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β-Catenin/LEF1 activated enamelin expression in ameloblast-like cells

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ABSTRACT

Enamelin is an ameloblast-specific matrix protein believed to play essential roles in enamel formation. However, mechanisms of enamelin transcription regulation are not clear. β-Catenin/LEF1 is a key transcriptional complex involved in tooth development. In this study, the role of β-catenin/LEF1 in enamelin expression was investigated. The 5′-flanking region of the mouse enamelin gene was analyzed and cloned. Co-transfection analysis and mutation assays revealed that two conserved LEF1 responsive elements located at −1002 and −597 bp upstream of the enamelin translation initiation site could augment transcriptional activity of the enamelin. The interaction between the enamelin elements and β-catenin/LEF1 was further confirmed by electrophoresis mobility shift assays and chromatin immunoprecipitation assays. In addition, LiCl treatment induced nuclear translocation of β-catenin and elevated endogenous enamelin expression in mouse ameloblast-like cells. The results suggested that Wnt/β-catenin signaling could function in enamelin gene expression by direct interaction through two conserved LEF1 responsive elements on the enamelin gene in ameloblast-like cells.

1. Introduction

Enamelin is an ameloblast-specific extracellular matrix protein that is essential for enamel formation. It is expressed by ameloblasts in the secretory, transition and early maturation stages of enamel development [1]. It has been suggested that enamelin may be a nucleator and/or a modulator for enamel matrix formation and crystal mineralization [2–4]. Mutations of the human enamelin gene cause amelogenesis imperfecta [5,6]. Lack of proper mineralized enamel formation has been found in *Enam*-null mice [4]. Investigations have been carried out on the regulatory mechanisms orchestrating spatiotemporal expression of enamelin. A 5.2 kb-long regulatory region upstream of the translation initiation site was identified to be necessary for the tooth-specific expression pattern of enamelin [7,8]. However, the precise *cis*-acting elements and *trans*-acting factors within this region have not been identified.

Several groups have reported that the canonical Wnt pathway plays key roles in inducing epithelial–mesenchymal interaction during odontogenesis. In the β-catenin and *Lef1* null mice, tooth morphogenesis was unable to progress beyond the bud stage [9–11]. In addition, apoptotic activity was significantly increased within the dental epithelium in *Lef1* null mutant mice [10]. A constitutively active mutation of β-catenin in oral epithelium caused formation of large, misshapen tooth buds and supernumerary teeth [12–14]. These observations indicate that Wnt/β-catenin signals direct multiple stages of tooth development and are essential for patterning tooth development. At the molecular level, Wnt/β-catenin is responsible for the regulation of *Fgf4* and *Fgf*8 and it is suggested that LEF1 acts as a relay of the Wnt signal to a cascade of FGF signaling activities [14,15]. Transient overexpression of *Wnt10* in a pluripotent fibroblast cell line induced *Dspp* mRNA expression, indicating that Wnt/β-catenin is also an upstream regulator of *Dspp* expression [16]. Despite these observations, whether Wnt/β-catenin is involved in the activation of enamelin, one of the most important matrix proteins in amelogenesis, has not been fully studied.

In this report, we investigated the potential role of β-catenin/LEF1 as a transcriptional activator of enamelin. A 5′-flanking region of the mouse enamelin and its deletions were analyzed and cloned. The activation and specific binding motifs of β-catenin/LEF1 on the enamelin gene were examined and the effects of Wnt/β-catenin activation on enamelin expression in ameloblast-like cells were evaluated.

2. Materials and methods

2.1. Reporter constructs

Enamelin genes have been characterized in mice and humans [17]. Putative regulatory elements in the 5′-flanking region of ena-

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melin were identified with MatInspector professional version 7.4.5 (http://www.genomatix.de). Alignment of 5′-flanking regions of human and mouse enamelin was carried out with BioEdit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The 5′-flanking region of the mouse enamelin gene was generated by PCR using TaKaRa LA Taq (TAKARA Bio Inc., Dalian, CHN) according to the manufacturer's instructions. The PCR primers are p6254: forward, 5′-CGGGTACCGGGCATTTATTCTCACTACTTTCT-3′ (*Kpn*I site underlined) and reverse: 5′-AAGACGCGTAGAGCCAAGGAGCAAGA-3′ (*Mul*I site underlined). For p2554, p2113, p1617, p1255 and p546, the common 3′-primer is 5′-GTAAGATCTTT ATTACCATCAACC ATACCCTTA-3′ (*Bgl*II site underlined), 5′-primers are, p2554: 5′-ATAGGTACCTATCACTAAACTTTGGAACTACGG-3′; p2113: 5′-CTT GGTACCGGTATAGGACTGAGCAATCTGAAG-3′; p1617: 5′-AATGG TACC AGCATTACACTAAGCAAACACACT-3′; p1255: 5′-CGAGGTAC CAAAGTCTAAATCCTAACAACGAAG-3′; and p546: 5′-ATCGGTACC AGCTTCTAAGTGGCATT-3' (*Kpn*I site underlined). The amplified products were extracted and purified with QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA). The purified fragments were ligated to a pGEM-T vector and cloned into a pGL3-basic vector (Promega, Madison, USA). Correct constructs were verified by DNA sequencing (3730XL sequencer, ABI, USA).

