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The Importance of Serine Phosphorylation of Ameloblastin on Enamel Formation

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ABSTRACT

FAM20C is a newly identified kinase on the secretory pathway responsible for the phosphorylation of serine residues in the Ser-x-Glu/pSer motifs in several enamel matrix proteins (EMPs). *Fam20C*-knockout mice showed severe enamel defects that are very similar to those in the ameloblastin- (*Ambn*) knockout mice, implying that the phosphoserines (pSers) may have critical role in AMBN function. To test this hypothesis, we generated amelogenin (*Amel*) promoter-driven *Ambn*-transgenic mice, in which ser^{48}, ser^{226} and ser^{227} were replaced by aspartic acids (designated as "D-Tg") or alanines (designated as "A-Tg"). The negative charge of aspartic acid is believed to be able to mimic the phosphorylation state of pSer, while alanine is a commonly used residue to substitute Ser due to their similar structure. Using Western immunoblotting and quantitative PCR methods, we identified transgenic lines expressing transgene somewhat higher (Tg+) or much higher (Tg++) than endogenous *Ambn*. The lower incisors collected from 7-d- and 7-wk-old mice were analyzed by histology, SEM, immunohistochemistry, and Western immunoblotting methods to examine the morphology and microstructure changes in enamel, as well as the expression pattern of EMPs. Both the A-Tg+ and A-Tg++ mice displayed severe enamel defects in spite of the expression level of transgenes, while the D-Tg+ and D-Tg++ mice showed minor to mild enamel defects, indicating that the D-Tg transgenes have less disturbed the enamel formation than did the A-Tg transgenes. Our results suggest that the phosphorylation state of pSers is likely an essential component for the integrity of AMBN function.

Key words: ameloblastin, phosphorylation, phosphoserine, FAM20C, enamel, transgene

INTRODUCTION

Dental enamel is the most highly mineralized hard tissue that is unique both in its composition and in its formation. Enamel formation is a strictly controlled step-wise process in which ameloblasts continuously secrete organic extracellular matrix while slowly moving away from the dentinal surface until the desired thickness of the matrix is achieved. In mammals, the enamel formation is artificially divided into presecretion, secretion and maturation stages (Hu et al. 2007). During the secretion stage, ameloblasts secrete the enamel matrix proteins (EMPs). These include amelogenin (AMEL), ameloblastin (AMBN), and enamelin (ENAM) and matrix metalloproteinase-20 (MMP20), a calcium-dependent peptidase that cleaves newly secreted EMPs into various derivative fragments (Bartlett et al. 2004; Simmer et al. 2012). Amelogenin represents 90% of the matrix deposited whilst ameloblastin and enamelin comprise the majority of the remaining 10% of the organic matrix.

In humans, the hypomineralization or hyploplastic classes of amelogenesis imperfecta (AI) have been related to mutations in *AMELX* (Hart et al. 2002; Kim et al. 2004; Stephanopoulos et al. 2005; Urzúa B et al. 2011), *ENAM* (Hart et al. 2003; Hu and Yamakoshi 2003; Kim et al. 2005; Stephanopoulos et al. 2005), and recently, *AMBN* (Poulter et al. 2014) genes. The mouse model in which exons 5 and 6 are deleted from *Ambn* showed severe hypoplastic AI that had no enamel formation on the tooth surface (Fukumoto et al. 2004). *Ambn* overexpression in transgenic mice resulted in abnormal enamel crystallite formation and enamel rod morphology (Paine et al. 2003; Chun et al. 2010), or a generally normal enamel structure when the transgene expression was only somewhat higher than normal (Chun et al. 2010).

Evolutionary analyses have classified AMEL, AMBN, and ENAM into a family named the secretory calcium-binding phosphoproteins (SCPP), which have one or more Golgi casein kinase phosphorylation sites that are recognized by their distinctive Ser-x-Glu/pSer (S-x-E/pS) motifs (Brunati et al. 2000; Kawasaki and Weiss 2003). Family with sequence similarity member 20-C (FAM20C) is a newly discovered kinase localized to the Golgi that is believed to be the genuine casein kinase phosphorylating SCPP proteins (Ishikawa et al. 2012; Tagliabracci et al. 2012). *Fam20C*-knockout mice exhibit severe enamel defects that are very similar to those in the *Ambn*- or *Enam*-knockout mice (Wang et al. 2012; 2013), suggesting that the phosphorylation of serines (pSers) in EMPs may be an essential post-translational modification required for their proper function.

