Int. J. Dev. Biol. 38: 405-420 (1994)

Original Article

Comparative analysis of *TGFßs, BMPs, IGF1, msxs, fibronectin, osteonectin* and *bone sialoprotein* gene expression during normal and *in vitro*-induced odontoblast differentiation

CATHERINE BÈGUE-KIRN[1]*, ANTHONY J. SMITH[2], MARIA LORIOT[3], CHRISTIAN KUPFERLE[1], JEAN VICTOR RUCH[1] and HERVÉ LESOT[1]

[1]*Institut de Biologie Médicale, Université Louis Pasteur, Faculté de Médecine, Strasbourg, France,* [2]*Department of Oral Pathology, St. Chad's Queensway, Faculty of Medicine and Dentistry, Birmingham, United Kingdom and* [3]*Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, U 184 INSERM, Institut de Chimie Biologie, Faculté de Médecine, Strasbourg, France*

ABSTRACT Immobilized TGFß1 and BMP2 are able to promote the differentiation of odontoblast-like cells in isolated mouse dental papillae cultured *in vitro*. These cells polarize and accumulate predentin-like matrix at their apical pole. Immobilized IGF1 mainly promoted polarization with disturbed matrix accumulation. *In situ* hybridization demonstrated that TGFß1 combined with heparin mirrored the physiological processes of odontoblast differentiation. Normal odontoblast and *in vitro* induced odontoblast-like cells expressed transcripts encoding for TGFß1 and 3, BMP2 and 4, bone sialoprotein and osteonectin whereas either ubiquitous expression or no expression could be detected for TGFß2, IGF1 or fibronectin mRNAs. Odontoblast-like cells obtained in the presence of IGF1 combined with heparin did not express TGFß1 transcripts and expressed weakly TGFß3 transcripts. Our results suggest that *in vivo* an epithelial-derived member of the TGFß family trapped by basement membrane-associated components interacts with competent preodontoblasts and promotes the polarization by triggering the transcription of growth factor gene(s) like TGFß itself and/or selector gene(s) like *msx2*.

KEY WORDS: *TGFβ, BMP, IGF, odontoblast-differentiation*

Introduction

Odontoblasts, secreting predentin-dentin components, are tall columnar post-mitotic cells organized in a single layer at the periphery of the dental papilla in front of the inner dental epithelium of the enamel organ. Terminal differentiation of odontoblast occurs after a minimal number of cell cycles (Ruch *et al.*, 1976, 1983) and follows a specific temporospatial pattern within each cusp. In addition to collagen type I and collagen type I trimer (Lesot, 1981), several non-collagenous proteins are secreted by odontoblasts (Butler *et al.*, 1992). These include osteonectin and bone sialoprotein which, in the developing tooth, appear as positive markers of odontoblast phenotype and are expressed by newly polarized and mature odontoblasts but not by preodontoblasts or by any cell type of the enamel organ (Fisher *et al.*, 1983; Chen *et al.*, 1992; Reichert *et al.*, 1992). The inner dental epithelium controls odontoblast differentiation by means of interactions mediated by constituents of the basement membrane (Ruch *et al.*, 1983; Lesot *et al.*, 1992). Fibronectin, expressed by dental papilla cells including preodontoblasts, is redistributed during odontoblast differentiation and might play a role in the cytoskeletal reorganization by interacting with a 165 kDa membrane protein (Lesot *et al.*, 1981, 1992). Immunohistochemistry and/or *in situ* hybridization suggested that many soluble growth factors, including members of the NGF, FGF, IGF and TGFß families (Vaahtokari *et al.*, 1991; Cam *et al.*, 1992; Mitsiadis *et al.*, 1992, 1993; Joseph *et al.*, 1993) might play a role in odontoblast differentiation. When studying expression patterns of TGFß1, 2 and 3 transcripts conflicting data have been reported about TGFß1. Pelton *et al.* (1990) demonstrated a strong TGFß2 and 3 hybridization signal in the odontoblast layer while TGFß1

Abbreviations used in this paper: BMP, bone morphogenetic protein; BSP, bone sialoprotein; cDNA, complementary deoxyribonucleic acid; DEAE, diethylaminoethyl; ECM, extracellular matrix; EDTA, ethylenediaminetetracetic acid; FN, fibronectin; IGF, insulin growth factor; ON, osteonectin; PBS, phosphate buffer saline; RNAse, ribonuclease; SSC, sodium saline citrate; TGFß, transforming growth factor beta.

***Address for reprints:** Institut de Biologie Médicale, Université Louis Pasteur, Faculté de Médecine, 11 rue Humann, F-67085 Strasbourg Cedex, France. FAX: 33.88257817.

0214-6282/94/$03.00

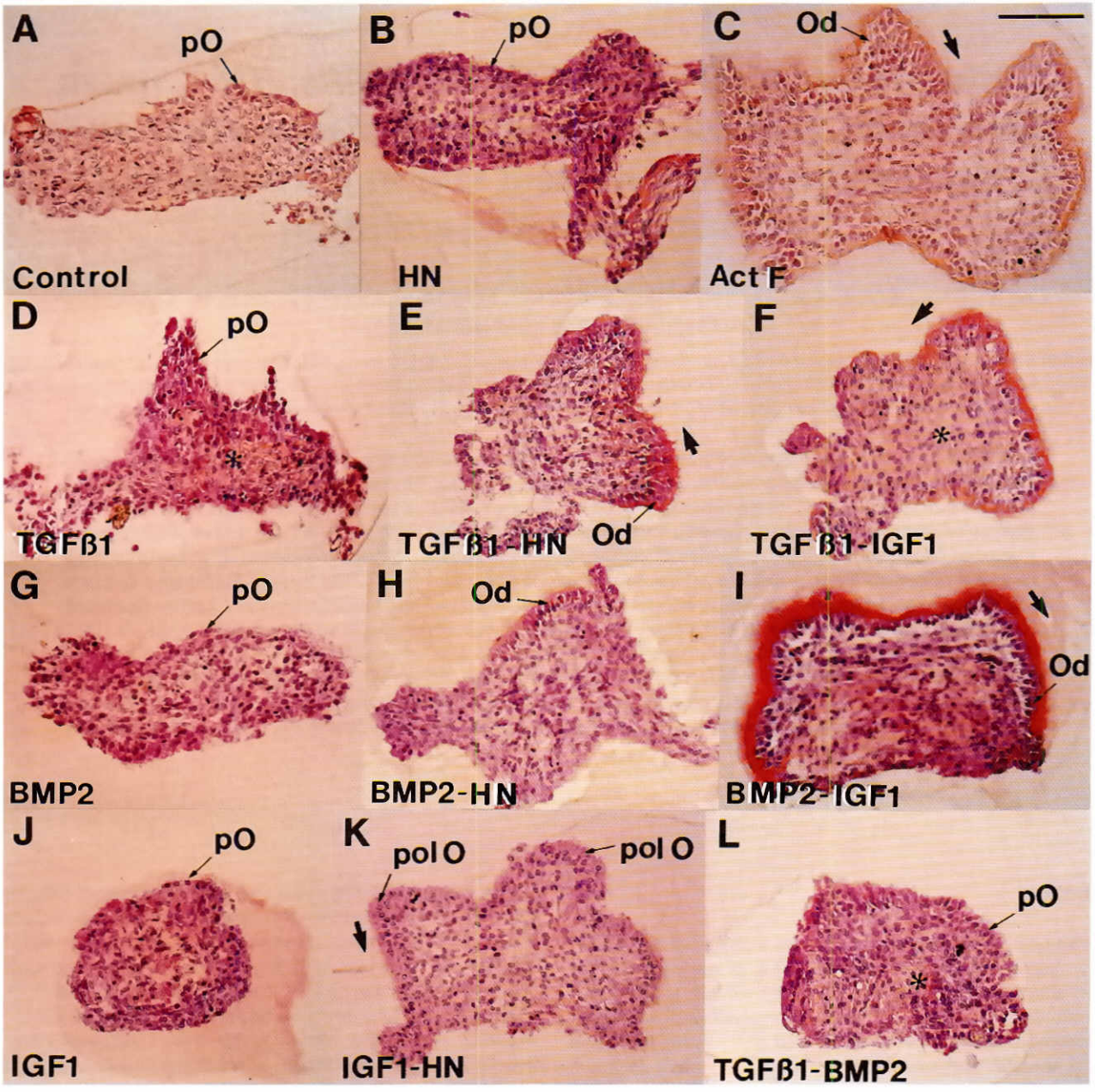


Fig. 1. Sections of day-17 trypsin-isolated dental papillae cultured for 6 days *in semi-solid control medium* **(A)** *or supplemented with either heparin: HN* **(B)**, *active dentin fraction: Act F* **(C)**, *TGFß1 alone* **(D)**, *TGFß1 combined with HN* **(E)**, *TGFß1 combined with IGF1* **(F)**, *BMP2 alone* **(G)**, *BMP2 combined with HN* **(H)**, *BMP2 combined with IGF1* **(I)**, *IGF1 alone* **(J)**, *IGF1 combined with HN* **(K)**, *or TGFß1 combined with BMP2* **(L).** *Odontoblast-like cells neither differentiated in control medium* **(A)**, *nor in presence of HN* **(B)**, *nor in the presence of the growth factors alone* **(D,G,J)**, *nor in the presence of TGFß1 combined with BMP2* **(L)**. *Polarized cells were observed either when the growth factors were combined with HN* **(E,H,K)** *or in the presence of active dentin fraction* **(C)** *or when TGFß1 or BMP2 were combined with IGF1* **(F,I** *respectively). BMP2-HN promoted differentiation of odontoblast-like cells in restricted areas* **(H)**, *and IGF1-HN allowed extended polarization without matrix deposition* **(K)**. **: Intrapulpal extracellular matrix, Od: odontoblast-like cells, pol O: polarizing odontoblast. Arrows indicate gradients of differentiation. Scale bar, 100 µm.*

mRNA was not expressed. However more recently, it has been shown that TGFß1 and 2 mRNA expression switched from the inner dental epithelium to the odontoblast layer in the area where predentin matrix secretion took place up to the appearance of secretory odontoblasts (Vaahtokari *et al.*, 1991; Heikinheimo *et al.*, 1993). Furthermore, secretory odontoblasts continue to express TGFß2 polypeptide (Heikinheimo *et al.*, 1993). While TGFß1 and 3 transcripts have been detected in odontoblasts, the presence of these molecules at the epithelio-mesenchymal junction has not been confirmed by immunolocalization (Cam *et al.*, 1990; Pelton *et*

