Combination of Runx2 and Cbfβ upregulates *Amelotin* gene expression in ameloblasts by directly interacting with cis-enhancers during amelogenesis

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Abstract. Amelotin (Amtn) is a recently identified enamel protein secreted by ameloblasts at late stage of enamel development. Runt-related transcription factor 2 (Runx2) in combination with the coactivator core-binding factor β (Cbfβ) regulates the early stages of tooth development. The aim of the present study was to investigate the role of Runx2 in the regulation of *Amtn* gene expression in ameloblasts. Immunohistochemistry was performed and the results revealed that Runx2 protein was predominantly expressed in the nuclei of ameloblasts during the transition stage and the maturation stage of enamel development, whereas Cbfβ was expressed in ameloblasts from the secretory stage to the maturation stage. Reverse transcription-quantitative polymerase chain reaction results demonstrated that *Runx2* knockdown decreased *Amtn* expression in ameloblast-lineage cells and co-expression of *Runx2* and *Cbfβ* in ameloblast lineage cells induced an upregulation in *Amtn* gene expression. Two putative Runx2-binding sites within the *Amtn* promoter were identified using bioinformatics analysis. Results of an electrophoretic mobility shift assay and chromatin immunoprecipitation indicated that Runx2/Cbfβ bound to specific DNA sequences. Site-directed mutagenesis of the Runx2 binding sites within the *Amtn* promoter resulted in decreased basal promoter activity and did not affect the overexpressed Runx2/Cbfβ. The results of the present study suggest that Runx2 upregulates *Amtn* gene expression via binding directly to Runx2 sites within the *Amtn* promoter during amelogenesis.

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Introduction

Enamel is an oral epithelial-derived hard tissue (1). During enamel development, ameloblasts synthesize and secrete a tissue-specific extracellular matrix that facilitates the initiation and orientation of hydroxyapatite crystallites (2,3). Amelotin (Amtn) is a recently identified enamel matrix protein that is expressed and secreted predominantly by ameloblasts during the transition and maturation stages (4). *Amtn* knockout mice have hypomineralized enamel (5) and, in transgenic mice, overexpression of Amtn disrupts the enamel microstructure, causing biomineralization defects (6). To understand the role of Amtn in amelogenesis, it is essential to study the molecular regulatory mechanisms of *Amtn* gene expression.

Runt-related transcription factor 2 (Runx2) is an important molecule in bone and tooth development (7,8). In mice, targeted disruption of *Runx2* results in cessation of bone and tooth development (9). In humans, *Runx2* mutation causes cleidocranial dysplasia, a genetic bone disorder (10). Dental abnormalities, including supernumerary teeth, are also associated with cleidocranial dysplasia caused by *Runx2* mutations (11). It has been demonstrated that Runx2 binds to the consensus sequences in a number of gene promoters, including *Bone Sialoprotein*, *Osteocalcin*, *Dentin Sialophosphoprotein* and *Ameloblastin* to regulate the expression of target genes (12,13). Runx2 is a dimeric transcription complex comprising α unit (Runx2) DNA binding and a stabilizing core-binding factor β subunit (Cbfβ). Cbfβ acts as a binding partner for all Runx proteins and conditional *Cbfβ* knockout mice exhibit impaired enamel formation (14).

The results of the present study suggest that Runx2 protein is upregulated in ameloblasts from the transition stage to the maturation stage. Runx2 specifically regulates *Amtn* gene expression in ameloblast lineage cells (ALCs) by interacting with two functional regions in the mouse *Amtn* promoter. Results of electrophoretic mobility shift (EMSA), chromatin immunoprecipitation (ChIP) and luciferase reporter assays revealed that Runx2 is associated with the transcription activity of the *Amtn* gene in mice via Runx2 binding sites.

Materials and methods

Immunolocalization of Runx2 in mouse mandibles. A total of 30 Kunming strain male mice (2-5 g; 5, 7 and 10 days old; provided by the Laboratory Animal Center, School of Medicine, Shandong University, Jinan, China) were housed under controlled conditions (22±1˚C; 50-60% humidity; 12 hour light-dark cycle). All protocols involving mice were reviewed and approved by the Ethical Committee of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). Mandibles were obtained from mice on postnatal days (PND) 5, 7 and 10 (10 mice/group). The dissected mandibles were fixed with 4% paraformaldehyde in PBS for 20 h at 4˚C and decalcified at 4˚C in a 10% (w/v) Na_2-EDTA solution (pH 7.0) for 10 days. Paraffin samples were prepared as previously described (15). For immunohistochemistry, sections were incubated overnight with rabbit polyclonal antibody against Runx2 (ab23981; 1:300; Abcam, Cambridge, MA, USA), or Cbfβ (ab72696; 1:300 dilution; Abcam) at 4˚C. Antibody binding was detected using the vectastain ABC Elite kit and a peroxidase substrate kit (both Vector Laboratories, Inc., Burlingame, CA, USA). For the control group, the primary anti-Runx2 antibody was replaced with non-immune rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and the nuclei were stained with hematoxylin for 2 min at room temperature.

