A Transgenic Animal Model Resembling Amelogenesis Imperfecta Related to Ameloblastin Overexpression*

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Genetic diseases that affect tooth enamel are grouped under the classification amelogenesis imperfecta. Human pedigrees and experiments on transgenic and null mice have all demonstrated that mutations to the secreted proteins amelogenin, enamelin, and enamelysin result in visibly, structurally, or mechanically defective enamel. In an attempt to better define a physiologic function for ameloblastin during enamel formation, we have produced transgenic mice that misexpress the ameloblastin gene. These transgenic animals exhibit imperfections in their enamel that is evident at the nanoscale level. Specifically, ameloblastin overexpression influences enamel crystallite habit and enamel rod morphology. These findings suggest enamel crystallite habit and rod morphology are influenced by the temporal and spatial expression of ameloblastin and may implicate the role of the ameloblastin gene locus in the etiology of a number of undiagnosed autosomally dominant cases of amelogenesis imperfecta.

In 1996 a new member of the non-amelogenin, non-enamelin class of enamel proteins was simultaneously characterized by three different groups of investigators, two groups using rat incisors and one group using porcine teeth. In the United States, Krebsbach et al. (1) named it ameloblastin; in Sweden, Cerny et al. (2) named it amel; and in a joint study between investigators in Japan and the United States, Hu et al. (3) named it sheathlin. A definitive physiologic role for the ameloblastin protein in tooth development remains unknown. Immunologic identification of ameloblastin during secretory amelogenesis, the developmental stage at which the extracellular matrix is deposited and organized, reveals an ameloblastin distribution (within the enamel extracellular matrix) that follows the ameloblast outline, resulting in a “fish-net” partitioning (3). Ameloblastin can also be immunolocalized to Tomes’ processes, the highly specialized plasma membrane component of secretory ameloblast cells (4). Localization to Tomes’ processes has also lead to speculation that ameloblastin has a role to play in crystal nucleation (4, 5). The ameloblastin molecule has a “DGEA” domain that has been identified in collagen type VI as a recognition site for a-2 b-1 integrin (2, 6). Ameloblastin also contains a trombospondin-like cell adhesion domain, “VTKG” (7). These findings have lead to the speculation that ameloblastin might serve as part of the linkage between ameloblasts and the enamel extracellular matrix (2). Ameloblastin may be critical for retaining a registration between the secretory ameloblasts and the enamel organic matrix because a single ameloblast is responsible for the creation of its corresponding matrix.

Amelogenesis imperfecta (AI) is an inherited dental disease that affects enamel. Linkage analysis was performed on three Swedish families that were affected with an autosomal dominant variant of amelogenesis imperfecta that was clinically noted as a localized hypoplastic enamel phenotype (8). DNA microsatellite markers linked all three families to a defect at chromosome 4q (8). In the human genome, both ameloblastin and enamelin are localized to chromosome 4q11-q21 (1, 9–11), thus making both genes potentially responsible for an amelogenesis imperfecta. A gene mutation in enamelin has recently been identified in a localized hypoplastic autosomal dominant amelogenesis imperfecta family pedigree (12). To date, no definitive evidence links the ameloblastin gene to defective enamel. The human enamelysin gene maps to chromosome 11q22.3, and although no linkage data yet have implicated enamelysin to an amelogenesis imperfecta phenotype, enamelysin null animals do have defective enamel and a weakened dentin enamel junction (13). Overexpressing a gene in a tissue-specific manner is an effective method for determining the role of a protein in the context of a developing animal. We targeted ameloblastin overexpression to ameloblasts by generating transgenic animals in which ameloblastin expression was under the control of the ameloblast-specific amelogenin promoter. With this transgenic animal model we provide evidence that altering the expression profile of ameloblastin has an adverse effect on enamel formation that results in imperfect enamel. These data suggest that ameloblastin has a significant physiologic role to play in enamel formation, and ameloblastin should be considered as a candidate gene when discussing the genetics of amelogenesis imperfecta.

