Mediator 1 contributes to enamel mineralization as a coactivator for Notch1 signaling and stimulates transcription of the alkaline phosphatase gene

Received for publication, February 24, 2017, and in revised form, June 17, 2017. Published, Papers in Press, July 3, 2017, DOI 10.1074/jbc.M117.780866

Keigo Yoshizaki1‡5, Lizhi Hu‡5, Thai Nguyen‡, Kiyoshi Sakai‡3, Masaki Ishikawa‡, Lizhi Hu‡5, Thai Nguyen‡, Kiyoshi Sakai‡3, Masaki Ishikawa‡, Ichiro Takahashi‡, Satoshi Fukumoto§, Pamela K. DenBesten**, Daniel D. Bikle‡, Yuko Oda‡5, and Yoshihiko Yamada‡6

From the 1Laboratory of Cell and Developmental Biology, NIDCR, National Institutes of Health, Bethesda, Maryland 20892, the 2Division of Oral Health, Growth, and Development, Kyushu University Faculty of Dental Science, Fukuoka 812-8582, Japan, the 3Departments of Medicine and Dermatology, the University of California San Francisco and the Veterans Affairs Medical Center, San Francisco, California 94158, the 4Division of Pediatric Dentistry, Department of Oral Health and Development Sciences, Tohoku University Graduate School of Dentistry, Sendai 980-8575, Japan, and the **University of California San Francisco School of Dentistry, San Francisco, California 94143

Edited by Alex Toker

Tooth enamel is mineralized through the differentiation of multiple dental epithelia including ameloblasts and the stratum intermediate (SI), and this differentiation is controlled by several signaling pathways. Previously, we demonstrated that the transcriptional coactivator Mediator 1 (MED1) plays a critical role in enamel formation. For instance, conditional ablation of Med1 in dental epithelia causes functional changes in incisor-specific dental epithelial stem cells, resulting in mineralization defects in the adult incisors. However, the molecular mechanism by which Med1 deficiency causes these abnormalities is not clear. Here, we demonstrated that Med1 ablation causes early SI differentiation defects resulting in enamel hypoplasia of the Med1-deficient molars. Med1 deletion prevented Notch1-mediated differentiation of the SI cells resulting in decreased alkaline phosphatase (ALPL), which is essential for mineralization. However, it does not affect the ability of ameloblasts to produce enamel matrix proteins. Using the dental epithelial SF2 cell line, we demonstrated that MED1 directly activates transcription of the Alpl gene through the stimulation of Notch1 signaling by forming a complex with cleaved Notch1–RBP-Jk on the Alpl promoter. These results suggest that MED1 may be essential for enamel matrix mineralization by serving as a coactivator for Notch1 signaling regulating transcription of the Alpl gene.

Tooth enamel is mineralized through the differentiation of multiple dental epithelia cells. Dental epithelial cells differentiate into multiple cell types including the inner enamel epithelium (IEE),7 the stratum intermediate (SI), the stellate reticulum, and the outer enamel epithelium. Ameloblasts, derived from the IEE, play a role in enamel mineralization (1, 2) by secreting enamel matrix proteins such as ameloblastin (AMBN), amelogenin (AMEL), and enamelin (ENAM) into the enamel layer at the secretory stage. These enamel matrix proteins are subsequently mineralized at the maturation stage, to form enamel.

SI cells are located adjacent to IEE cells and ameloblasts and support enamel mineralization, although their role in this process remains largely undefined. SI cells are considered to be involved in enamel mineralization through tissue-nonspecific alkaline phosphatase (ALPL), which is highly produced in SI cells. ALPL may be essential for mineralization, as Alpl KO mice show evidence of hypomineralization of both teeth and bone (3, 4). Other studies also show that Alpl is associated with enamel matrix calcification in teeth (5, 6).

The differentiation of SI cells is at least partly regulated by Notch signaling. NOTCH1 is expressed in SI cells, and the Notch ligands JAG1 and JAG2 are expressed in the adjacent IEE.

