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Ameloblastin and amelogenin expression in posnatal developing mouse molars

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Abstract: Ameloblastin and amelogenin are structural proteins present in the enamel matrix of developing teeth. Here we report the results of *in situ* hybridization analyses with DNA probes of ameloblastin and amelogenin expression in the mandibular first molars of ICR/Jcl mice from postnatal day 1 to day 15. Ameloblastin mRNA expression was observed in ameloblasts at day 2 while amelogenin mRNA was detected in secretory ameloblasts at day 3. Significant expression of both molecules was observed at days 4, 5 and 6, after which the levels decreased. Amelogenin expression ended on day 10, while ameloblastin mRNA was only weakly detected on day 12. Neither amelogenin nor ameloblastin expression was observed in day 15 mouse molars. Amelogenin and ameloblastin mRNAs were restricted to ameloblasts. We conclude that amelogenin and ameloblastin expression is enamel-specific, and suggest that these genes might be involved in the mineralization of enamel. It is possible that ameloblastin could participate in the attachment of ameloblasts to the enamel surface. In this case, the downregulation of expression may indicate the beginning of the maturation stage in which the ameloblasts tend to detach from the enamel layer. (J. Oral Sci. 47, 27-34, 2005)

Key words: amelogenin; ameloblastin; mouse molar; *in situ* hybridization.

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Introduction

In the developing tooth crown, two mineralized matrices, dentin and enamel, are formed adjacent to each other by two cell sheets, odontoblasts and ameloblasts. These cells are derivatives of the neural ectomesenchyme and the oral epithelium, respectively. The cell properties are the result of the mutual induction of several differentiation steps in both layers during development (1).

The ameloblast phenotype has been characterized by the expression of two main classes of proteins: the hydrophobic proteins known as amelogenins (2) and the non-amelogenin proteins. The latter include: 1) the anionic enamel proteins (enamelin, tuft protein, tuftelin and the sheath proteins: ameloblastin and sheathlin or amelin) (3-6); 2) the enamel proteinases (7-11); 3) the proteoglycans (12,13); and 4) the sulfated glycoconjugates (14,15). Recently dentin sialophosphoprotein (DSPP), initially described in dentin, has been found in the pre-ameloblasts and in the first layers of the immature enamel (16).

Amelogenin is the dominant constituent of the developing enamel matrix and appears to comprise about 80-90% of the total protein. It is highly conserved throughout most species studied (17) and is essential for normal enamel formation. In humans, a deletion mutation in the amelogenin gene results in defects of enamel formation, manifested as the disorder amelogenesis imperfecta (MIM 301200) (18). The *in vitro* study of developing mouse molars has demonstrated that inhibition of amelogenin translation by antisense oligonucleotides results in dysmorphology of developing enamel (19), and amelogenin null mice display chalky-white discoloration and a disorganized hypoplastic enamel (20).

Amelogenin is composed of several peptides, and

although only one or two genes are present in the genome, the complexity is generated by alternative splicing of pre-mRNA and partial degradation and aggregation of proteins (21,22). The biological function of the amelogenins is not yet clear: they could have a signal transduction function during tooth development (23-25), they could contribute to enamel matrix and mantle dentin architecture (26,27), they could be responsible for ion transport (28), or they could regulate apatite crystal growth (29,30) during enamel biomineralization. Amelogenins are also implicated in cementogenesis, in preventing abnormal resorption of cementum (31) and they have a cell-adhesive activity that may play a role in periodontal regeneration (32).

In contrast to the uniform distribution of the amelogenins in the future mineralized enamel matrix among the immature crystallites in the rod and inter-rod enamel and the unmineralized prism sheath space, non-amelogenins accumulate in one of these subcompartments (33). A particular group of non-amelogenin proteins are the "sheathlins". Immunohistochemistry using antibodies has revealed that sheathlins concentrate in the prism sheath space (5). They were first recognized at the protein level as a group of polypeptides 13 to 17 kDa in size. They are synthesized in the ameloblast RER as a core protein with a molecular weight around 55 kDa, and are then post-translationally modified by O-linked glycosylation into a 65-70 kDa secretory form (34). They have a low affinity for hydroxyapatite *in vitro* (30). A cDNA encoding the full-length parent of the sheath proteins was termed sheathlin and shown to be the porcine homologue of rat ameloblastin/amelin (30,35,36), also showing a high homology with human ameloblastin (37,38). Ameloblastin displays potential integrin-binding motifs which have been located along the plasma membrane of the non-secretory face of Tomes' process, suggesting that these proteins and/or their cleavage products mediate the contact of ameloblasts to the mineralized matrix (5). Other reports note detection of ameloblastin in the region of root formation suggesting that this protein may play an important role in cementogenesis and in the generation of the prismatic structure and formation of the interprismatic enamel (37,39). Ameloblastin is also expressed transiently in the pre-odontoblasts before the initiation of amelogenesis (34,39) in the same way that DSPP is expressed by the pre-ameloblasts. This suggests that ameloblastin and DSPP may work as signaling molecules between ameloblasts and odontoblasts in the initiation of enamel and dentin development (36,40).

