambn*), enamelin (*Enam*), kallikrein related peptidase 4 (*Klk4*), matrix metallopeptidase 20 and Msh homeobox 2 (*Msx2*). Chromatin immunoprecipitation assay verified that *Msx2* was a transcriptional target of *Bcl11b*. However, overexpression of *Msx2* resulted in downregulation of enamel-associated genes, including *Ambn*, *Amelx*, *Enam* and *Klk4*. The present study suggested that *Bcl11b* serves a potentially important role in the regulation of ameloblast differentiation and enamel matrix protein expression. In addition, a complex feedback regulatory network may exist between *Bcl11b* and *Msx2*.

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Introduction

Tooth development is associated with the continuous interaction between mesenchymal cells and epithelial cells (1,2). As the hardest component of teeth, mammalian enamel serves an essential role in mastication and occlusion. In mammals, enamel development is categorized into various stages: Initiation, bud, cap and bell. Amelogenesis begins with the proliferation of restricted epithelia, which forms dental lamina, and subsequently invaginates into adjacent mesenchyme cells; these cells develop into a bud structure (referred to as the bud stage) (3,4). Following bud generation, tooth germ cells differentiate into inner enamel epithelium (IEE) cells and outer enamel epithelium (OEE) cells. Preameloblasts are provided by IEE cells and further develop into mature ameloblasts, which secrete enamel matrix proteins, including amelogenin, X-linked (*Amelx*), ameloblastin (*Ambn*) and enamelin (*Enam*); these secreted proteins form enamel (5). In rodent mature molars, ameloblasts are not present and are replaced by mineralized enamel, whereas in mature incisors, the dental epithelial stem cells in the labial cervical loop (laCL) maintain the capacity of self-renewal and differentiation, thus presenting immense potential for tooth regeneration (6,7).

Mammalian tooth development involves a complex signaling network, which includes various growth factors and transcription factors during the developmental process. Various transcription factors, including paired box 9, paired like homeodomain 2, Msh homeobox 1 (*Msx1*), Msh homeobox 2 (*Msx2*) and distal-less homeobox 2, are expressed in dental epithelium and mesenchyme, and participate in odontogenesis (8). B-cell CLL/lymphoma 11B (*Bcl11b*) (also known as COUP-TF-interacting protein 2) is a C2H2 zinc finger transcription factor, which has been demonstrated to serve crucial roles in the thymus (9), central nervous system (10), and during epidermal development (11). Mice

with *Bcl11b* knockdown die perinatally, thus indicating an essential role for *Bcl11b* in vertebrate development (12). It has previously been reported that *Bcl11b* serves functions during tooth development, including ameloblast formation (13), and asymmetric development of the laCL and lingual cervical loop (liCL) (14). Early genetic studies demonstrated that *Bcl11b* knockout mice exhibited delayed odontogenesis at the bud stage and subsequent cap and bell stages, as well as thinner enamel (13). Furthermore, abnormal development occurred at the laCL with improper differentiation of ameloblasts, and at the lingual side with ectopic formation of ameloblast-like cells in *Bcl11b* knockout mice (14).

The present study aimed to generate an overview regarding the regulatory effects of *Bcl11b* in enamel development. Initally, the protein expression levels of Bcl11b were detected, thus revealing that Bcl11b was expressed throughout rat embryonic odontogenesis. Notably, Bcl11b expression was not detected in the epithelia or epithelial-originated tissues of postnatal rat teeth; however, it was continuously expressed in murine postnatal tooth germ cells. Combined with a previous report regarding the *Bcl11b* expression pattern in mice (5), the present study potentially indicated that *Bcl11b* may not be essential in postnatal amelogenesis, based on the parallel morphology of rat and mouse teeth but different *Bcl11b* expression profile during late development. Furthermore, the expression levels of various enamel-associated genes were detected, including *Amelx*, *Ambn*, *Enam*, kallikrein-related peptidase 4 (*Klk4*), matrix metallopeptidase 20 (*Mmp20*) and *Msx2*, which were regulated by *Bcl11b*. These results suggest that *Bcl11b* has principal functions in embryonic odontogenesis, particularly in dental epithelial development but less noticeably in post-natal tooth development.

Materials and methods

Cell culture and transient transfection. HAT-7 cells are dental epithelial cells originating from rat cervical loop epithelium, which were provided by Professor Hidemitsu Harada's Laboratory (Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare, Logan, UT, USA) and 1% penicillin/streptomycin. Three small interfering (si)RNAs (si1, si2 and si3) that were designed to specifically target various sites of *Bcl11b* (NM_001277288.1), as well as a negative control siRNA, were purchased from RiboBio Co., Ltd. (Guangzhou, China). *Msx2* cDNA open reading frame (ORF) clone (cat. no. RR202436) and pCMV6-Entry plasmid (cat. no. PS100001) were purchased from OriGene Technologies, Inc. (Beijing, China). Cells were cultured in 6-well plates in culture medium for 24 h at 37˚C prior to transfection. siRNA was transfected at a concentration of 50 nM into HAT-7 cells (70-80% confluence) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc). HAT-7 cells were harvested 72 h post-transfection. In addition, 2 μg *Msx2* ORF plasmid was transfected using MegaTran 1.0 (cat. no. TT200002; OriGene Technologies, Inc.) into cells (70-80% confluence) in a 60-mm culture dish cultured in DMEM/F12 supplemented with 10% FBS. Transfected cells were harvested after a 72 h culture at 37˚C in an incubator containing 5% CO_2. RNA and protein samples were extracted from all transfected samples.

