Altering Biomineralization by Protein Design*\S

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To create a bioceramic with unique materials properties, biomineralization exploits cells to create a tissue-specific protein matrix to control the crystal habit, timing, and position of the mineral phase. The biomineralized covering of vertebrate teeth is enamel, a distinctive tissue of ectodermal origin that is collagen-free. In forming enamel, amelogenin is the abundant protein that undergoes self-assembly to contribute to a matrix that guides its own replacement by mineral. Conserved domains in amelogenin suggest their importance to biomineralization. We used gene targeting in mice to replace native amelogenin with one of two engineered amelogenins. Replacement changed enamel organization by altering protein-to-crystallite interactions and crystallite stacking while diminishing the ability of the ameloblast to interact with the matrix. These data demonstrate that ameloblasts must continuously interact with the developing matrix to provide amelogenin-specific protein to protein, protein to mineral, and protein to membrane interactions critical to biomineralization and enamel architecture while suggesting that mutations within conserved amelogenin domains could account for enamel variations preserved in the fossil record.

In mammals mesenchyme-derived bone is the dominant biomineralized tissue and is composed of substituted hydroxyapatite crystals with specific proteins distributed in an abundant collagen network (1–3). For bone, the mineral composite is dynamic with both the protein and mineral phases being constantly remodeled by cells. A notable exception to such a biomineralization theme is found in enamel, the vertebrate covering of teeth. In teeth, ectoderm-derived ameloblasts synthesize a short-lived, diverse repertoire of proteins that self-assemble to produce a matrix that directs its own replacement by the mineral phase (4, 5). Mature enamel is a bioceramic composed of less than 0.5% protein that is dispersed among the longest hydroxyapatite crystals in the vertebrate body (6–8). Unlike mesenchyme-derived bone, enamel does not contain collagen, does not have the capacity of self-repair, and does not undergo remodeling. Nonetheless, enamel lasts the lifetime of the organism due to its unique material properties and remarkable resistance to failure (9, 10). These properties are due in part to the cell-directed organization of crystallites into bundles called rods. Rods are further woven together during their formation by cell migration to form a unique three-dimensional lattice architecture that resists wear and deformation (9–14).

Amelogenin is the most abundant of the enamel matrix proteins and undergoes self-assembly to form nanospheres (15–17). Amelogenin nanospheres appear to control the habit of hydroxyapatite crystallites by directing mineral growth to the ends (c’ axis) of the crystallites by providing the space for crystallites to grow within and by buffering the local environment against protons liberated during mineral deposition (5, 7, 18–20). The amino acid sequence of amelogenin is highly conserved among diverse vertebrate species, suggesting that conserved modular domains impart specific functions during biomineralization (4, 16, 21, 22). To clarify the role that two of the conserved amelogenin domains contribute to the matrix that guides enamel biomineralization, we created knock-in mice by inserting an engineered amelogenin cDNA that lacked one of the two highly conserved domains. The resulting animals exhibited significant alteration to the organization of the enamel and to the cells that synthesize and interact with the organic matrix.

EXPERIMENTAL PROCEDURES

Compliance with Institutional Review Boards—All DNA manipulations complied with the University of Southern California Institutional Guidelines. The use of vertebrate animals in these studies complied with the University of Southern California Institutional Guidelines.

Homologous Knock-in for Engineered Amelogenin—The sequence of the mouse 180-amino acid amelogenin is shown in supplemental Fig. 1A, where the A- or B-domain is underlined for identification. In the case of the A-domain-deleted amelogenin, no other engineering was performed, and in the case of the B-domain-deleted amelogenin, three end-to-end hemagglutinin tags were used to replace the B-domain in order to aid in its subsequent identification. The targeting DNA construct (supplemental Fig. 1B) was engineered using standard recombinant DNA techniques (23) by adding either the A-domain- or B-domain-deleted amelogenin cDNA minigene after the second exon followed by the SV-40 poly-A-signal sequence withlox sites flanking either end of the Neo resistance gene. The length of the two homologous arms for the amelogenin genomic DNA, upstream or downstream to the engineered amelogenin-Neo minigene, corresponded to about 6 kbp at the 5’-end and 3.6 kbp at the 3’-end. The upper map depicts the wild type parental state, and the lower map depicts the knock-in state for the engineered amelogenin minigene. The physical
positions for the internal or external DNA hybridization probes are shown. The targeting construct was confirmed to nucleotide sequence across each restriction endonuclease junction by DNA sequence determination.

