Int. J. Dev. Biol. 36: 381-389 (1992)

Original Article

Immunolocalization of acidic and basic fibroblast growth factors during mouse odontogenesis

YVES CAM[1]*, MARIE-ROSE NEUMANN[1], LISA OLIVER[2], DANIEL RAULAIS[2], THIERRY JANET[3#] and JEAN-VICTOR RUCH[1]

[1]*Institut de Biologie Médicale, INSERM CJF 88-08, Université Louis Pasteur, Faculté de Médecine, Strasbourg,* [2]*INSERM Unité 118, Paris and* [3]*Laboratoire de Neurobiologie Ontogénique CNRS UPR 417 - Centre de Neurochimie, Strasbourg, France*

ABSTRACT **Acidic and basic fibroblast growth factors (aFGF and bFGF), are both known to bind to extracellular matrix components, particularly proteoheparan sulfates, and to regulate *in vitro* proliferation, differentiation and morphology of cells of neuroectodermal and mesodermal origins. Their patterns of distribution were studied during mouse odontogenesis by means of indirect immunofluorescence and immunoperoxidase histochemistry on frozen fixed sections and after Bouin's fixative and paraffin embedding. Localization of aFGF on frozen fixed sections was observed in the oral epithelium, dental lamina and oral mesenchyme (day-12 of gestation), the stellate reticulum and oral epithelium (day-14), the stratum intermedium and at the basal and apical poles of preameloblasts at bell stage. After birth aFGF epitopes were localized within the predentin-dentin area, the stratum intermedium and at the secretory pole of ameloblasts. There was no staining with anti-aFGF antibodies after Bouin's fixative and paraffin embedding. In contrast, using this protocol, intense stainings were found with anti-bFGF antibodies predominantly within dental and peridental basement membranes and mesenchyme: staining of the dental basement membranes was transient (bud and cap stage) and discontinuous; a preferential concentration of bFGF epitopes in the condensed dental mesenchyme of incisors (cap stage) and the dental papillae mesenchymal cells of molars (bell stage) was observed in the posterior and the cervical part of tooth germs. An intense immunostaining of the stellate reticulum with anti-bFGF antibodies was also found on paraffin sections from bud to bell stage. Localization of bFGF on frozen fixed sections was observed in the dental lamina, stellate reticulum and dental basement membranes at bud and cap stage, the stratum intermedium and at the secretory pole of ameloblasts after birth. Treatment of sections with NaCl (2-3M) solutions and heparitinase diminished, but did not abolish, specific immunostaining obtained with bFGF antibodies. Our results suggest that, among growth factors, a- and bFGFs might intervene in different ways during odontogenesis, particularly through binding to the cellular and/or extracellular matrix heparan sulfate-containing molecules, and may participate in the control of proliferation, determination and terminal differentiation of preodontoblasts and preameloblasts.**

KEY WORDS: *immunohistochemistry, acidic and basic fibroblast growth factors, mouse odontogenesis, tissue fixation*

Introduction

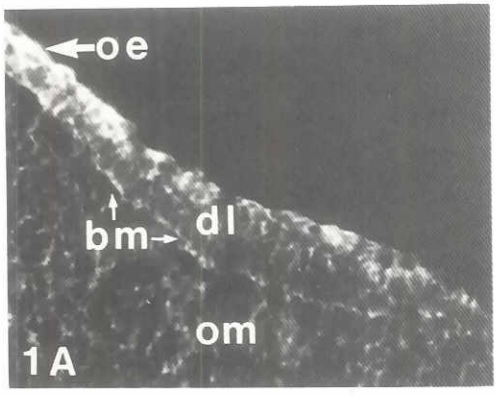
Odontogenesis is a well studied example of cell kinetic-dependent developmental processes that are mediated through homotypic and heterotypic cell interactions. Most of these interactions involve the temporal and spatial action of extracellular matrices first as solid substrata that interact with plasma membrane receptors and ligands and second as potential reservoirs for diffusible molecules such as growth factors (Cam *et al.*, 1987; Ruch, 1987, 1990; Slavkin, 1988; Thesleff *et al.*, 1990; Lesot *et al.*, 1990; Mark *et al.*, 1990; Kronmiller *et al.*, 1991a, b; Vainio *et al.*, 1991; Hall and Ekanayake, 1991).

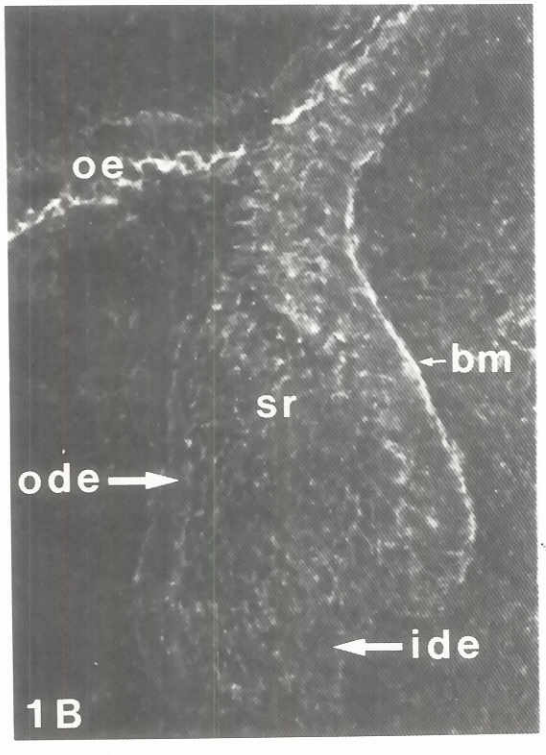
Abbreviations used in this paper: BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylene diamine tetra acetate; EGF, epidermal growth factor; HBSS, Hank's balanced salts solution; Ig, immunoglobulin; IGF, insulin like growth factor; K_d, dissociation constant; KGF, keratinocyte growth factor; NGF, nerve growth factor; PBS, phosphate buffered saline; RNA, ribonucleic acid; TGF, transforming growth factor; Tris, tris (hydroxymethyl) aminomethane.

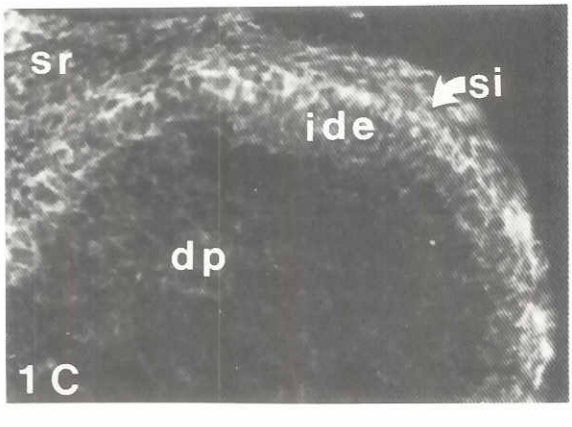
***Address for reprints:** INSERM CJF 88-08, Faculté de Médecine, Institut de Biologie Médicale, 11, rue Humann, 67085 Strasbourg-Cedex, France. FAX: 33-88242005.

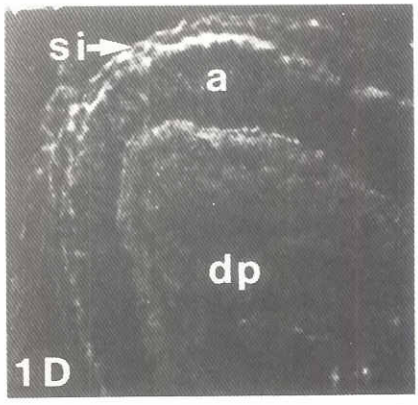
#Present address: Department of Anatomy and Cell Biology, University of Marburg, D-3550 Marburg, Germany.