Animals, histology and immunohistochemistry. C57BL/6 mice and Sprague Dawley rats were commercially purchased from the Animal Centre (Sichuan University, Sichuan, China). Animal care and handling was conducted in compliance with the guidelines of the Ethics Committee of West China College of Stomatology (Sichuan University) for animal research. All experimental protocols were approved by the Sichuan University Science Animal Care and Use Committee. Embryos and mandibles from postnatal offspring were collected at various time points, which were then used for sectioning and subsequent immunostaining. The first detection of a vaginal plug was considered embryonic day (E) 0.5 and the date of birth was considered postnatal day (P) 0.5. All tissues were treated with 4% paraformaldehyde and the postnatal mandibles were treated with 10% EDTA for demineralization. Subsequently, the tissues were dehydrated with a sequential concentration of alcohol and finally with xylene. The tissues were embedded in paraffin and were cut into 5-7 μm sections.

To examine Bcl11b protein expression by immunohistochemistry, sections were dried overnight in a 60˚C incubator, were dehydrated with sequential concentrations of alcohol, and were treated with 3% hydrogen peroxide for 15 min at room temperature to block endogenous peroxidase activity. Subsequently, the sections were incubated in slightly boiling 0.1 M sodium citrate buffer at 95˚C for 15 min. Sections were then incubated with goat serum working solution (ZSGB-BIO, Beijing, China) for 30 min, followed by 1 h (37˚C) and then overnight (4˚C) incubations with anti-Bcl11b antibody (1:300; cat. no. 12120; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-Sonic hedgehog (Shh; 1:500; cat. no. 06-1106; EMD Millipore, Billerica, MA, USA), which was used as a positive control. Following primary antibody incubation, the slides were treated with horseradish peroxidase-labeled anti-rabbit secondary antibody (ChemMate EnVision Detection kit; Dako, Carpinteria, CA, USA) for 30 min at 37˚C. Avidin biotin complex and 3,3'-diaminobenzidine substrate were used as a reporting system, and the nuclei were stained with hematoxylin. Finally images were captured under a light microscope (BX43F; Olympus Corporation, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assays were performed according to the manufacturer's protocol of the ChIP assay kit (cat. no. 17-10086; Upstate; Merck Millipore, Darmstadt, Germany), and all reagents were provided in the kit. Briefly, HAT-7 cells were plated in 100 mm dishes and harvested the next day at ~90% confluence. Cells were treated with a final concentration of 1% formaldehyde, 10X glycine was added to quench unreacted formaldehyde. After two washes with PBS, cells were collected for lysis and subsequent nuclear lysis. The DNA/chromatin complex obtained from nuclear lysis were sonicated into fragments and the protein/DNA complex was immunoprecipitated with Bcl11b antibody. Finally purified DNA was obtained and underwent polymerase chain reaction (PCR).

All PCR analyses were conducted as follows: Initial denaturation at 94˚C for 3 min followed by 32 cycles at

Table I. Primer sequences.

Gene	Accession number	Primer sequence (5'-->3')
Bcl11b	NM_001277288.1	F: GATCGGC AAGGAGGTGTA R: CATCATTAGT CAGCAAGTGTTC
Msx2	NM_012982	F: CTGAGGAAA CACAAGACCAA R: GCGATGGA GAGGTACTGT
Amelx	NM_001271078.1	F: AGCTTTTGC TATGCCCCTACC R: GATGAGGCTG AAGGGTGTGACT
Ambn	NM_012900.1	F: CTGCTCCTGT TCCTGTCCCTA R: GCTTCCCAACT GTCTCATTGTC
Enam	NM_001106001.1	F: GGTGTCTTC CCTCTCCCTAAA R: AGTGGTTTGC CATTGTCTTTCT
Mmp20	NM_001106800.1	F: GCCTTGCTG TCCTTGTCAC R: GAGGTGGTA GTTGCTCCTGAAG
Klk4	NM_001004101.1	F: CCGAACTACA ATGACCCTTCTT R: TCAGATGCTAC CGAGAGATTCA
GAPDH	NM_017008.4	F: TATGACTCTAC CCACGGCAAG R: TACTCAGCAC CAGCATCACC

Bcl11b, B-cell CLL/lymphoma 11B; *Msx2*, Msh homeobox 2; *Amelx*, amelogenin, X-linked; *Ambn*, ameloblastin; *Enam*, enamelin; *Mmp20*, matrix metallopeptidase 20; *Klk4*, kallikrein related peptidase 4.