Stem Cell Manipulations—The mouse ES cell line 129/RW4 was grown in "knock-out" Dulbecco’s modified Eagle’s medium (Invitrogen) with 15% fetal bovine serum, 2 mM glutamine, 30 μg/ml of penicillin, 50 μg/ml of streptomycin, 0.1 mM β-mercaptoethanol, 1X nonessential amino acid, and 1000 units/ml mouse leukemia inhibiting factor (Chemicon) on inactivated mouse primary fibroblast cells. The ES cell medium was changed every day and split every 2 days. 25 μg of the targeting DNA was mixed with 1.1 × 10⁷ ES cells in phosphate buffered saline (PBS)³ and incubated at room temperature for 5 min before electroporation. Electroporation was performed in a 0.4-mm cuvette at 230 V/cm and 500 microfarads with a Bio-Rad Gene Pulser. After electroporation, 5 × 10⁶ cells with 10 ml of ES cell medium were plated onto 100-mm dishes on top of a layer of feeder cells. Selection was performed 24 h after electroporation by adding 300 μg/ml G418 to the ES cell medium for 7 additional days. Individual G418 resistant cell clones were physically isolated and separated by trypsin digestion. The trypsinized ES cells were cultured in 96-well plates with a feeder cell layer for 3 days.

Genomic DNA was recovered from ES cells digested with EcoR1 and hybridized with an external probe in Southern blot analysis to identify those cells that had undergone homologous recombination. Two positive knock-in ES cell clones with the A-domain-deleted amelogenin minigene (supplemental Fig. 1C, left panel) and five positive knock-in ES cell clones with the B-domain-deleted amelogenin minigene (supplemental Fig. 1C, right panel) were selected by PCR and confirmed by Southern hybridization. Two positive clones corresponding to the A-domain-deleted knock-in and two positive clones corresponding to the B-domain knock-in were chosen for blastocyst injection after confirming their normal karyotype. Blastocyst injections were performed at the University of California Irvine Transgenic Mouse Facility.

Verification of Knock-in Animals—Genomic DNAs from knock-in mice were analyzed by Southern blot hybridization revealed a 6-kbp band corresponding to the wild type state and a 3.6-kbp band corresponding to the homologous recombination state (supplemental Fig. 1D). A colony of animals corresponding to either the ΔA or ΔB knock-in state were maintained in barrier cages and expanded using standard breeding methods until homozygous animals (e.g. ΔA/ΔA and ΔB/ΔB) were achieved. Animals were created, housed, and sacrificed according to a University of Southern California Institutional Animal Care and Use Committee in full compliance with federal guidelines. Sentinel animals deployed in the vivarium were used to monitor the health status of the colony.

Reverse Transcription and Polymerase Chain Amplification—Total RNA from the mandible of newborn knock-in or wild type mice was extracted using Trizol reagent (Invitrogen). First strand cDNA was synthesized from 1 μg of total RNA using a reverse transcription-PCR kit (Invitrogen) according the recommendation of the manufacturer and served as the template for amplification using standard reagents (Qiagen). Sets of oligodeoxynucleotide primers were synthesized and correspond to the nucleotide sequence from the first exon to the sixth exon of the amelogenin gene (NM_009666) and were used to amplify the first strand cDNA. Primers (supplemental Fig. 2A) correspond to the following nucleotide sequence: Primer 1, the first exon forward primer, 5′-ATC AAG CAT CCC TGA GCT TCA GAC; Primer 2, the sixth exon reverse primer, 5′-AAC CAT AGG AAG GAT ACG GC TGC CT; Primer 3, the sixth exon reverse primer, 5′-AGC TCA GGA AGA ATG GGG GAC A. We identified no alternatively spliced products between the knock-in-engineered amelogenin minigene with the endogenous amelogenin expressed by ameloblasts from the ΔA/ΔA and ΔB/ΔB sources.

Analysis of Amelogenin Protein—Incisors and molars from 3-day postnatal knock-in or wild type mice were homogenized in lysis buffer consisting of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethyisulfonil fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate. Protein concentration was measured with a Bio-Rad protein assay based on bovine serum albumin as the standard. Equal amounts of total proteins (20 μg) were mixed with 2× loading buffer and size-resolved by electrophoresis in a 15% polyacrylamide gel. Size-separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp.) and immunoblotted with anti-amelogenin primary antibody. An enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) was used to identify the antibody-antigen complex and a record produced by exposure to radiographic film followed by development in an automated film processor (supplemental Fig. 2B).

Expression Levels of Engineered Amelogenin Transcripts—Total RNA was isolated from newborn mouse mandibles using Trizol reagent (Invitrogen) and recovered by precipitation. Total RNA was resolved by electrophoresis using a 1% agarose gel in the presence of 5% formaldehyde and transferred to a nylon membrane (Nytran Plus, Schleicher & Schuell). An amelogenin cDNA was used as the hybridization probe and was radioisotope-labeled with Random-Primer-It II kit (Stratagene). The membrane was stripped by emersion in boiling 1× SSC (0.15 M NaCl and 0.015 M sodium citrate) for 15 s and used in a second round of hybridization with a radioisotope-labeled murine β-actin cDNA to permit the signal intensity to be normalized with respect to total RNA loaded per lane. Signal intensity was measured by laser densitometry using a Kodak Image Station 1000 (supplemental Fig. 2C).