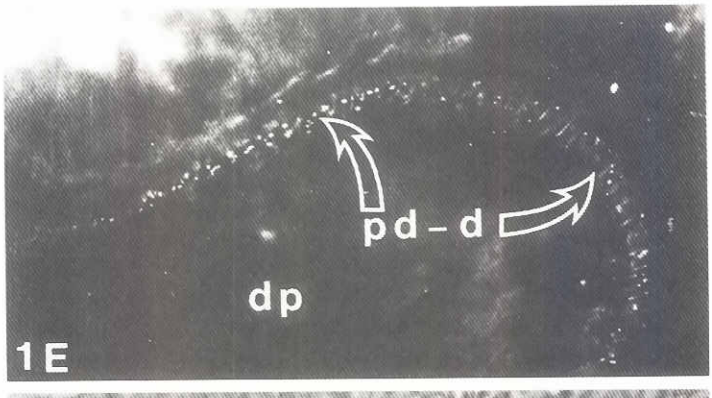
0214-6282/92/$03.00

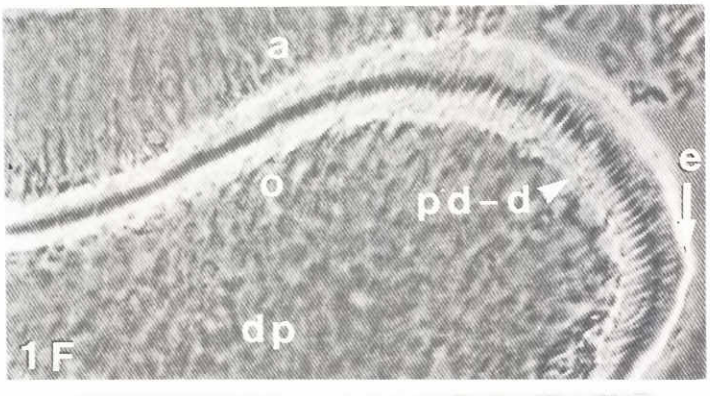

oe
bm
dl
om
1A

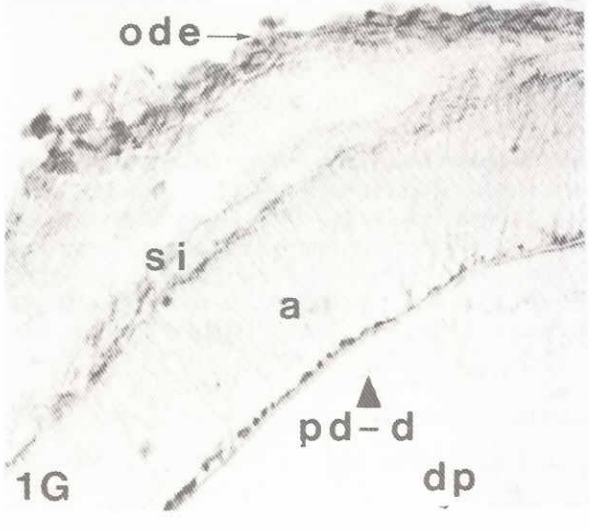

oe
bm
sr
ode
ide
1B


sr
si
ide
dp
1C


si
a
dp
1D


pd-d
dp
1E


a
e
pd-d
dp
1F


ode
si
a
pd-d
dp
1G

Several circulating and tissular growth factors and their receptors have been detected either at the mRNA and/or the protein level in murine dental tissues, especially pre-pro EGF (Snead *et al.*, 1989), EGF (Kronmiller *et al.*, 1991a), TGFα (Dixon *et al.*, 1991), NGF (Mitsiadis *et al.*, 1992), TGFs-ß$_1$, -ß$_2$, -ß$_3$ (Heine *et al.*, 1987; Lehnert and Akhurst, 1988; Cam *et al.*, 1990; D'Souza *et al.*, 1990; Pelton *et al.*, 1990; Vaahtokari *et al.*, 1991), EGF receptor (Partanen and Thesleff, 1987; Abbott and Pratt, 1988; Cam *et al.*, 1990; Wise *et al.*, 1990) and NGF receptors (Byers *et al.*, 1990). Growth hormone receptors (Zhang *et al.*, 1992) have also been localized in these tissues.

Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) are monomeric proteins sharing 55% structural homology that have no signal sequence in contrast to the other members of the FGF family, i.e., the protooncogene int-2, hst/k-FGF FGF-5, FGF-6 and KGF (reviewed by Goldfarb, 1990). Besides the *in vitro* growth-promoting activity towards fibroblasts originally described, different properties have been assigned to FGFs as have possible *in vitro* and *in vivo* roles such as chemotaxis, induction of proteases, including collagenases, synthesis and secretion of hormones, modulation of cell morphology and differentiation.

Expression of FGF genes at the RNA and the protein levels has been reported during chick, mouse and rat embryogenesis: both mRNAs and proteins have been localized in tissues of mesodermal and neurectodermal origins (Risau, 1986; Seed *et al.*, 1988; Wilkinson *et al.*, 1989; Gonzalez *et al.*, 1990; Fu *et al.*, 1991; Haub and Goldfarb, 1991).

Two types of receptors interacting both with aFGF and bFGF have been described so far. High affinity (K_d=2 to 15x10^{-11}) binding sites are membrane glycoproteins that have an external immunoglobulin-like domain and tyrosine kinase activity within their intracytoplasmic domain (Dionne *et al.*, 1990). Low affinity ($K_d \approx 2 \times 10^{-9}$) binding sites for bFGF have also been described (Baird and Ling, 1987; Vigny *et al.*, 1988; Bernfield and Sanderson, 1990; Kiefer *et al.*, 1990; Fayein *et al.*, 1990): these are heparan sulfate-containing proteoglycans located either within the extracellular matrix, especially basement membranes, or as constituents of plasma membranes, like syndecan. Other possible interactions or binding of FGFs have been evoked that involve type IV collagen, laminin and fibronectin (Thomson *et al.*, 1988; Feige *et al.*, 1989). The differential expression of two high affinity receptors, FGF-R_1 (the flg gene product) and FGF-R_2 (the bek gene product), in mesenchyme and epithelia respectively, has been recently demonstrated in rat and mouse embryos (Wanaka *et al.*, 1991; Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992).

In the present investigations, we used monospecific polyclonal antibodies to immunolocalize aFGF and bFGF during mouse molar and incisor development and demonstrated, by means of immunohistochemistry, that both factors are present in dental tissues at different stages of morphogenesis and during functional cytodifferentiation.

Results

Immunolocalization of aFGF

No significant staining of Bouin's fixed sections was found in the presence of specific anti-aFGF antibodies (not shown). In contrast, frozen fixed sections showed positive immunoreactivity within the oral epithelium, the dental lamina and the enamel organ of molars (Fig. 1A-B). Accumulation of aFGF was observed at the apical and the basal poles of preameloblasts (Fig. 1C-D) and ameloblasts (Fig. 1G) while a strong scattered specific immunostaining appeared at the predentin-dentin level (Fig. 1E). At bell stage and after birth cells of the stratum intermedium were also stained (Fig. 1C, D, G).