The consensus sequences of S-x-E/pS motifs are strictly conserved in EMPs (Hu et al. 2005; Al-Hashimi N et al. 2010). Three putative pSer residues have been identified in the AMBN of pig (Ser^{17}, Ser^{209} and Ser^{210}), mouse (Ser^{48}, Ser^{226} and Ser^{227}), and human (Ser^{17}, $Ser^{235}$and Ser^{236}). A recent bioinformatic study (Delsuc et al. 2015) indicated that these pSers are highly conserved in AMBN across the species during evolution and thus may have important roles for AMBN function. To examine the functional significance of the pSers in AMBN, we generated *Amel* promoter-driven *Ambn*-transgenic mice, in which $pSer^{48}$, $pSer^{226}$ and $pSer^{227}$ of the exogenous AMBN were replaced by alanines (Ala) or aspartic acids (Asp) to eliminate or mimic the phosphorylation state of pSers.

MATERIALS & METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M-Baylor College of Dentistry (Dallas, TX, USA) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The plasmid used for generating *Ambn* transgenic constructs was a gift from Dr. Jan C-C. Hu (Department of Biologic and Materials Sciences, University of Michigan School of Dentistry) (Chun et al. 2010). We mutated the cDNA sequences coding Ser^{48}, Ser^{226}, and Ser^{227} in the *Ambn* transgene into those encoding Ala (designated as A-Tg) or Asp (designated as D-Tg). The 7.5-kb *Ambn* transgenes were released from the mutated constructs by restriction digestion with *Not*I-*Srf*I and microinjected into fertilized C57BL/6 oocytes and transferred to recipients at the Transgenic Core Facility of the University of Texas Southwestern Medical Center at Dallas. In total, 6 lines of A-Tg mice and 5 lines of D-Tg mice were generated and mated with C57BL/6 mice. Germline transmission was determined by PCR analyses of genomic DNA obtained from tail biopsies using transgene specific primers, as described previously (Chun et al. 2010). The mice used for enamel morphometric analyses were fed with gel food after weaning.

Assessment of *Ambn* Tg Expression by Western Blotting and Quantitative PCR (qPCR)

To determine the protein expression levels of *Ambn* transgenes, the molars dissected from 5-day-old mice (3 for each group) were ground into powder in liquid nitrogen and extracted using RIPA buffer (ThermoFisher Scientific, Waltham, MA, USA) containing a proteinase inhibitor cocktail (Roche, Indianapolis, IN, USA). After bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific), equal amounts of lysates from wild type (WT) and

transgenic mice were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting using anti-AMBN antibody (1/1000) (SC-50534, Santa Cruz Biotechnology, CA, USA) and anti-β-ACTIN antibody (1/3000) (SC-2004, Santa Cruz Biotechnology). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The blots were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, NJ, USA), according to the manufacturers' instructions.

We also examined the expression levels of ENAM, and AMEL in the molar lysates by Western blot using anti-ENAM (1/1600) (Brookes et al. 2011) and anti-AMEL (1/400) (SC-32892, Santa Cruz Biotechnology).

To determine the transcriptional levels of *Ambn* transgenes, total RNAs were isolated from the molars of 5-d-old mice (3 for each group) using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and converted into cDNAs using a Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Quantitative real-time PCR was performed using a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) using SYBR Green Master Mix (Stratagene, La Jolla, CA, USA), as previously described (Wang et al. 2012). The Ct values were normalized to the reference gene 18s rRNA and expressed as fold-changes over the WT controls. The primers for qPCR analysis of *AMBN* and 18s RNA were purchased from SABiosciences (SABiosciences, Frederick, MD, USA).