Real-time PCR Amplification Analysis of Transcript Abundance—The isolation of mouse mandibular RNA and first strand cDNA synthesis was performed as described above. The sequences of primer pairs for amplifying the amelogenin gene and ameloblastin gene are shown below. The forward primer is listed followed by the reverse primer. The primers for amplifying the amelogenin gene were 5′-AGC TTC AGA CAG AAA CTC ACT GAG C and 5′-GGA GGC AGG CAA ACA AAA

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²The abbreviations used are: PBS, phosphate-buffered saline; SEM, scanning electron microscopy; TRAP, tyrosine-rich amelogenin peptide; RFP, red fluorescent protein; LAMP-3, lysosomal-membrane-associated glycoprotein 3.
TCC. The primers for amplifying the ameloblastin gene were 5′-TGG GAG CAC AGT GAA TGT CAG C and 5′-CCA CTA GCT TGT TGA GGA AAT GCC. The primers for amplifying the murine β-actin gene were 5′-GGG AAA TCG TGC GTG ACA TC and 5′-GCC GCA GTG GCC ATC TC. The PCR amplification procedure followed the instructions provided by the manufacturer (Bio-Rad).

Enamel Alteration Observed in Knock-in Amelogenin Replacement Animals—Images of freshly dissected mouse incisors and molars were prepared, and photographic records from the lateral, labial, or occlusal viewing orientations were prepared.

Transmission Electron Microscopy—The incisors and molars from 3–4-day-old postnatal mice were fixed in Karnovsky fixative (6% glutaraldehyde and 4% paraformaldehyde in 0.2M cacodylate buffer for 1–2 h at 4 °C and dehydrated with ascending concentrations of ethanol. The specimens were placed in gradually increased concentration of epon in propylene oxide medium and embedded in 100% epon. The blocks were placed in a 65 °C oven for 36–48 h. The embedded teeth were sectioned with a glass knife on a MT 2-B ultramicrotome to obtain semiultrathin sections. The blocks were sectioned further, stained with paragon dye, and examined until the tissue blocks reached a site judged suited for examination using a transmission electron microscope. The specimen blocks were transferred to a Reichert-Jung ultramicrotome and sectioned with a diamond knife to cut ultrathin sections (6 nm) for examination. The ultrathin sections (6 nm) were double-stained with uranyl acetate and lead citrate and examined with an electron microscope at 80 kV.

Scanning Electron Microscopy (Fractured Enamel)—The lower incisors from 6-week-old knock-in or wild type mice were carefully dissected from the mandible. The labial surface of the incisors was notched by sawing with a thin diamond wheel across the long axis at sites corresponding to the junction of the secretary and maturing zone of enamel. The notched site was fractured by physical force. The two pieces of the incisor were glued onto a metal stub and coated for 5 min with an intermittent pulse using gold-palladium alloy in Hummer V set with the high voltage control knob at “9” to provide a coating thickness of ~100 Å. The enamel surface was visualized in a Cambridge 360 scanning electron microscope at the University of Southern California Center for Electron Microscopy.

Scanning Electron Microscopy (Forming Enamel)—To visualize the impression of Tomes’ processes extending into the forming enamel, the incisors from 3-day postnatal knock-in and wild type mice were carefully dissected from the mandible. The surrounding soft tissue from the teeth and the pulp tissue were physically removed. The teeth were washed with PBS, pH 7.4, and digested with 1% dispase for 2 h. The dispase-digested enamel organs containing the epithelial layers that contribute to the forming enamel were physically removed from the underlying forming enamel. The resulting cell-free enamel matrix was washed three times with PBS, pH 7.4, at room temperature with agitation, fixed with Karnovsky fixative, critical point-dried, and sputter-coated with gold. The surfaces were visualized in a Cambridge 360 scanning electron microscope at the University of Southern California Center for Electron Microscopy.

Western blot analysis was used to characterize the amelogenin protein(s) from knock-in and wild type mice (supplemental Fig. 2B) and identified only the appropriate protein(s)
expressed from each corresponding genotype. A 21-kDa band corresponding to the A-domain-deleted amelogenin protein lacking 40 amino acids from the amino terminus of amelogenin was found in the ΔA/ΔA mice (supplemental Fig. 2B, lanes 1 and 2). A 26-kDa band corresponds to the wild type M180 amelogenin along with several minor bands corresponding to alternatively spliced are observed (supplemental Fig. 2B, lanes 3 and 4). The 27-kDa protein is expressed from the B-domain-deleted amelogenin protein found in the ΔB/ΔB mice (supplemental Fig. 2B, lanes 5 and 6). The ameloblasts and the enamel matrix from the teeth of the resulting homozygous-female or hemizygous-male knock-in mice exclusively express the engineered amelogenin transcript (supplemental Fig. 2A, lanes 1 and 2 and lanes 5, 6, 11, and 12, respectively) and the engineered protein (supplemental Fig. 2B, lanes 1 and 2 and lanes 5 and 6, respectively) with no wild type or alternatively spliced amelogenin contribution to the matrix.

