

Midkine (MK), a Heparin-binding Growth/Differentiation Factor, Is Regulated by Retinoic Acid and Epithelial–Mesenchymal Interactions in the Developing Mouse Tooth, and Affects Cell Proliferation and Morphogenesis

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Abstract. *Midkine (MK)* is the first cloned gene in a new family of heparin-binding growth/differentiation factors involved in the regulation of growth and differentiation. We have analyzed the expression of *MK* mRNA and protein during tooth development in mouse embryos and studied the regulation of *MK* expression and the biological effects of *MK* protein in organ cultures. *MK* expression was restricted and preferential in the tooth area as compared to the rest of the developing maxillary and mandibular processes suggesting specific functions for *MK* during tooth morphogenesis. *MK* mRNA and protein were expressed during all stages of tooth formation (initiation, morphogenesis, and cell differentiation), and shifts of expression were observed between the epithelial and mesenchymal tissue components. However, the expression of mRNA and protein showed marked differences at some stages suggesting paracrine functions for *MK*.

Tissue recombination experiments showed that *MK* gene and protein expression are regulated by epithelial–mesenchymal interactions, and, moreover, that dental tissue induces the ectopic expression of *MK* protein in non-dental tissue. The expression of *MK* gene and protein in the mandibular arch mesenchyme from the tooth region were stimulated by local application of retinoic acid in beads. Cell proliferation was inhibited in dental mesenchyme around the beads releasing *MK*, but this effect was modulated by simultaneous application of FGF-2. Morphogenesis and cell differentiation were inhibited in tooth germs cultured in the presence of neutralizing antibodies for *MK*, whereas the development of other organs (e.g., salivary gland, kidney) was unaffected. These results suggest important roles for *MK* in the molecular cascade that regulates tooth development.

MIDKINE (MK)¹ is a novel heparin-binding growth/differentiation factor encoded by a retinoic acid (RA) responsive gene (Kadomatsu et al., 1988; Tomomura et al., 1990). *MK* shares ~50% sequence homology with heparin-binding growth-associated molecule (HB-GAM) (Rauvala, 1989), also called pleiotrophin (PTN) (Li et al., 1990) or HBNF (heparin-binding neurite-promoting factor) (Böhlen and Kovesdi, 1991). The secreted forms of *MK* (13 kD) and *PTN* (15 kD) are unusually rich in cysteine residues and basic amino acids (Merenmies and

Rauvala, 1990; Tomomura et al., 1990), and display extensive sequence conservation between different species (Tsutsui et al., 1991; Muramatsu, 1994). Both *MK* and *PTN* are structurally unrelated to other heparin-binding factors, such as FGFs and hepatocyte growth factor (HGF) (Maruta et al., 1993; Muramatsu, 1994).

The biological functions of *MK* and *PTN* have remained largely contradictory. For example, both proteins stimulate neurite outgrowth from primary fetal rat neuron cultures (Rauvala, 1989; Böhlen and Kovesdi, 1991; Muramatsu and Muramatsu, 1991; Muramatsu et al., 1993), whereas controversy exists regarding the mitogenic effects of the proteins (Rauvala, 1989; Li et al., 1990; Muramatsu and Muramatsu, 1991; Maruta et al., 1993; Mitsiadis et al., 1995). Although the expression of *MK* increases at early stages of RA-induced differentiation of embryonal carcinoma cells (Kadomatsu et al., 1988), recent data have indicated that in several other cell lines RA is not a prerequisite for the induction of *MK* gene expression (Nurcombe et al., 1992). It is possible

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1. *Abbreviations used in this paper:* BMP, bone morphogenetic protein; ECM, extracellular matrix; HB-GAM, heparin-binding growth-associated molecule; HGF, hepatocyte growth factor; *MK*, midkine; NRS, normal rabbit serum; *PTN*, pleiotrophin; RA, retinoic acid; SF/HGF, scatter factor/hepatocyte growth factor.

that the contradictory effects of MK and PTN on cells are due to varying responses between cell types or, alternatively, to contamination by FGFs or other heparin-binding factors.

MK gene and protein are expressed in a variety of embryonic tissues (Kadomatsu et al., 1990; Muramatsu et al., 1993; Mitsiadis et al., 1995). Because of the preferential localization and unique distribution of MK protein in organs and tissues undergoing epithelial–mesenchymal interactions, we have suggested a role for MK in the regulation of organ development and cell differentiation (Mitsiadis et al., 1995).