Plain X-ray and Backscatter Scanning Electron Microscopy (SEM)

The lower jaws dissected from 7-week-old mice (3 for each group) were fixed in 4%

paraformaldehyde in PBS overnight and analyzed with plain X-ray radiography (Faxitron Bioptics, Tucson, Arizona, USA). Then, the mandibles were dehydrated through gradient concentrations of ethanol (70%–100%) and embedded in methylmethacrylate. The samples were cut at the position of the first lower molar to cut the incisor transversely, coated with carbon, and examined by field emission scanning electron microscopy (Philips XL30, FEI Company, OR, USA), as previously described (Wang et al. 2015)

Histology and Immunohistochemistry (IHC) Analyses

The mandibles dissected from 6-day-old mice (3 for each group) were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, then decalcified in 8% EDTA/PBS (pH 7.4) at 4°C for 4 d and embedded in paraffin. Five μm serial sections were prepared for H&E staining and IHC staining, as described previously (Wang et al. 2013). The primary antibodies used for IHC staining of AMBN, ENAM, and AMEL were the same as those used for Western blot analyses.

RESULTS:

Establishment of A-Tg and D-Tg *Ambn* Transgenic Lines

In total, 6 lines of A-Tg mice and 5 lines of D-Tg mice were confirmed germline transmission. We examined the expression levels of A-Tg and D-Tg in the transgenic lines using qPCR method followed by Western immunoblotting. Four A-Tg lines and three D-Tg lines were identified overexpressing the *Ambn* transgenes (Table S). The lines overexpressing the transgenes ~2-4 folds over WT mice were designated as A-Tg+ and D-Tg+, while those

overexpressing the transgenes ~6-8 folds over WT mice were designated as A-Tg++ and D-Tg++) (Fig. 1) (Table S).

The Gross Enamel Defects in the A-Tg and D-Tg *Ambn* Transgenic Mice

The 7-week-old A-Tg+ mice showed extensive enamel defects with discolored pits and dents on the surface of incisors (Fig. 2) and molars (data not shown). X-ray analysis revealed that the incisal edge of A-Tg+ mice appeared to have lost the characteristic chisel-like incisal tip exhibited by WT mice; a phenomenon that appeared even more prominent in the A-Tg++ mice (Fig. 2) (Table S). The enamel of D-Tg+ mice did not show apparent gross defects except for sporadic pits on the maxillary incisors (Fig. 2), while the enamel of D-Tg++ mice displayed a broader range of defects, which, however, appeared to be much milder than those in the A-Tg mice (Fig. 2) (Table S).

The Enamel Microstructure of A-Tg and D-Tg *Ambn* Transgenic Mice

Backscattered SEM analyses revealed a reduced thickness, disorganized enamel rods and interrod structure, mixed with an amorphous middle layer in the enamel of A-Tg+ and A-Tg++ mice compared with the well-organized enamel structure in WT mice (Fig. 3). The enamel microstructure of D-Tg+ mice appeared more ordered than in A-Tg mice but was less well organized than WT. The enamel microstructure of D-Tg++ mice showed more disturbance than D-Tg+ mice (Fig. 3), but overall was better organized compared with that of the A-Tg mice.

The Histological Defects of Enamel in A-Tg and D-Tg *Ambn* Transgenic Mice

The ameloblasts of 7-day-old A-Tg++ and D-Tg++ mice started to exhibit an abnormal

morphology at the presecretory stage (Figs. 4 and S) (Table S). At the secretory stage, the A-Tg+ mice showed malformed enamel matrix protruding into the ameloblast layer; the A-Tg++ mice displayed disturbed structures in both enamel matrix and ameloblasts. In contrast, the D-Tg+ and D-Tg++ mice exhibited a generally normal ameloblast morphology and enamel matrix microstructure, except for uneven staining and inconsistent thickness at some locations along the enamel matrix (Figs. 4 and S) (Table S).

