

## The expression of the epidermal growth factor (EGF) during odontogenesis: Immunohistochemical study in mice (*Mus musculus*)

## A expressão do fator de crescimento epidérmico (EGF) durante a odontogênese: Estudo imunohistoquímico em camundongos (*Mus musculus*)

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### ABSTRACT

*The objective of this study was to verify, through the technique of Immunohistochemistry, the expression of the epidermal growth factor (EGF) in the odontogenesis of the first upper molar in *Mus musculus* mice, relating it to the microscopic morphologic analysis. The sample consisted of 23 animals, with age from the 13<sup>th</sup> day of intrauterine life to the 20<sup>th</sup> day of postnatal life. The results showed EGF being expressed by several tissues and cells participating of the odontogenesis. However the dental follicle was the only place where the expression of EGF was constant for the whole process, characterizing it as one of the regulating tissues of odontogenesis.*

### UNITERMS

*Odontogenesis; tooth eruption; dental sac; EGF*

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### INTRODUCTION

Odontogenesis begins as a result of an interaction between the dental epithelium and the dental mesenchyme (originating from cells of the neural crest that migrated to the embryonic mesenchymal tissue).

The dental eruption is a localized process and has a precise time of movement of the tooth from its site of development within the alveolar bone to its functional position in the oral cavity. This displacement depends on two factors: osteoclastic activity (the reabsorption of bone to form an eruption pathway) and alveolar bone remodeling, these being functions inherent to specific portions of the dental follicle (MARKS & SCHROEDER<sup>9</sup>, 1996; WISE<sup>16</sup>, 1998).

The dental follicle, formed from mesenchymal soft tissue which surrounds the tooth, must be present if dental eruption is to occur, as seen by absence of the process in cases where the follicle has

been surgically removed prior to the start of eruption (CAHILL & MARKS<sup>1</sup>, 1980). In another article, the same authors demonstrated that, when a tooth is substituted by an inert object before the eruption, the process will usually still occur (MARKS & CAHILL<sup>8</sup>, 1984).

Bone resorption and bone formation are polarized around erupting teeth and these metabolic events depend on the adjacent parts of the dental follicle (MARKS & SCHROEDER<sup>9</sup>, 1996).

The dental follicle is essential for eruption, because it is the site of the influx of mononuclear cells which in turn form the osteoclasts needed to resorb alveolar bone to form an eruption pathway (WISE<sup>16</sup>, 1998).

The dental eruption is an excellent model for examining the osseous metabolism, as bone resorption and bone formation occur simultaneously and are spatially separated (VOLEJNIKOVA et al.<sup>15</sup>, 1997).

The two major challenges in the development of the study of molecular biology are: to determine the nature of the structural and regulatory genes involved in the specific processes of differentiation, and to elucidate the control mechanisms of sequential gene expression (RUCH<sup>13</sup>, 1985).

There are at least three ways whereby a few signaling molecules may achieve the induction and differentiation of different organ systems. They may be expressed singly in precisely the right place and time, or they may be expressed in combination with others of either a synergistic or antagonistic role. These different roles permit a considerable number of permutations to bring about the complex events associated with organogenesis (TEN CATE<sup>14</sup>, 1998).

A cascade of molecular events can initiate cellular events through monocyte recruitment and subsequent osteoclast formation. Questions began to be unmasked and it became possible to know more about the small proteins which induce cells in rest to suffer cellular division and, in some cases, differentiation (TEN CATE<sup>14</sup>, 1998; WISE<sup>16</sup>, 1998).

Human EGF (polypeptide isolated from human urine), and mouse EGF both have similar chemical characteristics and exhibit identical biological activities (CARPENTER & COHEN<sup>3</sup>, 1979). EGF is one of the most energetically stable proteins (molecular weight 6045).

EGF interacts with other molecules (Figure 1), such as transforming growth factor beta 1 (TGF- $\beta_1$ ), which enhances or inhibits the response of most cells to other growth factors (DERYNCK<sup>4</sup>, 1986; LEHNINGER et al.<sup>6</sup>, 1995), and interleukin-1 alpha (IL-1 $\alpha$ ), which increases the bone reabsorption, stimulating the production of CSF-1, a molecule also responsible for the chemotaxis for mononuclear cells (WISE et al.<sup>20</sup>, 1997).

