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The Role of Amelogenin Protein in the Development of Human Primary Teeth

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ABSTRACT

Background: Tooth development involves reciprocal interactions between the oral epithelium and ectomesenchyme. The inner layer cells of the enamel organ (EO), known as the inner enamel epithelium (IEE), undergo differentiation into preameloblasts and subsequently mature ameloblasts. These ameloblasts play a crucial role in the secretion of enamel matrix proteins, including amelogenin. This experimental study aimed to analyze the expression profile of amelogenin in various histological structures of human primary developing teeth, employing a relatively large sample size.

Methods: This experimental study included 33 human fetuses aged between 13 and 23 weeks, with three samples obtained from each gestational age. The samples were categorized into three age groups of ≥13 and <16 gestational weeks (g.w.), <19 g.w., as well as ≥19 and ≤ 23 g.w. Hematoxylin and eosin staining, as well as immunohistochemistry staining, were performed on the samples. To assess the expression level of amelogenin in different histological structures of human primary teeth, a two-way ANOVA test was employed. The statistical significance threshold was set at P≤0.05.

Results: There were statistically significant differences regarding the expression level of amelogenin in various histological structures of human primary teeth in different fetal ages (13-23 weeks), except for the maxillary central incisor and mandibular central incisor.

Conclusion: Due to the profile of expression of amelogenin, it can be concluded that amelogenin expression could be associated with ameloblast and odontoblast differentiation, as well as enamel and dentin matrix deposition. In addition, the results of this study may shed light on the role of the cervical and coronal portions of the EO as stem cell reservoirs.

Keywords: Amelogenin, Embryology, Immunohistochemistry, Enamel organ, Odontogenesis

Introduction

Tooth development involves reciprocal interactions between the oral epithelium and ectomesenchyme. The enamel organ (EO) represents the epithelial component of tooth formation, while the ectomesenchymal component is comprised of odontoblasts (1, 2). The process of tooth development occurs in three stages: bud, cap, and bell (3). Various signaling pathways play a role in modulating tooth shape and pattern during development (4). The initial stage of tooth development, known as the bud stage, is characterized by the thickening of the oral epithelium, leading to the formation of the dental lamina. Subsequently, the epithelium invaginates into the underlying ectomesenchyme, giving rise to the EO during the cap stage. At this stage, the epithelial component extends downwards and surrounds the condensed mesenchyme, forming

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the dental papilla. In the bell stage, the coronal part of the developing tooth exhibits four distinguishable layers: the inner enamel epithelium (IEE), stratum intermedium (SI), stellate reticulum (SR), and outer enamel epithelium (OEE) (5). The IEE cells play a crucial role in inducing neighboring mesenchymal cells to differentiate into odontoblasts. Conversely, odontoblasts influence the differentiation of IEE cells into ameloblasts (4). The IEE cells undergo a series of differentiation, progressing from preameloblasts to mature ameloblasts responsible for the secretion of enamel matrix proteins, including amelogenin. Additionally, the IEE cells secret various growth factors (6). Amelogenesis, the process of enamel formation, can be categorized into four stages based on the morphology and function of ameloblasts, namely presecretory, secretory, transition, and maturation (7). Amelogenin, the most abundant extracellular enamel matrix (ECM) protein, is crucial for enamel formation. Furthermore, amelogenin has been implicated in the proliferation of cementoblasts (8). The ECM plays a significant role in controlling cell proliferation, migration, and differentiation (9).

The cervical loop represents a region where cells continue to divide, ultimately contributing to the formation of the tooth root(s). Within the labial cervical loop, stem cells are present, which have the capacity to differentiate into odontoblasts and potentially ameloblasts. Hence, the cervical loop is considered a reservoir for ameloblasts (10-12). A previous study on animals has demonstrated the self-renewal and differentiation capabilities of dental epithelial stem cells within the cervical loop (13).

In recent years, several animal studies have elucidated the significance of amelogenin in tooth formation (14-16). However, the precise expression profile of amelogenin during various stages of human primary tooth development remains uncertain. Therefore, the objective of this experimental study was to analyze the expression profile of amelogenin in various histological structures of developing human primary teeth, utilizing a relatively large sample size. Further investigation of the functions of amelogenin aimed to enhance our understanding of the molecular and cellular mechanisms underlying human tooth development.