Mutation of the potential TCF/LEF1 binding sites on p1255 was performed using QuickChange site-directed mutagenesis kit (Stratagene). The 5′-TTCAAAGCC-3′ site was changed to 5′-TTCGATGCC-3′ (Site 1) and the 5′-TTCAAAGAC-3′ site was changed to 5′-TTCGAT-GAC-3′ (Site 2). The mutations were confirmed by DNA sequencing.

2.2. Cell culture

A mouse ameloblast cell lineage (LS8) was kindly provided by Dr. Malcolm L. Snead (University of Southern California). The LS8 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). MG63 cells were maintained in our lab and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Neonatal ICR mice (postnatal day 1) were sacrificed following the guidelines of Peking University Animal Care Committee. Ameloblast-like cells were prepared from collected lower first molars as previously described [18,19]. The cells were cultured in MCDB153 (Sigma) supplemented with 0.1% fetal bovine serum for 48 h before the media were changed into serum-free MCDB153 medium and kept for 24 h before further experiments.

2.3. Luciferase assays

LS8 cells and MG63 cells were seeded into 24-well plates 24 h before transfection. The PCG-LEF1-HA construct was provided by Dr. Klaus Wolff (Department of Experimental Dermatology at the University of Vienna). Constitutively active β-catenin was obtained from Dr. Roel Nusse (Stanford University School of Medicine). Transfections were performed with Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's protocol. Total amount of DNA was kept constant among samples by using pcDNA3.1 plasmid DNA (Invitrogen). The pRL-TR vector was used as an internal control to normalize transfection efficiency. Ratio activity was obtained by comparison with firefly/renilla luciferase activity. The data were obtained from at least three independent experiments and each experiment was performed in triplicate.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from PCG-LEF1-HA transfected LS8 cells as previously described [20]. Protein concentrations were determined using BCA protein assay kit (PIERCE, USA). The oligonucleotides used in EMSA, which represented LEF1 binding sites in mouse enamelin 5′-flanking region, wild and mutant types, were synthesized with the sequences shown in Table 1. Double-stranded oligonucleotides were labeled with γ-^{32}P ATP and incubated with 10 μg of nuclear extracts at 4 °C for 30 min. For competition experiments, the binding reaction was performed under 100 or 200-fold molar excess of unlabeled wild type or mutant oligonucleotides. For supershift assays, 0.8 μg anti-LEF1 antibody (sc-8591, Santa Cruz Biotechnology, USA) was added to the binding reaction 15 min prior to the addition of the labeled probes. Protein–DNA complexes were resolved on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography.

2.5. Chromatin immunoprecipitation assay (ChIP)

ChIP assays were conducted as previously described [21]. The LS8 cells were cross-linked with 1% (v/v) formaldehyde at 37 °C for 10 min. DNA was sheared by sonication to an average fragment size of 100–2000 bp. Cross-linked proteins were incubated with anti-LEF1 (sc-8591, Santa Cruz Biotechnology, USA) or anti-β-catenin antibody (sc-7199, Santa Cruz Biotechnology, USA) overnight at 4 °C. The PCR primers are: forward, 5′-ATCCATATCATTC CAAAAC-3′ and reverse, 5′-TTGCCATCACAAGAGTTAG-3′ (product size: 278 bp for Site 1); forward, 5′-TCAGCAGTTTGTAGTCAT-3′ and reverse, 5′-CCACAAATACAATAGGAAA-3′ (product size: 201 bp for Site 2).

2.6. Real-time quantitative RT-PCR

Primary cultured mouse ameloblast-like cells were treated with LiCl (10 mM) for 24 h. Total RNA was isolated and used for reverse-transcription into cDNAs using SuperScript™ First-Strand (Invitrogen). Real-time PCR was performed using a SYBR super mix kit (TOYOBO, JPN). Enamelin primers are: forward, 5′-TGCAGAAATCC GACTTCTCCT-3′ and reverse, 5′-CATCTGGAATGGCATGGCA-3′ (product size: 114 bp).

2.7. Western blot analysis

LS8 cells were cultured overnight with complete medium, and then cultured with serum-free DMEM for 12 h. The starved cells were treated with LiCl (10 mM) for 0, 6, 12 or 24 h. Total protein extracts and nuclear protein extracts were prepared separately. Aliquots of 30 μg of cell lysate per sample were subjected to 10% SDS–polyacrylamide gel electrophoresis. The primary antibodies were: anti-LEF1 (1/1000 dilution; sc-8591, Santa Cruz Biotechnology, USA) and anti-β-catenin (1/1000 dilution; sc-7199, Santa Cruz Biotechnology, USA); anti-enamelin polyclonal antibody was prepared as previously described (1/1000 dilution) [22]; anti-β-actin (1/1000 dilution; sc-47778, Santa Cruz Biotechnology, USA) and anti-lamin B (1/1000 dilution; sc-6217, Santa Cruz Biotechnology, USA) were used as internal controls. The enhanced chemiluminescence (ECL) detection system was purchased from Amersham (RPN2109).

