Immunolocalization of FGF-10 in murine incisors and molar germs

Imunolocalização de FGF-10 em germes de incisivos e molares murinos

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ABSTRACT

Objective

The aim of this study was to identify the presence of FGF-10 in mouse dental germs by means of the immunohistochemical technique, from the initial development phase through to the more advanced phases.

Methods

Fetuses of five mice, on days 15.5, 16.5, 17.5, 18.5 and 19.5 of pregnancy, respectively, were collected. At time intervals of 0.5, 1.5, 2.5 and 3.5 days after birth, the mouse offspring were sacrificed. The heads of all the specimens were fixed and submitted to histotechnique and 3μm thick sections were obtained. The presence of FGF-10 was detected by means of the avidin-biotin-peroxidase immunohistochemistry technique.

Results

Immunostaining was detected in both epithelium and ectomesenchyme with intensity and spatial-temporal differences. A reduction in the presence of FGF-10 was observed in the cervical loop area, on the lingual side of the incisor crowns, and both sides of the molar crowns. With the increase in enamel matrix deposition, immunostaining on the secretory pole of ameloblasts also increased.

Conclusion

FGF-10 immunostaining could be related to cell proliferation in epithelium and cell differentiation in epithelium and ectomesenchyme. This could be related to morphological determination in intercuspal areas of molar germs and to continuous growth of the incisor crown. Decrease in FGF-10 in the cervical loop could be related to the termination of crown formation. Increase in FGF-10 expression in ameloblasts suggests a relationship with active enamel production. The results suggest the inclusion of the pattern of the presence of FGF-10 in future investigations into the cause of morphological anomalies, such as palato-gingival groove.

Indexing terms: *Immunohistochemistry. Odontogenesis. Tooth development.*

RESUMO

Objetivo

Identificar a presença de FGF-10 em germes dentários de rato pela técnica de imunohistoquímica.

Métodos

Os fetos de cinco ratos, nos dias 15,5; 16,5; 17,5; 18,5; 19,5, respectivamente, de gravidez, foram coletados. Em intervalos de tempo de 0,5; 1,5; 2,5 e 3,5 dias após o nascimento, as proles de ratas foram sacrificadas. As cabeças de todos os exemplares foram fixadas e submetidas à histotécnica e cortes com 3μm de espessura foram obtidos. A presença de FGF-10 foi detectada pela técnica de imunohistoquímica da avidina-biotina-peroxidase.

Resultados

A imunomarcação foi detectada no epitélio e no ectomesênquima com intensidade e diferenças espaço-temporais. Uma redução da presença de FGF-10 foi observada na área da alça cervical, no lado lingual de coroas de incisivo e em ambos os lados das coroas de molares. Com o aumento da deposição de matriz de esmalte, a imunomarcação no pólo secretor de ameloblastos também aumentou.

Conclusão

a presença de FGF-10 parece estar relacionada com a proliferação de células no epitélio e diferenciação de células no epitélio e ectomesênquima. Isso pode estar relacionado à determinação morfológica nas áreas intercuspídeas de germes dos molares e ao crescimento contínuo da coroa nos incisivos. A diminuição do FGF-10 em áreas alça cervical parece estar relacionada com o término de formação da coroa. O aumento de FGF-10 em ameloblastos parece estar relacionada com a produção ativa de esmalte.

Termos de indexação: Imunoistoquímica. Odontogênese. Germe de dente.

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INTRODUCTION

Odontogenesis is the result of a manifestation of an polygenic system, in which the development genes manifest themselves in a sequential, cyclic pattern[1-2]. Over 200 genes have been associated with odontogenesis[1-2] and dental defects[1,3].

Many molecules and some gene cascades have been identified in the initial phases of tooth development: bud, cap, bell, and to a lesser degree, root formation[4-10]. Families of molecules such as those of Wnt, Hh (hedgehog), FGFs (Fibroblast Growth Factor) and BMPs (Bone Morphogenetic Protein, of TGFßs - Transforming Growth Factor family) have been identified, in different proportions and sites, with tissue-specific and stage-specific functions[1-2]. They have been related to functions such as determining the fate of neural crest cells during tooth morphogenesis[11], stimulating interactions between the ectoderm and ectomesenchyme (cell induction, proliferation, condensation and differentiation); forming signaling centers, as well as inducing transcription factors[2]. Disturbances in gene expression patterns have been related to tooth anomalies such as tooth agenesis, supernumerary teeth and root malformation[1,3]. All these studies have brought knowledge that has led to achievements in bio dental engineering[2,12-14].

