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The Amelogenin C-Terminus Is Required for Enamel Development

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ABSTRACT

The abundant amelogenin proteins are responsible for generating proper enamel thickness and structure, and most amelogenins include a conserved hydrophilic C-terminus. To evaluate the importance of the C-terminus, we generated transgenic mice that express an amelogenin lacking the C-terminal 13 amino acids (CTRNC). MicroCT analysis of TgCTRNC29 teeth (low transgene number) indicated that molar enamel density was similar to that of wild-type mice, but TgCTRNC18 molar enamel (high transgene number) was deficient, indicating that extra transgene copies were associated with a more severe phenotype. When amelogenin-null (KO) and TgCTRNC transgenic mice were mated, density and volume of molar enamel from TgCTRNCKO offspring were not different from those of KO mice, indicating that neither TgCTRNC18 nor TgCTRNC29 rescued enamel's physical characteristics. Because transgenic full-length amelogenin partially rescues both density and volume of KO molar enamel, it was concluded that the amelogenin C-terminus is essential for proper enamel density, volume, and organization.

KEY WORDS: amelogenin, enamel, transgenic mice.

INTRODUCTION

Mature dental enamel is composed of at least 95% mineral by weight and is considered to be the hardest tissue in the body (Robinson and Kirkham, 1985). During tooth development prior to eruption into the oral cavity, ameloblast cells secrete an organic matrix that guides mineral formation, and the highly conserved amelogenin proteins constitute 90% of this secreted matrix (Termine *et al*., 1980; Delgado *et al*., 2007). Proteases are also secreted by ameloblasts, and these enzymes process the amelogenin and other less abundant enamel proteins as mineral crystals grow in size (Smith, 1998; Lu *et al*., 2008). By the time the tooth erupts into the oral cavity, the enamel protein content is reduced to less than 3%, and the ameloblast cells covering the enamel are finally lost (Robinson *et al.,* 1995; Shibata *et al.,* 1995). This process is strikingly different from that seen in bone, since biological repair or remodeling of enamel does not occur. In addition, enamel, an epithelial product, lacks collagen as a structural component of its organic matrix.

Because numerous individuals have been reported with the enamel defect amelogenesis imperfecta (AI) due to amelogenin (*AMELX)* gene mutations (Wright, 2006), it is accepted that amelogenins are required for enamel to develop normally. Mouse models with defective enamel have been generated with amelogenin null (KO) or knock-in mutations (Gibson *et al*., 2001; Zhu *et al*., 2006), or by over-expression of an amelogenin protein with a point mutation seen in several human AI kindreds (Collier *et al.,* 1997; Ravassipour *et al*., 2000; Gibson *et al*., 2007). In addition, when KO females are mated with transgenic males that express a single 180-amino-acid amelogenin, partial restoration of molar enamel volume, thickness, density, and organization of the AI phenotype was observed in transgene-positive/KO offspring, indicating the importance of the most abundant 180-amino-acid amelogenin (Li *et al.,* 2008).

Five *AMELX* mutations have been reported where a premature stop truncates the protein, and alters the C-terminus of the 180-amino-acid amelogenin (Lench and Winter, 1995; Kindelan *et al*., 2000; Greene *et al.,* 2002; Hart *et al*., 2002; Sekiguchi *et al.,* personal communication). This charged domain is completely conserved in mammalian species (Delgado *et al*., 2007) with characteristics of a mineral binding region (Aoba *et al*., 1987; Moradian-Oldak *et al*., 2002).