The dental follicle produces CSF-1 and MCP-1, which are chemotactic for monocytes. Both of these chemoattractants can be responsible for the influx of monocytes into the follicle necessary to initiate tooth eruption (QUE & WISE<sup>11</sup>, 1997).

Located in the stellate reticulum, the molecule parathyroid hormone-related protein (PTHrP) enhances the expression of monocyte chemotactic protein-one (MCP-1) as much as it does that of colony-stimulating factor-1 (CSF-1), both being chemoattractant factors for mononuclear cells. PTHrP can exert a paracrine effect on the cells of

the adjacent dental follicle, influencing the expression of molecules potentially related to the beginning of the eruption (WISE et al.<sup>21</sup>, 2000).

The potential tooth-eruption molecules (EGF, TGF- $\beta_1$ , IL-1 $\alpha$  and CSF-1) enhance the expression of the MCP-1 gene in the dental follicle cells in culture (QUE & WISE<sup>12</sup>, 1998).

Dental follicle cells in culture synthesized and secreted MCP-1 into the medium (WISE & HUANG<sup>17</sup>, 1999). The secretion of MCP-1 by the cells is enhanced when the cells are incubated with either TGF- $\beta_1$  or IL-1 $\alpha$ .

The aim of this study was to confirm, through the modern molecular biology techniques, the continuous and fundamental role of the dental follicle in the dental eruption, once classic papers have established that only in clinical experiments.

## MATERIALS AND METHOD

We used 23 Swiss white mice. It was necessary to use five embryos to cover the period from the 13<sup>th</sup> to the 18<sup>th</sup> day of intrauterine life, and 18 specimens were appraised from the birth until the 20<sup>th</sup> day of postnatal life. The heads were removed and the part of the maxilla containing the portions cor-

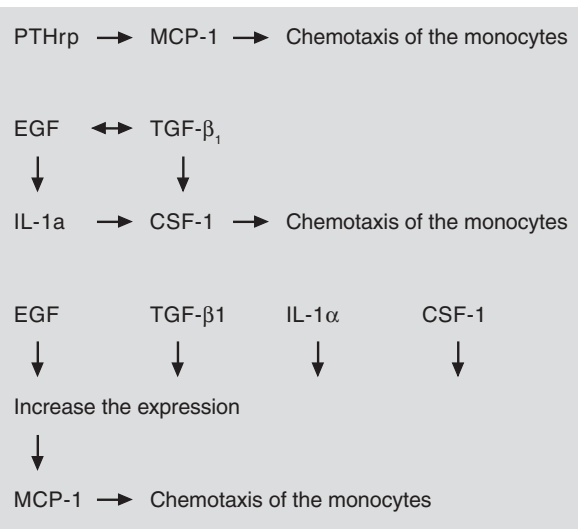


FIGURE 1 - Molecular interaction related to the dental eruption.

responding to the dental germs of the first upper molars were fixed in 10% formol for 48hr. Thereafter, the pieces were demineralized in solution of EDTA at 4°C. Then, all the pieces were dehydrated in alcohol, cleared in xylol and soaked in paraffin.

The cuts were made with a thickness of 3µm. The laminas were fixed in the stove at 60°C for 1hr and then stored at 37°C overnight. The sections were deparaffinized in the following sequence: xylol for 10 min. (3 times); absolute alcohol for 5 min.; absolute alcohol for 3 min. (twice); 95% alcohol for 3 min.; 70% alcohol for 3 min, then washed in distilled water for 5 min.

In the blockade of the endogenous peroxidase, H<sub>2</sub>O<sub>2</sub> was used to at 3% (3 ml of H<sub>2</sub>O<sub>2</sub> and 97 ml of methanol) for 20 min., in darkness. Afterwards, they were washed with phosphate-buffered saline (PBS) for 5 min.

