Role of MIZ-1 in AMELX gene expression

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ABSTRACT

Amelogenin (AMELX) is the main component of the developing tooth enamel matrix and is essential for enamel thickness and structure. However, little is known about its transcriptional regulation. Using gene expression analysis, we found that MIZ-1, a potent transcriptional activator of CDKN1A, is expressed during odontoblastic differentiation of hDPSCs (human dental pulp stem cells), and is essential for odontoblast differentiation and mineralization. We also investigated how MIZ-1 regulates gene expression of AMELX. Oligonucleotide-pull down assays showed that MIZ-1 binds to an MRE (MIZ-1 binding element) of the AMELX proximal promoter region (bp, −170 to −25). Combined, our ChIP, transient transcription assays, and promoter mutagenesis revealed that MIZ-1 directly binds to the MRE of the Amelx promoter recruits p300 and induces Amelx gene transcription. Finally, we show that the zinc finger protein MIZ-1 is an essential transcriptional activator of Amelx, which is critical in odontogenesis and matrix mineralization in the developing tooth.

1. Introduction

Tooth development is controlled by extensive “crosstalk” between epithelium and mesenchyme, involving ligand–receptor interactions that induce transcriptional changes that orchestrate cellular processes required for tooth development [1]. During tooth formation, the ectoderm thickens and forms a placode that buds into the underlying neural-crest derived mesenchyme. The epithelium then signals to the ectoderm to thicken and form a placode that buds into the underlying neural-crest derived mesenchyme, resulting in condensation around the epithelial bud [2–5]. After the bud stage, the epithelium starts to extend further into the mesenchyme, wrapping around the condensing mesenchyme via structures created at its center, known as “primary enamel knots,” that instruct the patterning of the tooth crown and regulate the location and height of tooth cups [2–5]. 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, cytodifferentiation occurs, in which the adjacent layer of epithelial cells differentiates into ameloblasts that secrete the enamel matrix, while the mesenchyme differentiates into odontoblasts, producing dentin [2–5].

Ameloblasts secrete the enamel-forming amelogenin family of proteins, which play a key role in regulating proper tooth enamel and replacement, by the mineral phase, generating a “woven” architecture [6]. Dental enamel is the hardest tissue in the body and cannot be replaced or repaired, because 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 [7,8]. Several mutations in the human X-chromosomal AMELOGENIN (AMELX) gene have been reported that lead to X-linked amelogenesis imperfecta 1 (AI 1) [9], 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 [7].

Amelogenin expression is and developmentally regulated at the temporal and spatial levels [10–14], at both the transcriptional and post-transcriptional levels [1]. Moreover, using transgenic mouse analysis, a 2263-nucleotide promoter element from the mouse X-chromosomal Amelx gene was demonstrated to recapitulate the spatiotemporal expression pattern of the endogenous Amelx gene [13]. 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

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region is likely long conserved for transcription of this important gene (Amelx).

Several transcription factors have been shown to regulate Amelx. Its promoter region contains a reversed CCAAT box four base pairs downstream from the C/EBPα-binding site. CCAAT/enhancer-binding protein α (C/EBPα) plays a strong role in developmental control of Amelx, while the tooth development regulator Msx2 interferes with C/EBPα binding at the mouse Amelx minimal promoter by protein–protein interaction [15]. Moreover, NF-Y synergistically acts with C/EBPα to activate mouse Amelx during amelogenesis [16], while the tooth germ proteins Foxj1 and Dlx2 function independently to activate the Amelx promoter [17].

MIZ-1 is a member of the POK family proteins, BTB/POZ domain protein having one or more Krüppel-like zinc-fingers [18]. Previous reports showed that MIZ-1 is a potent transcriptional activator of CDKN1A [18], and it interacts with various oncoproteins, such as c-MYC, BCL6, ZBTB4 and GFI-1, to transcriptionally repress genes involved in cellular differentiation and metabolism [18–20]. More recently, POK family proteins have also been characterized as transcriptional regulators of genes that control cell proliferation [21]. Although POK family proteins appear to play key roles in various cellular regulatory processes, functions of many POK family proteins remain largely unknown [21].