The developing tooth of rodents represents a suitable model organ to examine the molecular mechanisms involved in inductive, morphogenetic, and cytodifferentiation phenomena. Tooth development is regulated by sequential and reciprocal interactions between the cranial neural crest-derived mesenchyme and the oral ectoderm (Thesleff and Hurmerinta, 1981; Ruch, 1987; Lumsden, 1988). The first morphological sign of tooth development is a local thickening of the stomodeal epithelium in the embryonic day 10–11 (E10–E11) mouse embryos. This tissue has been shown to have the capacity to regulate tooth formation (Mina and Kollar, 1987; Lumsden, 1988). By E13 the dental epithelium forms a bud, around which mesenchymal cells condense. At this and subsequent developmental stages (cap and bell stages), the odontogenic potential is localized in the mesenchyme (Kollar and Baird, 1969; Ruch, 1987). Cells of the dental papilla that directly underlie the epithelium differentiate into odontoblasts and secrete the organic matrix of dentin. Epithelial cells in close relation with the dental papilla mesenchyme differentiate into ameloblasts that produce the enamel matrix.

During recent years, evidence has accumulated that growth factors (Mitsiadis et al., 1992; Vainio et al., 1993), transcription factors (Dollé et al., 1992; Karavanova et al., 1992; Jowett et al., 1993), and extracellular matrix molecules (Thesleff et al., 1988; Vainio et al., 1989, 1991) are involved in tooth development (reviewed in Thesleff et al., 1990, 1991, 1995). Results from tissue recombination experiments have indicated that the expression of several genes, such as syndecan-1, tenascin, *Egr-1* (or *Krox-24*), *msx-1*, *msx-2*, and *BMP-4*, depends on epithelial–mesenchymal interactions in the developing tooth (Vainio et al., 1989, 1991, 1993; Karavanova et al., 1992; Jowett et al., 1993).

In this paper, we describe the expression patterns of mouse *MK* gene and the distribution of MK protein during tooth development. The involvement of epithelial–mesenchymal interactions in the expression of *MK* gene and protein in tooth was studied by tissue recombination experiments. The role of RA as signal mediating *MK* expression in the mandibular arch mesenchyme from the tooth region was examined by its local application in resin or agarose beads. The effects of MK on cell proliferation were studied by using the same model system. Finally, we analyzed the effects of neutralizing antibodies against MK protein on morphogenesis and cell differentiation in organ cultures of tooth germs.

Materials and Methods

Animals and Tissue Preparation

Fl(CBA × C57BL or CBA × NMRI) mice were used at embryonic and

postnatal stages. Embryonic age was determined according to the vaginal plug (day 0) and confirmed by morphological criteria. The heads from the embryonic day 10 (E10) mouse embryos to the postnatal day 8 (PN8) pups were dissected in Dulbecco's phosphate-buffered saline, pH 7.4. Mandibles and mandibular molar tooth germs were removed under a stereomicroscope, fixed overnight at 4°C with 4% paraformaldehyde (PFA) in PBS, pH 7.4. After dehydration, the tissues were embedded in paraffin wax, and serially sectioned at 5 μm on silanized slides, dried overnight, and stored in airtight boxes at 4°C.

Immunohistochemistry and In Situ Hybridization on Tissue Sections

Affinity-purified rabbit anti–mouse MK polyclonal antibody (Muramatsu et al., 1993), polyclonal antisera to the MK protein, and rat monoclonal antibody 281-2 against mouse syndecan-1 core protein (kindly provided by Dr. Markku Jalkanen, Centre for Biotechnology, Univ. of Turku, Finland; Jalkanen et al., 1985) were used. Immunohistochemistry with these antibodies was performed as previously described (Mitsiadis et al., 1995).

For in situ hybridization, [³⁵S]UTP-labeled single-stranded sense (pSP64) and antisense (pSP65) *MK* RNA probes were prepared. In situ hybridization on paraffin sections was performed as described earlier (Mitsiadis et al., 1995).

Tissue Recombination Experiments

The region where the molar tooth germ develops was carefully dissected from the rest of the mandible of E11–E12 mouse embryos. This region contains both cells with dental potential and non-dental cells. Similarly, the mandibular tooth region of E13–E14 mouse embryos was isolated. Although these dissected tissues may also include some non-dental cells, when recombined and cultured they gave rise to teeth and not to cartilage or bone. Therefore the E13–E14 tissues will be called dental epithelium and dental mesenchyme. The distal parts of the forming hind limb of E11.5 embryos and the diastemal oral region of the mandible (where teeth do not develop) of E13 embryos were also dissected in Dulbecco's PBS. After dissection, tissues were incubated for 3 min in 2.25% trypsin/0.75% pancreatin on ice. Epithelial and mesenchymal tissues were mechanically separated under a stereomicroscope. Isolated E11–E14 mandibular arch epithelia from the tooth region were placed in contact with isolated E11–E14 mandibular arch mesenchyme from the tooth region on polycarbonate membranes (pore size 0.1 mm; Nuclepore Corp., Pleasanton, CA) supported by metal grids (Trowell-type). The recombinants were cultured for one day in DMEM supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD) in a humidified atmosphere of 5% CO₂ in air at 37°C. Similarly, limb mesenchyme and mandibular arch epithelia from the tooth region of the same embryonic age (E11.5) were cultured for one day as recombinants. Diastemal oral epithelium (E13) was recombined with either E13 mandibular mesenchyme from the diastemal region or with E13 dental mesenchyme and the explants were cultured for 3–4 d. After culture the explants were fixed overnight in 4% PFA, and then treated with antibodies or *MK* riboprobe as whole mounts. Some explants were dehydrated in ethanol and embedded in paraffin wax. 5-μm serial sections were analyzed by immunohistochemistry.

