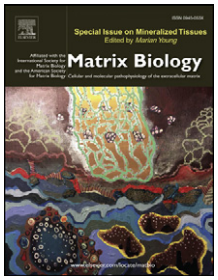

Multiple essential MT1-MMP functions in tooth root formation, dentinogenesis, and tooth eruption

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Abstract

Membrane-type matrix metalloproteinase 1 (MT1-MMP) is a transmembrane zinc-endopeptidase that breaks down extracellular matrix components, including several collagens, during tissue development and physiological remodeling. MT1-MMP-deficient mice (*MT1-MMP*$^{-/-}$) feature severe defects in connective tissues, such as impaired growth, osteopenia, fibrosis, and conspicuous loss of molar tooth eruption and root formation. In order to define the functions of MT1-MMP during root formation and tooth eruption, we analyzed the development of teeth and surrounding tissues in the absence of MT1-MMP. In situ hybridization showed that MT1-MMP was widely expressed in cells associated with teeth and surrounding connective tissues during development. Multiple defects in dentoalveolar tissues were associated with loss of MT1-MMP. Root formation was inhibited by defective structure and function of Hertwig's epithelial root sheath (HERS). However, no defect was found in creation of the eruption pathway, suggesting that tooth eruption was hampered by lack of alveolar bone modeling/remodeling coincident with reduced periodontal ligament (PDL) formation and integration with the alveolar bone. Additionally, we identified a significant defect in dentin formation and mineralization associated with the loss of MT1-MMP. To segregate these multiple defects and trace their cellular origin, conditional ablation of MT1-MMP was performed in epithelia and mesenchyme. Mice featuring selective loss of MT1-MMP activity in the epithelium were indistinguishable from wild type mice, and importantly, featured a normal HERS structure and molar eruption. In contrast, selective knock-out of MT1-MMP in Osterix-expressing mesenchymal cells, including osteoblasts and odontoblasts, recapitulated major defects from the global knock-out including altered HERS structure, short roots, defective dentin formation and mineralization, and reduced alveolar bone formation, although molars were able to erupt. These data indicate that MT1-MMP activity in the dental mesenchyme, and not in epithelial-derived HERS, is essential for proper tooth root formation and eruption. In summary, our studies point to an indispensable role for MT1-MMP-mediated matrix remodeling in tooth eruption through effects on bone formation, soft tissue remodeling and organization of the follicle/PDL region.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of degrading extracellular matrix (ECM) structural proteins and processing a number of bioactive molecules. MMPs play important roles in tissue development, physiological remodeling, and pathological processes when normal matrix remodeling is co-opted by pathogens or invasive tissue destructive processes [1–3]. Membrane-type 1 matrix metalloproteinase (MT1-MMP; also known as MMP14) is a cell-associated, membrane-bound MMP with substrate specificity for collagens (I, II, and III), gelatin, fibronectin, and other matrix molecules [4,5]. The widespread functions of MT1-MMP were revealed by targeted gene deletion in mice where severe deficits in connective tissue remodeling lead to dwarfism, soft tissue fibrosis, osteopenia, skeletal dysplasia, joint disease, and premature death [6].

The dentoalveolar complex is comprised of multiple connective tissues including dentin, cementum, periodontal ligament (PDL), and alveolar bone, with each tissue composed of a distinct ECM [7–9]. Tooth development encompasses several dynamic processes directed by and dependent on ECM production and modification, including cell–cell signaling and cell migration through ECM [10]. Odontogenesis requires secretion and mineralization of dentin matrix whereas root-periodontal formation depends on construction of a complex dentin–cementum–PDL–bone composite structure of mineralized ECM and unmineralized ECM. Eruption of the tooth, in turn, requires that the tooth bud breaches through the mineralized ECM of its bony crypt to assume its position in the oral cavity [11]. The function of the tooth over time further requires that the PDL–bone ECM is remodeled in response to occlusal loads [12].

MT1-MMP deficient mice (*MT1-MMP*$^{-/-}$) feature disrupted molar root formation and lack of eruption [13], though underlying mechanisms for these defects are not well understood. In order to define the functions of MT1-MMP during root formation and tooth eruption, we analyzed the development of teeth and surrounding tissues in the absence of MT1-MMP. Additionally, we further elucidated the dentoalveolar defects from loss of MT1-MMP by studying two mouse models with cell-specific ablation of the protease in distinct tissue compartments.

Results

MT1-MMP mRNA is widely expressed in the developing tooth and surrounding tissues

As a first step to determine the role(s) of MT1-MMP in tooth root formation, we identified mRNA localization in dentoalveolar tissues by in situ hybridization (ISH) in mice at 10 days postnatal (dpn), while the molar root is actively forming. Intense *MT1-MMP* mRNA expression was observed in several cell populations associated with the developing dentoalveolar complex, including cells in or around Hertwig's epithelial root sheath (HERS), the mesenchyme surrounding the root tip, odontoblasts in both molar and incisor teeth, developing periodontal ligament (PDL), and alveolar bone osteoblasts (Fig. 1).

Deletion of *MT1-MMP* prevents root growth and molar tooth eruption

MT1-MMP$^{-/-}$ mice were subsequently analyzed to correlate the observed MT1-MMP expression profile with potential functions of the enzyme during molar root formation. Radiography and micro-CT were used to survey bone and tooth development prior to root initiation (5 dpn), during active root growth and intra-osseous tooth eruption (14 dpn), and after completion of root formation with subsequent molar eruption into the oral cavity (26 dpn).

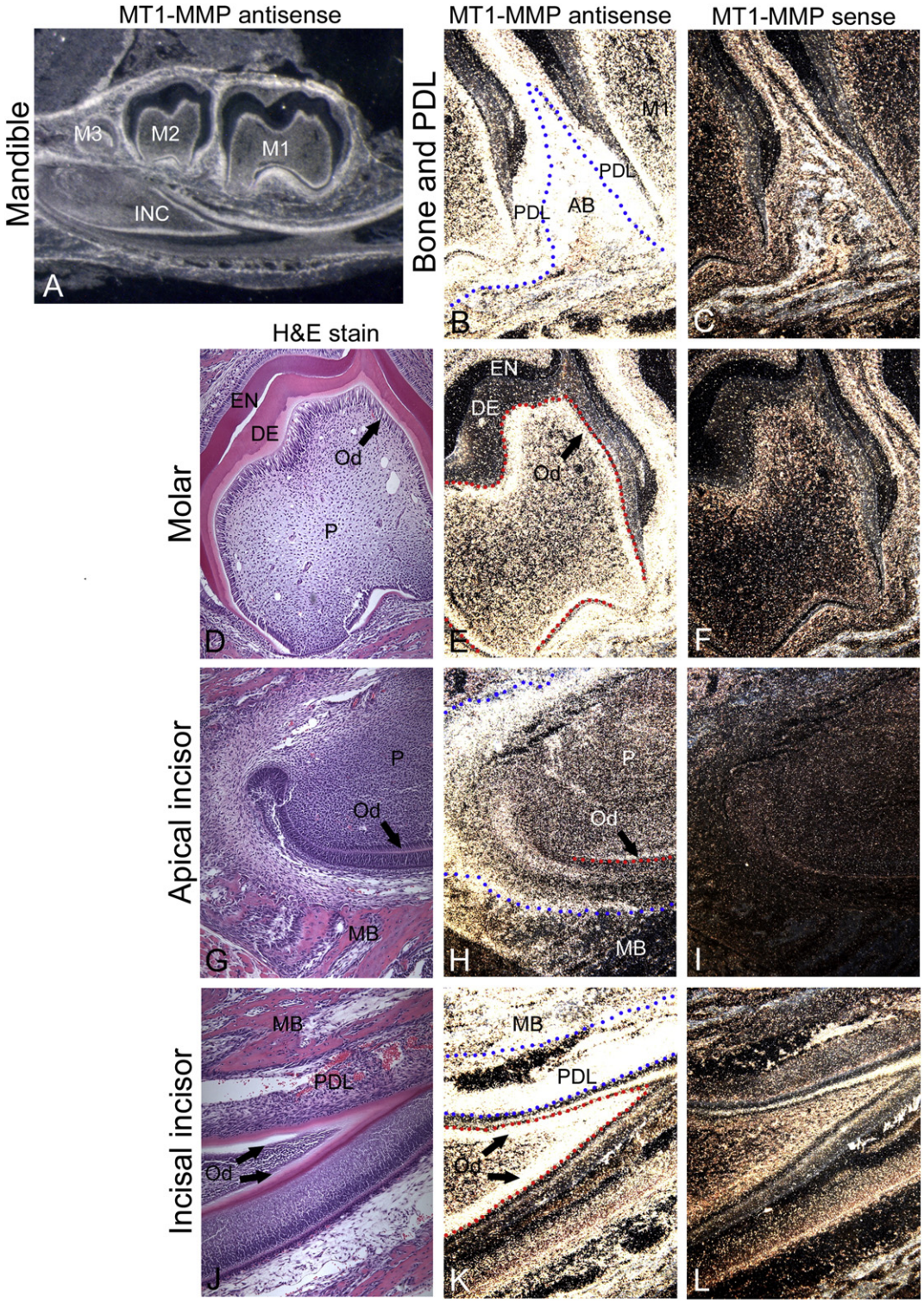
At 5 dpn, skulls and mandibles of *MT1-MMP*$^{-/-}$ mice were overtly smaller than controls (Supplementary Fig. 1). Corresponding radiography and microCT analysis revealed bone mineralization defects, however, molar crown formation appeared unperturbed. Defects in both mandible size and bone mineralization of *MT1-MMP*$^{-/-}$ mice became more apparent by 14 dpn (Supplementary Fig. 2) and 26 dpn (Fig. 2). At 14 dpn, first and second molar root development was delayed in *MT1-MMP*$^{-/-}$ mice, and third molar development also delayed. Basal bone formation apical to the molars as well as alveolar bone height in both the interradicular and interdental areas were reduced compared to WT. By 26 dpn, bone and tooth aberrancies in *MT1-MMP*$^{-/-}$ mice were even more accentuated (Fig. 2). All molars failed to erupt and had incomplete root development with diminished alveolar bone support. The third molar root also failed to form. By 26 dpn, all molars were fully erupted with alveolar bone support in control littermates.