Plasmid construction. Full-length mouse *Runx2* cDNA and *Cbfβ* cDNA were amplified by polymerase chain reaction (PCR) using DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China) from mouse ALCs (provided by Dr Toshihiro Sugiyama, Akita University, Akita, Japan). The following thermocycling conditions were used for the PCR: Initial denaturation at 94˚C for 10 min; 40 cycles of 94˚C for 15 sec, 60˚C for 30 sec and 72˚C for 1 min; and a final extension at 72˚C for 10 min. The following primer sequences were used: *Runx2* forward, 5'-AAGGATCCTGATGCGTATTCCTGTAG-3' and reverse, 5'-AATCTAGAATATGGCCGCCAAACAGA-3'; *Cbfβ* forward, 5'-GGCGCGGCCTGAGGGCGGGAAGA-3' and reverse, 5'-CGTTAAGTGGAGCACAGCTTATG-3'. Amplification products were cloned into a eukaryotic expression vector pcDNA3.1(+) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mouse *Amtn* promoter region of -1463/+196 was amplified by PCR using DNA polymerase (Takara Biotechnology Co., Ltd.) with mouse genomic DNA as a template. The reaction conditions of PCR were the same as above. The following primer sequences were used for amplification of the -1463/+196 region, forward, 5'-CCGGTACCCAGTGTAGTATGTCATTCT-3' and reverse, 5'-GCCGAGATCTGTTTCGATTTGCTACCT-3'. The amplification product was cloned into pGL3-basic reporter vector (Promega Corporation, Madison, WI, USA) to generate pGL3-Amtn (pWT). The region between bp -1463 and +196 of mouse *Amtn* promoter was analyzed using the online software JASPAR (http://jaspar.genereg.net/) to predict potential binding sites (16). Two putative Runx2 binding sites, AACCACT (site1: -1342/-1336) and AACCAAA (site2: -98/-92) were identified. The mutated *Amtn* promoter-driven luciferase reporter vectors pGL3-Amtn-mut1 (pmut1), pGL3-Amtn-mut2 (pmut2), and pGL3-Amtn-mut1+2 (pmut1+2) were created using a site-directed mutagenesis kit (Takara Biotechnology Co., Ltd.). The sequences of all promoter constructs were verified by DNA sequencing analyses which were carried out by Takara Biotechnology Co., Ltd.

DNA transfection. Immortalized mouse ALCs were cultured as previously described (17,18). All transfections were performed in 6-well plates. For Runx2 small interfering (si)RNA experiments, ALCs were transfected with 60 pmol Runx2 siRNA and control siRNA (sc-37146 and sc-37007; Santa Cruz Biotechnology, Inc.), respectively, using Lipofectamine® RNAi MAX (Invitrogen; Thermo Fisher Scientific, Inc.). Cultures were maintained for 36 h at 37˚C and cells were collected for reverse transcription-quantitative (RT-q) PCR and western blot analyses. For Runx2 overexpression experiments, ALCs were transfected with pcDNA3.1(+), pcDNA3.1-Runx2 (pRunx2), pcDNA3.1-Cbfβ (pCbfβ) or pRunx2 + pCbfβ using Lipofectamine® Plus (Invitrogen; Thermo Fisher Scientific, Inc.). At 36 h post-transfection, the cells were collected for RT-qPCR.

RT-qPCR. Total RNA from mouse ALCs was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed using the MMLV-RT system (Promega Corporation) for 15 min at 37˚C. The sequences of the primers used in qPCR are listed in Table I. Amplification was performed for using the SYBR® PrimeScript® kit (Takara Biotechnology Co., Ltd.) under the following thermocycling conditions: Initial denaturation 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec. GAPDH was used as an internal control. Relative gene expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (19). Each experiment was performed in triplicate and repeated three times.

Western blot analyses. At 36 h post-transfection, ALCs were collected and lysed in radioimmunoprecipitation assay buffer (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) and lysates were centrifuged at 10,000 x g for 7 min at 4˚C. The supernatant was collected and total proteins were quantified using a bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). A total of 30 μg protein samples were separated for each sample by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Following blocking in 5% bovine serum for 2 h at 20˚C, membranes were incubated with anti-Runx2 antibody (cat. no. ab23981; Abcam, diluted at 1:1,000) for 1 h at 37˚C. Membranes were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit antiserum (cat. no. A0545, Sigma Aldrich; Merck KGaA; 1:2,000) for 30 min at 37˚C. β-actin antibody (N-21; 1:1,500; Santa Cruz Biotechnology, Inc.) was used as an internal control. Blots were developed by using an Amersham enhanced chemiluminescence kit (GE Healthcare Life Sciences, Little Chalfont, UK). The experiment was performed three times.

Nuclear protein extraction and EMSA. 293T cells (Wuhan Boster Biological Technology, Ltd., Wuhan, China) were seeded in 60 mm plates at a density of $0.3x10^5$ cells/cm^2. Following 16 h culture, cells were transfected with 10 μg pcDNA3.1(+), 5 μg pRunx2 plus 5 μg pcDNA3.1(+) or 5 μg

Table I. Primers used in RT-qPCR and ChIP.