7 The abbreviations used are: IEE, inner enamel epithelium; MED1, Mediator 1; ALPL, alkaline phosphatase; CON, control; SI, stratum intermediate; AMBN, ameloblastin; AMEL, amelogenin; ENAM, enamelin; NICD, Notch1 intracellular domain; qPCR, quantitative polymerase chain reaction; DAPT, N-(2-

Phenylacetamido)Leucyl-L-Leucyl-L-Valinal; RNA pol II, RNA polymerase.
Med1 activates alkaline phosphatase gene transcription

and ameloblasts during dental epithelial differentiation (7). Previous studies have indicated that Notch signaling facilitates differentiation of the dental epithelial cell line HAT-7 into Alpl-expressing SI-like cells in vitro (8). Notch signaling also plays a role in enamel mineralization, as Jag2-deficient mice display enamel hypoplasia (9).

Notch signaling is activated by cleavage of the intracellular domain of Notch receptors through γ-secretase. The intracellular domain of Notch moves to the nucleus and activates the transcription of target genes such as the hairy enhancer of split homologues-1 (Hes1) through the interaction of transcription factors such as the recombinating binding protein suppressor of hairless (RBP-Jk). The transcription of target genes is promoted by the binding of the c-Notch–RBP-Jk complex to the RBP-Jk DNA-binding sites on their promoter. Notch1 signaling is activated by the overexpression of the Notch1 intracellular domain (NICD) or by chelating reagents such as EDTA through prevention of calcium-dependent dimerization of Notch proteins (10). In addition, the γ-secretase inhibitor DAPT inhibits Notch signaling by preventing the cleavage of Notch receptors. The transcription of Notch–RBP-Jk is activated by mastermind-like proteins, but the role of the transcriptional coactivator Mediator is not known, at least in mammalian systems.

Mediator is a multiprotein complex that facilitates transcription by bridging transcription factors and the RNA polymerase (pol II) transcriptional machinery. Mediator not only facilitates transcription in general but also controls the differentiation of a variety of cells by regulating fate-specific transcription factors (11–16). Mediator 1 (MED1) is one of the critical subunits for the Mediator complex (12), and the ablation of Med1 causes abnormalities in cell differentiation of a number of cell types, including hematopoietic cells (17, 18), luminal cells (19, 20), and epidermal keratinocytes (21, 22). We generated conditional knock-out (KO) mice, in which Med1 is removed from keratin 14 (Krt14)-expressing epithelia, and used these mice to show that Med1 ablation causes defects in hair differentiation leading to alopecia in the skin (23). The same conditional Med1 KO mice, in which Med1 was also removed from Krt14-expressing dental epithelia, showed disruption in the development of incisors (24). Med1 deletion causes defects in cell fate of incisor-specific adult stem cells, resulting in ectopic hair formation in the SI while reducing mineralization of the incisor enamel. Here, we investigated the role of MED1 in enamel mineralization using Med1 KO molaris in which hair was not generated but enamel mineralization was inhibited. We analyzed Med1 KO molars at the secretory stage (P7) and found changes in Notch signaling and SI differentiation in Med1 KO molars in vivo. These results led us to investigate the molecular mechanisms by which MED1 regulates Notch signaling and Alpl expression. We utilized the immortalized dental epithelial cell line SF2 that is derived from rat incisor and is capable of differentiating into the SI lineage (25, 26). We determined the impact of the overexpression or silencing of Med1 on Notch1-regulated SI differentiation and on Alpl gene transcription. Our study demonstrates that MED1 promotes SI differentiation and activates the gene transcription of Alpl via Notch signaling, which is required for enamel matrix mineralization.

Results

Med1 deficiency in dental epithelia causes defects in enamel matrix mineralization