Based on these aggregate observations of mandibular and radicular developmental defects in the absence of MT1-MMP, we sought to define the effects of MT1-MMP deletion on first molar tissue compartments including HERS, dentin, cementum, PDL, and surrounding alveolar bone.

Loss of MT1-MMP results in altered HERS structure and signaling

During root formation, growth of HERS defines the size and shape of the tooth root [14]. In WT mice, a well-defined apical HERS migration between 5 and 14 dpn precedes normal root maturation, which is complete by 26 dpn (Fig. 3). In

contrast, HERS in *MT1-MMP*$^{-/-}$ mice was disorganized and surrounded by a dense mass of cells from the dental papilla and follicle along its perimeter, with a diffuse boundary between mesenchyme and (HERS) epithelium. The mean HERS length in *MT1-MMP*$^{-/-}$ mice was reduced by 58% on the buccal molar aspect ($p = 0.02$) and 26% on the lingual molar aspect ($p = 0.26$). Furthermore,



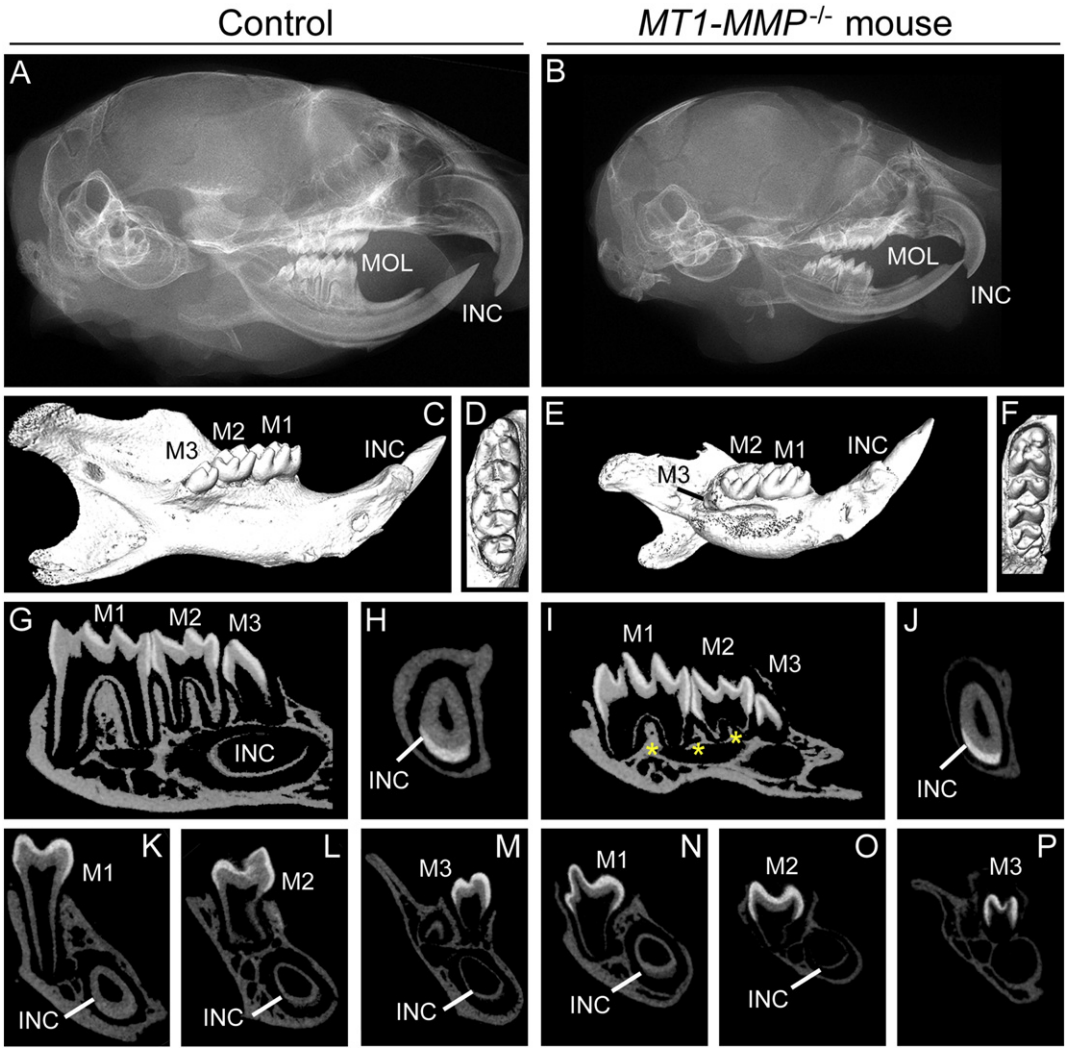


Fig. 2. Knockout of *MT1-MMP* prevents root growth and molar tooth eruption. Radiographs (A, B) and micro-CT (C–P) were used to analyze dentoalveolar development at 26 dpn (ages 5 and 14 dpn are shown in Supplementary Figs. 1 and 2). (A–F) *MT1-MMP*$^{-/-}$ mice feature a smaller cranium and mandible compared to WT controls at 26 dpn. The radiograph shows that molars (M1-3) in *MT1-MMP*$^{-/-}$ mice have not erupted, though the coronal eruption pathway has been cleared (shown in D and F). (G–P) *MT1-MMP*$^{-/-}$ mice exhibit failure of molar root formation compared to WT, with short roots and thin dentin. Third molar and incisor development is delayed compared to WT. *MT1-MMP*$^{-/-}$ mouse mandibles show severe lack of formation of tooth-associated bone of the alveolar ridge, interalveolar septum and interradicular septum regions (yellow stars in I).

Fig. 1. *MT1-MMP* mRNA is widely expressed in the developing tooth and surrounding tissues. In situ hybridization was used to localize *MT1-MMP* mRNA expression within mouse dentoalveolar tissues at 10 dpn (A, B, E, H, K), with H&E (D, G, J) shown for morphological reference, and sense panels (C, F, I, L) included as negative controls. (A) *MT1-MMP* expression is observed in molar (M1–M3) and incisor (INC) teeth and surrounding tissues. (B, C) Intense expression of *MT1-MMP* is present in developing Hertwig's epithelial root sheath (HERS), periodontal ligament (PDL), and alveolar bone (AB) around molar teeth. The blue dotted line marks the PDL-AB border. (D–F) Odontoblasts (Od) of the molar tooth express high levels of MT1-MMP, though little expression is found in dental pulp (P). The red dotted line outlines the odontoblast cell layer. Odontoblasts in the (G–I) apical incisor begin expressing *MT1-MMP* mRNA coincident with differentiation and cell layer organization. Osteoblasts (Ob) on the surface of mandibular bone (MB) exhibit *MT1-MMP* expression. Red dotted lines indicate the odontoblast layer and blue dotted lines demarcate bone surfaces. (J–L) Odontoblasts of the incisal incisor tooth retain strong *MT1-MMP* mRNA expression, and cells of the PDL and mandibular bone exhibit intense *MT1-MMP* gene expression.