Recombinant Proteins and Treatment of Beads

Recombinant MK protein (30 μg/ml) was stored at –70°C in 0.05 M Na-phosphate containing 1.0 M NaCl until use. Recombinant FGF-2 protein (10 μg/ml) was from Boehringer Mannheim Corp. (Indianapolis, IN). Recombinant BMP-2 protein (1.12 mg/ml) was a kind gift of Dr. E. Wang (Genetics Institute, Cambridge, MA). The protein was stored at –70°C in 0.5 mM arginine-HCl, 10 mM histidine (pH 5.6) until use.

Affi-gel blue agarose beads (100–200 mesh/75–150-μm diam; Bio Rad Labs., Hercules, CA) and heparin acrylic beads (100–200 mesh/100–250-μm diam; Sigma Chem. Co., St. Louis, MO) were used as carriers of MK, FGF-2, and BMP-2 protein (Vainio et al., 1993; Mitsiadis et al., 1995). Anion exchange resin beads (AG 1-X2, 100–200 mesh/106–205-μm diam; Bio Rad Labs.) and agarose beads were used as carriers of retinoic acid (RA). Beads were washed once with PBS and pelleted. Recombinant proteins were diluted into PBS, pH 7.4, to concentrations 5–250 ng/μl/5 μl/50 beads (FGF-2, 100–250; MK, 5–150; BMP-2, 250) and incubated for 40 min at room temperature. Beads used as carriers for RA were washed for 5 min with dimethyl-sulfoxide (DMSO; Merck Sharp & Dohme/Isotopes, Dorval, Canada). RA was diluted into DMSO to concentrations 5–100

ng/ μ l/500 μ l/50 beads and incubated for 30 min at room temperature. Beads were washed for 5–15 min in culture media and then transferred with a mouth-controlled capillary pipette on top of the explants. Control beads for MK were treated identically with 0.1% BSA in PBS, whereas DMSO beads were used as controls for RA.

Whole-mount In Situ Hybridization of Explants

For whole-mount in situ hybridization the explants were fixed in 100% methanol, cooled to -20°C , for 5 min, rehydrated with 75, 50, and 25% methanol in PBS (MeOH), pH 7.4, washed with PBS, and fixed overnight at 4°C with fresh 4% PFA. Tissues were then washed with PBS and processed for in situ hybridization. Whole mount in situ hybridization was performed according to Vainio et al. (1993).

Bromodeoxyuridine (BrdU) Labeling and Immunohistochemistry of the Explants as Whole Mounts

Cell proliferation was analyzed by using cell proliferation kits (Amersham, Boehringer Mannheim). After culture, the explants were labeled for 1 h with BrdU according to manufacturer's instructions and as described earlier (Mitsiadis et al., 1995). They were fixed in 4% PFA overnight at 4°C , treated to inhibit endogenous peroxidase with 3% H_2O_2 /PBS for 30 min at room temperature, washed in PBS, and used immediately for staining. Whole mount immunohistochemistry with antibodies against MK, syndecan-1 and BrdU was performed as earlier described (Vainio et al., 1993; Mitsiadis et al., 1995). When the color reaction was satisfactory, the explants were washed in tap water and mounted in Aquamount (BDH, Gurr, England).

Tooth Germ Organ Cultures

Molar tooth germs were carefully dissected from the mandibles of E13 and E16 mouse embryos. Developing salivary glands and kidneys were dissected from E12 embryos. The organs were cultured in Trowell-type cultures, and the basic culture medium (DMEM and 10% FCS) was supplemented with neutralizing antibodies against the MK protein (40 $\mu\text{g}/\text{ml}$). The neutralizing ability of these antibodies has been demonstrated either in neuronal cultures where they inhibited the neurite outgrowth evoked by MK, or by inhibition of the growth of Wilm's tumor cells, which secrete large amounts of MK (Muramatsu et al., 1993). In control cultures, normal rabbit serum was added to the medium in an amount identical to the anti-MK antibody. The organs were cultured for 6–7 d with three changes of fresh medium. After photography, they were fixed in fresh 4% PFA overnight, rinsed twice with PBS, dehydrated in ethanol, and embedded in paraffin. Immunohistochemistry was performed in 5- μm serial sections by omitting the primary antibody (anti-MK) and sections were counterstained with hematoxylin.

