Transcriptional regulation of *AMELX* and *DSPP* genes by MIZ-1 and ZBTB5

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Transcriptional regulation of *AMELX* and *DSPP* genes by MIZ-1 and ZBTB5

Directed by Professor Man-Wook Hur

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Hee Jin Noh

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Amelogenin (*AMELX*) is the main component of the developing enamel matrix and is essential for enamel thickness and structure. *Dentin sialophosphoprotein* (*DSPP*) plays an important role in the differentiation of odontoblast and mineral deposition. We recently found that POK family proteins are expressed during tooth development. To identify which POK proteins are potentially involved in tooth development, we investigated mRNA expression profiles of POK proteins during odontoblastic differentiation of hDPSC (human dental pulp stem cell). The mRNA expression pattern is variable depending on the POK gene and temporally regulated during odontoblast differentiation. To investigate which POK proteins regulate mineralization of differentiated odontoblast cells, hDPSCs

were transfected with expression vector of various POK proteins and siRNA against POK mRNA. Arizarin red staining of the odontoblast (day 11 or 12 after transfection) showed that MIZ-1, ZBTB2 significantly increased the mineral nodules but ZBTB5 decreased. We are in the process of investigating how POK family proteins regulate gene expression of *AMELX* and *DSPP.* In the mice MEF cells transfected with MIZ-1, ZBTB2 or ZBTB5 expression vectors, MIZ-1 increased but ZBTB5 repressed gene expression of *Amelx* and *Dspp*. However, ZBTB2 didn't affect expression of the two genes. By reporter assays, we found that ZBTB5 repressed longer (bp, -485 to +100) and minimal promoters (bp, -70 to +100) of *Amelx* gene. MIZ-1 activated minimal promoter of *Amelx* gene, but repressed longer promoter of *Amelx* gene. ZBTB2 repressed only minimal promoter. Our preliminary data suggest that POK proteins regulate *AMELX* and *DSPP* gene expression.

Key Words: POK, transcription factor, *AMELX*, *DSPP*, tooth development

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Ⅰ. INTRODUCTION

The tooth development is controlled by a ‘cross-talk’ between epithelium and mesenchyme. The cross-talk involves ligand–receptor interactions that induce the transcriptional changes necessary to orchestrate the cellular processes that are required for the progression of tooth development.[1] During tooth initiation the ectoderm thickens and forms a placode that buds to the underlying neural-crestderived mesenchyme. The epithelium signals to the mesenchyme, which then condenses around the epithelial bud. After the bud stage, the epithelium starts to extend further into the mesenchyme, wrapping around the condensing mesenchyme and a structure known as the primary enamel knot is created at its center. Enamel knots instruct the patterning of

tooth crown and determine the location and height of tooth cups. Cervical loops fold around the condensing mesenchyme. At the late-cap to early-bell stages, high levels of apoptosis occur within the enamel knot, leading to the eventual loss of the structure and silencing of the signaling center. During the bell stages, cyto-differentiation occurs. The adjacent layer of epithelial cells differentiates into ameloblasts which secrete the enamel matrix, while mesenchyme differentiates into odontoblasts producing dentin.[2-9]

Ameloblasts secrete the key enamel protein amelogenin. Amelogenin plays a key role in regulating proper enamel mineralization and is believed to regulate its own replacement by the mineral phase to create a woven hierarchical architecture that accounts for the unique material properties of enamel.[10] Dental enamel is the hardest tissue in the body and cannot be replaced or repaired, because the enamel secreting cells, ameloblasts, are lost at tooth eruption. Amelogenin proteins constitute 90% of the extracellular matrix secreted by ameloblasts, and these proteins are cleaved in a regulated process during enamel maturation.[11,12] Several mutations in the human X-chromosomal *amelogenin* (*AMELX*) gene have been reported that lead to X-linked amelogenesis imperfecta (AI)1.[13-18] AI 1 is an inherited enamel defect characterized by phenotypic variability in which patients present with hypoplastic defects (thin pitted or grooved enamel) and/or hypomineralization where the enamel mineral content is decreased.[11] Therefore, X-linked AI provides strong evidence that amelogenin is critical for normal enamel formation.

Amelogenin expression is ameloblast specific and developmentally regulated at the temporal and spatial level.[19-25] Also, amelogenin expression is regulated at both the transcriptional and post transcriptional levels.[1] A 2263-nucleotide proximal promoter element from the mouse X-chromosomal amelogenin gene has been demonstrated by transgenic mouse analysis to recapitulate the spatiotemporal expression pattern of the endogenous amelogenin gene.[22] Homologies (70% identity) in the 300-nucleotide region upstream of the transcription initiation site exist between the murine, bovine, and human X-chromosomal amelogenin gene, suggesting that this region is likely involved in the transcriptional regulation of tissue-specific amelogenin gene expression. The promoter contains a reversed CCAAT box that is four base pairs downstream from the C/EBPα binding site. The CCAAT/enhancer-binding protein α (C/EBPα) plays a key role in the developmentally regulated expression of the amelogenin gene at the transcriptional level. Msx2 interferes with the binding of C/EBPα to its cognate site in the mouse amelogenin minimal promoter by protein-protein interaction, although Msx2 itself does not bind to the same promoter fragment.[26] NF-Y and C/EBPα synergistically activate the mouse amelogenin gene in a manner that can contribute to its physiological regulation during amelogenesis.[27] Foxj1 and Dlx2 independently activate the amelogenin promoter.[28]

Odontoblasts synthesize and secrete type I collagen and major noncollagenous protein, Dentin sialophosphoprotein (Dspp).[29] DSPP is a phosphorylated protein representing a major component, and essential for dentinogenesis.[30, 31] DSPP is expressed predominantly in odontoblasts and

transiently in preameloblasts as well as at low levels in bone.[32-34] The DSPP genes of mouse, rat, human, and pig have been cloned.[35-38] The DSPP gene consists of five exons and four introns. The DSP domain is found at the NH_2 terminus (exons 1–4 and the part of exon 5), whereas the DPP sequence is located at the COOH region (remainder of exon 5).[38-40] Dspp processing by tolloid-related protein 1 or by bone morphogenic protein 1 yields mature dentin sialoprotein (Dsp) and phosphophoryn (pp) proteins,[41] which are critical for dentin mineralization.[42] The DSPP expression level in rat long bone is about one-four hundredth that of rat dentin, suggesting that the function of DSPP is involved in tooth formation and mineralization.[34] Mutations of the human DSPP gene are associated with dentinogenesis imperfecta type II and type III.[43,44] Patients with these diseases present with discolored teeth, enlarged pulp chambers that fill in with mineralized matrix, a wider predentin zone, decreased dentin width, hypomineralization, and the prevalence of pulp exposures. Dspp-deficient mice have teeth that display dentin mineralization defects that are similar to those in human dentinogenesis imperfecta type III, indicating that Dspp plays a critical role in odontoblast differentiation and dentinogenesis.[45,46] Homologies (88% identity) in the 400 nucleotide region upstream of the transcription initiation site exist between rat, mouse and human DSPP gene and higher homologies identity (94%) was found between rat and mouse in the -405 bp sequence region.[39,47-48] Sequence conservation in the proximal promoter region between different species often suggests the presence of functional regulatory elements. The DSPP proximal promoter region conserved sequences in rat, mouse and human genes are SRE,

MSX-1, Inf.1, DRSII, and GRE (glucocorticoid responsive element) sites, as well as CCAAT box sequence. Transactivating factors capable of binding to some of these known conserved *cis*-element sites are likely to control DSPP gene transcription. [49] The human DSPP gene promoter contains TATA and CAATT box sequences.[39] Transcription factors that recognize the CCAAT motif have been identified, such as CCAAT/enhancer-binding protein (C/EBP), CAAT-box transcription factor (CTF/NF-I), and CP1 (NF-Y/CBF).[50] Furthermore, C/EBPβ and NF-Y mediate DSPP expression and odontoblast differentiation.[51] Homeoproteins Msx2 and Dlx5 transcriptionally control the proximal promoter of DSPP gene and these genes are in opposite direction by competing with homeodomain response element (HRE, TAATT). [52]