The slices were incubated with the primary antibody "Anti-mouse EGF", product number E2635 (Sigma, St Louis, USA) also in darkness, with a concentration of 1:100 for about 1hr30min. and soon after, three baths with PBS followed. The negative controls stayed in PBS.

The Envision® "rabbit antibody" kit, product number 4003 (Dako, Copenhagen, Denmark) was used as a secondary antibody, its incubation being for 30 min., in darkness and at ambient temperature, followed by three baths with PBS. Diamino benzidine (DAB) was applied, left for about 4 to 5 min. and then washed with distilled water (twice). The

counterstain was accomplished being used the following technique: hematoxylin for 1 min., then a wash in tap water; 70% alcohol (bathed quickly); 95% alcohol (bathed quickly); absolute alcohol (three baths); xylol (three baths), where the laminas stayed until they were mounted with Permount® resin and coverslips.

## RESULTS

Once EGF was first isolated from an extract of the mouse submaxillary salivary glands (LEVI-MONTALCINI & COHEN<sup>7</sup>, 1960), we opted to use cuts of the same ones for positive (Figure 2) and negative controls.

The slice of positive control showed that the mucosal acinus did not mark for EGF. Only some serous acinus marked intensely and in a dispersed pattern. The interglobular intercalated, striated and excretory ducts presented an intense and continuous demarcation pattern, serving as the parameter for the classification showed in Picture 1.

The first phase, or intrauterine odontogenesis begins in the 13<sup>th</sup> day (Figure 3). EGF was mainly expressed in a more intense and continuous way in the tissues of ectodermal origin on the 15<sup>th</sup> day (bud stage) and the 18<sup>th</sup> day (bell stage), showed in Figure 4. The tissues of dental mesenchymal origin marked for the growth factor in a moderate and usually dispersed pattern (Table 1).

A second phase was defined starting from birth, in the moment when the dental germ presented mineralized tissue and the dental papilla was denominated dental pulp. The ameloblasts expressed EGF intensely for almost the whole second phase, which lasted until the 12<sup>th</sup> postnatal day. The odontoblasts expressed EGF until the 15<sup>th</sup> day, and later presented a negative staining. During almost the whole period the dental pulp expressed it in a moderate and dispersed way, and the dental follicle expressed it intensely, in a dispersed pattern on days one, two, seven, eighth (Figure 5). On the other days it was present in a moderate and dispersed fashion (Table 2).

The third phase is presented in Table 3, and it was defined starting from the moment that the dental follicle, richer in obliquely disposed collagen fibers characterized the periodontal ligament. From the 13<sup>th</sup> to the 15<sup>th</sup> postnatal day, the ameloblasts expressed EGF in an intense and continuous way, and later became negative. The radicular odonto-

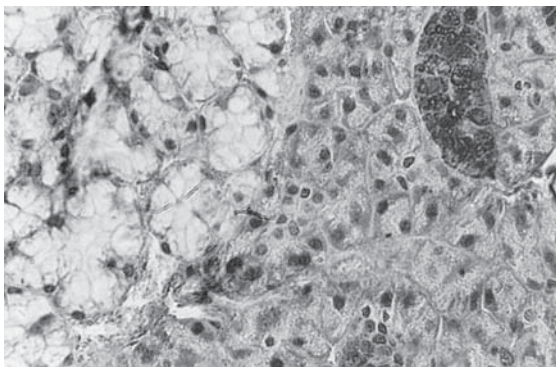


FIGURE 2 - Expression of EGF in the glandular tissue of the submandibular of adult mouse. Control positive. 80x.

Picture 1 - Pattern score for Immunohistochemistry

Absence	Faint	Moderate	Intense
-	+	++	+++

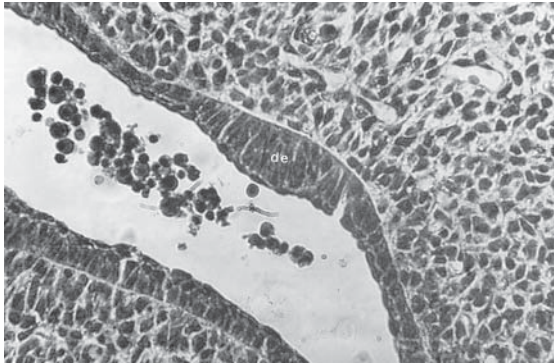


FIGURE 3 - Expression of EGF in the primary band (of) in the intra-uterine 13<sup>th</sup> day.