94˚C for 20 sec, 59˚C for 30 sec and 72˚C for 30 sec, and final extension at 72˚C for 2 min. The 2X PCR Master Mix (Thermo Fisher Scientific, Inc.) was used to conduct the PCR reaction. Primers (forward CTGAGGAAACAC AAGACCAA and reverse GCGATGGAGAGGTAC TGT) were used to amplify the *Msx2* promoter. *GAPDH* primer (forward TATGACTCTACCCACGGCAAG and reverse TACTCAGCACCAGCATCACC) was used as a control primer. Size of sonicated DNA fragments were confirmed by 2% agarose gel electrophoresis.

To ensure the ChIP assay had been successfully performed, anti-RNA Polymerase II was used as a positive control and normal mouse immunoglobulin G was used to replace the Bcl11b antibody as a negative control, thus revealing nonspecific immunoprecipitation. *GAPDH* primer was used as a primer control.

RNA preparation and reverse transcription-quantitative (RT-q)PCR. RNA extraction was performed using RNAiso (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RT was performed using the First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). qPCR was conducted using SYBR Green I kit (Thermo Fisher Scientific, Inc.), and all quantification cycle (Cq) values were normalized to *GAPDH* levels (15). For qPCR, a reaction volume of 10 μl was prepared as follows: 5 μl SYBR Green I master mix, 100 ng cDNA, 0.2 μl forward and reverse primers (0.4 μM final concentration), and water. Cycling conditions were as follows: Denaturation at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec, and finally 1 cycle at 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. All qPCR products were examined by melt curve analysis and 2% agarose gel electrophoresis. The primers used are listed in Table I.

Protein extraction and western blotting. Protein extraction was conducted using a total protein extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Protein samples were treated with 4X loading buffer and incubated at 100˚C for 5-10 min. Briefly, protein concentration was determined using the Bicinchoninc Acid Protein Assay kit ((Nanjing KeyGen Biotech Co., Ltd.). Equal amounts of protein (30 ng) for each sample were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Laboratories, Inc., Hercules, CA, USA). The membranes were then incubated with 5% fat-free milk at room temperature for 2 h, followed by primary antibody incubation at 4˚C overnight. After three washes, the membranes were incubated with appropriate secondary antibodies at room temperature for 2 h. Finally, Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (WBKLS0500; EMD Millipore) was used for visualization combined with the LAS-3000 imaging system (Bio-Rad Laboratories, Inc.). The primary antibodies used for western blotting were as follows: Anti-Bcl11b (1:1,000; cat. no. 12120; Cell Signaling Technology, Inc.), anti-Msx2 (1:500; cat. no. sc-17731; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-amelogenin (1:500; cat. no. sc-32892; Santa Cruz Biotechnology, Inc.), anti-ameloblastin (1:500; cat. no. sc-50534; Santa Cruz Biotechnology, Inc.), anti-Klk4 (1:500; cat. no. sc-20622; Santa Cruz Biotechnology, Inc.), anti-Mmp20 (1:1,000; cat. no. ab76109; Abcam, Cambridge, UK) and anti-β-actin (1:2,000; cat. no. ab3280; Abcam). In addition, anti-mouse, anti-rabbit and anti-goat antibodies (1:10,000; cat. nos. ZB-2305, ZB-2301 and ZB-2306; ZSGB-BIO) were used as secondary antibodies.

Cell proliferation assay. HAT-7 cells were seeded in a 48-well plate, transfected with *Bcl11b*-specific siRNA for 48 h, and were incubated with 5-ethynyl-2'-deoxyuridine (EdU; final concentration, 20 μM; RiboBio Co., Ltd.) for 2 h at 37˚C. Images were acquired under a Leica DMI6000 B inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The number of fluorescent cells was counted and used to calculate proliferation rate (fluorescent cells/total cells).

Statistical analysis. Student's t-test was used to perform all statistical analyses, which were analyzed using Excel 2010