A great variety of methods have been used to characterize amelogenin expression in the developing teeth of a number of animal species (1). In the mouse, high-sensitivity RT-PCR has demonstrated amelogenin expression at E10.5 (27) and E14 (23,24), but these results indicated that gene expression was detected but not necessarily demonstrated *in situ*. Other *in situ* hybridization methods have showed amelogenin expression in the secretory ameloblasts of the first mandibular molars of mice (1), while other studies have described amelogenin proteins in pre-ameloblasts and the cell surfaces of odontoblasts adjacent to the forming mantle dentin extracellular matrix prior to biomineralization (24).

In contrast to the well-defined pattern of amelogenin expression during tooth development, ameloblastin mRNA localization has only been characterized in postnatal rats (40-42). In the present study, we compared the ameloblastin and amelogenin expression patterns in postnatal growing mouse molars by *in situ* hybridization using DNA biotinylated probes. Characterization of the expression of these genes in all stages of molar development is important for understanding the potential functions of these molecules in normal and pathological tooth development.

Materials and Methods

All experimental procedures involving the use of animals were reviewed and approved by the Animal Welfare Committee of the University of Chile Dental School.

In situ hybridization of mouse molars

Preparation of sections. Postnatal (PN) days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 15 *Mus musculus* ICR/Jcl heads (Laboratory Clea Japan, Japan) were collected on ice (day 0 = vaginal plug and day 1 = day of birth). The mandibular processes were dissected and fixed in ice-cold 4% paraformaldehyde PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na7HPO4, 1.4 mM KHPO4) for 12 h at 4°C. For demineralization we used EDTA 4.13% pH 7.4 (adjusted with NaOH) with microwave irradiation exposure for periods between 1 and 8 hours (43). The tissues were processed for histology and embedded in paraffin. The wax blocks were cut into 7 µm frontal tissue sections and mounted on silanized glass slides. The slides were stored at 4°C. Prior to hybridization the sections were deparaffinized with xylene and washed in ethanol. They were then incubated in 10.9 mM citrate buffer, pH 6.0, for 20 min at 94°C, and finally washed in distilled water.

Synthesis of DNA probes. The bovine amelogenin cDNA was introduced into the pBluescript II KS(-((Stratagene, La Jolla, CA, USA) and was digested with EcoRI, and the 845-bp restriction fragment (nucleotides 74-919, GenBank Accession # U82698) was used as a DNA probe. The

mouse ameloblastin cDNA cloned in pCR®II-TOPO® (Life Science Products, Renaissance®) was digested with Hind III, and the 1113-bp restriction fragment (nucleotides 249-1302, NM_009664 or GI:6753043) was used as a DNA probe. The restriction fragments were purified from agarose gels using the Concert Rapid Gel Extraction Kit (Gibco, BRL), labeled with a Random Primer Biotin Labeling Kit (NEN®, Life Science Products, Renaissance®), followed by ethanol precipitation.

Hybridization. The probes in the hybridization medium were distributed evenly over the section by placing a coverslip on top. The slides were placed in an oven and heated at 90°C for 10 min. The hybridization mixture contained 1 ng/µl labeled amelogenin-probe or labeled ameloblastin-probe, 50% deionized formamide, 100 µg/ml heparin, 1 × Denhardt's solution, 5 × saline-sodium citrate (SSC) buffer For ISH procedures, 1 mg/ml of yeast tRNA, 0.1% Tween 20, 10 mM EDTA, and 0.1% MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5). Controls were carried out by using the hybridization mixture without probes under the same conditions. The slides were incubated for 18 h at 37°C in a humid chamber.