Previously, we reported that Med1 KO mice develop ectopic hair formation and hypomineralization of incisor enamel (24). Here, we re-evaluated the impact of Med1 deletion on molar enamel mineralization. Ten-week-old floxed Med1 mice containing the Krt14Cre transgene (KO) were compared with control (CON) littermates that had floxed Med1 alleles but no Cre. Med1 was removed from dental epithelial cells in Med1 KO teeth, as shown in our previous study (24). The Krt14Cre transgene is expressed in all dental epithelia cell lineages in the developing tooth (27). A stereomicroscopic analysis of molars and incisors of CON mice showed translucent enamel but less of it in Med1 KO molars (Fig. 1A). Moreover, the cusps of the KO molars and the tips of the KO incisors were rounded (Fig. 1A), suggesting increased wear. Microradiographic imaging of KO incisors showed an impaired calcified structure as compared with CON incisors (Fig. 1B, left panel). A micro-CT-scan of CON molars showed a thick radio-opaque enamel layer over dentin, whereas KO molars had a flat occlusal plane, likely due to attrition, with a marked decrease in the thickness of the enamel covering the dentin (Fig. 1B, right panel). Scanning electron microscopic analysis of CON incisors revealed highly organized enamel crystals over dentin, whereas the Med1 KO incisors almost completely lacked these crystals (Fig. 1C). H&E staining indicated that KO incisors lacked a mineralized layer, although the matrix of the enamel layer was intact (Fig. 1D). These results indicate that enamel matrix mineralization is inhibited in Med1 KO teeth, whereas enamel matrix proteins are present.

Med1 ablation specifically reduced Alpl expression in the SI layer but did not affect enamel matrix protein expression in ameloblasts

First, we evaluated the impact of Med1 ablation on the differentiation of dental epithelial cells by examining the molars at P7. The molars were dissected from Med1 KO and CON mice, and dental epithelial tissues were separated from mesenchymal tissues. RNA was isolated from epithelial tissues, and the mRNA levels of the KO epithelia were compared with those of CON epithelia using qPCR (Fig. 2A). The expression levels of the enamel matrix proteins secreted by ameloblasts, including Amel, Ambn, Enam, and matrix metalloproteinase-20 (Mmp20) were similar in the KO and CON molars. The levels of Calb1, a calcium-binding protein that is abundant in the maturation stage of ameloblasts, were also similar (Fig. 2A). However, mRNA levels of Alpl, which is specifically expressed in the SI layer, were down-regulated in Med1 KO molars compared with CON molars (Fig. 2A). The protein levels of AMBN, AMEL, and ALPL in Med1 KO and CON molars (P7) were evaluated by immunostaining (Fig. 2B). Both AMBN and AMEL were similarly deposited in the enamel matrix layer of the KO and CON molars (Fig. 2B). However, the relative immunostaining of the SI marker, ALPL, detected in the SI layer adjacent to the ameloblasts, was significantly reduced in the KO molars as compared with the CON molars (Fig. 2B). The analyses of both mRNA and
Med1 activates alkaline phosphatase gene transcription

protein indicated that Med1 ablation impairs SI differentiation but does not affect ameloblast differentiation, as indicated by the relatively normal levels of enamel matrix proteins.

**Notch signaling is down-regulated in Med1 KO dental epithelial cells**

Because Notch1 is expressed in SI cells, NOTCH1 and its signaling may regulate SI cell differentiation, resulting in Alpl induction. To test this possibility, we examined the expression levels of Notch receptors (Notch1 and Notch2) and Notch ligands (Jag1 and Jag2) and its target gene, Hes1, by qPCR using mRNA from dental epithelial tissues dissected from P7 molars of Med1 KO and CON mice (Fig. 3). qPCR analyses showed that mRNA levels of Notch1 and Hes1 were down-regulated in KO molars compared with CON molars (Fig. 3 A). In contrast, the levels of Notch2 and Jag1 were not changed, and the decrease in Jag2 was not statistically significant (Fig. 3A). Immunostaining showed that NOTCH1 protein was also down-regulated in KO molars at P7 compared with CON molars, where NOTCH1 was specifically expressed in the SI layer but not in ameloblasts (Fig. 3B, left panels). The active form of Notch1, NICD, was also reduced in KO molars at P7 compared with CON molars, where it was exclusively detected in the nucleus of SI cells (Fig. 3B, right panels). In contrast, the localization and expression levels of NOTCH2, JAG1, and JAG2 proteins did not change in Med1 KO molars compared with CON molars (Fig. 3C). These results demonstrated that the ablation of Med1 Notch1 signaling in SI cells by reducing Notch1 expression and activation.