2.8. Immunofluorescence

The LS8 cells treated as described above were subjected to immunocytochemical analysis of β-catenin. Collected cells were rinsed twice with ice-cold PBS and fixed with 4% (w/v) paraformaldehyde for 15 min at 4 °C, and then the slides were treated with 0.5% (v/v) Triton X-100 for 30 min and immersed in blocking agents for 1 h at room temperature. Samples were incubated with anti-β-catenin primary antibody (1/300 dilution) overnight at 4 °C, followed by a secondary antibody fluorescence-conjugated anti-

Table 1
Oligonucleotides[a] used in EMSA.

Oligonucleotide name		Sequences 5′→3′	
Wt1 sense	−1009	5′-AAGTTTCTATACTTCAAAGCCCAAATTCACA-3′	−979
Wt1 antisense		5′-TGTGAATTTGGGCTTTGAAGTATAGAAACTT-3′	
Mut1 sense		5′-AAGTTTCTATAC***GTTGCCT***CCCAAATTCACA-3′	
Mut1 antisense		5′-TGTGAATTTGGG***AGGCAAC***GTATAGAAACT-3′	
Wt2 sense	−604	5′-TTAAGAACATATTTCAAAGACAGGTTAACA-3′	−575
Wt2 antisense		5′-TGTTAACCTGTCTTTGAAATATGTTCTTAA-3′	
Mut2 sense		5′-TTAAGAACATAT***GTTGCCT***ACAGGTTAACA-3′	
Mut2 antisense		5′-TGTTAACCTGT***AGGCAAC***ATATGTTCTTAA-3′	

[a] LEF1 binding sites are underlined; substitution mutations are presented in boldface italic letters.

rabbit IgG-FITC (1/400 dilution, Sigma, USA). Nuclei were counterstained with Hoechst 33342 (1/500 dilution). Fluorescent images were collected by confocal laser microscopy (OLYMPUS FV1000, JPN). Slides were processed at the same time to ensure homogeneity of the staining procedures.

2.9. Statistical analysis

The data are presented as mean ± SD. Comparisons between two groups were made by two-tailed Student *t*-test. A significant difference was noted when $p < 0.05$.

3. Results

3.1. Analysis and construction of the 5′-flanking region of mouse enamelin gene

The mouse enamelin gene is 24,651 bp long and located on chromosome 5 (Enam ID: 13801). Its cDNA sequence contains 10 exons. Translation initiates in exon 3 and terminates in exon 10. Bioinformatic analysis with MatInspector software showed that there are 10 putative binding sites for TCF/LEF1 within a 6 kb region upstream of the mouse enamelin translation initiation site (assigned +1) (Fig. 1A). Alignment of the 5′-flanking region sequences of mouse and human enamelin (ENAM ID: 10117) indicated that there were two species-conserved TCF/LEF1 binding elements, positioned at −1002 to −986 bp (named as Site 1) and −597 to −581 bp (named as Site 2), respectively (Fig. 1B).

In order to understand whether β-catenin/LEF1 regulates enamelin gene expression, enamelin 5′-flanking regions were obtained by PCR and cloned into pGL3-basic luciferase vector. The p6254 construct contains a ~6 kb region upstream of the enamelin translation initiation site. p2554, p2113, p1617, p1255 and p546 were 5′ truncated constructs and obtained by PCR.

3.2. Activation of enamelin reporters by β-catenin/LEF1 and identification of the responsive elements on the enamelin 5′-flanking region

We firstly investigated the functionality of those cloned reporters in LS8 cells. Consistent with the transgenic analysis, the 6 kb region in the reporter construct p6254 was sufficient to drive the reporter gene in LS8 cells. Sequential deletions of the region between −6254 and −1255 in enamelin 5′-UTR gave rise to an increase (>4.5-fold) in transcriptional activity. However, further deletion between p1255 and p546 resulted in a sharp decrease of p546 reporter activity in LS8 cells (Fig. 2A). Therefore, we presume that the −1255/−546 region of the mouse enamelin 5′-flanking region functioned as a core promoter in LS8 cells. To determine whether β-catenin/LEF1 could activate the enamelin 5′-flanking region, co-transfection assays were performed in both LS8 cells and MG63 cells, a non-ameloblast osteosarcoma cell line. The same set of reporter constructs was transiently transfected with β-catenin/LEF1. The reporter activity of p6254 was 2.5-fold above that of the pGL3-basic vector in LS8 cells. Progressive deletions to p1255 led to an 11-fold increase in reporter activity in LS8 cells, in comparison with 1.08-fold in MG63 cells (Fig. 2B). The results suggested that the regulatory sequence between 1255 and 546 bp was required for β-catenin/LEF1 activation in ameloblast-like cells. The activation was disrupted by mutations of either Site 1 or Site 2 in p1255, indicating that both sites were necessary for β-catenin/LEF1 activation of the enamelin (Fig. 2C).