Post-hybridization washing. The coverslips were removed by floating the slides in TBST (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for 15 min at room temperature. The biotin-labeled DNA probe was developed using phosphatase-coupled streptavidin in the presence of 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium salt (NBT-BCIP, Dako®), to form a blue precipitate over the hybridized DNA. Tissue sections were dehydrated in an ascending ethanol series followed by xylene, then mounted on cover slips with Permount® (Prolab). All slides were examined with a DLMS Leica light microscope.

Results

In situ hybridization analyses of mouse amelogenin and ameloblastin expression were performed on postnatal day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, and 15 molars. The day of birth was considered as postnatal day 1.

No amelogenin or ameloblastin mRNA was observed in the first mandibular molar on day 1 (Figs. 2 and 3). At this stage of development a thin layer of predentin separates the odontoblasts and the pre-ameloblasts (Fig. 1). The first hybridization signals for the ameloblastin mRNA were detected in pre-ameloblasts at the tip of the cusp at day 2 of development, coinciding with the appearance of a dentin matrix (Figs. 4 and 6). Amelogenin mRNA was detected in secretory ameloblasts on the cusp at day 3 and more strongly at day 4, coinciding with the start of enamel matrix formation for ICR/Jcl first mandibular molars (Fig. 8).

On days 4 and 5 the signals for both molecules were strong throughout the different stages of ameloblast development. By day 5, the hybridization of both ameloblastin and amelogenin could be seen as continuous lines covering the ameloblast layer.

After day 7 the first notable differences were observed between ameloblastin and amelogenin expression (Figs. 11 and 12), such that the level of amelogenin mRNA gradually decreased in the ameloblasts until it was no longer detectable at day 10 (Fig. 14). The ameloblastin expression waned more slowly (Fig. 15) than the amelogenin expression, and by day 12 only a very weak hybridization signal was detected in the molars (data not shown). No transcripts for either gene were detected in the developing molars on day 15, shortly before the fusion of the oral and dental epithelia (data not shown).

Discussion

Amelogenin and ameloblastin are proteins exclusively expressed in the teeth (37). The localization of amelogenin mRNA transcripts by *in situ* hybridization in developing teeth has been broadly described in diverse animal species such as the pig, rat, mouse and hamster. In general, amelogenin expression is abundant in ameloblasts, but is not observed in odontoblasts or other cells of the dental pulp, in Hertwig's epithelial root sheath, or any other cells in the vicinity of the developing cementum (1). Recent work has demonstrated that some alternative splice forms of amelogenins are expressed in the periodontal region of mouse tooth roots (29) and in young rat odontoblasts (25).

Our present data show that in the first mandibular molar, amelogenin mRNA is expressed only in the ameloblasts. The first signal was observed at day 3, the beginning of the ameloblast secretory stage in ICR/Jcl first mandibular molars (44). Amelogenin was abundant between days 4 and 7, and ended by day 10. These results agree with those obtained for maxillary first molars (1,41), although a difference exists with regard to the method of mouse age determination. However, no transcript was detected in any other surrounding tissue. Some differences have been noted in reports about the endpoint of amelogenin expression in different species and among different teeth in the same species. It has been reported that in growing rat molar ameloblasts, amelogenin RNA can still be detected in the maturation phase, when no enamel matrix is produced (35). Other studies of maxillary mouse molars have only reported the presence of the transcript up until the early maturation stages (1). Our studies on mouse first