FGFs expression, is associated with initiation eventsinducing the expression of other genes[2] by stimulating cell proliferation in the epithelium[15-16]. The expression of FGF-10 would thus maintain a stem cell compartment[17-18] that would supply epithelial cells to the inner dental epithelium, which would remain as cells of the stratum intermediate[18] or would differentiate into ameloblasts[15]. The cessation of FGF-10 expression would interrupt crown formation and lead to root development[4,8,17]. Its expression has also been related to tooth morphogenesis as it would induce epithelial cell proliferation[1,15].

FGF-10 has been related to cell proliferation[15,19-21] and fate[15], in early tooth developmental phases. The pattern of FGF-10 presence, from early to later stages of tooth development, might help us to understand the relationship between this molecule and events in tooth development. In addition, its distribution pattern might help with the understanding of anomalies of tooth morphology in future approaches. The aim of this study was to investigate the presence of FGF-10 in incisor and molar germs in mice, from the initial embryonic days of development (E): E15.5, E16.5, E17.5, E18.5, E19.5 to the more advanced phases 0.5, 1.5, 2.5 and 3.5 days after birth.

METHODS

The experiment was approved by the UFMS Ethics Committee on the Use of Animals, Protocol No. 159/07. Five female mice were anesthetized, on days 15.5, 16.5, 17.5, 18.5 and 19.5 of pregnancy, respectively, using a combination of Ketalar® (0.08ml/100g), Virbaxil® (0.04/100g), prior to the caesarean section, when the fetuses were collected. At time intervals of 0.5, 1.5, 2.5 and 3.5 days after birth, the mouse offspring were sacrificed. Their heads were fixed in 10% formaldehyde for 48 hours and submitted to histotechnique, embedded in paraffin, alternating their sectional planes: the first was in the front section plane and the second was in the sagittal plane. The sections, about 3µm thick, were applied to silanized slides.

The presence of FGF-10 was detected by the avidin-biotin-peroxidase immunohistochemistry technique[19]. After removing the paraffin from the sections with xylol and rehydrating them through a series of graded ethanol baths, antigen recovery was performed through humid heat treatment in citrate buffer for two and a half minutes. After cooling for 20 minutes, endogen peroxidase was blocked by 10 volume-H_2O_2 baths of 10 minutes each. Next, the sections were washed with distilled water, neutralized with Phosphate Buffered Saline (PBS) and dried thoroughly. The slides were incubated with rabbit anti-mouse antibody FGF-10 (200µg/ml, H-121, Santa Cruz Biotechnology) diluted in PBS and albumin at $1/100$ concentration for 18 hours, at 4ºC. After being washed with PBS, the sections were thoroughly dried in order to receive a pre-prepared solution of biotinylated antibodies for an exposure of 40 minutes. Before being exposed to streptavidin for 30 minutes, the slides were properly washed and dried and were stained with chromogenic diaminobenzidine (DAB) solution and incubated for 30 minutes. After being washed, the sections were quickly stained with hematoxylin, washed and passed through ammonia water, dehydrated through a series of graded ethanol baths, immersed in xylol to be assembled with Entellan.

Positive control of the reactions was obtained using rabbit ovary sections; and the negative control was obtained by replacing the primary antibodies with PBS-BSA 1%.

RESULTS

The FGF-10 immunostaining pattern in incisor germs is summarized in Chart 1.

In early incisor crown development, at E15.5, the cap phase could be identified. Immunostaining was evident in the proximal pole of inner epithelium cells. In the ectomesenchyme, it was identified in the cells facing the basal membrane and also between more distant cells.

Both E16.5 and E15.5 incisor germs presented very similar immunostaining patterns, with moderate immunostaining in both tissues. As the cells of the inner epithelium were proliferating, staining was more intense and covered a larger area of cytoplasm in their secretory pole. Epithelial cells also presented immunostaining in the distal pole, although less intensely. Longitudinal sections showed a predominance of moderate immunostaining of epithelial and ectomesenchymal cells on the labial side of the tooth germ crown. The ectomesenchymal cells that were condensed and turned towards the epithelium presented more intense immunostaining.

At E17.5, incisors were in the bell stage. In the ectomesenchyme, moderate immunostaining was observed in the secretory poles of more differentiated cells (Figure 1A) and in non-differentiated cells of the papilla, further away from the enamel organ. On the proximal side of the transverse sections of these incisor germs, weak immunostaining could be observed, predominantly in epithelial cells (Figure 1B), while weaker on the lingual side; almost no immunostaining was seen (Figure 1C). In longitudinal sections, the predominance of FGF-10 immunostaining distribution could be followed all the way through the labial side of the developing crown (Figure 1D). This could also be observed in the other periods analyzed: E18.5, E19.5, 0.5, 1.5, 2.5 (Figure 2) and 3.5 days after birth.