MATERIALS AND METHODS

DNA Cassette—Briefly, the 2.3-kb amelogenin promoter (14) was used to express the rat ameloblastin transgene (GenBank™ accession U35097) (1). Also included, as part of the promoter, is intron 1 of amelogenin, which is included to ensure that in vivo RNA processing events are appropriate and functional (15). Located at the amino terminus of the transgene product are the mouse dentin sialophosphoprotein (DSPP) signal peptide to ensure transport to the extracellular space (16) and the vesicular stomatitis virus glycoprotein (VSV-G)(17) and human c-Myc epitopes (Roche Applied Science) (18), which are used to follow transgene expression (19). Finally, the mouse DSPP 3′ untranslated region follows the transgene coding region. For this particular construct, the selection of the mouse DSPP

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signal peptide, the DSPP 3′-untranslated region, and the inclusion of multiple VSV-G and c-Myc epitopes were primarily based on ease of construction from available plasmids and relevance to other ongoing transgenic studies.2

Transgenic Animals—All vertebrate animal manipulation complied with institutional and federal guidelines. Transgenic mouse lines were prepared as described elsewhere (15). Animals were analyzed for transgene status by Southern blot hybridization of genomic DNA (14). Hybridization was to random primed 32P-labeled PCR-generated DNA to identify and distinguish the protein corresponding to the transgene following five additional matings among transgenic animals, and transgenic animals appeared to develop normally. Aged-matched, non-transgenic animal controls were taken from siblings or parent-founder lines; this was to reduce any genetic variability. Aged-matched, non-transgenic animal controls were taken from the same breeding stock and used for S.E.M. studies.

Immunolocalization—Tissue sections of 4-day postnatal heterozygous transgenic mouse pups or their normal (non-transgenic) littermates were prepared as described previously (14). An anti-c-Myc monoclonal antibody (Roche Applied Science, catalogue no. 1 667 149) was used to demonstrate tissue-specific expression of the introduced ameloblastin transgene. Concentration and reaction conditions used for this antibody were as recommended by the manufacturer. Polyclonal rabbit antibodies against recombinant rat ameloblastin were prepared, purified, and used as previously described at a dilution of 1:2,000 (4, 14). Immunohistochemistry methodology has been described elsewhere (20).

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**Fig. 1.** A, schematic of the ameloblastin transgene. Significant features are identified. Restriction enzymes *Pvu*I and *Bam*HI were used to remove the pGEM 7zf (+) vector backbone. B, the predicted translated and unmodified transgene product. The introduced signal peptide and epitopes are underlined. a—g, the design origins of the regions are (a) first amino acid of dentin sialophosphoprotein after the signal peptide, (b) and (d) an interrupted VSV-G epitope, (c) part of the pGEM 7zf (+) multicloning site employed in the construction of the transgene construct, and (e) an interrupted region containing multiple restriction sites for cloning manipulations. C, the nucleotide sequence from region e of panel B. Four blunt-end restriction sites are identified.

**Scanning Electron Microscopy**—Methodology for sample preparation and imaging by S.E.M. were previously reported (21–23). Six-week-old animals were sacrificed for S.E.M. imaging. A single animal from each of five unique transgene lines was subjected to S.E.M. analysis, and all lines gave similar results. The figures are representative of the defects noted in all of these transgenic animals.

**RESULTS**

**Establishment of Transgenic Lines**—The details of the transgene DNA construct are illustrated (Fig. 1). The mouse X-chromosomal-derived amelogenin promoter is used to drive the expression of the ameloblastin gene that is marked as a transgene by the presence of the c-Myc epitope. No gross abnormalities were detected in the dentition of any of the transgenic mouse lines at the time of the eruption of the incisor or molar teeth. No gross abnormalities were observed for the molar or the incisor teeth at 6 weeks of age (Fig. 2). The diet of the animals was constant for both non-transgenic and transgenic animals, and transgenic animals appeared to develop normally with no evidence of malnutrition.