Name	Direction	Primer sequence (5' to 3')	Experiment
Amtn	Forward	CAAATCAGGTTTTTCCTTCCATAA	RT-qPCR
	Reverse	CCAGGGTGAACGGGAGAGTA	
Klk4	Forward	GTCAGCAGCCGGATCATACAAGG	RT-qPCR
	Reverse	GCACCAAGACTCCCGAGCAGAAA	
ALP	Forward	AACCTGACTGACCCTTCGC	RT-qPCR
	Reverse	CAATCCTGCCTCCTTCCAC	
GAPDH	Forward	TGTGTCCGTCGTGGATCTGA	RT-qPCR
	Reverse	TTGCTGTTGAAGTCGCAGGAG	
Runx2 (1)	Forward	TTCTCCATCAGTGTCCACCTT	ChIP
	Reverse	TGGGGTCACTGTCACTCATAA	
Runx2 (2)	Forward	ATACCCTCTAGTCAGATCG	ChIP
	Reverse	GCTGATTTGTGTTTGTAG	

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation; Amtn, amelotin; Klk4, kallikrein-related peptidase 4; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2.

pRunx2 plus 5 μg pCbfβ. After 24 h, nuclear proteins from transfected cells were isolated using a nuclear protein extraction kit (Pierce; Thermo Fisher Scientific, Inc.). Protein concentration of the nuclear extracts was determined using a bicinchoninic acid assay. EMSA was performed using a lightshift chemiluminescent EMSA kit (Pierce; Thermo Fisher Scientific, Inc.). Probes were labeled using the Biotin 3' end DNA labeling kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and incubated with nuclear protein extracts in the binding buffer from the EMSA kit for 20 min at room temperature. For the competition assay, nuclear proteins were pre-incubated with a 100-fold molar excess of unlabeled probe or mutation probe in the binding buffer. For the supershift experiments, 10 μg of anti-Runx2 antibody or normal rabbit IgG was incubated with nuclear extracts for 30 min at room temperature prior to the binding reaction. DNA-protein complexes were resolved in 5% native polyacrylamide gels, transferred onto Nylon N^+ membranes, and cross-linked for the detection of biotin-labeled DNA using chemiluminescence. Each experiment was repeated three times.

ChIP. ChIP analysis was performed using the EZ-ChIP kit (EMD Millipore, Billerica, MA, USA). The ALCs were seeded at a density of $0.7x10^5$ cells/cm^2 in a 150 mm^2 dish and cultured for 24 h at 37˚C. Proteins and DNA were cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by the addition of glycine. Cell pellets were resuspended in 1 ml of 1% SDS lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitors and fragmented using a sonic dismembrator (Soniprep 150, Sanyo, London, UK). For immunoprecipitation, antibodies against Runx2 or normal rabbit IgG (negative control) were used. The precipitated and input DNAs were subjected to PCR with two pairs of primers both specific to Runx2 listed in Table I, which generates 165-bp and 179-bp DNA fragments. The reactions were performed under the following conditions: 94˚C for 1 min; 40 cycles of 94˚C for 15 sec, 56˚C for 30 sec and 72˚C for 15 sec; and a final extension at 72˚C for 10 min. PCR products were visualized on a 2% agarose gel following electrophoresis and analyzed by Matlab 8.6 software (MathWorks, Inc., Natick, MA, USA). Each experiment was performed three times.

Luciferase reporter assay. ALCs were cultured in 24-well plates at a density of $1x10^5$ cells/cm^2 for 16 h at 37˚C, following which cells were transfected with 400 ng/well of the indicated reporter plasmids plus 50 ng of pRL-TK. In all transfection experiments, the amount of plasmid DNAs was normalized as necessary with the pcDNA3.1(+) expression plasmid so that the total DNA was constant in each group. Following 30 h transfection, cells were harvested and luciferase activity was measured using a Luciferase Assay Reagent (Promega Corporation). To obtain the relative luciferase activity, luciferase activity values were divided by Renilla luciferase activity values. Each experiment was performed in triplicate and repeated three times.

Statistical analysis. Data were analyzed using two-way analysis of variance and are expressed as the mean ± standard deviation. The statistical differences between two groups were evaluated using Student's t test. Dunnett's test for multiple comparisons was applied when the overall F test result was significant. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Runx2 and Cbfβ localize in ameloblasts during amelogenesis. A detailed immunohistochemistry analysis of Runx2 expression in the first mandibular molars revealed that Runx2 is expressed in ameloblasts in a stage-dependent manner during amelogenesis (Fig. 1). Strong staining was also observed in