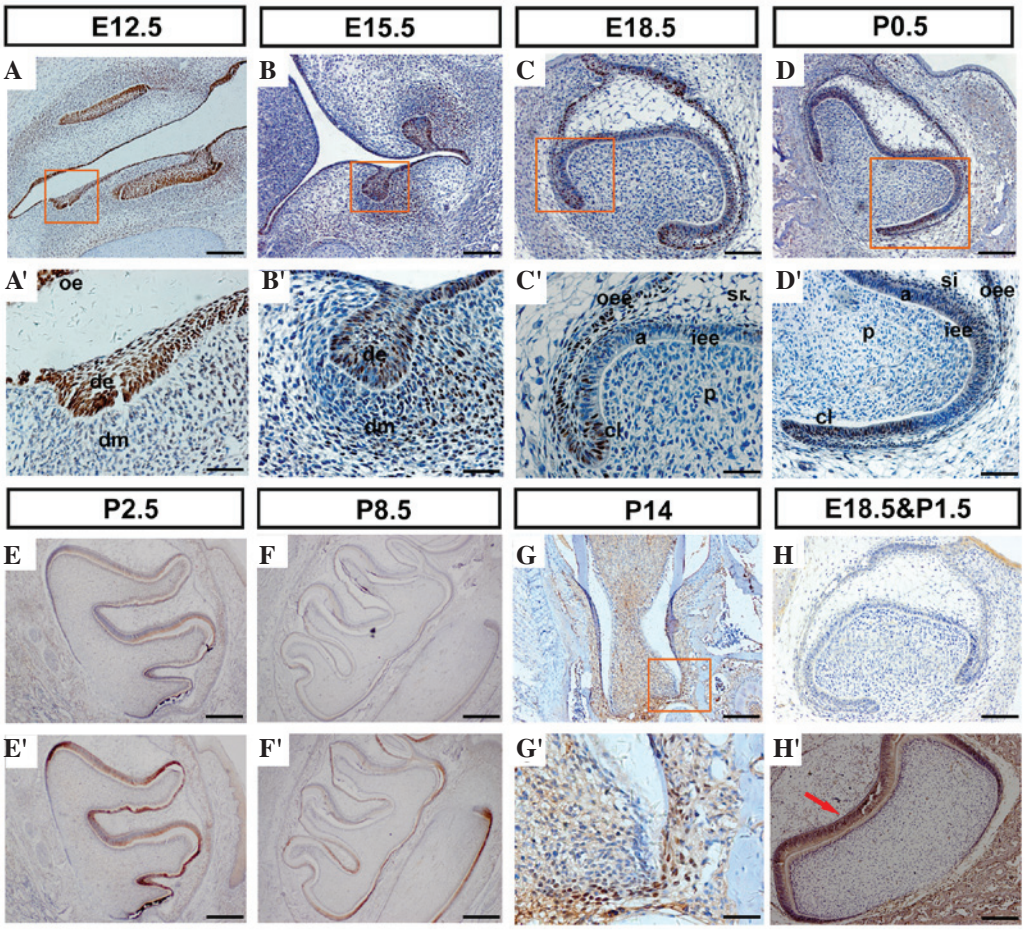

Figure 1. Bcl11b expression pattern in rat teeth. Bcl11b immunostaining appeared brown in all panels colored by 3,3'-diaminobenzidine. Bcl11b expression in rat developing molars at (A) E12.5, (B) E15.5, (C) E18.5 and (D) P0.5 (D). (A', B', C', D' and G') are partial magnifications of (A, B, C, D and G) respectively, as indicated by red rectangles. No detectable Bcl11b expression was detected in (E) P2.5 molars, and (F) P8.5 molars and incisors. Sonic hedgehog expression was used as a positive control in (E') P2.5 molars, and (F') P8.5 molars and incisors. Bcl11b was expressed in mineralized mouse teeth at (G and G') P14. Msx2 was more strongly expressed in (H') mature ameloblasts (red arrow) compared with in (H) embryonic tooth germs. [Scale bars: (A, B, E, E', F, F') 200 μm; (C, D, G', H, H') 100 μm; (A', B', C', D') 50 μm; (G) 500 μm] . Bcl11b, B-cell CLL/lymphoma 11B; oe, oral epithelium; de, dental epithelium; dm, dental mesenchyme; oee, outer enamel epithelium; iee, inner enamel epithelium; a, ameloblast; sr, stellate reticulum; p, papilla; cl, cervical loop; si, stratum intermedium; E, embryonic day; P, postnatal day.

