MMP20 Overexpression Disrupts Molar Ameloblast Polarity and Migration

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Abstract

Ameloblasts responsible for enamel formation express matrix metalloproteinase 20 (MMP20), an enzyme that cleaves enamel matrix proteins, including amelogenin (AMELX) and ameloblastin (AMBN). Previously, we showed that continuously erupting incisors from transgenic mice overexpressing active MMP20 had a massive cell infiltrate present within their enamel space, leading to enamel mineralization defects. However, effects of MMP20 overexpression on mouse molars were not analyzed, although these teeth more accurately represent human odontogenesis. Therefore, MMP20-overexpressing mice (*Mmp20*^{+/+}Tg⁺) were assessed by multiscale analyses, combining several approaches from high-resolution micro-computed tomography to enamel organ immunoblots. During the secretory stage at postnatal day 6 (P6), *Mmp20*^{+/+}Tg⁺ mice had a discontinuous ameloblast layer and, unlike incisors, molar P12 maturation stage ameloblasts abnormally migrated away from the enamel layer into the stratum intermedium/stellate reticulum. TOPflash assays performed in vitro demonstrated that MMP20 expression promoted β -catenin nuclear localization and that MMP20 expression promoted invasion through Matrigel-coated filters. However, for both assays, significant differences were eliminated in the presence of the β -catenin inhibitor ICG-001. This suggests that MMP20 activity promotes cell migration via the Wnt pathway. In vivo, the unique molar migration of amelogenin-expressing ameloblasts was associated with abnormal deposition of ectopic calcified nodules surrounding the adherent enamel layer. Enamel content was assessed just prior to eruption at P15. Compared to wild-type, *Mmp20*^{+/+}Tg⁺ molars exhibited significant reductions in enamel thickness (70%), volume (60%), and mineral density (40%), and MMP20 overexpression resulted in premature cleavage of AMBN, which likely contributed to the severe defects in enamel mineralization. In addition, *Mmp20*^{+/+}Tg⁺ mouse molar enamel organs had increased levels of inactive p-cofilin, a protein that regulates cell polarity. These data demonstrate that increased MMP20 activity in molars causes premature degradation of ameloblastin and inactivation of cofilin, which may contribute to pathological Wnt-mediated cell migration away from the enamel layer.

Keywords: enamel, cell polarity, cell migration, ameloblastin, cofilin, β -catenin

Introduction

Dental enamel development proceeds in stages. During the presecretory stage, preameloblasts are cuboidal cells that elongate, become polarized, and extend Tomes' processes as they enter the secretory stage. This is when ameloblasts secrete large amounts of protein into the forming enamel as long thin enamel ribbons initiate at the dentin surface and start extending outward to form the full thickness of the enamel layer. Each ameloblast is responsible for approximately 10,000 ribbons that will eventually coalesce into a single enamel rod. The mineralized ribbons and eventual rods are not straight and, in rodent incisors, will crisscross (decussate). Because the rod is the mineralized trail of the ameloblast that formed it, the ameloblasts must move in different directions relative to one another to achieve the decussating enamel rod pattern. Once enamel full thickness is achieved, ameloblasts retract their Tomes' processes and transition to the maturation stage. This is when they shorten and start removing enamel matrix proteins as the mineralized ribbons grow in width and thickness and coalesce into rods to form the fully mineralized enamel layer (reviewed in Bartlett 2013).

Matrix metalloproteinase 20 (MMP20; enamelysin) is expressed by ameloblasts during the secretory stage through the early to mid-maturation stage. Inactivating *MMP20* mutations cause severe enamel malformation, termed *amelogenesis imperfecta*. Therefore, MMP20 is essential for proper enamel development. Twelve different human *MMP20* mutations are currently known to cause autosomal recessive amelogenesis imperfecta (Kim et al. 2005; Ozdemir et al. 2005; Papagerakis et al. 2008; Lee et al. 2010; Wright et al. 2011; Gasse et al. 2013; Wang et al. 2013; Seymen et al. 2015; Gasse et al. 2017; Kim et al. 2017). MMP20 cleaves enamel matrix proteins

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during early enamel development (Ryu et al. 1999; Nagano et al. 2009), and recent literature suggests that MMP20 may also facilitate ameloblast movement by cleaving cadherin extracellular domains (Bartlett and Smith 2013; Guan and Bartlett 2013). Cadherins are part of the adherens junction (AJ) complex responsible for ameloblast cell-cell attachment, and these junctions must be cleaved and/or removed if ameloblasts are to move in different directions relative to one another.