**Transgene Expression Is Restricted to Secretory Ameloblast Cells and the Developing Enamel Matrix**—Using a monoclonal antibody against the c-Myc epitope (18), 4-day-old mouse incisors were chosen for immunohistochemical detection of the transgene protein because at this developmental stage of mouse tooth formation ameloblastin expression is robust (4). The transgenic protein product was identified in the transition zone (Fig. 3) of developing enamel of a lower incisor tooth using the c-Myc epitope “tag” (Fig. 4). This strategy allowed us to identify and distinguish the protein corresponding to the transgene, as opposed to protein derived from the endogenous wild-type ameloblastin gene. Four-day-old mouse incisors were cho-
sen for immunohistochemical detection of the transgene protein. At this developmental stage of mouse tooth formation, high levels of ameloblastin expression are expected (4). The transgenic protein was identified in the cytoplasm of ameloblasts and also within the newly secreted enamel organic extracellular matrix (Fig. 4). Using an antibody to amelogenin (4), no gross disturbances to the expression pattern for the endogenous amelogenin gene were observed (data not shown).

**Ameloblastin Gene Expression in Transgenic and Non-transgenic Animals Using Polyclonal Antibodies against Rat Ameloblastin**

—Using polyclonal antibodies to rat ameloblastin (1, 4), 4-day-old mouse incisors were chosen for immunohistochemical detection of both the endogenous and transgenic ameloblastin proteins. The transgenic protein is identified in the cytoplasm of ameloblasts and also within the newly secreted enamel organic extracellular matrix (Fig. 5). Of particular note are the greater levels of ameloblastin expression observed in transgenic animals (Fig. 5C) when compared with non-transgenic animals (Fig. 5B). This increased level of ameloblastin expression is consistent with expression levels observed for the amelogenin gene whose promoter is regulating expression of the transgene.

Localization of the ameloblastin protein is identical in transgenic animals when compared with non-transgenic animals. It is present in the cytoplasm of secretory ameloblasts, and it appears evenly distributed in the enamel matrix in the transition zone of the incisor (Fig. 5, Bc and Cc) and in the maturing enamel (Fig. 5, Bb and Cb) and mature enamel (Fig. 5, Ba and Ca). In addition, immunoreactivity is observed at the dentin-enamel junction, a unique junction linking the enamel to the underlying dentin (Fig. 5, Ca and Cb). Within the maturing enamel, ameloblastin immunoreactivity is significantly less than is observed at either the dentin-enamel junction or the ameloblast cells (Fig. 5, Bc and Cc). This decrease in observed ameloblastin is consistent with the history of ameloblastin processing, namely the hydrolysis of ameloblastin protein subsequent to its delivery to the enamel extracellular matrix environment with a consequential loss of epitopes.

**Enamel Rod and Interrod Structure by S.E.M.**—S.E.M. analysis of 6-week postnatal mouse incisor teeth showed the consequences of the ameloblastin protein up-regulation on enamel morphology. A 6-week-old mouse incisor indicating the transition zone of the enamel from which all S.E.M. images were collected for this study is provided in Fig. 3. Incisor teeth were fractured coronally through the enamel transition zone (Fig. 3). There was no acid etching of the samples done prior to S.E.M. analysis. The results shown are representative of findings observed consistently in each of five independent transgenic founder lines. Because in each of the multiple founder lines the observed phenotype is relatively constant, it appears unlikely that transgene integration disrupted a gene required for enamel formation.

The enamel formed in the mice homozygous for the transgene appeared to be more porous (Fig. 6, E and F). There was severe enamel rod dysmorphology (Fig. 6, C–F) when compared with age-matched non-transgenic control animals (Fig. 6, A and B). Although some regions of transgenic enamel appeared relatively normal (Fig. 6, C compared with A), perhaps more apparent in the incisor of transgenic animals were vast areas of enamel showing no rod architecture and with interrod enamel
dominating (Fig. 6D). Another feature noted in the enamel of these incisor teeth of transgenic animals was the absence of an aprismatic surface layer (as noted in the majority of sections studied) (Fig. 6, D–F). This feature would equate to an increase in surface porosity at this stage of development.