There are nearly 200 human BTB/POZ domain-containing proteins. BTB/POZ domain proteins having one or more Krüppel-like zinc-fingers are classified as the POK family. The N-terminal POZ domain has an important role in forming homo- or hetero-dimers and interacting with other proteins, while the C-terminal Krüppel-like zinc-finger domain (C_2H_2) recognizes and binds to specific DNA sequences.[53-56] POK family proteins that have been relatively well characterized include B-cell CLL/lymphoma 6 (BCL6), factor binding IST protein-1 (FBI-1)/leukemia-/lymphoma-related factor, hypermethylated in cancer 1 (HIC1), promyelocytic leukemia zinc-finger (PLZF) and Myc-interacting zinc-finger-1 (MIZ-1). Aberrant expression of some POK family proteins, including BCL6, FBI-1 and HIC1, has been associated with cancers, such as leukemia and various spontaneous malignant tumors.[57-60] PLZF controls the development of invariant natural killer T cell

effector function and the maintenance of spermatogonial stem cells.[61,62] MIZ-1, a potent transcriptional activator of CDKN1A, interacts with various oncoproteins, such as c-MYC, BCL6, ZBTB4 and GFI-1, to repress transcription of genes involved in cellular differentiation and metabolism.[58, 63-65] Recently, some novel POK family proteins have been characterized as transcriptional regulators of genes that control cell proliferation.[66-73] Although POK family proteins appear to play key roles in the various cell regulatory progress described above, the functions of many of the abovementioned POK family proteins remain largely unknown.[55]

Dentin is a calcified tissue that is characteristically similar in composition to bone.[46] Recently, some of POK proteins were shown to be related to bone formation or degradation. FBI-1, also known as OCZF(osteoclast derived zinc finger), regulates osteoclastogenesis.[74-76] ZBTB16 regulate a Runx2-independent pathway during the dexamethasone induced osteogenic differentiation of DFCs.[77,78] FAZF participates in the regulation of osteoblastic differentiation.[79] ZNF450, FAZF are induced by BMP protein that plays regulatory roles in bone or tooth development.[79,80] Gene expression profile analysis of tooth bud epithelium at initiation stage (E10.5) and bell stage (E16.5) showed that Zbtb16, Zbtb7a and Zbtb7b were up-regulated by 5, 3.1 and 2.8 fold, respectably at E16.5. [81] These results potentially suggest that POK proteins may regulate tooth development.

I recently found that POK family proteins are expressed in hDPSC (human dental pulp stem cell) during odontoblastic differentiation. mRNA expression of the tested POK genes is temporally regulated and is variable

depending on the POK gene during odontoblastic differentiation of hDPSC. I investigated whether POK family proteins regulate *AMELX* and *DSPP* expression during tooth development. I found that POK family protein members such as MIZ-1 and ZBTB5 may regulate odontoblastic differentiation by transcriptionally regulating *AMLEX* and *DSPP* genes.

Ⅱ. METHERIALS AND METHODS

1. Cell culture and transient transfection assays

HEK293, MEF, LS8, hDPSC (CEFO Research Center, Seoul, Korea) cells were maintained in Dulbecco's modified Eagle's medium(DMEM), supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin and grown at 37 ℃ in a humidified, 5% CO2 incubator.[82] All cell culture media and supplements were from Gibco-BRL. For odontoblastic induction, hDPSCs were cultured in DMEM supplemented with 10% fetal bovine serum, antibiotics, 50 mg/mL ascorbic acid (Sigma-Aldrich, St-Louis, Mo, USA), 10 mmol/l sodium b-glycerophosphate (Sigma-Aldrich, St-Louis, Mo, USA), and 10 nmol/l dexamethasone (Sigma-Aldrich, St-Louis, Mo, USA).

Various combinations of the plasmids pGL2-minimal-*Amelx*-Luc, pGL2-longer-*Amelx*-Luc, pcDNA3.1, pcDNA3.1-MIZ-1, pcDNA3-ZBTB2 or pcDNA3-ZBTB5 were transiently transfected into HEK293 using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA). After 24 to 36 h of incubation, transfected cells were harvested and analyzed for luciferase activity using a Microplate LB 96V luminometer (EG&G Berthold). All reactions were performed in triplicate. Reporter activity was normalized to co-transfected β-galactosidase activity, or total cellular protein, to determine transfection efficiencies.

2. Electroporation

Electroporation was performed using a Neon Transfection System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. MEF cells were washed with PBS and resuspended in electroporation buffer containing plasmid DNA, and the cells then electroporated (condition: volts 1350 V, pulse width 30 ms, pulse number 1) using a 100 μl tip. After electroporation, cells were suspended in DMEM medium and cultured

3. Alizarin Red Staining

hDPSCs were incubated with odontoblastic induction medium for 14 days, and mineralization was assessed at 0, 7, and 14 days by staining with alizarin red (Sigma-Aldrich, St-Louis, Mo, USA). hDPSCs cultured in DMEM supplemented with 10% bovine serum were set as the control. Briefly, fix cells 1ml 10% formaldehyde for minutes at room temperature. The cells were washed with distilled water. 1% alizarin red (pH 5.5) applied to the cells for 30 minutes at room temperature with gentle agitation and then the cells were washed with distilled water and allowed to dry.

4. Total RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using 2 μg of total RNA, random hexamers (10 pmol) and Oligo-dT(10 pmol), and Superscript reverse transcriptase II (200 units) in a total volume of 20 μl using a reverse transcription kit (Invitrogen, Carlsbad, CA, USA). PCR was performed using

the following amplification conditions: 94 ℃ denaturation for 3 min, 25, 30, 35 or 40 cycles of amplification reaction (94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s), and a final extension reaction at 72 ℃ for 5 min. The oligonucleotide primers sets used in semi-RT-PCR analysis were listed in Table1.

5. Quantitative real-time PCR (qPCR) analysis

Quantitative RT-PCR reactions were conducted with SYBR Green PCR Master Mix (Applied Biosystems) using gene-specific primers in an ABI PRISM 7300 RT-PCR System. All reactions were performed in triplicate. 18S ribosomal RNA was measured as a control. I designed quantitative real time PCR primers. The oligonucleotide primers sets of RT-qPCR were used as listed in Table2.

6. Knock down of endogenous MIZ-1, ZBTB2 and ZBTB5 expression by siRNA

siRNA against MIZ-1, ZBTB2 and ZBTB5 mRNA were synthesized in duplex and purchased from Bioneer (Bioneer, Daejeon, South Korea). siRNA (100 pmoles) were transfected into hDPSCs using Lipofectamin RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA). The siRNA were used as listed in Table3.

7. Western blot analysis

Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer. Cell extracts (30 mg) were separated using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto ImmunBlot Polyvinylidene Difluoride (PVDF) membranes (BioRad, Philadelpia, PA, USA) and blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA) or bovine serum albumin (Sigma-Aldrich, St-Louis, Mo, USA). Membrane blots were incubated with antibody against GAPDH, FLAG, or MIZ-1 followed by incubation with anti-mouse or rabbit secondary antibodies conjugated to Horseradish peroxidase (Vector Laboratories). Protein bands were visualized using an ECL kit (PerkinElmer, Waltham, MA, USA).

8. Biotin-Streptavidin oligonucleotide pull-down assay

Cells were lysed in HKMG (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40 and 1 mM dithiothreitol) buffer and the extracts incubated with 1 g biotinylated double-stranded oligonucleotides for 16 hr. Oligonucleotide probes were annealed by heating at 95 ◦C for 5 min, cooled slowly to room temperature and pull-down procedures performed as reported elsewhere. Oligonucleotides sequences were (only top strands are shown): *Amelx* promoter element #1, 5'-AGA AAG AAC ACC AGC GAT TG-3' (20mer); element #2, 5'-CAA GAA TGG GGA TTC AAT CC-3' (20mer); element #3, 5'-TTG CTA GAA CTG AGA CGT CG-3' (20mer) ; element #4, 5'-CGA CTA TAT GCA CTA ATC AC-3' (20mer); element #5, 5'-

CAT GAT ATA AAT TGG GGC AC-3' (20mer) ; and putative MRE, 5'-TTT CAT TCA GAA ACC TGA TTG GCT GTT CAA-3' (30mer).