We showed previously that *Mmp20*-ablated mice have ameloblasts that abnormally form and retract their Tomes' processes repeatedly (Bartlett, Yamakoshi, et al. 2011). MMP20 appeared to have signaling properties that define when ameloblasts enter the maturation stage. Therefore, we asked how potential increased cell signaling would affect ameloblasts if MMP20 was overexpressed. Even so, the resulting massive migration of cells into the incisor enamel space was surprising because this invasion nearly replaced the enamel layer. We attributed this result to increased cadherin cleavage with subsequent release of β -catenin from the intracellular portions of the cleaved cadherins (Shin et al. 2016). However, rodent incisors continuously erupt, whereas mouse molars and adult human dentition erupt just once. We therefore examined mouse molars to determine if MMP20 overexpression had a similar effect on enamel from molars compared to previous results observed in mouse incisors.

Materials and Methods

Mice

All animals used in this study were housed in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facilities and were treated humanely based on protocols approved by the Forsyth Institute Institutional Animal Care and Use Committee (IACUC) and The Ohio State University IACUC. Experimental protocols were designed along university and National Institutes of Health (NIH) guidelines for the humane use of animals.

The MMP20 catalytic domain was deleted from both alleles of the *Mmp20*-ablated (*Mmp20*^{-/-}) mice (Caterina et al. 2002). Mice with a transgene expressing high levels of MMP20 were previously identified as Tg24⁺ *Mmp20*^{+/-} (Shin et al. 2014). In this article, we simplify the nomenclature to *Mmp20*^{+/-}Tg⁺.

Histology and In Situ Hybridization

Mandibles harvested from mice at postnatal day 6 or 12 (P6 and P12, respectively) were formalin-fixed, decalcified in a solution of 20% sodium citrate/4% formic acid, paraffin embedded, and microtome sectioned. Sections were deparaffinized in xylene and stained by hematoxylin and eosin (H&E). In situ hybridization with antisense amelogenin (*Amelx*) probe was performed on deparaffinized histological sections and visualized with fast red dye using hematoxylin as a counter-stain (Zweifler et al. 2016).

Micro-Computed Tomography (μ CT)

Mandibles from P15 mice were formalin-fixed and scanned in a μ CT 50 (Scanco Medical) at 70 kVp, 76 μ A, 0.5 Al Filter,

900-ms integration time, and 6- μ m voxel size. Mandibles from P45 mice were fixed and scanned in a μ CT 40 (Scanco) at 70 kVp, 76 μ A, 0.5 Al Filter, 300-ms integration time, and 10- μ m voxel size. DICOM images were uploaded to AnalyzePro 1.0 (AnalyzeDirect) and calibrated to a standard curve of 5 hydroxyapatite (HA) standards of known densities (mg HA/cm³) for quantitative analysis. Assessed incisor enamel included only the most mesial/incisal unerupted 300 μ m. Enamel was segmented at 1,550 mg/cm³ HA in wild-type (WT) and manually traced at the point of delamination in *Mmp20*^{+/-}Tg⁺ mice. Molar enamel thickness was calculated using cortical bone algorithms for the most median 25 axial slices (150 μ m), as measured from the cementum-enamel junction to highest cusp tip. Incisor enamel thickness was calculated from 10 linear measurements made perpendicularly across the midpoint of the enamel layer in the frontal plane. In *Mmp20*^{+/-}Tg⁺ mice, enamel volume and density measurements included ectopic enamel deposits. However, only adherent enamel was measured to determine enamel thickness.