**Med1 regulates Alpl expression and Notch signaling in SF2 cells**

The role of MED1 in SI differentiation and Notch signaling was examined using the dental epithelial cell line SF2. SF2 cells differentiate into the SI lineage, as indicated by the increased expression of Alpl with time in culture (Fig. 4A, left panel, vector control (Vec)). When Med1 was overexpressed, Med1 expression increased more than 50-fold (Fig. 4A, right panel (Med1)), and Alpl was further induced at the 96-h time point (Fig. 4A, left panel). When Med1 was silenced by siRNA (siMed1), Med1 was efficiently down-regulated compared with cells transfected with control siRNA (siCON) (Fig. 4B right panel), and the induction of Alpl expression was reduced (Fig. 4B, left panel). Med1 silencing also reduced the mRNA expression levels of Notch1 and Hes1 at 96 h (Fig. 4C). Med1 overexpression increased the protein level of NICD (Fig. 4D, left panel), although total NOTCH1 did not change (Fig. 4D). Med1 silencing (siMed1) reduced NICD but did not change total NOTCH1 (Fig. 4D, right panel). When SF2 cells were treated with EDTA, Notch signaling was activated, as shown by the increased levels of NICD, in a time-dependent manner (Fig. 4E). However, Med1 silencing by siMed1 delayed the increase in NICD levels with EDTA treatment (Fig. 4E). These results
confirm the *in vivo* results that MED1 regulates Notch signaling and the expression of *Alpl*.

**Med1 regulates SI differentiation and Alpl expression through Notch signaling**

We next examined the involvement of Notch signaling in SI differentiation and *Alpl* expression using the γ-secretase inhibitor DAPT, which prevents the cleavage of NOTCH1 and thus inhibits Notch signaling. When the SF2 cells were treated with DAPT, the levels of NICD were reduced in a dose-dependent manner with no change in NOTCH1 itself (Fig. 5A). In addition, DAPT reversed the MED1-induced *Alpl* and *Hes1* expression at the 96-h time point (Fig. 5B). To further confirm the involvement of Notch signaling in the *Alpl* induction, we transfected SF2 cells with the NICD expression vector, which mimics the action of NICD. The NICD transfection increased the expression of *Alpl* (Fig. 5C, *left panel*) and *Hes1* (Fig. 5C, *right panel*).
Med1 functions as a coactivator for Notch1 signaling to control gene transcription of Alpl

We examined whether MED1 was directly recruited into the promoter region of Alpl using chromatin immunoprecipitation (ChIP). Med1-overexpressing SF2 cells were fixed and immunoprecipitated with antibodies against MED1, RNA pol II, RBP-Jk, and IgG (control). The recruitment of MED1 and other proteins into the complex was detected by qPCR using primer sequences spanning the proximal region (site 1, −452 bp to −353 bp) containing the consensus DNA-binding sequences (GTGGGAA) for the RBP-Jk–binding site. The recruitment of MED1 into the distal region of the promoter lacking RBP-Jk (site 2, −2.5 to −2.4 kb) was also examined as a control. MED1, RBP-Jk, and RNA pol II were recruited into the proximal region of the Alpl promoter but not into the distal sites (Fig. 6A). When the cleavage of NOTCH1 was prevented by DAPT treatment, the recruitment of MED1 and pol II was inhibited, whereas the recruitment of RBP-Jk was not affected (Fig. 6A, and B, DAPT). These results suggest that MED1 is recruited into the RBP-Jk–binding site on the Alpl promoter only when NICD is present, whereas RBP-Jk does not require NICD for binding to this site. To confirm this proposition, we next examined whether MED1 directly interacts with NICD in the nucleus. Nuclear extracts were prepared from SF2 cells overexpressing MED1 and NICD, and transcriptional complexes were pulled down using antibodies against either MED1 or NICD (Fig. 6C). Both NICD and MED1 were detected in the MED1 pulldown complex, as compared with the IgG control (Fig. 6C, upper panels). Likewise, both NICD and MED1 were found in NOTCH1 complexes but not in the IgG control (Fig. 6C, lower panels). These results indicate that MED1 physically interacts with NICD in the nucleus. We next examined MED1 regulation of RBP-Jk transcriptional activity. The promoter reporter construct containing tandem repeats of the RBP-Jk–binding DNA sequence was transfected into SF2 cells, and transcriptional activity was evaluated by luciferase activity. SF2 cells showed basal RBP-Jk transcriptional activity after transfection with the reporter construct. When Med1 was overexpressed, the RBP-Jk activity further increased (Fig. 6D). However, DAPT treatment abrogated both basal and Med1-induced transcriptional activity (Fig. 6D). These results indicated that MED1 facilitates the transcriptional activity of RBP-Jk through Notch signaling. We propose a model to show the MED1 regulation of Alpl transcription through Notch signaling (Fig. 6E). First of all, MED1...
stimulates the expression and activation of Notch1. In the activation of NOTCH1 signaling, the NICD is cleaved by specific proteases, such as γ-secretase, in either a ligand– or non-ligand–dependent manner. The cleaved NOTCH1 is then translocated into the nucleus and binds to RBP-Jk on the Alpl promoter. MED1 then binds to NICD and forms a complex.
activates machinery including RNA pol II. This study demonstrated that hair generation and enamel defects in the incisor (24). This study incisor-specific stem cells that switch cell fate, resulting in ectopic
Discussion