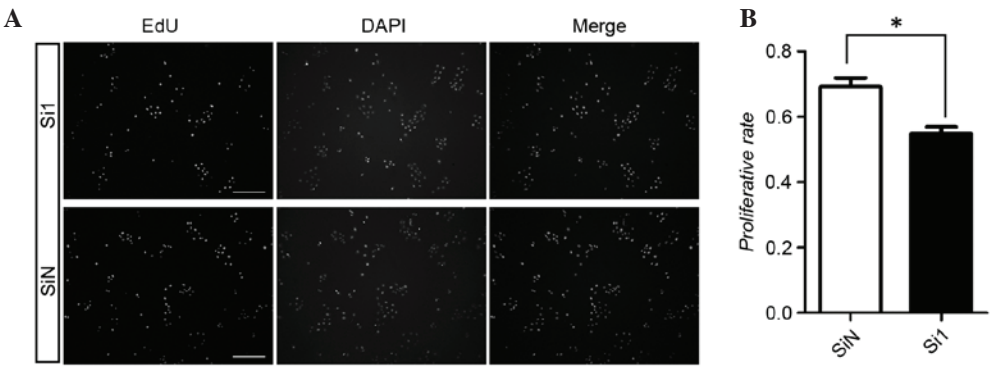

Figure 2. Cell proliferation of *Bcl11b* knockdown cells. (A) EdU-labeled cells were detected and 4',6-diamidino-2-phenylindole was used to stain cell nuclei in siRNA-treated cells and control cells. Scale bars, 50 μm. (B) Counting of EdU-positive cells following 48 h siRNA transfection. *P<0.05, n=3. *Bcl11b*, B-cell CLL/lymphoma 11B; EdU, 5-ethynyl-2'-deoxyuridine; SiN, negative control siRNA-transfected cells; Si1, *Bcl11b* siRNA-transfected cells. siRNA, small interfering RNA.

(Microscoft Corporation, Redmond, WA, USA). Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference (16).

Results

Bcl11b expression pattern in early tooth germ cells. To determine the expression pattern of Bcl11b in rat teeth, the

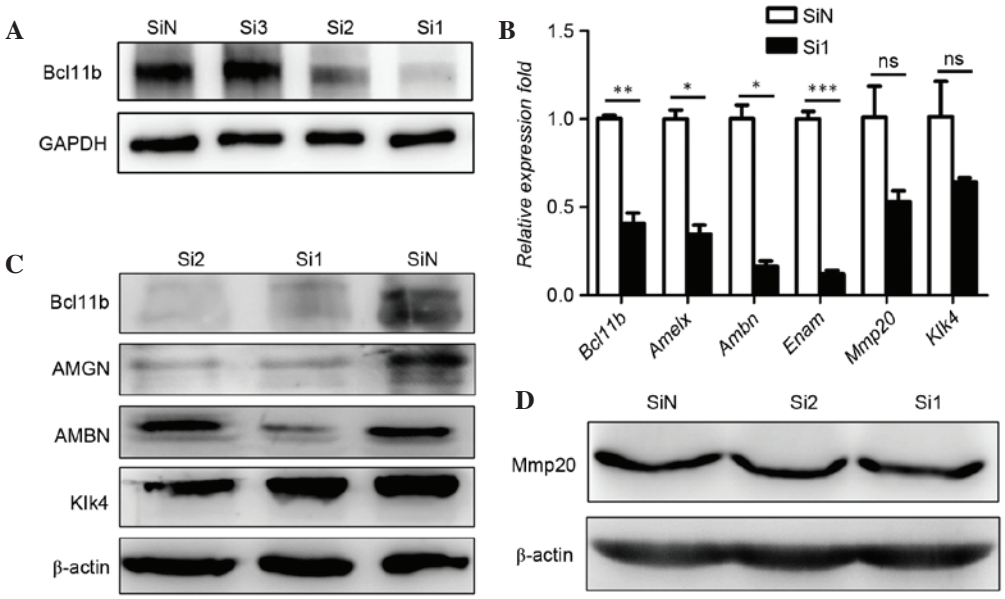

Figure 3. Enamel-associated genes and proteins were downregulated following knockdown of *Bcl11b*. (A) Si1 and Si2 were efficient siRNA molecules, as determined by western blotting. Total protein was extracted from HAT-7 cells 72 h post-transfection. (B) Quantitative polymerase chain reaction and (C and D) western blotting demonstrated that enamel-associated genes and proteins were downregulated by *Bcl11b* knockdown. Statistical significance was determined using Student's t-test. *P<0.05, **P<0.005, ***P<0.001, n=3. ns, no significance; *Bcl11b*, B-cell CLL/lymphoma 11B; *Amelx*/AMGN, amelogenin, X-linked; *Ambn*/AMBN, ameloblastin; *Enam*, enamelin; *Mmp20*, matrix metallopeptidase 20; *Klk4*, kallikrein related peptidase 4; SiN, negative control siRNA-transfected cells; Si1/2/3, *Bcl11b* siRNA-transfected cells. siRNA, small interfering RNA.

present study detected Bcl11b protein expression during the initiation stage and in postnatal tooth germ cells. The results demonstrated that Bcl11b was highly expressed in the thickened dental lamina at E12.5 (Fig. 1A), as detected by anti-Bcl11b immunohistochemistry; however, expression levels were much lower in the mesenchyme (Fig. 1A'). Bcl11b was also expressed in the tooth bud at E15.5 (Fig. 1B) as well as in the adjacent mesenchyme. During the later cap and bell stages, Bcl11b exhibited high expression in OEE cells, cervical loops, and stratum intermedium (SI) cells; however, levels were reduced in IEE cells and ameloblasts, and expression was negative in the mesenchyme and stellate reticulum cells (Fig. 1C and C'). In the neonatal molars, Bcl11b was detected in the IEE, OEE and SI cells (Fig. 1D and D'), similar to the late cap stage. However, no obvious expression was detected in the postnatal mineralizing tooth (Fig. 1E and F), which was confirmed by positive control Shh expression (Fig. 1F and F') and nuclear-specific location of Bcl11b. In addition, Bcl11b was detected in mineralized mouse teeth and was restricted to the root area (Fig. 1G and G'). Msx2 was negatively expressed in embryonic tooth germs at E18.5 (Fig. 1H); however, it was strongly expressed in mature ameloblasts at P1.5 (Fig. 1H').