For better understanding of the importance of the C-terminus, we generated transgenic mice that directed ameloblasts to express amelogenin lacking the terminal 13 amino acids. The phenotype of the mice was evaluated, and we attempted rescue experiments to better understand the role of the C-terminus, by evaluating amelogenesis in its absence.
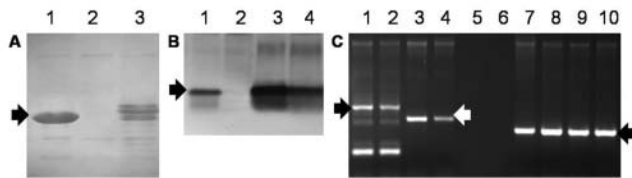
Figure 1. Western blot and RT-PCR of extracts of TgCTRNCKO and WT teeth. **(A)** Western blot showing transgene expression in molars from a TgCTRNC18KO male (lane 1), several bands in a het female (lane 3), and none in a KO male (lane 2), as expected. The Western blot was probed with rat anti-amelogenin antibody, which recognized transgenic and WT amelogenins (arrowhead). **(B)** Western blot of amelogenin proteins. TgCTRNC protein in molars (lane 1) and incisors (lane 2) of a TgCTRNCKO male and WT molars and incisors (lanes 3, 4). **(C)** Gel with *Amelx* RT-PCR products from molar and incisor RNA. Lane 1: WT molar. 2: WT incisor. 3: TgCTRNCKO molar. 4: TgCTRNCKO incisor. 5-6: empty. 7-10: same RNA samples as lanes 1-4 with β-actin primers. Arrowheads indicate principal amelogenin and β-actin PCR products.

MATERIALS & METHODS

Vector Construction

To generate the C-terminal deletion, we subcloned a region of the previously used M180 expression vector (Gibson *et al.,* 2007). A 5′ primer was designed to begin at the HindIII site (underlined), and an in-frame termination codon was added (in bold), removing 13 codons. The 3′ primer, which included a XhoI site (underlined) for cloning, was designed so that the polyadenylation signal would be included in the PCR product.

   5′ primer: 5′GC*AAGCT***TAA**AAGATCAGAAAATGAGAAGAG
   3′ primer: 5′C*CTCGAG*ATGCAGATTAGATCCCTGGGTT
Following PCR with these primers, the product was digested with HindIII and XhoI and inserted into the expression vector, replacing the wild-type sequence.

Transgenic Mice

All procedures were performed after approval by the University of Pennsylvania IACUC. Mice were generated at the UPenn Transgenic Core Facility, and maintained in an AAALAC-accredited facility. Founders and transgene-positive offspring were identified by PCR of tail DNA as described previously (Chen *et al*., 2003; Li *et al.,* 2008). Dot blot analysis of transgene copy number in some strains was done as described previously (Gibson *et al.,* 2007). Because the murine *Amelx* gene is solely on the X-chromosome, female *Amelx* null (KO) mice were mated with transgenic (TgCTRNC) males for molecular analysis and evaluation of phenotypic rescue in transgenic-positive male offspring with a KO background, *e.g.,* TgCTRNCKO.

Molecular Analysis of Mice

For evaluation of transgenic RNA in teeth, transgenic males were mated with KO females. First mandibular molar and incisor teeth were dissected from 4-day-old male offspring that were PCR-positive for the transgene, or from 4-day-old WT mice, followed by semi-quantitative RT-PCR, as described previously, with 24 cycles (Li *et al.,* 2008). These transgenic/KO pups have an *Amelx*-null background.

For transgenic protein in teeth from matings similar to those described above, protein extracts and Western blots were prepared and probed with an anti-rat polyclonal antibody (Gibson *et al.,* 2001). Image J software (Rasband, 2008) was used for analysis of band intensity with images of RT-PCR gels and Western blots.

Microscopic Analysis of Teeth

Mandibles were fixed overnight in 4% paraformaldehyde, and light microscopic images were recorded. For histologic examination of ameloblasts and developing dental tissues, the mandibles were decalcified, embedded in paraffin, sectioned with identical orientation, stained with hematoxylin/eosin, and images were recorded. For immunohistochemistry, paraffin sections were incubated with anti-amelogenin antibody (Kamiya Biomedical Co., Seattle, WA, USA); negative controls lacked primary antibody.

Scanning electron micrographic analysis of tooth surfaces and fractured internal enamel and dentin surfaces of incisors and molars was completed at 20 kV (JEOL JSM T330A, JEOL, Inc., Peabody, MA, USA).

MicroCT

Scans for volume and density of enamel and dentin of molars and incisors were performed as described (Li *et al.*, 2008), with a Skyscan portable x-ray microtomograph (MicroPho-tonics, Allentown, PA, USA). The images were processed by three-dimensional reconstruction software and analyzed for determination of enamel, dentin, and whole-tooth density and volume. Hydroxyapatite standards were used for instrument calibration.