Previously, we reported that Med1 deletion causes defects in incisor-specific stem cells that switch cell fate, resulting in ectopic hair generation and enamel defects in the incisor (24). This study demonstrated that Med1 ablation causes enamel hypoplasia through not only defects in stem cell commitment but also through defects in Notch1-mediated SI differentiation. Med1 ablation results in specific enamel defects in which the enamel matrix layer is formed but not mineralized. Med1 deletion prevented the differentiation of SI but did not block commitment of dental epithelial stem cells to the IEE/ameloblast lineage. The matrix proteins were normally secreted and formed the enamel matrix layer in Med1 KO molars, although Notch components and Alpl expression are reduced in the presumptive SI layer. These results suggest that Med1 ablation may cause enamel hypomineralization through defects in Alpl expression during post-embryonic development. However, further studies are needed to prove that MED1 is required for enamel mineralization directly through stimulation of Alpl gene transcription in SI cells.

We demonstrated that MED1 regulates gene transcription of Alpl in vitro, and this regulation was mediated through Notch signaling. We suggest that MED1 is essential for enamel mineralization through the regulation of Alpl in the SI layer at the secretory stage. MED1 may also function at the maturation stage in which SI cells are differentiated into the papillary layer cells and ALPL becomes involved in enamel matrix mineralization. However, MED1 may have other roles in the enamel mineralization process. Med1 is also expressed in ameloblasts, especially at the maturation stage in the adult tooth, which may affect the enamel mineralization processes in Med1 KO mice in addition to the Alpl expression defect in SI cells. Our previous study demonstrated that Med1 deficiency disturbs the nuclear polarization of ameloblasts and reduced gene expression at the maturation stage of adult incisors (24).

As noted above, we demonstrated that MED1 regulates enamel mineralization and Notch signaling. However, a recent study shows that Mediator regulates cell-specific gene transcription by forming a super-enhancer in which several different fate transcription factors are recruited to induce lineage-specific transcription (16). Therefore, it is still possible that MED1 regulates other signal pathways or transcription factors required for enamel mineralization together with Notch signaling.

We also demonstrated that MED1 regulates Notch1 signaling through transcriptional regulation. However, the expression of NOTCH1 and NICD was reduced in Med1 KO molars (P7) as well as in Med1-silenced SF2 cells. MED1 also may influence the activation of NOTCH1 by regulating γ-secretase, which cleaves NOTCH1 to activate Notch signaling. In fact, our preliminary results showed that components of γ-secretase are down-regulated in siMed1 cells (data not shown).

Taken together, our in vivo and in vitro results suggest that MED1 facilitates enamel mineralization through the regulation of Notch1-mediated Alpl expression in SI cells. ALPL is critical for mineralization in bone and tooth (3, 4). The tissue-nonspecific ALPL promotes extracellular matrix mineralization by increasing the local availability of phosphate needed for hydroxyapatite crystal formation in bone and tooth.

MED1 acts as a coactivator for Notch1 signaling and directly regulates the transcription of Alpl. MED1 is specifically recruited into the RBP-Jk–binding site of the Alpl promoter, indicating that MED1 controls Notch1 transcriptional activity.