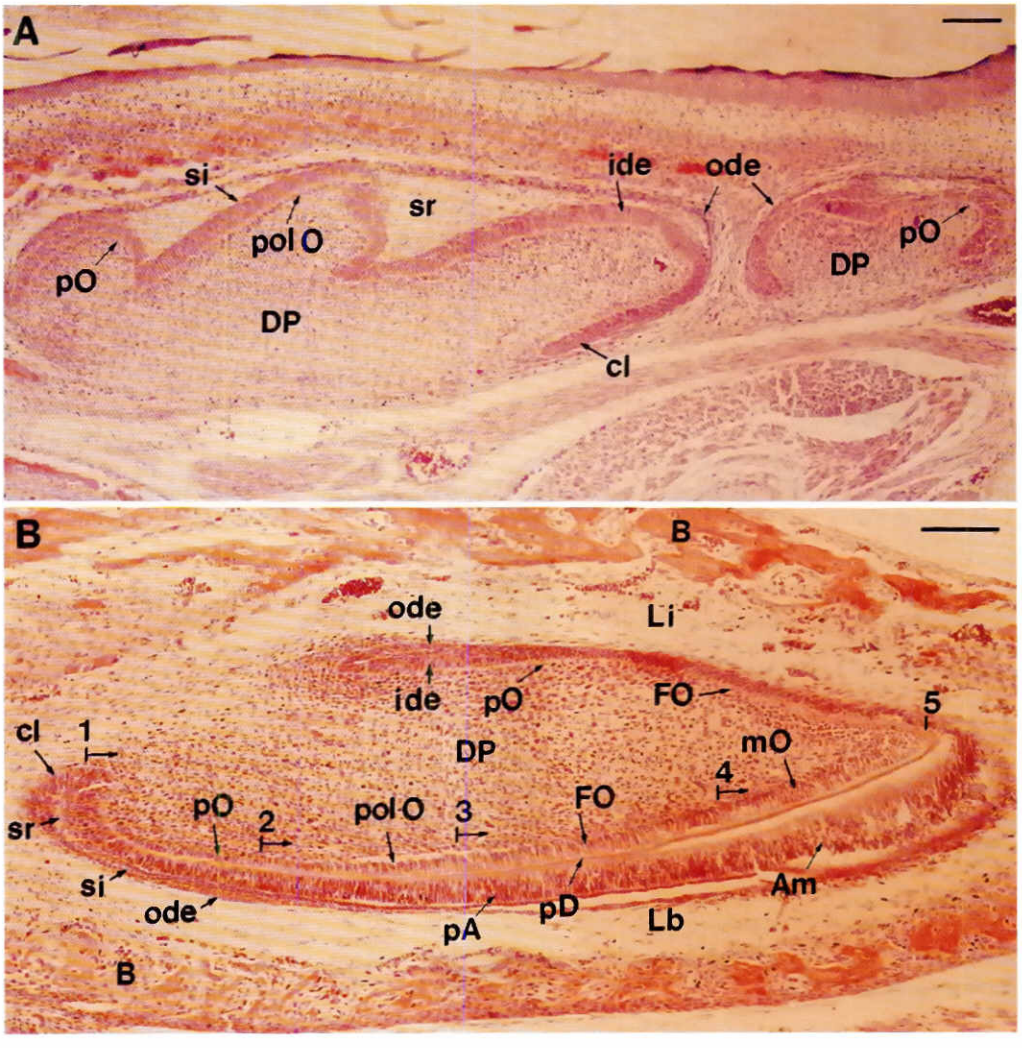


Fig. 2. Sagittal sections of first, second lower molars (A) and lower incisor (B) of newborn mice. *The labial (Lb) incisor pre-odontoblasts (pO) - odontoblasts (O) according to the gradient of differentiation are subdivided into: 1→2 posterior zone: area of pre-odontoblasts (pO), 2→3 area of polarizing odontoblasts (pol O), 3→4 area of functional odontoblasts (FO) secreting predentin (pD), 4→5 anterior zone: area of mature odontoblasts (mO). The enamel organ is composed of preameloblasts (pA) and ameloblasts (Am) forming the inner dental epithelium (ide) and of the outer dental epithelium (ode), the stratum intermedium (si) and the stellate reticulum (sr). cl: cervical loops, Li: lingual side, B: alveolar bone, DP: dental papillae. Scale bar, 100 μm.*

al., 1991). Bone morphogenetic proteins (BMPs) are a group of molecules which belong to the TGFß superfamily. In addition to their initially discovered effect in promoting chondrogenesis and osteogenesis (Wozney *et al.*, 1988), BMPs are likely to be involved in several developing systems (Kingsley, 1994). In particular, BMPs have been suggested to play major roles in epithelio-mesenchymal interactions at different stages of tooth morphogenesis (Lyons *et al.*, 1990; Vainio *et al.*, 1993; Heikinheimo, 1994). BMP2 transcripts have been shown to be confined to mesenchymal cells of the dental papillae, and to increase strongly in odontoblasts during their differentiation process (Lyons *et al.*, 1990; Vainio *et al.*, 1993). BMP4 transcripts were detected in odontoblasts and transiently in ameloblasts (Vainio *et al.*, 1993). Human BMP6 (also known as Vgr-1 in the mouse) proteins immunolocated in early enamel epithelium shifted to dental papilla mesenchyme with a predominance in developing and functional odontoblasts (Heikinheimo, 1994).

Furthermore, members of the TGFß superfamily have been suggested to play a role in the induction of the expression of transcription factors such as msx1 and msx2 (formerly Hox7 and Hox8) (Vainio *et al.*, 1993). Transcripts for msx1 were exclusively detected in the mesenchyme of the dental papillae whereas differentiating odontoblasts expressed high levels of *msx2* transcripts (MacKenzie *et al.*, 1992; Jowett *et al.*, 1993).

We have shown previously that the differentiation of odontoblast-like cells could be promoted *in vitro* in isolated dental papillae, in the absence of both the inner dental epithelium and the basement membrane, by a dentin matrix fraction added to the semi-solid culture medium (Bègue-Kirn *et al.*, 1992). Predentin-dentin contains several growth factors including TGFßs, BMPs and IGFs (Kawai and Urist, 1989; Finkelman *et al.*, 1990). Furthermore, since the positive effects of the dentin matrix fraction were abolished after its preincubation with a blocking antibody directed against TGFß1 and 2, it was postulated that active dentin fraction induced odontoblast differentiation by means of trapped growth factors (Bègue-Kirn *et al.*, 1992).

In this paper, we studied the effects of TGFß1, BMP2 and IGF1 on isolated dental papillae at the histological level. Furthermore, we have investigated the effect of these growth factors at the level of expression of TGFß1, 2 and 3, BMP2 and 4, IGF1, msx1 and 2, ON, BSP and FN mRNA transcripts by *in situ* hybridization analysis of cultured isolated dental papillae. The expression of these transcripts in experimental-induced odontoblast-like cells was compared to that observed *in vivo* in odontoblasts of intact teeth of

TABLE 1

TGFßs, BMPs, IGF1, msx1 AND 2, FIBRONECTIN (FN), OSTEONECTIN (ON) AND BONE SIALOPROTEIN (BSP) TRANSCRIPT DISTRIBUTIONS IN NEWBORN MOUSE TEETH

Tissues	TGFß1	TGFß2	TGFß3	BMP2	BMP4	IGF1	msx1	msx2	FN	ON	BSP
DP	0	0.5	0.5↑	1	0.5	0	0.5	0.5	1↑	0	0
pOd	1	0.5-1	0	1	2	0	0.5	2	1	0.5	0
polarizing Od	2	0.5-1	0.5	2	1	0	1	1	1	1	1
functional Od	1	0.5	1	2	1	0	2	0.5	0	2	2
mature Od	1	0.5	2	1	1	0	2	0.5	0	2	2
soc	1	0.5	2	1	1	0	2	0.5	1	2	2
pAm/pOd	0.5	0.5	0	0	0.5	0	0	2	0	0	0
pAm/ pol Od	1	2	2	0	1	0	0	2	0	0	0
polarizing Am	2	1↑	2	0	1	0	0	0.5	0	0	0
functional Am	2	0.5	0	1	1	0	0	0.5	0	0	0
ODE	1	1	0	0	0	0	0	1↑	0	0	0
SI	1	0	0	0	0	0	0	2↑	0	0	0
SR	0	0	0	0	0	0	0	0.5	0	0	0
alveolar bone	1	0	0	0.5	0.5	0.5	0.5	0	1	2	2
perident. mes.	0	0.5	2	0	0.5	2	1	0	0	0	2

2: strong positive signal; 1: positive signal; 0.5: weak "ubiquitous" signal; 0: no signal ;↑: graded distribution. DP: dental papillae; pOd: preodontoblasts; pol Od: polarizing odontoblasts; Od: odontoblasts; soc: sub-odontoblastic cells; pAm/pOd: preameloblasts in front of preodontoblasts; pAm/pol Od: preameloblasts in front of polarizing odontoblasts; Am: ameloblasts; ODE: outer dental epithelium; SI: stratum intermedium; SR: stellate reticulum; perident. mes.: peridental mesenchyme.

newborn mice. One further aim of our investigation was to correlate growth factor induced odontoblast-like differentiation with transcriptional modifications occurring *in vivo* in preameloblasts putatively involved in odontoblast terminal differentiation.

Results

Histological aspects of dental papillae cultured in the presence of growth factors

Controls

Differentiation of functional odontoblast-like cells was not observed either when 20 isolated dental papillae (DP) were cultured in control medium (Fig. 1A) or in 22 DP enclosed in control medium supplemented with heparin (Fig. 1B).

Effects of active dentin fraction

As shown previously the active dentin fraction (Bègue-Kirn *et al.*, 1992) induced gradients of differentiating odontoblast-like cells (Fig. 1C).

Effects of BMP2, IGF1 or TGFß1

Odontoblast differentiation never occurred when either BMP2, IGF1 or TGFß1 were added alone to the semi-solid medium in 10, 10 and 13 DP respectively (Fig. 1G,J,D). Nevertheless, a significant increase in extracellular matrix production was observed in 12/13 DP when TGFß1 was added to the culture medium (Fig. 1D) in contrast to BMP2 and IGF1 (Fig. 1G,J).

Effects of BMP2, IGF1 or TGFß1 combined with heparin

BMP2 combined with heparin induced both polarization and apical matrix deposition in very restricted areas in 8/12DP (Fig. 1H).

IGF1 combined with heparin initiated and promoted cytological polarizations in 8/11DP in extended areas. However matrix accumulation at the apical poles of these cells was very limited (Fig. 1K).

TGFß1 combined with heparin promoted the differentiation of well-polarized and functional odontoblast-like cells and typical gradients of differentiation were observed in 14/21 DP (Fig. 1E).

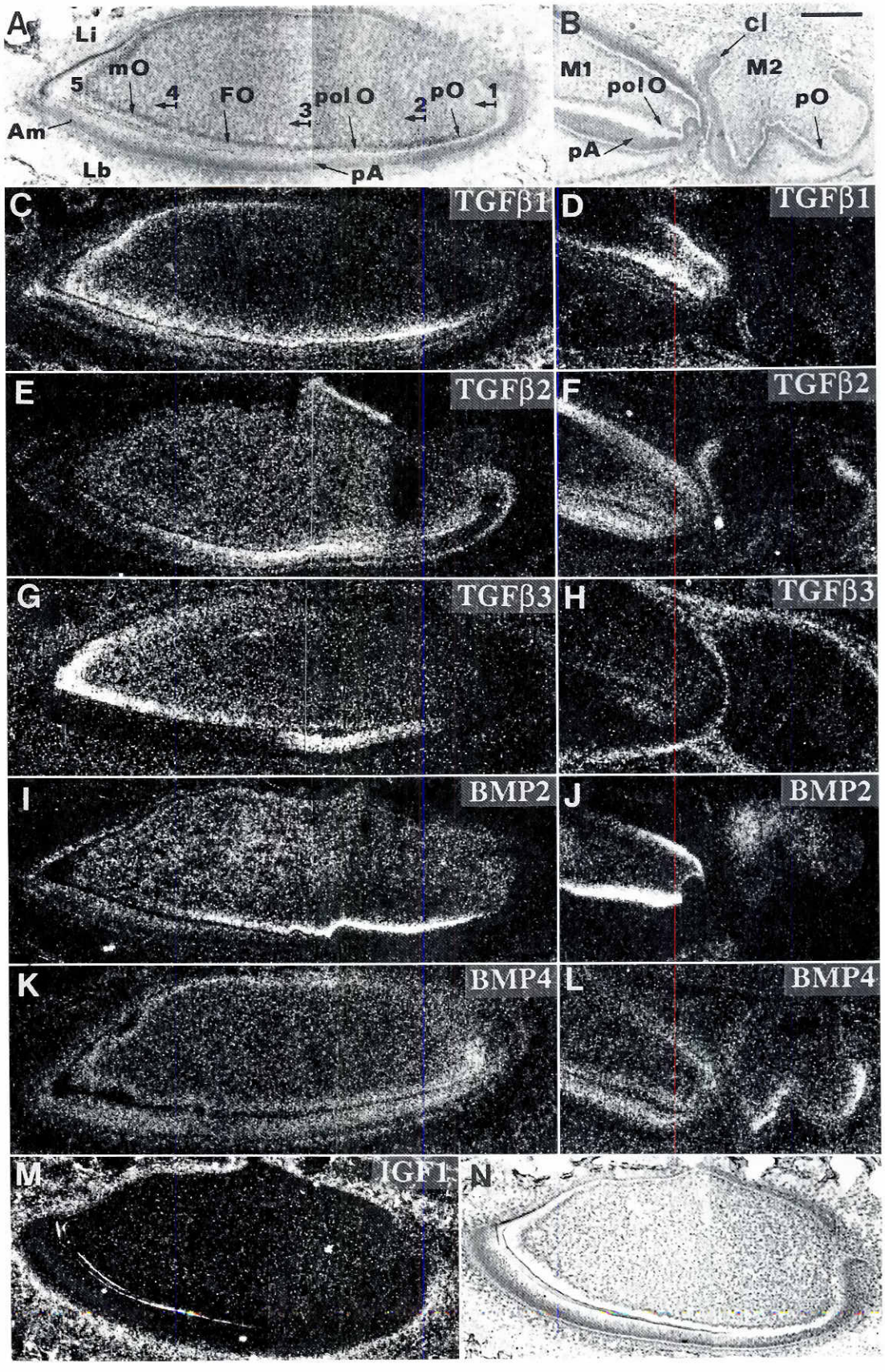
Effects of growth factors combinations in the absence of heparin

TGFß1 combined with BMP2 never allowed odontoblast-like differentiation in 13DP (Fig. 1L).