Statistical Analysis

We used unpaired *t* tests and ANOVA with the Bonferroni *post hoc* test to detect differences ($p < 0.05$ was considered significant) between groups of teeth analyzed for enamel and dentin density and volume, as measured by microCT (GraphPad Software, San Diego, CA, USA). Groups that lacked a normal distribution due to phenotypic heterogeneity are indicated in Figs. and Tables.

RESULTS

Generation and Molecular Analysis of Transgenic Mice

The transgenic expression vector was identical to that used previously to express the 180-amino-acid amelogenin in ameloblasts (Gibson *et al.,* 2007), but with a deletion of the C-terminal 13 amino acids. Five independent transgenic mouse lines were generated that harbored the CTRNC transgene. Copy number for several strains was determined by dot blot: TgCTRNC2 had 2 copies, TgCTRNC18 had 28 copies, and TgCTRNC29 had 2 copies (not shown).

To detect transgene expression in developing teeth, we mated KO females with transgenic males to eliminate the background of multiple WT amelogenin proteins and RNAs in the male offspring. This is necessary, since there are at least 15 alternative splice products in WT mice (Hu *et al.*, 1997; Bartlett *et al.*, 2006; Li *et al.*, 2006). Western blots of TgCTRNCKO molars from male offspring

indicated that all mouse lines expressed TgCTRNC amelogenin (Fig. 1A). ImageJ analysis of 7 Western blots indicated that the TgCTRNC18 transgene was approximately 75% as active as the WT *Amelx* gene in het (heterozygous) female mice when the major M180 amelogenin band was analyzed; TgCTRNC29 and TgCTRNC2 mice expressed approximately 25% of that expressed by WT mice, as could be predicted by the transgene copy number.

A higher transgene expression level was detected in TgCTRNCKO molars compared with incisors (Fig. 1B), as had previously been observed in all other strains examined thus far with this vector (Li *et al.*, 2008). A light band of transgenic CTRNC protein could be detected in incisor extracts at long exposure times. Equal amounts of tooth RNA were converted to cDNA, amplified by PCR for 24 cycles, and analyzed by gel electrophoresis, which indicated an average molar/incisor ratio of 1.4/1 for WT and 1.9/1 for TgCTRNCKO for 4-day-old mice (Fig. 1C). The results were substantially unchanged when this experiment was repeated beginning with different mouse pups.

Therefore, by different methods involving protein and RNA analysis, more transgenic amelogenin was expressed in molars compared with incisors. This result allowed for the phenotypic examination of developing teeth with various amounts of transgenic amelogenin present, either in the presence (in transgenic mice with all WT amelogenins) or absence of the WT complement of proteins (in transgenic/KO mice).

Phenotype of Transgenic Teeth

The TgCTRNC18KO ameloblasts appear normal (Fig. 2A), and amelogenin protein is detected in the ameloblasts and the enamel layer of TgCTRNC18KO molars (Figs. 2B, 2C), indicating that the protein is secreted into the developing enamel matrix as expected.
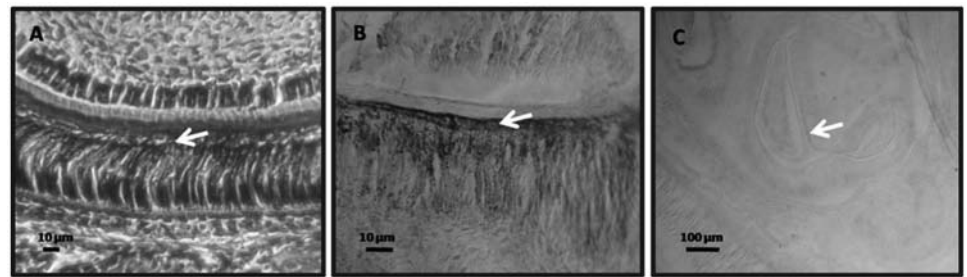