Our results indicate that MED1 has a critical role in the mineralization of enamel through Alpl. Krt14-specific Med1 KO mice did not show defects in dentin and bone, as these tissues do not have Krt14-expressing epithelia, although Med1 is expressed in osteoblasts, odontoblasts, and dental mesenchyme cells in teeth (data not shown). The specific deletion of Med1 from these cells may reveal a role for MED1 in mineralization in these tissues as well.

In summary, our results reveal that MED1 functions as a coactivator for Notch signaling and directly regulates gene transcription of Alpl in tooth enamel organ. Alpl expression in the SI appears to be necessary for enamel matrix mineralization during tooth development. This observation suggests a role for the SI cells in enamel matrix mineralization that has not been explored previously.
Experimental procedures

Mouse model of Med1 conditional KO mice

To delete the expression of Med1 in dental epithelial cells, floxed Med1 mice were mated with Krt14Cre mice as described previously (23). Floxed Med1 mice with the Krt14Cre transgene (Med1 KO) and control floxed Med1 mice without Krt14-Cre (CON) were used for this study. Genotyping was performed by PCR as described previously (23). All of the experiments were approved by the Institutional Animal Care and Ethics Committee at the San Francisco Department of the Veterans Affairs Medical Center.

Tissue preparation, histological analysis, and immunostaining

The heads of postnatal day 7 (P7) and 10-week-old control and Med1 KO mice were excised and fixed with 4% paraformaldehyde in PBS overnight at 4 °C. For histological and immuno-
histochemical analyses, P7 heads were decalcified with 250 mM EDTA/PBS for 7 days and processed for embedding in paraffin. Next, 8-μm sections were sliced using a microtome. The sections were stained with Harris hematoxylin (Sigma-Aldrich) and eosin Y (Sigma-Aldrich) for a detailed morphological analysis of the incisors. For immunohistochemistry, sections were dehydrated, rehydrated, and then pretreated in 10 mmol/liter citrate buffer, pH 6.0 (Sigma-Aldrich), for 20 min using a microwave for antigen retrieval. The specimens were blocked using Power Block (BioGenex) and reacted with primary antibodies to ALPL (R&D System), NOTCH1, NOTCH2, NICD, JAG1, and JAG2 (Cell Signaling), AMBN (Santa Cruz Biotechnology), and AMEL (Abcam) overnight at 4 °C. Primary antibodies were detected using Alexa Fluor 488 (Invitrogen). Nuclear staining was performed with DAPI (Sigma-Aldrich). The molars from P7 Med1 KO and CON mice were dissected. Epithelial tissues were separated from the P7 molars under a microscope. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol. Total RNA (1 μg) was used for reverse transcription to generate cDNA. First-strand cDNA was synthesized at 42 °C for 60 min using random primers with SuperScript III (Invitrogen). PCR amplification was performed using the primers listed in Table 1. Real-time qPCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories) and a CFX96 thermal cycler (Bio-Rad Laboratories). Gene expression was normalized to the housekeeping gene Gapdh in each sample.

**Immunoprecipitation (IP) Assay**

SF2 cells were plated in 60-mm dishes at a density of 2 × 10^6 cells in 3 ml of the media/dish and then transfected with the Med1 and NICD expression vectors for 24 h. The cells were then harvested for nuclear extraction using a nuclear extract kit (Active Motif). The nuclear fraction was analyzed using a Cignal RBP-Jk (Invitrogen) kit (Qiagen). The RBP-Jk reporter plasmid was transfected into SF2 cells either with mock vector or the Med1 expression vector. Activity was determined using a Dual-Luciferase reporter assay system (Promega) using a luminometer (Berthold). The firefly luciferase activity was normalized for Renilla luciferase activity as an internal control.
**Med1 activates alkaline phosphatase gene transcription**

**Chromatin immunoprecipitation assay**

To examine whether MED1 interacts directly with the promoter region of *Alpl*, ChIP assays were performed in SF2 cells either with or without DAPT using a ChIP-IT express kit (Active Motif) according to the manufacturer’s protocol. Cross-linked chromatin lysates were sonicated, incubated with antibodies against MED1 (Santa Cruz Biotechnology), RNA pol II (Active Motif), RBP-Jk (Santa Cruz Biotechnology), and control IgG (Active Motif) at 4 °C overnight, and then precipitated with protein G–magnet beads. Cross-linking was reversed using a reverse cross-linking buffer, and DNA fragments were analyzed by PCR using the following primer pairs, specific for their respective promoter region, on an agarose gel. For the *Alpl* promoter site 1, the forward primer used was TCTGCTTC-TACCTCAGGTT, and the reverse primer was CATCCTT-GTCTGTAACC. For the *Alpl* promoter site 2, the forward primer was AGTTGTGAAAGGAAAGTC, and the reverse primer was ATGTTGAAAGGAAAGTC.