Immunolocalization of bFGF

On frozen sections fixed according to protocols 1, 2a and 3a (Figs. 2C, H and 3C) and on paraffin-embedded sections after Bouin's fixative (Figs. 2B, 3A and B), the distribution of bFGF was similar in the oral epithelium and the stellate reticulum (compare Fig. 2B-C), in some dental basement membranes (compare Fig. 3A and C) and between ameloblasts (compare Figs. 2H and 3E). Intense immunoreactivity was observed after Bouin's fixative in oral, dental and peridental mesenchymes: positive reactivity in the dental mesenchyme was found during dental lamina formation and at the bud stage (Fig. 2A-B); this reactivity was progressively restricted to clusters of cells in the incisors (Fig. 3B and D) and to some areas of the dental papilla in the posterior part of the molars (Figs. 2D-G and 3E). The stellate reticulum was also intensely stained after Bouin's fixative (Figs. 2G, 3D and 3E).

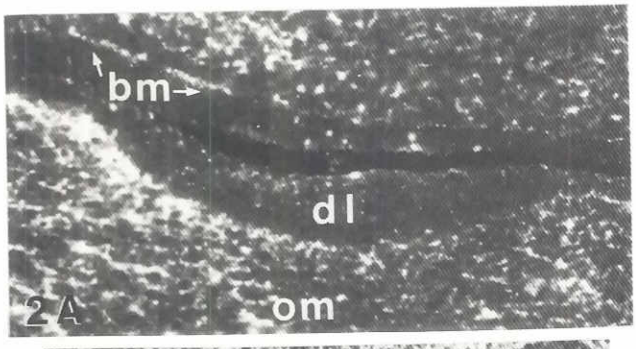
bFGF immunostaining following treatment with NaCl (2M-3M) or digestion with heparitinase

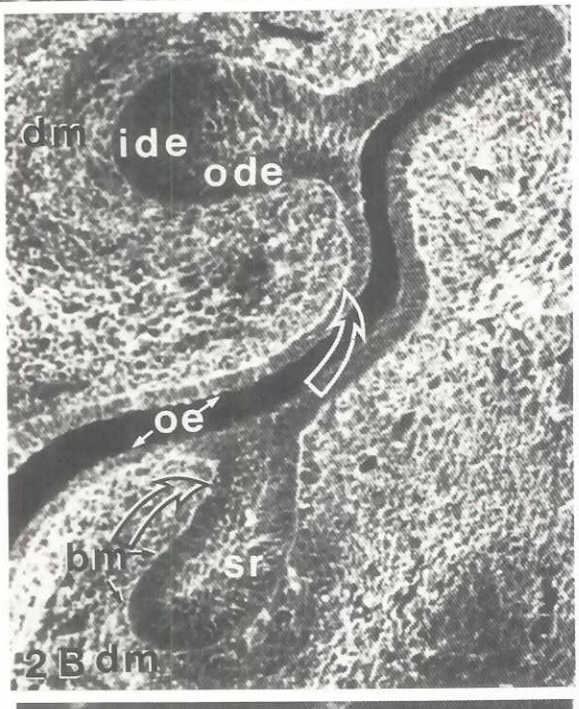
When acetone-fixed sections were treated with increasing concentrations of NaCl (2M and 3M) (Protocol 2b), bFGF immunostaining progressively decreased except in the stratum intermedium (not shown). Digestion of acetone-fixed sections by heparitinase (Protocol 2c) resulted in slight but significant decrease of staining especially in the basement membranes and the epithelial tissues (not shown).

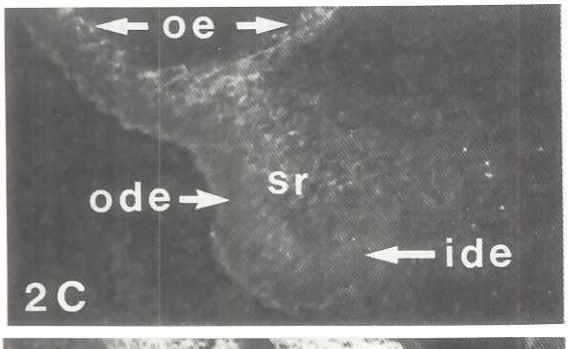
Discussion

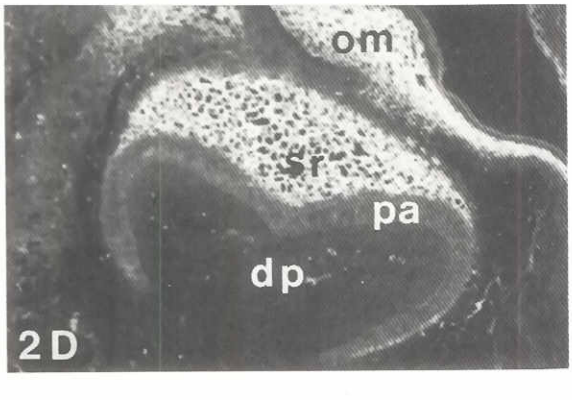
Despite some similarities between immunolocalizations of aFGF and bFGF, for instance at the secretory pole of ameloblasts and within the stratum intermedium after birth, our results stress that the stainings of aFGF and bFGF appear different. In particular the

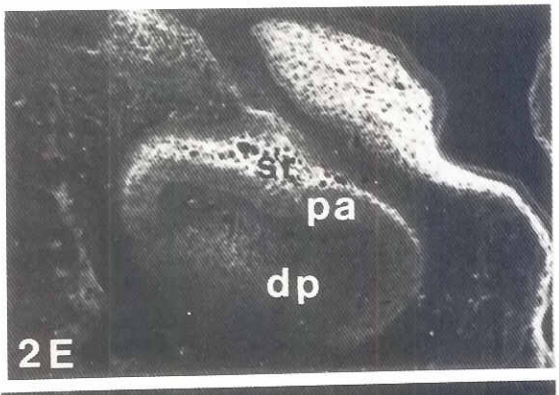
Fig. 1. Immunolocalization of aFGF on frontal, frozen fixed, sections *(A-E: staining by the indirect immunohistochemical method; F: phase contrast micrograph; G: staining by the immunoperoxidase technique; A-C: protocol 3a; D-G: protocol 2a).* **(A)** *Section of a day-12 mandible showing intense immunostaining in the oral epithelium (oe) and the dental lamina (dl); the basement membrane (bm) and the oral mesenchyme (om) stained positively. (x270).* **(B)** *Section of a day-14 lower molar (cap stage) showing bright immunoreactivity of the (oe) and the (bm) facing the labial peridental mesenchyme; the enamel organ, constituted by the outer dental epithelium (ode), the inner dental epithelium (ide) and the stellate reticulum (sr) exhibited a pronounced but uneven staining; immunostaining persisted in some areas of the peridental mesenchyme. (x315).* **(C)** *Section of a day-17 lower molar (bell stage); immunostaining predominated in the stratum intermedium (si) and (ide); the stellate reticulum remained positive while some areas of the dental pulp (dp) stained faintly. (x265).* **(D)** *Section of a day-19 lower molar showing intense immunoreactivity of the (si) and at the non secretory pole of ameloblasts (a); the secretory pole of (a) also stained positively. (x265).* ***(E-G)*** *Sections of day-22 molars.* **(E)** *Bright immunostaining was seen at the secretory pole of (a) while the predentin-dentin area (pd-d) exhibited a pronounced but dotted staining (arrows). (x360).* **(G)** *A faint and discontinuous immunoreactivity of (pd-d) remained (arrowhead) when the immunoperoxidase technique was used; intense immunostaining in (ode) and at the secretory pole of (a) was seen and staining persisted in (si) and at the non-secretory poles of (a) (x465).* **(F)** *(x360); (e): enamel.*