Figure 2. Histology and immunohistochemistry of 4-day-old TgCTRNC18KO teeth. (**A**) Histology of a section through a molar showing a phenotypically normal ameloblast layer. (**B**) Immunohistochemistry of TgCTRNC18KO molar with amelogenin localization in ameloblasts and enamel layer. (**C**) Negative control. Arrows indicate Tomes' process region of ameloblasts.
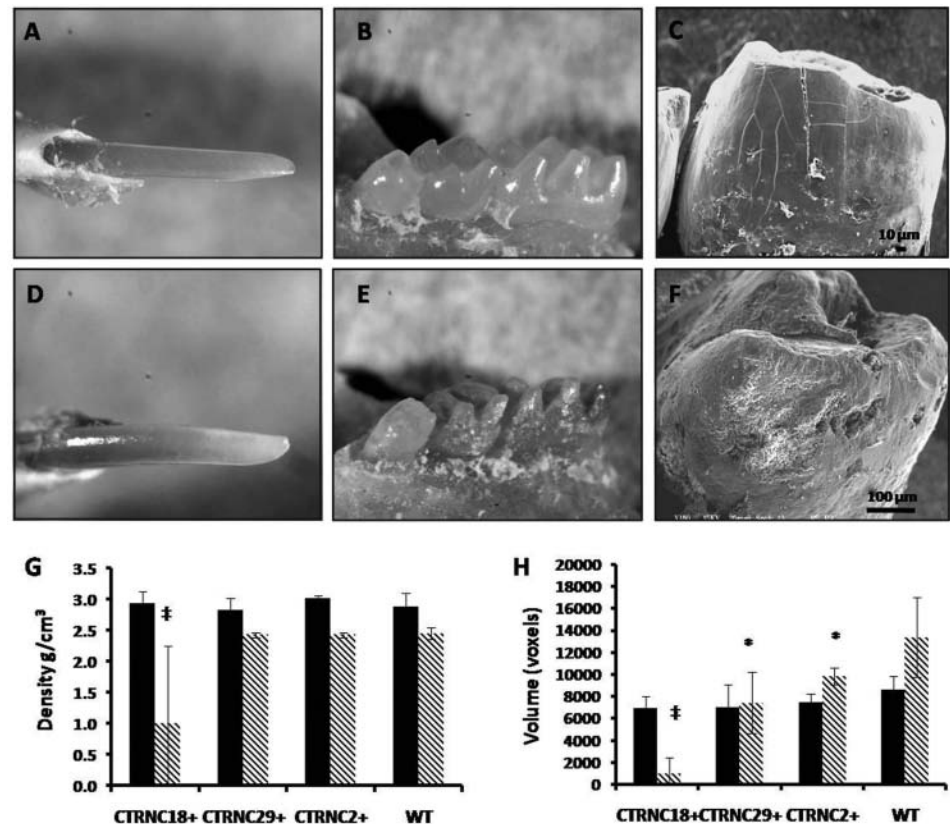

Figure 3. Photographs, SEM, and microCT of TgCTRNC18 and WT teeth. Gross photographs of WT incisor (**A**), molars (**B**), and SEM of normal molar (**C**); photographs of TgCTRNC18 incisor (**D**), molars showing rough surfaces (**E**), and SEM showing rough surfaces (**F**). MicroCT density (**G**) and volume (**H**); incisor enamel indicated by solid bars and molar enamel by striped bars. See Appendix Table 1 for N and estimates of variability.

The mandibles of the TgCTRNC mice were analyzed grossly and by scanning electron microscopy, which revealed rough and pitted surfaces in TgCTRNC18 molars (Figs. 3E, 3F) compared with those of WT mice (Figs. 3B, 3C), while the transgenic incisors appeared similar to those of WT mice (Figs. 3A, 3D). TgCTRNC2 and 29 molars and incisors appeared similar to those of WT mice.