TOPflash Assays

The ameloblast-lineage cells (ALCs) used here were stably transfected to express MMP20, but the presence of doxycycline (Dox) suppresses this expression (Shin et al. 2016). These ALCs were transfected with 800 ng/well TOPflash plasmid, a gift from Randall Moon (Veeman et al. 2003) (Addgene), and with 40 ng/well of *Renilla* luciferase (Promega) in a 24-well plate. TOPflash contains 7 T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) β -catenin binding sites. Once bound, β -catenin drives expression of the TOPflash firefly luciferase gene, which is quantified in a plate reader relative to the uninducible *Renilla*-expressing plasmid. Cells were incubated for 6 h, after which transfection medium was replaced with Dulbecco's modified Eagle's medium (DMEM) and incubated overnight. ALCs were treated for 48 h with or without 1 μ M ICG-001 and/or broad-spectrum protease inhibitor (except MMPs) or with 10 μ M of the MMP inhibitor GM6001 and then lysed. Lysates were incubated with firefly luciferase assay reagent and then with Stop & Glo substrate for the *Renilla* luciferase assay. Results are presented as firefly/*Renilla* activity ratio.

Transwell Invasion Assay

Invasion assays were performed as previously described for ALCs that do not inducibly express MMP20 (Guan et al. 2016). Briefly, cells were added onto filter well inserts with a top layer of Matrigel (8- μ m pore size; Corning). Cells were cultured with broad-spectrum protease inhibitors diluted 1:3,500 that excluded MMP inhibitors (Thermo Fisher Scientific) and were treated with or without 1 μ g/mL Dox for 48 h. Cells on the filter top were wiped off, and the filter was fixed and stained. The total number of cells that migrated to the opposite side of the filter was determined by averaging the numbers of cells counted in 5 random microscope fields (magnification $\times$ 200). Experiments were independently repeated 3 times.

Figure 1. Matrix metalloproteinase 20 (MMP20)-overexpressing mouse molars feature dysplastic ameloblasts. (A, E) Relative to wild-type (WT) molars at postnatal day 6 (P6), (B, F) *Mmp20*^{+/+}Tg⁺ mice have abrupt ameloblast depolarization at the cervical margin of their molars. (C, G) While WT mice had a smooth and continuous layer of ameloblasts near their P12 molar cusp tips, (D, H) *Mmp20*^{+/+}Tg⁺ mice showed a discontinuous and disorganized ameloblast cell layer. (H) White arrows on the left side of the panel point to apparent mineralized nodules present within the enamel organ near the enamel layer. (A–D) Boxes inside panels are (E–H) magnified in panels beneath. Sections were examined from 3 P6 mice and 3 P12 mice per genotype. Am, ameloblasts; De, dentin; En, enamel space; Od, odontoblasts.

Immunoblots

First molars were removed from P6 mouse pups. Mineral was dissolved by submerging in 2 mL of 0.17 M HCl/0.98% formic acid for 2 to 3 h at 4°C and centrifuged at 3,500 g for 5 min at 4°C. The supernatants were then dialyzed against water overnight and lyophilized. The lyophilized proteins were weighed and eluted into sample buffer. Protein (25 μg) was loaded in each lane, run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a membrane. Immunoblots were performed with antiserum specific for ameloblastin (R&D Systems) diluted 1:2,500 in Tris-buffered Saline with Tween 20 or with antiserum for cofilin or p-cofilin (Cell Signaling Technology), each diluted 1:3,000 in TBST.

Statistical Analyses

Results are expressed as mean ± standard deviation (SD). μCT data were analyzed using Student's *t* test. TOPflash and invasion assay results were assessed by 1-way analysis of variance (ANOVA) Scheffé's *F* test. Statistical analyses were computed using GraphPad Prism 6.00 (GraphPad Software).