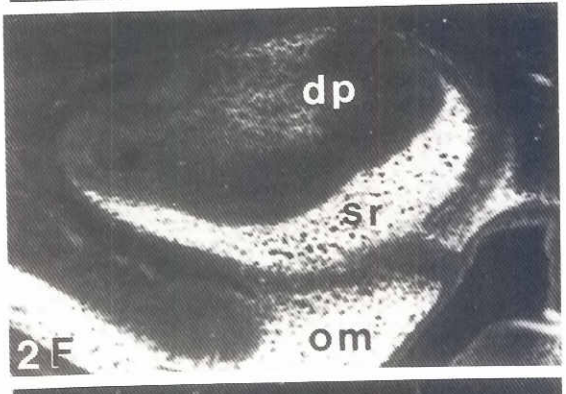

bm
dl
om
2A

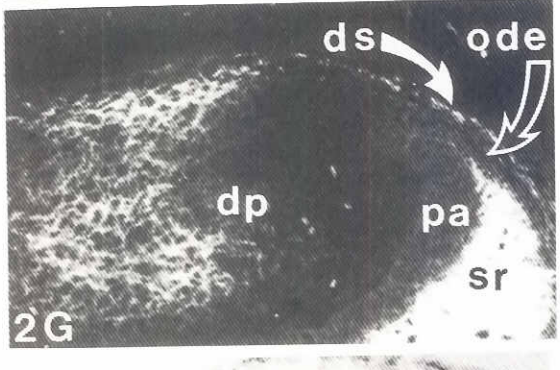

dm
ide
ode
oe
bm
sr
2B dm

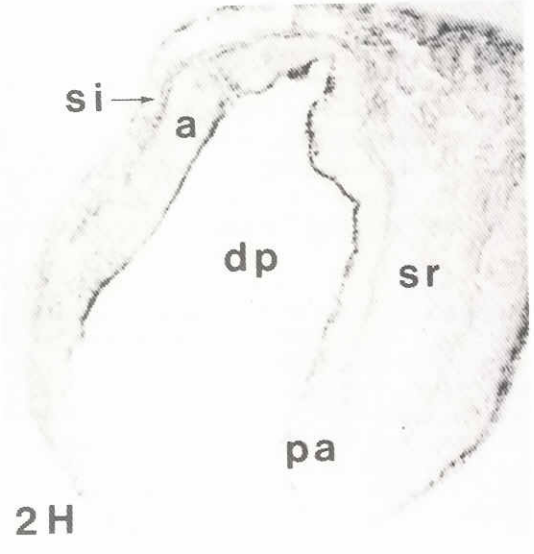

oe
ode
sr
ide
2C


om
sr
pa
dp
2D


sr
pa
dp
2E


dp
sr
om
2F


ds
ode
dp
pa
sr
2G


si
a
dp
sr
pa
2H

intense mesenchymal staining observed with bFGF antibodies is not observed with aFGF antibodies, nor is the very intense staining in stellate reticulum. Simultaneous expression of aFGF and bFGF genes has been reported by Hebert *et al.* (1990) in mouse embryos ranging from day-11 to day-17 of gestation: however bFGF was expressed more abundantly than aFGF in facial tissues and limbs. Fu *et al.* (1991) have also demonstrated the simultaneous presence of aFGF and bFGF within extracts of diverse tissues of rat embryos and stressed the similar pattern of immunostaining of both factors.

The specific localization of aFGF epitopes found in the predentin-dentin zone after birth leads to different hypotheses. Given that odontoblastic cell processes and nervous fibers are known to cross this matrix, such a localization might be related to the neurotropic or neurotrophic activities of FGFs previously reported (reviewed by Goldfarb, 1990). The fact that aFGF is an acidic protein might also contribute to the process of mineralization of the dentin matrix (Weiner and Addadi, 1991). In addition, other growth factors, such as IGFs I and II and TGFßs have been extracted from human dentin (Finkelman *et al.*, 1990).

The distribution of bFGF epitopes on Bouin's fixed sections in dental and peridental mesenchyme and in association with oral and dental basement membranes is consistent with observations by Gonzalez *et al.* (1990).

Our investigations stress the importance of tissue fixation and paraffin embedding with regard to immunostaining with monospecific polyclonal antibodies directed against either aFGF or bFGF. Surprisingly, incubation of Bouin's fixed paraffin-embedded sections with anti-recombinant aFGF resulted in the absence of staining, while a wide distribution of bFGF was detected on such sections. We suggest that this condition of fixation and/or paraffin embedding (which includes dehydration and toluene treatment) leads either to an exclusive unmasking of bFGF epitopes belonging to the 45% of the amino-acid sequence not shared with aFGF, or more probably, to the denaturation of specific epitopes of aFGF. Therefore, a single method of tissue treatment for both antibodies would not be satisfactory. Recent reports concerning immunohistochemical reactivity obtained with different anti-bFGF antibodies on similarly or differently fixed tissues have also illustrated such variations (Grothe and Unsicker, 1990; Kardami *et al.*, 1990).

Treatment of frozen fixed sections with NaCl (2M-3M) solutions and digestion with heparitinase were designed to check whether bFGF was linked to low affinity heparan sulfate binding sites in preference to other low affinity or to high affinity binding sites. The failure of heparitinase to delete specific immunostaining argues in favor of the presence of all types of binding sites. Progressive diminution of staining after incubation in NaCl solutions favors the hypothesis of a linkage of bFGF to low affinity heparin-containing binding sites. The distribution of bFGF that we observed in dental tissues from the stage of dental lamina formation to late bell stage is not identical to that reported for syndecan, a putative low affinity binding transmembranous protein for bFGF that might promote subsequent binding to high affinity membrane receptors (Thesleff *et al.*, 1988; Salmivirta *et al.*, 1991; Vainio *et al.*, 1991); other low affinity heparin-containing binding sites might be involved.

The cellular immunolocalizations of aFGF and bFGF observed in dental epithelia and mesenchyme might be relevant to the temporal and spatial related expression of the $FGFR_1$ (flg) and $FGFR_2$ (bek) genes so far reported (Peters *et al.*, 1992; Orr-Urtreger *et al.*, 1992) (Cf. Table 1). The extracellular immunostainings of aFGF and bFGF, particularly their associations with dental basement membranes, are also important and need further investigations, since dental basement membranes are known to play crucial roles during odontogenesis (Ruch, 1990).

The comparison of the distribution of FGFs and their receptors with other growth factors during murine odontogenesis is outlined in Table 1. aFGF and bFGF overlap partially NGF, NGF receptor, EGF receptor and $TGFß_1$. Although it is well known that tooth histomorphogenesis and cytodifferentiations involve cell kinetic-dependent developmental processes which are mediated through epithelial-mesenchymal interactions (see Ruch, 1990 for review), we do not know how the network of growth factors operates. A more complete study of the patterns of expression of growth factors and their receptors combined with functional analyses will be necessary to elaborate a unifying concept (Nathan and Sporn, 1991).

Materials and Methods

Collection and preparation of tissues

Stages of mouse embryos are referred to day zero (vaginal plug). Day-12 to day-15 heads, day-16 to day-18 mandibles and day-17 to day-19 isolated lower first molars as well as post-natal (day-19 to day-22) mandibular molars and incisors were dissected in cold HBSS (Gibco, Paisley, Scotland).