BMP2 combined with IGF1 promoted initiation and propagation of odontoblast-like differentiation. This pattern extended towards the basal part of cusps in 7/8DP (Fig. 1I). Furthermore, the morphology of the pulps was well maintained.

TGFß1 combined with IGF1 induced partial cytological and functional polarization over extended areas (Fig. 1F): secretion occurred both at the apical poles and lateral sides of the incom-

Fig. 3. TGFß1, 2 and 3; BMP2 and 4 RNA expression observed on consecutive sections of postnatal lower incisor, first (M1) and second (M2) upper molars and IGF1 RNA expression observed in the lower post-natal incisor (A,B and N are bright-field images of C,F and M respectively). *No signal was detected in dental tissues with IGF1 probe* **(M)**. *Preodontoblasts (pO) expressed TGFß1, TGFß2, BMP2 and BMP4 transcripts* **(C,E,I,K)**. *Functional odontoblast (FO) expressed TGFß1, TGFß3 and BMP2 transcripts strongly* **(C,D,G,H,I,J)**. *TGFß2 transcripts were essentially detected in epithelial structures* **(E,F)** *like cervical loops (cl) in M2. Preameloblasts (pA) transcribed TGFß1, TGFß3 and BMP4* **(C,D,G, H,K,L)**. *Ameloblasts (Am) displayed signals for TGFß1, BMP2, BMP4 RNAs* **(C,I,K)**. *The preodontoblast-odontoblast layer is subdivided into: preodontoblasts (pO): 1→2, polarizing odontoblasts (pol O): 2→3, functional odontoblasts (FO): 3→4 and mature odontoblasts (mO): 4→5 according to Fig. 2B. Scale bar, 200 µm.*


A
Li
mO
5
4
FO
3
polO
2
pO
1
Am
Lb
pA
B
cl
M1
M2
polO
pO
pA
C
TGFβ1
D
TGFβ1
E
TGFβ2
F
TGFβ2
G
TGFβ3
H
TGFβ3
I
BMP2
J
BMP2
K
BMP4
L
BMP4
M
IGF1
N

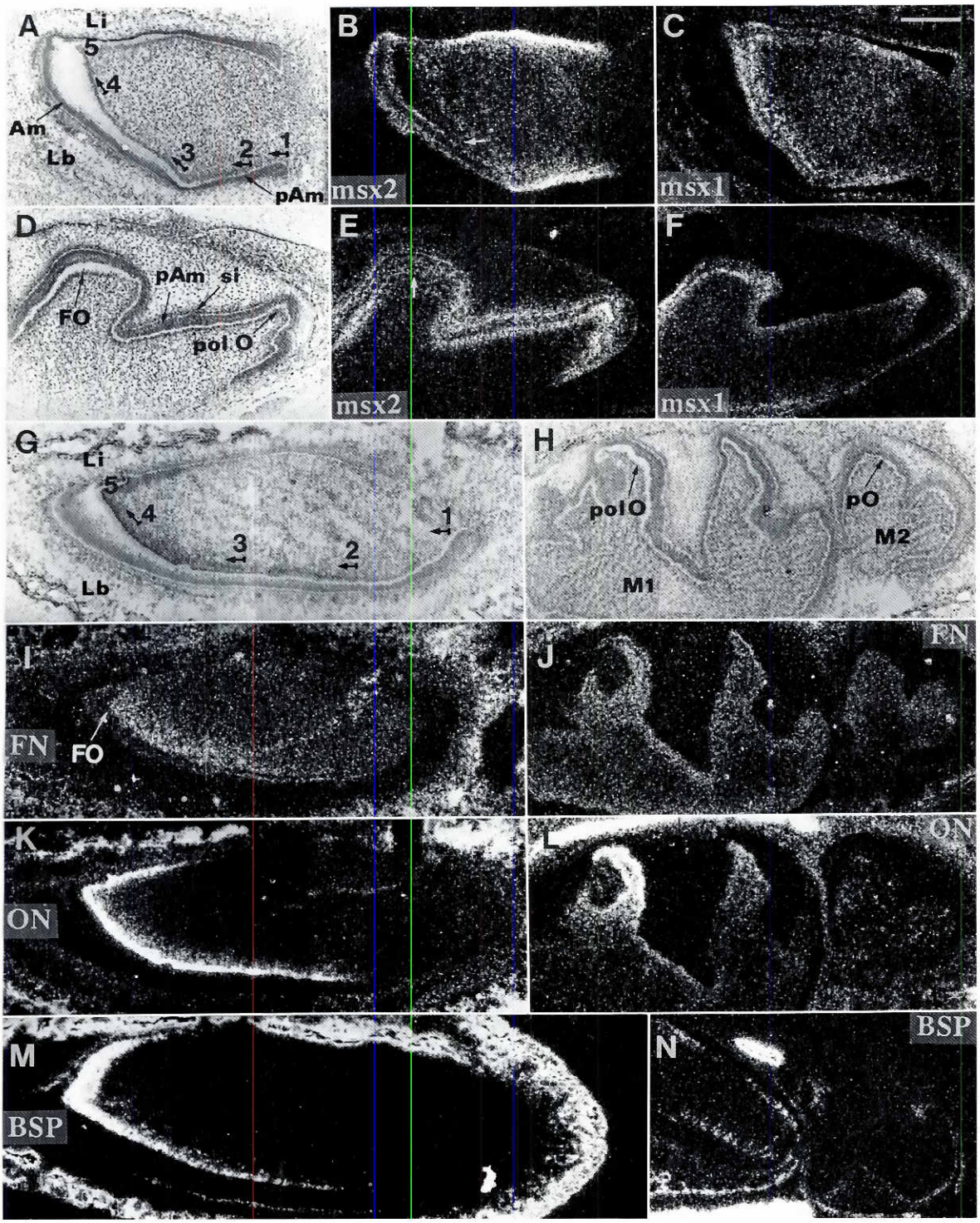


Fig. 4. Expression of msx1 and 2, fibronectin (FN), osteonectin (ON) and bone sialoprotein (BSP) transcripts in newborn dental tissues. *Labeling with msxs* (**B,C,E,F**) *probes are on consecutive sections of the lower incisor and first lower molars* (**A** *and* **D** *are bright-field images of* **C** *and* **E** *respectively). Labeling with FN and ON probes are on consecutive sections of lower incisor, lower first (M1) and second molars (M2)* (**G** *and* **H** *are bright-field images of* **K** *and* **J** *respectively). Labeling with BSP probe was performed on sections of lower incisor and superior first (M1) and second (M2) molars consecutive to bright-field images A and B of the Figure 3. Preodontoblasts (pO) expressed msx2 and FN transcripts* (**B,E,I,J**) *and msx1, ON and BSP transcripts were detected strongly in odontoblasts* (**C,F,K-N**). *Functional odontoblasts (FO) expressed few msx2 transcripts see arrows* (**B,E**). *While msx1, FN, ON and BSP transcripts were never detected in epithelial structures* (**C,F,I-N**), *msx2 transcripts showed significant hybridization signals in preameloblasts (pAm) and stratum intermedium: si* (**B,E**). *The preodontoblast-odontoblast layer is subdivided into: preodontoblasts (pO): 1→2, polarizing odontoblasts (pol O): 2→3, functional odontoblasts (FO): 3→4 and mature odontoblasts (mO): 4→5 according to Fig. 2B. Ameloblasts: Am. Scale bar, 200 μm.*

pletely polarized cells in 8/10DP. Furthermore, significant accumulation of extracellular matrix could be observed in the whole explant.

In situ hybridization

mRNA detection in teeth of newborn mice (day 19)

In first molars, which are at the late bell stage, functional odontoblasts were present in all the cusps. The dental papillae were composed of distinct peripheral odontoblasts and pulp cells. The first terminally differentiated ameloblasts were located at the tip of the main cusps and elsewhere the inner dental epithelium was composed of preameloblasts (Fig. 2A). In second molars, which are at the early bell stage, terminal differentiation of odontoblasts and ameloblasts was not yet initiated (Fig. 2A). In incisors, which are continuously growing teeth with an asymmetry between their labial and lingual sides, functional odontoblasts were present on both labial and lingual sides, whereas functional ameloblasts were restricted to the labial side (Fig. 2B). Preodontoblasts, polarizing odontoblasts, functional odontoblasts and mature odontoblasts can be distinguished histologically (see Fig. 2). The distribution of TGFß1, 2 and 3, BMP2 and 4, IGF1, fibronectin (FN), osteonectin (ON), bone sialoprotein (BSP) as well as *msx1* and *msx2* mRNA transcripts was investigated by *in situ* hybridization of (^{35}S)-labeled antisense riboprobes on serial cryosections of newborn mice head. The distribution of all the investigated transcripts is summarized in Table 1 and illustrated in Figs. 3 and 4. The pattern of expression of all the transcripts was similar in molars and incisors for corresponding cells. The various labeling patterns will be described first in the mesenchymal components, and then in the epithelial components of the developing teeth.

Mesenchymal expression patterns for growth factor transcripts

The signals for TGFß1 and BMP2 transcripts increased significantly as odontoblasts polarized (Fig. 3C,D,I,J). TGFß1 signal intensity showed a slight decrease in functional and mature odontoblasts (Fig. 3C) whereas BMP2 mRNAs decreased considerably in mature odontoblasts to reach the weak signal intensity of the other cells of the dental papilla. Interestingly, the strong labeling for BMP2 mRNAs in odontoblasts was restricted to the labial side of the incisor (Fig. 3I). TGFß3 mRNA signal increased progressively along the gradient of differentiation (Fig. 3G). An antero-posterior increasing gradient in TGFß3 transcripts was also observed in other papilla cells. In contrast to TGFß1 and 3, the signal for TGFß2 transcripts remained at a constant ubiquitous level throughout the odontoblast layer except in a few preodontoblasts and polarizing odontoblasts on the incisor labial side (Fig. 3E). BMP4 transcripts were expressed at higher levels in preodontoblasts and decreased in functional and mature odontoblasts (but remained higher than in other dental papilla cells) (Fig. 3K,L). IGF1 mRNA was not detected in dental papilla cells but was expressed by peridental mesenchymal cells (Fig. 3M).