Methods

Sample collection and staining method

Informed consent was obtained from the parents prior to the study. Human fetal cadavers at the gestational ages of 13 to 23 weeks (three samples for each gestational age) were collected from Fatemieh Hospital, Hamadan, Iran. The gestational age of each embryo was determined by obstetricians and measured carefully using the crown-rump length before fixation (17). Subsequently, the heads of the samples were removed and immersed in 10% neutral buffered formalin as a fixative for two days. Decalcification was then performed using a diluted nitric acid solution (5%). The samples were embedded in paraffin, and sections of 4-µm thickness were cut from the blocks for hematoxylin and eosin staining and immunohistochemistry staining following established protocols (18, 19). The histologic slides were deparaffinized, rehydrated using xylene and ethanol, and subjected to heat-induced antigen retrieval in 0.01M citrate buffer for 20 minutes using a microwave. The primary antibody used for immunohistochemistry was Anti-AMG Rabbit polyclonal antibody (1:170, ab153915; Abcam, UK). The slides were incubated with antibodies for 1 hour at room temperature. The omission of the primary antibody served as the negative control. Considering the developmental timeline of human primary tooth development, the primary incisor tooth germ is typically in the bell stage during the 14*th* gestational week (g.w.) (20-22). Dentin formation has been observed in the cusp tip of both the central and lateral mandibular incisors by the 16th g.w. (23), and functional ameloblasts have been detected by the 18*th g.w.* (20, 23). Thus, the samples were categorized into three age groups: ≥13 and < 16 g.w, <19 g.w., as well as ≥19 and ≤ 23 g.w. The cytoplasmic immunostaining was subsequently analyzed and the percentage of positive cells was recorded for odontoblasts, cervical loop, and each layer of EO in the primary central incisor and primary first molar of all samples in both jaws (24).

Statistical Analysis

The statistical analysis was performed using SPSS software (version 20.0; SPSS Inc. Chicago, IL). A two-way ANOVA analysis was conducted to compare mean differences *between* groups and assess the effect of the g.w. and types of teeth on the expression level of amelogenin in odontoblasts, cervical loop, and distinct layers of EO. Tukey's post-hoc test was employed to determine the significance of the differences among groups. The significance level was set at P≤0.05.

Ethical approval

This experimental study was granted ethical approval by the Ethics Committee of Hamadan University of Medical Sciences (approval No.IR.UMSHA.REC.1398.043).

Results

Histologic analysis for the expression level of amelogenin in all samples

In this study, 25 and 8 samples were obtained from males and females, respectively. Table 1 provides details on the observed differences among the groups.

The post-hoc analysis revealed a significant difference between the maxillary central incisor and mandibular first molar (P<0.001), as well as the maxillary central incisor and maxillary first molar (P<0.001) regarding the amelogenin expression level in the IEE layer. However, no significant difference was observed between the maxillary central incisor and mandibular central incisor (P<0.948) in terms of the amelogenin expression level in the IEE layer. Furthermore, the post-hoc analysis demonstrated a significant difference between the maxillary central incisor and mandibular first molar (P<0.001), as well as the maxillary central incisor and maxillary first molar (P<0.001) in terms of the amelogenin expression level in the SI layer. However, no significant difference was found between the maxillary central incisor and mandibular central incisor regarding the amelogenin expression level in the SI layer (P<0.945). The post-hoc analysis further revealed significant differences among other cell types in terms of amelogenin expression levels.

In the SR layer, there was a significant difference between the maxillary central incisor and mandibular first molar (P<0.001), as well as the maxillary central incisor and maxillary first molar (P<0.001) regarding the amelogenin expression level. However, no significant difference was observed between the maxillary central incisor and mandibular central incisor in terms of the amelogenin expression level (P<0.540).