Northern blot analysis was used to determine the mRNA expression level for each of the knock-in replacement amelogenin minigenes. The amelogenin messenger RNA expression level from the ΔA/ΔA and ΔB/ΔB mouse teeth was observed to be as robust as the messenger RNA levels observed in wild type mouse enamel (supplemental Fig. 2C).

To independently confirm that there was no difference in the transcript abundance between the A-domain-deleted or B-domain-deleted engineered minigene knock-in animals compared with the wild type animal, real time reverse transcription and amplification was performed, and the threshold cycle function was used to establish transcript abundance (supplemental Table 1). We chose ameloblastin gene transcript abundance as an internal standard since ameloblastin is an enamel-specific product that is expressed during the same developmental period as is amelogenin. As an alternative to ameloblastin, we also used β-actin as an internal standard to identify variation in transcript abundance from the knock-in replacement versus the wild type animals. Based upon either the ameloblastin or β-actin internal standard, there was no statistical difference between the abundance of the ΔA or ΔB transcripts compared with the abundance of the wild type amelogenin transcript.

These data indicate that each of the two replacement-engineered amelogenin minigene was expressed at levels comparable with the wild type endogenous amelogenin as shown by Northern blot analysis (supplemental Fig. 2C), by real time polymerase chain amplification (supplemental Table 1), and by Western blot analysis (supplemental Fig. 2B). The finding that the knock-in replacement amelogenin is expressed as robustly as the endogenous amelogenin is consistent with the fact that the engineered replacement amelogenin uses the same transcriptional regulatory elements as does the endogenous amelogenin gene.

The teeth created with either of these engineered amelogenin proteins exhibited visible changes to the enamel. The enamel covering the incisor teeth is thinned in both the homozygous A-domain-deleted amelogenin (ΔA/ΔA) and in the homozygous B-domain-deleted amelogenin (ΔB/ΔB) compared with the incisors from wild type animals (supplemental Fig. 3). The incisors from ΔA/ΔA mice fractured during mastication (supplemental Fig. 3, B1), whereas the 6-week molars exhibited a shallow cusp structure (supplemental Fig. 3, B2) compared with wild type mouse molars (supplemental Fig. 3, C1 and C2). The incisors from the ΔB/ΔB animals also are fragile with incisal edge fractures occurring with mastication (supplemental Fig. 3, D1) compared with incisors from wild type animals (supplemental Fig. 3, C1 and C2). The molars from the ΔB/ΔB animals also showed accelerated wear (supplemental Fig. 3, D2). These findings indicate that the enamel from the ΔA/ΔA and ΔB/ΔB animal is more prone to fracture and more easily abraded with mastication than the wild type enamel.

Using transmission electron microscopy, we examined the relationship of the enamel protein matrix to the forming mineral crystallites. In wild type enamel, amelogenin protein assemblies as 15–20-nm nanospheres aligned along the developing hydroxyapatite crystallites (Fig. 1, B1) (15, 26). Images taken near the secretory end piece of ameloblasts from 4-day postnatal ΔA/ΔA mouse incisors showed an absence of 15–20 nm nanospheres (Fig. 1, A1). Amelogenin lacking the A-domain does not assemble into amelogenin multimers having the shape of nanospheres but, rather, remains monomeric (22). In addition, we observed numerous but irregularly arranged short-length crystallites in the ΔA/ΔA mouse incisors (Fig. 1, A1) compared with those from the wild type enamel (Fig. 1, B1). The images of enamel from ΔB/ΔB mouse incisors displayed larger nanospheres with heterogeneous diameters aligned along the mis-arranged crystallites (Fig. 1, C1).

Enamel architecture was surveyed using fractured cross-sections from 6-week-old mouse incisors observed by scanning electron microscopy (SEM) to visualize the fundamental building blocks of enamel, the enamel rods, and crystallite structures. We observed the enamel deposition of ΔA/ΔA animals (Fig. 1, A2) was reduced in thickness compared with the wild type mouse enamel (Fig. 1, B2), and the ΔA/ΔA enamel lacked an architecture of woven rods of crystallites. The enamel rod reflects the organization of the enamel matrix imposed by a single ameloblast with adjacent rows of ameloblasts weaving their matrix column past one another during development of the matrix. In the case of the enamel from ΔA/ΔA animals, the rod organization is disrupted, with the rod and interrod boundaries features missing (Fig. 1, A3).