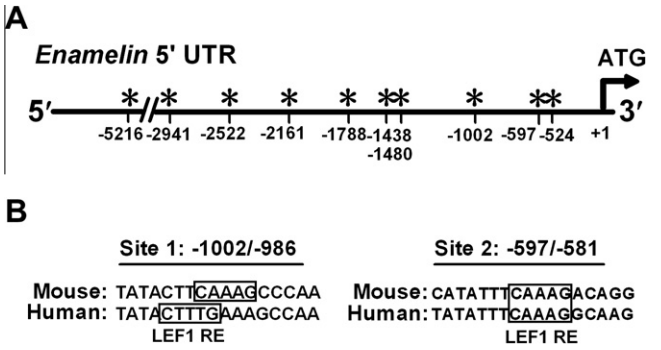

Fig. 1. Analysis and cloning of the enamelin 5′-flanking region. (A) Diagram of the mouse enamelin 5′-flanking region (5′-UTR). Translation initiation site is numbered as +1. TCF/LEF1 binding elements on 6 kb-long enamelin regulatory region are denoted by an asterisk (*). (B) Two conserved TCF/LEF1 responsive elements (RE) on enamelin genes of human and mouse. Site 1 from −1002 to −986 and Site 2 from −597 to −581 on the mouse enamelin gene are presented. LEF1 core binding sequences of Site 1 and Site 2 are boxed.

3.3. Binding of β-catenin/LEF1 to responsive elements on the enamelin 5′-flanking region

EMSAs were carried out to determine whether LEF1 could specifically interact with the *cis*-acting elements on the enamelin 5′-flanking region. Strong protein–DNA complexes were shown when the labeled wild type enamelin probes were incubated with LEF1 overexpressed nuclear extracts (Fig. 3A, lane 3 and B, lane 3). Unlabeled wild type enamelin probes with 100- or 200-fold molar excess inhibited binding of LEF1 to the labeled wild type probes (Fig. 3A, lanes 4–5 and B, lanes 4–5). No inhibition was found with 100-, or 200-fold molar excess mutant probes (Fig. 3A, lanes 6–7 and B, lanes 6–7). In addition, a supershift band was detected when LEF1 antibody was added into the Site 1 reaction mix (Fig. 3A, lane 8). No supershift was formed when the antibody was incubated with the Site 2 reaction mix; instead the protein–DNA complex disappeared (Fig. 3B, lane 8).

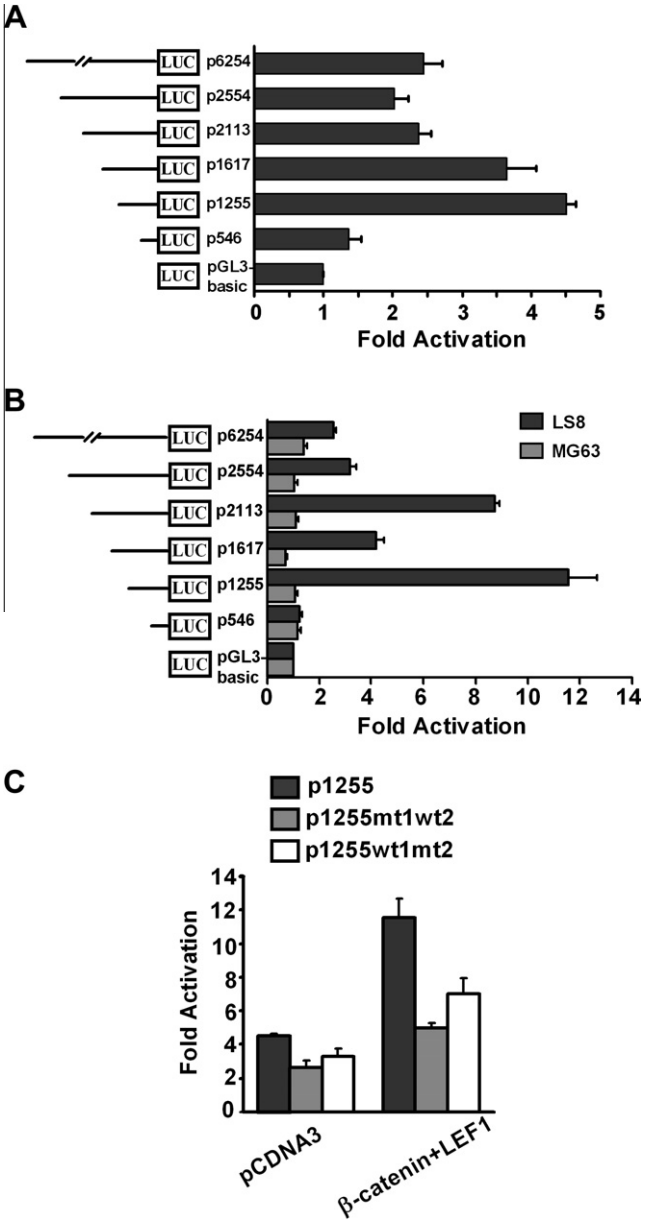

Fig. 2. Activation of enamelin transcription by β-catenin/LEF1 and identification of the responsive elements on the 5′-flanking region of mouse enamelin gene. (A) Luciferase reporter assays performed on serial deletions of the mouse enamelin gene in LS8 cells. The 6 kb enamelin regulatory region and deletions were subcloned into pGL3-basic luciferase vector (Luc). Equal amounts of various reporter constructs were transiently transfected into LS8 cells. The fold activation of luciferase activity was normalized to pGL3-basic controls. (B) The 5′-flanking regions of enamelin gene transactivated by β-catenin/LEF1 complex. Co-transfection assays were performed with the series enamelin reporter constructs in combination with constitutively active β-catenin and LEF1 in LS8 cells and MG63 cells, respectively. The total amount of DNA was kept constant with pCDNA3.1. The p1255 construct showed highest reporter activity of 11-fold specifically in LS8 cells. (C) Mutations of LEF1 binding Site 1 or Site 2 in p1255-luc reduced the reporter activities and β-catenin/LEF1 activation. The data represent the mean ± SD of three independent experiments, each experiment performed in triplicate.