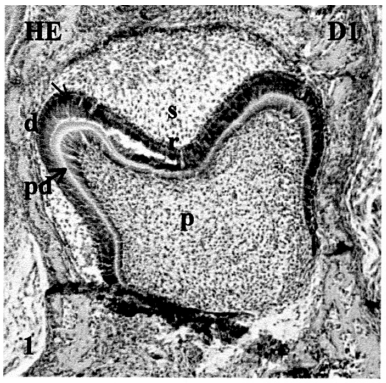

Fig. 1

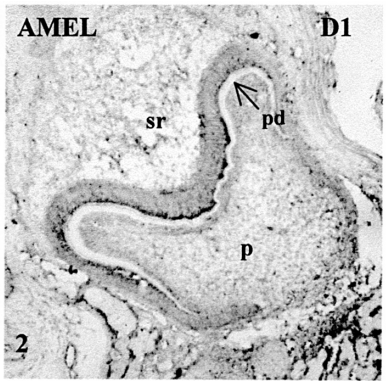

Fig. 2

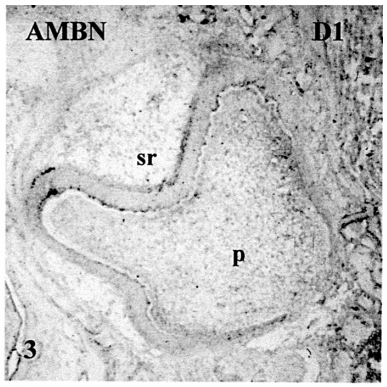

Fig. 3

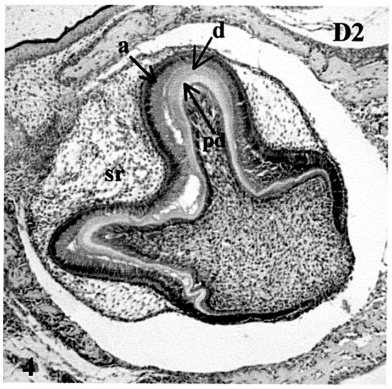

Fig. 4

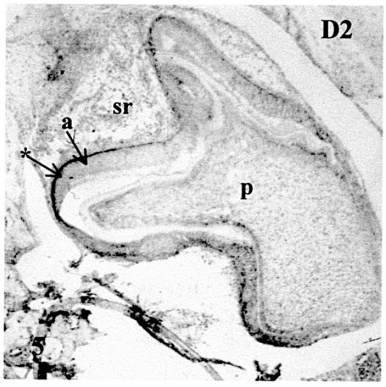

Fig. 5

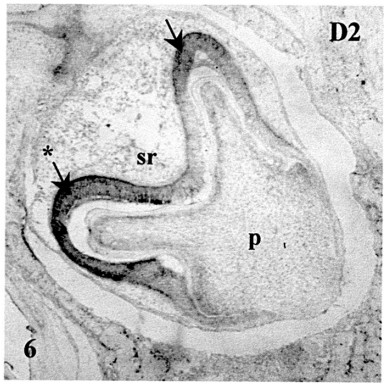

Fig. 6

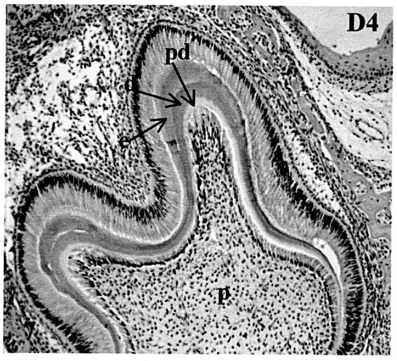

Fig. 7

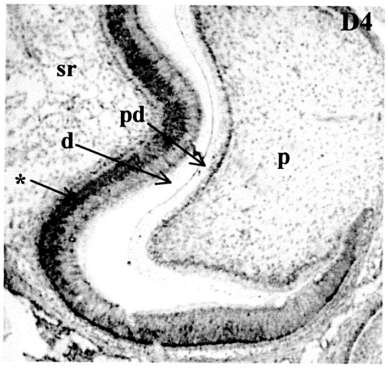

Fig. 8

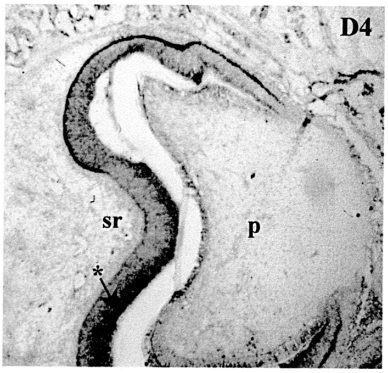

Fig. 9

Fig. 1 7 μm section of the first mandibular molar of a newborn mouse (D1). Predentin is produced by odontoblasts. (HE × 40)

Fig. 2 Amelogenin *in situ* hybridization on 7 μm section of a D1 first mandibular molar revealed with NBT-BCIP. No transcript expression can be detected. (× 40)

Fig. 3 Ameloblastin HIS on 7 μm section of the first mandibular molar of a D1 mouse revealed with NBT-BCIP. Ameloblastin mRNA was not observed at this stage.