The enamel from ΔB/ΔB mouse incisors (Fig. 1, C2) was reduced in overall thickness compared with the wild type (Fig. 1, B2). The arrangement and size of enamel rods was also abnormal in the ΔB/ΔB incisor teeth (Fig. 1, C2 and C3), indicating reduced interaction between the ameloblast and its column of matrix, resulting in diminished weaving of the matrix during development, a condition that is preserved with mineral replacement of the matrix. Within a rod of the ΔB/ΔB mouse incisor enamel, the enamel crystallites exhibited irregular size and organization, suggesting a loss of control over the crystallite habit and packing (27) (Fig. 1, C2 and C3).

For amelogenin, homologous recombination occurs on the X-chromosome. Because the X-chromosome is randomly inactivated in females (28), we were able to demonstrate the impact of the engineered amelogenin by evaluating the enamel architecture from ΔA/Wt or ΔB/Wt females. In such heterozygous females, some ameloblasts express the knock-in-engineered
The results show that altered enamel architecture is found only where the engineered amelogenin protein is expressed and that normal enamel architecture is "rescued" when the engineered amelogenin is silent. For this reason all data shown for the knock-in replacement amelogenin were performed on homozygous (females) or hemizygous (male) animals as noted.

To test the possibility that the ameloblast cells from the ΔA/ΔA or ΔB/ΔB mice lost their ability to synthesize and secrete amelogenin due to the changes we engineered into the A- and B-domain of amelogenin, we mated mice bearing a transgene (TRAP-RFP) composed from the tyrosine rich amelogenin peptide (TRAP) fused in-frame to a red fluorescent protein (RFP) with either of the two knock-in animals. Offspring bearing both the knock-in-engineered amelogenin replacement and the TRAP-RFP transgene demonstrate that amelogenin is synthesized and secreted using fluorescent microscopy. Tissue sections taken from the resulting animals demonstrate the accumulation of RFP-TRAP protein within the secretory pathway of ameloblasts and showed that TRAP-RFP was secreted into the enamel extracellular space of the ΔA/ΔA mice (supplemental Figs. 4, A3 and A4), the ΔB/ΔB mice (supplemental Fig. 4, C3 and C4), and the Wt/Wt mice (supplemental Fig. 4, B3 and B4), suggesting that the secretory process for ameloblasts from the knock-in animals did not suffer from deleterious effects from the engineered amelogenin.

To ensure that cell death did not contribute to the alterations observed in the forming matrix, we performed an apoptosis assay that demonstrated there was no change in the number of apoptotic figures occurring in either of the knock-in animals compared with those occurring in the wild type control animals (data not shown). Similarly, no gross differences in the cytoskeleton were observed between the inci...
Ameloblasts were examined in knock-in animals using immunohistochemistry (supplemental Fig. 4, A2 and C2) and real time reverse transcription-PCR (supplemental Table 1). These assays revealed no substantial differences in the amount or in the distribution of ameloblastin for either the ΔA/ΔA or ΔB/ΔB knock-in animals (supplemental Fig. 4, A2 and C2, respectively) compared with wild type animals (supplemental Fig. 4, B2), suggesting that the alteration to the ameloblasts’ processes are unlikely to be due to a loss of ameloblastin protein with its RGD motif.

Protein-to-protein interaction between amelogenin with CD63 (lysosomal-membrane-associated glycoprotein 3, LAMP-3) has been reported based on the use of the yeast two-hybrid assay with amelogenin used as the bait protein (32). CD63 has been shown to shuttle to the cell membrane where cooperative interactions between integrins and CD63 have been observed that result in changes to cell signaling (33–36). In addition, a tetraspanin family member, LAMP-1, has been proposed as the receptor for amelogenin where amelogenin splice variant isoforms serves in a signaling capacity during development (37, 38). Therefore, we used an antibody to CD63 to examine its distribution on ameloblasts during enamel development. In the case of wild type control enamel, CD63 is localized to ameloblast processes and to membrane-bound vesicles located in the cytoplasm of the ameloblasts (Fig. 2, B2, arrow). In the case of the enamel from animals with either of the two-engineered replacement amelogenin proteins, we observed a distinctive change in distribution of CD63 antigens. In the case of the enamel from ΔA/ΔA animals, CD63 is located on vesicles near the secretory end piece and throughout the cytoplasm (Fig. 2, A2, arrow). In the instance of the ΔB/ΔB animals, CD63 localization is lost from the secretory end piece, although its distribution remains pronounced in the cytoplasm (Fig. 2, C2, arrow).