In the development of incisor germs at E18.5E, 19.5E, 0.5, 1.5, 2.5 and 3.5 days after birth, immunostaining followed the pattern observed in the previous phases. The outer epithelium cells also presented immunostaining in the distal pole, facing the dental follicle. As the enamel layer increased, so did the extent of immunostaining at the secretory pole of the ameloblasts (Figure 3).

TheFGF-10 immunostaining pattern in molar germs is summarized in Table 1.

In molar development at E15.5, the early cap stage structure was typically organized. In maxillary and mandibular molar crowns, at E16.5, development of the same pattern of immunostaining observed in E15.5 molar germs was identified.

Development of the maxillary and mandibular molar crowns at E17.5, 18.5 and 19.5 presented a very similar immunostaining pattern. The folding of inner epithelium (enamel knots) could be observed in areas related to the localization of future cusps. In areas related to the cervical loop, immunostaining diminished in both the epithelium and ectomesenchyme (Figure 2).

After birth, on days 0.5 and 1.5, the first maxillary molar germ presented a similar pattern (Table 2).

After birth, on days 2.5 and 3.5, molar sections presented a similar pattern with moderate immunostaining in both ameloblasts and odontoblasts at the secretory pole. The length of the secretory pole presenting staining grew with the increase in enamel deposition (Figure 4).

Chart 1. FGF-10 immunostaining intensity pattern in incisor germs.

		Cell pole	E 15.5	E16.5	E17.5	E18.5 E19.5 Ab 0.5 Ab 1.5	Ab 2.5 Ab 3.5
Epithelium	Inner epithelium	basal	moderate	moderate		weak	
	pre-ameloblasts	secretor				weak, moderate to intense	
	ameloblasts	basal secretor					moderate moderate
	intermediate layer			moderate	moderate	moderate moderate	moderate
	stellate reticulum			moderate	moderate	moderate	
	outer epithelium					weak	weak
Ectomesenchyme	papilla cells next to basal membrane		moderate	moderate			
	other papilla cells			weak	weak	moderate	
	pre odontoblasts	secretory basal				moderate	moderate moderate
	odontoblasts	secretory basal					moderate moderate
	other pulp cells					weak	weak
	cervical loops cells						
	epithelial sheath cells						
side of germ crown with prevalent immuno-staining	labial			X	X	X	X
	lingual						

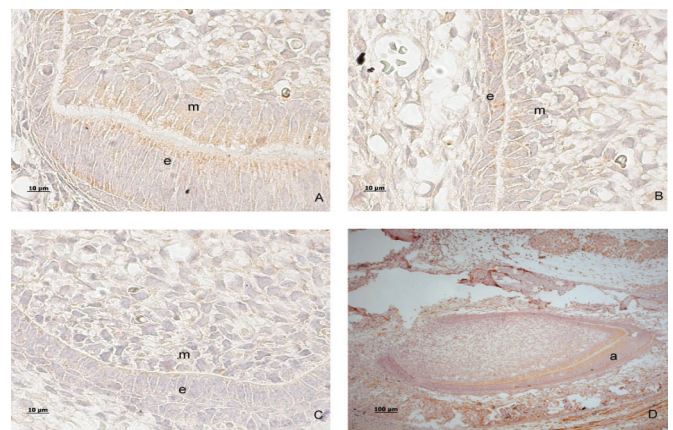Note: E - embryonic stage; Ab - after birth period. UFMS/FOB-USP (2010).