Similarly, in the OEE layer, a significant difference in amelogenin expression level was found between the maxillary central incisor and mandibular first molar (P<0.001), as well as the maxillary central incisor and maxillary first molar (P<0.001). However, no significant difference was detected between the maxillary central incisor and mandibular central incisor regarding the amelogenin expression level in the OEE layer (P<0.240). The post-hoc analysis also identified a significant difference between the odontoblasts of the maxillary central incisor and mandibular first molar (P<0.001), as well as the odontoblasts of the maxillary central incisor and maxillary first molar (P<0.001) regarding the amelogenin expression level. However, no significant difference was observed between the maxillary central incisor and mandibular central incisor in terms of the

Table 1. Analysis of amelogenin expression profile in different histological structures of the tooth germ

Source of Variation	*df*	Mean square	F	P-value
Gestational week (IEE)	2	59613.962	292.217	0.000
Types of teeth (IEE)	3	2625.705	12.871	0.000
Interaction (IEE)	6	138.308	0.678	0.668
Gestational week (SI)	2	63310.002	322.793	0.000
Types of teeth (SI)	3	2585.423	13.182	0.000
Interaction(SI)	6	144.433	0.736	0.621
Gestational week (SR)	2	12687.717	651.605	0.000
Types of teeth (SR)	3	488.650	25.096	0.000
Interaction (SR)	6	94.634	4.860	0.000
Gestational week (OEE)	2	67345.758	437.516	0.000
Types of teeth (OEE)	3	2472.724	16.064	0.000
Interaction (OEE)	6	196.240	1.275	0.274
Gestational week (odontoblasts)	2	68265.573	379.358	0.000
Types of teeth (odontoblasts)	3	3212.023	17.852	0.000
Interaction (odontoblasts)	6	231.902	1.289	0.267
Gestational week (cervical loop)	2	62475.735	324.015	0.000
Types of teeth (cervical loop)	3	2412.475	12.512	0.000
Interaction (cervical loop)	6	148.649	0.771	0.594

df: degrees of freedom and F: variation between sample means/variation within the samples.

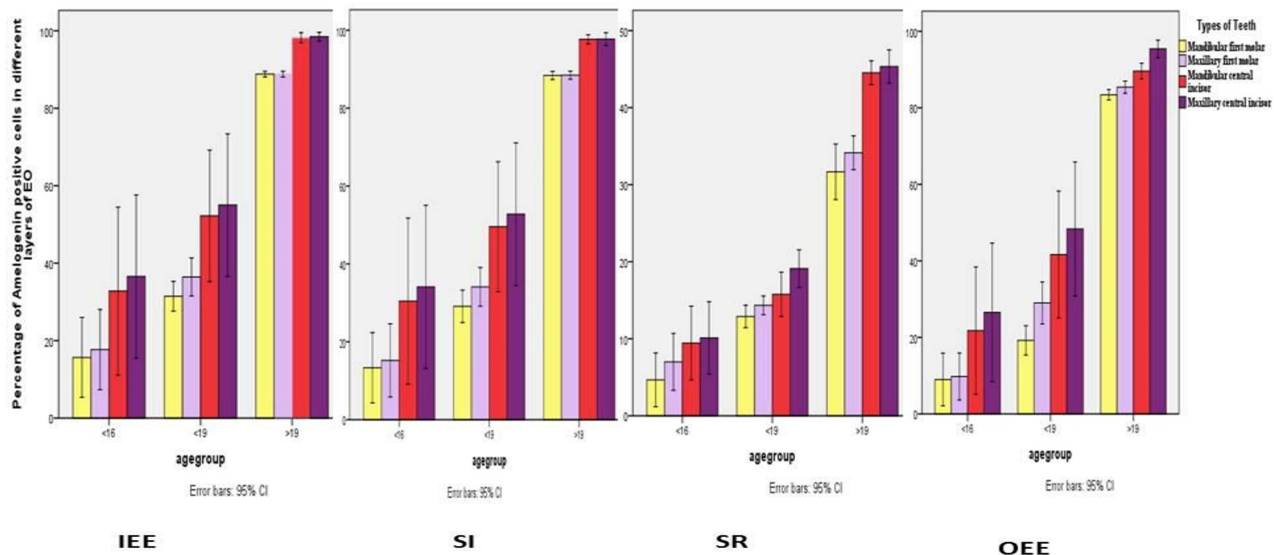

Figure 1a. Histograms depicting the percentage of amelogenin expression in different layers of EO (a) and in odontoblasts and cervical loop (b). IEE: inner enamel epithelium, SI: stratum intermedium, SR: stellate reticulum, OEE: outer enamel epitheliu

amelogenin expression level in the odontoblasts (P<0.846). Furthermore, a significant difference in amelogenin expression level was found in the cervical loop between the maxillary central incisor and mandibular first molar (P<0.001), as well as the maxillary central incisor and maxillary first molar (P<0.001). However, no significant difference was detected between the maxillary central incisor and mandibular central incisor regarding the amelogenin expression level in the cervical loop (P<0.910) (Figure 1: A, B).