ENAM and AMEL Expression in the A-Tg and D-Tg *Ambn* Transgenic Mouse Molars

Both the A-Tg and D-Tg mice showed reduced amounts of ENAM intermediate cleavage fragments migrating at about 50-80 kDa compared with those in the WT mice. The A-Tg mice had a similar amount of 32 kDa ENAM fragments with the WT mice, while the D-Tg++ mice displayed an increased amount of these fragments (Fig. 5). Both A-Tg and D-Tg mice showed less than normal amounts of AMEL cleavage fragments (Fig. 5).

DISCUSSION

In previous studies, we demonstrated that inactivation of FAM20C, a kinase localized to the Golgi apparatus that phosphorylates the Ser residues in enamel matrix proteins, led to severe enamel defects that are similar to those in the *Ambn*-knockout mice (Wang, Wang, Lu, et al. 2012; Wang, Jung, et al. 2013), suggesting that the phosphorylation state of pSers may be essential to AMBN function. In this study, we generated *Ambn* transgenic mice in which the pSers in the *Ambn* transgene were eliminated by replacing the pSers with Ala, or mimicked by substituting the pSers with Asp. Ala is the most commonly used amino acid to substitute Ser

residues due to their similar structures (Dayhoff et al. 1978), while Asp, with its negatively charged side chain, is frequently used for mimicking pSer (Thorsness and Koshland 1987; Pearlman et al. 2011).

Previous study (Chun et al. 2010) showed that overexpression of wild type *Ambn* led to minor or mild enamel defects when the transgene dosage was slightly higher (~3 folds based on the Western blot) or much higher (~6-9 folds based on the Western blot) than normal. As transgene is randomly inserted into the genomic DNA, no two transgenic lines are genetically identical whereas their phenotypes could be very similar. In this regard, the mice overexpressing WT *Ambn* (Chun et al. 2010) can serve as controls in comparison with those overexpressing the mutant versions. In our study, D-Tg^{+} and D-Tg^{++} mice showed minor and mild enamel defects when the transgene dosage was slightly higher (~3 folds) and much higher (~7 folds) than normal. The correlation between the gene dosage and enamel phenotype in D-Tg mice was very similar to that in the controls. In contrast, overexpression of A-mutant *Ambn* consistently led to severe enamel defects in spite of the expression levels, though the severity of defects increased with gene dosage (Table S). These results suggest that the enamel defects in D-Tg mice are mainly associated with the transgene dosage, while those in A-Tg mice are mainly due to the mutation. Theoretically, it might be assumed that compared to a Ser-to-Asp substitution, a Ser-to-Ala substitution would have a smaller effect on protein conformation or function due to the similarity in structure between Ser and Ala, but only if the posttranslational modifications (such as phosphorylation) associated with the Ser residue are not of significant importance. Since aspartic acids can (partially) mimic the phosphorylation state of pSers, our results outlined above (i.e. D-Tg++ mice were relatively unaffected) suggests that the

phosphorylation of the pSers may be an important functional post translational modification.

To date, the genetically engineered AI mouse models have faithfully recapitulated the phenotypes and inheritance manners of human AI. The *Ambn*- and *Enam*-mutant subjects develop autosomal dominant AI in a dose-dependent manner, while those bearing *Amel* mutations show X-linked traits. *Ambn* knockout (Fukumoto et al, 2004) and rescue (Chun et al, 2010) studies suggest that the AI caused by AMBN truncation was most likely due to haploinsufficiency. However, it remains unclear whether point mutations of AMBN can interfere with WT protein in a dominant negative manner, as point mutation of *Ambn* has not been reported in humans and animals. In our study, the overdose of A-mutant AMBN caused severe enamel defects, while overexpression of D-mutant protein only resulted in mild defects at the similar dosages, suggesting that the A-mutant AMBN had interfered with WT protein in an unknown manner (such as dominant negative) whereas the D-mutant AMBN acted more like the WT version.