Knockdown of Bcl11b expression inhibits HAT-7 cell proliferation. The present study analyzed the effects of *Bcl11b* expression on dental epithelial cell proliferation using the EdU assay. The results indicated that transfection with *Bcl11b*-specific siRNA statistically attenuated HAT-7 proliferation (Fig. 2). The proliferative rate was represented by EdU-positive cells. These results suggest that Bcl11b may promote dental epithelial cell proliferation during tooth development.

Bcl11b regulates the expression of enamel-associated genes and proteins. The present study aimed to determine whether *Bcl11b* exerts an effect on enamel-associated gene and protein expression. Two efficient siRNAs: Si1 and Si2, were screened from the three siRNA molecules designed to knockdown *Bcl11b* expression (Fig. 3A). Following transfection of HAT-7 cells with *Bcl11b*-specific siRNA the expression levels of enamel matrix genes and proteins, including *Amelx*, *Ambn* and *Enam*, were significantly downregulated. In addition, two enamel matrix enzymes, *Klk4* and *Mmp20*, exhibited slight decreases at the gene and protein level (Fig. 3B-D); however, these findings were not significant (P=0.074 and 0.087).

Msx2 is associated with enamel-related gene regulation. As shown in Fig. 4A, knockdown of *Bcl11b* significantly downregulated the expression of *Msx2*. To determine whether *Bcl11b* regulated enamel expression through *Msx2*, the binding of *Bcl11b* to the promoter region of *Msx2* was determined in HAT-7 cells using a ChIP assay. *Msx2* promoter enrichment in *Bcl11b* immunoprecipitation was determined using PCR (Fig. 4B and C). As shown in Fig. 4C *Msx2* was significantly enriched in *Bcl11b* immunoprecipitation, compared with the anti-polymerase II positive control. These results indicate that *Msx2* is a target gene of the transcription factor *Bcl11b* (Fig. 4B and C).

The present study also transfected an *Msx2* ORF cDNA clone into HAT-7 cells; pCMV6-Entry plasmid was used as a control. The results demonstrated that all enamel-associated genes, including *Ambn*, *Amelx*, *Enam* and *Klk4,* were downregulated by overexpression of *Msx2* (Fig. 4D). Notably, *Bcl11b* was also significantly decreased. These results suggest that a feedback loop may exist between *Bcl11b* and *Msx2*. However, further experiments are required.