FIGURE 4 - Expression of EGF in the dental organ (eo), dental papilla (dp) and the dental follicle (df), in the intra-uterine 18<sup>th</sup> day. Out seen of the alveolar-bone. 16x.

Table 1 - Expression of EGF from 13<sup>o</sup> to the 18<sup>o</sup> intrauterine day

	13 <sup>th</sup> day	15 <sup>th</sup> day	16 <sup>th</sup> day	17 <sup>th</sup> day	18 <sup>th</sup> day
Dental lamina			++		
Dental epithelium	+				
Dental mesenchyme	++				
Outer enamel epithelium		+++	++	++	+++
Stellate reticulum		++	++	++	++
Stratum intermedium		++			
Inner enamel epithelium		+++	++	++	+++
Dental papilla		++	++	+	++
Dental follicle		++	++	++	++

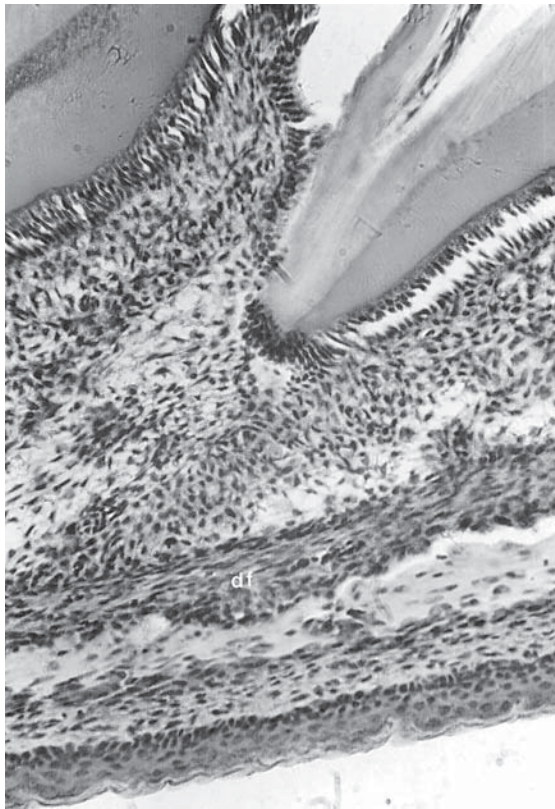


FIGURE 5 - Dental follicle (df) expressing EGF intensity in the 8° powder - native day. 80x.

blasts expressed EGF starting from the 14<sup>th</sup> day, and were directly involved with root formation, as well the pulpal endothelium. On the 17<sup>th</sup> day, radicular odontoblasts and pulpal endothelium both reached an intense and continuous staining. The periodontal ligament maintained the same characteristics of staining of the dental follicle during the first two phases: moderate to intense and always with the dispersed pattern.

## DISCUSSION

Odontogenesis happens starting from an interaction between the dental epithelium and the dental mesenchyme. Phenomena such as the blood pressure exercised by the dental papilla, proliferation of the dental pulp and dentin formation (CAHILL et al.<sup>2</sup>, 1988); root growth (CAHILL & MARKS<sup>1</sup>, 1980); changes in the periodontal ligament (HARRIS & DMYTRYK<sup>5</sup>, 1988) and alveolar bone remodeling (MARKS & SCHROEDER<sup>9</sup>, 1996) are essential for the process of dental eruption.

The dental follicle, derived from the dental mesenchyme, assumes a fundamental role in the regulation of the dental eruption, as much by being the place in which a constant expression and interaction of the fundamental molecules for the evolution of the cellular alterations occurs, as by being a vehicle for the entrance and transport of the mononuclear cells necessary for events such as reabsorption and bone formation (WISE & LIN<sup>19</sup>, 1995; WISE<sup>16</sup>, 1998).