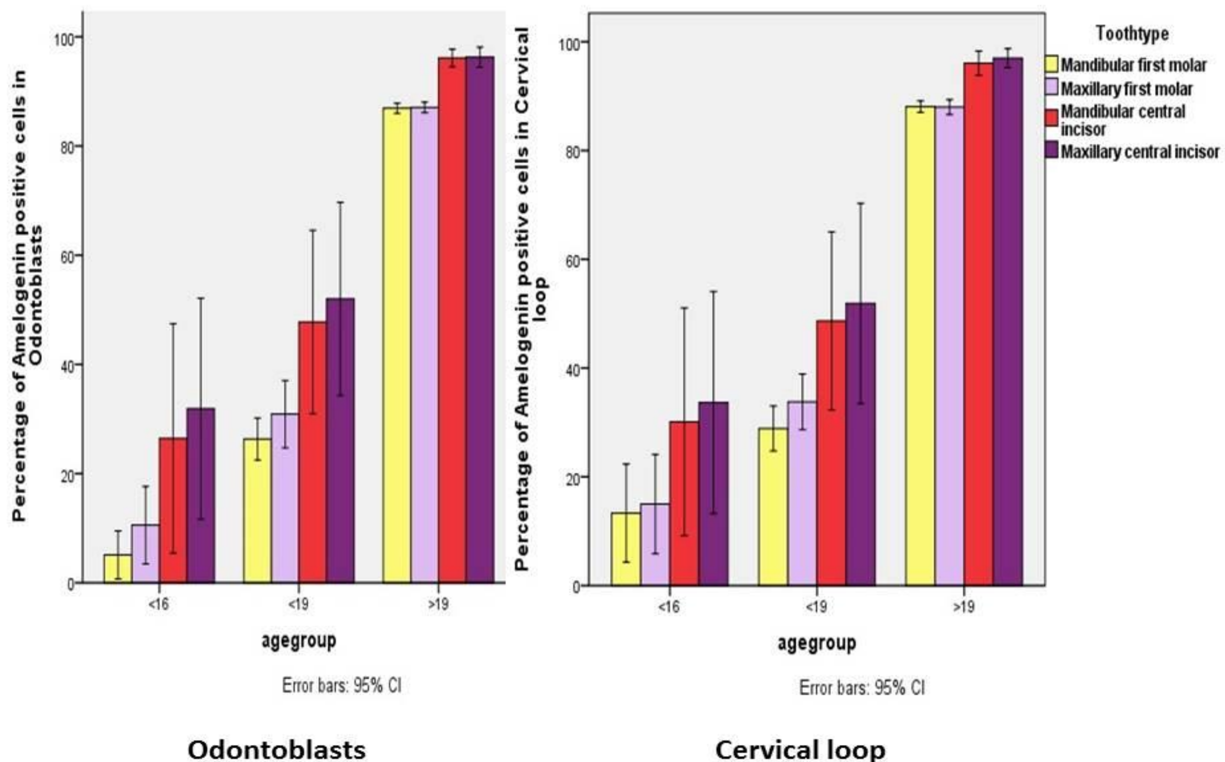

Figure 1b. Histograms depicting the percentage of amelogenin expression in different layers of EO (a) and in odontoblasts and cervical loop (b). IEE: inner enamel epithelium, SI: stratum intermedium, SR: stellate reticulum, OEE: outer enamel epitheliu

Discussion

In this study, the expression level of amelogenin was investigated in various structures of human developing primary teeth (Figure 2: A-L). Consistent with previous research (25), our findings demonstrated an increase in amelogenin expression from the bud stage onwards in distinct structures of human developing primary teeth. In addition, there was a noticeable increase in the number of immune-positive cells in different histological structures of human developing primary teeth with the progression of gestational weeks. These findings suggest that amelogenin may play a role in cell proliferation and differentiation during human tooth development. Interestingly, no significant differences were observed between different histological structures of maxillary and mandibular central incisors in terms of amelogenin expression levels. This aligns with previous studies indicating that both maxillary and mandibular central incisors initiate differentiation at the same time during the development of human primary teeth (23). In our study, the expression of amelogenin was detected in the enamel matrix of the maxillary primary central incisor from the 18th g.w., and in the maxillary primary first molar from the 21st g.w. This finding supports previous research showing amelogenin expression in human deciduous teeth starting from the 18th g.w. (20).