The observation that the ameloblasts appeared to be less competent to interact with the enamel matrix prompted us to examine the ability of selected cells to interact with the enamel extracellular matrix. We choose to examine amelogenin in detail since it is the most abundant protein in the forming

Amelogenin protein lacks canonical integrin binding motifs; however, another rodent enamel protein, ameloblastin, does contain an RGD motif and has been shown to be involved with attachment of the ameloblasts to the matrix (29, 30). Therefore, we used anti-integrin α6 antibodies (31) to examine the distribution of integrin molecules localized to the ameloblasts’ processes. In wild type mouse ameloblasts, the ameloblasts’ processes extend into the organic enamel matrix with α6 integrin localized throughout the membrane extension that composes the secretory end piece (Fig. 2, B1). Tomes’ processes in the ΔA/ΔA mice are observed to be shorter, and the α6 integrin is restricted to a flat apical membrane extension for these ameloblasts (Fig. 2, A1). This finding is consistent with the morphology of Tomes’ processes seen in the forming matrix by SEM when the ameloblasts are removed (Fig. 1, A4 and A5). The morphology and arrangement of Tomes’ processes in ΔB/ΔB animals is also abnormal, with shortened Tomes’ processes (Fig. 2, C1) to which the α6 integrin is distributed at a multitude of foci in the secretory end piece. This finding is consistent with the morphology of Tomes’ processes seen in SEM images of the forming matrix when the ameloblasts are removed (Fig. 1, C4 and C5).

We also examined ameloblastin expression in the knock-in animals using immunohistochemistry (supplemental Fig. 4, A4 and C5). Tomes’ processes left in the forming matrix of incisor teeth from the ΔA/ΔA mice is irregular with squat, wide depressions that represent the altered shapes of the enamel rods surrounded by irregular fields of interrod enamel (Fig. 1, A4 and A5). These features suggest that several Tomes’ processes contributed to each impression perhaps due to their coalescence or that the shape of Tomes’ processes was altered, which led to a concomitant loss of rod and interrod boundaries. The impression of Tomes’ processes left in the forming enamel matrix from ΔB/ΔB mouse incisors (Fig. 1, C4 and C5) revealed multiple small and irregular shapes compared with wild type mouse incisors. These features suggest a loss of polarity to Tomes’ processes that result in a loss of rod to interrod boundaries. In both the ΔA/ΔA- and the ΔB/ΔB-forming enamel the alterations to the Tomes’ processes result in distortions to the rod and interrod boundaries that are seen in the arrangement of rods and crystallites from the fractured cross-sections of more mature enamel imaged by SEM (Fig. 1, A3 and C3, respectively). The observed changes to the morphology of Tomes’ processes in either of the engineered amelogenin animals demonstrate a loss of normal enamel rod-interrod architecture seen in the wild type amelogenin where the bundles of crystallites are woven into a tissue with unique materials properties (Fig. 1, B3 and B4).

Light-microscopic examination of tissue sections from incisors of 3-day postnatal animals was performed to directly observe the arrangement of ameloblasts and their secretory ends, the Tomes’ processes. In the wild type mice, the ameloblasts engaged in secretion of the enamel organic matrix are polarized with their cell bodies arranged in an orderly fashion with regularly arranged Tomes’ processes extending into the forming matrix (supplemental Fig. 4, B1). In the case of the ΔA/ΔA or ΔB/ΔB incisors, the ameloblast cells are polarized and appear normal except that the Tomes’ processes are shortened (supplemental Fig. 4, A1 and C1, respectively), confirming the altered impressions left by Tomes’ processes and seen by scanning electron microscopy (Fig. 1, A4 and A5, and C4 and C5, respectively).

Based upon these changes to the secretory ends of ameloblasts, we sought to determine whether changes to the cell surface receptors for various extracellular matrix molecules are observed in the knock-in condition. Amelogenin protein lacks
matrix. We used HeLa cells, Madin-Darby canine kidney cells, and a mouse ameloblast-like cell line (39) to measure the ability of these epithelial cells to recognize recombinant-produced wild type, ΔA-M180, or ΔB-M180 amelogenin protein coated to plates in assays that measured cell binding efficiency. Emdogain is a commercial preparation of proteins recovered from developing porcine enamel that contains amelogenin, ameloblastin, and other enamel proteins used here to replicate a complete matrix protein repertoire (40). The data reveals that each of the three cells tested plated less efficiently on amelogenin protein lacking the A-domain or the B-domain compared with wild type M180 mouse amelogenin to which each of the cell lines efficiently interacted (Fig. 2D).

**DISCUSSION**

Enamel is a unique tissue, being the only ectoderm-derived hard tissue in the vertebrate body. Enamel does not contain collagen. Enamel is a bioceramic composite with high aspect ratios for the length and width of the hydroxyapatite crystallites (8). The crystallites are organized under the control of a single ameloblast cell into bundles composed from thousands of crystallites arranged parallel to one another (4). In mice, the bundles of crystallites are further arranged by their long axis into alternating rows that are themselves woven together by a third group of crystallites whose long axis is normal to the direction of the alternating rows. This arrangement of the crystallites into bundles and the weaving of the crystallite bundles is genetically controlled and exhibit a species-specific architecture that has been used to identify fossil sources and their evolutionary relatives (41–43). The arrangement of the bundles of crystallites within enamel also contributes to the remarkable materials properties of enamel, such as wear and fracture resistance that are essential to mastication and nutrition (9, 10). Of further interests is the biological control over mineral deposition in a process occurring at physiologic pH and temperature.