MicroCT analysis revealed that enamel density for molars was similar in WT mice for TgCTRNC29 and TgCTRNC2, both with 2 copies of the transgene (Fig. 3G). However, for TgCTRNC18 with many transgene copies, enamel density and
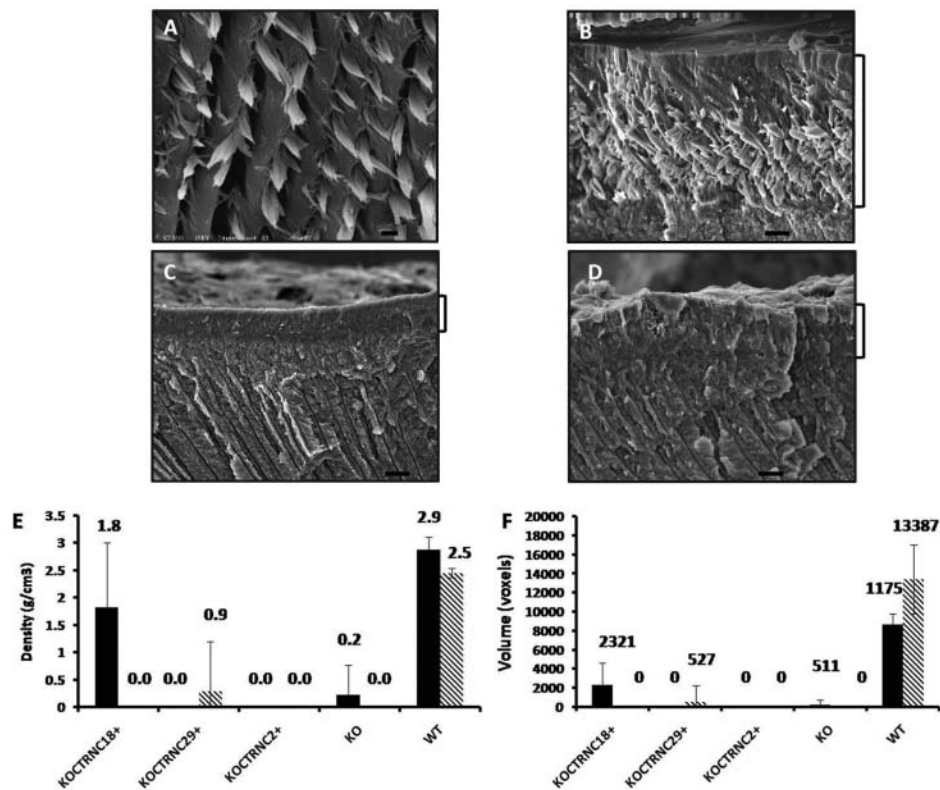
Figure 4. SEM and microCT of teeth. SEM from fractured teeth: WT incisor **(A),** WT molar **(B),** KO molar **(C),** TgCTRNC18KO molar **(D)**. Magnification bars = 10 μm; brackets on B, C, and D indicate the enamel thickness from the enamel surface to the dentin-enamel junction. MicroCT analysis for density **(E)** and volume **(F)** of incisor enamel (solid bars) and molar enamel (striped bars). See Appendix Table 2 for N and estimates of variability.

volume of molars were reduced (Figs. 3G, 3H). Density and volume of molar and incisor dentin did not vary significantly (Appendix Table 1).

Amelogenin Lacking the C-Terminus is Unable to Rescue the KO Enamel Phenotype

Male offspring from mating KO females to transgenic males have KO genetic background and are either positive or negative for the transgene. These mice inherited the Y chromosome from the male parent, and thus have no normal X-chromosomal *Amelx* genes. Gross examination of molars and incisors indicated that TgCTRNC18KO molars and incisors (Appendix Figs. B, D) were worn and discolored, similar to those in KO mice (Appendix Figs. A, C). SEM analysis revealed that, compared with WT mice (Figs. 4A, 4B), molars (Fig. 4D) and incisors (Appendix Fig. F) of TgCTRNC18KO mice had disorganized aprismatic and hypoplastic enamel similar to that observed in KO mouse molars and incisors (Fig. 4C, Appendix Fig. E).

By microCT analysis, significant rescue was not observed in enamel density or volume for either TgCTRNC2KO or TgCTRNC29KO (Figs. 4E, 4F). Incisors from TgCTRNC18KO mice presented with heterogeneity, since 2 mice had incisors with no measurable enamel, while 7 mice had incisors with a small amount. Both the KO and transgenic mice had mixed genetic backgrounds, which may have contributed to phenotypic heterogeneity. Densities for molar and incisor dentin for KO and transgenic mice were

slightly increased compared with those in WT mice (Appendix Table 2).