ChIP assay was performed to test whether β-catenin/LEF1 would interact with enamelin *in vivo*. The protein–DNA mix immunoprecipitated with an antibody against β-catenin or LEF1 was used as a template for PCR. Specific products of 278 bp and 201 bp were obtained, which contained Site 1 and Site 2, respectively (Fig. 3C).

3.4. Elevation of enamelin expression by activation of Wnt/β-catenin pathway in ameloblast-like cells

To investigate the function of β-catenin/LEF1 on endogenous enamelin, we mimicked the activation of Wnt/β-catenin pathway using LiCl in ameloblasts. In the presence of 10 mM LiCl, enamelin transcription was upregulated 1.5-fold after 24 h in the primary cultured ameloblast-like cells (Fig. 4A). To further analyze the responses of enamelin to Wnt activation, we performed Western-blotting and immunofluorescence in LS8 cells. Enamelin expression at the protein level was markedly elevated at 24 h after treating with LiCl in LS8 cells and enhanced expression of nuclear β-catenin and LEF1 were detected after 6 h treatment, peaking at 12 h (Fig. 4B). Immunocytochemical analysis also showed strong nuclear staining of β-catenin at 6 h (Fig. 4C, b1–b3) and lasted up to 24 h with LiCl treatment (Fig. 4C, c1–c3, d1–d3).

4. Discussion

The importance of enamelin for proper enamel formation is well recognized. Results from studies using transgenic mouse

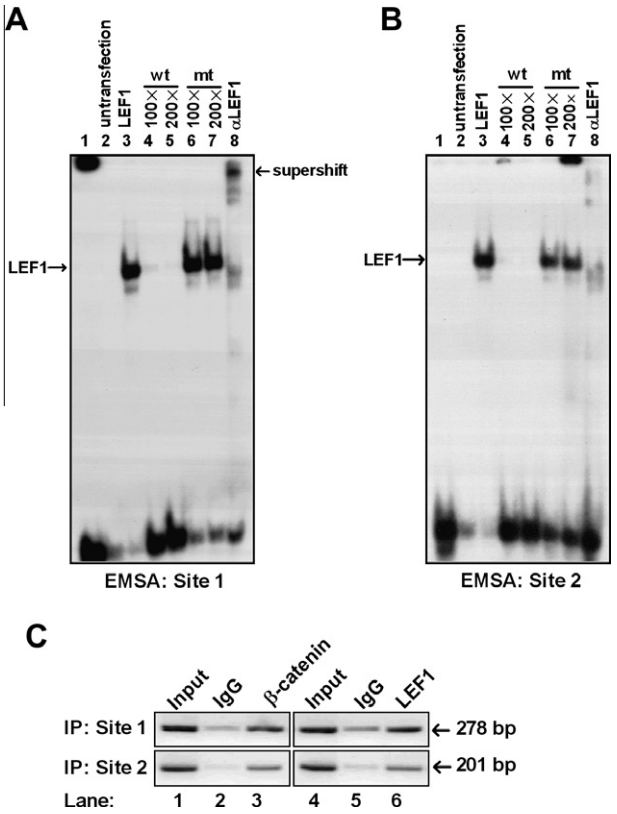

Fig. 3. Specific binding of β-catenin/LEF1 to responsive elements on the enamelin 5′-flanking region. (A) and (B) LEF1 formed a complex with the enamelin 5′-flanking region. Nuclear extracts were prepared from LS8 cells and subjected to EMSAs. Extracts were incubated with ^{32}P-labeled double-stranded oligonucleotides, Site 1 and Site 2. The arrows on the left margin of (A) and (B) mark DNA–protein complexes. A supershift band was observed when anti-LEF1 antibody was added into Site 1 reaction buffers (A, lane 8). The DNA–protein complex disappeared when LEF1 antibody was incubated with Site 2 reaction buffers (B, lane 8). Wt: wild type; mt: mutant type; αLEF1: anti-LEF1 antibody. (C) β-catenin/LEF1 recruited to the enamelin 5′-flanking region. LS8 cells were lysed and subjected to ChIP analysis using β-catenin or LEF1 antibodies. The both β-catenin–DNA mix and LEF1–DNA mix were used as the templates for PCR. Specific products of 278 bp and 201 bp were obtained, which contained Site 1 and Site 2 (C, lane 3 and lane 6), respectively. Total DNA before immunoprecipitation were used as inputs (C, lane 1 and lane 4). Products amplified from DNA precipitated by normal IgG were used as the negative controls (C, lane 2 and lane 5).

models suggested that a 5.2 kb regulatory region is required for tooth-specific expression of enamelin [7,8]. However, the identity of the critical regulatory elements and transcription factors within this region remained unclear. Wnt/β-catenin signaling has been implicated in the control of dental epithelial cell differentiation in tooth development [9,10,23]. In this study, we provide important information regarding to the functional significance of Wnt/β-catenin signaling in regulating enamelin expression in ameloblast-like cells.