Some specimens were fixed in Bouin's solution, dehydrated in a series of increasing concentrations of ethanol and toluene before embedding in paraffin (Prolabo, Paris) at 54-56°C. Other specimens were promptly frozen in OCT compound (Tissue Tek II, Miles, Elkhart, IN, USA) on an aluminium foil floating on liquid nitrogen and stored at -70°C.

Serial sections of Bouin's fixed and frozen sections were cut at 5 µm in a microtome (Leitz 1515, Wetzlar, Germany) and at 8 µm in a cryostat (Slee, Mainz, Germany), respectively.

Paraffin-embedded and frozen sections were mounted on glass slides which had been sterilized and coated with chromalum-gelatin; the former sections were dried at 37°C and kept at 4°C; the latter were air dried and kept at -40°C.

Fig. 2. Immunolocalization of bFGF by means of the indirect immunofluorescence method (A-G) and the immunoperoxidase technique (H) on frontal sections of molars fixed and incubated as follows: A-B, D-G (Protocol 3b; anti b_t antibody); C (Protocol 2a; anti-b_{1-24} antibody); H (Protocol 1; anti b_{bb} antibody). (A) *Section of a day-12 mandible; immunostaining was predominant in the oral mesenchyme (om) and the basement membranes (bm); the dental lamina (dl) was faintly stained. (x270).* **(B)** *Section through day-14 upper and lower molars (cap stage) showing intense immunoreactivity of the peridental mesenchyme and the basement membranes (bm) bordering the outer dental (ode) and oral (oe) epithelia (arrows); the stellate reticulum (sr) and the dental mesenchyme (dm) exhibited pronounced immunostaining; the (oe) stained positively. (x215).* **(C)** *Section of a day-14 lower molar fixed differently from that shown in B: the (oe) and the (sr) still stained positively while the basement membranes and the mesenchymes remained negative (see Discussion). (x260).* **(D-G)** *Sections of day-18 molars (bell stage) at different levels (D - E: anterior and posterior parts of lower molar, respectively (x115); F: posterior part of upper molar (x115); G: enlargement of F (x235)).* **(D)** *Section showing intense immunoreactivity of the (om) and the (sr); the dental sac stained positively while the (si), (ide) and (ode) were negative.* **(E)** *Section showing the same bright staining in the (om) and the (sr) as in Fig. D; some areas of the dental pulp (dp) exhibited positive staining.* **(F-G)** *Sections showing intense immunostaining in the (om) and the (sr); the dental sac (ds) and part of the dental pulp exhibited pronounced staining.* **(H)** *Section of a day-21 molar; immunostaining was primarily seen at the secretory pole of ameloblasts (a); positive staining was found between and at the non-secretory pole of (a), in (si) and (ode). (x125).*

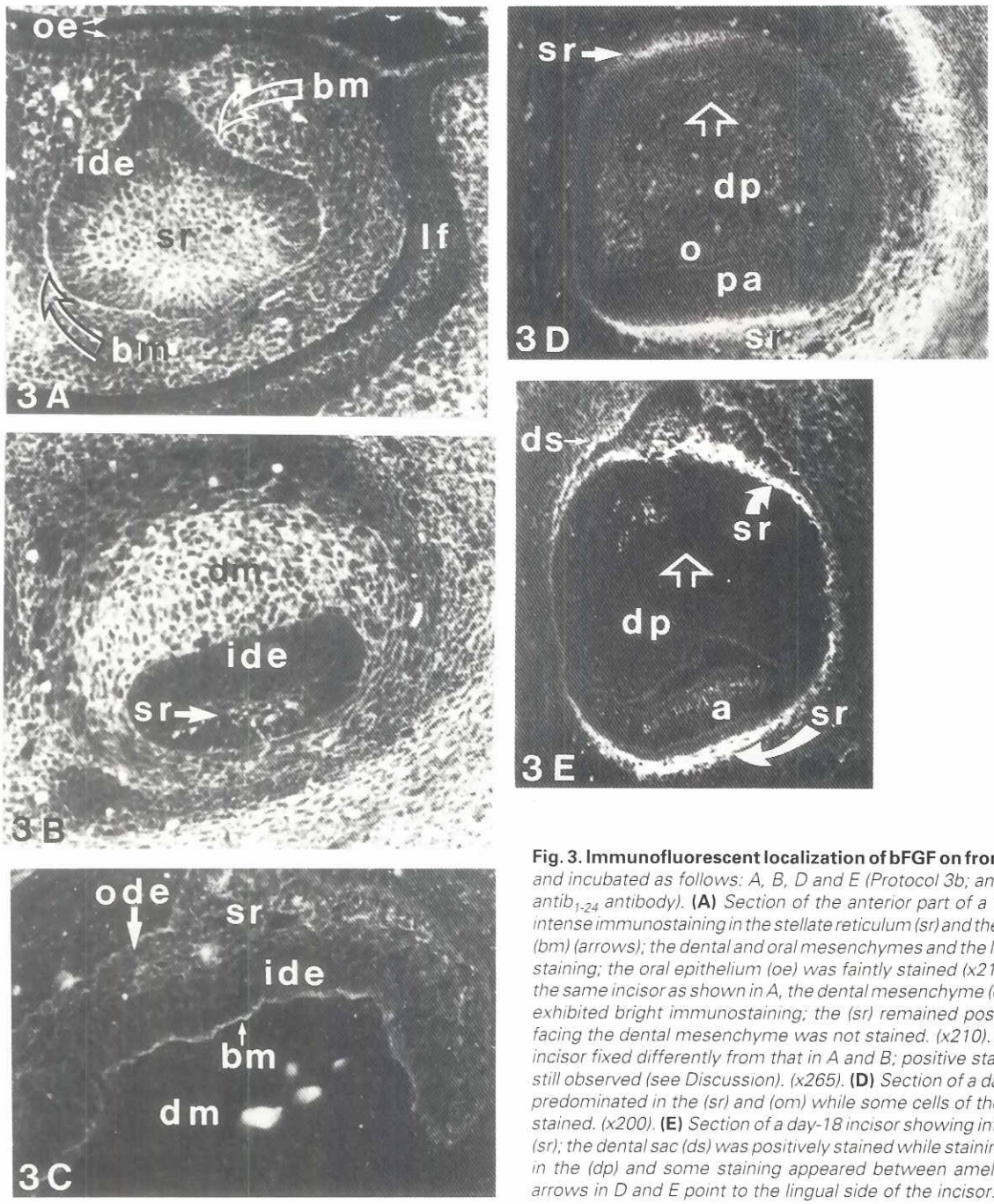


Fig. 3. Immunofluorescent localization of bFGF on frontal sections of incisors *fixed and incubated as follows: A, B, D and E (Protocol 3b; anti-b, antibody); C (Protocol 3a; antib$_{1\text{-}24}$ antibody).* **(A)** *Section of the anterior part of a 14-day lower incisor showing intense immunostaining in the stellate reticulum (sr) and the dental basement membranes (bm) (arrows); the dental and oral mesenchymes and the lip furrow (lf) exhibited positive staining; the oral epithelium (oe) was faintly stained (x210).* **(B)** *In the posterior part of the same incisor as shown in A, the dental mesenchyme (dm) and the oral mesenchyme exhibited bright immunostaining; the (sr) remained positively stained while the (bm) facing the dental mesenchyme was not stained. (x210).* **(C)** *Section of a 14-day lower incisor fixed differently from that in A and B; positive staining of the (sr) and (bm) was still observed (see Discussion). (x265).* **(D)** *Section of a day-16 incisor; immunostaining predominated in the (sr) and (om) while some cells of the dental pulp (dp) were faintly stained. (x200).* **(E)** *Section of a day-18 incisor showing intense immunoreactivity of the (sr); the dental sac (ds) was positively stained while staining remained in clusters of cells in the (dp) and some staining appeared between ameloblasts (a). (x170). The open arrows in D and E point to the lingual side of the incisor.*

Sources of specific antibodies

First we used polyclonal antibodies raised in rabbits against the synthetic (1-24 amino terminal) fragment of bovine bFGF — conjugated to BSA (Sigma, St-Louis, MO, USA) — and prepared as described by Baird and Ling (1987) and Gonzalez *et al.* (1990); these antibodies detected bFGF and did not recognize less than 1% of aFGF: they were designated "anti-$b_{1\text{-}24}$".

