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M180 Amelogenin Processed by MMP20 is Sufficient for Decussating Murine Enamel

ABSTRACT

Amelogenin (AMELX) and matrix metalloproteinase-20 (MMP20) are essential for proper enamel development. *Amelx* and *Mmp20* mutations cause amelogenesis imperfecta. MMP20, a protease secreted by ameloblasts, is responsible for processing enamel proteins, including AMELX, during the secretory stage of enamel formation. Of at least 16 different amelogenin splice products, the most abundant isoform found in murine ameloblasts and developing enamel is the M180 protein. To understand the role of MMP20 processing of M180 AMELX, we generated *Amelx*KO/*Mmp*20KO (DKO) mice with an amelogenin (M180Tg) transgene. We analyzed the enamel phenotype by SEM to determine enamel structure and thickness, μCT, and by nanoindentation to quantify enamel mechanical properties. M180Tg/DKO mouse enamel had 37% of the hardness of M180Tg/*Amelx*KO teeth and demonstrated a complete lack of normal prismatic architecture. Although molar enamel of M180Tg/*Amelx*KO mice was thinner than WT, it had similar mechanical properties and decussating enamel prisms, which were abolished by the loss of MMP20 in the M180Tg/DKO mice. Retention of the C-terminus or complete lack of this domain is unable to rescue amelogenin null enamel. We conclude that among amelogenins, M180 alone is sufficient for normal enamel mechanical properties and prism patterns, but that additional amelogenin splice products are required to restore enamel thickness.

KEY WORDS: matrix metalloproteinase-20, knockout mouse, transgenic mouse, amelogenesis, imperfecta, ameloblasts, tooth calcification.

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INTRODUCTION

Development and mineralization of enamel, a highly organized, mostly inorganic structure, depend on numerous structural and proteolytic proteins. Amelogenins constitute 90% of the enamel organic matrix secreted by ameloblasts. The murine amelogenin gene encodes an abundant M180 amelogenin protein with distinct regions that are important for proper enamel mineralization (Wright *et al*., 2003). The amelogenin primary RNA transcript is extensively alternatively spliced (Simmer *et al.*, 1994), and the splice variants may have different functions in developing enamel. Following secretion, amelogenins assemble into nanospheres, which occupy spaces between the enamel crystallites, to separate and support them (Fincham *et al*., 1995), and to bind mineral crystals and guide mineral growth (Iijima and Moradian-Oldak, 2004).

Ameloblasts also secrete proteases, which process amelogenins and other structural enamel proteins during mineralization (Smith, 1998; Lu *et al*., 2008). During crystal growth, the proteases matrix metalloproteinase-20 (MMP20) and kallikrein-4 (KLK4) process the enamel proteins (Bartlett and Simmer, 1999) until the overall protein content in the enamel is reduced from 30% to less than 3%. MMP20 is the predominant secretory-stage enzyme (Ryu *et al*., 1999) that cleaves the hydrophilic C-terminus from the hydrophobic region of the full-length amelogenin protein (Nagano *et al*., 2009) soon after secretion (Simmer and Hu, 2002), while the other enamel protease KLK4 is secreted by maturation-stage ameloblasts (Hu *et al*., 2002) to process the remaining enamel proteins.

Mice that do not make any amelogenin protein (*Amelx*KO) develop hypoplastic enamel lacking prismatic structure (Gibson *et al*., 2001). These defects can be partially rescued by mating the *Amelx*KO mice with mice expressing an amelogenin transgene (M180) (Li *et al*., 2008). M180 is the most abundant splice variant in mice, consisting of 180 amino acids, 13 of which are normally cleaved from the C-terminus by *Mmp20* immediately after secretion. Amelogenin isoform processing by proteases during enamel formation has been characterized in recombinant porcine amelogenin (Nagano *et al*., 2009). Mice that contain M180 in an *Amelx*KO background do not carry the other alternatively spliced amelogenins. Transgenic mice that lack the C-terminus of M180 in an *Amelx*KO background display a phenotype similar to that of

*Amelx*KO mice and have disorganized, aprismatic enamel (Pugach *et al*., 2010), indicating that the C-terminus is essential for the formation of prismatic enamel, which has been suggested by numerous studies (Moradian-Oldak *et al*., 2000, 2002; Paine *et al*., 2000; Beniash *et al*., 2005; Margolis *et al*., 2006; Fang *et al*., 2011; Wiedemann-Bidlack *et al*., 2011).