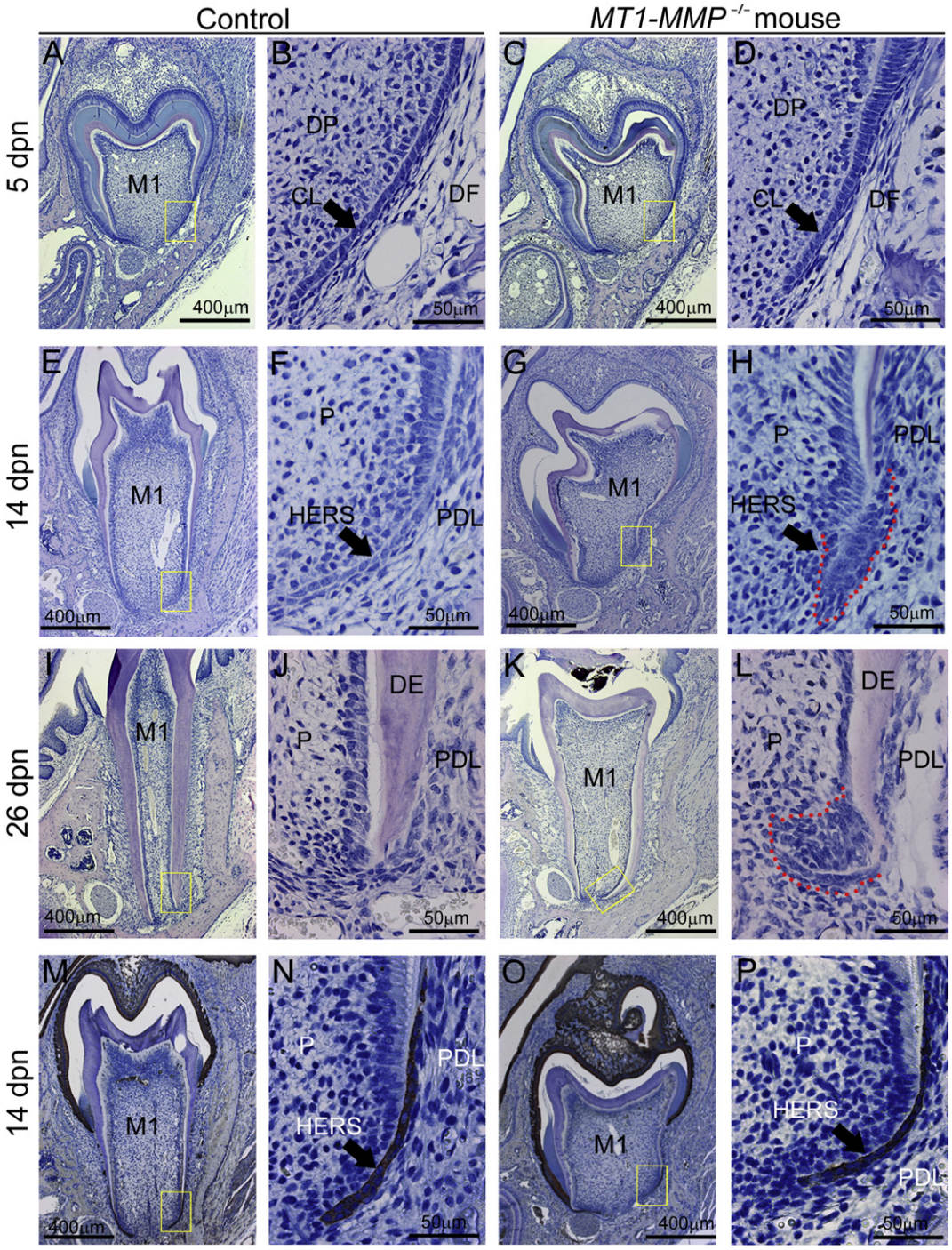


Fig. 3. Loss of MT1-MMP alters the structure of Hertwig's epithelial root sheath (HERS). HERS was analyzed by H&E staining of the first mandibular molar at 5, 14, and 26 dpn. (A–D) At 5 dpn, the shape of the cervical loop (CL) separating dental papilla (DP) and dental follicle (DF) is similar in WT controls and *MT1-MMP*$^{-/-}$ mice, though the structure appears shorter in total length. (E–H) At 14 dpn, the WT molar root is lengthening while the tooth erupts. In the *MT1-MMP*$^{-/-}$ mouse, root length is severely reduced. Compared to the elongated bilayer of HERS in WT, the HERS structure in *MT1-MMP*$^{-/-}$ mice is short, blunted, and surrounded by an accumulated mass of cells (indicated by red dotted line) from pulp (P) and/or periodontal ligament (PDL). (I–L) By 26 dpn, the WT molar has completed root growth and the HERS structure has disappeared. In contrast, in the *MT1-MMP*$^{-/-}$ mouse, a dysmorphic HERS structure exists and is surrounded by a dense mass of cells (indicated by red dotted line). (M–P) Cytokeratin 14 immunostaining was used to identify epithelial cells, revealing that the mass of cell surrounding the HERS structure in *MT1-MMP*$^{-/-}$ mice is mesenchymal in origin.

the characteristic inflection of HERS in relation to the root and apical foramen was blunted in the *MT1-MMP*$^{-/-}$ mouse (132° on the buccal aspect, 112° on the lingual aspect) compared to controls (146° on the buccal aspect, 143° on the lingual aspect), showing significant changes ($p = 0.03$ for the buccal aspect, $p < 0.0001$ for the lingual aspect).

Based on structural alterations in HERS of *MT1-MMP*$^{-/-}$ mice, we analyzed cellular processes and signaling pathways previously suggested to be important for HERS function and root formation including proliferation and apoptosis [15–17], Wnt signaling [18], and NFIC expression [19]. PCNA immunohistochemistry indicated similar proliferation potential in 14 dpn WT and *MT1-MMP*$^{-/-}$ mouse molars, and similar numbers of TUNEL-positive apoptotic cells in the region of WT and *MT1-MMP*$^{-/-}$ HERS (Supplementary Fig. 3A–D). However, a clear difference in apoptotic cell number was observed in the alveolar bone of *MT1-MMP*$^{-/-}$ mandibles. *Wnt*-signaling appeared unaffected, as no difference was observed in the pattern of β-catenin immunostaining in odontoblasts (Supplementary Fig. 3E–H). However, NFIC localization was reduced in the dental papilla immediately surrounding HERS in *MT1-MMP*$^{-/-}$ mice coincident with abnormal cellular aggregation (Supplementary Fig. 3 I–L).

MT1-MMP$^{-/-}$ mice feature defective dentin formation and mineralization

Dentinogenesis is required for crown formation as well as root development and elongation during tooth eruption. Considering the diminished root formation in *MT1-MMP*$^{-/-}$ mice, we further analyzed dentinogenesis. WT mouse molars at 14 dpn displayed a highly organized odontoblast cell layer and well developed dentin with a smooth, regular border demarcating the mineralized dentin from the unmineralized predentin (Fig. 4A, B). In contrast, *MT1-MMP*$^{-/-}$ mouse molars displayed regions of grossly disorganized odontoblasts forming a dystrophic dentin matrix with protein deposition in coronal dentin (Fig. 4C, D). Coronal odontoblasts here were entrapped in an ECM composed of types I and XII collagen with unusually organized and oriented fibers extending into the pulp (Supplementary Fig. 4C, D, asterisks). Increased interglobular dentin was also characteristic of this area. Ectopic calcification visualized by von Kossa and Goldner's trichrome staining were found in the pulp and associated with disorganized odontoblasts (Supplementary Fig. 4K, L, asterisks). These histologic aberrations were associated with significantly reduced total dentin thickness (by 55–71%) in molar roots ($p < 0.0001$), significantly increased lingual predentin layer (by 56–85%; $p < 0.01$), and an increased predentin:-dentin ratio (increased by 4- to 7-fold) by 26 dpn (Fig. 4E–H and M, N). Despite this diminution in dentin content, odontoblast differentiation appeared normal initially as induction of the markers dentin sialoprotein (DSP) and tissue nonspecific alkaline phosphatase (TNAP) [20,21] was unaltered (Fig. 4 I–L).

Loss of MT1-MMP disrupts periodontal organization

The periodontium forms in step with the root, and we therefore examined cementogenesis and PDL formation in *MT1-MMP*$^{-/-}$ mice. Acellular cementum was present on the cervical root aspects of WT and *MT1-MMP*$^{-/-}$ mice (Fig. 5A–D), as indicated by immunostaining for bone sialoprotein (BSP) and osteopontin (OPN) [22]. The acellular cementum layer in *MT1-MMP*$^{-/-}$ mice was thicker in WT controls at 14 and 26 dpn (Fig. 5A, B vs. C, D), whereas cellular cementum was absent on the shortened molars of *MT1-MMP*$^{-/-}$ mice by 26 dpn (data not shown).