The enamel defects of *Fam20C* null mice are very similar to those in the *Ambn* null mice; both showed severe defects in ameloblasts and enamel matrix and thus impeded enamel formation on the dentin surface. In contrast, the A-Tg mice did not show severe DEJ defects, and the enamel defects were milder than those in *Ambn* null and *Fam20C* null mice, suggesting that the endogenous AMBN may alleviate the enamel defects, or the mutant protein did not eliminate AMBN function. It remains elusive the role of pSers in proper mineralization, cell-amblastin interaction and ameloblastin-amelogenin interactions; stepwise and tremendous works are needed in future studies using knockin, cell biology, biochemistry, and biophysics approaches.

A limitation of this study is that the transgenic mice also express endogenous *Ambn* as well as the A-Tg and D-Tg transgenes, which added complexity to the interpretation of the results.

It remains unclear how the mutant AMBN proteins interacted with the endogenous AMBN and how the latter's function may have been affected in the transgenic mice. A further limitation is that we cannot rule out the possibility that AMBN undergoes a toxic gain of function when Ser are replaced by Ala. If this were the case, one other possible explanation for the results obtained may be due to the toxic effects of A-Tg on amelogenesis (acting either in the extracellular matrix or intracellularly). D-Tg may simply be a harmless bystander while amelogenesis proceeds relatively normally due to the presence of WT AMBN.

These limitations can be overcome in future studies where the effect of endogenous AMBN has been eliminated by generating knockin mice or crossbreeding the A-Tg and D-Tg mice with *Ambn*-knockout mice and thus may provide a more clear answer to the role of pSers in AMBN function.

The A-Tg and D-Tg mice also displayed less amounts of 20-kDa AMEL fragments and 50-80 kDa ENAM fragments in the molars compared with those in the WT mice. However, it remains unclear whether the reduction of these intermediate fragments has contributed to the enamel defects, because we did not identify a consistent correlation between the severity of enamel defects and the degree by which expression of these proteins were reduced. The enamelin data obtained in this study contrasted with previous data obtained using this specific enamelin antibody. Brookes et al. (2011) showed that the antibody failed to recognize enamelin at 32 kDa whereas in our hands clear cross reactivity was observed. This interesting difference may be due to the fact that we have analyzed developing mouse molars whereas Brookes et al. analyzed rat lower mandibular incisors. This raises a possibility that enamelin is processed differently in rats and mice. Alternatively, it may be processed differently

depending on tooth type.

In summary, our results suggest that the pSers in S-x-E motifs are likely essential components for normal AMBN function.

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FIGURE LEGENDS

Fig 1. Evaluation of *Ambn* A-Tg and D-Tg expression levels in the lower molars from 5-d-old mice.

(A) Western immunoblotting identified A-Tg and D-Tg lines in which the total AMBN expression levels were somewhat higher (A/D-Tg+) or much higher (A/D-Tg++) than WT mice. **(B)** Quantitative PCR analysis confirmed that the transcriptional levels of *Ambn* transgenes were ~2-3 fold or ~5-7 fold higher than WT in each line. **(C)** Immunohistochemistry staining demonstrated the location of AMBN protein in both ameloblasts and enamel matrix in the lower incisor from 7-d-old mice. Scale bars: 100 μm.

Fig 2. Gross enamel defects in the lower incisors of 7-wk-old *Ambn* A-Tg and D-Tg transgenic mice.

(A) The A-Tg+ mice showed extensive discolored dents and pits on the enamel surfaces (arrows). The A-Tg++ mice displayed chalky white enamel with patchy lesions on the enamel surfaces (arrows). The enamel of D-Tg+ mice was generally normal except for sporadic discolored lesions on the surfaces (arrow). The enamel surfaces (arrow) of the D-Tg++ mice were more affected than the D-Tg+ mice but not to the same extent as in A-Tg+ and A-Tg++ mice. **(B)** Lateral X-rays of WT and D-Tg+ incisors both displayed a similar smooth enamel surface, enamel of the A-Tg+, A-Tg++, and D-Tg++ mice appeared ridged with evidence of hypoplasia (arrows), with the A-Tg++ mice being the worst affected.

Fig 3. The enamel microstructure of 7-wk-old A-Tg and D-Tg *Ambn* transgenic mice.

(A)-(E) Backscatter SEM analyses of the cross-sections obtained from a cut through the mesial roots of the first lower molar. **(F)-(J)** Higher magnification of the boxed areas in A-E.