Mesenchymal expression patterns for ECM proteins transcripts

BSP and ON transcripts were both exclusively and increasingly detected in polarizing, functional and mature odontoblasts (Fig. 4K,L,M,N). FN transcripts were detected in preodontoblasts and polarizing odontoblasts but were absent in functional and mature odontoblasts (Fig. 4I,J). Furthermore, a graded distribution of FN transcripts decreasing in labio-lingual and antero-posterior directions was observed in dental papilla cells.

Mesenchymal expression patterns for msx1 and 2 transcripts

msx1 transcripts were expressed at higher levels in functional and mature odontoblasts (Fig. 4C,F). In contrast, *msx2* transcripts were weakly expressed in functional and mature odontoblasts but showed increased hybridization signals in preodontoblasts and polarizing odontoblasts (Fig. 4B,E).

Epithelial expression patterns for growth factor transcripts

TGFß1 transcript levels increased along the inner dental epithelium, from preameloblasts facing polarized odontoblasts towards functional ameloblasts at the tip of the incisor (Fig. 3C). TGFß2 and TGFß3 mRNAs were detected with strong intensity in preameloblasts and polarizing ameloblasts and diminished progressively in functional and mature ameloblasts (Fig. 3E,G). In second molars, a strong expression of TGFß2 transcripts was observed in epithelial loop cells (Fig. 3F). The inner dental epithelium exhibited a rather constant labeling for BMP4 transcripts (Fig. 3K,L), whereas BMP2 transcripts were only detected in functional ameloblasts (Fig. 3I). IGF1 mRNAs could not be detected in any of the dental epithelial components.

Epithelial expression patterns for ECM proteins transcripts

None of the ON, BSP and FN probes showed hybridization signals in the dental epithelial tissues (Fig. 4 I-N).

Epithelial expression patterns for msx1 and 2 transcripts

msx1 mRNA was never detected in dental epithelium (Fig. 4C,F). In contrast, msx2 transcripts were strongly expressed in preameloblasts and progressively decreased towards polarizing ameloblasts and functional ameloblasts (Fig. 4B,E).

mRNA detection in dental papillae cultured for 6 days

The patterns of expression of the various transcripts are illustrated in Figs. 5 and 6 and semi-quantitative evaluations of the signal intensities as a function of the experimental conditions are summarized in Fig. 7.

Growth factor transcripts

In culture conditions where well-differentiated functional odontoblast-like cells were observed (active fraction of dentin, IGF1-BMP2, TGFß1-IGF1 and TGFß1-heparin), the TGFß1 signals were slightly enhanced in peripheral areas corresponding to odontoblast-like cells (Figs. 5B,G,L, 6F and 7A). In cultures performed in the presence of BMP2-heparin where differentiation occurred in restricted areas, TGFß1 signals were readily detected in large peripheral areas (Fig. 5Q).

On the other hand, IGF1-heparin did not produce any increase of TGFß1 transcript signal in such peripheral cells (Fig. 5V). BMP2 and 4 mRNAs were strongly and uniformly detected in odontoblast-like cells and in other dental papilla cells whatever the culture conditions (Figs. 6K,L,O,P and 7A,B). In cultures performed in the presence of active fraction of dentin TGFß3 mRNAs were preferentially expressed in odontoblast-like cells (Figs. 5C, 6H and 7A).

In cultures performed in the presence of either IGF1-BMP2, TGFß1-heparin, BMP2-heparin or IGF1-heparin, TGFß3 transcripts were homogeneously distributed in the whole explants (Fig. 5H, M, R, W). The strongest signals for TGFß2 and IGF1 mRNAs were observed in control cultures (compare Fig. 6C,U with 6G,Y).

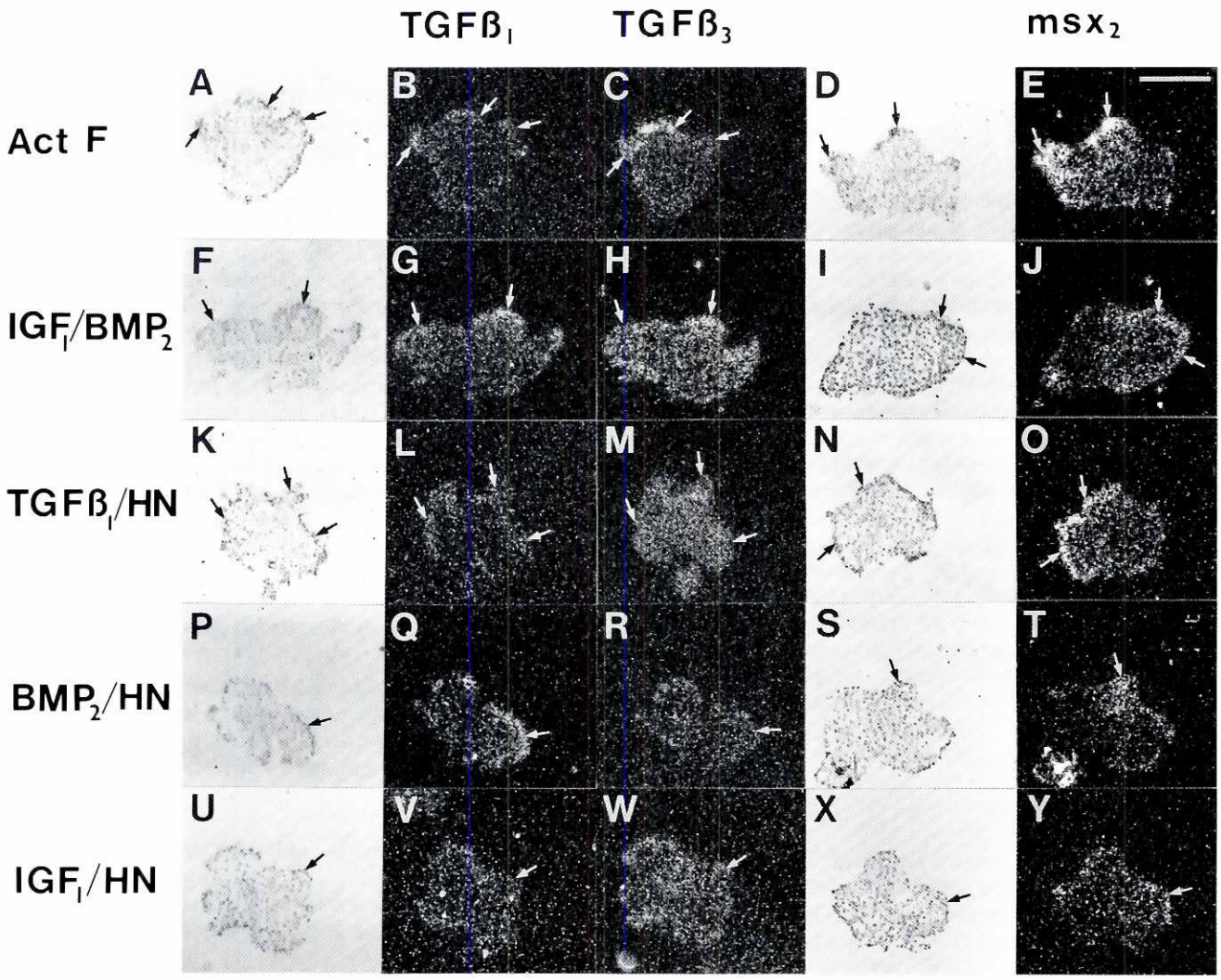


Fig. 5. Expression of TGFß1 and 3 and msx2 transcripts in day-17 dental papillae cultured for 6 days in the presence of active dentin fraction: *Act F* **(A-E)**, *IGF1 combined with BMP2* **(F-J)**, *TGFß1 combined with heparin: HN* **(K-O)**, *BMP2 combined with HN* **(P-T)** *and IGF1 combined with HN* **(U-Y)**. *Labeling with TGFß1* **(B,G,L,Q,V)** *and TGFß3* **(C,H,M,R,W)** *probes was on consecutive sections* (**A,F,K,P** *and* **U** *are bright-field images of* **B,G,L,Q** *and* **W** *respectively). Msx2 mRNA detection on dark-field images* **(E,J,O,T,Y)** *correspond to adjacent bright-field microphotographs* **(D,I,N,S,X)**. *TGFß1 mRNA was preferentially expressed by odontoblast-like cells* **(B,G,L,Q)**, *TGFß3 mRNA was homogeneously expressed by all the dental papillae cells* **(H,M,R,W)** *except when dental papillae were cultured in the presence of Act F* **(C)**. *Msx2 mRNA was detected strongly in odontoblast-like cells* **(E,J,O,T)** *and polarized odontoblast-like cells* **(Y)**. *Arrows indicate odontoblast-like cells or polarized odontoblast-like cells in cultures performed in the presence of IGF1-HN. Scale bar, 200 µm.*

ECM protein transcripts

ON and BSP mRNAs were always detected in functional odontoblast-like cells (Figs. 6N,W and 7C) and also, to a lower extent, in cultures performed in the presence of IGF1-heparin where polarized cells were obtained in large areas without predentin accumulation (Fig. 1K). In addition, both ON and BSP transcripts were also detected in presumptive bone cells as shown in control cultures (Fig. 6J,R). FN mRNA was always faintly detected in dental papillae cells but never in odontoblast-like cells (Fig. 6X and 7C).

msx1 and 2 transcripts

An increased hybridization signal for *msx2* mRNA was observed under all the conditions leading to functional odontoblast-like cells (Figs. 5E,J,O,T, 6AG and 7C). In the presence of IGF1-heparin where peripheral cells did not deposit predentin-like ECM the *msx2* signal was also slightly increased (Figs. 5Y and 7C). In contrast, msx1 mRNAs were faintly detected in odontoblast-like cells as well as in other dental papilla cells and in control cultures (Figs. 6AE, AA and 7C,D).

Preliminary data of mRNA detection in dental papillae cultured for 24 hours

The TGFß1,2 and 3, BMP2, *msx1* and *2* probes were hybridized to sections of control cultures and cultures performed during 24 h in the presence of either active dentin fraction, or TGFß1-heparin, or BMP2-heparin or IGF1-heparin. Semi-quantitative evaluations of the signal intensities as a function of the experimental conditions

are summarized in Fig. 7 and the patterns of expression of the several transcripts are illustrated in Fig. 8.

Under these conditions, except in controls, the signal for TGFß1 mRNA was increased in peripheral cells corresponding to the potential odontoblasts (Figs. 7E and 8G-J). In the presence of active dentin fraction (Figs. 7E and 8L) potential odontoblasts showed some increase in signal for TGFß2 mRNA. A significant signal for TGFß3 mRNA was always observed in peridental mesenchyme (Fig. 8P-T).

BMP2 signals, rather homogeneous in controls, decreased in the main cusps areas corresponding to preodontoblasts and subperipheral cells in the presence of BMP2-heparin (Figs. 7E and 8X). In contrast, when dental papillae were cultured in the presence of either the active fraction of dentin or TGFß1-heparin, BMP2 transcripts were not homogeneously distributed but formed a gradient with higher amounts in main cusp areas (Fig. 8V,W). BMP2 mRNA was never detected in peridental mesenchyme.

No significant variation in signal intensity could be detected for msx1 mRNAs (Figs. 7E,F and 8AE-AI). Higher levels of *msx2* signals were observed in the whole dental papillae in the presence of IGF1-heparin (Fig. 8AN) and to a lesser extent in the presence of the active dentin fraction and BMP2-heparin (Fig. 8AK, AM). In contrast, in the presence of TGFß1-heparin *msx2* signals were lower than those observed in controls (Figs. 7E,F and 8AL).