Polyclonal antibodies specific for bFGF were also prepared by immunizing mice with bFGF previously purified from bovine brain by heparin-sepharose chromatography, as described by Pettmann *et al.* (1985) and Janet *et al.* (1987). These antibodies were designated "anti-b_{bb}".

Rabbit polyclonal antisera directed against human recombinant aFGF and bFGF were purified by affinity chromatography. Briefly, to purify antibodies to aFGF, 8 ml of sera were dialyzed against 35 mM NaCl (Merck, Darmstadt, Germany) in 25 mM Tris (Sigma), pH 8.8 at room temperature for 3 h, then passed over a DEAE Sepharose (Kabi-Pharmacia, Paris, France) column; the IgG fraction was dialyzed against PBS, then loaded onto a bFGF Aminolink (Pierce, Eurochimie BV, The Netherlands) column to remove any cross reacting antibodies. The IgG fraction was cycled over an aFGF-Aminolink column several times over-night at 4°C; the column was washed extensively with PBS and the bound antibody was eluted in 0.1M glycin (Sigma), pH 2.8 and immediately buffered in Tris, pH 8.0. To purify antibodies to bFGF, a similar procedure was used, i.e. an IgG fraction was

TABLE 1

COMPARISON OF FGFs AND THEIR RECEPTORS WITH OTHER ONCOGENES, GROWTH FACTORS AND THEIR RECEPTORS IN DEVELOPING MURINE TEETH (RNA(m) or PROTEIN (p)

Developmental stages	Dental lamina		Bud stage		Cap stage		Bell stage	
Molecules	**Epi**	**Mes**	**Epi**	**Mes**	**Epi**	**Mes**	**Epi**	**Mes**
N-myc					+(m)	-(m)		
c-myc					-(m)	+(m)		
int-2			-(m)	+(m)	-(m)	+(m)	-(m)	+(m)
aFGF	++(p)	+(p)			++(p)	+(p)	++(p)	-(p)
bFGF	+(p)	++(p)			++(p)	++(p)	++(p)	++(p)
FGFR1							+(m)	+(m)
FGFR2							+(m)	-(m)
Syndecan			+(m,p)	++(m,p)	+(m,p)	++(m,p)	++(m,p)	++(m,p)
prepro EGF							+(m)	+(m)
TGFa					+(p)	+(p)		
EGFR			++(p)	-(p)	+(p)	+(p)	++(p)	++(p)
NGF			+(p)	+(p)	++(p)	+(p)	++(p)	+(p)
NGF R	+(m,p)	+(m,p)	++(m,p)	+(m,p)	++(p)	+(p)	++(p)	+(p)
TGF ß1	-(m)	-(m) +(p)	+(m)	±(m) +(p)	++(m,p)	+(m) ++(p)	+(m) ++(p)	+(m) +(p)
TGFß2			-(m)	-(m)			-(m,p)	+(m)++(p)
TGF ß3	-(m)	-(m)	-(m)	-(m)	+(m)		+(m,p)	+(m,p)

Epi: tissue components of enamel organ; Mes: dental mesenchyme. The intensities of immunostaining (p) and *in situ* hybridization (m) were evaluated from the descriptions by Abbott and Pratt (1988); Byers *et al.*, (1990); Cam *et al.*,(1990); Dixon *et al.*, (1991); D'Souza *et al.*, (1990); Gonzalez *et al.*, (1990); Heine *et al.*, (1987); Hirning *et al.*, (1991); Lehnert and Akhurst (1988); Mitsiadis *et al.*, (1992), Pelton *et al.*, (1990, 1991); Snead *et al.* (1989); Vaahtokari *et al.*, (1991); Vainio *et al.*, (1991); Wilkinson *et al.*, (1989). (±) means discrepancies between authors.

passed over an aFGF Aminolink column and purified by cycling over a bFGF-Aminolink column. Specificity of affinity purified antibodies for their respective antigen, either aFGF or bFGF, was assessed by ELISA and Western-blot experiments; these preparations were designated "anti-a," and "anti-b," respectively.

Immunohistochemical procedures

The procedures of fixation, permeabilization and enzymatic digestion of sections that resulted in reproducible positive or negative immunostaining were as follows (see Table 2).

Protocol 1

Frozen sections were fixed in 4% (w:v) paraformaldehyde (Serva, Heidelberg, Germany) freshly prepared in PBS pH 7.2, and rinsed in PBS containing 0.1% (w/v) BSA and merthiolate (50 mg/ml); then sections were permeabilized with methanol alone or methanol containing 0.6% (v/v) (final concentration) H_2O_2 (Prolabo) when peroxidase-conjugated secondary antibodies were to be used. Sections were subsequently incubated with bovine testes hyaluronidase (Sigma), diluted at 1 mg/ml in 0.1M sodium acetate (Merck) buffer pH 5.5 for 30 min at 37°C.

Protocol 2

Frozen sections were fixed and permeabilized with cold acetone for 3 min and then incubated with pepsin (Sigma) diluted to 100 ng/ml in 0.5 M acetic acid (Merck) for 15 min at room temperature (protocol 2a). Some sections were further incubated either in 2M and 3M NaCl solutions in PBS, pH 7.2 for 2 h at room temperature (protocol 2b) or in the presence of heparitinase from Flavo bacterium heparinum (Seikagaku Kogyo, Tokyo, Japan) diluted (40 µg/ml) in 50 mM Tris, pH 7.5 containing 5 mM EDTA (Merck) for 45 min at 37°C (protocol 2c).

Protocol 3

Frozen sections were fixed and permeabilized with methanol and digested with hyaluronidase as described in protocol 1 (protocol 3a). Once deparaffinized in toluene and rehydrated in decreasing concentrations of

TABLE 2

HISTOCHEMICAL PROTOCOLS USED FOR IMMUNOLOCALIZATION OF FGFS

Protocol Number	Type of section	Fixation	Permeabilization	Enzymatic and/or chemical treatment
1	Frozen	Paraformaldehyde	Methanol or Methanol+H_2O_2	Hyaluronidase
2a	Frozen	cold acetone	cold acetone	Pepsin
2b	Frozen	cold acetone	cold acetone	Pepsin + NaCl {2M / 3M}
2c	Frozen	cold acetone	cold acetone	Pepsin+heparitinase
3a	Frozen	Methanol	Methanol	Hyaluronidase
3b	Paraffin-embedded	Bouin's	Methanol	Hyaluronidase

TABLE 3

SOURCES AND DILUTIONS OF ANTIBODIES AND PROTOCOLS USED FOR IMMUNOLOCALIZATION OF FGFs

Antibodies to FGFs	Anti-b_{1-24}	Anti-b_{bb}	Anti-b_r	Anti-a_r
Dilutions	1: 40	1: 30	1: 40	1: 100
Protocols	1, 2a-b-c, 3a	1, 2a	2a-b, 3b	2a-b-c, 3a-b

ethanol, Bouin's fixed sections were also permeabilized with methanol and digested with hyaluronidase (protocol 3b).