9. Chromatin immunoprecipitation (ChIP)

Cells were fixed with formaldehyde (final 1%) to crosslink proteins to DNA promoters. ChIP procedures were then performed as reported elsewhere.[74] For detection of transcription factor binding, chromatin was immunoprecipitated with antibodies against MIZ-1 and FLAG-tag. Immunoglobulin G (IgG) and 3'-untranslated region (UTR) were used as negative controls. PCR reactions were conducted using the following oligonucleotide primer sets designed to amplify the regions of interest: element #1 of *Amelx* promoter (forward, 5'-AAC ACC AGC GAT TGT GGA AT-3' ; reverse, 5'- GTC AAG TTT CTC CAG TGT AC-3'), element #4 of *Amelx* promoter (forward, 5'- 5'-AAC ACC AGC GAT TGT GGA AT-3'; reverse, 5'- ATT TAT ATC ATG CAG GGC AC-3'), *Amelx* 3'-UTR (forward, 5'-AGG AGC AGG CCT TCA AGA AA-3'; reverse, 5'-TCT TTT CCT CTA CTC GGT TG-3')

Table 1. Nucleotide sequences of semi-quantitative RT-PCR oligonucleotide primers of POK protein genes, *AMELX* and odontoblastic marker genes, such as *DSPP* and *DMP-1*

Oligopeptide		Sequence
Kr-pok	Sense	5’- GAAGATGATGATGATGAGGAGGA-3’
	Antisense	5’- CTCACAGAG AAGGCCTATT CAGA-3’
ZBTB5	Sense	5’-TTA ATTAAATCAG GGCAGTT-3’
	Antisense	5’-TCC TGCAATGAAGGATG-3’
FBI-1	Sense	5’-TTCA CCAGGCAGGA CAAGCT-3’
	Antisense	5’-AAGAACCAC ATGCGCGTG-3’
Th-POK	Sense	5’-AAGCTGGT GCGCAAACG-3’
	Antisense	5’-ACACAGGC GAGAAGCCCTT-3’
MIZ-1	Sense	5’-CAGC CGTCACTCAG CTCA-3’
	Antisense	5’- ATCAGC AAAGCTGTGA AGCAAGT-3’
BCL6	Sense	5’-AATGAGTGTGACTGCCGCTTCT-3’
	Antisense	5’-CAAGACCGTCCATACCGGTG-3’
ZNF509	Sense	5’-GATCGGATCCGACCCTGTTGCTACCCAC-3’
	Antisense	5’-GATCCTCGAGAGAGTGGCGTGATTTTAAAAA-3’
KAISO	Sense	5’-GATT CTGCCCACAA AGGA-3’
	Antisense	5’-A ATCTAACCCA GGCCCTGTT-3’
ZBTB2	Sense	5’-GATCGGTACCGATTTGGCCAACCATGGACT-3’
	Antisense	5’-GATCGCTAGCTCAAACTTGGTCAGGGTGAGA-3’

GAPDH	Sense	5’-ACCACAGTCCATGCCATCAC-3’
	Antisense	5’-TCCACCACCCTGTTGCTGTA-3’
AMELX	Sense	5’-TCCCCCAGCAACCAATGAT-3’
	Antisense	5’-GAACATCGGAGGCAGAGGTG-3’
DSPP	Sense	5’-GAGGATAAAGGACAACATGGAAT-3’
	Antisense	5’-AAGAAGCATCTCCTCGGC-3’
DMP-1	Sense	5’-ACAGGCAAATGAAGACCC-3’
	Antisense	5’-TTCACTGGCTTGTATGG-3’

Table 2. Nucleotide sequences of RT-qPCR oligonucleotide primers of *Amelx* and *Dspp* mRNA

Oligopeptide		Sequence
Amelx	Sense	5’-CCCCTGTCCCCCATTCTT-3’
	Antisense	5’-TCCCGCTTGGTCTTGTCTGT-3’
Dspp	Sense	5’-CGAATCTAACGGAAGTGACGAAA-3’
	Antisense	5’-CTCCACGGCTGCCACTCT-3’
18S	Sense	5’-CCCCTTCATTGACCTCAACTAC-3’
	Antisense	5’-TCTCGCTCCTGGAAGATGG-3’

Table 3. Nucleotide sequences of siRNA against MIZ-1, ZBTB2 and ZBTB5 mRNA

Oligopeptide		Sequence
MIZ-1	Sense	5’-GAAGGCCGAGAUCAGCAAA(dTdT)-3’
	Antisense	5’-UUUGCUGAUCUCGGCCUUC(dTdT)-3’
ZBTB2	Sense	5’-CUCCACUCCAAUGAGGUU(dTdT)-3’
	Antisense	5’-AACCUCAUUGGGAGUGGAG(dTdT)-3’
ZBTB5	Sense	5’-GUGUGUACUUGUAGAGAGU(dTdT)-3’
	Antisense	5’-ACUCUCUACAAGUACACAC(dTdT)-3’

Ⅲ. RESULTS

1. Odontoblastic differentiation of hDPSCs

DPSCs are mesenchymal stem cells (MSC) that are present in the core region of the pulp.[86] These cells can differentiate into odontoblast-like cells, pulpal fibroblasts, adipocytes, and neural-like cells. hDPSCs abundantly express *AMELX* and *DSPP* proteins in differentiating odontoblasts. To test odontoblastic differentiation capability of hDPSCs, hDPSCs were cultured with induction medium(DMEM supplemented with 10% FBS, antibiotics, 50 μg/ml ascorbic acid, 10 mmol/l sodium β-glycerophosphate, and 10 nmol/l dexamethasone) for 14 days. Alizarin red staining was used to evaluate calcium-rich deposits in the cell cultured with normal medium or induction medium on day 0, 7 and 14 post induction. The early hDPSCs show flat, spindle-shape, fibroblast-like morphology. At day 0, there was no difference in staning of the hDPSCs grown in normal medium and odontoblasic induction medium. At day 7, the hDPSCs cultured in normal growth medium, are elongated and proliferate. But the hDPSCs grown in odontoblasic induction medium are polygonal in shape with more regular dimensions and the cells show mild in alizarin red staining, indicating an initiation of mineral deposit. The mineral deposit was markedly increased on 14 day of culture in odontoblastic differentiation medium (Figure 1A, B).

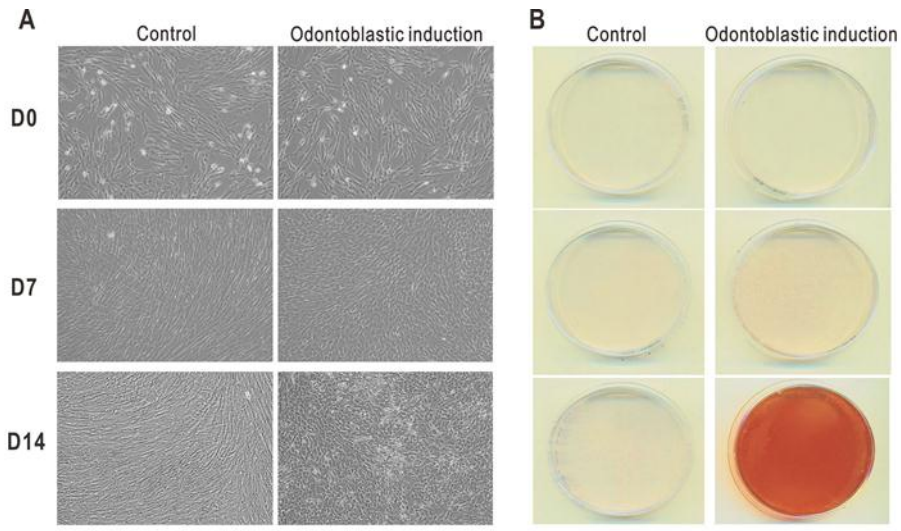

Figure 1. Odontoblastic differentiation of hDPSCs and mineralization. (A) The microscopic images of the hDPSCs cultured with normal growth medium(DMEM supplemented with 10% FBS, antibiotics) or with odontoblasic induction medium(DMEM supplemented with 10% FBS, antibiotics, 50 μg/ml ascorbic acid, 10 mmol/l sodium β-glycerophosphate, and 10 nmol/l dexamethasone) for 14 days. The microscopic images were taken at day 0, 7 and 14. (scale bar, 50 μm) (B) The mineralization of hDPSC was analyzed by alizarin red S staining of the cells at day 0, 7 and 14, post induction.

2. mRNA expression of POK, *AMELX* and *DSPP* genes during odontoblastic differentiation of hDPSCs

To examine mRNA expression profiles of POK, *AMELX* and odontoblastic marker gene, such as *DSPP* and DMP-1 during odontoblastic differentiation of hDPSCs, hDPSCs were cultured in odontoblasic induction medium for 20 days. Total RNA was isolated daily and mRNA level was measured by semi-quantitative RT-PCR. Consistently, the expression of *AMELX* and odontoblast-related genes, such as DMP-1 and *DSPP*, was significantly enhanced by culturing the cell in the odontoblasic induction medium (Figure 2A). Interestingly, I found that POK proteins were highly expressed during odontoblast differentiation. The mRNA expression pattern is variable depending on the POK gene and expression is temporally regulated during odontoblast differentiation. Also, the expression patterns of POK genes were largely similar to *AMELX* and *DSPP* genes (Figure 2B). The data suggested that POK proteins may regulate expression of the *AMELX* and *DSPP* genes.