Results

MK Gene Expression in the Tooth Germ Is Developmentally Regulated

In situ hybridization analysis showed a ubiquitous expression of MK transcripts in both oral epithelium and mesenchyme of the maxillary and mandibular processes of E10–E12 mouse embryos. No specific hybridization signal was detected with sense probes at this or subsequent developmental stages (data not shown). In the thickened oral epithelium, which represents the initiation of tooth development at E11, MK mRNA expression was downregulated in the basal cell layer (Fig. 1 a).

At E13, the dental epithelium forms a bud, around which the neural crest-derived mesenchymal cells condense. MK gene expression was weak in the dental epithelium and strong in the condensing mesenchyme (Fig. 1 b). MK transcripts were also detected in non-dental epithelium and in mesenchyme of the forming bone.

During cap stage of molar morphogenesis (E14–E15), MK gene expression became progressively restricted to the tooth

germ. It was strongly expressed in mesenchymal cells forming the dental papilla and weakly in dental epithelium (Figs. 1 c and 3 a). Transcripts were also detected in osteogenic areas of the mandible.

By E16 the dental epithelium—also called enamel organ—acquires the bell configuration. Strong hybridization signal for MK was detected in mesenchymal cells of the dental papilla and of the dental follicle (surrounding the enamel organ). The expression of MK was moderate in the enamel organ, except in some cells of the inner enamel epithelium (Fig. 1 d). At E18–E19 (late bell stage), MK gene expression was upregulated in the mesenchymal cells located at the tip of the cusps which differentiate into odontoblasts. The hybridization signal persisted in the dental papilla, whereas a weak signal was detected in dental epithelium (stratum intermedium and preameloblasts) (Fig. 1 e).

At PN8, the morphology of the crown of the molar is stabilized by the deposition of minerals in the dentin and enamel matrices. MK expression had been downregulated in the dental papilla mesenchyme except in the basal part of the developing tooth, where the roots start to form (Fig. 1 f).

Distribution of MK Protein Does Not Entirely Correlate with mRNA Expression

At E10–E11, MK immunoreactivity was detected in the oral epithelium of the maxillary and mandibular processes. The staining was found on cell surfaces and in the basement membrane. In the thickened dental epithelium, reactivity was mostly observed in its anterior part. In the mesenchyme, the MK staining was very faint and punctuated (Fig. 2 a). Hence, the patterns of MK distribution did not correspond to that of MK mRNA which was expressed both in mesenchyme and epithelium. By E12, MK immunoreactivity was detected also on the surfaces of condensing mesenchymal cells in close contact with dental epithelium (data not shown), whereas the staining in more peripheral mesenchymal cells remained punctuated.

During bud and early cap stages of molar development (E13 and E14, respectively), MK staining was particularly strong in the developing tooth as compared to the rest of the mandible. MK was detected on the surfaces of dental epithelial cells and of mesenchymal cells forming the dental papilla, as well as in the basement membrane (Fig. 2, b and c). Faint MK staining was found in osteogenic and chondrogenic areas of the mandible, whereas the majority of the tissues were negative for MK (Fig. 2 c). The pattern of MK protein distribution in the non-dental mesenchyme of the mandible did not correspond with that of mRNA expression, which, unlike the protein, was intense in this tissue.

At E15 (late cap stage), the distribution pattern of MK protein changed. Staining was seen only on the surfaces of cells of the inner dental epithelium and in the basement membrane separating these cells from the dental papilla (Fig. 3 b), and this pattern persisted in the epithelium during the early bell stage (E16). In the dental papilla, only cells underlying the inner dental epithelium exhibited MK reactivity. During bell stage, cell surface immunoreactivity appeared in mesenchymal cells located in the areas of the forming cusps, whereas the staining was punctuated in the rest of the dental papilla (Fig. 2 d). Hence, during the late cap/early bell stage, the expression of MK mRNA and protein were oppo-