Mice lacking MMP20 develop hypoplastic, hypomature enamel that separates from the dentin (Caterina *et al*., 2002) and has decreased mineral content and hardness (Bartlett *et al*., 2004), presumably due to failure to cleave the enamel matrix proteins properly. Like *Amelx*KO mice, *Mmp20*KO mice produce a thin layer of disorganized enamel (Caterina *et al*., 2002). *Mmp20*KO murine enamel was approximately 37% softer than wild-type enamel, contained around 53% less mineral, and had 7% to 16% higher water and protein content (Bartlett *et al*., 2004). While amelogenin-null enamel mineral is plate-like, *Mmp20*KO enamel has a disrupted prism pattern (Bartlett *et al*., 2006), demonstrating that both genes are essential for production of full-thickness enamel with decussating prisms.

Here we determined whether the presence of the C-terminus, in the absence of the protease that generates it, would be sufficient to rescue the enamel defects in AKO mice. The M180/DKO mice do not make any alternatively spliced amelogenins or *Mmp20*, so the M180Tg would be unable to be initially cleaved at the C-terminus. By overexpressing M180 in *Amelx* and *Mmp20* double-knock-out mice, we sought to elucidate the relative contribution of this most abundant amelogenin splice variant to the enamel phenotype, particularly in terms of thickness, mechanical properties, and prism pattern.

MATERIALS & METHODS

Transgenic and Knockout Mice

All procedures were performed after approval by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice were generated at the UPenn Transgenic Core Facility and maintained in an AAALAC-accredited facility. *AmelxKO* and *Mmp20KO* mice were generated by introduction of a deletion into the coding region as described previously (Gibson *et al*., 2001; Caterina *et al*., 2002). The C-terminus truncated (CTRNCTg) and M180 transgenic mice were generated as previously described (Pugach *et al*., 2010; Li *et al*., 2008). To determine the genotype of *Amelx*KO, *Mmp2*0KO, and transgene-positive offspring, we isolated high-molecular-weight genomic DNA from mouse tails (Chen *et al*., 2003). The amelogenin, *Mmp20*, CTRNCTg, and M180Tg PCR primers and conditions used have been described (Caterina *et al*., 2002; Li *et al*., 2008; Pugach *et al*., 2010).

Protein Analysis

For protein analysis, first molar mandibular teeth were dissected from transgene-positive or knockout offspring at post-natal day 4. To identify transgenic and endogenous protein in teeth from M180Tg/*Amelx*KO/*Mmp20*KO and CTRNCTg/*Amelx*KO matings, we prepared protein extracts and SDS-PAGE gels as previously described (Chen *et al.*, 2003) and stained them with Coomassie blue (Bio-Rad, Hercules, CA, USA).

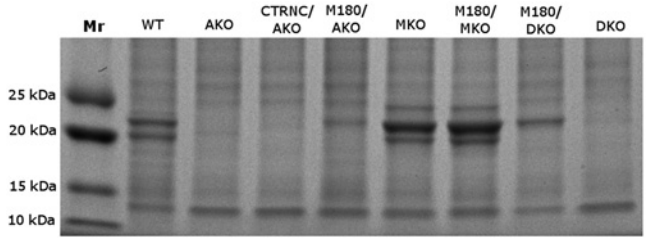

Figure 1. SDS-PAGE of four-day-old molar extracts stained with Coomassie blue. Non-amelogenin enamel proteins are evident by comparing AKO and DKO with the other lanes. An additonal band at approximately 24 kDa (either alternative splice variant or uncleaved protein) is noticeable in the MKO and M180/MKO lanes. The absence of several lower-M_r bands in MKO, M180/MKO, and M180/DKO that appear in WT and M180/AKO illustrates the differences in amelogenin cleavage. CTRNC/AKO mice expressed only the cleaved form of M180Tg and no other splice variants, and CTRNCTg is visible as a faint band slightly below 23 kDa.