Results

MMP20-Overexpressing Mouse Molars Feature Dysplastic Ameloblasts

To determine if MMP20-overexpressing mouse molars have a similar phenotype to incisors, we first performed H&E staining to examine molar morphology. First, mandibular molars from P6 WT mice had a well-defined ameloblast layer in the secretory stage of development, consisting of tall columnar cells with nuclei present near their basal (proximal) ends (Fig. 1A, E). In contrast, molars from P6 *Mmp20*^{+/+}Tg⁺ mice had an ameloblast layer that appeared to initially have tall columnar

cells near the newly formed enamel at the cervical margin, but these cells abruptly shortened as the enamel space enlarged (Fig. 1B, F). Interestingly, the cells forming the enamel between the cusp tips appeared dysplastic, and in places, it was difficult to identify the ameloblast layer (Fig. 1B).

Examination of first molars in the maturation stage of enamel formation at P12 yielded similar observations. WT molars had ameloblasts that were characteristically shortened relative to secretory stage ameloblasts, although the ameloblast layer remained well-defined (Fig. 1C, G). The layer of maturation stage ameloblasts in P12 *Mmp20*^{+/+}Tg⁺ mice was apparent at certain locations but unclear or absent in others, indicating dramatic disorganization. The dysplastic ameloblast morphology persisted between the cusp tips (Fig. 1D, H). However, unlike the secretory stage, apparent nodules of ectopic mineralized matrix were present within the cell layer(s) adjacent to the enamel space (Fig. 1H, white arrows).

Reduced and Ectopic Enamel Formation in MMP20-Overexpressing Mouse Molars

At P15, mouse first mandibular molars have not yet erupted into the oral cavity. However, enamel formation is nearly complete (Lungova et al. 2011). μCT analyses on P15 first molars showed that, compared to the organized, smooth, and highly mineralized enamel layer in WT mouse first molars, *Mmp20*^{+/+}Tg⁺ mouse molars had a patchy, exceptionally thin, and poorly-defined enamel layer that appeared to be delaminating from dentin in some regions (Fig. 2A–D). This thin enamel layer was surrounded by a disorganized zone of mineralized nodules consistent with the observed forming nodules in unerupted molars (Fig. 1H).

Only attached enamel was included in thickness measurements, while both attached and ectopic enamel mineral were included in volume and mineral density analyses. Compared to

WT controls, *Mmp20*^{+/+}Tg⁺ mouse molars at P15 exhibited 70% reduced enamel thickness ($P < 0.0001$), 60% reduced enamel volume ($P = 0.0001$) (Fig. 2F, G), and 40% reduced enamel mineral density ($P < 0.0001$) (Fig. 2E). In patterns remarkably similar to molars, mesial/incisal portions of *Mmp20*^{+/+}Tg⁺ mouse incisors had 70% decreased enamel thickness ($P < 0.0001$), 40% reduced enamel volume ($P = 0.004$), and 40% reduced enamel mineral density ($P = 0.0003$) (Fig. 2H–J). At P45, after first molars had erupted and were in occlusion for about 3 wk, *Mmp20*^{+/+}Tg⁺ mouse molars featured virtually no detectable enamel compared to the intact enamel layer in WT mouse molars (Appendix Fig. 1A–D).

Enhanced Wnt Signaling May Promote Dysplastic Ameloblast Migration in MMP20-Overexpressing Mouse Molars

Amelx in situ hybridization identified ameloblasts within the secretory stage enamel. P6 first molars from WT mice showed strong *Amelx* expression in the ameloblast layer but not in any other region of the enamel organ (Fig. 3A, B). Molars from *Mmp20*^{+/+}Tg⁺ mice also expressed *Amelx* in the ameloblast layer. However, at this stage, the *Amelx*-positive ameloblast layer was already uneven, disruptions and gaps were observed, and many *Amelx*-positive ameloblasts had apparently migrated into the adjoining stratum intermedium or perhaps even beyond into the stellate reticulum (Fig. 3C, D). This dysplasia was prominent between the cusp tips.