The TgCTRNC18/het female mice with one active *Amelx* gene had no enamel, while the 2 TgCTRNC29/het females had enamel volume measurements by microCT intermediate between KO and TgCTRNC-positive mice with a WT background (not shown).

DISCUSSION

In a study of 52 mammalian amelogenin genes, the C-terminal 10 amino acids encoded by exons 6 and 7 were identical (Delgado *et al.*, 2007). The C-terminal deletion presented a dominant-negative phenotype in TgCT-RNC18 transgenic mice where all WT amelogenins were expressed, and the enamel defect was severe when high levels of transgenic protein were present. It is unlikely that the phenotype was due primarily to protein over-expression, since transgenic mice that express TRAP (tyrosine-rich amelogenin peptide), LRAP (leucine-rich amelogenin peptide), or M180 had minimal disruption in their enamel structure (Chen *et al.*, 2003; Paine *et al.*, 2004; Gibson *et al.*, 2007).

TgCTRNC18 molars had decreased enamel thickness and abnormal enamel near the surface, but organization below the surface was similar to that in WT mice. Both density and volume were substantially affected when the amount of transgenic protein present was elevated, as in TgCTRNC18 molars.

MMP20, the enamelysin protease that processes enamel proteins during the secretory stage, begins processing by cleaving the C-terminus from the 180-amino-acid amelogenin (Simmer and Hu, 2002). Mice that lack MMP20 have enamel defects, and it is assumed that the amelogenin C-terminus is retained longer than appropriate in these mice (Caterina *et al.,* 2002). Three human *MMP20* mutations lead to the hypomaturation type of AI, where the enamel has retained excess organic material (Wright, 2006). We assumed therefore that the amelogenin C-terminus is required both to be present upon secretion and to be appropriately cleaved during the secretory stage of amelogenesis for enamel to develop normally.

By adsorption affinity and inhibition of crystal growth assays, full-length native and recombinant amelogenins bind mineral *in vitro,* and binding is dependent on the C-terminus (Aoba *et al.,* 1987; Moradian-Oldak *et al.,* 2002). The 180-amino-acid amelogenin also has regions that direct assembly *in vitro* into structures that have been termed "nanospheres", which are approximately 20-nm spherical structures when analyzed by dynamic light-scattering, or atomic force or transmission electron microscopy (Fincham *et al.,* 1995). The amelogenin C-terminus is required for

normal nanosphere organization *in vitro*, since nanospheres that assembled from amelogenins lacking the C-terminus were larger than expected (Moradian-Oldak *et al.*, 2002). In addition, nanospheres can form supramolecular structures *in vitro*, referred to as nanochains, and these assemblies are blocked when the C-terminus is not present (Moradian-Oldak and Goldberg, 2005). Although the protein complement of nanospheres *in vivo* is not clear, mineral formation is promoted in the *in vitro*-assembled nanospheres in a concentration-dependent manner (Wen *et al.,* 2000).

KO mice, which express none of the amelogenin proteins, lack prismatic enamel structure and have only a thin enamel layer (Gibson *et al.*, 2001). When TgM180 mice that express the most abundant 180-amino-acid amelogenin were mated with KO mice, enamel volume, thickness, and density, measured by microCT, improved significantly in transgene-positive male offspring, revealing partial phenotypic rescue (Li *et al.*, 2008). However, when the TgCTRNC mice were mated with KO for a similar rescue experiment, no obvious rescue was observed. Because neither high- nor low-copy-number transgenic mice were able to rescue the KO phenotype of either molars or incisors, we find that the lack of the C-terminus is inconsistent with the development of a normal enamel layer, regardless of the amount of protein produced.

In conclusion, we describe a mouse model in which a single amelogenin, which is able to rescue the KO phenotype, loses that function with deletion of 13 amino acids at the C-terminus. Using this *in vivo* approach, we examined enamel development in the presence of the mutated transgene, both with and without the normal complement of amelogenin proteins, which revealed a dominant-negative effect. It will be interesting in future studies to evaluate nanosphere size in the transgenic and rescue mouse models, and the associated effect on crystal sizes and enamel rod development.

ACKNOWLEDGMENTS

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