All types of sections were subsequently rinsed in PBS containing 0.1% (w/v) BSA and non-specific binding was prevented by blocking epitopes with PBS containing 0.5% (w/v) BSA and 1.5% (v/v) normal goat serum (NGS) (Gibco) for 15 min at room temperature.

Depending on the size of sections, 5 to 50 µl of primary antibodies diluted in PBS containing 1% BSA were incubated on the slides overnight at 4°C (see Table 3).

Sections were rinsed in PBS containing 0.1% BSA and incubated with either fluoroisothiocyanate (FITC) or peroxidase conjugated anti-rabbit (or mouse according to primary antibody) IgGs (H+L) (Cappel Labs, Cochranville, USA) diluted 1:40 in PBS containing 1% BSA for 1 h at room temperature. Sections that had been treated with FITC-conjugated secondary antibodies were mounted in glycerol: PBS (9:1, v/v), viewed in a Leitz Orthoplan microscope equipped with epifluorescence and photographed with an Orthomat automatic camera. Sections processed for immunoperoxidase staining were rinsed with PBS alone and incubated with 4-chloro-1 naphthol (Merck) diluted first to 14% (w/v) in absolute ethanol and then to 1:400 (v/v) in PBS containing 0.02% (v/v) (final concentration) H_2O_2. These sections were finally rinsed, mounted in glycerol-gelatin (Sigma), viewed and photographed.

Control sections were obtained by using either rabbit/mouse preimmune or commercial IgGs (Cappel Labs) instead of primary antibodies.

Acknowledgments

We appreciate the kind gift of specific antibodies to bFGF peptide by Dr. A. Baird (The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA, USA). We would like to thank Drs. M. Sensenbrenner and G. Labourdette (Laboratoire de Neurobiologie Ontogénique CNRS UPR 417, Centre de Neurochimie, Strasbourg) and Drs. M. Vigny, J.C. Jeanny and Y. Courtois (INSERM U118, Paris) for their valuable advice and support. We thank Dr. P. Simpson for reading and Mrs M. Martiny for typing our manuscript. The technical assistance of A. Ackermann is also gratefully acknowledged. Y. Cam, M.R. Neumann and J.V. Ruch are indebted to Fondation pour la Recherche Médicale (France) for special funding.

References

ABBOTT, B.D. and PRATT, R.M. (1988). EGF receptor expression in the developing tooth is altered by exogenous retinoic acid and EGF. *Dev. Biol. 128:* 300-304.

BAIRD, A. and LING, N. (1987). Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells *in vitro*: implications for a role of heparinase-like enzymes in the neovascular response. *Biochem. Biophys. Res. Commun. 142:* 428-435.

BERNFIELD, M. and SANDERSON, R.D. (1990). Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. *Phil. Trans. R. Soc. Lond. B 327:* 171-186.

BYERS, M.R., SCHATTEMAN, G.C. and BOTHWELL, M. (1990). Multiple functions for NGF receptor in developing, aging and injured rat teeth are suggested by epithelial, mesenchymal and neural immunoreactivity. *Development 109:* 461-471.

CAM, Y., MEYER, J.M., STAUBLI, A. and RUCH, J.V. (1987). Epithelial-mesenchymal interactions: effects of a dental biomatrix on odontoblasts. *Arch. Anat. Microsc Morph. Exp. 75:* 75-89.

CAM, Y., NEUMANN, M.R. and RUCH, J.V. (1990). Immunolocalization of transforming growth factor $ß_1$ epitopes in mouse incisors and molars with a demonstration of *in vitro* production of transforming activity. *Arch. Oral Biol. 35, 10:* 813-822.

DIONNE, C.A., CRUMLEY, G., BELLOT, F., KAPLOW, J.M., SEARFOSS, G., RUTA, M., BURGESS, W.H., JAYE, M. and SCHLESSINGER, J. (1990). Cloning and expression of two distinct high affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J. 9, 9:* 2685-2692.

DIXON, M.J., GARNER, J. and FERGUSON, M.W.J. (1991). Immunolocalisation of epidermal growth factor (EGF), EGF receptor and transforming growth factor alpha (TGFα) during murine palatogenesis *in vivo* and *in vitro*. *Anat. Embryol. 184:* 83-91.

D'SOUZA, R.N., HAPPONEN, R.P., RITTER, N.M. and BUTLER, W.T. (1990). Temporal and spatial patterns of transforming growth factor ß1 expression in developing rat molars. *Arch. Oral Biol. 35:* 12, 957-965.

FAYEIN, N.A., COURTOIS, Y. and JEANNY, J.C. (1990). Ontogeny of basic fibroblast growth factor binding sites in mouse ocular tissues. *Exp. Cell Res. 188:* 75-88.

FEIGE, J.J., BRADLEY, J.D., FRYBURG, K., FARRIS, J., COUSENS, L.C., BARR, P.J. and BAIRD, A. (1989). Differential effects of heparin, fibronectin and laminin on the phosphorylation of basic FGF by protein kinase C and the catalytic subunit of protein kinase A. *J. Cell Biol. 109:* 3105-3114.

FINKELMAN, R.D., MOHAN, S., JENNINGS, J.C., TAYLOR, A.K., JEPSEN, S. and BAYLINK, D.J. (1990). Quantitation of growth factors IGF-I, SGF-II, and TGFß in human dentin. *J. Bone Min. Res. 5, 7:* 717-723.

FU, Y.M., SPIRITO, P., YU, Z.X., BIRO, S., SASSE, J., LEI, J., FERRANS, V.J., EPSTEIN, S.E. and CASSCELLS, W. (1991). Acidic fibroblast growth factor in the developing rat embryo. *J. Cell Biol. 144:* 6, 1261-1273.

GOLDFARB, M. (1990). The fibroblast growth factor family. *Cell Growth Differ. 1:* 439-445.

GONZALEZ, A.M., BUSCAGLIA, M., ONG, M. and BAIRD, A. (1990). Distribution of basic fibroblast growth factor in the 18-day rat fetus: localization in the basement membranes of diverse tissues. *J. Cell Biol. 110:* 753-765.

GROTHE, C. and UNSICKER, K. (1990). Immunocytochemical mapping of basic fibroblast growth factor in the developing and adult rat adrenal gland. *Histochemistry 94:* 141-147.

HALL, B.K. and EKANAYAKE, S. (1991). Effects of growth factors on the differentiation of neural crest cells and neural crest cell derivatives. *Int. J. Dev. Biol. 35:* 367-387.

HAUB, O. and GOLDFARB, M. (1991). Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development 112:* 397-406.

HEBERT, J.M., BASILICO, C., GOLDFARB, M., HAUB, O. and MARTIN, G.R. (1990). Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev. Biol. 138:* 454-463.

HEINE, U.I., MUNOZ, E.F., FLANDERS, K.C., ELLINGSWORTH, L.R., LAM, H.Y.P., THOMPSON, N.L., ROBERTS, A.B. and SPORN, M.B. (1987). Role of transforming growth factor ß in the development of the mouse embryo. *J. Cell Biol. 105:* 2861-2876.

HIRNING, U., SCHMID, P., SCHULZ, W.A., RETTENBERGER, G. and HAMEISTER, H. (1991). A comparative analysis of N-myc and c-myc expression and cellular proliferation in mouse organogenesis. *Mech. Dev. 33:* 119-126.