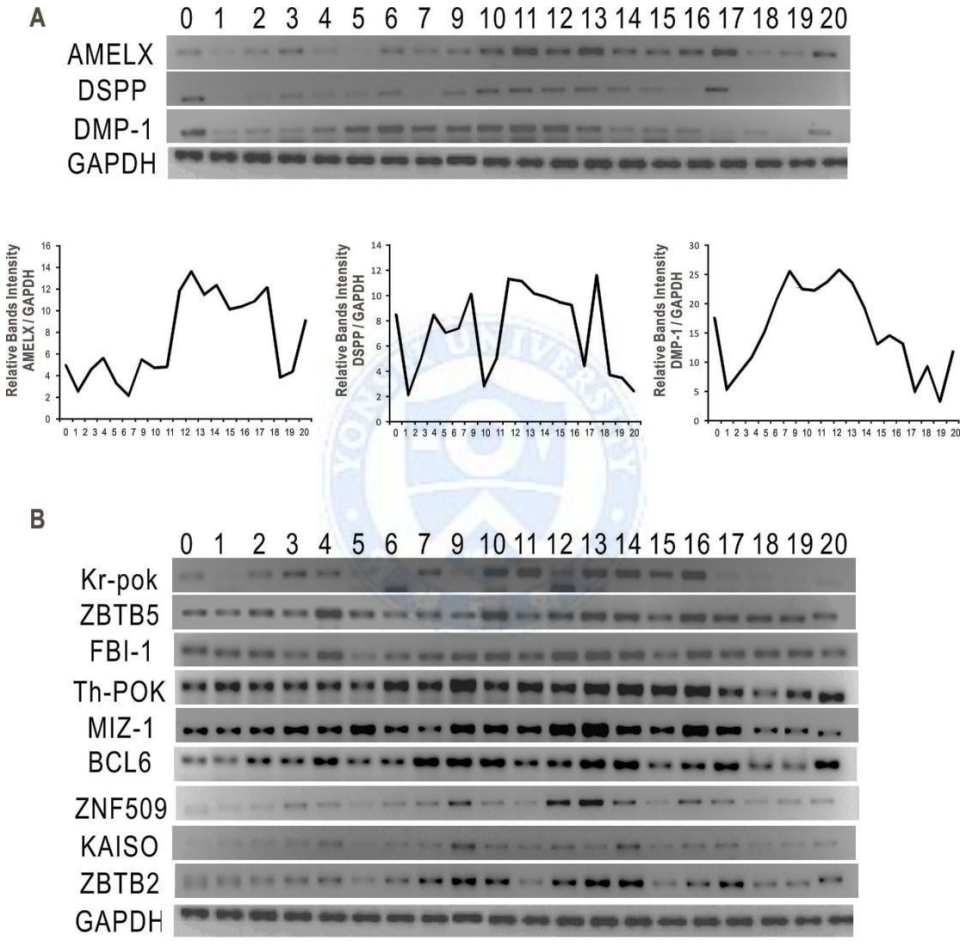
A
0 1 2 3 4 5 6 7 9 10 11 12 13 14 15 16 17 18 19 20
AMELX
DSPP
DMP-1
GAPDH
Relative Bands Intensity AMELX / GAPDH
Relative Bands Intensity DSPP / GAPDH
Relative Bands Intensity DMP-1 / GAPDH
B
0 1 2 3 4 5 6 7 9 10 11 12 13 14 15 16 17 18 19 20
Kr-pok
ZBTB5
FBI-1
Th-POK
MIZ-1
BCL6
ZNF509
KAISO
ZBTB2
GAPDH

Figure 2. The mRNA expression pattern of POK family genes, *AMELX*, *DSPP* and *DMP-1* during odontoblastic differentiation. Messenger RNA expression was analyzed by semi-quantitative RT-PCR. (A) The mRNA expression of AMELX, DSPP and odontoblastic marker gene (DMP-1) in hDPSCs over 20 days of odontoblastic differentiation. The mRNA transcript levels of *AMELX*, *DSPP* and DMP-1 genes were quantified. (B) The mRNA expression of POK proteins in hDPSCs during odontoblastic differentiation for 20 days. GAPDH was used as internal control. mRNA level normalized with GAPDH mRNA.

3. MIZ-1, ZBTB2 and ZBTB5 regulate mineralization in hDPSCs

The above data showed that POK proteins may regulate mRNA expression of *AMELX* and *DSPP* genes, and thereby may also regulate odontoblast differentiation and mineralization. I investigated the possibility by gain or loss of function of POK proteins experiments. hDPSCs were transfected with expression vector of various POK proteins and siRNA against POK mRNA. At day 11, the hDPSCs cultured in odontoblast differentiation medium only were not stained by alizarin red, but ectopic MIZ-1 significantly increased mineral deposit compared to control, ectopic ZBTB2 or ZBTB5 (Figure 3A). At day 12, the hDPSCs grown with odontoblast differentiation medium only were significantly stained by alizarin red, but knock down of MIZ-1 or ZBTB2 decreased mineral deposit. Interestingly, loss of ZBTB5 highly increased the mineral deposit (Figure 3B). These results suggested that MIZ-1, ZBTB2 and ZBTB5 may affect differentiation of hDPSCs and mineralization. MIZ-1 may be a key protein promoting odontoblast differentiation. And ZBTB5 may be a protein inhibiting odontoblast diffentiation.

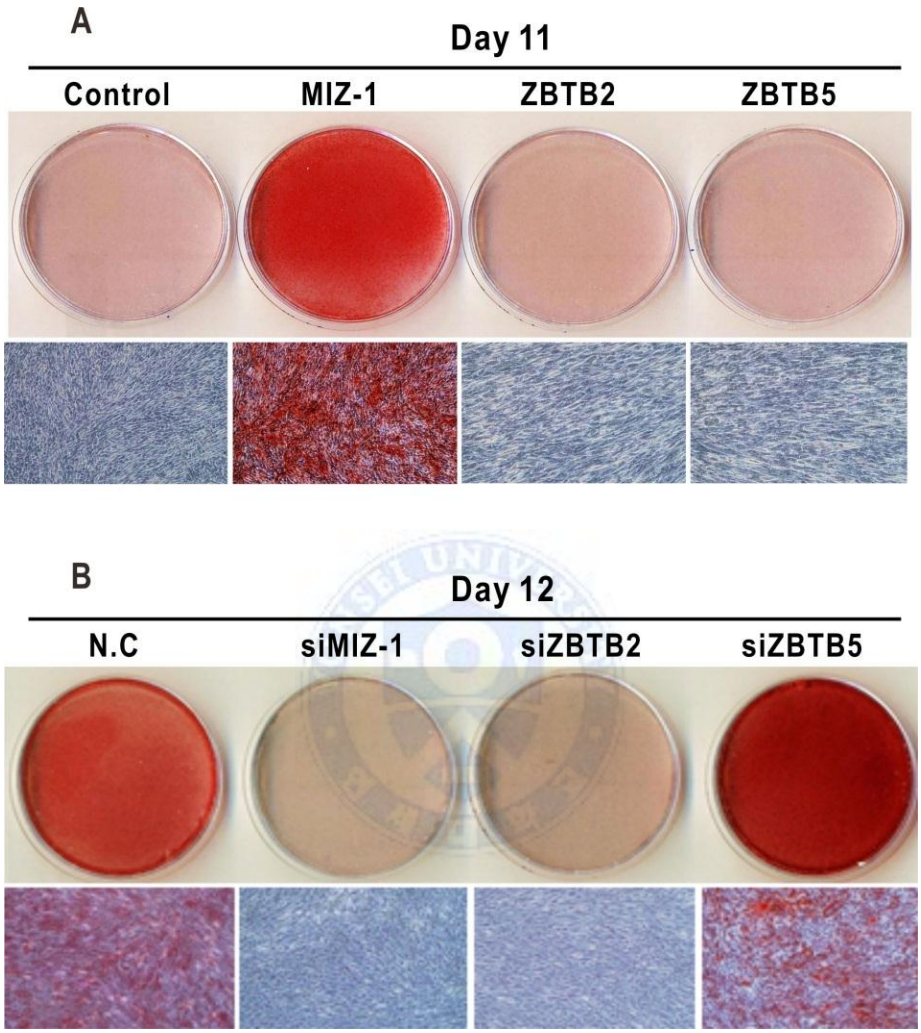
A
Day 11
Control
MIZ-1
ZBTB2
ZBTB5
B
Day 12
N.C
siMIZ-1
siZBTB2
siZBTB5

Figure 3. The POK proteins regulate mineralization of differentiated hDPSCs. (A) Alizarin Red-S (AR-S) staining for the mineralization of hDPSCs overexpressing MIZ-1, ZBTB2 or ZBTB5. hDPSCs transfected with expression vector of MIZ-1, ZBTB2 or ZBTB5 were cultured with odontoblastic induction medium (DMEM supplemented with 10% fetal bovine serum, antibiotics, 50 μg/ml ascorbic acid, 10 mmol/l sodium β-glycerophosphate, and 10 nmol/l dexamethasone) for 11 days, and then the cells were fixed and stained with AR-S. Control, cells was transfected with pcDNA3.1. (B) Alizarin Red-S (AR-S) staining of hDPSCs transfected with siRNA against MIZ-1, ZBTB2 or ZBTB5 mRNA. The cells were cultured with odontoblastic induction medium for 12 days, fixed and stained with AR-S.