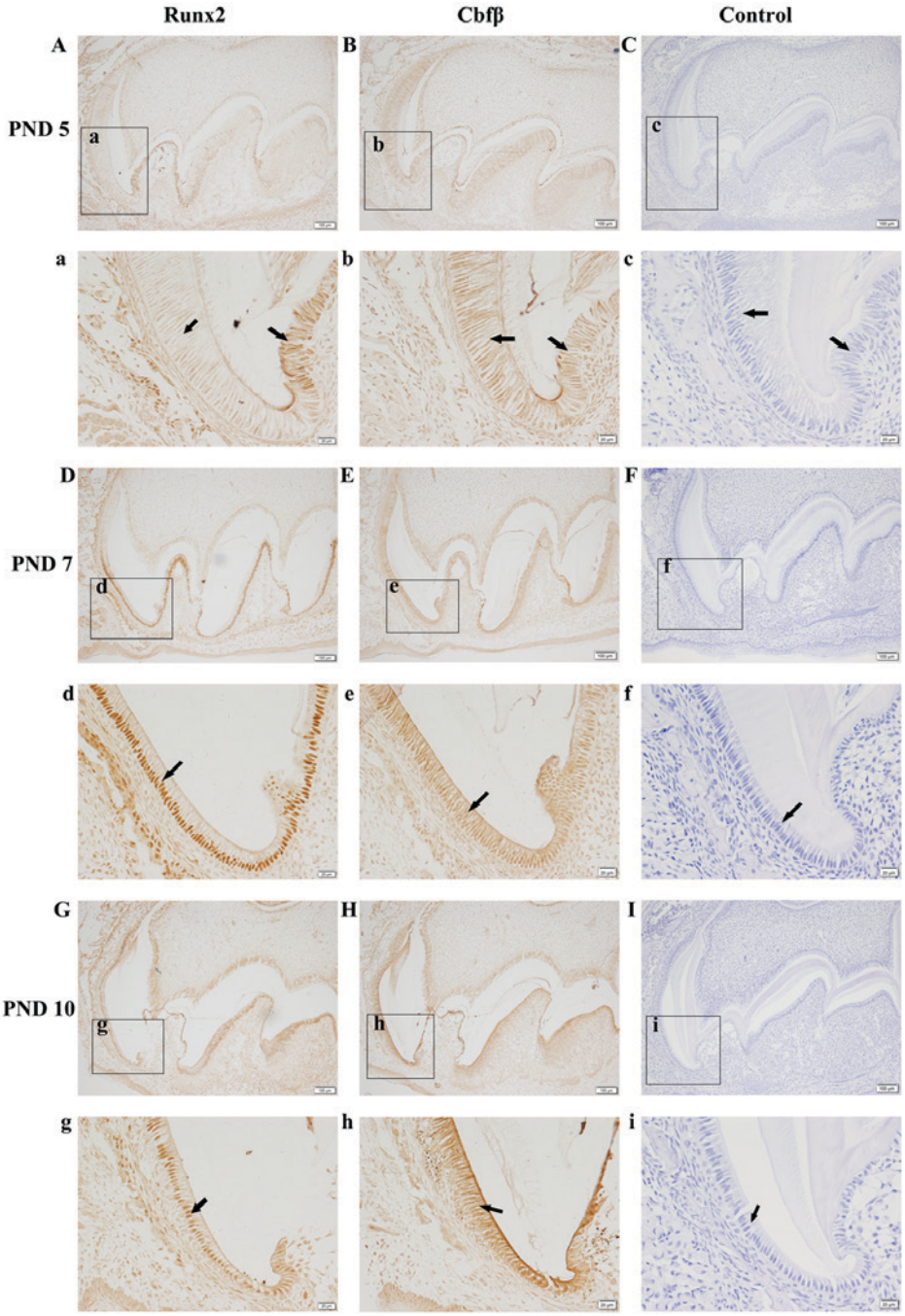

Figure 1. Identification of Runx2 and Cbfβ in amelogenesis by immunohistochemistry. Runx2 staining was investigated in mandibular molars of mice at *postnatal day* (A) 5, (D) 7 and (G) 10. Cbfβ protein in molars was observed in mandibular molars of mice at postnatal day (B) 5, (E) 7, and (H) 10. The staining on the control section was treated with non-immune rabbit IgG replacing primary antibody and stained again by hematoxylin staining (C, F and I). Scale bars=20 μm (a-i) and 100 μm (A-I). Arrows indicate where investigated protein was stained in ameloblasts by immunohistochemistry. Runx2, runt-related transcription factor 2; Cbfβ, coactivator core-binding factor β; PND, postnatal day; Ig, immunoglobulin.

osteoblasts in bone tissue surrounding the enamel organ. In the late secretory stage, a faint Runx2 expression was observed in the nucleus of ameloblasts (Fig. 1Aa). The signal increased when ameloblasts progressed to the transition stage (Fig. 1Dd) and continued to be observed at the maturation stage (Fig. 1Gg). Runx2 is known to function by forming a heterodimer with Cbfβ (20), so Cbfβ expression was also investigated using immunohistochemistry. Strong staining for Cbfβ was observed in ameloblasts at the secretory stage and was evenly distributed in the nucleus and cytoplasm of first mandibular molars from PND 5 mice (Fig. 1Bb). With enamel development, Cbfβ protein accumulated in the nucleus of ameloblasts at the transition (Fig. 1Ee) and maturation stages (Fig. 1Hh). These results suggest that Runx2 and Cbfβ are co-localized in the nucleus of ameloblasts from the late secretory stage to the maturation stage. In the control section of incisors, no positive signal was observed (Fig. 1C, F and I).