Figure 1. A, B, C cross-section of incisor germ at E17.5. A) labial side of germ crown presenting moderate FGF-10 immunostaining in both ectomesenchymal (m) and epithelium (e) cells. In epithelial cells, immunostaining is observed at both poles, moderate at the secretory pole and weak at the opposite pole; B) proximal side of germ crown epithelium-ectomesenchyme interface. Epithelial cells show weak immunostaining; C) lingual side of germ crown epithelium-ectomesenchyme interface. Very weak to no immunostaining can be observed in epithelium cells (x1000). D) Longitudinal cut of incisor germ at E17.5. Moderate immunostaining can be seen all through the labial contour at germ crown epithelium-ectomesenchyme interface. The basal poles of ameloblasts (a) show weak immunostaining. A, B andC =1000X andD = 100X. UFMS/FOB-USP (2010).
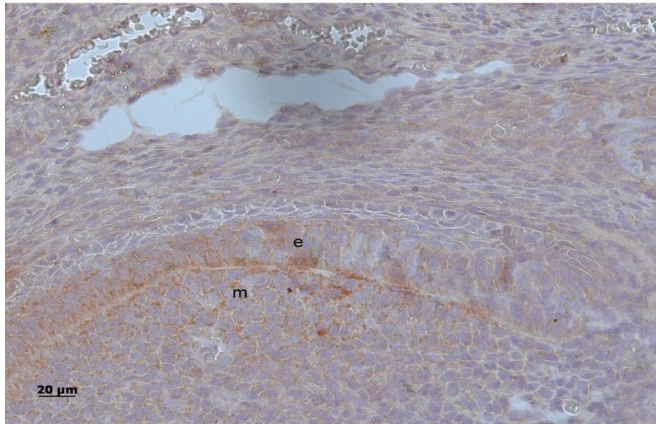
Figure 2. Immunostaining in E19.5 molar germ.Immunostaining diminishes towards cervical loop. Epithelium (e); ectomesenchyme (m).1000X. UFMS/FOB-USP (2010).
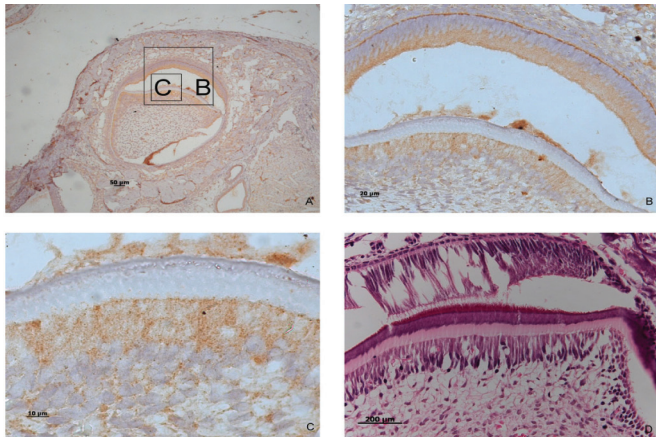

Figure 3. A) FGF-10 immunostaining in incisor germ, 2.5 after birth. Moderate immunostaining on the labial side of the germ crown, which becomes weaker towards the lateral side of the crown and diminishes to almost nothing on the lingual side of the crown. B) extent of immunostaining at the secretory pole of the epithelium cells. C) ectomesenchyme. D: H.E. cut demonstrating the extent of enamel layer and the secretory pole of cells with intracellular materialA = 100X, B and D = 400Xand C = 1000X. UFMS/FOB-USP, 2010.
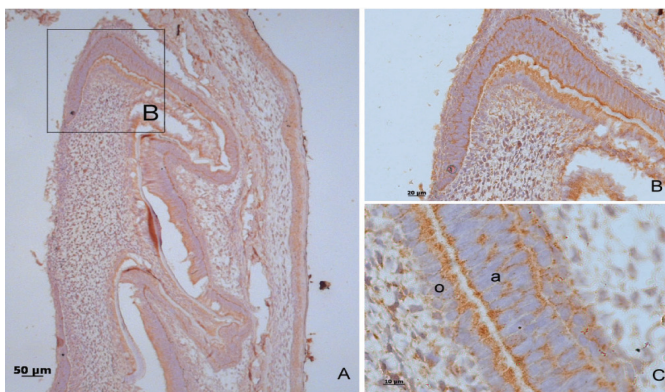

Figure 4. Immunostaining in 3.5 molar germ, after birth. A) moderate immunostaining is observed at the epithelium-ectomesenchyme interface, in epithelial cells of intermediate layer and among ectomesenchymal cells; B) immunostaining decreases towards epithelial sheath; C) the secretory pole of ameloblasts (a) and odontoblasts (o) presents moderate immunostaining. The basal pole of ameloblasts presents weaker immunostaining. The inner epithelial cells present moderate immunostaining A = 100X, B = 400X and D = 1000X. UFMS/FOB-USP, 2010.

Table 1. FGF-10 immunostaining intensity pattern in molar germs.

		Cell pole	E 15.5	E16.5 / E17.5 / E18.5 / E19.5	Ab 0.5 / Ab 1.5	Ab 2.5 / Ab 3.5
Epithelium	Inner epithelium			moderate		
	pre-ameloblasts	basal				weak
		secretory				moderate
	ameloblasts	basal				
		secretory				moderate
	intermediate layer		moderate	moderate	moderate	moderate
	stellate reticulum		moderate	moderate	moderate	moderate
	outer epithelium					
Ectomesenchyme	papilla cells next to basal membrane		moderate	moderate		
	pre-odontoblasts	secretory			moderate	
		basal				
	odontoblasts	secretory				moderate
		basal				
	other pulp cells follicle cells cervical loop cells		weak	weak	weak	weak
	cervical loop cells			none (E16.5) to weak	weak	
	epithelial sheath cells					weak to none

Note: E: embryonic stage;Ab: after birth period. UFMS/FOB-USP (2010).