JANET, T., MIEHE, M., PETTMANN, B., LABOURDETTE, G. and SENSENBRENNER, M. (1987). Ultrastructural localization of fibroblast growth factor in neurons of rat brain. *Neurosci. Lett. 80:* 153-157.

KARDAMI, E., MURPHY, L.J., LIU, L., PADUA, R.R. and FANDRICH, R.R. (1990). Characterization of two preparations of antibodies to basic fibroblast growth factor which exhibit distinct patterns of immunolocalization. *Growth Factors 4:* 69-80.

KIEFER, M.C., STEPHANS, J.C., CRAWFORD, K., OKINO, K. and BARR, P.J. (1990). Ligand affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA 87:* 6985-6989.

KRONMILLER, J.E., UPHOLT, W.B. and KOLLAR, E.J. (1991a). Expression of epidermal growth factor mRNA in the developing mouse mandibular process. *Archs. Oral Biol. 36:* 6, 405-410.

KRONMILLER, J.E., UPHOLT, W.B. and KOLLAR, E.J. (1991b). EGF antisense oligodeoxynucleotides block murine odontogenesis *in vitro*. *Dev. Biol. 147:* 485-488.

LEHNERT, S.A. and AKHURST, R.J. (1988). Embryonic expression pattern of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. *Development 104:* 263-273.

LESOT, H., KUBLER, M.D., FAUSSER, J.L. and RUCH, J.V. (1990). A 165 kDa membrane antigen mediating fibronectin-vinculin interaction is involved in murine odontoblast differentiation. *Differentiation 44:* 25-35.

MARK, M.P., BAKER, J.R., KIMATA, K. and RUCH, J.V. (1990). Regulated changes in chondroitin sulfation during embryogenesis: an immunohistochemical approach. *Int. J. Dev. Biol. 34:* 191-204.

MITSIADIS, T.A., DICOU, E., JOFFRE, A. and MAGLOIRE, H. (1992). Immunohistochemical localization of nerve growth factor (NGF) and NGF receptor (NGF-R) in the developing first molar tooth of the rat. *Differentiation 49:* 47-61.

NATHAN, C. and SPORN, M. (1991). Cytokines in context. *J. Cell Biol. 113:* 981-986.

ORR-URTREGER, A., GIVOL, D., YAYON, A., YARDEN, Y. and LONAI, P. (1991). Developmental expression of two murine fibroblast growth factor receptors, flg and bek. *Development 113:* 1419-1434.

PARTANEN, A.M. and THESLEFF, I. (1987). Localization and quantitation of ^{125}I-epidermal growth factor binding in mouse embryonic tooth and other embryonic tissues at different developmental stages. *Dev. Biol. 120:* 186-197.

PELTON, R.W., DICKINSON, M.E., MOSES, H.L. and HOGAN, B.L.M. (1990). *In situ* hybridization analysis of TGF β_3 RNA expression during mouse development: comparative studies with TGF β_1 and β_2. *Development 110:* 609-620.

PELTON, R.W., SAXENA, B., JONES, M., MOSES, H.L. and GOLD, L.I. (1991). Immunohistochemical localization of TGFß1, TGFß2 and TGFß3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. *J. Cell Biol. 115:* 4, 1091-1105.

PETERS, K.G., WERNER, S., CHEN, G. and WILLIAMS, L.T. (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development 114:* 233-243.

PETTMANN, B., WEIBEL, M., SENSENBRENNER, M. and LABOURDETTE, G. (1985). Purification of two astroglial growth factors from bovine brain. *Febs Lett. 189:* 102-108.

RISAU, W. (1986). Developing brain produces an angiogenesis factor. *Proc. Natl. Acad. Sci. USA 83:* 3855-3859.

RUCH, J.V. (1987). Determinisms of odontogenesis. In *Cell Biology Reviews* (Ed. E. Barberâ-Guillém). Springer, Berlin, vol. 14, pp. 1-112.

RUCH, J.V. (1990). Patterned distribution of differentiating dental cells: facts and hypotheses. *J. Biol. Buccale 18:* 91-98.

SALMIVIRTA, M., ELENIUS, K., VAINIO, S., HOFER, U., CHIQUET-EHRISMANN, R., THESLEFF, I. and JALKANEN, M. (1991). Syndecan from embryonic tooth mesenchyme binds tenascin. *J. Biol. Chem. 266:* 12, 7733-7739.

SEED, J., OLWIN, B.B. and HAUSCHKA, S.D. (1988). Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Dev. Biol. 128:* 50-57.

SLAVKIN, H.C. (1988). Gene regulation in the development of oral tissues. *J. Dent. Res. 47:* 1142-1149.

SNEAD, M.L., LUO, W., OLIVER, P., NAKAMURA, M., DON-WHEELER, G., BESSEM, C., BELL, G.I., RALL, L.B. and SLAVKIN, H.C. (1989). Localization of epidermal growth factor precursor in tooth and lung during embryonic mouse development. *Dev. Biol. 134:* 420-429.

THESLEFF, I., JALKANEN, M., VAINIO, S. and BERNFIELD, M. (1988). Cell surface proteoglycan expression correlates with epithelial-mesenchymal interaction during tooth morphogenesis. *Dev. Biol. 129:* 565-572.

THESLEFF, I., VAAHTOKARI, A. and VAINIO, S. (1990). Molecular changes during determination and differentiation of the dental mesenchymal cell lineage. *J. Biol. Buccale 18:* 179-188.

THOMSON, J.A., ANDERSON, K.D., DI PETRO, J.M., ZWIEBEL, J.A., ZAMETTA, M., ANDERSON, W.F. and MACIAG, T. (1988). Site directed neovessel formation *in vivo*. *Science 241:* 1349-1352.

VAAHTOKARI, A., VAINIO, S. and THESLEFF, I. (1991). Associations between TGFß$_1$ RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. *Development 113:* 985-994.

VAINIO, S., JALKANEN, M., VAAHTOKARI, A., SAHLBERG, C., MALI, M., BERNFIELD, M. and THESLEFF, I. (1991). Expression of syndecan gene is induced early, is transient, and correlates with changes in mesenchymal cell proliferation during tooth organogenesis. *Dev. Biol. 147:* 322-333.

VIGNY, M., OLLIER-HARTMANN, M.P., LAVIGNE, M., FAYEIN, N., JEANNY, J.C., LAURENT, M. and COURTOIS Y. (1988). Specific binding of basic fibroblast growth factor to basement membrane-like structures and to purified heparan sulfate proteoglycan of the EHS tumor. *J. Cell Physiol. 137:* 321-328.

WANAKA, A., MILBRANDT, J. and JOHNSON, E.M., Jr. (1991). Expression of FGF receptor gene in rat development. *Development 111:* 455-468.

WEINER, S. and ADDADI, L. (1991). Acidic macromolecules of mineralized tissues: the controllers of crystal formation. *TIBS 16:* 252-256.

WILKINSON, D.G., BHATT, S. and McMAHON, A.P. (1989). Expression pattern of the FGF-related proto-oncogen int-2 suggests multiple roles in fetal development. *Development 105:* 131-136.

WISE, G.E., RUDICK, V.L., BRUN-ZINKERNAGEL, A.M. and FAN, W. (1990). Ultrastructural and immunocytochemical characterization of cultured cells from rat molar stellate reticulum. *Arch. Oral Biol. 35:* 8, 603-613.

ZHANG, C.Z., YOUNG, W.G. and WATERS, M.J. (1992). Immunocytochemical localization of growth hormone receptor in rat maxillary teeth. *Arch. Oral Biol. 2:* 77-84.

Accepted for publication: June 1992