Fig. 4 7 μm section of the first mandibular molar of a day 2 mouse. The predentin and dentin matrix can be seen. (HE × 40)

Fig. 5 7 μm section of the first mandibular molar of a day 2 mouse treated by amelogenin HIS revealed with NBT-BCIP. No stain is observed. (× 40)

Fig. 6 7 μm section of the first mandibular molar of a day 2 mouse treated by ameloblastin HIS revealed with NBT-BCIP. The first hybridization signals for ameloblastin mRNA can be seen in the pre-ameloblasts. (× 40)

Fig. 7 7 μm section of the first mandibular molar of a day 4 mouse. The enamel matrix can be seen. (HE × 40)

Fig. 8 7 μm section of the first mandibular molar of a day 4 mouse. Amelogenin HIS revealed with NBT-BCIP. Amelogenin mRNA was detected in the secretory ameloblasts on the cusp. (× 40)

Fig. 9 7 μm section of the first mandibular molar of a day 4 mouse (D1). Ameloblastin HIS revealed with NBT-BCIP. (× 40)

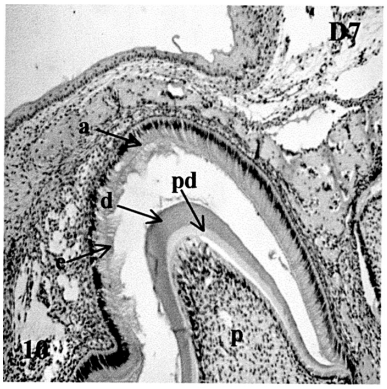

Fig. 10

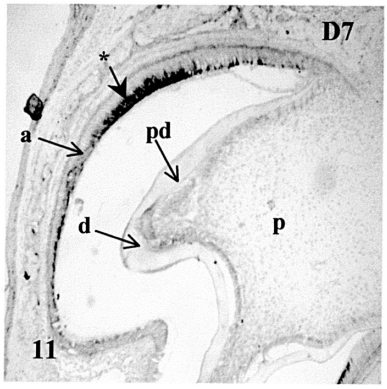

Fig. 11

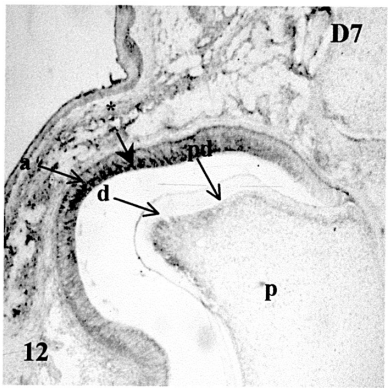

Fig. 12

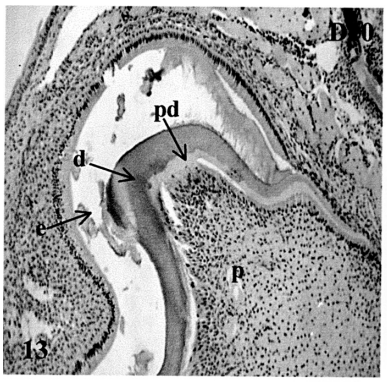

Fig. 13

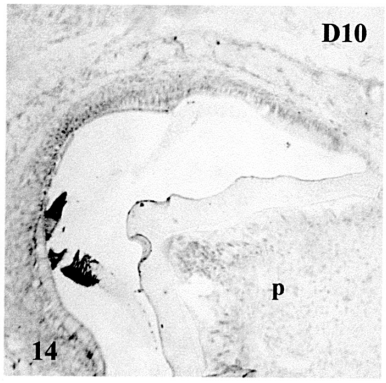

Fig. 14

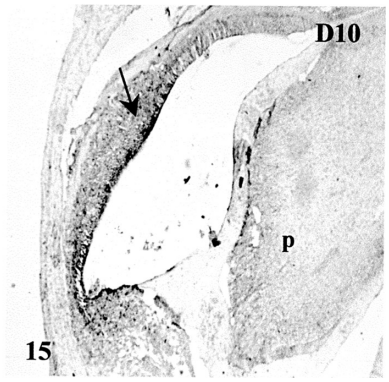

Fig. 15

Fig. 10 7 μm section of the first mandibular molar of a day 7 mouse. (HE × 40)

Fig. 11 Amelogenin HIS on a 7 μm section of the first mandibular molar of a day 7 mouse, revealed with NBT-BCIP. The amelogenin mRNA signals gradually decreased in the ameloblasts. (× 40)

Fig. 12 7 μm section of the first mandibular molar of a day 7 mouse. Ameloblastin HIS revealed with NBT-BCIP. Ameloblastin expression waned more slowly than amelogenin expression. (× 40)