Table 2 - Expression of EGF from the birth to the 12<sup>th</sup> postnatal day

	Zero	1 <sup>st</sup> day	2 <sup>nd</sup> day	5 <sup>th</sup> day	6 <sup>th</sup> day	7 <sup>th</sup> day	8 <sup>th</sup> day	9 <sup>th</sup> day	11 <sup>th</sup> day	12 <sup>th</sup> day
Outer enamel epithelium	+++									
Stellate reticulum	++	++	+++							
Ameloblasts	+++	+++	+++	+++	+++	+++	++	+++	+++	+++
Odontoblasts		+++	++	+++	-	-	-	-	-	-
Dental pulp	++	++	+	++	++	++	++	++	+	++
Dental follicle	++	+++	+++	++	++	+++	+++	++	++	++

EGF inhibits early morphogenesis and cell differentiation of mouse embryonic molar teeth in organ culture, while at the same time stimulating cell proliferation in the tissues surrounding the tooth germs. The inhibitory effect of EGF in tooth morphogenesis occurs during the developmental stage, so that the capstaged molar teeth are affected by EGF in organ culture, but the teeth that have reached bell stage develop and differentiate in the presence of EGF (PARTANEN & THESLEFF<sup>10</sup>, 1987). The authors believe that EGF stimulates or maintains the proliferation of undifferentiated cells during the embryonic development.

In the beginning of the first phase (13<sup>th</sup> intrauterine day), dental mesenchyme with a moderate and dispersed staining assumes a dominant role in dental epithelium. Tissues involved in the crown formation expressed EGF intensely until the first days of the second phase. The ameloblasts expressed EGF intensely for almost the whole second phase, which lasted until the 12<sup>th</sup> postnatal day. This exercised a paracrine effect in the dental follicle (WISE et al.<sup>18</sup>, 1992).

Odontoblasts presented a negative staining since the sixth<sup>th</sup> postnatal day, but the radicular odontoblasts expressed EGF starting from the 14<sup>th</sup> during the root formation.

The dental follicle, and later the periodontal ligament were the only tissues that acted throughout odontogenesis in a constant way, characterizing them as tissues capable of regulating the whole process.

EGF can exercise a physiological role in the dental eruption, and the dental follicle can be the structure in which the growth factor acts. The reduction of EGF expression in dental follicle suggests that the role of EGF will decrease at the end of the process (WISE et al.<sup>18</sup>, 1992).

In the near future, studies with knockout gene mice (null mice) will be able to elucidate the role of the molecules that begin the dental eruption in cascade (WISE & HUANG<sup>17</sup>, 1999). Understanding the effects of the absence of these genes on odontogenesis and dental eruption will extend the possibility of the discovery of new treatments for problems in which odontogenesis occurs but dental eruption is absent.

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**Table 3 - Expression of EGF from the 13<sup>th</sup> to the 20<sup>th</sup> postnatal day**

	13 <sup>th</sup> day	14 <sup>th</sup> day	15 <sup>th</sup> day	17 <sup>th</sup> day	19 <sup>th</sup> day	20 <sup>th</sup> day
Ameloblasts	+++	+++	+++	-		
Odontoblasts	-	-	-	-		
Radicular odontoblasts		+	++	+++	++	++
Dental pulp	++	++	++	++	++	++
Periodontal ligament	++	++	++	+++	++	++
Pulpal endothelium				+++	+++	++

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## RESUMO

O objetivo deste estudo foi verificar, por intermédio da técnica de imunohistoquímica, a expressão do fator de crescimento epidérmico (EGF) na odontogênese do primeiro molar superior em camundongos *Mus Musculus*, correlacionando-o com a análise morfológica microscópica óptica. A amostragem constou de 23 animais com a idade variando do 13º dia de vida intra-uterina até o 20º dia de vida pós-natal. Os resultados mostraram o EGF sendo expresso por vários tecidos e células participantes da odontogênese. Entretanto, o folículo dentário foi o único local onde a expressão do EGF permaneceu constante por todo o processo, caracterizando-o como um dos tecidos reguladores da odontogênese.

## UNITERMOS

Odontogênese; erupção dentária; folículo dentário; EGF

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