# Human Ameloblastin Labeling in Fetuses

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**Abstract**

Human teeth enamel, the hardest tissue in the body, is produced at embryo development during amelogenesis. Its lack of regeneration after the teeth eruption and its destruction susceptibility turn it as a good subject of study. Ameloblastin an abundant, phosphorylated, proline/glutamine-rich protein, secreted during enamel formation is a structural matrix component that participates on the maintaining of prismatic structure of the enamel crystals. It also, has been involved in the animal amelogenesis as adhesion protein, expressed at pre-secretory stage that diminishes in the secretory stage. However its function and distribution in human being remains unclear. In order to contribute to the understanding of ameloblastin distribution during amelogenesis, the present study explores its location in the different structures on human fetal tooth by immunohistochemical labeling using a polyclonal antibody against ameloblastin in 5µm human jaws fetuses' sections. Thus, at pre-secretory stage ameloblastin was detected at the apical membrane and also at basal end of the ameloblast, as well as in the stellate reticulum. At the secretory stage is located at the secretory ameloblast, enamel, and dentin and in the odontoblasts. This ameloblastin location can be related with the enamel crystals organization in both pre-secretory and secretory stages.

**Keywords:** ameloblastin; immunohistochemistry; ameloblast; dental enamel

## 1. INTRODUCTION

Tooth development or odontogenesis is the advanced process regulated by several sequential and reciprocal interactions between the epithelial and mesenchymal tissues and polarized growth. Through complex morphogenetic-events that culminates in the formation of three different mineralized tissues: enamel, dentin and cementum (Thesleff, 2003). The odontogenesis starts with intra and inter cellular interactions that induce differentiation of odontoblasts and ameloblasts, which will form dentin and enamel respectively (Gutiérrez-Cantú et al., 2011; Thesleff et al., 1995). The ameloblast differentiation begins once a small predentin layer is formed and then is followed by an enamel matrix deposition and mineralization (Simmer et al., 2010).

The outer structure of the tooth dedicated to mastication (Zanolli et al., 2017), the enamel, surrounds the dental crown (Sa et al., 2014; Yahyazadehfar et al., 2013), a highly well organized and mineralized tissue that is not regenerated once the tooth erupts in human being (Lawn et al., 2010; Zhao et al., 2013). Enamel structure is described as prismatic, scilicet with hydroxyapatite crystallites tightly packed into bundles in an organized pattern with complex orientations (Gasse & Sire, 2015), these physical properties make it the hardest tissue in the vertebrates (Dahal et al., 2014; Nakayama et al., 2015; Simmer et al., 2010).

The enamel is chemically constituted by 95% of inorganic matrix, 1-2% of organic matrix and 3-5% water (Gómez de Ferraris, 2009; Gutiérrez-Cantú et al., 2011). Enamel extracellular matrix is essential for the normal development of its architecture and composition (Wright et al., 2011), this matrix is rich in enamel proteins that really affect the growth of enamel crystals (Diekwisch et al., 2009; Lu et al., 2011; Zanolli et al., 2017), including amelogenins, ameloblastins, enamelins and tuftelins (Zeichner-David et al., 2006).

Ameloblastin is 60-70 kDa protein synthetized by the ameloblasts that are present in the enamel organic matrix (Dhamija et al., 1999; Lee et al., 1996; Nanci et al., 1998),

it is a proline rich protein that is secreted by secretory granules (Zalzal et al., 2008). It is the second most abundant enamel protein after amelogenin and represents almost the 5% of total protein amount in the organic matrix (Moradian-Oldak, 2012). Once the maturation stage is complete, enamel achieves its final hardened form where it ultimately contains less than 1% protein by weight (Lefevre & Manly, 1938).

Apparently, ameloblastin has both roles, as a structural component of the enamel matrix, as well as a growth factor or signaling molecule during tooth growth (Fong et al, 1996a; Nakamura et al., 2006; Tamburstuen et al., 2011). Previous studies in animals and human being have suggested the ameloblastin presence mainly in secreting ameloblasts and in the adjacent newly secreted enamel extracellular matrix (Fong et al., 1996b), but, it has also detected in pulpal mesenchymal cells during early odontogenesis, as well as in differentiating odontoblasts prior to mantle dentin mineralization (Fong et al., 1998).