It has been reported that 5′ regulatory sequence of mouse enamelin from −5200 bp to −3900 bp was specifically involved in tooth development, whereas the region of −3900 to −1 corresponded to basal promoter activity [7,8]. In our study, the reporter assay demonstrated that the p6254 reporter (containing the previously reported 5.2 kb region) possessed specific promoter activity. However, the deletion of −6254 to −2554 (containing the previously reported −5.2 to −3.9 kb region) did not result in significant changes in luciferase activity; while the deletion of p6254 to p1617 resulted a marked increase in luciferase activity specifically in LS8 cells (Fig. 2A), suggesting that negative regulators may bind to this region. Further deletion between −1255 and −546 diminished the promoter activity to a basal level, indicating that the core promoter region of enamelin may be located between −1255 and −546 bp. According to our results of primer extension assay (data are shown in Supplementary Fig. 1), the transcription initiation site of the mouse enamelin is located at −702 bp upstream of translation initiation site. The −1255/−546 bp region is resided within the predicted putative basal promoter (PBP) of the mouse enamelin reported [7]. Notably, there are two species-conserved binding elements for LEF1, which are positioned at −1002 to −986 bp (Site 1) and −597 to −581 bp (Site 2), respectively (Fig. 1). Generally, the evolutionary conservation indicates a potentially important role in gene transcriptional regulation. Co-transfection assays showed that the −1255/−546 region responded to β-catenin/LEF1 specifically in LS8 cells (Fig. 2B). The EMSA and ChIP analysis further indicated that β-catenin/LEF1 could specifically be recruited and interact with these elements (Fig. 3). As we know, the core promoter is required for proper initiation of gene transcription. The identification of the core promoter of enamelin will form a basis to study the transcription regulation of enamelin and therefore provide important information for further investigation. Our results demonstrated that β-catenin/LEF1 was critical for the specific