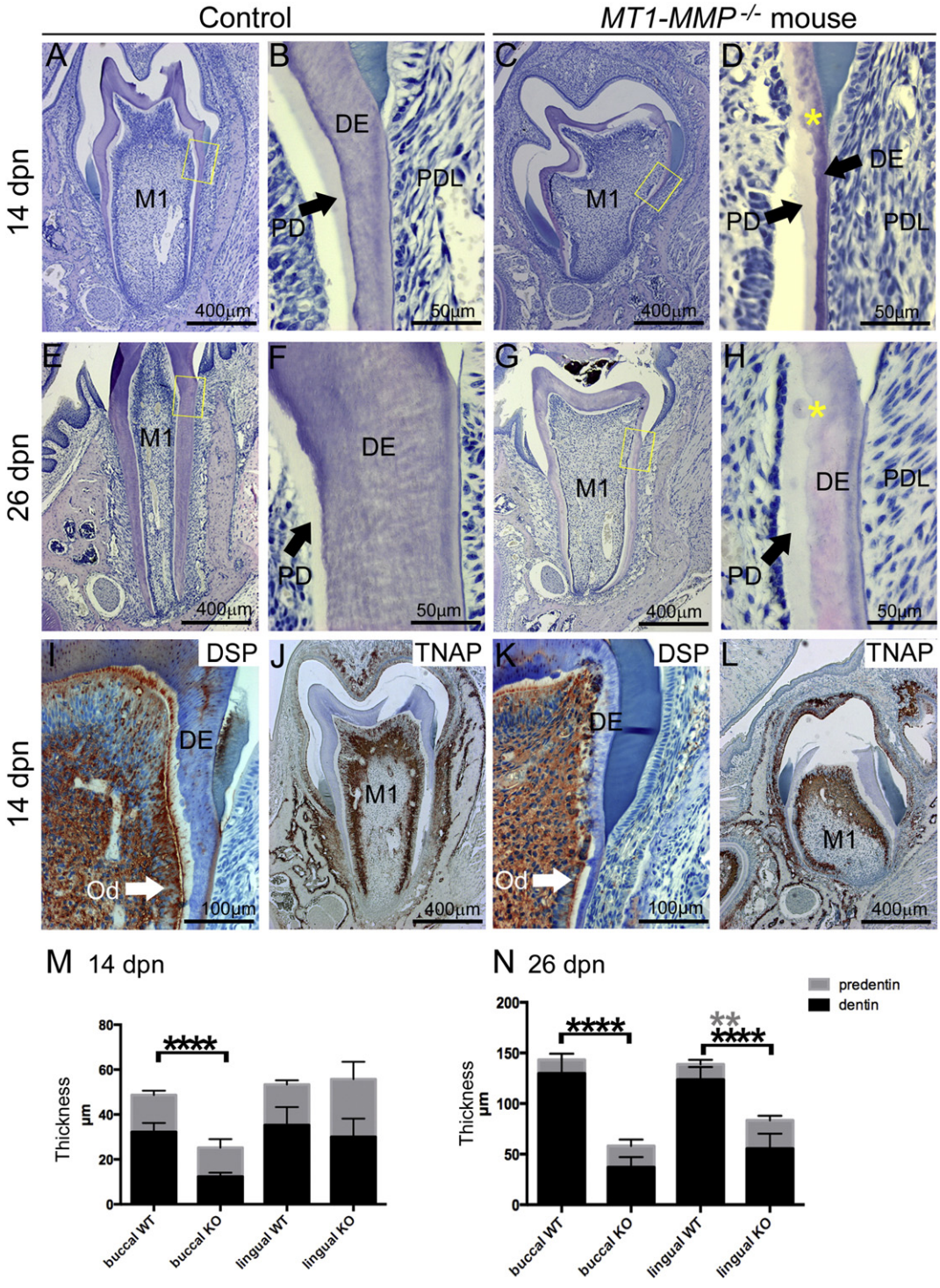
Immunostaining for two ECM proteins associated with PDL organization, collagen type XII (COL XII) [23] and periostin (POSTN) [24], revealed reduced localization of both markers in the PDL of the *MT1-MMP*$^{-/-}$ mice (Fig. 5E–H). Collagen fibers of the PDL remained poorly organized at 26 dpn, with near absence of insertion of Sharpey's fibers at the alveolar bone surface (Fig. 5K, L and Supplementary Fig. 5).

Loss of MT1-MMP results in decreased alveolar bone formation

Directed processes of basal bone apposition and coronal resorption are required for tooth eruption. Given the dramatic differences in bone architecture between control and MT1-MMP-deficient mice (Fig. 2 and Supplementary Figs. 1, 2), we examined mandibular and alveolar bone in further detail by histology. Deficient bone resorption coronal to the tooth can impede the formation of the eruption pathway and contribute to failure of tooth eruption [25]. Tartrate resistant acid phosphatase (TRAP) staining at 5 and 14 dpn, however, revealed numerous osteoclasts coronal to the forming tooth in both WT and *MT1-MMP*$^{-/-}$ mice (Fig. 6A–D). The TRAP staining pattern in conjunction with 3-dimensional images generated by microCT analysis showed clearance of bone coronal to the tooth (Supplementary Figs. 1 and 2; Fig. 2), consistent with an eruption pathway for the developing molars in *MT1-MMP*$^{-/-}$ mice.

Based on the evidence supporting eruption pathway formation, we considered other factors essential to eruption, such as basal bone apposition [26]. Mandibular bone did not exhibit radiographic or histologic distinctions in *MT1-MMP*$^{-/-}$ mice

compared to WT at 5 dpn, prior to root formation (Fig. 3A, C and Supplementary Fig. 1). In contrast to bone of WT mice at 14 or 26 days of age, basal and alveolar bone in *MT1-MMP*$^{-/-}$ littermates remained immature and poorly organized, and in particular, alveolar bone surrounding molars was thin, with a woven appearance (Fig. 3E, G, I, K, Fig. 2, and Supplementary Fig. 2). Immunostaining for TNAP, which is abundant in WT osteoblasts, revealed decreased TNAP in alveolar bone osteoblasts in *MT1-MMP*$^{-/-}$ mice, as well as fibrotic areas at the PDL–bone interface where cells were disorganized



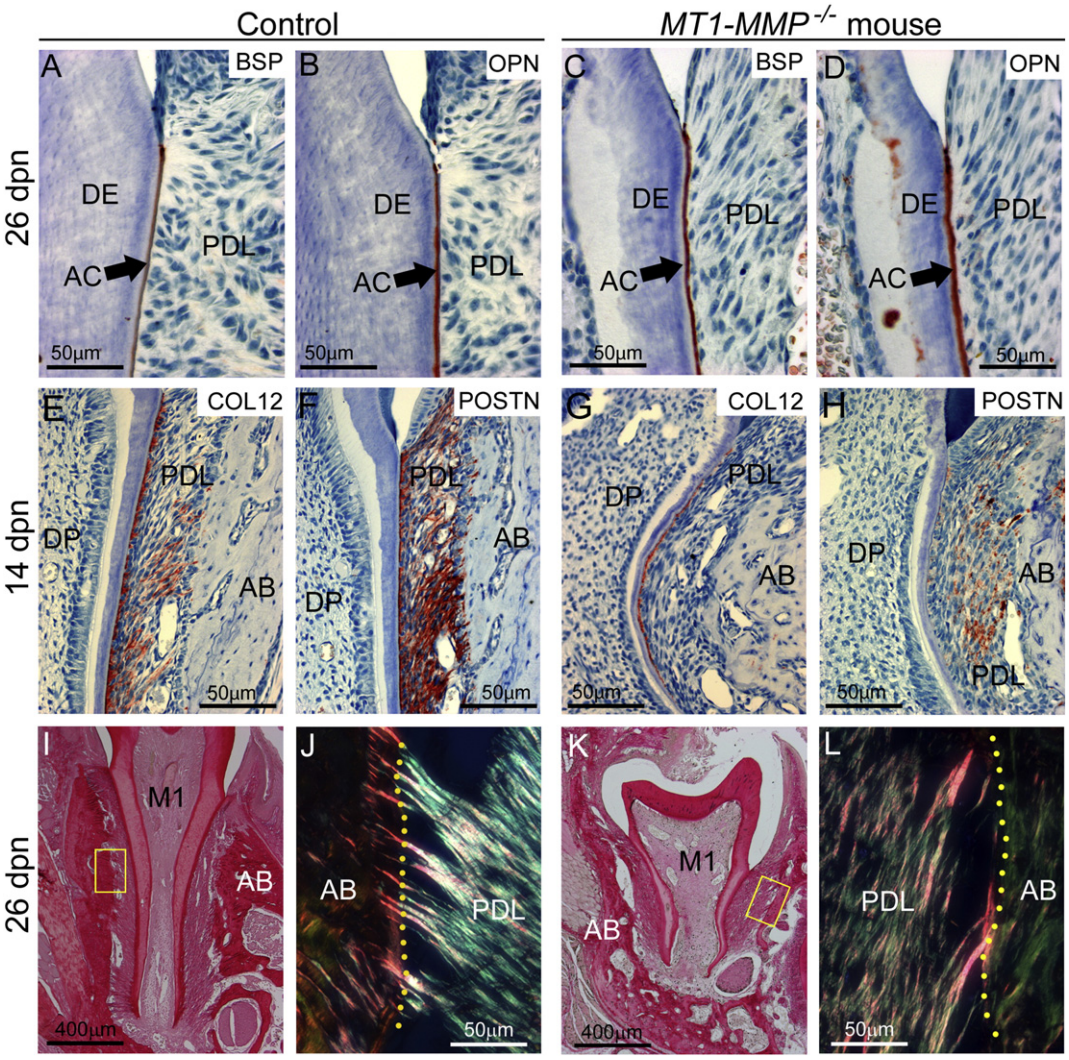


Fig. 5. Loss of MT1-MMP disrupts periodontal structure. Immunostaining and selective staining approaches were used to analyze periodontal development in WT control and *MT1-MMP*$^{-/-}$ mice. (A–D) BSP and OPN immunostaining indicates the presence the acellular cementum (AC) layer on the molar root surface in controls and *MT1-MMP*$^{-/-}$ mice. The PDL begins to organize in WT by 14 dpn, as shown by increased localization of ECM markers (E) collagen type XII (COL12) and (F) periostin (POSTN), whereas the *MT1-MMP*$^{-/-}$ mouse PDL shows little localization of (G) COL12 or (H) POSTN, though strong COL12 staining near the root surface reflects collagen fiber insertion into cementum. Picrosirius red staining (under non-polarized light) indicates the presence of fibrillar collagen in 26 dpn (I) WT and (K) *MT1-MMP*$^{-/-}$ first molars (M1), PDL, and alveolar bone (AB). Under polarized light, (J) WT PDL shows organized collagen fibrils in the PDL, and numerous insertions of Sharpey's fibers into the alveolar bone (yellow dotted line indicates the PDL–bone border). (K) *MT1-MMP*$^{-/-}$ PDL shows less organization, with loose collagen fibers running mostly parallel to the root surface, and few Sharpey's fibers that are also poorly embedded.