DISCUSSION

In the present study, immunolocalization of FGF-10 was observed in incisor and molar germs in all the phases analyzed, in both the epithelium and ectomesenchyme, with a variation in intensity and localization in these phases.In this study, FGF-10 was present in areas related to epithelial and ectomesenchymal cell proliferation in the early developmental stages, (E15.5; E16.6) as previously reported[15]; and in cell differentiation areas (from E17.5 to day 3.5 after birth) in incisors and molars. The expression of FGF-10 has been identified in the mesenchyme, and is related to epithelialstem cell proliferation, maintenance and fate[8,17-18,20-21]. Other studies have described the presence of FGF-10 only in the ectomesenchyme and related to epithelium cell proliferation[15,21] and differentiation[15]. The immunostaining intensity observed in the ectomesenchyme was higher in the area at the epithelial-mesenchymal interface than in the remaining part of the papilla. FGF-10 expression in the mesenchyme has previously been reported and is related to the induction of cell proliferation in the epithelium[15,18,20].

In longitudinal sections of the incisor, immunostaining in both the ectomesenchyme and epithelium could be seen. It occurred predominantly on the labial side of the crown, where pre-ameloblasts and pre-odontoblasts could be observed, as previously reported[18]. Very weak to no immunostaining was observed in the epithelium of the crown on the lingual side. FGF-10 expression was related to the maintenance of an adult epithelium stem cell for crown formation[4,8]. Thus, the presence of FGF-10 on the labial side of incisor crowns, observed in all periods analyzed, could be associated with continuous tooth growth which demands crown development with enamel deposition on the labial side of the crown. In molars, moderate FGF-10 immunostaining was also observed in the stellate reticulum and inner epithelium, from E15.5 through all other periods analyzed. These areas were related to stem cell proliferation and maintenance and to ameloblast precursor cell proliferation, respectively[20], while the crown was forming. At E17.5, epithelial cells presented moderate immunostaining in intercuspal areas and in the stellate reticulum. Cell proliferation is necessary to promote growth and is also associated with morphological determination. Areas of cell proliferation around areas in which cell apoptosis is identified are related to cusp determination[2]. The determination of some morphological tooth anomalies could also involve these events, but with some disturbances of localization or intensity in gene expression cascades. FGF-10 deficient mice fail to form a cervical loop and present interruption of dental epithelial development in their explants[17]. Therefore, studies on palato-gingival groove, for example, should explore the pattern of FGF-10 presence.

The extent of FGF-10 immunostaining increased at the secretory pole of ameloblasts, as the extent of the enamel layer increased. The presence of FGF-10 has previously been related to epithelial cell fate[15,18,21], but this study showed a later stage of tooth development, up to 3.5 days after birth, when FGF-10 may have some relationship with cell activity, such as active enamel production.

In molars, FGF-10 presented moderate immunostaining at E15.5, E16.5 in mesenchymal cells. This could be related to cell proliferation areas, such as areas of enamel knots, as previously reported[2,15].

CONCLUSION

Immunostaining of FGF-10 was identified in both the epithelium and ectomesenchyme in all tooth germ stages analyzed and could be associated with cell proliferation in the epithelium and cell differentiation in the epithelium and ectomesenchyme. It could be related to morphological determination in the intercuspal areas of molar germs and to continuous growth of the crown in incisors. A decrease in the presence of FGF-10 was observed in the cervical loop area on the lingual side of the incisor crown and on both sides of molar crowns. This could be related to the termination of crown formation. With the increase in enamel matrix deposition, immunostaining at the secretory pole of ameloblasts also increased, in later phases of development, which suggests a relationship with active enamel production. The findings suggest that the pattern of FGF-10 presence should be analyzed when studying dental morphological anomalies, such as the palato-gingival groove.

Acknowledgments

Collaborators

JP ENNES coordinated the research project and participated in all phases of the development of the article. VS LARA was responsible for the standardizing the antibody, checking the slides, photomicrography and the composition of the article. IA TOZETTI supervised the development of the immunohistochemical technique, the checking of the slides and took part in the composition of the article. DM ANTUNES supervised the histotechnique, checking of the slides and took part in the composition of the article. GB BORGATO participated in the development of the immunohistochemical technique of the specimens from 0.5 to 3.5 days, the checking of the slides, photomicrography and the composition of the article

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