To identify a mechanism for this pathology, we asked if β -catenin contributed to MMP20-mediated cell migration. ALCs that inducibly express MMP20 when Dox is not present (Shin et al. 2016) were transiently transfected with TOPflash plasmid containing 7 β -catenin-responsive TCF/LEF binding sites linked to the firefly luciferase reporter gene. In the absence of Dox, cells were treated or not with a broad-spectrum protease inhibitor that does not inhibit MMPs (PI), the β -catenin inhibitor ICG-001, and/or a general MMP inhibitor (GM6001). Results suggest that the broad-spectrum protease inhibitor was necessary for MMP20-mediated Wnt

Figure 2. Reduced and ectopic enamel formation in matrix metalloproteinase 20 (MMP20)-overexpressing mouse molars. By micro-computed tomography (μ CT) analysis, (A, B) unerupted wild-type (WT) molars at P15 show normal enamel thickness, volume, and mineralization while (C, D) *Mmp20*^{+/+}Tg⁺ mouse molars have a very thin, patchy, and poorly defined enamel layer that appears to be delaminating (white arrows) in some regions. This thin enamel layer in *Mmp20*^{+/+}Tg⁺ mice is surrounded by a disorganized zone of mineralized nodules (white *). The observation of thin and delaminating enamel surrounded by disorganized ectopic nodules was consistent between molars and incisors, although the molars had many more mineralized nodules. Scale bar: 500 μ m. (E, G) Compared to WT controls, *Mmp20*^{+/+}Tg⁺ mouse molars exhibit 70% reduced enamel thickness (**** $P < 0.0001$), 60% reduced enamel volume (*** $P = 0.0001$), and 40% reduced enamel mineral density (**** $P < 0.0001$). (H, J) Compared to WT incisors, *Mmp20*^{+/+}Tg⁺ mouse incisors show 70% decreased enamel thickness (*** $P < 0.0001$), 40% reduced enamel volume (** $P = 0.004$), and 40% reduced enamel mineral density (*** $P = 0.0003$). Circular data points are from WT teeth and square data points are from MMP20-overexpressing teeth. Small hash marks indicate the standard deviation and large hash marks indicate the mean. Teeth were analyzed from 3 mice per genotype.

signaling, indicating other unidentified secreted proteinase(s) degrade MMP20; that the β -catenin inhibitor eliminated MMP20-mediated Wnt signaling; that inhibition of MMP activity by GM6001 inhibited Wnt signaling; and, importantly, that inhibition of MMP activity in the absence of the broad-spectrum protease inhibitor did not induce Wnt signaling (Fig. 3E), indicating that the other secreted proteinases play no role in the β -catenin-mediated Wnt pathway initiation.

Figure 3. Dysplastic ameloblast migration in matrix metalloproteinase 20 (MMP20)-overexpressing P6 mouse molars. In situ hybridization of molar sections from 2 wild-type (WT) overexpressing mice and 1 MMP20-overexpressing mouse (**A, B**) reveals that within enamel organs, only the organized and continuous ameloblast layer expresses amelogenin (*Amelx*) transcripts. In contrast, (**C, D**) in *Mmp20*^{+/+}Tg⁺ mouse molar enamel organs, the ameloblast layer is discontinuous, and it appears that *Amelx*-expressing ameloblasts have migrated into the stratum intermedium and perhaps the stellate reticulum layers of the enamel organ. Am, ameloblasts; De, dentin; En, enamel space. (**E**) TOPflash assay demonstrating that Wnt signaling through β -catenin is enhanced in ALC cells when MMP20 is overexpressed. Note that when either MMP20 (GM6001) or β -catenin (ICG-001) is inhibited, a significant reduction in luciferase activity occurs. Importantly, when

Next, we performed transwell invasion assays with MMP20-inducible ALCs. These cells migrated through a Matrigel-coated membrane. MMP20 expression was induced (no Dox, Dox -) or suppressed (with Dox, Dox +) with or without the presence of the β -catenin inhibitor ICG-001. Induction of MMP20 expression facilitated ALC invasion through the membrane, and inhibition of β -catenin eliminated this enhanced invasion (Fig. 3F). Thus, MMP20-mediated migration may depend on β -catenin and the Wnt pathway.