Discussion

Odontoblast terminal differentiation occurs according to a tooth specific pattern and implies both temporospatial regulated availability of epigenetic signals and expression of specific competence (Ruch, 1990). Terminal differentiation of odontoblasts is characterized by several steps implying withdrawal from the cell cycle, elongation, cytological polarization and transcriptional and translational modifications, enabling the cells to deposit predentin and dentin components. It is not known yet if all the neural crest-derived dental papilla cells are potential odontoblasts and whether during normal odontogenesis only cells in immediate proximity with the inner dental epithelium will overtly differentiate or if distinct dental pulp cells and odontoblastic lineage exist. A paracrine role of a temporospatial specific inner dental epithelium inducing terminal differentiation of odontoblasts through matrix-mediated interactions is generally accepted, involving matrix molecules like FN (Lesot *et al.*, 1992) and basement membrane trapped growth factors (Ruoslahti and Yamaguchi, 1991). How many steps are involved in the initiation of odontoblast differentiation and whether these steps are interdependent is still unclear.

Knowing that EDTA-soluble constituents of dentin could promote the differentiation of odontoblast-like cells *in vitro* and that these effects might be due to TGFßs, BMPs and/or IGFs, we compared in this study, both at the histological and transcriptional levels, odontoblast-like cells induced *in vitro* by various growth factors to odontoblasts in the *in vivo* situation.

Growth factor induction of odontoblast-like cells

While growth factors (TGFß1, BMP2, IGF1) added alone to the culture medium never allowed the differentiation of odontoblast-like cells, when combined with heparin (HN) they had positive albeit differential effects: TGFß1-HN (like active dentin fractions) induced gradients of cytological and functional differentiation, BMP2-HN allowed polarized secretion in restricted areas and IGF1-HN induced extensive cytological differentiation without apical matrix deposition. The effects of IGF1 combined with BMP2 and IGF1 combined with TGFß1 were similar to those of active dentin fraction and TGFß1-HN while TGFß1 combined with BMP2 had no effect.

At the histological level, even in culture conditions allowing the promotion of differentiation gradients, the histo-cytological arrangement of odontoblast-like cells was not strictly equivalent to the odontoblast layer *in vivo*. Indeed, the layer of odontoblast-like cells was not perfectly aligned and a little matrix was also deposited in between odontoblast-like cells.

The differentiation of odontoblasts according to temporospatial gradients might be correlated with the progressive, kinetic-dependent (endogenous) expression of competence to respond to epigenetic signals. In our experimental conditions, immobilization in semi-solid culture medium of inducer(s) combined with heparin allowing their interaction with cells progressively expressing competence, might explain the formation of gradients. Both TGFß1 and IGF1 when combined with heparin, induced gradients of differentiation but in contrast to TGFß1, IGF1 only partly allowed polarized matrix deposition. Considering the more restricted effects of BMP2-HN we might postulate that the immobilization of BMP2 was incomplete and/or that its half-life was shorter. The results obtained when IGF1 was combined either with BMP2 or TGFß1 in the absence of heparin are difficult to explain. We do not know if these growth factors interact and if their diffusion was restricted after combination. However, IGF1 and TGFß1 or BMP2 might cooperate to promote matrix secretion (Dennis and Rifkin, 1991; Martin *et al.*, 1992; Davidson *et al.*, 1993). The absence of odontoblast-like induction by TGFß1 combined with BMP2 could result from diffusion. The importance of modulation of TGFß1 activity by either extracellular components (Ruoslahti and Yamaguchi, 1991; Schubert, 1992; Streuli *et al.*, 1993) or the growth factor context by itself have already been reported (Davidson *et al.*, 1993).

Transcriptional modulation during odontoblast differentiation in vivo

As far as preodontoblasts and odontoblasts are concerned, the analysis of several transcripts in teeth of newborn mice allowed us to subdivide them into five categories:

1) *transcripts significantly increased in preodontoblasts before initiation of terminal differentiation*:
TGFß2, BMP4 and *msx2* transcripts were high in preodontoblasts and diminished during polarization. This suggests that the corresponding proteins might be involved in some events initiating odontoblast terminal differentiation. TGFß2 transcripts have been previously weakly detected in odontoblasts (Pelton *et al.*, 1990; Heikinheimo *et al.*, 1993). An increased expression of BMP4 and *msx2* in preodontoblasts has already been described by Vainio *et al.* (1993) and MacKenzie *et al.* (1992). On the other hand, TGFß2 polypeptides were immunolocalized in secretory odontoblasts and underlying cells (Pelton *et al.*, 1990; Heikinheimo *et al.*, 1993), whereas BMP4 and *msx2* immunolocalizations have not been reported.
2) *Transcripts detected in polarizing and functional odontoblasts:*
TGFß3, msx1, BSP and ON transcripts were detected from the first polarizing odontoblasts to the oldest odontoblasts located at the tip of the cusps with a progressive increase in signal intensity along the gradient of differentiation. These transcripts can thus be considered as markers of the functional odontoblast phenotype. Our results confirm previous observations concerning TGFß3, *msx1* and BSP transcript distribution (Pelton *et al.*,

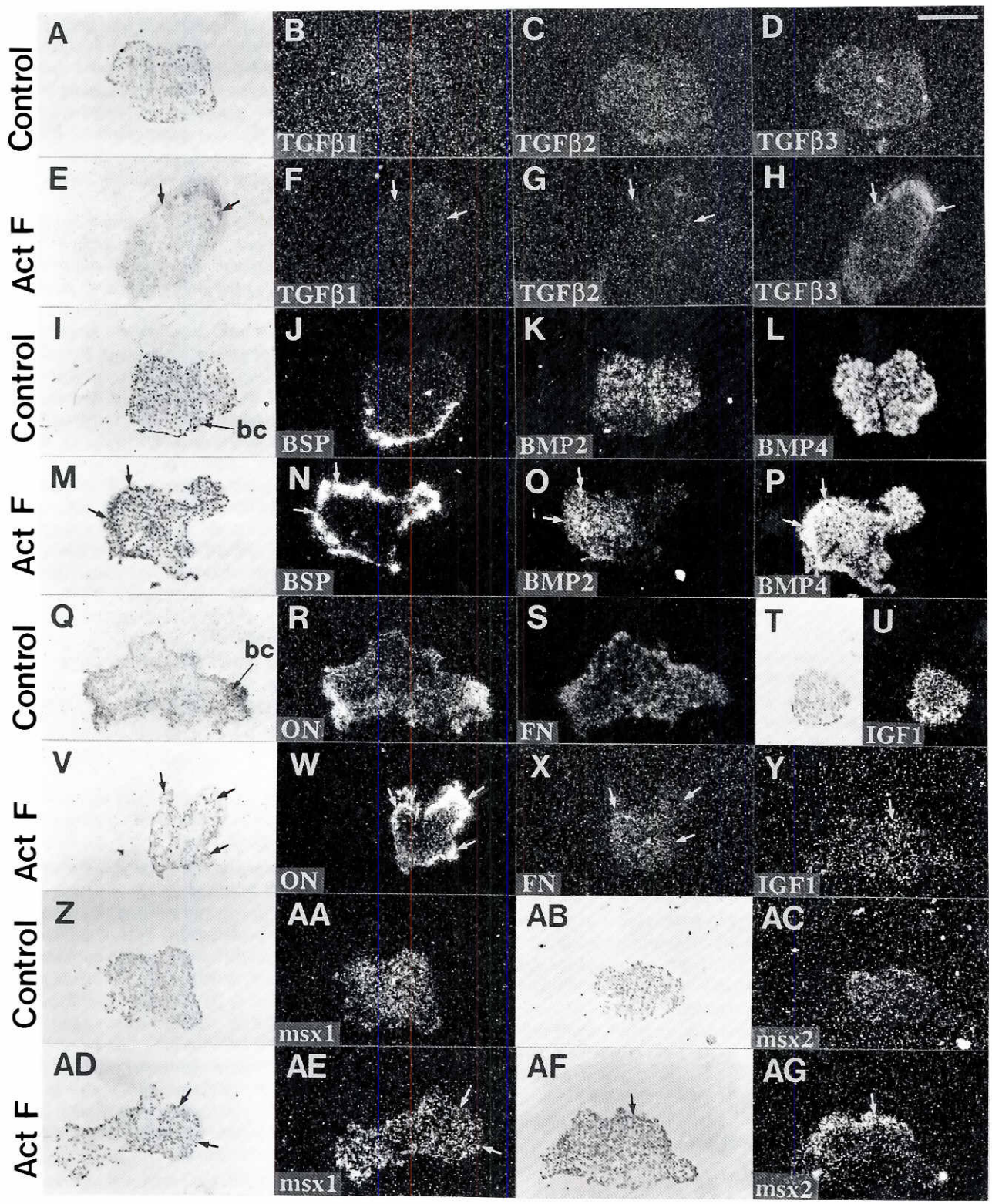


Fig. 6. Expression of TGFß1, 2 and 3; bone sialoprotein (BSP); BMP2 and 4; osteonectin (ON); fibronectin (FN); IGF1 and msx1 and 2 transcripts in Day-17 dental papillae *cultured for 6 days either in control conditions: Control* **(A-D, I-L, Q-U, Z-AC)** *or in the presence of active dentin fraction: Act F* **(E-H, M-P, V-Y, AD-AG)**. *Labeling with either TGFß1, 2 and 3 or BSP, BMP2 and 4 or ON and FN probes are on consecutive sections* **(A,E,I,M,Q,T,V,Z, AB, AD** *and* **AF** *are bright-field images of* **B,H,K,P,R,U,X, AA, AC, AE** *and* **Y** *respectively). Odontoblast-like cells expressed significant levels of TGFß3, BSP, BMP2 and 4, ON and msx2 transcripts but few or no TGFß1 and 2, IGF1, FN and msx1 transcripts. Arrows indicate odontoblast-like cells. bc: presumptive bone cell. Scale bar, 200 μm.*