Furthermore, our investigation revealed amelogenin expression in various structures of developing teeth. Although amelogenin was traditionally believed to be exclusively expressed by ameloblasts with an epithelial origin, recent studies have identified different isoforms of amelogenin in other tissues (15, 26). A previous study on developing mice showed strong amelogenin staining in the oral epithelium, dental lamina, IEE, and SI layers, as well as in the extracellular matrix, odontoblasts, dental papilla, and alveolar bone trabeculae at different gestational weeks. Weak staining was also

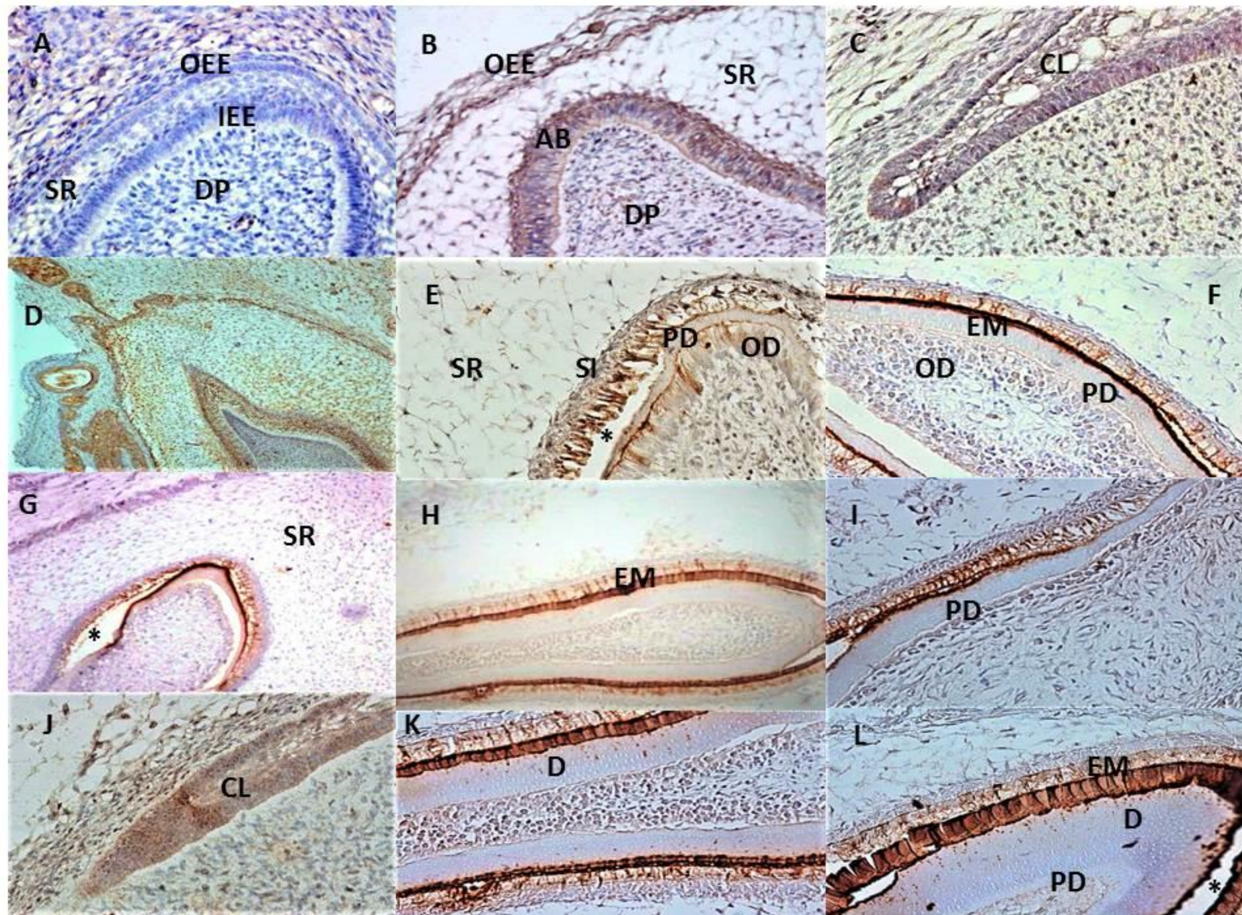

Figure 2. Expression patterns of amelogenin in the human primary EO, odontoblasts, and cervical loop in different fetal ages (A-L) (X100 or X400). (A) 13g.w. (MCI) (B) 15 g.w. (MaCI) (C) 16 g.w. (MFM). (D) 17 g.w. (MaCI). (E) 18 g.w. (MCI). (F) 19

observed in the SR cells and the cervical loop, while minimal staining was detected in the OEE layer (26).