Here, we found that MIZ-1 is expressed in hDPSCs (human dental pulp stem cells) during odontoblastic differentiation, and is temporally regulated during odontoblastic differentiation of hDPSCs. MIZ-1 modulates AMELX expression and thus, odontoblastic differentiation.

2. Materials and methods

2.1. Cell culture and transient transfection assays

Human embryonic kidney (HEK293) and dental pulp stem cells (hDPSCs, CEPO Research Center, Seoul, Korea), and murine embryonic fibroblasts (MEFs) and LS8 ameloblast-like cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco-BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 µg/ml streptomycin, and 100 units/ml penicillin, at 37 °C in a humidified atmosphere containing 5% CO2 incubator. To induce odontoblast differentiation, hDPSCs were cultured in DMEM supplemented with 10% FBS, a P/S (penicillin/streptomycin) solution, 2 mM β-glycerol phosphate, and 0.1 mM ascorbic acid, 10 mM lactic acid, and 10 mM l-dexamethasone.

Various combinations of the plasmids pGL2-Amelx-Luc –485 bp, pGL2-Amelx-Luc –70 bp, pcDNA3.1, and pcDNA3.1-MIZ-1 were transiently transfected into murine LS8 cells using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA), cultured for 24–36 h, and analyzed for luciferase activity, using a Microplate LB 96V luminometer (EG & G Berthold, Wildbad, Germany). All reactions were performed in triplicate and presented as means ± SDs. Reporter activity was normalized to coexpressed β-galactosidase activity, or total cellular protein.

2.2. Electroporation

Electroporation was performed using a Neon Transfection System (Invitrogen), according to the manufacturer’s instructions. MEF cells were washed with PBS, resuspended in electroporation buffer containing plasmid DNA, and electroporated (1350 V, pulse width 30 ms, pulse number 1), using a 100 µl tip. After electroporation, cells were cultured in DMEM medium and allowed to recover for 72 h.

2.3. Odontoblastic differentiation of hDSPPs and alizarin red staining

hDSPPs were cultured in 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). The cells were grown in 10 cm dishes, fixed with 1 ml 10% formaldehyde for several minutes at room temperature, washed with distilled water, and stained with 1% alizarin red (pH 5.5) for 30 min at room temperature.

2.4. Total RNA isolation and RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). cDNA was synthesized using 2 µg of total RNA, random hexamers (10 pmol), oligo-dT (10 pmol), and Superscript reverse transcriptase II (200 units), in a total volume of 20 µl, using a reverse transcription kit (Invitrogen). PCR was then performed using the following cycling conditions: 94 °C denaturation for 3 min, followed by 25, 30, 35, or 40 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s), and a final extension at 72 °C for 5 min. The following oligonucleotide PCR primers were:

- MIZ-1 forward, 5′-CAGCCGTCATCAGCTCA-3′, reverse, 5′-ATCAGCAAAGCTGTAAGCAAGT-3′; GAPDH forward, 5′-ACCACTGCTCATGCCATC-3′, reverse, 5′-TCCACCCCGCTGTCGCTGTA-3′; AMELX forward, 5′-TCCCCAGGACACCTAATG-3′; reverse, 5′-GAACATCGAGAGGAGGTG-3′; DMP-1 forward, 5′-ACAGCGAAATGAAGACCC-3′, reverse, 5′-TTACTGCGCTTGATAGG-3′.

2.5. Quantitative real-time PCR (qRT-PCR) analysis

Quantitative qRT-PCR reactions were conducted with SYBR Green PCR Master Mix, using an ABI PRISM 7300 RT-PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate; 18S ribosomal RNA was used as a control. The following oligonucleotides were used as PCR primers:

- Amelx forward, 5′-CCCTGTCTGCCCATCCTTT-3′, reverse, 5′-TCCGGCTTTGCTGCTTGT-3′; 18S forward, 5′-CCCTTCATTGTACCTCAAATC-3′, reverse, 5′-TCTGCTCCTGGAAGATGG-3′.