**Enamel Crystallites as Observed by S.E.M.**—Individual crystallites were visualized by S.E.M. and compared with crystallites from non-transgenic mice. All samples were from the transition zone of incisor teeth (Fig. 3). Within the enamel of transgenic animals it was clear that regions of relatively normal rod and interrod structure could be found directly neighboring homogeneous regions of interrod-only enamel (Fig. 7, E, right side versus left side). For transgenic animals, in some regions where rod enamel architecture was apparent, crystallite dimensions were comparable with non-transgenic animals (Fig. 7, C and D when compared with A and B). Individual enamel rods of transgenic animals had either relatively normal enamel crystallites (Fig. 7, D and G for the enamel rod identified with a solid circle) or relatively abnormal crystallites (Fig. 7, G for the enamel rod identified with a solid square) predominating. These “abnormal” enamel crystallites, seen only in the enamel of transgenic animals, had a diameter approximately twice that of the enamel crystallites for the non-transgenic controls. In addition, the c axis of these abnormal crystallites measured ~1 μm, as opposed to being a continuous inorganic unit structure spanning the entire enamel thickness.
Fig. 5. Expression of ameloblastin in incisor teeth of 4-day-old transgenic and non-transgenic mice, using polyclonal antibodies to rat ameloblastin. A, transgenic control animal exposed to secondary, but not primary, antibody. B, non-transgenic animal. C, transgenic animal. All three sections were prepared under identical conditions. Aa–d, Ba–d, and Ca–d, enlarged regions from panels A–C taken at the approximate regions identified as a, b, c, and d in panel A. a and b, mature enamel; c, transition zone ameloblasts and enamel; d, ameloblasts of the secretory zone (prior to any evidence of the enamel matrix). The red line in panels Aa, Bb, Ca, and Cb is to the dentin-enamel junction with dentine being superior (between the arrow-head and the red arrow as identified in panels Aa and Cb) and enamel being inferior (between the red and black arrows as identified in panels Aa and Cb). The mature (M) and growing ends (GE) of the incisor are identified, as are the odontoblasts (Od), ameloblasts (Am), and cells of the stratum intermedium (Si). The bar scale in panel A is also true for panels B and C. The bar scale in panel Aa is also true for panels Ab–d, Ba–d, and Ca–d.

Fig. 6. Enamel rod and interrod morphology. Scanning electron microscopy images of 6-week-old mouse incisor teeth that were fractured coronally through the transitional zone. Fractured samples were not acid-etched during their preparation. A and B, non-transgenic control pups; C–F, transgenic animals that are overexpressing ameloblastin. Arrow is to the dentin-enamel junction in each panel. Arrowhead is to the junction between presumed interrod (IR) enamel (superior) and enamel with a predominantly rod-like (R) architecture. Enamel rod, interrod, and dentin (De) are identified. A scale bar is included in each panel.
pressing animals have an increase in interrod enamel at the expense of rod enamel.

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FIG. 7. Heterogeneous nature of the enamel crystallite habit in the maturing enamel of transgenic animals. Scanning electron microscopy images of 6-week-old mouse incisor teeth that were fractured coronally through the transitional zone. Fractured samples were not acid-etched during their preparation. B, an enlarged region identified in panel A, D, an enlarged region identified in panel C, G and H, enlarged regions identified in either panels F or G, A and B, non-transgenic control pups. C–H, transgenic animals. A black triangle spans the width of a single crystallite in panels B and D; the same dimension triangle covers approximately half of the diameter of individual crystallites shown in panel H. An image of an enamel rod composed of "normal" crystallites (G, solid circle) that is neighboring an enamel rod of "abnormal" crystallites (G, solid square) is presented. Arrows point to the extremes of the c axis for a single crystallite with an approximate c axis length of 1 μm (H). A scale bar is included in each panel.
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