Altered Ameloblastin and Cofilin Levels in Secretory Stage Enamel Organ from MMP20-Overexpressing Mice

Immunoblots identified the relative quantities of MMP20, ameloblastin (AMBN), or cofilin (CFL1) present in extracted secretory stage molar enamel among mouse genotypes. Matrix proteins from *Mmp20*-ablated (-/-), WT (+/+), and *Mmp20*^{+/+}Tg⁺ (Tg+) mice were probed for MMP20, AMBN, cofilin, or phosphorylated cofilin (p-cofilin). MMP20 was not detected in matrix proteins from *Mmp20*^{-/-} mice, was detected in WT mice, and was expressed at high levels in *Mmp20*^{+/+}Tg⁺ mice (Fig. 4A). These data confirmed the expected MMP20 expression levels as a function of genotype. AMBN is an enamel matrix protein essential for proper enamel development in both mice (Fukumoto et al. 2004) and humans (Poulter et al. 2014) and is an MMP20 substrate (reviewed in Bartlett 2013). Therefore, we asked whether the cleavage pattern of AMBN was altered in *Mmp20*^{+/+}Tg⁺ mice. AMBN immunoblots demonstrated that *Mmp20*^{-/-} mice had an abundance of uncleaved AMBN present within their secretory stage enamel (Fig. 4B). In contrast, AMBN from WT mice was mostly cleaved. AMBN was not detected in enamel extracted from the *Mmp20*^{+/+}Tg⁺ mice, indicating that *Mmp20*^{+/+}Tg⁺ mice cleave AMBN into small fragments at a more rapid rate than in WT mice.

CFL1 can polymerize or depolymerize actin filaments, and inhibition of its activity causes a loss of cell polarity. In general, CFL1 is inhibited by Ser3 phosphorylation (Bravo-Cordero et al. 2013). Because MMP20-overexpressing ameloblasts lose their cell polarity, we asked if these mice had greater amounts of CFL1 Ser3 phosphorylation within their secretory stage enamel organs. Three replicate immunoblots demonstrated that overall amounts of CFL1 were unchanged. However, significantly greater amounts of p-cofilin

MMP20 is inhibited and no broad-spectrum protease inhibitor (PI) is present, luciferase activity is not induced, indicating that only MMP20 is responsible for the β -catenin-mediated luciferase induction. (**F**) The ALC invasion assays show that inhibition of MMP20 expression (doxycycline [Dox]) or the presence of a β -catenin inhibitor (ICG-001) results in reduced cell invasion compared to when MMP20 is overexpressed. Invasion was normalized to the Dox-treated control. TOPflash results were from 3 replicates performed on 3 different dates, and invasion assay results were from 2 replicates performed on 3 different dates. Error bars represent standard deviation. Data for panels E and F were assessed for significance by 1-way analysis of variance with Scheffe's F test.

were present in *Mmp20*^{+/+}Tg⁺ mouse enamel organs ($P < 0.05$), suggesting that CFL1 inhibition may play a role in the loss of ameloblast polarity in these mice (Fig. 4C).

Discussion

We show that MMP20 overexpression in mouse molar ameloblasts results in a discontinuous ameloblast layer, ameloblasts that abnormally migrate away from the enamel layer into the stratum intermedium/stellate reticulum, and results in a significant reduction of enamel thickness, volume, and mineral density. Wnt signaling induction may participate in the abnormal migration of ameloblasts, resulting in the formation of atypical calcified nodules adjacent to the enamel space (Fig. 5). These nodules are present just prior to eruption. So, it appears that the reduced enamel epithelium protects the nodules and enamel as the molars erupt, but soon after eruption completes, *Mmp20*^{+/+}Tg⁺ mouse molars have virtually no detectable enamel because it and the nodules abrade away.