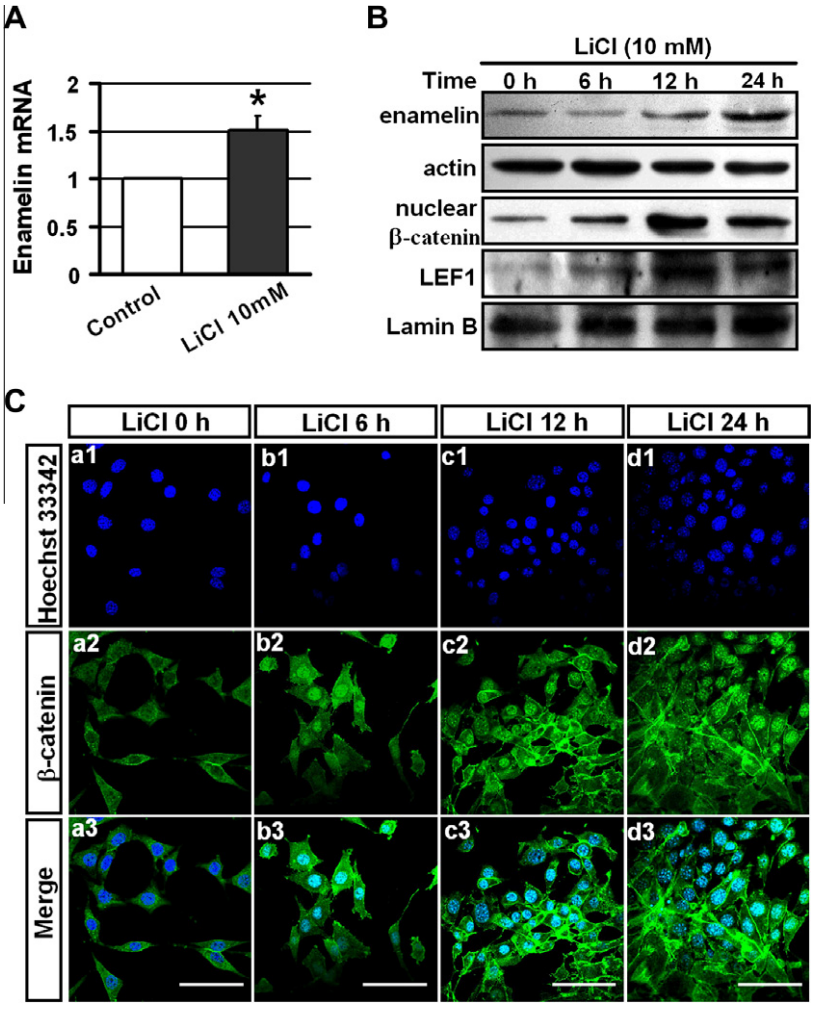

Fig. 4. Elevation of endogenous enamelin expression and translocation of β-catenin following LiCl treatment in ameloblast-like cells. (A) Enamelin expression at mRNA level in primary cultured ameloblast-like cells treated with LiCl was analyzed by real-time quantitative RT-PCR. Cells without LiCl treatment were used as a control. Similar data were obtained from three independent experiments. *$p < 0.05$. (B) Whole cell lysates and nuclear extracts were prepared from LS8 cells treated with LiCl and then subjected to Western blot analysis with anti-enamelin, anti-β-catenin and anti-LEF1 antibodies. Anti-actin and anti-lamin B antibodies were loaded as internal controls. Similar data were obtained from three separate experiments. (C) Immunocytochemical analysis for β-catenin was performed in LS8 cells treated with LiCl at different time points. β-Catenin was detected in the cell membrane and cytoplasm when LS8 cells were maintained without LiCl stimulation (C, a1–a3). Nuclear β-catenin staining was observed 6 h after LiCl treatment (C, b1–b3), and maintained until 24 h after the treatment (C, c1–c3, d1–d3). Similar data were obtained from three separate experiments. Bars = 50 μm.

activation of enamelin core promoter (−1255/−546) in LS8 cells. In addition, it also suggested that the tissue-specific coactivators may be involved in this region that synergistically activate enamelin with β-catenin/LEF1, while the distal regulatory region (−6254 to −2113) may contain negative regulatory elements that suppress β-catenin/LEF1 activation. Further studies will include making a construct containing only −5200 to −3900 bp of enamelin and compare its transcriptional activity and specificity with other regions.

Previous *in vivo* studies showed that, in the late bell stage, enamelin transcripts was detected in pre-ameloblasts on mouse molars [1] and β-catenin/LEF1 were upregulated within inner enamel epithelium [10,24]. A recent study reported that *Wnt3a*, *Wnt10a* and *Axin2* are expressed in pre-ameloblasts and ameloblasts in the continuously growing mouse incisors, while LEF1 was not detected in ameloblasts [25]. Although β-catenin, the mediator of the Wnt pathway, was not characterized, *Dkk3*, an antagonist for Wnt signaling, was also detected in the pre-ameloblasts and ameloblasts of mouse teeth [25,26]. It seemed that the roles of Wnt/β-catenin signals in secretory stage ameloblasts remain controversial. In our current study, elevation of enamelin expression was detected with the activation of Wnt/β-catenin pathway in ameloblast-like cells (Fig. 4). A further *in vivo* study should be performed to confirm the importance of Wnt/β-catenin pathway in differentiation of ameloblasts.

In summary, this study showed that β-catenin/LEF1 complex could activate enamelin gene transcription. Two key elements at −1002 to −986 and −597 to −581 bp of mouse enamelin directly respond to β-catenin/LEF1 signaling in ameloblast-like cells. This suggests that Wnt/β-catenin can upregulate enamelin expression by direct binding to enamelin 5′-flanking region in ameloblast-like cells.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.111.