4. MIZ-1 and ZBTB5 regulate *Amelx* and *Dspp* gene

I found that MIZ-1 and ZBTB5 affect mineralization of hDPSCs and expression of MIZ-1 and ZBTB5 tend to increase 1-3 days prior to expression of Amelx and Dspp genes. I suggested that MIZ-1, ZBTB2 and ZBTB5 might regulate odontoblast differentiation by regulating transcription of *AMELX* and *DSPP* genes. I investigated whether MIZ-1, ZBTB2, and ZBTB5 regulate *Amelx* and *Dspp* genes. MEF cells were transfected with MIZ-1, ZBTB2 or ZBTB5 expression vectors and isolated mRNA after 48 hr. By qRT-PCR (quantitative real-time PCR) analysis, I found that MIZ-1 increased but ZBTB5 repressed endogenous *Amelx* gene expression (Figure 4A). But ZBTB5 repressed endogenous *Dspp* gene expression (Figure 4B). ZBTB2 didn't affect expression of the *Amelx* and *Dspp* (Figure 4A, B). ZBTB2 may influence the two genes expression by other regulatory mechanism or the assay MEF system I am using may be inappropriate for this study, MEF, and may lack of certain regulation factor. These data suggested that MIZ-1 and ZBTB5 regulate the mRNA expression of *Amelx* and *Dspp* genes and thereby mineralization of hDPSCs.

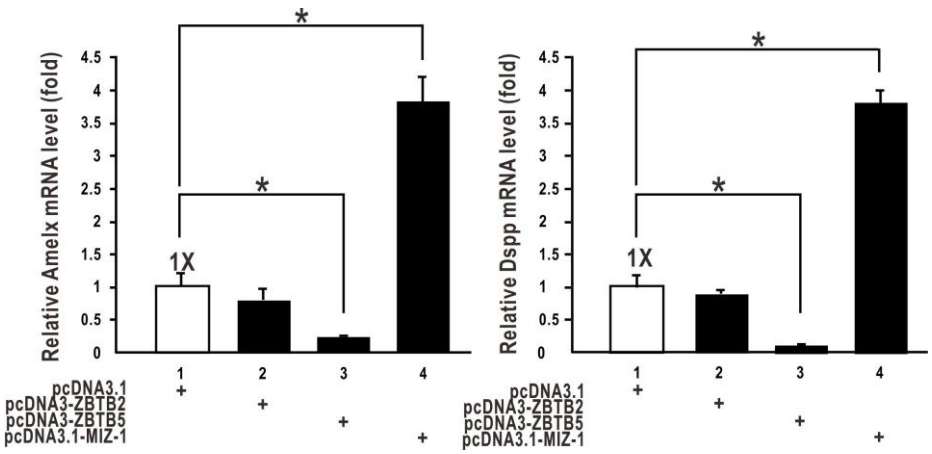

Figure 4. MIZ-1 and ZBTB5 regulate endogenous *Amelx* and *Dspp* mRNA expression. (A, B) RT-qPCR analysis. MEF cells were transfected with the expression vector of MIZ-1, ZBTB2 or ZBTB5 by electroporation. Total mRNA was isolated from the cells. The endogenous mRNA expression of *Amelx* (A), *Dspp*(B) were measured by RT-qPCR at 48 hr post transcription. Normalized to 18S ribosomal RNA.

5. ZBTB5 represses Amelx promoter via -485 to -71 bp region, and MIZ-1 activates Amelx promoter by acting on the -70 to +100 bp region

AMELX is necessary for tooth development and matrix mineralization. The above data showed that MIZ-1 and ZBTB5 regulated mRNA expression of *Amelx*. To explore the regulatory mechanism of *Amelx* by the two POK proteins, I analyzed potential transcription factor binding sites on the *Amelx* promoter and then examined the effects of MIZ-1, ZBTB2 or ZBTB5 on *Amelx* promoter activity. Promoter analysis using the MacVector program revealed binding element for MIZ-1 (bp, -472 to -454 and -406 to -401), Sp1 (bp, -472 to -463, -443 to -434, -160 to -151, -11 to -2 and +6 to +15), p53 (bp, -343 to -334 and +9 to +18) and CCAAT box (bp, -58 to -54). Previously, deletion analysis of mouse *Amelx* promoter has shown that the bp, -70 to +51 region functions as a minimal promoter.[50] I prepared two different *Amelx* promoter reporter fusion constructs with long 5' upstream regulatory region (bp, -485 to +100) or minimal promoter (bp, -70 to +100) (Figure 5A). ZBTB5 significantly repressed repoter transcription with long 5' upstream regulatory region, but not so effctively on the minimal promoter (Figure 5B). MIZ-1 activated the minimal promoter of *Amelx* gene, but repressed *Amelx* gene promoter with long 5' upstream regulatory region (Figure 5C). ZBTB2 repressed only the promoter with long 5' upstream region (Figure 5D). These results suggested that the sequence located between bp, -485 to -70 region may be involved in repression of transcription by ZBTB5 and -70 to +100 region may be involved in activation of transcription by MIZ-1.

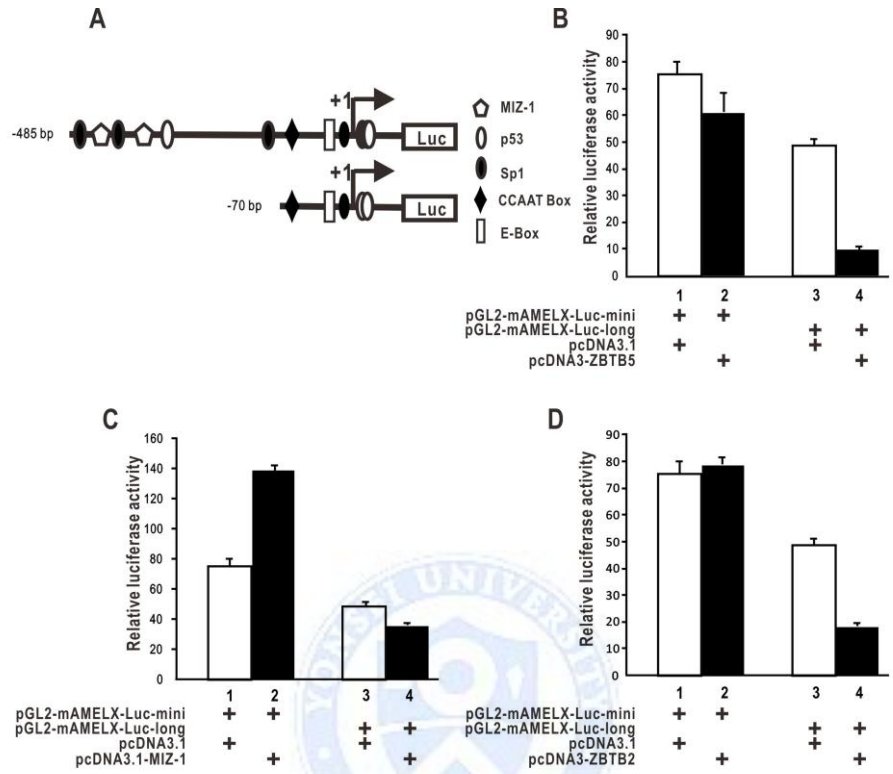

Figure 5. Transcriptional regulation of *Amelx* by MIZ-1, ZBTB2 and ZBTB5. (A) Diagram of the *Amelx* promoter–luciferase gene fusion reporter constructs tested. +1 (Tsp), transcription start point. ⬠, MIZ-1; ⬯, p53; ⬮, Sp1; ⧫, CAAT Box; ▯, E-Box. (B-D) Transient transcription assays. The reporter plasmid and the expression vector of ZBTB5 (B), MIZ-1 (C), or ZBTB2 (D) were transiently co-transfected into MEF cells and analyzed for luciferase activity. Luciferase activity was measured 48 hr after transfection and normalized with co-expressed β-galactosidase activity. Data presented are the average of three independent assays. Error bars represent standard deviations.