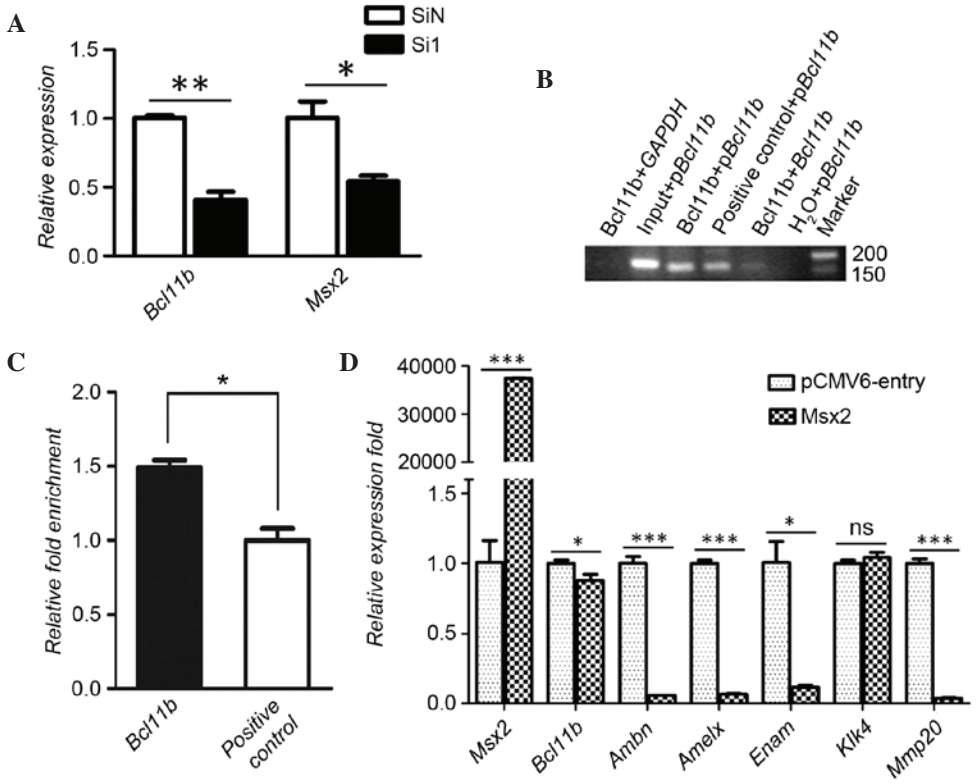

Figure 4. *Msx2* regulates enamel-associated genes. *Msx2* is a target gene of the transcription factor *Bcl11b*, as verified by ChIP assay. (A) Knockdown of *Bcl11b* led to a significant decrease in *Msx2* expression. *P<0.05, **P<0.005, n=3. (B) PCR products of ChIP assay were verified by 2% agrose gel electrophoresis. Bcl11b, anti-Bcl11b antibody; input, cell lysate; Positive control, anti-RNA polymerase II antibody; *GAPDH, GAPDH* primer; *pBcl11b*, *Bcl11b* promoter primer; *Bcl11b*, *Bcl11b* primer. (C) Analysis of relative fold enrichment of ChIP. Quantitative PCR result between the *Bcl11b* experimental group and the positive control group. Student's t-test was used for statistical analysis. *P<0.05, n=3. (D) Relative expression of enamel-associated genes following *Msx2* overexpression. *P<0.05, ***P<0.001, n=3. ns, not significant; *Bcl11b*, B-cell CLL/lymphoma 11B; *Msx2*, Msh homeobox 2; *Amelx*, amelogenin, X-linked; *Ambn*, ameloblastin; *Enam*, enamelin; *Mmp20*, matrix metallopeptidase 20; *Klk4*, kallikrein related peptidase 4; PCR, polymerase chain reaction.

Discussion

The present study demonstrated that *Bcl11b* regulates enamel-associated gene and protein expression, via its transcriptional target *Msx2*, thus suggesting that *Bcl11b* serves a role in sustaining differentiation of epithelial cells.

Bcl11b-null mice exhibited a reduction in early tooth germ size, as well as aberrant ameloblast proliferation and differentiation, and reduced stellate reticulum (13). Furthermore, asymmetric development of incisor laCL and liCL was disrupted by ectopic proliferation of epithelial cells, thus suggesting that *Bcl11b* was crucial for epithelial cell proliferation and differentiation (14). At the molecular level, *Bcl11b* is expressed at all stages throughout embryonic odontogenesis in mice and rats, particularly in the epithelium; however, its expression is reduced during late postnatal rat tooth development (Fig. 1). Notably, Bcl11b protein was continuously expressed in mouse teeth until adulthood (Fig. 1G and G'). In mineralized mouse molars, Bcl11b appeared in preameloblasts, ameloblasts, dental follicle cells (DFCs) and periodontal ligament cells (PLCs). In mineralized mouse incisors, Bcl11b was similarly located in preameloblasts, ameloblasts, DFCs and PLCs (data not shown); and in the adult cervical loop area it was strongly expressed in OEE of the laCL and liCL, whereas it was lowly expressed in IEE and mature ameloblasts (5). Mouse and rat teeth share similarities in shape and regulation pattern during

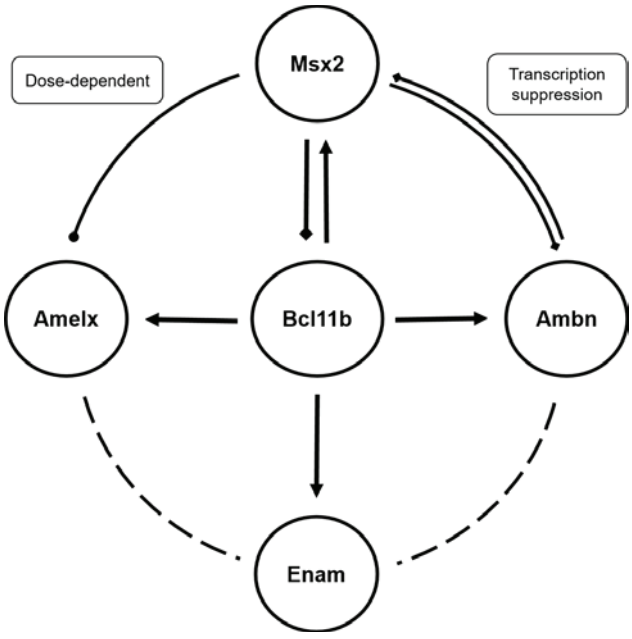

Figure 5. Regulatory network among *Bcl11b*, *Msx2* and enamel matrix proteins. *Bcl11b* promoted expression of enamel matrix proteins *Amelx*, *Ambn* and *Enam*, and Msx2. *Msx2* regulated Amelx and Ambn in a dose-dependent manner. In turn, *Msx2* inhibited enamel matrix protein expression, thus indicating the existence of a negative feedback loop between *Msx2* and *Bcl11b*. *Bcl11b*, B-cell CLL/lymphoma 11B; *Msx2*, Msh homeobox 2; *Amelx*, amelogenin, X-linked; *Ambn*, ameloblastin; *Enam*, enamelin.