Fig. 4. *MT1-MMP*$^{-/-}$ mice feature defective dentin formation and mineralization. Dentinogenesis was analyzed by H&E and immunostaining of the first mandibular molar at 14 and 26 dpn. Yellow boxes in A, C, E, and G indicate locations of higher magnification images in B, D, F, and H. (A–D, M) At 14 dpn, compared to WT control, *MT1-MMP*$^{-/-}$ molar root dentin (DE) thickness is significantly reduced (on the buccal aspect, $p < 0.05$), and regions of increased interglobular dentin (yellow stars) are observed in the crown. (E–H, N) At 26 dpn, *MT1-MMP*$^{-/-}$ dentin defects are more pronounced, including significantly reduced thickness of dentin, increased predentin (PD), and residual interglobular dentin patterns (yellow stars) compared to controls. (I–L) Two odontoblast markers, dentin sialoprotein (DSP) and tissue-nonspecific alkaline phosphatase (TNAP), display comparable localization in controls and *MT1-MMP*$^{-/-}$ mice, indicating that odontoblast differentiation is not initially defective. ** Indicates significant difference, $p < 0.01$ and **** indicates significant difference, $p < 0.0001$. M1 = first molar; PDL = periodontal ligament.

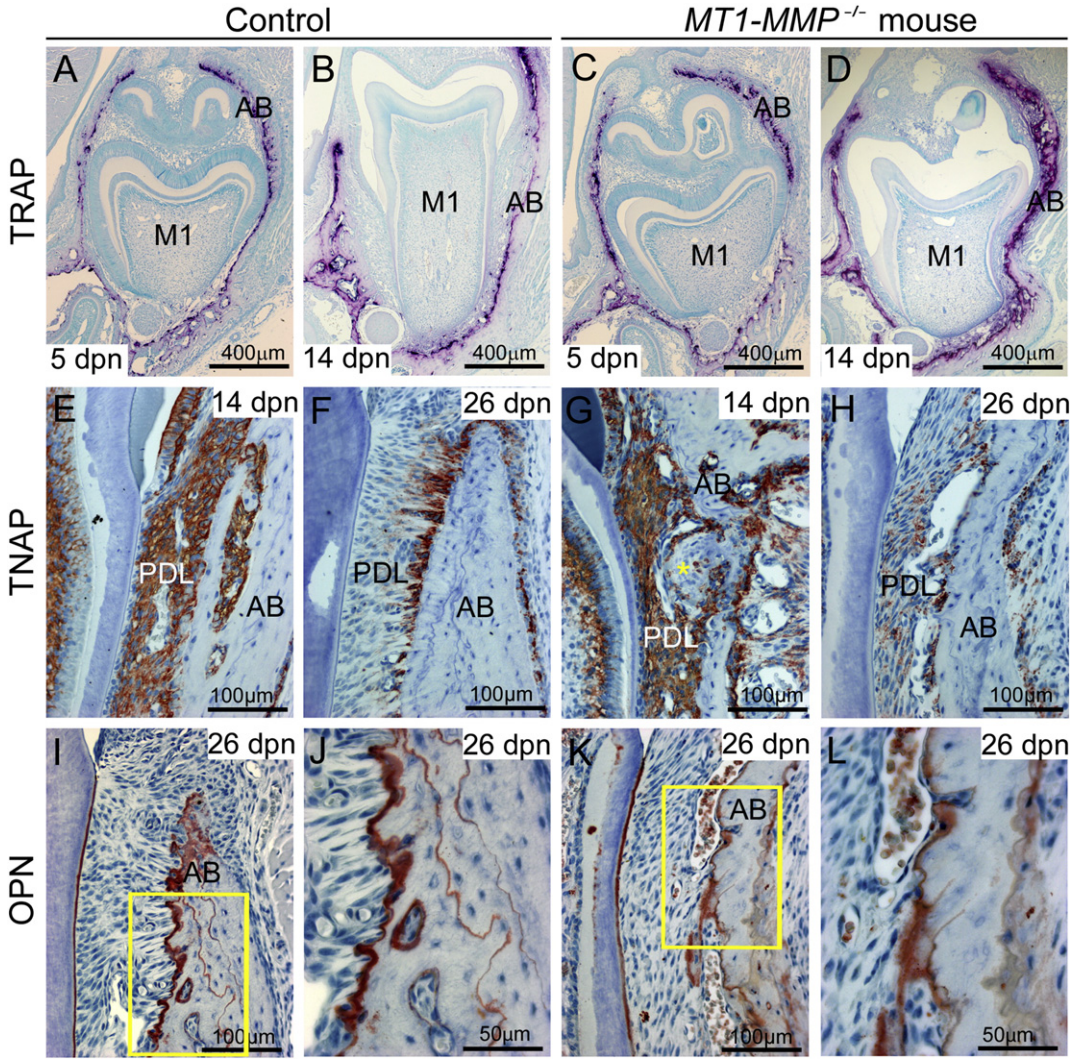


Fig. 6. Alveolar bone dysplasia in *MT1-MMP*$^{-/-}$ mice. Immunostaining and selective staining were used to analyze alveolar bone modeling and remodeling in WT and *MT1-MMP*$^{-/-}$ mice. (A–D) TRAP staining (red–purple color) at 5 and 14 dpn reveals the presence of numerous osteoclasts on alveolar bone (AB) coronal to the forming tooth in both WT and *MT1-MMP*$^{-/-}$ mice, corresponding to the open eruption pathway observed by micro-CT and histology. (E, F) Immunostaining indicates widespread TNAP in the WT periodontium at 14 dpn, and selectively localized TNAP to alveolar bone surfaces at 26 dpn. (G, H) In *MT1-MMP*$^{-/-}$ mice, TNAP localization in the PDL space is disrupted in large fibrotic areas at the bone surface (yellow star), and by 26 dpn, TNAP localization adjacent to bone is severely decreased compared to WT controls. (I, J) Matrix protein OPN localizes to reversal lines in bone, indicating cycles of remodeling in WT alveolar bone. (K, L) In *MT1-MMP*$^{-/-}$ mice, OPN immunostaining reveals a conspicuous lack of remodeling and little osteoblastic activity, resulting in an adynamic appearance to alveolar bone.

and no TNAP was detected (Fig. 6E–H). The matrix protein OPN localizes to reversal lines in bone [27] and immunohistochemistry clearly revealed cycles of apposition in WT alveolar bone (Fig. 6I, J). However, in *MT1-MMP*$^{-/-}$ mice, OPN immunostaining demonstrated a conspicuous lack of apposition and an adynamic appearance of alveolar bone (Fig. 6K, L).

Conditional ablation of MT1-MMP in dental mesenchyme but not epithelium affects root and alveolar bone formation

In light of diminished molar root growth and lack of eruption, and the observed aberrations of HERS structure, dentinogenesis, periodontal, and bone formation in *MT1-MMP*$^{-/-}$ mice, we next aimed to