6. Oligonucleotide pull-down assays of MIZ-1 binding on *Amelx* promoter

To study how MIZ-1 regulate the transcription of *Amelx*, I divided the 5' upstream regulatory region of *Amelx* promoter (bp, - 485 to +100) into 5 regions, based on putative transcriptional factor binding sites. #1 element (bp, -471 to -334) included MIZ-1, Sp1 and p53 binding sites. #2 element (bp, -422 to -276) included MIZ-1 and p53 binding sites. #3 element (bp, - 301 to -155). #4 element (bp, -170 to -25) lncluded Sp1 and CCAAT box. #5 element (bp, -36 to +100) included E-box, Sp1 and p53 binding sites (Figure 6A). I examined which regulatory elements of the *Amelx* promoter were important for MIZ-1 binding using oligonucleotide pull down assays in HEK293A cells. MIZ-1stongly bound to #1 (bp, -471 to -334) and #4 (bp, -170 to -25) elements (Figure 6B). Although overall homology between #1 (bp, -471 to -334) and #4 (bp, -170 to -25) is only 40%, there are highly homologous region overlapping with each other #1; bp, -380 to -359, #4; bp, -70 to -49. The #4 highly homologous region (called MRE) is bound by MIZ-1 and may potentially important in transcription activation of *Amelx* gene (Figure 6C). Oligonucleotide pull down assays showed that MIZ-1 bound to MRE, which may be critical in transcriptional activator of *Amelx* gene (Figure 6D, E).

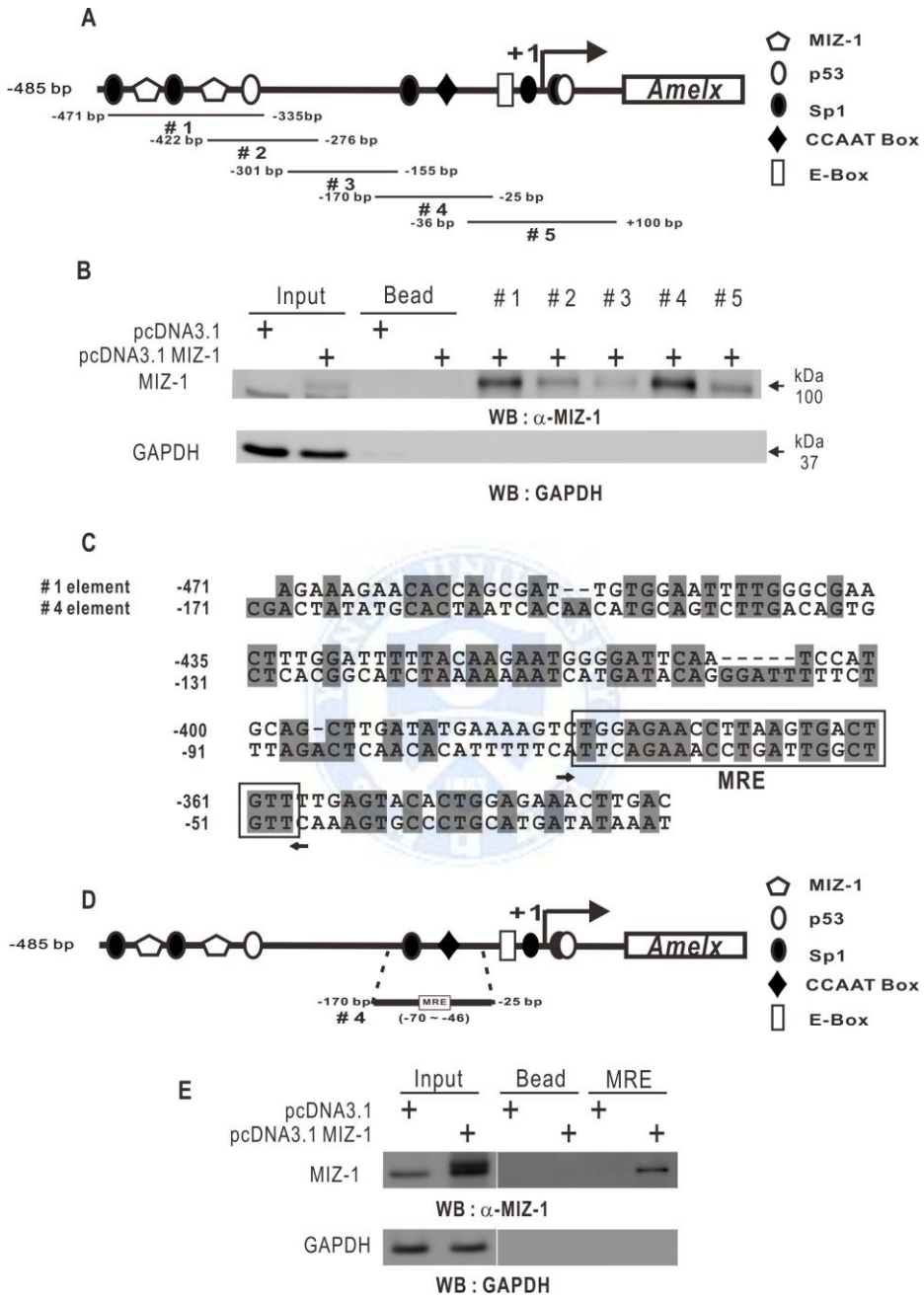
A
-485 bp
+1
Amelx
-471 bp
-335bp
1
-422 bp
-276 bp
2
-301 bp
-155 bp
3
-170 bp
-25 bp
4
-36 bp
+100 bp
5
MIZ-1
p53
Sp1
CCAAT Box
E-Box
B
Input
Bead
1
2
3
4
5
pcDNA3.1
pcDNA3.1 MIZ-1
MIZ-1
kDa
100
WB : α-MIZ-1
GAPDH
kDa
37
WB : GAPDH
C
1 element
4 element
-471
-171
AGAAAGAACACCAGCGAT--TGTGGAATTTTGGGCGAA
CGACTATATGCACTAATCACAACATGCAGTCTTGACAGTG
-435
-131
CTTTGGATTTTTACAAGAATGGGGATTCAA-----TCCAT
CTCACGGCATCTAAAAAAATCATGATACAGGGATTTTTCT
-400
-91
GCAG-CTTGATATGAAAAGTCTGGAGAACCTTAAGTGACT
TTAGACTCAACACATTTTTCATTCAGAAACCTGATTGGCT
MRE
-361
-51
GTTTTGAGTACACTGGAGAAACTTGAC
GTTCAAAGTGCCCTGCATGATATAAAT
D
-485 bp
+1
Amelx
-170 bp
MRE
-25 bp
4
(-70 ~ -46)
MIZ-1
p53
Sp1
CCAAT Box
E-Box
E
Input
Bead
MRE
pcDNA3.1
pcDNA3.1 MIZ-1
MIZ-1
WB : α-MIZ-1
GAPDH
WB : GAPDH

Figure 6. MIZ-1 binds to the MRE of *Amelx* promoter. (A) Diagram of the *Amelx* promoter structure. Key regulatory elements such as MIZ-1 binding element, p53-binding elements, Sp1-binding CCAAT box and E-box are indicated. ⬠, MIZ-1; ⬯, p53; ⬮, Sp1; ⧫, CAAT Box; ▯, E-Box. (B) Oligonucleotide pull-down assays of MIZ-1 binding on 5 regulatory elements of *Amelx* promoter. Streptavidin agarose beads linked to biotinylated oligonucleotides probes (#1 to #5) were incubated with the HEK293 cell lysates with ectopic MIZ-1 expression. The precipitates were analyzed by western blotting using anti-Miz-1 antibody (C) Nucleotide alignment of homology of #1; bp, -380 to -359, #4; bp, -70 to -49 elements nucleotide sequence by MacVoctor program. (D) Diagram of the *Amelx* promoter structure. MRE, MIZ-1 binding element (bp, -70 to -46). (E) Oligonucleotide pull-down assays of the MRE of *Amelx* promoter. The oligonucleotide pull-down assay is described above.