μCT Analysis of Teeth

Scans for volume and density of enamel in molars (n = 6) were performed as described (Pugach *et al*., 2010), with a microtomograph imaging system (μCT 40, Scanco Medical AG, Brüttisellen, Switzerland) with 16-μm resolution at 70 kVp. The images were processed by three-dimensional reconstruction software (μCT Evaluation Program v6.0, Scanco Medical) and analyzed for determination of enamel density and volume. Hydroxyapatite standards were used for instrument calibration.

Scanning Electron Microscopy Analysis of Enamel

Molars dissected from adult (6 wks and 3 mos old) mouse mandibles (n = 6) were dehydrated through graded ethyl alcohols and embedded in eponate 12 resin (Ted Pella, Inc., Redding, CA, USA). Embedded teeth were sectioned with a Buehler Isomet low-speed saw (Buehler, Lake Bluff, IL, USA) to produce 200-μm-thick sections, which were polished with 600-grit SiC paper, 15-μm polishing paper, and with 1-μm and 0.25-μm diamond suspensions (Buehler). Sections were etched with 20% phosphoric acid for 10 sec prior to being mounted for scanning electron microscopic (SEM) analysis. SEM analysis of enamel mesiodistal cross-sections from molars was completed at 15 kV (FEI Quanta 200 FEG, Hillsboro, OR, USA). Enamel structure was analyzed and measurements of molar enamel thickness were calculated with Image J. Molar enamel thickness was measured on the thickest part (center) of distal and mesial enamel, adjacent to the respective cusps.

Nanomechanical Property Measurements of Teeth

Mandibles were dissected from mouse heads as described (Li *et al*., 2008). Elastic modulus and hardness of molar enamel were determined in adult (6 wks and 3 mos old) mice (n = 6) by a Nanoindenter XP (MTS Systems, Oak Ridge, TN, USA). Half-mandibles were embedded in Acrymount embedding resin

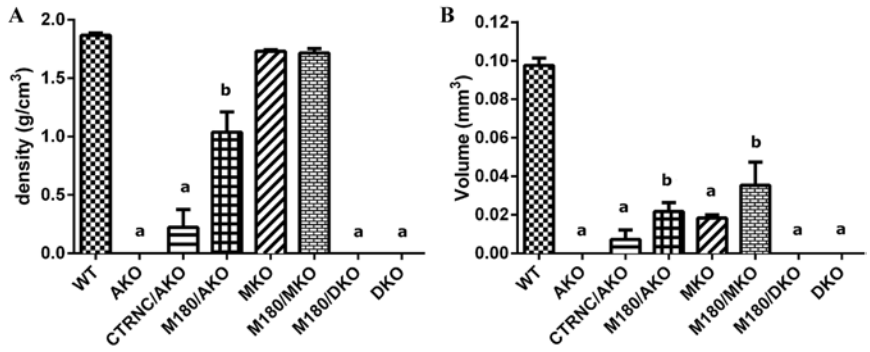

Figure 2. μCT of adult molar enamel from WT, *Amelx*KO (AKO), CTRNC/*Amelx*KO (CTRNC/AKO), M180Tg/*Amelx*KO (M180/AKO), *Mmp20*KO (MKO), M180Tg/*Mmp20*KO (M180/MKO), M180Tg/*Amelx*KO/*Mmp20*KO (M180/DKO), and *Amelx*KO/*Mmp20*KO (DKO) mouse models. **(A)** Density. **(B)** Volume. Although in some groups there is no column value for density or volume, there is actually enamel present; it was below the measurement threshold of the μCT system and thus was recorded as a 0 value. [a]Significantly different from WT and [b]. [b]Significantly different from WT and [a] ($p < .05$).