References

[1] J.C. Hu, X. Sun, C. Zhang, J.P. Simmer, A comparison of enamelin and amelogenin expression in developing mouse molars, Eur. J. Oral Sci. 109 (2001) 125–132.
[2] C. Robinson, S.J. Brookes, R.C. Shore, J. Kirkham, The developing enamel matrix: nature and function, Eur. J. Oral Sci. 106 (Suppl 1) (1998) 282–291.
[3] T. Tanabe, T. Aoba, E.C. Moreno, M. Fukae, M. Shimuzu, Properties of phosphorylated 32 kD nonamelogenin proteins isolated from porcine secretory enamel, Calcif. Tissue Int. 46 (1990) 205–215.
[4] J.C. Hu, Y. Hu, C.E. Smith, M.D. McKee, J.T. Wright, Y. Yamakoshi, P. Papagerakis, G.K. Hunter, J.Q. Feng, F. Yamakoshi, J.P. Simmer, Enamel defects and ameloblast-specific expression in Enam knock-out/lacz knock-in mice, J. Biol. Chem. 283 (2008) 10858–10871.
[5] M.H. Rajpar, K. Harley, C. Laing, R.M. Davies, M.J. Dixon, Mutation of the gene encoding the enamel-specific protein, enamelin, causes autosomal-dominant amelogenesis imperfecta, Human Mol. Genet. 10 (2001) 1673–1677.
[6] J.W. Kim, F. Seymen, B.P. Lin, B. Kiziltan, K. Gencay, J.P. Simmer, J.C. Hu, ENAM mutations in autosomal-dominant amelogenesis imperfecta, J. Dent. Res. 84 (2005) 278–282.
[7] Y. Hu, P. Papagerakis, L. Ye, J.Q. Feng, J.P. Simmer, J.C. Hu, Distal cis-regulatory elements are required for tissue-specific expression of enamelin (Enam), Eur. J. Oral Sci. 116 (2008) 113–123.
[8] P. Papagerakis, Y. Hu, L. Ye, J.Q. Feng, J.P. Simmer, J.C. Hu, Identifying promoter elements necessary for enamelin tissue-specific expression, Cells Tissues Organs 189 (2009) 98–104.
[9] C. van Genderen, R.M. Okamura, I. Farinas, R.G. Quo, T.G. Parslow, L. Bruhn, R. Grosschedl, Development of several organs that require inductive epithelial–mesenchymal interactions is impaired in LEF-1-deficient mice, Genes Dev. 8 (1994) 2691–2703.
[10] T. Sasaki, Y. Ito, X. Xu, J. Han, P. Bringas Jr., T. Maeda, H.C. Slavkin, R. Grosschedl, Y. Chai, LEF1 is a critical epithelial survival factor during tooth morphogenesis, Dev. Biol. 278 (2005) 130–143.
[11] J. Chen, Y. Lan, J.A. Baek, Y. Gao, R. Jiang, Wnt/beta-catenin signaling plays an essential role in activation of odontogenic mesenchyme during early tooth development, Dev. Biol. 334 (2009) 174–185.
[12] E. Jarvinen, I. Salazar-Ciudad, W. Birchmeier, M.M. Taketo, J. Jernvall, I. Thesleff, Continuous tooth generation in mouse is induced by activated epithelial Wnt/beta-catenin signaling, Proc. Natl. Acad. Sci. USA 103 (2006) 18627–18632.
[13] F. Liu, E.Y. Chu, B. Watt, Y. Zhang, N.M. Gallant, T. Andl, S.H. Yang, M.M. Lu, S. Piccolo, R. Schmidt-Ullrich, M.M. Taketo, E.E. Morrisey, R. Atit, A.A. Dlugosz, S.E. Millar, Wnt/beta-catenin signaling directs multiple stages of tooth morphogenesis, Dev. Biol. 313 (2008) 210–224.
[14] X.P. Wang, D.J. O'Connell, J.J. Lund, I. Saadi, M. Kuraguchi, A. Turbe-Doan, R. Cavallesco, H. Kim, P.J. Park, H. Harada, R. Kucherlapati, R.L. Maas, Apc inhibition of Wnt signaling regulates supernumerary tooth formation during embryogenesis and throughout adulthood, Development 136 (2009) 1939–1949.
[15] K. Kratochwil, J. Galceran, S. Tontsch, W. Roth, R. Grosschedl, FGF4, a direct target of LEF1 and Wnt signaling, can rescue the arrest of tooth organogenesis in Lef1(−/−) mice, Genes Dev. 16 (2002) 3173–3185.
[16] T. Yamashiro, L. Zheng, Y. Shitaku, M. Saito, T. Tsubakimoto, K. Takada, T. Takano-Yamamoto, I. Thesleff, Wnt10a regulates dentin sialophosphoprotein mRNA expression and possibly links odontoblast differentiation and tooth morphogenesis, Differentiation 75 (2007) 452–462.
[17] J.C. Hu, C.H. Zhang, Y. Yang, C. Karrman-Mardh, K. Forsman-Semb, J.P. Simmer, Cloning and characterization of the mouse and human enamelin genes, J. Dent. Res. 80 (2001) 898–902.
[18] A. Nakata, T. Kameda, H. Nagai, K. Ikegami, Y. Duan, K. Terada, T. Sugiyama, Establishment and characterization of a spontaneously immortalized mouse ameloblast-lineage cell line, Biochem. Biophys. Res. Commun. 308 (2003) 834–839.
[19] T. Suzawa, N. Itoh, N. Takahashi, T. Katagiri, N. Morimura, Y. Kobayashi, T. Yamamoto, R. Kamijo, Establishment of primary cultures for mouse ameloblasts as a model of their lifetime, Biochem. Biophys. Res. Commun. 345 (2006) 1247–1253.
[20] J.D. Dignam, R.M. Lebovitz, R.G. Roeder, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei, Nucleic Acids Res. 11 (1983) 1475–1489.
[21] Y. Shang, X. Hu, J. DiRenzo, M.A. Lazar, M. Brown, Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription, Cell 103 (2000) 843–852.
[22] P. Lv, X.J. Gao, Y. Gao, H.T. Jia, Immunohistochemical localization of enamelin in developing rat tooth germ, Beijing Da Xue Xue Bao 39 (2007) 37–40.
[23] M. Lohi, A.S. Tucker, P.T. Sharpe, Expression of Axin2 indicates a role for canonical Wnt signaling in development of the crown and root during pre- and postnatal tooth development, Dev. Dyn. 239 (2010) 160–167.
[24] N. Obara, Y. Suzuki, M. Takeda, Gene expression of beta-catenin is up-regulated in inner dental epithelium and enamel knots during molar tooth morphogenesis in the mouse, Cell Tissue Res. 325 (2006) 197–201.
[25] M. Suomalainen, I. Thesleff, Patterns of Wnt pathway activity in the mouse incisor indicate absence of Wnt/beta-catenin signaling in the epithelial stem cells, Dev. Dyn. 239 (2010) 364–372.
[26] K. Fjeld, P. Kettunen, T. Furmanek, I.H. Kvinnsland, K. Luukko, Dynamic expression of Wnt signaling-related Dickkopf1, -2, and -3 mRNAs in the developing mouse tooth, Dev. Dyn. 233 (2005) 161–166.