**Statistical analysis**

All of the experiments in this study were repeated at least three times, and reproducibility was confirmed. The data were analyzed using Prism 6 software (GraphPad Software Inc.), and *p* < 0.05 was considered statistically significant.

**Author contributions**—K. Y. designed the study, performed the experiments and the data analysis, and wrote the manuscript. L. H., T. N., K. S., M. I., T. T., and S. F. contributed in conducting the experiments and the data analysis. Y. O., L. H., T. N., and D. D. B. generated and bred the transgenic mice. Y. O., D. D. B. P. D., and Y. Y. initiated and designed the study, performed the data analysis, and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**References**

1. Yoshizaki, K., and Yamada, Y. (2013) Gene evolution and functions of extracellular matrix proteins in teeth. *Orthod. Waves* 72, 1–10
2. Fukumoto, S., and Yamada, Y. (2005) Review: Extracellular matrix regulates tooth morphogenesis. *Connect Tissue Res.* 46, 220–226
3. Gasque, K. C., Foster, B. L., Kuss, P., Yadav, M. C., Liu, J., Kiffer-Moreira, T., van Elsas, A., Hatch, N., Somerman, M. J., and Millán, J. L. (2015) Improvement of the skeletal and dental hypophosphatasia phenotype in *Alpl−/−* mice by administration of soluble (non-targeted) chimeric alcaline phosphatase. *Bone* 72, 137–147
4. McKee, M. D., Yadav, M. C., Foster, B. L., Somerman, M. J., Farquharson, C., and Millán, J. L. (2013) Compounded PHOSPHO1/ALPL deficiencies reduce dentin mineralization. *J. Dent. Res.* 92, 721–727
5. Kawanou, S., Saito, M., Handa, K., Morotomi, T., Toyono, T., Seta, Y., Nakamura, N., Uchida, T., Toyoshima, K., Ohashi, M., and Harada, H. (2004) Characterization of dental epithelial progenitor cells derived from cervical-loop epithelium in a rat lower incisor. *J. Dent. Res.* 83, 129–133
6. Yadav, M. C., de Oliveira, R. C., Foster, B. L., Fong, H., Cory, E., Narisawa, S., Sah, R. L., Somerman, M., Whyte, M. P., and Millán, J. L. (2012) Enzyme replacement prevents enamel defects in hypophosphatasia mice. *J. Bone Miner. Res.* 27, 1722–1734
7. Cai, X., Gong, P., Huang, Y., and Lin, Y. (2011) Notch signalling pathway in tooth development and adult dental cells. *Cell Prolif.* 44, 495–507
8. Harada, H., Ichimori, Y., Yokohama-Tamaki, T., Oshtima, H., Kawanou, S., Katsube, K., and Wakisaka, S. (2006) Stratum intermedium lineage diverges from ameloblast lineage via Notch signaling. *Biochem. Biophys. Res. Commun.* 340, 611–616
9. Mitsiadias, T. A., Graf, D., Luder, H., Gridley, T., and Bluteau, G. (2010) BMPs and FGFs target Notch signalling via jagged 2 to regulate tooth morphogenesis and cytodifferentiation. *Development* 137, 3035–3035
10. Rand, M. D., Grimm, L. M., Arvanavis-Tsokonas, S., Patriub, V., Blacklow, S. C., Sklar, J., and Aster, J. C. (2000) Calcium depletion dissociates and activates heterodimeric notch receptors. *Mol. Cell. Biol.* 20, 1825–1835
11. Blazek, E., Mittler, G., and Meisterernst, M. (2005) The mediator of RNA polymerase II. *Cell* 14, 553–557
12. Bourbon, H. M., Aguilera, A., Ansari, A. Z., Asturias, F. I., Berk, A. J., Bjorklund, S., Blackwell, T. K., Borggreve, T., Carey, M., Carlson, M., Conaway, J. W., Conaway, R. C., Emmons, S. W., Fondell, J. D., Freedman, L., et al. (2004) A unified nomenclature for protein subunits of mediator complexes linking transcriptional regulators to RNA polymerase II. *Mol. Cell Biol.* 10, 307–319