Amelogenin is the most abundant protein of forming enamel, although other enamel proteins are known (4, 44) and others have only been recently identified (45). Of the known enamel proteins, amelogenin is the only one known to self-assemble into nanospheres that appear to encase the long axis of the hydroxyapatite crystallites based on either transmission electron microscopy or by atomic force microscopy (22, 46). Comparison of the amino sequence of amelogenin among diverse vertebrates identified several highly conserved domains that are implicated in the function of amelogenin to control crystallite organization and to control nanosphere assembly (21). We
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sought to examine the role of these highly conserved domains, anticipating their functional significance to enamel formation.

A knock-in gene targeting strategy allowed us to create mice that express a replacement-engineered amelogenin protein that lacked one of these two conserved domains implicated in biomineralization control. Ameloblasts from the teeth of these knock-in animals solely express the engineered amelogenin in the developing enamel. The resulting teeth show alterations from the normal condition of the enamel biocomposite at several levels of hierarchical organization, from the nanoscale to the mesoscale.

Transmission electron microscopy verified the altered self-assembly of the engineered amelogenin in developing enamel biomineralization. As anticipated, defects in the assembly of the enamel organic matrix created defects in the mineral phase that it replaces. The newly formed enamel from ΔA/ΔA animals lacked nanospheres structures. We interpreted the smaller but more numerous crystallites in the ΔA/ΔA to reflect additional nucleation sites unmasked by the absence of nanospheres and the irregular crystal shape in the ΔA/ΔA mice to result from the failure of nanospheres to guide growth to the long axis of the crystallite (6, 20, 47, 48). The B-domain of amelogenin contains a unique hydrophilic domain that serves to locate amelogenin nanospheres to the surface of forming crystallites (20, 49, 50) as well as serving to prevent amelogenin nanospheres from fusing to one another in vitro (22). The B-domain of amelogenin, thus, serves the important function of stabilizing the amelogenin assemblies and maintaining the amelogenin nanospheres as bead-like chains aligning along the crystallite ribbons (22). The absence of the B-domain in the ΔB/ΔB mice is interpreted to permit the collapse of the nanospheres into misshapen aggregates that further perturb forming crystallites (Fig. 1, C1).

Amelogenin also contains a lectin-like motif at the amino acid positions 34–45, which after secretion may allow amelogenin to interact with membrane glycoproteins resident to the Tomes’ processes of ameloblasts (51). Because ameloblasts secrete a rod of enamel matrix proteins, the ameloblasts simultaneously move away from the dental enamel junction toward what will become the tooth surface (52, 53). Ameloblast movement in combination with the lectin-like interaction between the wild type amelogenin with the ameloblast membrane is projected to create tension at the cell-matrix interface causing both the Tomes’ processes to elongate and the nanospheres to align along the path of ameloblast movement (26). The amelogenin in the ΔA/ΔA mouse lacks this lectin-like motif and, thus, is unable to directly interact with the membrane of ameloblasts, consistent with allowing them to shorten as is observed for Tomes’ processes from ΔA/ΔA mice (Fig. 1, A5). In the case of the ΔB/ΔB mouse (Fig. 1, C5) that retains the amelogenin lectin-like motif, permitting amelogenin to interact with the ameloblast membrane, we observe irregular size and arrangement for the Tomes’ processes consistent with the collapse of the nanospheres (22).

Amelogenin self-assembly to form nanospheres is essential to enamel biomineralization with proper self-assembly of the matrix being used to guide crystallite orientation and growth. The assembly of the organic matrix is evident at higher order scale features by also being responsible for organizing the crystallites into rods that are woven among their ameloblast neighbors during formation of the enamel organic matrix (13, 17, 27, 54). The absence of appropriate weaving of the matrix by ameloblasts and the interference with the organization of the crystallite bundles within a rod culminates in a defective enamel product that is subject to increased wear and fracture (supplemental Figs. 3, B1, B2, D1, and D2 and Fig. 1, A2, A3, C2, and C3).