Runx2 regulates Amtn expression in ALCs. During tooth development, Kallikrein-related peptidase 4 (Klk4) and alkaline phosphatase (ALP) are predominantly expressed during the late stages of enamel formation (21,22). Therefore, the

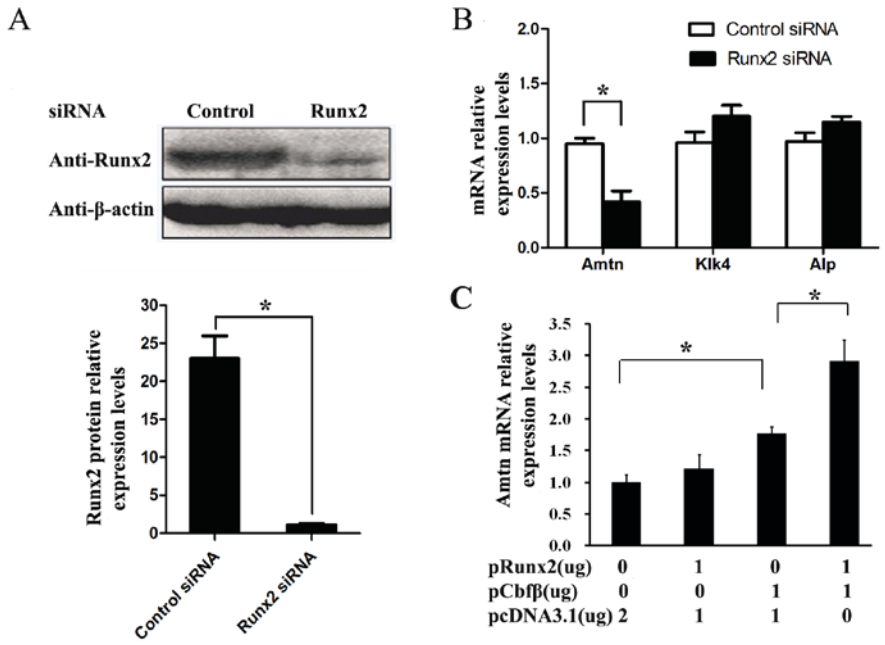

Figure 2. The regulation of Runx2 on Amtn gene expression in the ALCs. ALCs were transfected with Runx2 siRNAs or control siRNAs for 36 h. (A) The expression level of Runx2 was significantly decreased at the level of protein as determined by western blotting. β-actin was used as a loading control. (B) The mRNA levels of *Amtn*, *Klk4*, and *Alp* were analyzed by RT-qPCR. (C) ALCs were transfected with 1 μg pRunx2, 1 μg pCbfβ, or 1 μg pRunx2 plus 1 μg pCbfβ. After 36 h transfection, the mRNA level of *Amtn* gene was analyzed by RT-qPCR. *P<0.05. Runx2, runt-related transcription factor 2; Amtn, amelotin; ALCs, ameloblast lineage cells; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

expression of *Amtn*, *Klk4*, and *ALP* in ALCs by was examined using RT-qPCR. When ALCs were treated with siRNA targeting *Runx2*, the expression of Runx2 was significantly decreased at the protein level as determined by western blotting (Fig. 2A). A significant reduction in Amtn mRNA expression was observed, whereas the expression of Klk4 and Alp was not affected by siRNA targeting Runx2 (Fig. 2B). These data suggest that Runx2 may participate in *Amtn* gene expression. Next, the effect of overexpressed Runx2 on *Amtn* gene expression in ALCs was investigated. As shown in Fig. 2C, Runx2 overexpression alone had no significant effect on *Amtn* gene expression, whereas *Amtn* gene expression was upregulated by Cbfβ overexpression in a dose-dependent manner. Combined treatment with overexpressed Runx2 and Cbfβ further elevated *Amtn* expression in ALCs compared with overexpressed Cbfβ alone. These results suggest that Cbfβ is essential for Runx2-induced *Amtn* gene expression in ALCs.