In despite of all the studies made to understand the role of ameloblastin in odontogenesis, several questions remain unsolved. Thus, the main propose of the present work is to identify the ameloblastin location in human fetal tooth germs at different stages of development and tooth structures in order to relate and make a comparative analysis with the findings done by other researchers in both human being or in animal models.

## 2. MATERIALS AND METHODS

### *2.1. Ethical considerations*

The Education, Research and Ethics Committee of the Zacatecas General Hospital, the institution that made the donation, provided permits for the use of human fetuses in this investigation.

*2.2. Obtaining samples.*

Five fetuses between 14 and 22 weeks of intrauterine life were analyzed. Jaws were dissected and separated into upper, lower and segmented into left and right sections, obtaining 20 samples. A macroscopic evaluation was done to verify that there was no apparent malformation. The specimen dissection was performed to obtain five upper and five lower jaws, which were divided into right and left, in order to get 20 samples, which were fixed in neutral formalin at 10%.

*2.3. Sample preparation.*

2.3.1. Immunohistochemistry.

The specimens were processed for paraffin embedding technique. Samples were decalcified with 10% ethylenediaminetetracetic acid (EDTA) for 15 days, periapical radiographs were taken to see the progress of decalcification (Gnatus, Zeyco Model Timex 70Kvps cts, wall scisors). Once they were decalcified, the samples were washed in PBS at 1% for 30 minutes. Then the samples were embedded in paraffin and 5 μm serial sections were made.

After the sections were made, the antigens recovery was done according with Campos-Navarro et al., 2017; briefly, sections were treated with sodium citrate at 0.01M (pH 6.0) in cycles of 25, 10, 8, 5 and 5 seconds, respectively with 2 minutes intervals between each cycle. Each cycle was made in microwave, and at the end of the last cycle, they were maintained for 10 minutes in the sodium citrate buffer at room temperature. The endogenous peroxidase was blocked with 5% skim-milk (Difco). Primary antibody was polyclonal anti-ameloblastin (1:200) (Santa Cruz Biotechnology). The reaction was revealed with the Dako LSAB system HRP (Dako, Glostrup, Denmark). Then, sections were counterstained with hematoxylin (Allen, 1992), dehydrated, cleared and assembled with Entellan resin (Fermont, Mexico City, Mexico). The images were obtained with a Leica DM 1000 microscope and then processed with Leica Acquire system.

The sample size calculation was conducted according to the Cummins and Hulley formula (Hulley et al., 2001) obtaining 20 samples to analyze.

## 3. RESULTS

### *3.1. Ameloblastin location in the pre-secretory stage.*

The figures 1a to 1d were obtained from the dental germ zone represented by the green circle in the molar tooth germ scheme in early bell stage of development (Figure 1g). While figures 1e to 1f were obtained from the dental germ zone represented by blue circle (Figure 1g). The scheme was taken and modified from Gómez de Ferraris, 2009.

Immunostaining of both pre-secretory stages, early and late, was found in the ameloblasts, identified the ameloblastin presence in the tooth germ structures, it also was recognized in the stellate reticulum, pre-secretory ameloblasts, stratum intermedium and dental papilla. The pre-secretory stage of the ameloblasts is observed in the early bell stage of development.

Figure 1a and 1b show the pre-secretory stage dyed with Hematoxylin-Eosin (H-E), which shows the ameloblasts (A), the stellate reticulum (SR), stratum intermedium (SI) and the dental papilla (P).

Ameloblastin was identified in the early pre-secretory stage showed a strong reaction in the ameloblasts and linear staining in the dentinoenamel junction. A high ameloblastin labeling was observed in the basal end of the ameloblasts, decreasing in the apical end, stratum intermedium and dental papilla; meanwhile a small amount is observed in the stellate reticulum (Figure 1c and 1d). In late pre-secretory stage, the ameloblastin recognition was increased in the ameloblast cytoplasm, stratum intermedium and the dental papilla, meanwhile in the stellate reticulum remains slight (Figure 1e and 1f).