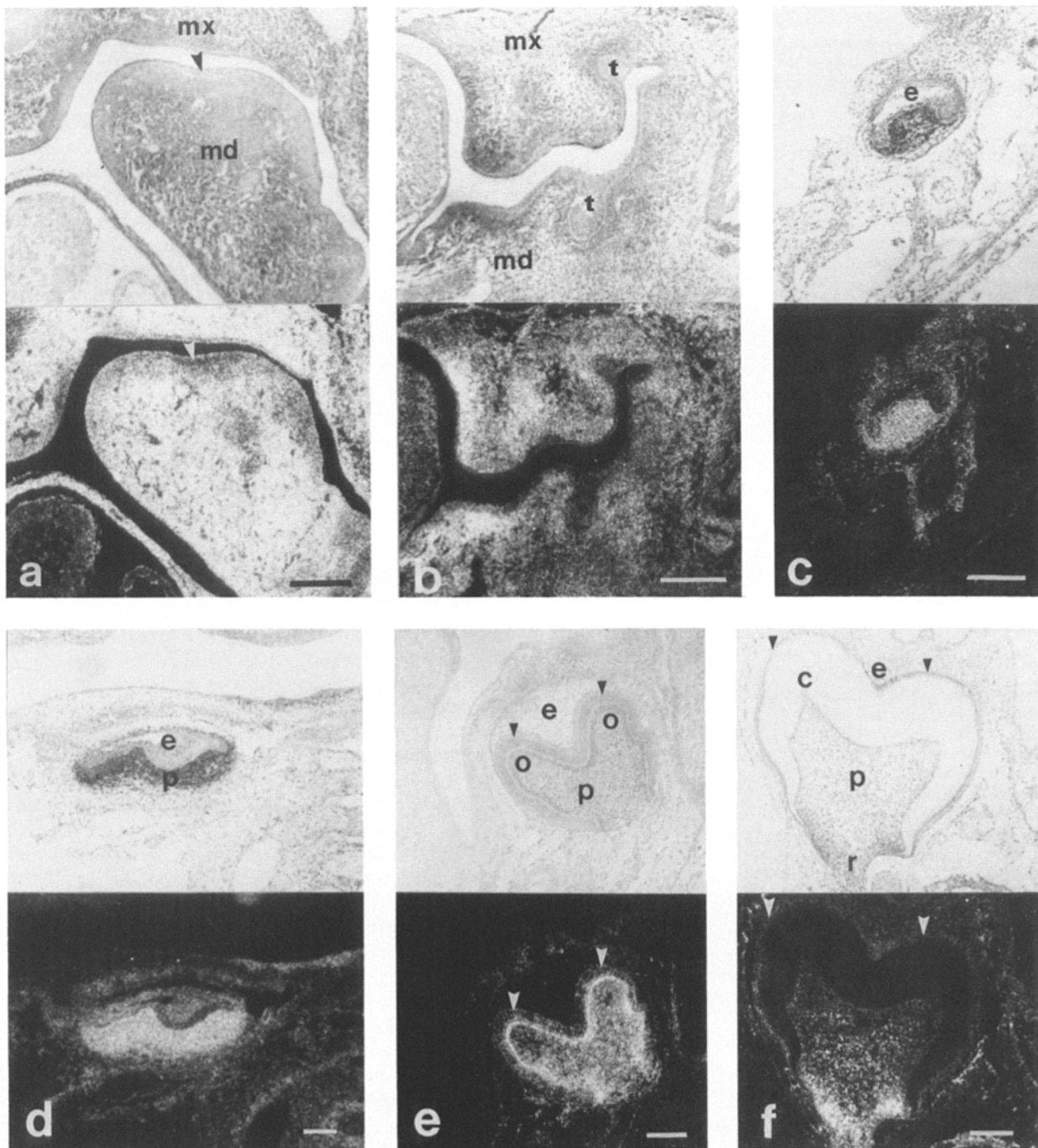


Figure 1. Localization of *MK* mRNA by in situ hybridization in embryonic and postnatal mouse mandibular and maxillary processes and tooth germs (E11-PN8). Bright and dark-field micrographs. (a) *MK* transcripts are detected throughout the epithelium and mesenchyme of the mandibular and maxillary processes, but they are downregulated in the basal layer of the thickened dental epithelium at E11 (arrow). (b) *MK* gene is expressed throughout the mandibular and maxillary processes at E13. Strong hybridization signal is observed in the mesenchyme of bud staged molar tooth germs, whereas in dental epithelium the signal is faint. (c) *MK* mRNA expression has been progressively restricted to dental mesenchymal cells at cap stage (E15). (d) *MK* gene is expressed in the mesenchyme and dental epithelium at the early bell stage (E16). The signal is absent from several cells of the inner dental epithelium. (e) At E19 (late bell stage), *MK* mRNA expression is evident in polarizing odontoblasts and dental papilla mesenchyme. Weak signal is also present in the enamel epithelium (preameloblasts; arrowheads). (f) *MK* mRNA expression is downregulated in cusps, but persists in the lower part of the dental papilla mesenchyme during root formation (PN8). Gene expression is lost from ameloblasts during advancing differentiation (arrowheads). *c*, cusp; *e*, enamel epithelium; *md*, mandibular process; *mx*, maxillary process; *p*, dental papilla mesenchyme; *o*, odontoblasts; *r*, forming root area; *t*, tooth bud. Bars, 200 μ m.