Runx2 binds to the putative Runx2 binding sites in the presence of Cbfβ. To assess whether Runx2 activates *Amtn* expression by binding to Runx2 sites. Two putative Runx2 binding sites, AACCACT (site1: -1342/-1336) and AACCAAA (site2: -98/-92), were identified and underlined (Fig. 3A). EMSA was performed using nuclear extracts from 293T cells and probes corresponding to the two putative Runx2 binding sequences. As negative controls, the nuclear extracts from 293T cells transfected with pcDNA3.1(+) did not bind to the oligonucleotide (lanes 1 and 8, Fig. 3B). No oligonucleotide binding was observed for nuclear extracts from the cells transfected with pcDNA3.1 -Runx2 (lanes 2 and 9, Fig. 3B), whereas the nuclear extracts from 293T cells cotransfected with *Runx2* and *Cbfβ* plasmids exhibited strong binding to the oligonucleotide (lanes 3 and 10, Fig. 3B). Binding specificity was confirmed by preincubating nuclear extracts with a 100-fold excess of unlabeled wild type and mutant oligonucleotides. As presented in Fig. 3B, the DNA-Runx2/Cbfβ complex was strongly inhibited by the addition of a 100-fold molar excess of unlabeled wild type probes (lanes 4 and 11), but only partially inhibited by a 100-fold molar excess of unlabeled mutated probes (lanes 5 and 12). The DNA-Runx2/Cbfβ complex was further verified using anti-Runx2 antibody (lanes 6 and 13, Fig. 3B), but not by normal IgG (lanes 7 and 14, Fig. 3B). These results suggest that the Runx2/Cbfβ complex may specifically bind to the *Amtn* promoter to regulate *Amtn* gene transcription.

Runx2 binds to the endogenous Amtn promoter in vivo. To confirm that Runx2 binds to the *Amtn* promoter, an *in vivo* ChIP assay was performed (Fig. 3C). When the endogenous *Amtn* gene chromatin from ALCs was randomly fragmented by sonication, antibodies against Runx2 was able to precipitate down the *Amtn* promoter sequences, in which a 165-bp fragment containing Runx2 site 1 and a 179-bp fragment containing Runx2 site 2 were amplified by PCR (Fig. 3C). As a negative control, normal IgG failed to precipitate the promoter sequences. As a loading control, the corresponding 165-bp and 179-bp fragments were also identified in isolated genomic DNA inputs. These results suggest that two Runx2 binding sites in the *Amtn* promoter may serve essential roles in mediating *Amtn* transcription in ALCs.

Runx2 activates Amtn promoter activity via Runx2 binding sites in the presence of Cbfβ. To further confirm that Runx2