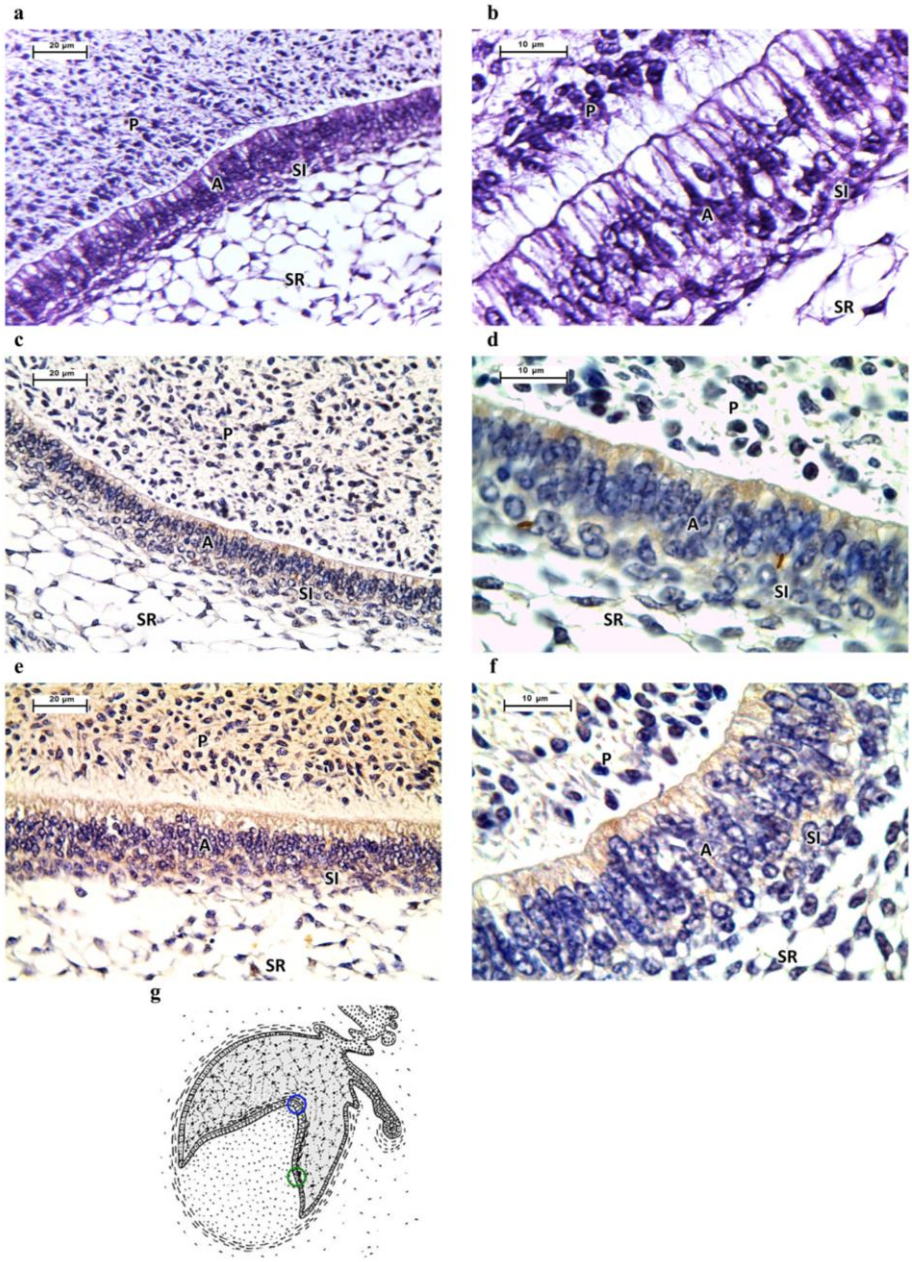


**Figure 1. Specific recognition of ameloblastin in human dental tooth germ in fetuses between 14 and 22 weeks of gestation during the pre-secretory stage.**
H-E staining controls (a and b). Immunostaining of ameloblastin (c, d, e and f). In the early pre-secretory stage the ameloblastin is in the basal and apical portion of the ameloblasts (A), stratum intermedium (SI), decreasing in the dental papilla (P) and with a little marking in the stellate reticulum (SR) in the early pre-secretory stage (c and d). In the late pre-secretory stage, the reaction increases throughout the cytoplasm

in the ameloblast, stratum intermedium and dental papilla (e and f). The figures on the left were were taken with the 40x objective and the figures on right with the 100X. Diagram of the early bell stage (g).