2.6. Knockdown of endogenous MIZ-1 mRNA by siRNA

100 pmoles of siRNA against MIZ-1 mRNA, synthesized in duplex and purchased from Bioneer (Daejeon, South Korea), were transfected into hDPSCs, using Lipofectamin RNAiMAX reagent (Invitrogen). Nucleotide sequences of siRNA against MIZ-1 were: forward, 5′-GAAGGCGGAGAUCGAAA-3′, and reverse, 5′-UUUGUCGUAUCGCGCUUC-3′.

2.7. Western blot analysis

Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer. Cell extracts (30 µg) were separated by 12% SDS-PAGE, transferred onto ImmunBlot Polyvinylidene Difluoride (PVDF) membranes (BioRad, Hercules, CA, USA), and blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA) or bovine serum albumin (Sigma-Aldrich). Membrane blots were incubated with antibodies against GAPDH or MIZ-1, followed by incubation with secondary antibodies conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA). Protein bands were visualized using an ECL kit (PerkinElmer, Waltham, MA, USA).

2.8. Oligonucleotide pull-down assays

Cells were lysed in HKMG buffer (10 mM HEPES, pH 7.9, 100 µM KCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40, and 1 mM DTT), and the extracts incubated with 1 µg biotinylated double-stranded oligonucleotides for 16 h. Oligonucleotide probes were annealed by heating at 95 °C for 5 min in annealing buffer (100 µl of 1× TE +0.1 M NaCl), cooled slowly to room temperature, and pulled-down, as reported elsewhere [22]. Oligonucleotides sequences within the Amelx promoter.
were as follows (only top strands are shown): Amelx promoter elements: MRE, 5′-TTTCATTCAAGAACTGATTGGCTGTTAA-3′. PCR-amplified products were used as templates for #1~#5 probe. The following oligonucleotides were used as PCR primers (only forward strand biotinylated): #1 forward, 5′-AGAAAGAACACCAGCGATTG-3′, reverse, 5′-ATTTATATCGACGTCTCAGT-3′. #2 forward, 5′-CAAGAATGGGGATCTCCAGTAC-3′, reverse, 5′-CATGATATAAATTGGGGCAC-3′. #3 forward, 5′-TTGCTAGAATGACGTCTCAGT-3′, reverse, 5′-ATTAGTGCATACTGTT-3′. #4 forward, 5′-CGACTATAGGATTCAATCC-3′, reverse, 5′-ATTATATATCGACGTCTCAGT-3′. #5 forward, 5′-CATGATATAAATTGGGGCAC-3′, reverse, 5′-ATGACCACAGTGAGAT-3′.

2.9. Chromatin immunoprecipitation (ChIP)

Cells were fixed with formaldehyde (final 1%) to crosslink proteins to DNA. For detection of MIZ-1, protein-bound chromatin was immunoprecipitated with an anti-MIZ-1 antibody, as we have described previously [22]. An anti-immunoglobulin G (IgG) antibody was used as the ChIP negative control. PCR reactions were conducted using the following oligonucleotide primer sets, designed to amplify region of interest: the MRE of the Amelx promoter forward, 5′-AACACCAGCGATGGGAAAT-3′, reverse, 5′-ATTATATCA TGCAGGGCAC-3′.

2.10. Statistical analysis

Student’s t-test was used for statistical analyses. P-values of < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Differentiation of hDPSCs into odontoblasts and mRNA expression profiles of MIZ-1, AMELX and dentin-forming DSPP genes

DPSCs are mesenchymal stem cells (MSCs) present in the core region of the tooth pulp [23] that differentiate into odontoblast-like cells, pulpal fibroblasts, adipocytes, and neural-like cells [24]. hDPSCs
abundantly express AMELX proteins during their differentiation into odontoblasts [17]. To test odontoblastic differentiation capability of hDPSCs, hDPSCs were cultured in induction medium for 14 days. Alizarin red staining was used to evaluate calcium-rich deposits in the cells cultured with normal medium or induction medium on days 0, 7, and 14 post-induction, exhibiting a flat, spindle-shape, and fibroblast-like morphology. At day 7, the hDPSCs cultured in normal growth medium were elongated in shape and continued to proliferate (Fig. 1A).