13. Kornberg, R. D. (2005) Mediator and the mechanism of transcriptional activation. *Trends Biochem. Sci.* 30, 220–226
14. Malik, S., and Roeder, R. G. (2005) Dynamic regulation of pol II transcription by the mammalian Mediator complex. *Trends Biochem. Sci.* 30, 220–223
15. Kagey, M. H., Newman, J. I., Bilodeau, S., Zhan, Y., Orlando, D. A., van Berkum, N. L., Ebmeier, C. C., Goossens, I., Rahl, P. B., Levine, S. J., Taatjes, D. J., Dekker, J., and Young, R. A. (2010) Mediator and cohesin connect gene expression and chromatin architecture. *Nature* 467, 430–435
16. Whyte, W. A., Orlando, D. A., Hnisz, D., Abraham, B. J., Lin, C. Y., Kagey, M. H., Rahl, P. B., Lee, T. I., and Young, R. A. (2013) Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153, 307–319
17. Yue, X., Iczue, A., and Borggreve, T. (2011) Essential role of Mediator subunit Med1 in invariant natural killer T-cell development. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17105–17110
18. Stampf, M., Yue, X., Schmitz, S., Luche, H., Reddy, J. K., and Borggreve, T. (2010) Specific erythroid-lineage defect in mice conditionally deficient for Mediator subunit Med1. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21541–21546
19. Jiang, P., Hu, Q., Ito, M., Meyer, S., Waltz, S., Khan, S., Roeder, R. G., and Zhang, X. (2010) Key roles for MED1 LxxLL motifs in pubertal mammary gland development and luminal-cell differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6765–6770
20. Jia, Y., Qi, C., Zhang, Z., Zhu, Y. T., Rao, S. M., and Zhu, Y. J. (2005) Peroxisome proliferator-activated receptor-binding protein null mutation results in defective mammary gland development. *J. Biol. Chem.* 280, 10766–10773
21. Oda, Y., Chalkley, R. J., Burlingame, A. L., and Bikle, D. D. (2010) The transcriptional coactivator DRIP/Mediator complex is involved in vitamin D receptor function and regulates keratinocyte proliferation and differentiation. *J. Invest. Dermatol.* 130, 2377–2388
22. Bikle, D., Teichert, A., Hawker, N., Xie, Z., and Oda, Y. (2007) Sequential regulation of keratinocyte differentiation by 1,25(OH)2D3, VDR, and its coregulators. *J. Steroid Biochem. Mol. Biol.* 103, 396–404
23. Oda, Y., Hu, L., Bul, V., Elalieh, H., Reddy, J. K., and Bikle, D. D. (2012) Coactivator MED1 ablation in keratinocytes results in hair-cycling defects and epidermal alterations. *J. Invest. Dermatol.* 132, 1075–1083
24. Yoshizaki, K., Hu, L., Nguyen, T., Sakai, K., He, B., Fong, C., Yamada, Y., Bikle, D. D., and Oda, Y. (2014) Ablation of coactivator Med1 switches the cell fate of dental epithelia to that generating hair. *PLoS ONE* 9, e99991
25. Nakamura, T., de Vega, S., Fukumoto, S., Jimenez, L., Unda, F., and Yamada, Y. (2008) Transcription factor epiprosin is essential for tooth morphogenesis by regulating epithelial cell fate and tooth number. *J. Biol. Chem.* 283, 4825–4833
26. Arakaki, M., Ishikawa, M., Nakamura, T., Iwamoto, T., Yamada, A., Fukumoto, E., Saito, M., Otsu, K., Harada, H., Yamada, Y., and Fukumoto, S. (2012) Role of epithelial-stem cell interactions during dental cell differentiation. *J. Biol. Chem.* 287, 10590–10601
27. Dassule, H. R., Lewis, P., Bei, M., Maas, R., and McMahon, A. P. (2000) Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* 127, 4775–4785