The ameloblasts pre-secretory stage was observed in the early bell stage of a developing tooth.

*3.2. Ameloblastin location in the secretory stage.*

The secretory stage of the ameloblasts is observed in the late bell stage. Images 2a, 2b, 2c and 2d, taken from the blue circle on the image 2e, diagram from a tooth germ of a molar in the late bell stage of development. The scheme was taken and modified from Gómez de Ferraris, 2009.

The immunostaining in the secretory stage, showed the ameloblastin in the secretory ameloblasts (A), the stellate reticulum (SR), the dental papilla (P), odontoblasts (O) and mantle dentine (MD).

Figures 2a and 2c show the H-E secretory stage; the stellate reticulum, ameloblasts, enamel, mantle dentin, odontoblasts and dental papilla are observed. During the secretory stage, the immunostaining showed greater intensity in the newly secreted enamel and Tomes' processes; in the cytoplasm of the ameloblast, in the mantle dentin and odontoblasts decreases slightly, but remains strong and, in stellate reticulum and papilla is much lower (Figure 2b and 2d).

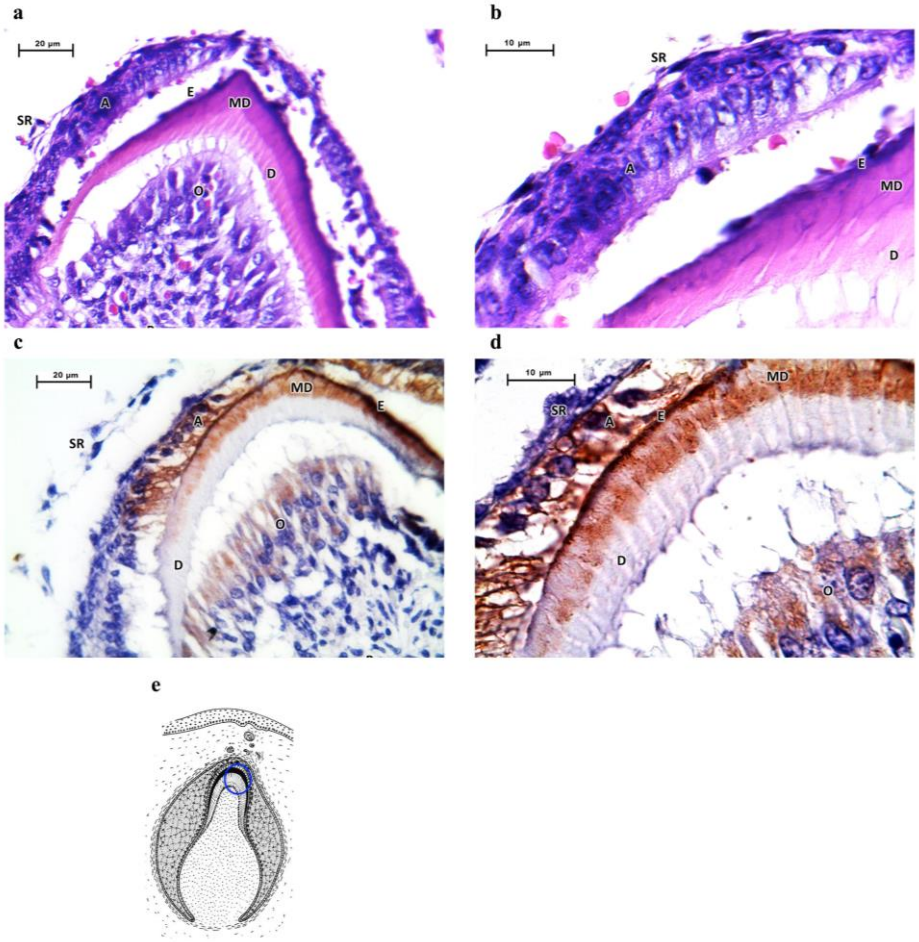


**Figure 2. Specific recognition of ameloblastin in human dental tooth germ in fetuses between 14 and 22 weeks of gestation during the secretory stage.**
The figures a and b show the stained controls with H-E. The immunostaining of ameloblastin is shown on the figures c and d. Figures a and c were taken at 40X and the figures b and d at 100X. There is more ameloblastin in the enamel (E), there is also a strong reaction in the cytoplasm of the ameloblasts (A), the mantle dentin (MD), and odontoblasts (O) and in the stellate reticulum (SR) and papilla (P) in minor proportion (b and d). Diagram of the late bell stage (e).

## 4. DISCUSSION

As describe before, embryological, teeth are ectodermal organs that form by sequential and reciprocal interactions between the odontogenic epithelium and neural

crest-derived mesenchyme (Jernvall & Thesleff, 2012).

Organogenesis involves three fundamental processes: *the initiation*, in which positional information is provided and interpreted to initiate organ formation at the right place; *morphogenesis*, cells build up an organ rudiment; and finally, *differentiation*, cells form organ-specific structures (Li et al., 2013; Peters & Balling, 1999).

As described previously, the enamel is a unique and highly mineralized structure that covers the teeth and is almost entirely free from organic components. The enamel biomineralization in animals uses a polyanionic protein matrix that controls the nucleation, growth and orientation of crystals. This formation is regulated by specialized epithelial cells derived from ectoderm known as ameloblasts (Shintani et al., 2006). The ameloblasts, during the amelogenesis keep on differentiation stages that are characterized by changes in cell morphology and function. Cell differentiation during enamel development results in pre-secretory, secretory, transitional, and maturation phase ameloblasts. Cell morphology and chemical composition of the extracellular matrix of the enamel are the basis for defining the different stages of amelogenesis (Smith, 1998). During the pre-secretory stage, pre-ameloblasts, polarize and develop a large capacity to increased protein synthesis. Also they play an important role in degradation and resorption of the basal lamina separating them from the predentin (Ronnholm, 1962).