The enamel surfaces of A-Tg+ and A-Tg++ mice were hypoplastic compared with WT and D-Tg mice. **(K)-(O)** Higher magnification of the boxed areas in F-J. Compared to the well-organized enamel rods in WT mice, the enamel rods and interrod structures in A-Tg+ mice and A-Tg++ mice were severely disturbed; an area of reduced structural detail (arrows) was located in the middle layer of enamel in these mice. In contrast, the enamel of D-Tg+ mice and D-Tg++ mice showed overall a better microstructure than that in A-Tg mice, except that the D-Tg mice showed a less defined interrod structure and the enamel rods of D-Tg++ mice were slightly disturbed. The D-Tg mice appeared to have a higher/accelerated mineralization in the enamel matrix at the stage shown in this section. The enamel-dentin junction in the D-Tg++ mice appeared to have a higher mineralization (arrow). Scale bars: 1mm in A-E, 250 μm in F-J, 10 μm in K-O.

Fig 4. Histology of the enamel defects in A-Tg and D-Tg *Ambn* transgenic mice.

(A)-(E) H&E staining on the sagittal sections of lower jaws from 7-d-old WT, A-Tg and D-Tg mice. In the A-Tg mice, the ameloblasts were separated from the enamel matrix at indicated locations in both molars and incisors (arrows). **(F)-(J)** Higher magnification of the black boxed areas (~ late secretory stage) in A-E. The A-Tg+ mice had malformed enamel matrix invading the ameloblast layer (arrows). The A-Tg++ mice showed disorganized enamel matrices and malformed ameloblasts (arrow). The D-Tg+ and D-Tg++ mice showed generally normal ameloblast structure and normal enamel matrix, except for uneven staining and inconsistent thickness of enamel matrix at indicated locations (arrows). **(K)-(O)** Higher magnification of the black boxed areas (~ presecretory or early secretory stage) in A-E. The ameloblasts of A-Tg++ mice showed disorganized morphology (arrow) compared with the

generally normal ameloblasts in the other lines of *Ambn* transgenic mice. Scale bars, 500 μm in A-E, 100 μm in F-O.

Fig 5. The expression patterns of ENAM and AMEL in 5-d-old A-Tg and D-Tg *Ambn* transgenic mice.

(A) Western immunoblotting of ENAM showed reduced amounts of 50-80 kDa intermediate fragments in the lower molars of A-Tg and D-Tg mice compared with the WT mice. The D-Tg++ mice appeared to have more 32-kDa fragments. **(B)** Western immunoblotting of AMEL showed less amounts of 20-kDa fragments in the lower molars of A-Tg and D-Tg mice than WT mice, and the reduction appeared reversely related to the expression levels of *Ambn* transgene. **(C)** Immunohistochemical staining of ENAM (left column) and AMEL (right column) at seceretory stage of the lower incisors from 7-d-old mice. Scale bars: 100 μm.

Fig S. Histology of the enamel defects in additional lines of A-Tg and D-Tg *Ambn* transgenic mice.

(A) H&E staining on the sagittal sections of lower jaws from 7-d-old A-Tg mice line 3 (~6.5 folds overexpression of A-Tg). The mutant mice showed disorganized enamel matrices (En) and malformed ameloblasts (Am). **(B)** H&E staining on the sagittal sections of lower jaws from 7-d-old A-Tg mice line 4 (~7.3 folds overexpression of A-Tg). The enamel matrices (En) were severely disorganized; the ameloblasts were malformed and peeled off from the enamel matrices. **(C)** H&E staining on the sagittal sections of lower jaws from 7-d-old D-Tg mice line 3 (~8.2 folds overexpression of D-Tg). The D-Tg mice showed uneven staining and inconsistent thickness of enamel matrix that protruded into the ameloblasts. **(D)** H&E staining

on the sagittal sections of lower jaws from 7-d-old D-Tg mice line 4 (the transgene was not overexpressed in ameloblasts). The D-Tg mice showed generally normal ameloblasts and enamel matrix.