7. MIZ-1 activates transcription of *Amelx* by binding to the MRE

The above data showed that MIZ-1 activates transcription of Amelx promoter and binds to the MRE (Figure 5, 6). Nucleotide sequence aligment of mammalian of Amelx genes showed that the MREs are highly conserved, which suggest that the MRE may be critical element in transcription of mammalian Amelx genes. The MRE also contains highly conserved CCAAT box (Figure 7A). To test presence of an element critical in transcription regulation, I divided the 5' upstream regulatory region of *Amelx* promoter (bp, - 485 to +100) into 5 regions, based on putative transcriptional factor binding sites. #1 element (bp, -471 to -334) included MIZ-1, Sp1 and p53 binding sites. #2 element (bp, -422 to -276) included MIZ-1 and p53 binding sites. #3 element (bp, - 301 to -155). #4 element (bp, -170 to -25) included Sp1 and CCAAT box. #5 element (bp, -36 to +100) included E-box, Sp1 and p53 binding sites. Oligonucleotide pull down assays showed that MIZ-1 strongly bound to #1 (bp, -471 to -334) and #4 (bp, -170 to -25) elements of *Amelx* promoter. To identify MIZ-1 binding site in #4 elements, I analyzed DNA sequences of #1 and #4 for highly homologous sites. The -380 to -359 bp region of #1 element was similar to the -75 to -46 bp region of #4 element, which was a MRE. To test the fuction of MRE – like element of #1 region and MRE element of #4 region, I introduced mutations into #1 or MRE region of the reporter constructs. To test whether MIZ-1 acts as transcriptional activator by binding to #1 region or MRE, I made promoter and reporter gene fusion constructs with mutation introduced at #1 (TGGAGAACCTTAAGTGA to TTTTTTTTGGATCCTTT) and MRE (TTCAGAAACCTGATTGG to

TTTTTTTTGGATCCTTT) (Figure 7B). LS8 cells, mouse ameloblast-like cells, were transfected with the reporter plasmids (pGL2-Amelx-Luc -485 bp, pGL2-Amelx -Luc-70 bp, pGL2-Amelx-Mt#1-Luc-485 bp, pGL2-Amelx-Mt #MRE-Luc-485 bp and pGL2-Amelx-Mt #1 & #MRE-Luc-485 bp) and the expression vector of pcDNA3.1 or MIZ-1. Transient transcription assays showed that transcription of Amelx-485bp promoter was up-regulated by ectopic MIZ-1in LS8 cells. Interestingly, MIZ-1 activate transcription of Amelx-70 bp promoter was potently (2.5 fold). Although the mutation of #1 region does not affect transcriptional activation by ectopic MIZ-1, the mutation of MRE promoter showed markedly decreased transcription and was not up-regulated by ectopic MIZ-1 (Figure 7C, D). The MRE is critical in transcriptional activation of Amelx gene by MIZ-1.

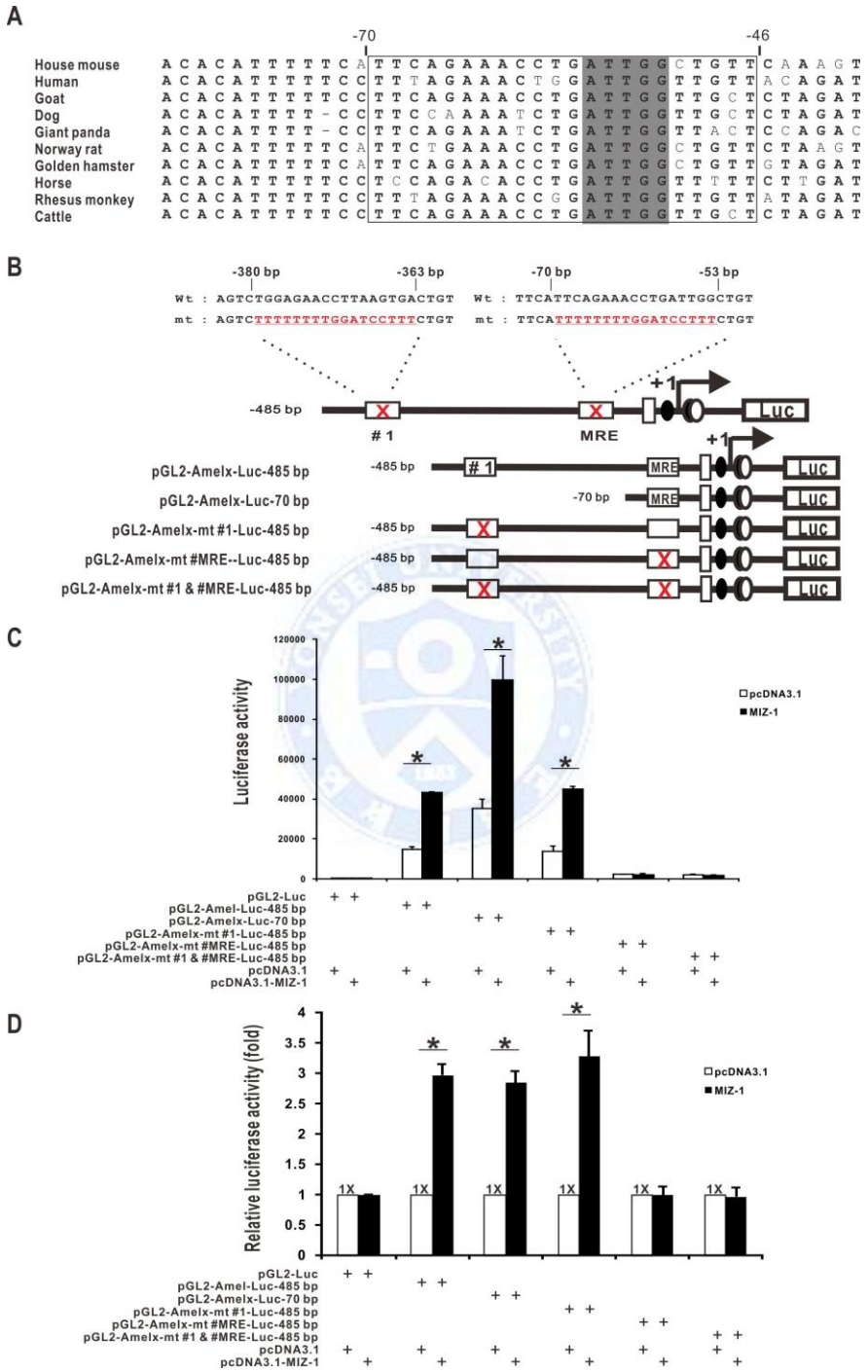
A
-70
-46
House mouse
Human
Goat
Dog
Giant panda
Norway rat
Golden hamster
Horse
Rhesus monkey
Cattle
B
-380 bp
-363 bp
-70 bp
-53 bp
Wt : AGTCTGGAGAACCTTAAGTGACTGT
mt : AGTCTTTTTTTTGGATCCTTTCTGT
Wt : TTCATTCAGAAACCTGATTGGCTGT
mt : TTCATTTTTTTTGGATCCTTTCTGT
-485 bp
1
MRE
+1
Luc
pGL2-AmeIx-Luc-485 bp
pGL2-AmeIx-Luc-70 bp
pGL2-AmeIx-mt #1-Luc-485 bp
pGL2-AmeIx-mt #MRE--Luc-485 bp
pGL2-AmeIx-mt #1 & #MRE-Luc-485 bp
C
Luciferase activity
pcDNA3.1
MIZ-1
pGL2-Luc
pGL2-AmeI-Luc-485 bp
pGL2-AmeIx-Luc-70 bp
pGL2-AmeIx-mt #1-Luc-485 bp
pGL2-AmeIx-mt #MRE-Luc-485 bp
pGL2-AmeIx-mt #1 & #MRE-Luc-485 bp
pcDNA3.1
pcDNA3.1-MIZ-1
D
Relative luciferase activity (fold)
1X

Figure 7. MIZ-1 directly binds to the MRE to activate *Amelx* gene expression. (A) Nucleotide sequence alignment analysis the Amelx genes of mammalian MREs using MacVoctor program. (B) Schematic representation of the Amelx promoter (bp, -485 to +100) and luciferase gene fusion reporter constructs. #1 region and MRE contatin potential binding sites for MIZ-1. Mutated nucleotides are shown in red. Tsp, +1, Transcription start point. (C, D) Transient transcription assays. The reporter plasmids and the expression vector of pcDNA3.1 or MIZ-1 were transiently co-transfected into LS8 cells and analyzed for luciferase activity. Luciferase activity was measured 48 hr after transfection and normalized with co-expressed β-galactosidase activity. Data presented are the average of three independent assays. Error bars represent standard deviations. (C) Raw luciferase activity of the cell extracts (D) relative luciferase activity of the reporter constructs.

Ⅳ. DISCUSSION

AMELX and *DSPP* are two essential genes for odontogenesis and matrix mineralization. Several evidences suggested that some of POK proteins may regulate odontogenesis. I found that some of the POK proteins were highly expressed during odontoblast differentiation. However, which or how POK proteins regulate *AMELX* and *DSPP* gene expression and influence odontogenesis was unknown. In this study, I found that POK gene mRNA expression pattern is variable depending on the POK gene and mRNA expression is temporally regulated during odontoblast differentiation. The *AMELX* and *DSPP* gene mRNA expression patterns were largely similar to those of POK genes. During hDPSCs differentiation into odontoblast, ectopic MIZ-1 or knock-down of ZBTB5 expression increased mineral deposit. In contrast, knock-down of MIZ-1 expression decreased mineral deposit. Also, other like Th-POk and Kr-pok increased mineral deposit during odontoblast differentiation (data not shown).