Changes to the integrin and CD63 distribution in the ameloblasts from either the ΔA/ΔA or the ΔB/ΔB animals is interpreted to reflect alteration to the ability of the Tomes’ processes to maintain receptor–ligand contacts with the extracellular matrix. The reductions to such contacts changes the morphology of the Tomes’ membrane extension and are observed in SEM images (Fig. 1, A4, A5, C4, and C5). Wang et al. (32) identified the interaction between amelogenin and CD63 (also known as LAMP-3, a member of the tetraspanin superfamily member of lysosomal membrane proteins) based on the yeast two-hybrid assay with amelogenin (32). CD63 can also be localized to the cell membrane location, where its cooperative interactions with integrins has been observed to result in changes to cell signaling (33–36). In addition, another tetraspanin family member, LAMP-1, has been proposed as the receptor for amelogenin when an amelogenin splice variant isoforms serves in a signaling capacity during development (37, 38, 55). The genetic approach used here eliminated alternatively spliced amelogenin expression by these knock-in animals (supplemental Fig. 2).

Although synthesis of the enamel matrix is rapid, so too is its degradation. Protein degradation accompanies mineral replacement during enamel biomineralization and is initiated simultaneously with secretion (6, 56–60). Degradation efficiency for the enamel matrix is emphasized by the observation that only minute amounts of amelogenin or any other enamel protein can be recovered from mature enamel (6). With such extensive degradation of the organic matrix inextricably linked with biomineralization, a relationship with the lysosomal compartment of the ameloblast as exemplified by CD63 is likely to accompany these events. Moreover, the cell signals that are initiated through such membrane-anchored receptor–ligand events may also be important to monitor synthesis of the matrix because enamel thickness is recognized as a regulated event determined by species as well as by gender (61, 62). For the mice in this study with either of the two engineered replacement amelogenin proteins, the change to such signals appears to result in reduced enamel thickness (supplemental Fig. 3A; Fig. 1, A2 and C2).

An unforeseen consequence from the amelogenin protein engineering was the reduced interaction between the ameloblast and the matrix. The change in cell-matrix interactions led to poor bundling of the crystallites into rods as well as affected the interrod enamel, events that further degrade enamel architecture and create a bioceramic composite tissue of inferior materials properties that fractured and/or wore prematurely (supplemental Fig. 3, B1, B2, D1, and D2). Our experimental strategy identified this heretofore underappreciated interaction between the matrix and the cell secretory end piece that is likely to be mediated through integrin α6 or CD63 or a combination of these.
Engineering changes in the highly conserved amelogenin protein sequence has led to altered assembly properties of the matrix with defective replacement by the mineral phase as well as altered membrane receptor interactions between the ameloblast and specific ligands located within the extracellular enamel matrix seen in this study. These pathways have also been illustrated by experiments of nature, in which humans inheriting single amino acid (e.g. Thr-21 and Pro-41) mutations within the A-domain of amelogenin are affected with defective enamel formation, a disease known as amelogenesis imperfecta (63, 64). Previously, we used plasmon resonance spectroscopy, dynamic light scatter, and atomic force microscopy to show that recombinant amelogenin that phenocopies these single amino acid mutations from man also results in defective self-assembly for amelogenin (22, 65). Based upon the present gene replacement studies with engineered amelogenin in mice, we predict that altered interactions between the ameloblasts and the mutated amelogenin in the matrix of humans affected with amelogenesis imperfecta contribute to the defective mineralization and materials properties observed in their enamel.

Taken together these findings provide insight into ectoderm-directed biomineralization while providing a molecular basis for interpreting changes in enamel rod-interrod architecture preserved as part of the fossil record. The creation of a competent enamel organic matrix based upon conserved protein domains within amelogenin capable of directing self-assembly into nanospheres is a requirement to direct crystallite habit and packing. Gibson et al. (66), using a knock-out strategy for amelogenin, demonstrated that enamel rods and interrod did not form in the absence of amelogenin, but that mineralization did occur. In our experimental strategy, we instead altered nanosphere formation by removing conserved domains essential for self-assembly. In the case of the ΔA-engineered amelogenin animals, this resulted in unmasking nucleating sites that led to numerous, short, ill-formed crystallites (Fig. 1, A1). In contrast, the collapse of amelogenin nanospheres, as seen in the ΔB-engineered amelogenin animals, also led to misshapen crystallites that altered crystallite stacking with one another (27) (Fig. 1, C1).

Current evidence suggests that for enamel to be organized into rods of crystallites requires the presence of amelogenin, an event restricted to vertebrates by evolution (67, 68). Such an argument is supported by the genetic knock-out of amelogenin in mice in which the loss of enamel rods is observed (66). Our observations further suggest that changes to highly conserved domains within enamel matrix proteins, such as amelogenin, can also alter enamel architecture and thickness. In addition, these observations provide a molecular basis for interpreting the changes in cell-matrix interactions that alter the shape and orientation of enamel rod and interrod that occurred during evolution of teeth and became preserved in the fossil record (21, 69, 70).

Acknowledgments—We are grateful to our colleagues at the Center for Craniofacial Molecular Biology and elsewhere who have added so much to our work and to our understanding. We thank the anonymous reviewers and the editor for helpful suggestions.

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