However, hDPSCs grown in odontoblastic induction medium were polygonal in shape, with more dimensions, and showed only mild alizarin red staining, indicating an initiation of mineral deposition (Fig. 1B). Using PCR, the mRNA expression profiles analyses of MIZ-1, AMELX, and the odontoblastic marker gene DMP-1, during odontoblastic differentiation of hDPSCs, showed that AMELX and DMP-1 were temporally regulated in odontoblastic induction medium.

Fig. 2. MIZ-1 plays a pivotal role in mineralization of differentiated hDPSCs, and increases Amelx transcription levels. (A) Alizarin Red-S (AR-S) staining for mineralization of hDPSCs overexpressing MIZ-1. hDPSCs transfected with MIZ-1 expression vector were cultured in odontoblastic induction medium for 11 days, and fixed and stained with AR-S. (B) AR-S staining of differentiated hDPSCs transfected with anti-MIZ-1 siRNA. The cells were cultured in odontoblastic induction medium for 12 days, fixed, and stained with AR-S. (C) RT-qPCR analysis. MEF cells were transfected with a MIZ-1 expression vector by electroporation, and endogenous Amelx mRNA measured by RT-qPCR at 48 h post transfection. mRNA levels were normalized to 18S ribosomal RNA.
Interestingly, MIZ-1 mRNA expression patterns were largely similar to those of the AMELX and DSPP genes (Fig. 1C).

3.2. MIZ-1 regulates mineralization in hDPSCs

Since the above data potentially suggested that MIZ-1 may regulate AMELX mRNA expression, and thereby, odontoblast differentiation and mineralization, we used a MIZ-1 gain vs. loss functional approach to examine this phenotype. hDPSCs were transfected with a MIZ-1 expression vector or anti-MIZ-1 siRNA. At day 11 after differentiation induction, hDPSCs cultured in odontoblast differentiation medium not only stained with alizarin red, but ectopic MIZ-1 significantly increased mineral deposition, compared to control cells (Fig. 2A). At day 12, hDPSCs grown in odontoblast differentiation medium stained significantly by alizarin red, although MIZ-1 knockdown decreased mineral deposition (Fig. 2B). These results suggested that MIZ-1 promotes differentiation of hDPSCs and mineralization. To further test whether MIZ-1 regulates Amelx, MEF cells were transfected with a MIZ-1 expression vector (Fig. 2C), with RT-qPCR analysis showing that ectopic MIZ-1 increased endogenous Amelx gene expression, and thereby, mineralization of hDPSCs (Fig. 2C).

3.3. MIZ-1 regulates Amelx expression and binds to the Amelx gene promoter

Intrigued by the finding that MIZ-1 regulates transcription of Amelx, we next investigated possible mechanisms of such regulation. We prepared two different Amelx promoter reporter fusion constructs with long 5’ upstream (bp, −485 to +100) or short (bp, −70 to +100) promoter regulatory regions (Fig. 3A) [25,26]. These were then ligated into pGL2-Amelx-Luc reporter plasmids, and transfected into ameloblast-like LS8 cells, along with a full-length MIZ-1 expression vector, showing that transcription of the two reporter constructs was activated similarly by MIZ-1 (Fig. 3B). To more distinctly identify the precise promoter regulatory region mediating transcriptional activation by MIZ-1, the Amelx promoter (bp, −485 to +100) was further divided into 5 regions partially overlapping with each other: #1 element (bp, −471 to −334), #2 element (bp, −422 to −276), #3 element (bp, −301 to −155), #4 element (bp, −170 to −25), and #5 element (bp, −36 to +100) (Fig. 3C). Oligonucleotide pull down assays using LS8 cell extracts showed that MIZ-1 strongly bound to the #4 element (bp, −170 to −25) (Fig. 3D). These results suggest that a sequence element located between bp, −70 to −25 of the Amelx promoter may be involved in transcriptional activation by MIZ-1.