Because several lines of evidences suggested that MIZ-1, ZBTB2 and ZBTB5 may regulate differentiation of hDPSCs and mineralization. I investigated how AMELX and DSPP gene were regulated by the above POK proteins, I used hDPSCs for transient transcription assays. But unfortunately I was unable to get enough luciferase activity to show that the MIZ-1 and ZBTB5 regulate the two genes. Instead, I used MEF cells which could be inappropriate for the study. The MEF cells may lack certain regulatory factor

to regulate the two genes. Circumvent this potential problem, I also used LS8 cells, mouse ameloblast-like cells.

ZBTB5 significantly repressed transcription of Amelx -485 bp promoter, but did not so effectively that of Amelx -70 bp promoter (Figure 5B). ZBTB5 may regulate the gene promoter by acting on 5' upstream regulatory region (bp, -485 to -70). MIZ-1 activated transcription of the Amelx -70 bp promoter of *Amelx* gene, but repressed transcription of Amelx-485 bp promoter of *Amelx* gene in MEFs. However, MIZ-1 activated transcription of the Amelx-70 bp promoter and Amelx-485 bp promoter of *Amelx* gene in LS8 cells. I suspect that there could be LS8 cells specific transcription factors that could bind to the 5' regulatory region (bp, -485 to -71) and play an activator role on Amelx promoter or lack repressor that bind the region.

MIZ-1 activates transcription mainly by acting on the -70 bp to +100 bp region. To find how MIZ-1 regulates Amelx promoter, the sequence from bp, -485 to +100 of Amelx was analyzed to find MIZ-1 binding site (5'-CCCACTCTCTGC-3' and 5'-ATCGAT-3') and p53 binding site which was also shown to be bound by MIZ-1 using MAC vector program.[83] Distal 5' regulatory element contains two MIZ-1 binding sites and the proximal -70 bp to +100 bp region has no putative MIZ-1 binding site. However, MIZ-1 activated transcription activity on Amelx -70 bp promoter, suggesting the presence of element important in transcription activation by MIZ-1.

To test presence of an element critical in transcription regulation, I divided the 5' upstream regulatory region of *Amelx* promoter (bp, - 485 to +100) into 5 regions, based on putative transcriptional factor binding sites. #1

element (bp, -471 to -334) included MIZ-1, Sp1 and p53 binding sites. #2 element (bp, -422 to -276) included MIZ-1 and p53 binding sites. #3 element (bp, - 301 to -155). #4 element (bp, -170 to -25) included Sp1 and CCAAT box. #5 element (bp, -36 to +100) included E-box, Sp1 and p53 binding sites. Oligonucleotide pull down assays showed that MIZ-1 strongly bound to #1 (bp, -471 to -334) and #4 (bp, -170 to -25) elements of *Amelx* promoter. To identify MIZ-1 binding site in #4 elements, I analyzed DNA sequences of #1 and #4 for highly homologous sites. The -380 to -359 bp region of #1 element was similar to the -75 to -46 bp region of #4 element, which was a MRE. To test the fuction of MRE – like element of #1 region and MRE element of #4 region, I introduced mutations into #1 or #4 region of the reporter constructs. Transient transcription assay showed that MIZ-1 regulate transcription of Amelx by binding MRE.

MRE includes CCAAT box, which is important element for transcription regulation by C/EBPα. Because the MRE is juxtaposed to MRE, affect transcriptional regulator by C/EBPα positively or negatively. ZBTB5 represses transcription of pGL2-Amelx -485 bp promoter, weakly and probabliy inhibit on the pGL2-Amelx -70 bp promoter. MIZ-1 activates transcription of *Amelx* gene expression by binding to the proximal Amelx promoter. Considering that MIZ-1 and ZBTB5 are interacted with each other, their interaction in transcriptional regulation of *Amelx* can be interesting.

Some of POK family proteins have been reported to play critical roles in various biological processes, including embryonic development, cell differentiation, inflammation, proliferation, and apoptosis. I found that some

of POK proteins may play critical rule in tooth development. After tooth injury, the dentin-pulp complex may need to undergo complete regeneration, including the differentiation of various cell types and the induction of new protein shch as *AMELX* and *DSPP.* MIZ-1 and ZBTB5 might play a critical role in *Amelx* and *Dspp* expressions and reparative regeneration.

Ⅴ. CONCLUSION

This study revealed that POK family proteins such as MIZ-1 and ZBTB5 may regulate odontogenic differentiation by transcriptionally regulating *AMLEX* and *DSPP* genes.

1. The POK proteins mRNA expression is temporally regulated during odontoblast differentiation and the expression patterns were largely similar for both *AMELX* and *DSPP* genes.
2. MIZ-1, ZBTB2 and ZBTB5 may regulate differentiation of hDPSCs and mineralization.
3. MIZ-1 and ZBTB5 regulate endogenous *Amelx* and *Dspp* gene expression in MEF cells.
4. MIZ-1 binds to *Amelx* promoter, #1 (bp, -471 to -334) and #4 (bp, -170 to -25) elements, and the MRE of #4 element (bp, -70 to -46) is more critical in transcriptional activation of *Amelx* gene.

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ABSTRACT (in Korean)

MIZ-1 과 ZBTB5 에 의한 *AMELX* 와 *DSPP* 유전자의 발현조절

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노 희 진

AMELX 와 *DSPP* 는 각각 법랑질과 상아질의 형성과정에서 중요한 유전자이다. 최근에 POK 계 단백질들이 치아형성 과정 동안에 발현됨을 발견되었다. 따라서 본 연구자는 어떤 POK 계 단백질이 치아형성과정에서의 관여하는지 알아보고자, 인간치수줄기세포(human dental pulp stem cell)에서 상아모세포로의 분화과정 동안의 POK 계 단백질의 mRNA 발현을 살펴보았다. POK 계 mRNA 발현 패턴이 POK 계 유전자에 따라서 다양한 발현을 나타났다. 어느 POK 계 유전자의 발현이 hDPSCs 의 분화과정에서 중요한 역할을 하는 지를 알아보기 위해, MIZ-1, ZBTB2 그리고 ZBTB5 를 과발현하거나, 발현을 낮춘 후 분화의 지표인 미네랄 축적을 alizarin red S 로

염색하여 확인하였다. MIZ-1 과 ZBTB2 이 축적을 촉진시키고, ZBTB5 는 억제시킨다. 따라서 치아형성과정에서 중요한 *AMELX* 와 *DSPP* 유전자의 발현이 MIZ-1, ZBTB2 또는 ZBTB5 에 의해 조절되는지를 조사하였다. MIZ-1 은 *AMELX, DSPP* 의 발현을 증가시키고, 반대로 ZBTB5 는 *AMELX, DSPP* 의 발현을 감소시킨다. ZBTB2 의 경우는 두 유전자 발현에 큰 영향을 미치지 않는다.

MIZ-1 과 ZBTB5 에 의한 *AMELX* 와 *DSPP* 의 유전자 발현 조절을 연구하였다. ZBTB5 의 경우 두 가지의 프로모터 모두에서 프로모터 활성을 억제한다. MIZ-1 는 짧은 구조 프로모터 활성을 증가시키지만, 긴 프로모터에서의 전사는 세포의 종류(MEF, LS8 cells)에 따라 다른 경향을 나타내지만, 짧은 프로모터 활성은 증가시키는 것을 확인하였다.

전사조절기전을 조사하기위해, *Amelx* promoter(bp, -485 에서 +100)를 5 가지의 지역으로 나누고 oligonucleotide pull down 실험을 했다. #1 (bp, -471 to -334 bp) 과 #4 (bp, -170 to -25 bp) 번 지역에서 강한 결합을 확인하였고, #1 과 #4 의 시컨스 유사성을 확인해본 결과 높은 유사성을 지닌 위치(#1; bp, -380 to -359, #4; bp, -70 to -49)를 찾았다. 특히 #4 번에서의 높은 유사성을 지닌 부위 (#4; bp, -70 to -49) 에서의 MIZ-1 결합 또한 확인하였고, MRE 라고 명명했다. 높은 유사성을 지닌 #1 번의 유전자 변형은 *Amelx* 의 프로모터 활성에 영향을 미치지는 않지만,

MRE 의 변이에 따라 MIZ-1 에 의한 프로모터 활성이 사라지는 것을 확인하였다. 따라서 MRE 는 MIZ-1 에 의한 *Amelx* 의 전사적인 발현 활성화에 중요한 역할을 할 것으로 생각된다.

ZBTB5 는 *AMELX* 와 *DSPP* 의 전사적인 발현을 조절하여 치아형성과정을 억제하고, MIZ-1 은 *AMELX* 와 *DSPP* 의 전사적인 발현을 증가하여 치아형성과정을 촉진시키는 것으로 예상된다.

핵심되는 말: POK 계 단백질, 전사인자, 치아형성과정, AMELX, DSPP