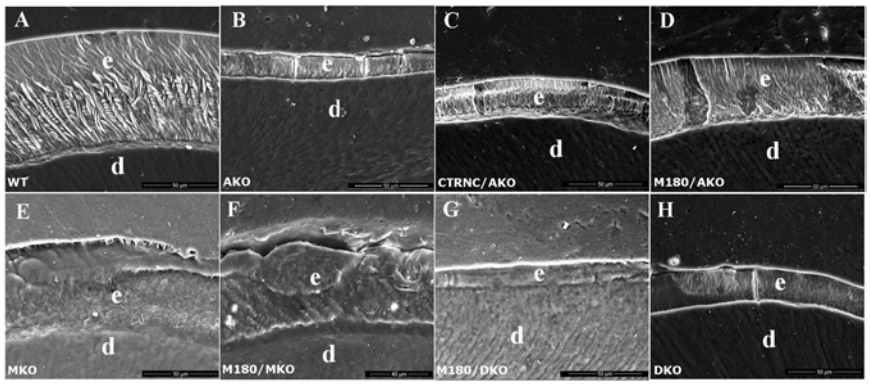

Figure 3. SEM analysis of polished, then etched, six-week-old adult molar enamel. SEM images of molar enamel, with enamel (e) and dentin (d) indicated, from **(A)** WT, **(B)** *Amelx*KO (AKO), **(C)** CTRNC/*Amelx*KO (CTRNC/AKO), **(D)** M180Tg/*Amelx*KO (M180/AKO), **(E)** *Mmp20*KO (MKO), **(F)** M180Tg/*Mmp20*KO (M180/MKO), **(G)** M180Tg/*Amelx*KO/*Mmp20*KO (M180/DKO), and **(H)** *Amelx*KO/*Mmp20*KO (DKO) mouse models. Note that the M180Tg/AKO (D) gained a prism pattern but did not attain full thickness.

(Electron Microscopy Sciences, Hatfield, PA, USA) and ground from the mesial side with 400-grit SiC paper until the interior of the first molar was exposed to reveal longitudinal cross-sections of molars. The exposed interior of first molars was further polished by 800- and 1200-grit SiC papers, and 1-μm and 0.25-μm diamond suspensions. Nanoindentations were performed with a Berkovich diamond tip, with a trapezoidal force profile with peak loads at 300 μN. Twenty indentations were made in the enamel of each tooth, in mesial cusps of 1st molars, in mature enamel. Each indentation yielded a load-deformation curve, from which the elastic modulus, and hardness, were determined according to the method of Oliver and Pharr (1992; Doerner and Nix, 1986).

Statistical Analysis

We used analysis of variance (ANOVA) with the Tukey *post hoc* test to detect differences ($p < .05$) between groups of teeth analyzed for enamel density and volume, as measured by μCT, enamel thickness as measured by SEM, and nanomechanical properties (GraphPad Software, San Diego, CA, USA).

RESULTS

Protein Analyses of Transgenic/KO Mice

SDS-PAGE analysis was used to detect transgenic and endogenous protein in developing molars (Fig. 1). Wild-type (WT) and *Mmp20*KO (MKO) four-day-old mouse molars expressed endogenous amelogenins, at least 16 of which were alternative splice products in WT mice (Simmer *et al*., 1994). M180Tg/*Mmp20*KO (M180/MKO) molars in the secretory stage expressed both transgenic and endogenous amelogenins, while M180Tg/ *Amelx*KO (M180/AKO) and M180Tg/doubleKO (M180/DKO) molars expressed only transgenic amelogenin of approximately 23 kDa with no alternative splice variants. Without MMP20, the endogenous amelogenin splice variants and transgenic M180 in M180/MKO mouse molars could not be normally cleaved, as indicated by the darker stained band approximately 23 kDa compared with that of WT mice (Fig. 1). In the M180/DKO molars, only M180Tg remained uncleaved, and no other amelogenin splice variants were present. In only the MKO and M180/MKO mouse enamel was an additional band visible at approximately 24 kDa, which has been previously reported (Caterina *et al*., 2002) and is presumably an uncleaved amelogenin alternative splice variant that is rapidly cleaved by *Mmp20* during the secretory stage in WT enamel (Fig. 1).