3.4. MIZ-1 activates Amelx transcription via direct binding to the Amelx promoter MREs

We next analyzed the Amelx promoter nucleotide sequence for potential MIZ-1-binding element (MRE) using MacVector (Ver. 7.2) (Fig. 3A) [27]. One such element (bp, −70 to −49; 5′-TTCAGAAACCTGATTGG-3′), resembling the MIZ-1 binding consensus motif (5′-CCCACTCTCTGC-3′ or 5′-ATCGAT-3′), was identified, and oligonucleotide pull-down assays showed robust MIZ-1 binding (Fig. 3B). ChIP assays of MIZ-1 binding to the region flanking the MRE of the Amelx gene promoter further confirmed the DNA-protein interactions.

Fig. 4. MIZ-1 binds to an Amelx promoter MRE and induces transcription. (A) Diagram of the Amelx promoter structure. One MRE (MIZ-1 binding element) was found (bp, −70 to −49) using MacVector (ver. 7.2). (B) Oligonucleotide pull-down assays of the Amelx promoter MRE in LS8 cells. (C) ChIP-PCR assays of MIZ-1 binding to the MRE site located within the proximal Amelx promoter in LS8 cells. (D) Co-immunoprecipitation of MIZ-1, p300 and c-Myc. hDPSC cell lysates of mineralization at days 0 and 7 post-induction were immunoprecipitated using an anti-p300 and anti-c-Myc antibody, and analyzed by western blot using anti-MIZ-1 antibody. (E) Structures of the four luciferase gene fusion reporter constructs with or without MRE mutation. +1, (Tsp), transcription start point. (F) Transient transcription assays. Reporter plasmids and MIZ-1 expression vector were transiently cotransfected into LS8 cells and analyzed for luciferase activity 48 h later, with normalization to coexpressed β-galactosidase activity. Error bars represent standard deviations.
interaction in LS8 cells (Fig. 3C). Previous reports showed that MIZ-1 can bind to both p300 and c-Myc [28–30]. Although MIZ-1 interacts with p300 to activate transcription, its complexation with c-Myc represses transcription of target genes. Accordingly, we investigated changes in molecular interaction between MIZ-1 and p300, or c-Myc, during differentiation of hDPSC. hDPSC differentiation results in MIZ-1 upregulation, and c-Myc downregulation, and the interaction between MIZ-1 and p300 is significantly increased, while MIZ-1 interaction with c-Myc is little changed. The results suggested that transcription of Amelx promoter may be directly regulated by MIZ-1 binding (Fig. 4D). We further tested whether MIZ-1 could directly activate transcription of the Amelx promoter, by binding to the MRE element, using transient transfection and transcription assays in LS8 cells (Fig. 4E, F). We prepared two additional promoter and reporter gene fusion constructs, with mutations introduced into the MRE element by site-directed mutagenesis (5′-TTCAGAACCTGATTGG-3′ was mutated to 5′-TTTTTTTGGATCCTTT-3′) (Fig. 4E). Transient transfection assay of LS8 cells transfected with the reporter plasmid and a full-length MIZ-1 expression vector demonstrated that MIZ-1 activated transcription of the Amelx gene promoter constructs both with short and long promoter sequence similarly; MIZ-1, however, could not activate transcription of the two MRE-mutated constructs (Fig. 4F). These data suggest that MIZ-1 binds to the MRE and activates Amelx transcription.

In summary, we show that MIZ-1 is expressed during odontoblast differentiation activates transcription of Amelx by direct binding to the MRE of proximal promoter MRE. MIZ-1 is critical for odontogenesis and matrix mineralization during tooth development.

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