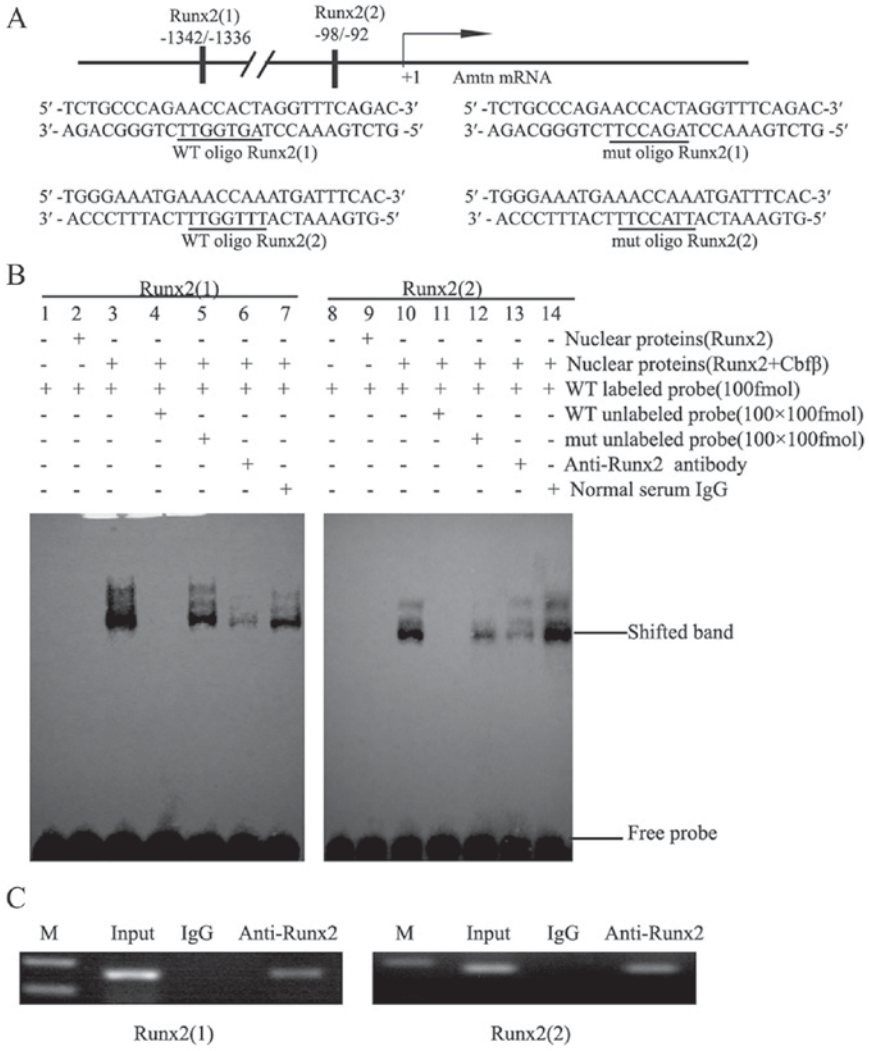

Figure 3. Runx2 directly binds to the motifs in the promoter of *Amtn* gene. (A) Design of the putative Runx2-binding sites (WT) and mutated (mut) Runx2 binding sites in the *Amtn* promoter for EMSA. (B) Detection of binding activity of Runx2 to WT and mut binding sites by EMSA. 293T cells were transfected with pcDNA3.1(+), pRunx2, and pRunx2 plus pCbfβ. Nuclear extracts from the transfected 293T cells were incubated with biotin-labeled wild-type (WT) probe of site 1 (Lanes 1-3) or site 2 (Lanes 8-10) in the absence of competitive probes. Competitive assays using 100-fold molar excess of unlabeled WT probes of site 1 and site 2 (Lanes 4 and 11) or unlabeled mutated probes of site 1 and site 2 (Lanes 5 and 12) were performed. (C) Verification of Runx2 binding with the site 1 and site 2 in the mouse *Amtn* promoter by chromatin immunoprecipitation assay. The sonicated nuclear extracts were subjected to immunoprecipitation with either an anti-Runx2 antibody or non-immune rabbit IgG. A pair of primers for site 1 or site 2 in the *Amtn* promoter was used for PCR. DNA extracted from genomic DNA served as a loading control (input), while DNA extracted from the non-immune rabbit IgG immunoprecipitated sample (IgG) served as a negative control. Runx2, runt-related transcription factor 2; Amtn, amelotin; IgG, immunoglobulin G; EMSA, electrophoretic mobility shift assay; Cbfβ, coactivator core-binding factor β; PCR, polymerase chain reaction.

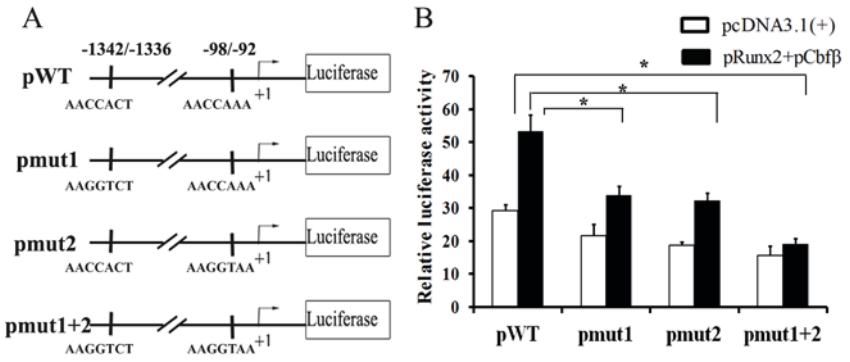

Figure 4. Regulation of *Amtn* promoter activity by Runx2 in the ALCs. (A) Mutation of putative Runx2 binding sites (site 1: -1342/1336; site 2: -98/-92) in mouse *Amtn* promoter. Site 1 was mutated from AACCACT to AAGGTCA (pmut1); site 2 was mutated from AACCAAA to AAGGTAA (pmut2); site 1 and site 2 were double mutated (pmut1+2). (B) ALCs were transfected with the indicated dose of pRunx2 plus pCbfβ. Promoter activities were measured with luciferase activity normalized to Renilla luciferase activity. *P<0.05. Amtn, amelotin; Runx2, runt-related transcription factor 2; ALCs, ameloblast lineage cells; Cbfβ, coactivator core-binding factor β.

regulates *Amtn* expression via the Runx2 binding sites, the -1463 bp to +196 bp region was cloned into the pGL3-basic vector (pGL3-Amtn). Two putative Runx2 binding sites in pGL3-Amtn (pWT) were mutated to create pGL3-Amtn-mut1 (pmut1), pGL3-Amtn-mut2 (pmut2), and pGL3-Amtn-mut1+2 (pmut1+2) (Fig. 4A). These constructs were transfected into ALCs and the promoter activities were analyzed in the presence or absence of overexpressed Runx2 and Cbfβ. To investigate Runx2/Cbfβ complex on *Amtn* promoter activity, a luciferase reporter assay was performed. The effects of Runx2/Cbfβ complex on the mutant *Amtn* promoter activity were observed. Mutation of either Runx2 site within the *Amtn* promoter caused statistically significant changes in promoter activity (Fig. 4B). Compared with pWT, the basal promoter activity of pmut1 or pmut2 was lower in the absence of Runx2/Cbfβ overexpression and the promoter activity was further decreased following mutation of both sites. Co-transfection with Runx2 and Cbfβ significantly enhanced the promoter activity of pWT, but only partially activated the promoter activity of pmut1 or pmut2. Furthermore, mutation of the two Runx2 binding sites completely eradicated the promoter activity in the presence of Runx2 and Cbfβ. These results suggest that Runx2/Cbfβ complex regulates *Amtn* gene expression via Runx2 binding sites in the *Amtn* promoter.

Discussion

The present study investigated the regulatory mechanism of Runx2 on *Amtn* gene expression during amelogenesis and the following results were obtained: i) Runx2 is predominantly expressed in the nuclei of ameloblasts at the transition and maturation stages; ii) Runx2 and Cbfβ are co-localized in the nucleus of ameloblasts at the transition and maturation stages; iii) Runx2 regulates *Amtn* but not *Klk4* and *ALP* gene expression; iv) Runx2 binds to specific motifs in the *Amtn* promoter in the presence of Cbfβ; and, v) Mutation of the Runx2 binding sites decreased or eliminated the activation of *Amtn* promoter activity by the Runx/Cbfβ complex.