In our study, we observed an increase in the expression level of amelogenin in odontoblasts with the progression of gestational weeks. This finding is consistent with earlier animal studies that have also reported the presence of amelogenin in odontoblasts. Amelogenin has been identified in odontoblasts before the mineralization of mantle dentin (27), along the cell surface (28), in young odontoblasts (29), and during predentin deposition (25). However, once mantle dentin formation is complete, the expression level of amelogenin decreases in the SI cells and odontoblasts (30). Another animal study has shown intense amelogenin staining in the predentin, intercellular spaces within odontoblasts, and dental pulp cells (25). However, amelogenin expression has not been detected in mature functional odontoblasts in another study (15).

Previous research has demonstrated that interactions between the SI cells and ameloblasts play a crucial role in controlling ameloblast differentiation and function during the secretory stage (31, 32). In addition, amelogenin has been shown to regulate the differentiation of ectomesenchymal cells in the dental papilla into odontoblasts (33). A study on developing human teeth has reported amelogenin staining in the IEE cells (preameloblasts), the cervical loop, and predentin of a deciduous molar at the 18th g.w. Strong amelogenin positivity was observed in the deposited enamel matrix, functional ameloblasts, and differentiating odontoblasts in that study (15). Amelogenin has also been identified in dentin (34), and previous studies have suggested that ameloblasts secrete amelogenin into the predentin matrix (35, 36). It is believed that amelogenin can diffuse from the enamel matrix to both the odontoblastic layer and dentin (16). Furthermore, studies have demonstrated that amelogenin is synthesized by odontoblasts and expressed in the dentin of human tooth germs before the deposition of the enamel matrix (36, 37).

In our current study, we observed strong amelogenin staining in ameloblasts, odontoblasts, deposited enamel matrix, the cervical loop of central incisors, and first molars in both jaws. Moreover, the immune positivity of the SI cells increased with gestational weeks. Some cells in the SR layer and the OEE layer also exhibited staining in these primary human tooth germs (during the 18-23 g.w.). These findings further support the role of amelogenin in the differentiation of ameloblasts and odontoblasts, as well as human primary tooth development.

A previous animal study has reported spot-like staining of amelogenin in predentin (25). However, weak staining was observed in the SR cells and the OEE cells at the 30th g.w. (15). Similarly, in our present study, spot-like staining of amelogenin was also observed in the predentin.

Epithelial-mesenchymal interactions play a crucial role in the differentiation of dental pulp stem cells into odontoblasts and the differentiation of epithelial cells of the EO into ameloblasts (30, 36). These cells subsequently synthesize the extracellular matrices of dentin and enamel, respectively (30). The expression of amelogenin in both ameloblasts and odontoblasts in our study suggests the involvement of amelogenin in epithelial-mesenchymal interactions during tooth development.

A previous animal study has indicated that the cervical loop contains stem cells and serves as a reservoir for ameloblasts and SI cells (38, 39). In our study, an increased immune positivity of amelogenin was also observed in the labial cervical loop cells as gestational weeks progressed. This finding further supports the role of the cervical loop as a reservoir for stem cells.

Conclusion

Our study provides detailed insights into the expression of amelogenin during human primary tooth development. A potential association was observed between amelogenin expression and the differentiation of ameloblasts and odontoblasts, as well as the deposition of enamel and dentin matrices. Additionally, our findings suggest the cervical and coronal portions of the EO may act as reservoirs for stem cells. Thus, in the process of tooth development, inductive signals from both the dental epithelium and underlying mesenchyme contribute to amelogenesis. Further studies are needed to fully elucidate the role of amelogenin in human primary tooth development.

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Conflicts of interest

The authors disclose any conflicts of interest related to the work in this manuscript.

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