While the pathological phenotype of reduced enamel thickness, volume, and mineralization appears quite similar between *Mmp20*^{+/+}Tg⁺ molars and incisors, at the histological level, the ameloblast behaviors in these 2 teeth are dissimilar in certain respects. We previously showed that *Mmp20*^{+/+}Tg⁺ mouse incisors featured highly irregular cyst-like rows of ameloblasts that appeared to weave in and out of the section plane (Shin et al. 2016). In addition, we observed a massive cell infiltrate that included fibroblast-like cells and dividing cells that occupied most of the space where the incisor enamel should have formed. This occupied enamel space included ectopic bone-like calcifications containing collagen with embedded cells that resembled osteocytes. However, in *Mmp20*^{+/+}Tg⁺ mouse molars, we observed *Amelx*-expressing ameloblasts migrating away from the enamel layer. This resulted in large numbers of ectopic mineral nodules deposited into areas normally occupied by the stratum intermedium/stellate reticulum of the enamel organ.

While both teeth featured dramatic enamel pathology, it remains uncertain as to why molar and incisor ameloblasts exhibit divergent migration patterns due to MMP20 overexpression. Although murine amelogenesis proceeds similarly in molars and continuously erupting incisors, many aspects of enamel development differ in potentially important ways. Unlike molars, ameloblasts on rodent incisors move with the incisor as it initiates at the most distal apical position in the jaw, traveling in the mesial direction under the molars, until just before the incisor erupts in the anterior of the oral cavity (Smith and Warshawsky 1975). During this process, ameloblasts move with the incisor while simultaneously moving relative to each other to form the decussating enamel rod pattern.

of AMBN present within forming enamel was inversely correlated with the quantity of MMP20 expression, with *Mmp20*^{+/+}Tg⁺ mice exhibiting virtually undetectable levels of AMBN. (C) *Mmp20*^{+/+}Tg⁺ mouse enamel organs expressed similar overall levels of cofilin (CFL1, 18.56 kDa) but significantly increased ($P < 0.05$) quantities of phosphorylated CFL1 (p-cofilin) compared to WT. Numbers represent the average of 3 immunoblots assessed for molar enamel organ cofilin levels by scanning densitometry. Panels A and B were from 3 mice per genotype, and panel C was from 9 mice per genotype.

Figure 4. Altered ameloblastin (AMBN) and phosphorylated cofilin levels in secretory stage enamel of matrix metalloproteinase 20 (MMP20)-overexpressing mice. Immunoblots of harvested P6 mouse molar enamel organs demonstrated that (A) MMP20 (active, propeptide removed: ~46 and ~41 kDa) was not expressed by *Mmp20*^{-/-} mice, was expressed in *MMP20*^{+/+} wild-type mice, and was present in greater than normal amounts in *MMP20*^{+/+}Tg⁺ mice. (B) The relative quantity

Figure 5. Schematic depicting molar enamel organ organization in wild-type (WT), matrix metalloproteinase 20 (MMP20)–overexpressing, and *Mmp20* null mice. MMP20 overexpression is hypothesized to cleave extracellular cadherin domains, which disrupts the adherens junctions (AJs) and releases intracellular AJ proteins that help bind the AJ to the cytoskeleton. These released proteins include the cell signaling molecules α -catenin, β -catenin, and p120-catenin. β -Catenin may then travel to ameloblast nuclei and initiate cell migration. Each ameloblast is responsible for the formation of a single enamel rod. In WT mice, limited cadherin cleavage by MMP20 may be essential for ameloblast movement necessary to form the characteristic rodent decussating enamel rod pattern. However, excessive cadherin cleavage may release too much β -catenin, causing ameloblasts to depolarize and invade adjacent tissues. These aberrant ameloblasts may initiate deposition of mineralized nodules along their invasion path. Furthermore, premature degradation of enamel matrix proteins in MMP20-overexpressing mice likely contributes to the formation of thin hypomineralized enamel. In contrast, molars from *Mmp20* null mice have ameloblasts that stay connected, but they bend and swirl around, and this can create room for nodules to form on the enamel surface (Hu et al. 2016). This is much more pronounced in incisors compared to molars (Bartlett, Skobe, et al. 2011). Even so, we have previously demonstrated by both quantitative polymerase chain reaction and immunoblots that molars from null mice have enamel organs with significantly greater quantities of E- and N-cadherins than do molars from wild-type mice (Guan and Bartlett 2013). It is unclear why ameloblasts bend and swirl in null mouse teeth. Perhaps ameloblasts have difficulty with directional migration and pile into one another, as suggested by the dysplastic rod pattern in null mice, or perhaps the half normal enamel thickness of null teeth cause ameloblasts to bend and swirl because the enamel area that they blanket has become much smaller.