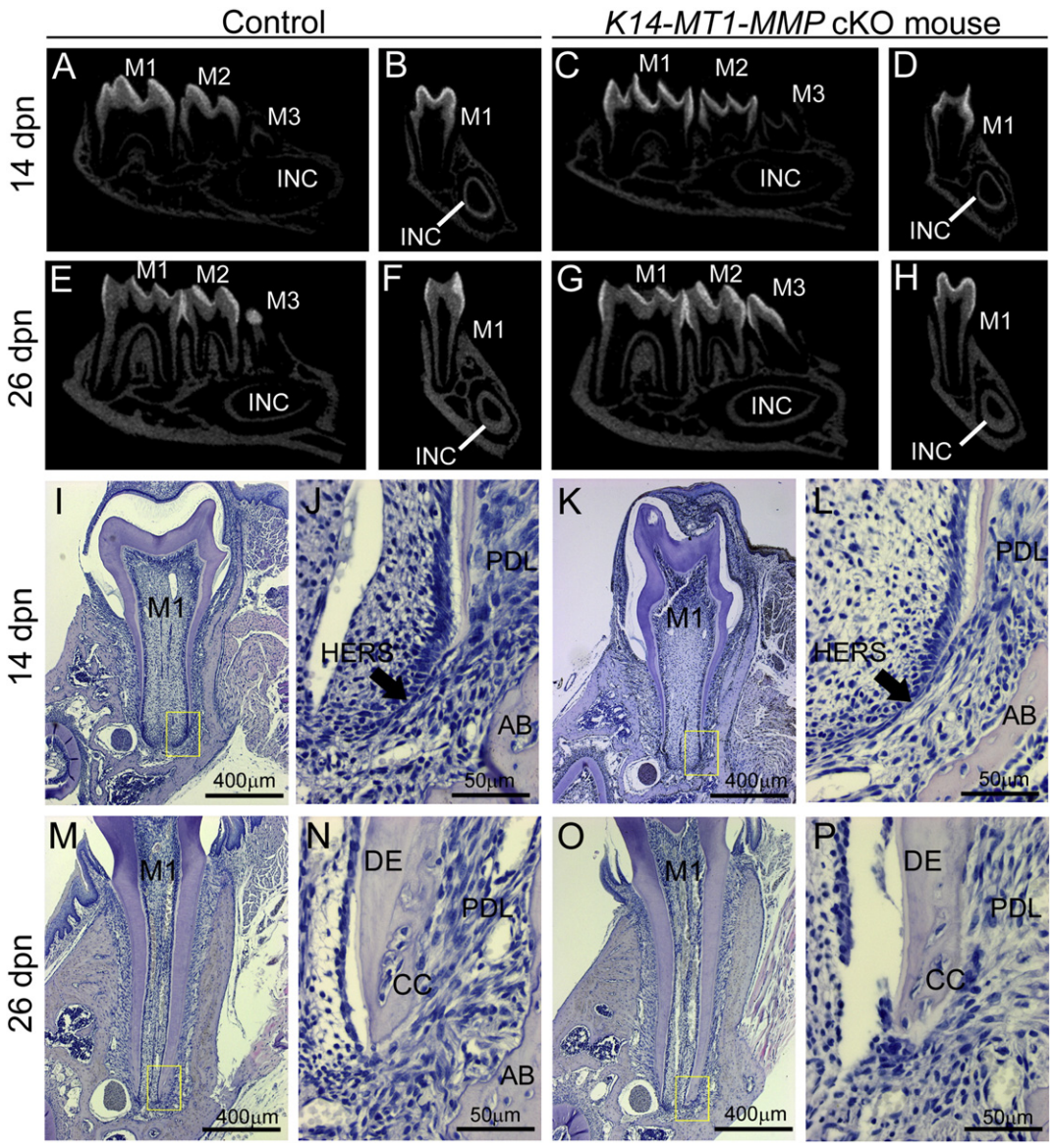


Fig. 7. Conditional knockout of *MT1-MMP* in the dental epithelium does not affect root formation or eruption. Tooth root development and eruption in *K14-MT1-MMP* cKO mice were analyzed by micro-CT and histology at 14 and 26 dpn. Yellow boxes in I, K, M, and O are shown at higher magnification in J, L, N, and P, respectively. (A–H) Micro-CT reveals comparable size and mineralization in WT and *K14-MT1-MMP* cKO mandibles and teeth at 14 and 26 dpn. (I–P) H&E staining of the first mandibular molar of WT and *K14-MT1-MMP* cKO mice shows comparable tooth morphology at 14 and 26 dpn stage, including structure of Hertwig's epithelial root sheath (HERS), root length, dentin (DE) structure and thickness, periodontal ligament (PDL) organization, alveolar bone (AB), and presence of cellular cementum (CC).

determine which tissue compartment(s) required MT1-MMP enzyme activity. To this end, we generated two types of *MT1-MMP* conditional knockout mice. The keratin 14 (*K14*)-*Cre* line has been used to selectively delete floxed alleles from the oral epithelium and its derived tissues, including HERS [19,28,29]. Micro-CT and histology revealed that *K14-Cre*$^{+}$; *MT1-MMP*$^{flox/flox}$ (*K14-MT1-MMP* cKO) mice displayed normal HERS structure, root development and size, and tooth eruption in molars (Fig. 7).

The Osterix (*Osx*)-*Cre* line has been used to selectively delete floxed alleles in committed osteoblasts and odontoblasts [30,31], though the spatiotemporal expression of *Osx* in the wider periodontium has not been completely resolved [32–34]. While the *Osx-Cre* transgene has been reported to cause delayed or defective skeletal and craniofacial mineralization resulting from Osterix loss-of-function [35–37], studies including analysis of molar teeth have not identified similar dental

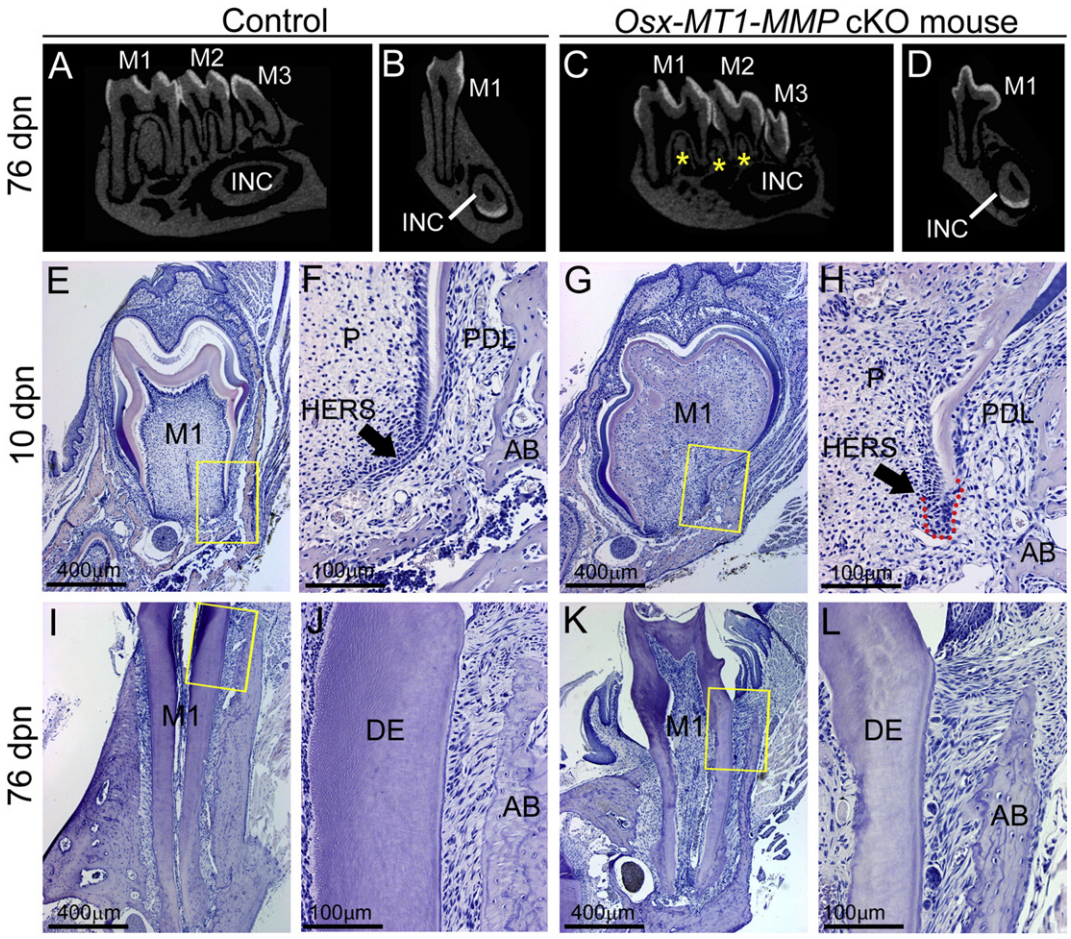


Fig. 8. Conditional knockout of *MT1-MMP* in the dental mesenchyme affects root and alveolar bone formation. Tooth root development and eruption in *Osx-MT1-MMP* cKO mice were analyzed by micro-CT and histology at 10 and 76 dpn. Yellow boxes in E, G, I, and K are shown at higher magnification in F, H, J, and L, respectively. (A–D) Micro-CT reveals lack of alveolar bone formation (yellow stars) and shorter molar roots in the *Osx-MT1-MMP* cKO mice, compared to controls. (E–H) At 10 dpn, *Osx-MT1-MMP* cKO mice display severely deficient crown and root dentin, including abnormal crown shape, defective circumpulpal dentin production, thin dentin, dysregulated dentin–pulp border, disrupted odontoblast layer, and numerous cells embedded in the osteodentin-like matrix in the pulp space (P). Compared to the elongated bilayer of HERS in WT, the HERS structure in *Osx-MT1-MMP* cKO mice is short, blunted, and surrounded by an accumulated mass of cells (red dotted line). (I–L) At 76 dpn, *Osx-MT1-MMP* cKO mice display reduced mandible alveolar bone (AB) formation, short roots, and thin and aberrant dentin (DE). However, molar teeth in *Osx-MT1-MMP* cKO are noted to erupt into the oral cavity.

defects [30,38,39]. To rule out dental alterations from the *Osx-Cre* transgene, several control genotypes were analyzed (Supplementary Fig. 6). *Osx-Cre*$^{+}$; *MT1-MMP*$^{flox/flox}$ (*Osx-MT1-MMP* cKO) mice displayed nearly all of the phenotypic characteristics of the *MT1-MMP*$^{-/-}$, including short molar roots and reduced alveolar bone (Fig. 8A–L). Notably, in *Osx-MT1-MMP* cKO, the HERS structure was defective and surrounded by a mass of accumulated cells strongly resembling the phenotype of *MT1-MMP*$^{-/-}$ mice (Fig. 8F, H). When considered along with the lack of HERS phenotype in *K14-MT1-MMP* cKO mice, these data strongly implicate the mesenchymal component in dentin and root formation defects observed in the absence of MT1-MMP. Additionally, *Osx-MT1-MMP* cKO featured overt defects in crown and root dentin, including abnormal coronal morphology, defective circumpulpal dentin production, thin dentin, disorganized dentin–pulp border, disrupted odontoblast layer, and numerous cells embedded in the osteodentin-like matrix (Fig. 8E–L). Despite crown and root defects and alveolar bone alterations, molar teeth in *Osx-MT1-MMP* cKO erupted into the oral cavity.