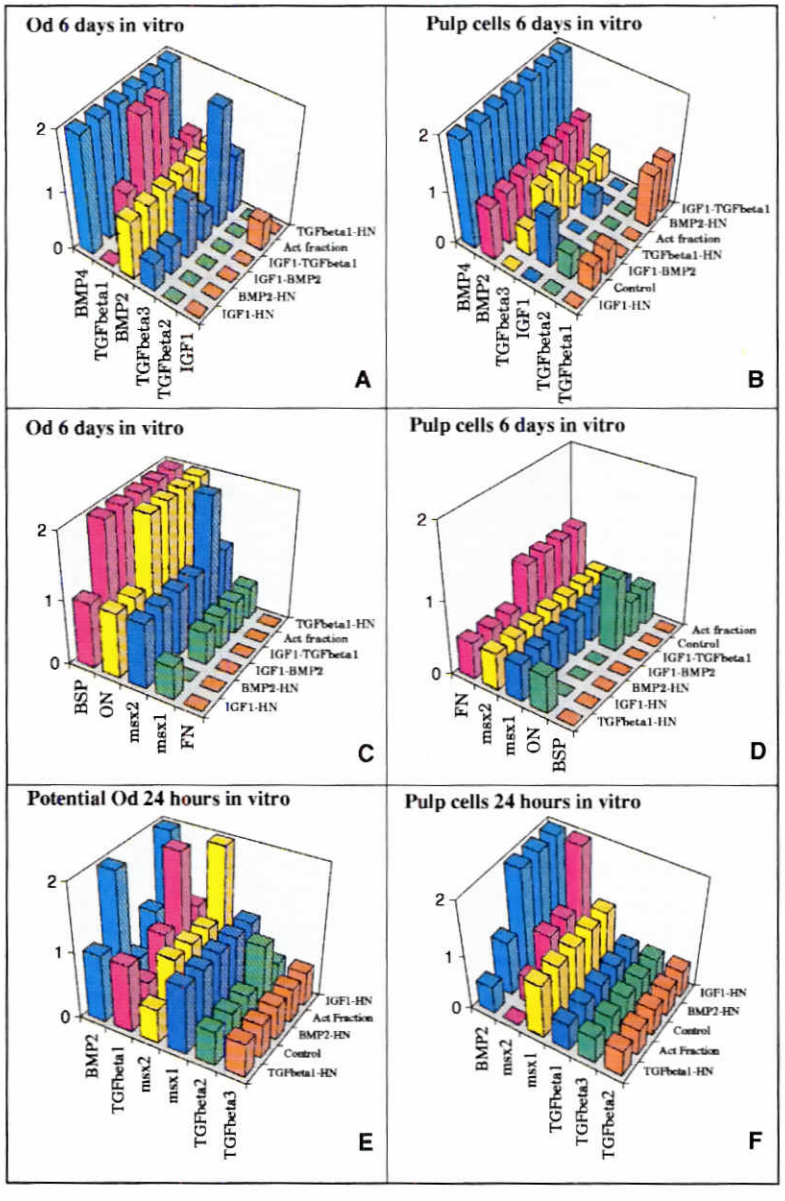
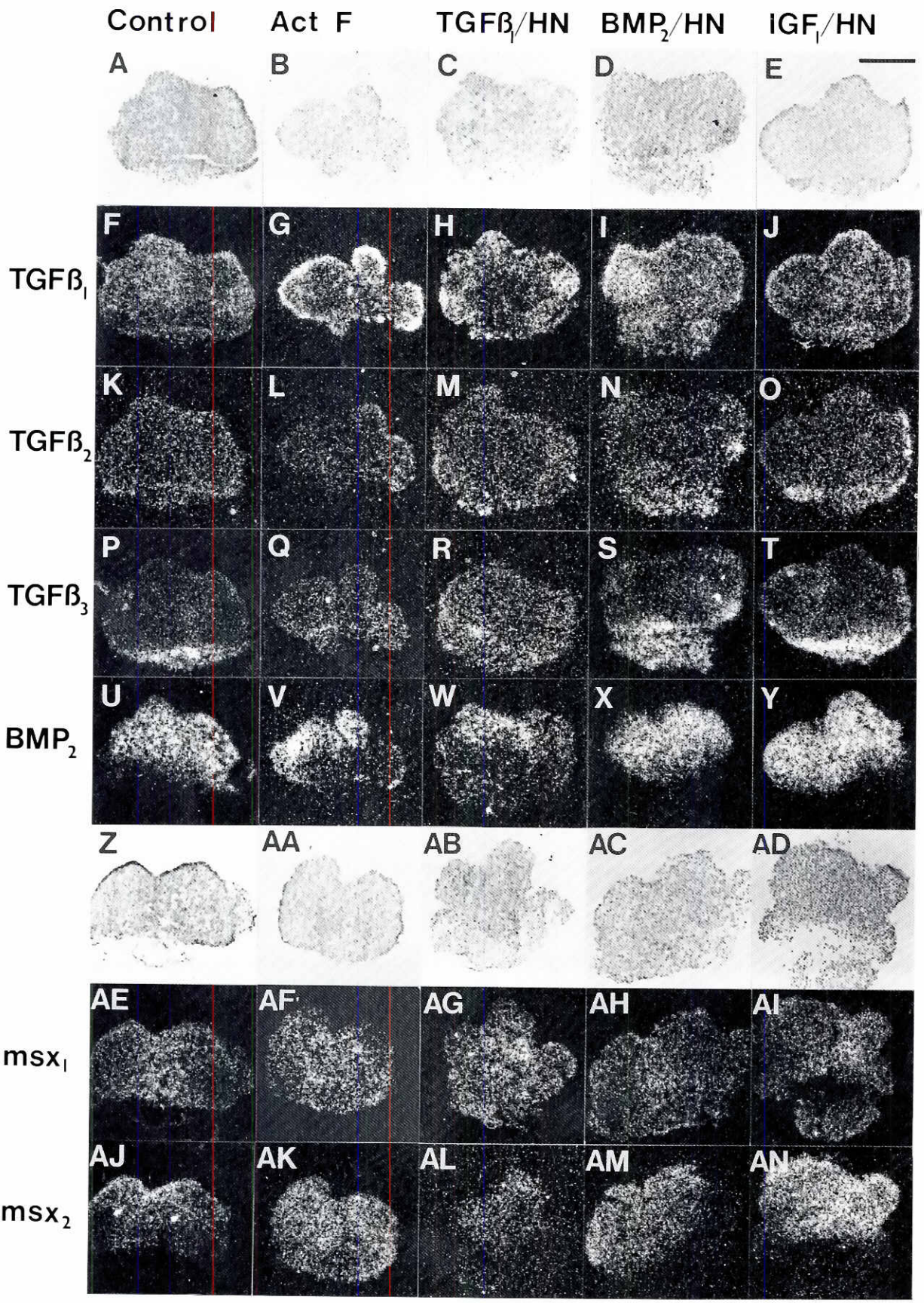


Fig. 7. Semi-quantitative evaluations of transcripts expression in Day-17 dental papillae cultured either for 6 days (A-D) or 24 h (E-F). *Diagrams* **A,C,E** *summarize signal intensities observed at the periphery of the explants corresponding to either odontoblast-like cells after 6 days of culture* **(A,C)** *or potential odontoblasts after 24 h culture* **(E)** *and diagrams* **B,D,F** *show signal intensities in pulp cells. An arbitrary scale was chosen to define signal intensities on Y axis: 0= no signal, 0.5= ubiquitous signal, 1= positive signal, 2= strong positive signal. Probes are on X axis: TGFß1, 2 and 3, BMP2 and 4, IGF1, msx1 and 2, osteonectin (ON), bone sialoprotein (BSP) and fibronectin (FN) and experimental conditions are on Z axis. To optimize optical discrimination of data it was necessary to change from one diagram to the other the color attributed to the probes. HN: heparin, Act Fraction: active dentin fraction, Od: odontoblasts.*

1990; Chen *et al.*, 1992; MacKenzie *et al.*, 1992) and ON protein detection (Reichert *et al.*, 1992). TGFß3 polypeptides have not been immunodetected in cells of the odontoblastic lineage (Pelton *et al.*, 1991).

3) *Transcripts highly detected during initial steps of odontoblast terminal differentiation and decreasing in functional-mature odontoblasts:*
TGFß1 and BMP2 transcripts, first intensely expressed in polarizing odontoblasts, showed a progressive decrease in more differentiated cells. This decrease was more pronounced for BMP2 transcripts. Our *in situ* hybridization data for TGFß1 and BMP2 transcripts are in agreement with those already reported (Vaahtokari *et al.*, 1991; Vainio *et al.*, 1993). To date, TGFß1 polypeptides could not be immunodetected in cells of the odontoblastic lineage (Cam *et al.*, 1990; Pelton *et al.*, 1991). Recently, BMP immunoreactivity has been localized in mature odontoblasts, predentin and dentin with a monoclonal antibody directed against purified BMP (Lianjia *et al.*, 1993). However specific BMP2 immunolocalization has never been reported.

4) *Transcripts detected before and during odontoblast differentiation and disappearing in functional odontoblasts:*
FN transcripts were apparently absent in terminally differentiated odontoblasts but were detected in dental papilla cells, preodontoblasts and polarizing odontoblasts. FN had not been analyzed previously at the transcriptional level in tooth germs and our present results are in agreement with the data obtained by immunohistochemistry (Lesot *et al.*, 1981, 1992; Lukinmaa *et al.*, 1991). The cDNA probe used in our study includes the EIIIB sequence (also called EDB) present only in some of the several FN isoforms (Georges *et al.*, 1993). Since an FN probe lacking the EIIIB fragment (not shown) only allowed very weak signal detection in dental papillae cells we may postulate that the major


Control
Act F
TGFß1/HN
BMP2/HN
IGF1/HN
A
B
C
D
E
TGFß1
F
G
H
I
J
TGFß2
K
L
M
N
O
TGFß3
P
Q
R
S
T
BMP2
U
V
W
X
Y
Z
AA
AB
AC
AD
msx1
AE
AF
AG
AH
AI
msx2
AJ
AK
AL
AM
AN

FN isoform expressed by dental mesenchymal cells is the fibronectin EIIIB$^+$.

5) *Transcripts never detected in cells of the odontoblast lineage:* IGF1 transcripts were not detected in preodontoblasts/odontoblasts or in other dental papillae cells while a strong signal existed in peridental mesenchyme. A previous study performed at the late cap stage, also did not reveal any signal for IGF1 transcripts within dental tissues (Ferguson *et al.*, 1992). However, IGF1 has recently been immunolocalized in differentiating odontoblasts and secretory odontoblasts (Joseph *et al.*, 1993). This discrepancy might be explained by 1) a low copy number of IGF1 transcripts, 2) a short half-life characterizing transcripts encoding for growth factors involved in the control of cell differentiation (Heyner *et al.*, 1989).

During odontogenesis, growth factors and their corresponding transcripts were often observed to localize to different cells, thereby indicating that complex patterns of transcription, translation and secretion exist and that growth factors are acting through several mechanisms including autocrine and paracrine modes of action (Pelton *et al.*, 1991). We have shown here that, whatever the differentiation stage, at least one of the various TGFß transcript isoforms is always expressed in the odontoblastic lineage. This suggests that TGFß molecules might have a role in initiation of odontoblast differentiation, but also later in regulation of the extracellular matrix deposition at the epithelio-mesenchymal junction. Furthermore, the complexity of the expression patterns described in the present work suggests coordinated roles for growth factors, extracellular components (FN) and transcription factors (as putative target of growth factor signalling pathways) during odontoblast differentiation. Molecules such as BMP4, which are highly expressed by preodontoblasts appear important in the initiation of odontoblast differentiation, whereas *msx2* which is strongly expressed in areas of undifferentiated and polarizing odontoblasts, might be more involved in the polarization process.

Transcriptional modulations in in vitro induced odontoblast-like cells: comparison with functional odontoblasts

The differentiation of odontoblast-like cells accumulating extracellular matrix at their apical pole and demonstrating significative signals for TGFß1, TGFß3, BMP2, BMP4, *msx2*, ON and BSP could be induced by means of an active dentin fraction, TGFß1-HN, IGF1-BMP2 and IGF1-TGFß1. In terms of hybridization signal intensity, odontoblast-like cells phenotypes were different from that of the normal odontoblast for *msx2* expression which appear to be less important *in vivo* and for *msx1* expression which was not up-regulated *in vitro*. However, accurate quantitative comparisons cannot be performed by means of *in situ* hybridization and at a qualitative level the odontoblast-like cells induced by the active dentin fraction, TGFß1-HN, IGF1-BMP2, IGF1-TGFß1, appear to be very similar to normal odontoblasts. Polarized cells induced by IGF1-HN accumulated less matrix at their apical pole although they expressed transcripts encoding for ON and BSP. These cells neither expressed TGFß1 nor TGFß2 and ubiquitous levels of TGFß3 were detected. The absence of matrix deposition by polarized cells in the presence of IGF1-HN might be linked to the fact that these cells were not able to express TGFß molecules.

Early effects of several growth factors on gene expression in potential odontoblasts

Our preliminary results indicated that the expression of TGFß1 mRNAs is induced *in vitro* in preodontoblasts (corresponding to the most peripheral cell layer) in all culture conditions promoting polarization. Thus, TGFß1 mRNA strong expression by preodontoblasts appeared to be related to early events preceding *in vitro* cell differentiation.

msx2 transcripts were differentially expressed after the different growth factors treatments. While *msx2* transcripts were detected at high levels in the presence of BMP2 and more significantly in the presence of IGF1, the signal was weaker in the presence of TGFß1. The up-regulation of *msx2* transcription in polarized cells after 6 days might be indirect and for example due to the cytokine context.