We propose this complex natural migration pattern contributes to the dissimilar pathological migration pattern of ameloblasts in *Mmp20*^{+/+}Tg⁺ mouse incisors versus molars. An additional difference between the teeth lies in the more pronounced papillary layer in maturation stage ameloblasts in incisors compared to molars. The papillary layer contains capillaries, a potential source for endothelial cells and fibroblasts to migrate into the incisor enamel space. Our observation that fibroblasts were present within the incisor (Shin et al. 2016), but not molar enamel space of *Mmp20*^{+/+}Tg⁺ mice, supports this interpretation.

Amelogenin (AMELX), AMBN, and enamelin (ENAM) are the major structural proteins secreted by ameloblasts into the forming enamel, and all have been identified as MMP20 substrates (reviewed in Bartlett 2013). We previously showed that *Mmp20*^{+/+}Tg⁺ mice had smaller amounts of high molecular weight enamel matrix proteins (mostly amelogenins) and

larger amounts of amelogenin cleavage products, suggesting more rapid and thorough cleavage of enamel proteins (Shin et al. 2014). Here we demonstrated that enamel organs from *Mmp20*^{+/+}Tg⁺ mouse molars also had nearly undetectable levels of AMBN. Given that decreased *Ambn* expression results in decreased enamel mineral content (Teepe et al. 2014), we propose that this rapid cleavage/removal of both AMELX and AMBN may contribute to the severe enamel mineralization defect in *Mmp20*^{+/+}Tg⁺ mice.

We assessed CFL1 phosphorylation because it is an essential actin-binding/regulating protein involved in polarization, proliferation, and migration. CFL1 defects inhibit proper morphological changes in cells, and genetic ablation of *Cfl1* in mice is embryonically lethal (Ohashi 2015). Localized CFL1 inactivation was associated with functional defects in human, mouse, and zebrafish podocytes (Ashworth et al. 2010). In addition, *Cfl1* knockdown was shown to induce polarity defects in mouse neurons with associated attenuation of axon formation (Garvalov et al. 2007). CFL1 maintains cell polarity, but its inhibited phosphorylated form does not. We propose that increased CFL1 phosphorylation levels observed in *Mmp20*^{+/+}Tg⁺ mouse enamel organs support its involvement in the loss of ameloblast cell polarity.

In conclusion, we report that MMP20 function in enamel formation requires the appropriate expression level. Too little MMP20 activity causes amelogenesis imperfecta, and too much MMP20 activity causes ameloblasts present within developing mouse molars to induce Wnt signaling, phosphorylate CFL1, and pathologically

depolarize. Molar ameloblasts uncharacteristically migrate into adjacent tissue layers, resulting in a discontinuous ameloblast layer containing abnormal mineralized nodules. In addition, premature degradation of enamel matrix proteins in overexpressing mice likely contributes to the formation of thin, hypomineralized enamel.

Author Contributions

M. Shin, contributed to conception, design, data acquisition, and analysis, drafted and critically revised the manuscript; M.B. Chavez, A. Ikeda, contributed to design, data acquisition, and analysis, critically revised the manuscript; B.L. Foster, contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; J.D. Bartlett, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave

final approval and agree to be accountable for all aspects of the work.

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