Runx2 is essential in the early stages of bone and tooth development. In addition to the skeletal defects in *Runx2* knockout mice, developing teeth fail to advance beyond the bud stage (23). Elevated *Runx2* expression was detected in the ameloblasts at the late stages of enamel development, which suggests that Runx2 may serve a role in enamel maturation. The results of the present study are consistent with a previous report that Runx2 is elevated at the late stage of enamel development (24). Runx2 is a master transcription factor of bone and serves a role in all stages of bone formation (25). It is essential for the initial commitment of mesenchymal cells to the osteoblastic lineage and also controls the proliferation, differentiation and maintenance of these cells (26). Enamel and bone are mineralized tissues with similar developmental processes (27,28). Enamel-related gene products (ERPs) are detected in non-enamel tissues such as bone (29). It has been reported that individual ERPs affect individual transcription factor (Runx2, Sp7, bone sialoprotein and Msh homeobox 2) cascades to affect downstream effects on osteoblast differentiation, mineralization and calvarial bone development (29). In the present study, *Runx2* was highly expressed in ameloblasts and osteoblasts, but weakly expressed in pre-odontoblasts, supporting the critical role of Runx2 in enamel development. It has been reported that Cbfβ, a partner protein of Runx2 transcription factor, is expressed in the secretory stage of ameloblasts during tooth development by *in situ* hybridization (14), which is consistent with the findings of the current study that Cbfβ protein is expressed in cytoplasm and nucleus of secretory ameloblasts as detected by immunohistochemistry. It is well established that the Runx2/Cbfβ complex in the nucleus serves an essential role in bone formation (30). The present study indicated that Cbfβ protein accumulates in the nucleus of ameloblasts at the transition and maturation stages, which suggests that Runx2/Cbfβ complexes may serve an important role in the later stage of enamel development.

Amtn is a recently identified enamel matrix protein (4,31). Due to the temporal-spatial expression pattern of *Amtn* being similar to that of Runx2 in late stage enamel development, it was hypothesized that *Amtn* gene expression may be regulated by Runx2. This hypothesis was verified using *Runx2* knockdown, in which *Runx2* siRNA abrogated *Amtn* gene expression. The overexpression experiment further confirmed that co-expression of Runx2 and Cbfβ augments *Amtn* gene expression in the ALCs. These results may explain the similar spatial-temporal expression pattern of *Amtn* as that of *Runx2* during amelogenesis. A notable discovery is that Runx2 downregulates *Amtn* gene transcription activity. Runx2 may act as either a positive regulator or a repressor for expression of the targeted genes, and Runx2 may downregulate expression of the targeted genes by interacting with mothers against decapentaplegic homolog (Smad)3 via Smad binding site (32). It is possible that Runx2 may interact with unknown factors binding to the promoter to downregulate *Amtn* gene expression at the late maturation stage, when *Amtn* expression evidently decreases (4). The findings of the present study suggest that the *Amtn* gene is specifically upregulated by the Runx2/Cbfβ complex as a physiological consequence of the late secretory and maturation stages.

Runx2 regulates gene expression by binding to the consensus core sequences AACCACA in the promoter of target genes. To evaluate the regulation of *Amtn* promoter activity by Runx2, mouse *Amtn* promoter (-1463/+96) was analyzed and it was identified that it contains two putative Runx2 binding sites, AACCACT (-1342/-1336) and AACCAAA (-98/-92), which are similar to the AACCACA sequences. The binding of Runx2 to the AACCACT element was demonstrated using EMSA and ChIP assays, which is consistent with previous reports that Runx2 binds to the AACCACT sequences in the promoter of bone- and tooth-associated genes, including dentin sialophosphoprotein, osteocalcin and collagenase 3 (33,34). Another putative Runx2 binding site AACCAAA (-98/-92) also demonstrated strong binding to Runx2. To the best of our knowledge, this is a new identified sequence binding to Runx2. In the present study, the *Amtn* promoter was trans-activated by Runx2 in a sequence-specific manner, suggesting that these binding sites are essential for activating the promoter activity of *Amtn* gene.

In conclusion, the present study demonstrated that the expression of mouse *Amtn* gene in amelogenesis is mediated by Runx2/Cbfβ complex. Runx2/Cbfβ can bind to the two Runx2 binding motifs AACCACT (-1342/-1336) and AACCAAA

(-98/-92) in the *Amtn* promoter and regulate *Amtn* gene expression. The present study may contribute to elucidation of the mechanism of tooth enamel development and prevention of clinical tooth enamel disease.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YG and XL designed the experiments. XL, ZX, QC, CX and YS performed the experiments. YW and LZ analyzed data. XL and YG wrote the manuscript.

Ethics approval and consent to participate

All protocols involving mice were reviewed and approved by the Ethical Committee of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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