odontogenesis, with a different expression pattern of Bcl11b. However, teeth in newborn *Bcl11b* mutant mice (P0) exhibited indistinguishable differences in morphogenesis and mineralization compared with wild type mice (13), which may suggest a nonessential role for *Bcl11b* in late embryonic and postnatal tooth development. Furthermore, the present study revealed that in *Bcl11b* siRNA-transfected HAT-7 cells, proliferation was reduced compared with the control, which was consistent with the reduced proliferation of cells in the laCL of P21 *Bcl11b* mutant mouse incisors (5). These findings may suggest that *Bcl11b* maintains the proliferation of non-differentiated epithelial cells.

Msx2, together with *Msx1* and *Msx3*, belongs to the *Msx* homeobox gene family, lack of which may cause defects in tooth cusp morphogenesis and amelogenesis (17,18). Msx2 protein was markedly expressed in secretory ameloblasts (Fig. 1H'), and has been reported to control expression of the extracellular matrix gene laminin 5α3 to mediate terminal ameloblast differentiation (17). These findings suggested that *Msx2* serves a significant role in enamel development. The present study demonstrated that the expression of enamel-associated genes, such as *Amelx*, *Ambn*, *Enam* and *Mmp20*, were decreased post-transfection with *Msx2* overexpression DNA (Fig. 4D), and *Bcl11b* expression was also downregulated. Previous studies have demonstrated that *Ambn* prevented *Msx2* expression (19) and *Msx2* transcriptionally suppressed *Ambn* (20), thus presenting a reciprocal regulatory mechanism between *Msx2* and *Ambn*. A dose-dependent relationship has also been identified between *Msx2* and enamel-related genes. In *Msx2*$^{-/-}$ mice, *Amelx* and *Enam* were significantly decreased, whereas in *Msx2*$^{+/-}$ heterozygous mice *Amelx* exhibited increased expression (18). Furthermore, knockdown of *Bcl11b* also led to diminished *Msx2* expression (Fig. 4A), and results of a ChIP assay suggested that *Msx2* was a target gene of the transcription factor *Bcl11b* (Fig. 4B) (13). Therefore it may be hypothesized that a feedback regulatory network exists between the two molecules for proper expression and function during amelogenesis (Fig. 5). In addition, *Msx2* is highly expressed in osteoclasts and regulates bone resorption in the alveolar compartment (21), which may provide evidence regarding the regulatory mechanism between *Msx2* and *Bcl11b*, since *Bcl11b* was continuously observed in the alveolar areas in the postnatal secretory and mature stages (data not shown).

Amelogenin is the most abundant component of the enamel matrix, and together with other non-amelogenin matrix proteins, ameloblastin and enamelin, is directly responsible for enamel formation (22). Aberrant expression of these proteins results in grievous amelogenesis imperfecta, which is characterized by disorganized hypoplastic enamel, thinner enamel and chalky-white discoloration (23-25). Adhesion of ameloblastin to ameloblasts maintains the differentiation fate of secretory ameloblasts but inhibits proliferation (23). *Bcl11b* is a transcriptional suppressor in the central nervous, tumor and cutaneous systems (11,26); however, it has been reported that *Bcl11b*-null mice exhibit defects in enamel (13) and knockdown of *Bcl11b* in the present study induced decreased enamel matrix expression. A previous study reported that *Bcl11b* was detected on promoter regions of amelogenin, enamelin and laminin 5α3, which control ameloblast terminal differentiation (13,17). Furthermore, it has been reported that *Msx2* acts as a dose-dependent transcriptional suppressor that regulates amelogenin expression through antagonistic protein-protein interaction with CCAAT/enhancer-binding protein α, which binds to its homologous region on the mouse amelogenin promoter (27). Taken together, *Bcl11b* positively regulates enamel development, and its role in ameloblast differentiation may be mediated via *Msx2* and enamel matrix proteins. In addition, the present data revealed that *Bcl11b* appeared to positively regulate *Mmp20* and *Klk4,* and their expression is consistent with enamel proteins; thus suggesting that enamel proteinase expression relies on their enamel matrix substrates.

In conclusion, there is a complex regulatory network among *Bcl11b*, *Msx2* and enamel matrix proteins (Fig. 5). *Bcl11b* serves a critical role in early epithelial development and amelogenesis (13,14), and promotes the expression of enamel matrix proteins, which are deemed to be markers of ameloblasts. These findings suggested that *Bcl11b* has a role in mediating ameloblast differentiation. However, in the present study, *Bcl11b* was revealed to possess a spatiotemporal differential regulatory role during rat enamel development. These results indicated that postnatal *Bcl11b* may not be as essential as it is during embryonic tooth development.

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