Fig. 13 7 μm section of the first mandibular molar of a day 10 mouse. (HE × 40)

Fig. 14 Amelogenin HIS on a 7 μm section of the first mandibular molar of a day 10 mouse revealed with NBT-BCIP. The level of amelogenin expression is no longer demonstrable at day 10. (× 40)

Fig. 15 7 μm section of the first mandibular molar of a day 10 mouse. Ameloblastin HIS revealed with NBT-BCIP. A very weak ameloblastin hybridization signal was detected in the molars at the day 12. (× 40)

a: ameloblasts
AMBN: *in situ* hybridization ameloblastin probe
AMEL: *in situ* hybridization amelogenin probe
d: dentin
D1: molar of newborn mouse
e: enamel
HE: hematoxylin-eosin stain
NBT-BCIP: 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium salt
o: odontoblasts
p: pulp
pd: predentin
sr: stellate reticulum

mandibular molars show that amelogenin expression has disappeared by the time the ameloblast morphology indicates the beginning of the maturation stage, two days before it occurs in the maxillary molar, according to the findings of Hu et al., 2001 (1). We did not observe amelogenin expression after day 12.

In the case of ameloblastin expression, our results show that in the developing first mandibular molar, the transcripts are detected earlier than the amelogenin transcripts, and expression is ameloblast specific. Previous studies have described the transient expression of a variant of amelobastin, amelin, in the pre-odontoblasts (34,39). The expression of this variant has also been demonstrated along the developing root in rat molars, although confined to the cellular cementum. Presumably expression is limited to cells derived from the disaggregation of the epithelial root sheath (40,41). As with amelogenin, we did not detect any hybridization signals in odontoblasts or in other cells. This could be related either to the sensitivity of the probe in detecting various alternatively spliced transcripts, or to differences in the expression patterns among different animal species.

Some *in situ* hybridization experiments have shown that ameloblastin mRNA appears concomitantly with amelogenin mRNA (41,45). However, our observations show that the DNA probe detects ameloblastin expression before amelogenin expression. Both ameloblastin and amelogenin mRNAs were highly expressed by cells of the inner enamel epithelium during mouse tooth formation. The expression of RNA for amelogenin gradually decreased in the post-secretory ameloblasts, but the RNA of ameloblastin remained until a more advanced developmental stage.

The pattern of ameloblastin expression coincides with ameloblast morphological changes. At the beginning of ameloblastin expression the ameloblast is firmly bound to the newly formed enamel matrix, and at the end of its expression, at the maturation stage, the ameloblast has shrunk to almost half its height, and tends to detach from the enamel layer in histological sections (1). It has been demonstrated that ameloblastin possesses two putative cell-binding domains (35), and the temporo-spatial expression pattern of ameloblastin mRNA corroborates the possibility that this protein acts as the mediator of the interactions between ameloblasts and the enamel matrix.

It has been reported that in the rat molar, ameloblastin expression continues until the time of fusion of the dental and oral epithelium (41). Our results show that in the ICR/Jcl mouse first mandibular molar, the expression of ameloblastin ends before molar eruption when the dental root is beginning to form. It is important to observe that the expression of amelogenin, ameloblastin and enamelin (1) ceases at the cement-enamel junction, where the inner and outer enamel epithelia are joined, indicating that some signals coming from the stratum intermedium and/or stellate reticulum may be needed to support the expression of enamel proteins. Further investigations would be necessary to determine the molecular interactions that mediate this process.

Enamel matrix proteins are presumed to play a critical function in the nucleation, shaping, spacing and organization of enamel crystals. Two ameloblastin transcripts of 2.0 and 1.6 kb were observed in rat ameloblasts during the formation of the enamel matrix. It is not yet determined whether the two transcripts resulted from alternative mRNA splicing, the use of multiple transcription start sites, or the use of two different polyadenylation sites (35). The encoded proteins are rich in proline, glycine, leucine and alanine residues and contain the peptide domain DGEA, an integrin recognition sequence. They also contain potential phosphorylation sites, including a tyrosine kinase site, three protein kinase C sites and casein kinase II phosphorylation sites that are shared by proteins involved in mineralization (36). The persistent expression of ameloblastin in the cytoplasm of mature ameloblasts, and the presence of these phosphorylated proteins in the enamel matrix, the key elements for normal enamel biomineralization (46), support the notion that ameloblastin could also play a role in enamel mineralization.

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