Phenotype of Transgenic/Double-KO Enamel

Phenotype analysis of enamel by μCT in adult M180/DKO mice and controls revealed that molars from AKO, M180/DKO, and DKO mice had lower enamel density and volume than all other groups and were not different from each other (Fig. 2). While the μCT density and volume values of AKO, M180/DKO, and DKO appear to be zero in Fig. 2, they were below the detectable threshold and thus were recorded as a zero value, when in fact enamel was present on these mouse molars, as shown in Fig. 3.

To investigate the mature enamel structure of M180/DKO mice and controls, we analyzed cross-sections of etched molar enamel by SEM. WT enamel displayed the characteristic decussating prisms that extended through almost the full thickness of the enamel layer (Fig. 3A). In contrast, AKO molar enamel

displayed no prisms but some flat crystals (Fig. 3B), while CTRNC/AKO enamel had a structure similar to that of AKO (Fig. 3C). M180/AKO molar enamel had an improved decussating prismatic structure, although this pattern did not extend through the entire thickness of the enamel layer (Fig. 3D). MKO molar enamel exhibited prisms with no inter-weaving that seemed to be obscured by organic matter (Fig. 3E), while M180/MKO molar enamel exhibited a prismatic enamel pattern that was improved over that of MKO enamel but still was obscured by organic material (Fig. 3F). Both M180/DKO and DKO molar enamel displayed neither prisms nor visible crystals (Figs. 3G, 3H).

Molar enamel thickness was measured from SEM images. Enamel thickness of CTRNC/AKO, M180/AKO, and M180/DKO was 24%, 34%, and 25% that of WT, respectively (Fig. 4A).

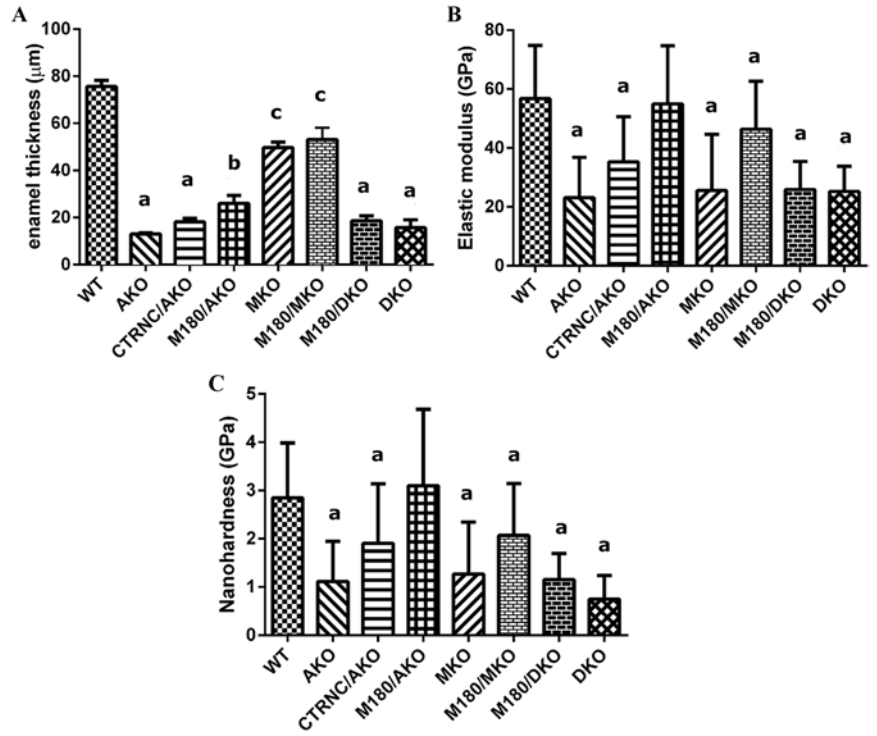

Figure 4. Enamel thickness and nanomechanical properties of WT, *Amelx*KO (AKO), CTRNCTg/AmelxKO (CTRNCTg/AKO), M180Tg/*Amelx*KO (M180Tg/AKO), *Mmp20*KO (MKO), M180Tg/*Mmp20*KO (M180/MKO), M180Tg/*Amelx*KO/*Mmp20*KO (M180/DKO), and *Amelx*KO/*Mmp20*KO (DKO) mouse models. **(A)** Molar enamel thickness, **(B)** enamel elastic modulus of molars, and **(C)** enamel hardness of molars. [a]Significantly different from WT, [b], and [c]. [b]Significantly different from WT, [a], and [c]. [c]Significantly different from WT, [a], and[b] ($p < .05$).