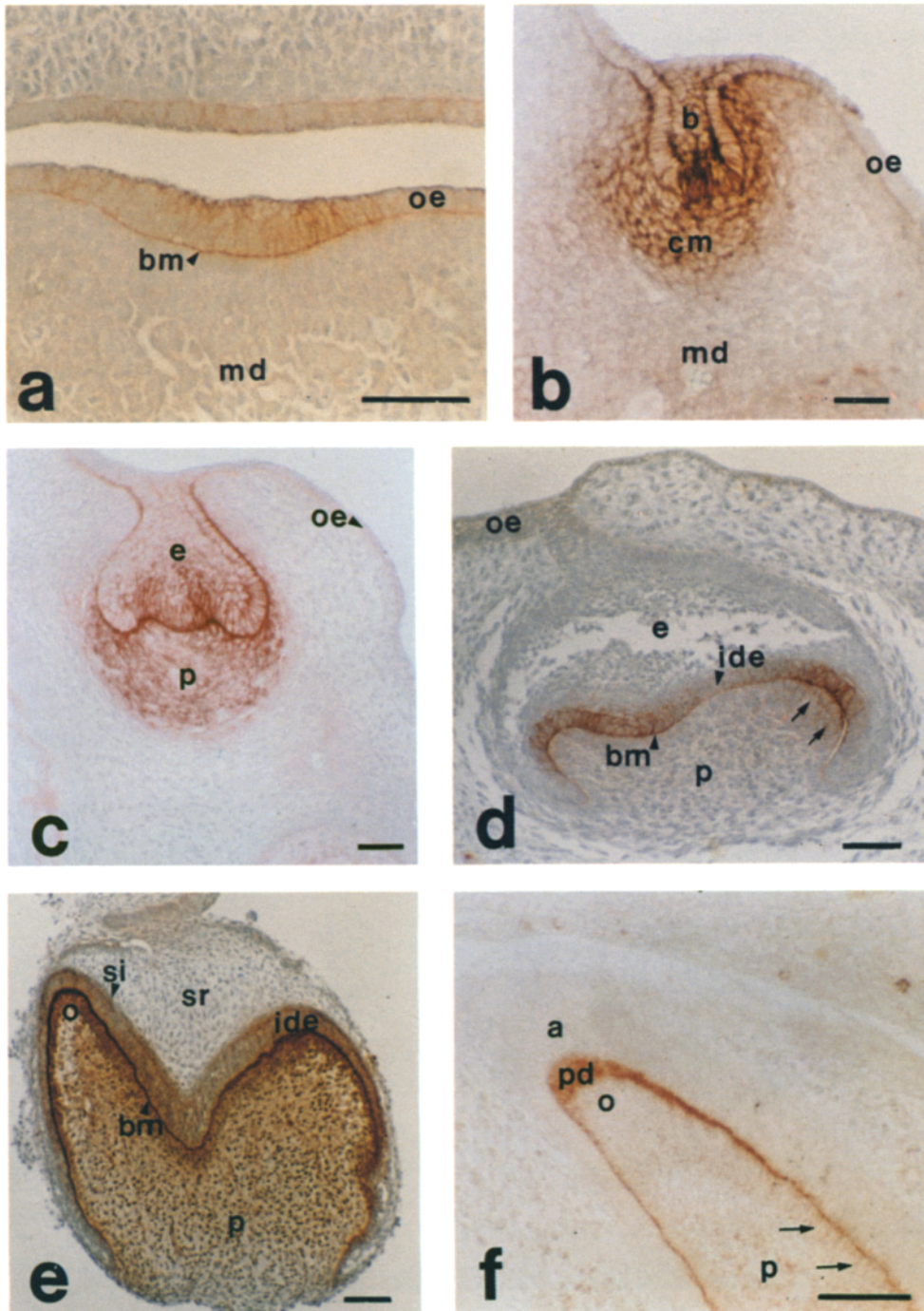


Figure 2. MK protein distribution in the developing first molar of the mouse. (a) MK protein is localized in the thickened epithelium of the mandibular process of an E11 embryo. Intense staining is localized on the cell surfaces of epithelial cells and in the basement membrane, whereas in mesenchyme the staining is faint and punctuated. (b) At bud stage (E13), MK protein is expressed on the cell surfaces of both dental epithelial bud and condensed dental mesenchyme. (c) Early cap stage at E14. MK protein distribution persists in both dental epithelium and mesenchyme. The surrounding tissues of the mandible are negative. (d) Early bell stage at E16. MK protein is localized on the surfaces of cells of the inner dental epithelium, in the basement membrane, and in mesenchymal cells underlying the enamel epithelium in the cuspal area (arrowheads). In dental papilla, protein expression was decreased considerably. (e) Bell stage at E18. MK protein is localized in inner dental epithelium, dental papilla mesenchyme and basement membrane. Note the positive staining in the nuclei of cells of the dental papilla mesenchyme. (f) Higher magnification of a cuspal area at PN1 shows that MK protein expression is lost from odontoblasts after terminal differentiation. The protein is localized on the surfaces of differentiating odontoblasts (arrows). MK is also found in the predentin matrix. *a*, ameloblasts; *b*, dental epithelial bud; *bm*, basement membrane; *cm*, condensed mesenchyme; *e*, enamel epithelium;

ide, inner dental epithelium; *md*, mandibular process; *oe*, oral epithelium; *o*, odontoblasts; *p*, dental papilla mesenchyme; *pd*, predentin; *si*, stratum intermedium; *sr*, stellate reticulum. Bars, 50 μ m.