Hypotheses concerning the physiological control of odontoblast differentiation

Our data concerning the *in vitro* induction of odontoblast differentiation suggest that up-regulation of *msx2* transcription as well as up-regulation of transcription of members of the TGFß family (TGFß1, 3; BMP2, 4) are prerequisites for terminal differentiation of odontoblasts allowing polarization and apical accumulation of matrix respectively. These up-regulations could be "induced" *in vitro* by immobilized members of the TGFß superfamily. In physiological conditions a stage-specific inner dental epithelium triggers odontoblast terminal differentiation through matrix mediated interactions. What kind of epithelial-derived molecules are involved in this process? It has been shown (Vainio *et al.*, 1993) that young dental epithelium (E11) induced transcription of BMP4 and *msx1* in dental mesenchyme and that BMP4-releasing agarose beads induced expression of *msx1* and *2* transcripts as well as BMP4 transcripts by an autoregulating loop system. Our *in vivo in situ* hybridization studies of course do not allow us to directly conclude that the inner dental epithelium releases such growth factors during odontoblast differentiation, but since TGFßs and BMPs transcripts are present such a possibility exists. However, the expression of these transcripts was not restricted to only those preameloblasts corresponding to preodontoblasts able to overtly differentiate. In order to explain how certain cells within the odontoblastic layer can respond to an epithelially-derived message while their neighbors do not, several, possibly complementary, control mechanisms may be hypothesized: the formation of gradients of odontoblast differentiation could require a progressive and continuous emergence of a few competent cells responding to the epithelially-derived mes-

Fig. 8. Expression of TGFß1, 2 and 3; BMP2; msx1 and 2 transcripts in Day-17 dental papillae cultured for 24 h *either in control condition* **(A,F,K,P,U,Z,AE,AJ)** *or in the presence of active dentin fraction: Act F* **(B,G,L,Q,V,AA,AF,AK),** *TGFß1 combined with heparin: HN* **(C,H,M,R,W,AB,AG,AL),** *BMP2 combined with HN* **(D,I,N,S,X,AC,AH,AM)** *or IGF1 combined with HN* **(E,J,O,T,Y,AD,AI,AN).** *Labeling with TGFß1, 2 and 3 and BMP2 probes are on consecutive sections (***A-E** *are bright-field images of* **K,L,W,S,O** *respectively) as well as labeling with msx1 and 2 probes (***Z-AD** *are bright-field images of* **AE, AF, AG, AH, AN** *respectively). Potential odontoblast-like cells expressed high levels of TGFß1 transcripts* **(G-J).** *Peridental mesenchymal cells expressed TGFß1, 2 and 3 and msx1 transcripts. Msx2 showed variable mRNA expression in the presence of either Act F or growth factors* **(AK-AN).** *Msx2 expression was decreased in the presence of TGFß1-HN* **(AL)** *and significantly increased in the presence of IGF1-HN* **(AN).** *Scale bar, 200 µm.*

sage. This competence might imply the progressive expression of 1) matrix molecules with affinity for growth factors or with potentializing activities or 2) membrane receptors (Lesot *et al.*, 1994). The maturation process could also imply the ability to transduce the signal towards the cytoplasm or the nucleus (Lesot *et al.*, 1994). The possible role of cell kinetics in the expression of competence has previously been suggested (Ruch *et al.*, 1983; Ruch, 1990).

As a simplified working hypothesis we might suggest the following: an epithelial-derived member of the TGFß family trapped by basement membrane associated components (paracrine pathway), interacts with competent preodontoblasts and regulates the transcription by the preodontoblasts of genes encoding for proteins belonging to the TGFß family and homeoproteins like Msx2. The MSX2 transcription factor, might in turn modulate the expression of genes encoding for components involved in cytoskeletal assembly. TGFßs could regulate matrix production (autocrine mode of action) including transitory synthesis of FN which co-regulates processes involved in polarization by interaction with the 165 kDa membrane receptor. Clearly the mechanisms involved in odontoblast differentiation are much more complicated and synergistic interactions with other growth factors (IGFs, FGFs) probably exist. Use of molecular technologies (oligodeoxynucleotides antisense strategies, gene knockout, etc.) combined with appropriate tissue recombinations will probably allow elucidation of the molecular network involved in the control of odontoblast differentiation.

Materials and Methods

Dentin matrix components

Total EDTA-soluble extracellular matrix components prepared from rabbit incisor dentin according to Smith and Leaver (1979) were purified by DEAE-Cellulose chromatography as previously described (Bègue-Kirn *et al.*, 1992). 3.6 µg of the DEAE-Cellulose fraction number 5, eluted in the presence of a linear gradient of NaCl (0-0.7 M) approximatively between 0.45 and 0.55 M NaCl, were added to the 12 µl of semi-solid medium enclosing the Day-17 dental papilla as described below.

Growth factors

50 ng recombinant simian Transforming Growth Factor ß1: rsTGFß1 (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA, USA) were added to the semi-solid medium alone or in combination as described below.

15 ng of recombinant human Bone Morphogenetic Protein 2: rhBMP2 (Genetics Institute, Cambridge, MA, USA) were added to the semi-solid medium as for TGFß1.

50 ng recombinant Insulin Growth Factor 1: rIGF1 (Chemicon, Temecula, CA, USA) were added to the semi-solid medium as for the other growth factors.

Tissue cultures

Swiss Day-17 pregnant mice (vaginal plug day= day 0) were sacrificed by cervical dislocation. The embryos were removed and the mandibular first molars were isolated and incubated for 1 h at 4°C in 1% trypsin (Difco 1:250) in Hanks' solution as previously described (Ruch *et al.*, 1976). Isolated, selected dental papillae (initiation of cusp formation) were embedded individually in 12 µl of culture medium (RPMI-1640 supplemented with ascorbic acid (0.18 mg/ml), L-Glutamin (2 mM), kanamycin (0.1 mg/ml), fetal calf serum (20%) and Difco agar (0.5%). Dentin fraction or growth factors were added to the 12 µl of control medium before gelling as follows:

- dentin matrix components (3.6 µg) fractionated on DEAE-cellulose,
- TGFß1 (50 ng) alone or combined with heparin (1.2 µg),
- BMP2 (15 ng) alone or combined with heparin (1.2 µg),
- IGF1 (50 ng) alone or combined with heparin (1.2 µg),
- TGFß1 (50 ng) combined with either BMP2 (15 ng) or IGF1 (50 ng),
- BMP2 (15 ng) combined with IGF1 (50 ng).

Finally, the solidified 12 µl blocks including the dental papillae were deposited on Millipore filters (Bègue-Kirn *et al.*, 1992) and cultured according to Trowell (1954) in the presence of 2.5 ml of liquid control medium at 37°C in 95% air and 5% CO_2 for 1 or 6 days. The liquid control medium was changed every other day.

Histology

Tissues were fixed in Bouin's solution, embedded in paraffin and cut serially in 5 µm thick sections. Staining of sections was performed with Mallory's Alun hematoxylin.

In situ hybridization

Tissues

Newborn Swiss mouse heads were dipped in methyl-2-butane cooled in dry ice and 7 µm thick sagittal cryosections were performed.

Cultured Day-17 dental papillae were frozen in an atmosphere of liquid nitrogen after embedding in Tissue-Tek and 7 µm thick sections cut.

In both cases consecutive sections were stuck to different gelatin and chrome Alun treated slides, in order to compare the labeling patterns of different probes. Slides were stored at -80°C before hybridization.

Probe synthesis

Antisense riboprobes were labeled with (α-^{35}S)CTP (Amersham) using the appropriate SP6, T3 or T7 *in vitro* transcription system. The probes were cleaved to an average length of 150 nucleotides by partial alkaline hydrolysis and used at a final concentration of 50 000 cpm/µl in the hybridization buffer.

Murine TGFß1, TGFß2 and TGFß3 probes were kind gifts from Dr. A.J. Akhurst and have already been described (Fitzpatrick *et al.*, 1990). These three cDNAs were specific for each isoform and were shown not to provide any cross-hybridization.

Murine BMP2 and 4 probes were kindly provided by Dr. B.L.M. Hogan and have been described respectively by Lyons *et al.* (1990) and Jones *et al.* (1991). These probes were devoid of domains conserved in the TGFß family in order to avoid cross-reactivity with closely related genes.

Murine IGF1 probe was previously described (Mathews *et al.*, 1986) and kindly provided by Drs. J.M. Waters and W.G. Young.

Murine fibronectin probe was provided by Drs. E. Georges-Labouesse and R. Hynes and corresponds to the III7b-EIIIB-III8a cDNA fragment of fibronectin (Hynes *et al.*, 1992; Georges *et al.*, 1993).

Murine BSP probe was provided by Drs. L.W. Fisher and M.F. Young. Murine BSP clone 1 cDNA contains about 100bp untranslated 5' DNA, the coding region terminates near the stop region and has been described by Young *et al.* (1994).

Murine osteonectin (also named SPARC) cDNA, kindly provided by Dr. P. Basset, extending from nucleotides (-) 63 to (+) 1162 was selected for *in situ* hybridization analyses (Mason *et al.*, 1986).

Murine msx1 and 2 probes were generously provided by Dr. R. Hill and were described respectively by Hill *et al.* (1989) and Monaghan *et al.* (1991).

In situ hybridization

The cryosections were dipped in cold acetone for 3 min, fixed in 4% formaldehyde in PBS for 15 min and washed twice in PBS. The tissues were submitted to an acetylation treatment and rinsed twice in 2xSSC. The tissue RNAs were denatured by heating in 50% formamide/1xSSC (60°C, 10 min) followed by rapid cooling by two successive passages in 50% and 70% ethanol (-20°C). Finally, the sections were dehydrated in absolute ethanol and air dried.

The hybridization step was perfomed according to Décimo *et al.* (1994). The sections were washed as follows: 2xSSC, 50% formamide, 60°C, 90 min; H_2O, 2 min; 2xSSC, RNAse T_1 2 U/ml (Boehringer), RNAse A 20-40 µg/ml, 37°C, 30 min; H_2O, 2 min; 2xSSC, 50% formamide, 60°C, 90 min; H_2O, 2 min. After dehydration in absolute ethanol the slides were air dried,

dipped in Kodak NTB2 emulsion (diluted 1:2 in distilled water) and autoradiographically exposed for 3 days to 4 weeks depending on the probe. After development the slides were stained with hematoxylin and examined using bright-field and dark-field optics.

Acknowledgments

We thank Dr. R. Hill of the MRC Human Cytogenetics Unit, Edhinburgh, Scotland, for providing the murine msx1 and 2 probes; Dr. R.J. Akhurst of the Duncan Guthrie Institute of Medical Genetics in Glasgow, Scotland, UK, for providing the murine TGFß1, 2, and 3 probes; Dr. B.L.M. Hogan of the Vanderbilt University Medical School in Nashville, TN, USA, for providing the murine BMP2 and 4 probes; Drs. J.M. Waters and W.G. Young of the University of Queensland, Brisbane, Australia, for providing the murine IGF1 probe; Drs. E. Georges-Labouesse and J.O. Hynes, respectively from LGME, Strasbourg France, and MIT, MA, USA, for providing the murine fibronectin III7b-EIIIB-III8a cDNA fragment; Drs. L.W. Fisher and M.F. Young from the NIH, Bethesda, MD, USA, for providing the murine BSP clone 1 probe; and Dr. P. Basset from the LGME, Strasbourg, France for providing the murine osteonectin probe. We are also grateful to Dr. J.M. Wozney from Genetics Institute Inc., Cambridge, MA, USA, for providing the human BMP2 protein; Dr. J. Lane from Bristol-Myers Squibb Pharmaceuticals Research Institute, Seattle, WA, USA for providing the human TGFß1 protein; and Dr. P. Dollé from the LGME, Strasbourg for reviewing the manuscript. We also thank G. Vetter for photographic work. The research was supported by grants from the Commission of the European Communities (SC1.-CT91-0680 (WNSN)) and the Ministère de la Recherche et de l'Espace (92 C 0320).