Nanomechanical Properties of Transgenic/ Double-KO Enamel

Nanomechanical properties of mature enamel were determined by nanoindentation of adult molars. Molar enamel elastic modulus of CTRNC/AKO, M180/AKO, and M180/DKO was 62%, 97%, and 46% that of WT, respectively (Fig. 4B), while enamel hardness of CTRNC/AKO, M180/AKO, and M180/DKO was 67%, 108%, and 40% that of WT, respectively (Fig. 4C).

DISCUSSION

In the current study, the phenotype of M180/DKO enamel was analyzed according to structure, thickness, and mechanical properties. In all of these analyses, the molar enamel of M180/DKO mice was significantly compromised compared with that of M180/AKO mice and was most similar to *Amelx*KO and CTRNC/AKO enamel, suggesting that the presence of the uncleaved C-terminus of M180 (M180/DKO) is insufficient for normal enamel structure, thickness, and mechanical properties. Presumably, the secreted amelogenin protein (with its C-terminus) could not properly participate in the formation of higher order assemblies of nanospheres and subsequent mineral crystal orientation and growth. Expression of the other major enamel protease, KLK4, begins during the maturation stage, at around post-natal day 6 (Hu *et al*., 2002; Simmer *et al*., 2011), so it is unlikely that M180Tg was cleaved during the secretory stage at post-natal day 4 by KLK4 in the absence of MMP20.

Structural analyses of molar enamel by genotype suggest that mice with molar enamel with decussating prisms (WT and M180/AKO) had significantly higher mechanical properties than the other mouse genotypes, without decussating prisms, or evidence of prisms. These data are consistent with prior suggestions that decussating enamel prism structure may be essential for maintaining hardness (Bartlett *et al*., 2004) and that MMP20 is necessary for decussating enamel prisms (Bartlett *et al*., 2011). In further agreement with analysis of the present data, prior studies of mechanical properties of *Amelx*KO (Li *et al*., 2008) and *Mmp20*KO enamel (Bartlett *et al*., 2004) found that mature enamel in both knockout models did not exhibit decussating prisms and had enamel hardness approximately 40% to 50% that of WT mice.

Analysis of the nanomechanical property data from molar enamel in this study indicates an almost complete recovery of mechanical properties to WT values in M180/AKO mice, even though enamel density, volume, thickness, and structure were not completely rescued. Conversely, when the M180 transgene was overexpressed in the double-KO mice, the mechanical property rescue of M180/AKO enamel was eliminated, since the mechanical properties of M180/DKO molar enamel did not differ significantly from those of AKO. Interestingly, while M180/DKO molar hardness was 40% that of WT, *Amelx*KO mice lacking the C-terminus of M180Tg (but retaining MMP20 activity) had molar hardness 67% that of WT. This suggests that MMP20 has additional important functions beyond cleaving M180 at the C-terminus that could affect the enamel phenotype in *Mmp20*KO mice, including cleaving M180 at the N-terminus to form

tyrosine-rich amelogenin peptide (TRAP) (Ryu *et al*., 1999), and processing of other enamel proteins, ameloblastin (Chun *et al*., 2010), and enamelin (Yamakoshi *et al*., 2006), during the secretory stage.

In conclusion, analysis of the data from the present study suggests that the presence of the C-terminus on M180 and MMP20 expression is sufficient to rescue enamel structural defects and mechanical properties, but not thickness, of AKO enamel. This is consistent with the previous report of a mouse model which overexpressed 2 *Amelx* splice variants (M180Tg and LRAPTg) in AKO mice, since the presence of both M180Tg and LRAPTg increased enamel thickness beyond that of the M180Tg alone (Gibson *et al*., 2011). We have provided further evidence that both *Amelx* and *Mmp20* are required for enamel prism structure, mechanical properties, and thickness, and that processing of full-length amelogenin by MMP20 has a crucial role in enamel formation.

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