site; *MK* transcripts were found in dental mesenchyme, whereas the protein was mainly detected in the epithelium.

During the advanced bell stage (E18-E19), MK protein was localized in both dental epithelium and mesenchyme (dental pulp). In epithelium, MK immunoreactivity was easily observed in inner dental epithelium (preameloblasts), whereas the staining was scarcely detectable in other cell populations of the enamel organ. In mesenchyme, MK staining was detected in the processes of the differentiating odontoblasts. Interestingly, an intranuclear staining was observed

in odontoblasts and pulpal fibroblasts. The basement membrane separating preameloblasts from the dental pulp was also stained (Fig. 2 e). At this stage of tooth development, the pattern of MK protein distribution was quite similar to that of *MK* gene expression.

When the deposition of predentin matrix started in the newborn mouse (PN0-PN1), MK staining was abolished from dental epithelium and functional odontoblasts. The staining persisted in the center of the dental pulp, and the predentin was positive for MK (Fig. 2 f). At PN8, MK im-

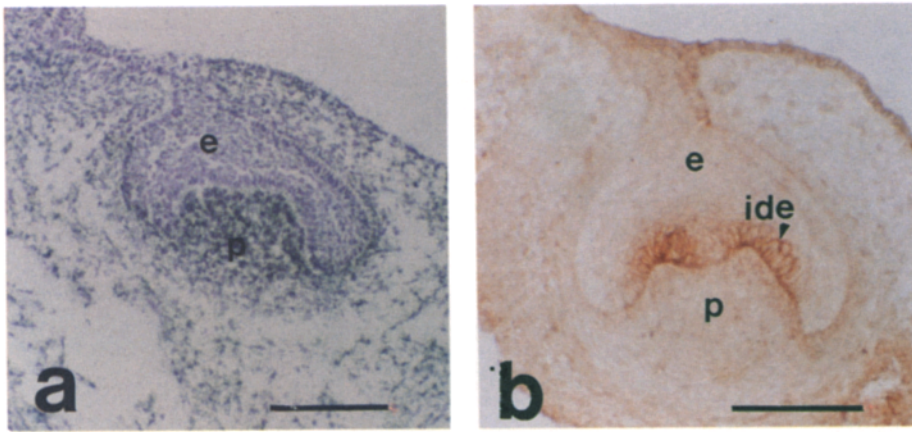


Figure 3. Comparison between *MK* mRNA expression and protein distribution in the first molar tooth of an E15 mouse embryo (cap stage). (a) *MK* transcripts are expressed in dental papilla mesenchyme. (b) The distribution of *MK* protein does not correspond to the pattern of gene expression. Strong staining is localized on the surfaces of cells of the inner dental epithelium, whereas in dental mesenchyme the staining is faint. Abbreviations: *e*, enamel epithelium; *ide*, inner dental epithelium; *p*, dental papilla mesenchyme. Bars, 200 μ m.