Discussion

MT1-MMP is essential during development in both humans and mice for dynamic remodeling of connective tissues, which in turn display profound defects in *MT1-MMP*-deficiency [3,6,40]. We document here that MT1-MMP is widely expressed in the tooth and surrounding connective tissues during development and postnatal growth. Consistent with this expression, we demonstrate that loss of MT1-MMP in mice impairs tooth root formation and eruption in association with multiple defects in dentoalveolar tissues. Defective root formation is associated with aberrant structure and function of Hertwig's epithelial root sheath (HERS) [19,41], and is further disrupted by lack of alveolar bone apposition/remodeling, or periodontal ligament (PDL) formation and integration into the alveolar bone [11]. For the first time, we have identified a significant defect in dentin formation and mineralization caused by the loss of MT1-MMP. Conditional ablation of MT1-MMP from the dental epithelium did not recapitulate root or eruption defects seen in *MT1-MMP*$^{-/-}$ mice, while selective ablation of MT1-MMP from the mesenchyme did recapitulate root and bone development, and dentinogenesis defects, indicating important functional roles for MT1-MMP activity in the dental mesenchyme for proper tooth root formation.

Defective root formation resulting from the loss of MT1-MMP activity

Previous work has demonstrated the general importance of MT1-MMP in tooth root growth and tooth eruption in mice [13], however, the extent of pathological changes and cellular involvement remained unclear to date. Here we systematically analyzed tissue compartments contributing to root development and eruption in the absence of MT1-MMP. Additionally, we employed selective epithelial and mesenchymal ablation of MT1-MMP in order to segregate the physiological significance of epithelial expression from those of the adjacent mesenchymal compartment in the etiopathology of dentoalveolar defects caused by the absence of MT1-MMP.

HERS is a bilayer structure derived from the epithelial enamel organ, and its directed growth defines the size and shape of the tooth root [7,14]. In *MT1-MMP*$^{-/-}$ mice, HERS displayed a progressive alteration in shape and angle suggestive of a functional defect related to the lack of root elongation, in association with an accumulated mass of mesenchymal cells surrounding the defective HERS. Conditional *K14-Cre*-mediated ablation of *MT1-MMP* in the epithelium, however, did not recapitulate the altered HERS structure and root formation. Normal HERS structure and root formation in *K14-MT1-MMP* cKO mice suggested that the underlying defect is in the surrounding mesenchyme of the papilla and/or dental follicle, and reproduction of the HERS defect in *Osx-MT1-MMP* cKO mice confirmed this observation. Based on these data, we propose that lack of remodeling and associated cellular defects due to collagen accumulation in these mesenchymal compartments physically impairs proper growth and function of HERS. This hypothesis is supported by previous findings that *MT1-MMP*$^{-/-}$ PDL fibroblasts accumulate massive amounts of collagen, which is routed into phagolysosomes to compensate for the lack of MT1-MMP-mediated pericellular matrix degradation [13].

During root growth, HERS cells proliferate, and a portion undergo apoptosis, while the inner enamel epithelium layer (IEE) of HERS induces adjacent dental papilla cells to differentiate into odontoblasts. We analyzed these functions of HERS in *MT1-MMP*$^{-/-}$ mice and identified no alterations in proliferation or apoptosis either in HERS, or in surrounding cells. Epithelial-mesenchymal signaling between HERS and papilla is further involved with root formation, and two critical signaling events are canonical Wnt activation [18] and odontoblast expression of the transcription factor, NFIC, as a result of epithelial SMAD4-induced sonic hedgehog (SHH) signaling [19,42,43]. Immunostaining for β-catenin, an indicator of Wnt activation, did not document altered Wnt activity in roots of *MT1-MMP*$^{-/-}$ mice. However, reduced NFIC localization in the nuclei of mesenchymal cells surrounding HERS suggests a signaling defect that may be related to the root growth defect. While MT1-MMP has been demonstrated to process several types of signaling molecules, including those affecting Wnt and Notch signaling pathways [5], it is presently unclear whether there is a direct effect of MT1-MMP proteolytic activity on processing of signaling molecules in the dental mesenchyme, or whether the accumulated collagen surrounding HERS negatively affects secreted signals between cells.

MT1-MMP mRNA is abundant in odontoblasts, and loss of MT1-MMP activity caused defects in both crown-associated and root dentin. The crown dentin of *MT1-MMP*$^{-/-}$ mice featured regions of dystrophic matrix secretion associated with disrupted odontoblast organization and embedding of cells in ECM. Moreover, thin dentin, interglobular mineralization patterns, ectopic matrix accumulation and mineralization, abnormal induction of COL XII expression, and decreased collagen organization, all suggested an important function for MT1-MMP in proper maintenance of the pulp-dentin border during dentinogenesis Consistent with this notion, older *MT1-MMP*$^{-/-}$ mice display overt fibrosis of the dental pulp. Molar roots of *MT1-MMP*$^{-/-}$ mice presented thinner dentin and wider predentin, although odontoblast differentiation and early function appeared

grossly normal, as indicated by histological analysis and expression of markers (TNAP and DSP). In contrast, the reduced NFIC induction, especially in root odontoblasts, would be expected to negatively impact odontoblast function, and as such could contribute to the shortened roots. Observations of severe defects in molar crown and root dentin in *Osx-MT1-MMP* cKO mice support an important function for odontoblast-expressed MT1-MMP in dentinogenesis. The discrepancy in severity of defects in the cKO versus the systemic knockout mouse however raises questions about how *Osx*-negative cells affect dentin synthesis and pulp homeostasis.

Failure of tooth eruption in *MT1-MMP*$^{-/-}$ mice

Coincident with root formation, teeth erupt from their bony crypts into their functional (occlusal) positions within the oral cavity. Failure of eruption in mice and humans can result from dysfunction in either coronal bone resorption or apical bone formation [11,26,44–59]. Micro-CT imaging and TRAP staining of histological sections from *MT1-MMP*$^{-/-}$ mice indicated no defect in osteoclast activation or function that would explain failure of eruption, pointing towards other causes. Formation of bone was severely affected by loss of MT1-MMP, showing persistent disorganization and woven appearance throughout the mandible, strikingly reduced alveolar bone formation, and an adynamic appearance and lack of alveolar bone apposition adjacent to the tooth root. Pockets of fibrotic cells, excessive ECM and aberrant osteoblasts were further identified at the alveolar bone surface. Together these data point towards a major diminution in bone formation and bone organization as being a significant contributor to lack of molar eruption. Conditionally ablating MT1-MMP in osteoblasts in *Osx-MT1-MMP* cKO mice also affected bone formation and remodeling, but to a lesser extent than complete gene-knock-out. Greater alveolar bone formation was evident and molar tooth eruption occurred in *Osx-MT1-MMP* cKO compared to *MT1-MMP*$^{-/-}$ mice, suggesting that non-*Osx*-expressing cells (e.g., pulp and PDL cells) significantly affect the root formation and tooth eruption.

The negative effects of loss of MT1-MMP on bone formation and mineralization are likely manifold. While an osteopenic skeletal phenotype was apparent in the original description of *MT1-MMP*$^{-/-}$ mice [6], subsequent work has identified regulatory roles for MT1-MMP in osteoblast differentiation, osteocyte function, and osteogenesis-related signaling pathways [5,60–65]. A more direct effect on mineralization may result from enzymatic activity of MT1-MMP on ECM-modifying factors such as transglutaminase 2 (TG2), present in bone, teeth, and the PDL [66,67]. Cleavage of TG2 by MT1-MMP was shown to alter its cross-linking and ATPase activity in osteoblasts, and inhibition of MT1-MMP decreased osteoblast mineralization, in vitro [68], though the function of TG2 in skeletal mineralization remains unclear [69].