Secretory stage ameloblasts are tall columnar epithelial cells that are characterized by histological structures called Tomes' processes (Fong et al., 1996a); and their main function is a massive production and secretion of enamel proteins (Moradian-Oldak, 2012).

Ameloblast that secrete various proteins of extracellular matrix involved in process of enamel formation (Shintani et al., 2006). Enamel proteins are classified as amelogenins, that are the most abundant (90%) and non-amelogenins, as ameloblastin

(Gutiérrez-Cantú et al., 2011; Krebsbach et al., 1996; Shintani et al., 2006). Both types of proteins play a major role in the mineralization of the enamel, regardless of the percentage in which they are presented. The ameloblastin is a protein that plays a critical role in controlling the growth of mineral crystals during the enamel formation and the adhesion between ameloblasts and enamel matrix. Given its importance, this protein has been extensively studied in animal specimens as mouse and rat (Fukumoto et al., 2004; Hirose et al., 2013; Lu et al., 2011; Nakayama et al., 2015; Nanci et al., 1998)**,** also in pigs (Chun et al., 2010; Hu et al., 1997; Nakamura et al., 2006) and crocodile (Shintani et al., 2006), showing that its presence is widespread in animal evolutionary scale. It has been performed the identification of the ameloblastin at genetic level, its characterization *in vitro* (Dhamija et al., 1999) as well as its monitoring *in* vivo (Jacques et al., 2014) to try to identify which events are involved in the development.

To contribute to research in humans and determine the distribution and importance of the protein, in this study were analyzed samples of human fetal tooth germs between 14-22 weeks gestation, period in which we can follow the different stages of tooth formation.

Lee et al. (1996) initiated studies on humans comparing the ameloblastin between rat and human demonstrating its presence in the pre-secretory, secretory and maturation stage by immunohistochemistry, the results obtained are in agreement with this observation, because ameloblastin was found in pre-secretor as well as in secretory stage. In contrast, MacDougall et al. in 2000 proposed that ameloblastin appears until secretor stage. On the other side, Hatakeyama and collaborators in 2009, found a high ameloblastin expression during the pre-secretory stage following of a decreased in the secretory stage (Hatakeyama et al., 2009), while the results showed an increasing of ameloblastin expression during secretory stage. To clarify this, more studies are necessary.