References

BÈGUE-KIRN, C., SMITH, A.J., RUCH, J.V., WOZNEY, J.M., PURCHIO, A., HARTMANN, D. and LESOT, H. (1992). Effects of dentin proteins, transforming growth factor ß1 (TGFß1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast *in vitro*. *Int. J. Dev. Biol. 36:* 491-503.

BUTLER, W.T., D'SOUZA, R.N., BRONCKERS, A.L.J.J., HAPPONEN, R-P. and SOMERMAN, M.J. (1992). Recent investigations on dentin specific proteins. In *Proceedings of the Finnish Dental Society* (Ed. M. Närhi). Finnish Dental Society, Helsinki, pp. 369-376.

CAM, Y., NEUMANN, M.R. and RUCH, J.V. (1990). Immunolocalization of transforming growth factor ß1 and epidermal growth factor receptor epitopes in mouse incisors and molars with a demonstration of *in vitro* production of transforming activity. *Arch. Oral Biol. 35:* 813-822.

CAM, Y., NEUMANN, M.R., OLIVER, L., RAULAIS, D., JANET, T. and RUCH, J.V. (1992). Immunolocalization of acidic and basic fibroblast growth factors during mouse odontogenesis. *Int. J. Dev. Biol. 36:* 381-398.

CHEN, J., SHAPIRO, H.S. and SODEK, J. (1992). Developmental expression of bone sialoprotein mRNA in rat mineralized connective tissues . *J. Bone Miner. Res. 7:* 987-997.

DAVIDSON, J.M., ZOIA, O. and LIU, J.M. (1993). Modulation of transforming growth factor-beta 1 stimulated elastin and collagen production and proliferation in porcine vascular smooth muscle cells and skin fibroblasts by basic fibroblast growth factor, transforming growth factor-alpha, and insulin-like growth factor-I. *J. Cell. Physiol. 155:* 149-156.

DÉCIMO, D., GEORGES-LABOUESSE, E. and DOLLÉ, P. (1994). *In situ* hybridization of nucleic acid probes to cellular RNA. In *Gene Probes: A Practical Approach* (Eds. B.D. Hames and S. Higgins). Oxford University Press, Oxford. (In press).

DENNIS, P.A. and RIFKIN, D.B. (1991). Cellular activation of latent transforming growth factor ß requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proc. Natl. Acad. Sci. USA 88:* 580-584.

FERGUSON, M.W.J., SHARPE, P.M., THOMAS, B.L. and BECK, F. (1992). Differential expression of insulin-like growth factors I and II (IGFI and II), mRNA, peptide and binding protein 1 during mouse palate development: comparison with TGFß peptide distribution. *J. Anat. 181:* 219-238.

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/IGF-II, and TGF-ß in human dentin. *J. Bone Miner. Res. 5:* 717-723.

FISHER, L.W., WHITSON, S.W., AVIOLI, L.W. and TERMINE, J.D. (1983). Matrix sialoprotein of developing bone. *J. Biol. Chem. 258:* 12723-12727.

FITZPATRICK, D.R., DENHEZ, F., KONDAIAH, P. and AKHURST, R.J. (1990). Differential expression of TGF beta isoforms in murine palatogenesis. *Development 109:* 585-595.

GEORGES, E.N., GEORGE, E.L., RAYBURN, H. and HYNES, R.O. (1993). Fibronectin mutations in mice. In *Cell Adhesion Molecules* (Eds. M.E. Hemler and E. Mihich). Plenum Press, New York, pp. 29-39.

HEIKINHEIMO, K. (1994). Stage-specific expression of decapentaplegic-Vg-related genes 2, 4, and 6 (bone morphogenetic proteins 2, 4 and 6) during human tooth morphogenesis. *J. Dent. Res. 73:* 590-597.

HEIKINHEIMO, K., HAPPONEN, R.P., MIETTINEN, P.J. and RITVOS, O. (1993). Transforming growth factor ß2 in epithelial differentiation of developing teeth and odontogenic tumors. *J. Clin. Invest. 91:* 1019-1027.

HEYNER, S., SMITH, R.M. and SCHULTZ, G.A. (1989). Temporally regulated expression of insulin and insulin-like growth factors and their receptors in early mammalian development. *BioEssays 11:* 171-176.

HILL, R.E., JONES, P.F., REES, A.R., SIME, C.M., JUSTICE, M.J., COPELAND, N.G., JENKINS, N.A., GRAHAM, E. and DAVIDSON, D.R. (1989). A new family of mouse homeobox containing genes: molecular structure, chromosomal location and developmental expression of *Hox7. Genes Dev. 3:* 26-37.

HYNES, R.O., GEORGE, E.L., GEORGES, E.N., GUAN, J.L., RAYBURN, H. and YANG, J.T. (1992). Toward a genetic analysis of cell-matrix adhesion. In Cold Spring Harbor Symposia on Quantitative Biology Vol. LVII . Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 249-258.

JONES, C.M., LYONS, K.M. and HOGAN, B.L.M. (1991). Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development 111:* 531-542.

JOSEPH, B.K., SAVAGE, N.W., YOUNG, W.G., GUPTA, G.S., BREIER, B.H. and WATERS, M.J. (1993). Expression and regulation of insulin-like growth factor-I in the rat incisor. *Growth Factors 8:* 267-275.

JOWETT, A.K., VAINIO, S., FERGUSON, M.W.J., SHARPE, P.T. and THESLEFF, I. (1993). Epithelial-mesenchymal interactions are required for *msx1* and *msx2* gene expression in the developing murine molar tooth. *Development 117:* 461-470.

KAWAI, T. and URIST, M.R. (1989). Bovine tooth-derived bone morphogenetic protein. *J. Dent. Res. 68:* 1069-1074.

KINGSLEY, D.M. (1994). What do BMPs do in mammals? Clues from the mouse short-ear mutation. *Trends Genet. 10:* 16-21.

LESOT, H. (1981). Collagen trimer synthesis by cultured embryonic mouse molars. *Eur. J. Biochem. 116:* 541-546.

LESOT, H., FAUSSER, J.L., AKIYAMA, S.K., STAUB, A., BLACK, D., KUBLER, M.D. and RUCH, J.V. (1992). The carboxy-terminal extension of the collagen binding domain of fibronectin mediates interaction with a 165 kDaa membrane protein involved in odontoblast differentiation. *Differentiation 49:* 109-118.

LESOT, H., OSMAN, M. and RUCH, J.V. (1981). Immunofluorescent localization of collagens, fibronectins, and laminin during terminal differentiation of odontoblasts. *Dev. Biol. 82:* 371-381.

LESOT, H., SMITH, A.J., TZIAFAS, D., BÈGUE-KIRN, C., CASSIDY, N. and RUCH, J.V. (1994). Effects of biologically active molecules on dental tissue repair: comparison of reactionary and reparative dentinogenesis with the induction of odontoblast differentiation *in vitro*. *Cells and Materials.* (In Press).

LIANJIA, Y., YUHAO, G. and WHITE, F.H. (1993). Bovine bone morphogenetic protein-induced dentinogenesis. *Clin. Orthop. 295:* 305-312.

LUKINMAA, P-L., MACKIE, E.J. and THESLEFF, I. (1991). Immunohistochemical localization of the matrix glycoproteins-tenascin and the ED-sequence-containing form of cellular fibronectin in human permanent teeth and periodontal ligament. J. *Dent. Res. 70:* 19-26.

LYONS, K.M., PELTON, R.W. and HOGAN, B.L.M. (1990). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development 109:* 833-844.

MACKENZIE, A., FERGUSON, M.W.J. and SHARPE, P.T. (1992). Expression patterns of the homeobox gene, *Hox 8*, in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development 115:* 403-420.

MARTIN, J.L., BALLESTEROS, M. and BAXTER, R.C. (1992). Insulin-like growth factor-I (IGF-I) and transforming growth factor-beta 1 release IGF-binding protein-3 from human fibroblasts by different mechanisms. *Endocrinology 131:* 1703-1710.

MASON, I.J., TAYLOR, A., WILLIAMS, J.G., SAGE, H. and HOGAN, B.L.M. (1986). Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial «culture shock» glycoprotein of Mr 43000. *EMBO J. 5:* 1465-1472.

MATHEWS, L.S., NORSTEDT, G. and PALMITER, R.D. (1986). Regulation of insulin-

like growth factor I gene expression by growth hormone. *Proc. Natl. Acad. Sci. USA 83:* 9343-9347.

MITSIADIS, T.A., COUBLE, P., DICOU, E., RUDKIN, B.B. and MAGLOIRE, H. (1993). Patterns of nerve growth factor (NGF), proNGF, and p75NGF receptor expression in the rat incisor: comparison with expression in the molar. *Differentiation 54:* 161-175.

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.

MONAGHAN, A.P., DAVIDSON, D.R., SIME, C., GRAHAM, E., BALDOCK, R., BHATTACHARYA, S.S. and HILL, R.E. (1991). The Msh-like homeobox genes define domains in the developing vertebrate eye. *Development 112:* 1053-1061.

PELTON, R.W., DICKINSON, M.E., MOSES, H.L. and HOGAN, B.L.M. (1990). *In situ* hybridization analysis of TGFß3 RNA expression during mouse development: comparative studies with TGFß1 and ß2. *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:* 1091-1105.

REICHERT, T., STÖRKEL, S., BECKER, K. and FISHER, L.W. (1992). The role of osteonectin in human tooth development: an immunohistological study. *Calcif. Tissue Int. 50:* 468-472.

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

RUCH, J.V., KARCHER-DJURICIC, V. and THIEBOLD, J. (1976). Cell division and cytodifferentiation of odontoblasts. *Differentiation 5:* 165-169.

RUCH, J.V., LESOT, H., KARCHER-DJURICIC, V., MEYER, J.M. and MARK, M. (1983). Epithelial-mesenchymal interactions in tooth germs: mechanisms of differentiation. *J. Biol. Buccale 11:* 173-193.

RUOSLAHTI, E. and YAMAGUCHI, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell 64:* 867-869.

SCHUBERT, D. (1992). Collaborative interactions between growth factors and the extracellular matrix. *Trends Cell Biol. 2:* 63-66.

SMITH, A.J. and LEAVER, A.G. (1979). Non-collagenous components of the organic matrix of rabbit incisor dentin. *Arch. Oral Biol. 24:* 449-454.

STREULI, C.H., SCHMIDHAUSER, C., KOBRIN, M., BISSELL, M.J. and DERYNCK, R. (1993). Extracellular matrix regulates expression of TGF-ß1 gene. *J. Cell Biol. 120:* 253-260.

TROWELL, O.A. (1954). A modified technique for organ culture *in vitro*. *Exp. Cell Res. 46:* 246-248.

VAAHTOKARI, A., VAINIO, S. and THESLEFF, I. (1991). Associations between transforming growth factor ß1 RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. *Development 113:* 985-994.

VAINIO, S., KARAVANOVA, I., JOWETT, A. and THESLEFF, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell 75:* 45-58.

WOZNEY, J.M., ROSEN, V., CELESTE, A.J., MITSOCK, L.M., WHITTERS, M.J., KRIZ, R.W., HEWICK, R.M. and WANG, E.A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science 242:* 1528-1534.

YOUNG, M.F., IBARAKI, K., KERR, J.M., LYU, M.S. and KOZAC, C.A. (1994). Murine bone sialoprotein (BSP): cDNA cloning, mRNA expression, and genetic mapping. *Mammal. Genome 5:* 108-111.

Accepted for publication: June 1994