Considering the reduced bone formation and excess matrix accumulation in MT1-MMP-deficient mice, we may ask whether defective collagen metabolism in the PDL is responsible for the lack of tooth eruption. A functional periodontium depends on stable insertion of Sharpey's fibers into the acellular cementum and alveolar bone, organization of PDL collagen fibrils to accommodate and disperse forces arising from occlusion, as well as ability of the PDL–bone complex to remodel in response to changing physiological cues and functional demands [70]. Nearly all of these attributes of a functional periodontium are defective in *MT1-MMP*$^{-/-}$ mice, which exhibit lack of directed PDL collagen fibrils, reduced Sharpey's insertion into bone, and adynamic bone. The PDL is comprised of a complex and dynamic ECM featuring predominantly collagenous fibers, especially types I and III, with lesser amounts of types IV, V, VI, and XII [9]. Collagens I and III are known substrates for MT1-MMP [5], therefore it is perhaps not surprising that this tissue is severely affected by deletion of MT1-MMP activity, which is essential for collagen cleavage, and in turn, in the rapid remodeling of the PDL. This concept is supported by previous observations of age-dependent accumulation of phagocytosed collagen fibrils in PDL fibroblasts in *MT1-MMP*$^{-/-}$ mice [13]. In contrast to the defective PDL–bone interface in *MT1-MMP*$^{-/-}$ mice, tooth root cementum formed and had normal fringe collagen fiber organization and insertion of Sharpey's fibers. This contrasting outcome likely results from lack of remodeling of the cementum under normal circumstances, compared to active remodeling required for the PDL–bone interface, especially during tooth movement such as eruption [9,11].

Despite this dramatic failure of PDL development caused by lack of MT1-MMP, current prevailing theories do not hold that PDL plays a significant role in tooth eruption. This is based in part on experiments demonstrating eruption of rootless teeth and replacement of tooth germs with inanimate objects [11,71]. However, changes occurring in the newly organizing PDL may contribute to tooth eruption. For example, large-scale migration of progenitor cells from the coronal to apical follicle region [72] may contribute to the motive force of tooth eruption by their apical dislocation, as well as by contributing to osteoblast populations synthesizing newly forming alveolar bone. Migratory capabilities of follicle cells would be expected to be disturbed in the absence of MT1-MMP, as collagen content increases dramatically in the follicle during root formation and tooth eruption [73]. MMPs have been shown to aid cell migration in several contexts, and MMP activity is

associated with cell protrusions involved in directing cell motility, with MMPase activity especially essential in densely cross-linked three dimensional ECM [74–77]. Findings in the *Osx-MT1-MMP* cKO mice support this concept because deletion of MT1-MMP in committed osteoblasts was not sufficient to inhibit tooth eruption, allowing for an important contribution to tooth eruption of non-*Osx* expressing cells in the periodontium, e.g. subsets of cells in the PDL.

MT1-MMP, vanishing bone diseases, and failure of tooth eruption

A missense mutation in *MT1-MMP* in humans is associated with the rare condition, Winchester syndrome, one of a group of "vanishing bone" syndromes featuring marked osteolysis and joint destruction [40]. Case reports on Winchester syndrome have reported delayed or lack of tooth eruption [78–80], consistent with our findings in *MT1-MMP*$^{-/-}$ mice. However, further dental manifestations, such as effects on tooth structures, have not been reported. To date, dental effects have not been reported in closely related vanishing bone diseases, such as multicentric osteolysis with nodulosis and arthropathy (MONA), associated with mutations in MMP-2 [81].

Ultimately, most causes for primary failure of tooth eruption in humans remain unidentified and poorly understood [82,83]. These studies on tooth formation and eruption in the absence of MT1-MMP point to a role for collagen metabolism in tooth eruption, possibly through effects on bone formation, as well as remodeling and organization of the follicle/PDL region. Further studies will elucidate functions of MT1-MMP and other regulators of ECM remodeling on tooth formation and eruption, and enhance diagnosis and interventions into cases of failure of eruption in human patients.

Experimental procedures

Mice

Generation and genotyping of *MT1-MMP* deficient (*MT1-MMP*$^{-/-}$) mice have been described previously [6]. *MT1-MMP*$^{-/-}$ mice and control littermates were euthanized at 5, 14, and 26 days postnatal (dpn) and skulls and mandibles were collected. For tissue-specific ablation, a Cre-recombinase recognition target (LoxP)-mediated gene excision strategy was used to conditionally knock out *MT1-MMP*. Keratin 14 (*K14*)-*Cre* mice [84] were crossed with mice harboring a floxed *MT1-MMP* allele [85] to ablate MT1-MMP from the oral epithelium and its derived tissues. These *K14-Cre*$^{+}$; *MT1-MMP*$^{flox/flox}$ (*K14-MT1-MMP* cKO) mice were compared to control littermates (*MT1-MMP*$^{flox/flox}$ and *MT1-MMP*$^{flox/+}$) at 14 and 26 dpn (n = 3–5 samples each per age). Osterix (*Osx*)-*Cre* mice [86] were crossed with *MT1-MMP*$^{flox/flox}$ mice to ablate MT1-MMP from mesenchymal cells including osteoblasts and odontoblasts. These *Osx-Cre*$^{+}$; *MT1-MMP*$^{flox/flox}$ (*Osx-MT1-MMP* cKO) mice were compared to control littermates (including *Osx-Cre*$^{+}$; *MT1-MMP*$^{flox/+}$, *MT1-MMP*$^{flox/flox}$, and *MT1-MMP*$^{flox/+}$) at 10 and 76–79 dpn (n = 3 cKO samples per age and n = 1–3 of the various control genotypes per age, for a total of 9 controls).

Radiography and microcomputed tomography

Conventional radiography was performed using a Faxitron cabinet x-ray (Faxitron Bioptics, LLC, Tucson, AZ) Kodak PPL film was exposed at 30 kV for 40 s. For microcomputed tomography (microCT), mandibles were scanned at a 10 μm voxel resolution, 70 keV, 80 μA, 300 ms exposure time in a Scanco Medical μCT 50 (Scanco Medical AG, Brüttisellen, Switzerland). Z-stacks were exported as DICOM files and reoriented using ImageJ software (1.48r), with identical sectioning planes chosen for comparison. DICOM stacks were rendered as 3D isoimages using Amira software (version 5.6.0; FEI, Hillsboro, OR).

Histology and staining

Dissected mandibles were fixed with 4% formaldehyde in PBS, demineralized in 20% EDTA at 4 °C, processed for paraffin embedding, and serial sectioned at a thickness of 5 μm. Hematoxylin and eosin (H&E) and picrosirius red staining were performed as described previously [22].

Non-decalcified hemi-mandibles were processed and embedded in methyl methacrylate for von Kossa and Goldner's trichrome staining, as described previously [70]. Tartrate resistant acid phosphatase (TRAP) staining (Wako Chemicals, Japan) was used to identify osteoclast-like cells [70], while TdT-mediated dUTP--biotin nick end-labeling (TUNEL) assay (Promega, Madison, WI, USA) was used to visualize apoptotic cells.

In situ hybridization and immunohistochemistry

In situ hybridization (ISH) for *MT1-MMP* mRNA was performed as described previously [87]. Immunohistochemistry (IHC) was performed on paraffin sections using an avidin-biotinylated peroxidase enzyme complex (ABC) based kit (Vector Labs, Burlingame, CA) with 3-amino-9-ethylcarbazole (AEC) or 3,3′-diaminobenzidine (DAB) chromogenic substrates (Vector Labs), as described in more detail previously [22]. Primary antibodies included: Rabbit polyclonal anti-cytokeratin 14 (Covance, Berkeley, CA, USA), rabbit polyclonal anti-Proliferating Cell Nuclear Antigen (PCNA) (Santa Cruz, CA, USA), rabbit polyclonal

anti-β-catenin (H-102, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-nuclear factor IC (NFIC; a gift from Dr. J.C. Park, Seoul National University, Korea) [88], rabbit polyclonal anti-dentin sialoprotein (DSP) LF-153 (a gift from Dr. Larry Fisher, NIDCR/NIH, Bethesda, MD, USA) [89], rat anti-tissue nonspecific alkaline phosphatase (TNAP, R&D Systems, Minneapolis, MN, USA) [90], rabbit polyclonal anti-collagen type 1a1 (COL1A1) LF-68 (a gift from Dr. Larry Fisher) [91], rabbit polyclonal anti-collagen type 12 (COLXII) KR-33 (a gift from Dr. Manual Koch, University of Cologne, Germany) [92], rabbit polyclonal anti-bone sialoprotein (BSP; a gift from Dr. Renny Franceschi, University of Michigan, Ann Arbor, MI, USA) [70], polyclonal rabbit anti-osteopontin (OPN) LF-175 (a gift from Dr. Larry Fisher, NIDCR/NIH) [70], and rabbit polyclonal anti-periostin (POSTN; Abcam, Cambridge, MA).

TNAP tissue nonspecific alkaline phosphatase

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Author contributions

BLF and KH designed the project, BLF, HX, TNS, SSY, and TY performed experiments. BLF, KH, HX, and TNS analyzed the data. BLF, HX, and TNS wrote the manuscript. All authors read, contributed to discussion, and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Abbreviations used:
BSP, bone sialoprotein; COL1A1, collagen type Ia1; COL XII, collagen type 12; DSP, dentinsialoprotein; ECM, extracellular matrix; HERS, Hertwig's epithelial root sheath; K14, cytokeratin14; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 matrix metalloproteinase; NFIC, nuclear factor 1C; OPN, osteopontin; OSX, osterix; PCNA, proliferating cell nuclear antigen.PDL, periodontal ligament.POSTN, periostin.TNAP, tissue nonspecific alkaline phosphatase.

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