The results obtained in this research show a high expression of ameloblastin on the

early pre-secretory stage that increases in the late stage, with expression peak in the secretion stage as seen by Fukumoto et al. in 2004, which propose the high protein levels presence in the secretory-stage. In the pre-secretory stage, the ameloblastin is distributed mainly in the basal portion of ameloblast and decreases both in the apical portion as in the dental papilla. Lee et al. in 1996, point out that at this stage the ameloblastin is confined to the distal portion of the cytoplasm in the ameloblasts. In the secretory stage, several studies showed that the distribution of ameloblastin is restricted to the interface between the Tomes' process and in the secreted extracellular matrix (Fong et al., 1996a; Fong et al., 1996b; Krebsbach et al., 1996; Lee et al., 1996). Additionally, the ameloblastin was distributed throughout the cytoplasm in the ameloblast, mantle dentin and odontoblasts also in the papilla and the stellate reticulum, but in smaller amounts. The observations made by MacDougall and his group found ameloblastin within rounded structures located at the distal end of the cell body, in contrast in the present work the protein was distributed in whole cytoplasm. As well as MacDougall group this research also found ameloblastin associated at the ameloblast apical pole and in the enamel that surrounds this area, at the vicinity of the dentinal-enamel junction. Faint immunostaining was also present in the predentin region and within the developing odontoblast cell layer facing mantle dentin.

The sequential expression of enamel proteins, including ameloblastin, is essential for normal enamel mineralization, because the non-amelogenins proteins are acidic and they are deposited along amelodentinal union, they also perform a nucleating and regulatory function in the mineralization (Atsawasuwan et al., 2013; Grandin et al., 2012; Lee et al., 1996). In the pre-secretory stage the ameloblastin only is expressed in the ameloblasts. However, in the secretory stage the odontoblasts are also found. This differs from the findings of Lee et al. on this stage the ameloblastin, apparently, it is not confined to the ameloblasts, but it may be present in developing odontoblasts and it can play an important role in the formation of dentin, particularly in dentin formed at the dentinoenamel junction (Fong et al., 1996a; Fukumoto et al., 2004; Hao

et al., 2005).

The enamel and dentin are joined at the edge enamel dentin, a robust interface that contributes to the strength of the tooth that takes part of the dentinoenamel union, which main function is to transfer mechanical forces from the brittle and hard enamel to the soft and durable dentin (Deshpande et al., 2010). Deshpande et al. have postulated that during the early development of tooth germ there are interactions between the dentin and the enamel proteins that regulate growth and organization of calcium crystals in the dentinoenamel union. They show that the collagen fibrils guide the amelogenin assembly within filamentous structures oriented along the axes of collagen fibers (Deshpande et al., 2010). There has been demostrated that the amelogenin-calcium phosphate complex leads the orientation of deposition of amorphous mineral particles along the fibers and suggest that these interactions between collagen and amelogenin play an important role in the formation of dentin at the dentino-enamel junction (Deshpande et al., 2010; Gutiérrez-Cantú et al., 2011). According with this, if the production of ameloblastin acts as a core for amelogenin, the results in this study suggest that the secretion precedes the deposition and the arrangement amelogenin to assist in enamel and dentin mineralization. These results contribute to know the tooth mineralization in early development, as several researches seek the cell niches within dental tissues in order to propose in future novel therapeutic solutions (Mitsiadis & Orsini, 2016). More studies are needed to confirm this assumption.

## 5. CONCLUSION

The results in this study show that the ameloblastin is present during the pre-secretory stage in the development of the enamel in human fetal tooth, not only in the ameloblasts, but also in the papilla and in less amounts in stellate reticulum, and increases in the secretory stage, being located even in the mantle dentin and

odontoblasts, therefore here is substantiated that the ameloblastin is not exclusive from the enamel, but is involved in the formation of other hard tissues of the tooth.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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