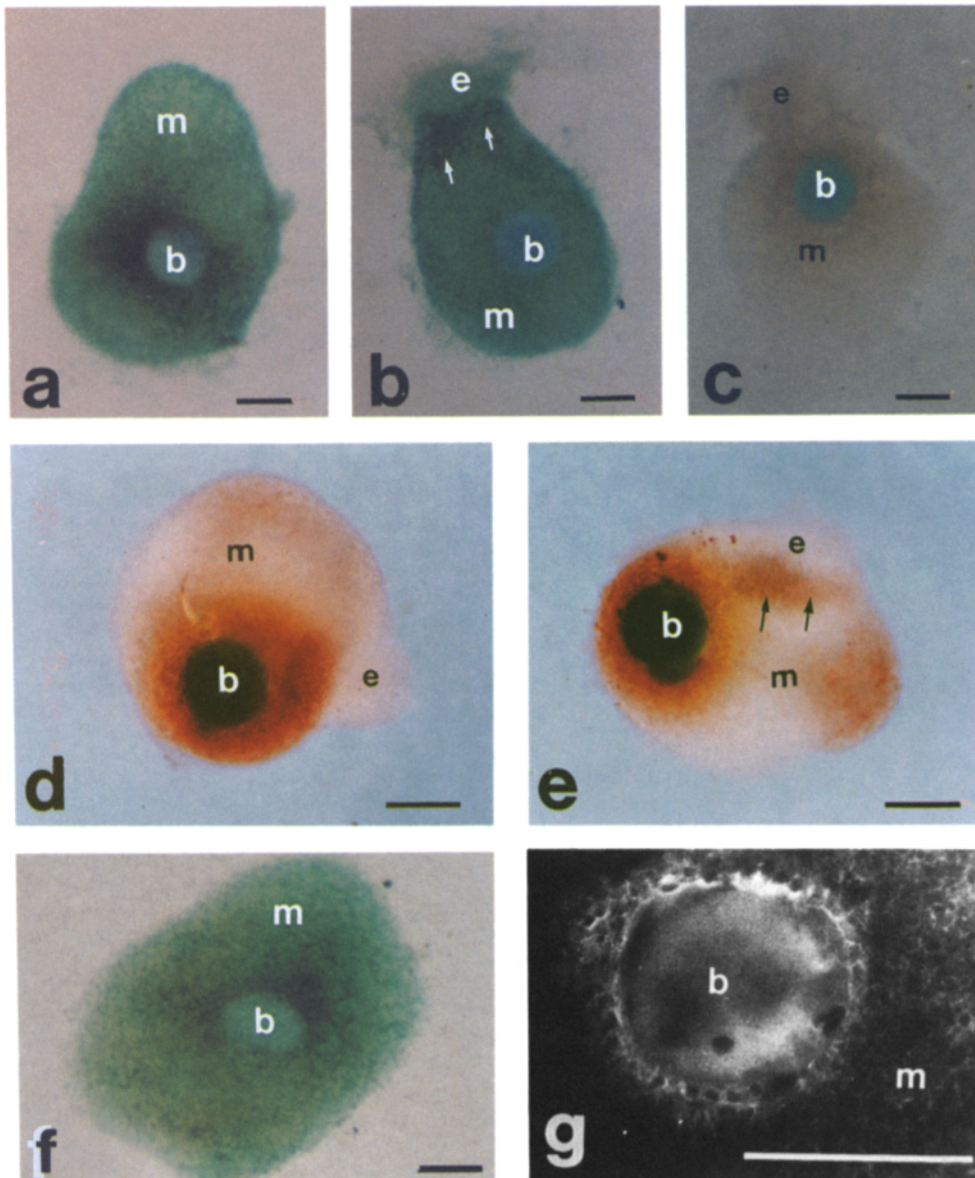


Figure 4. Stimulation of *MK* gene and protein expression in mandibular mesenchyme from the tooth region by RA. The mesenchyme (E11-E12) was cultured for 24–36 h in contact with mandibular arch epithelium from the dental region and beads soaked either in RA (5–100 ng/ μ l) (a and c–e) or DMSO (b). *MK* gene and protein expression was monitored by whole mount in situ hybridization and whole mount immunohistochemistry, respectively. (a) *MK* gene expression in mesenchyme (E12) is intense around the RA releasing bead. (b) DMSO bead has not affected *MK* gene expression, whereas epithelium has stimulated the expression of *MK* transcripts in the adjacent E12 mesenchyme (arrowheads). (c) RA bead. *MK* sense probe as control. (d) RA bead has induced *MK* protein expression in the E11 mesenchyme. The recombinant epithelium is negative and has not yet induced *MK* protein expression in the adjacent mesenchyme. (e) RA bead has stimulated *MK* protein expression in the E12 mesenchyme. At this stage, the epithelium induces the expression of *MK* protein in the mesenchyme (arrowheads). The positive staining in the periphery of the explant represents a site of cartilage formation. (f) Up-regulation of *MK* gene expression in mesenchymal cells (E12) surrounding a *MK* releasing bead. (g) Distribution of the *MK* protein on the surfaces of cells surrounding a heparin acrylic *MK* bead. *b*, bead; *e*, epithelium; *m*, mesenchyme. Bars, 100 μ m.

munoreactivity was absent from all dental cells in the crown region, whereas staining was found in mesenchymal cells located in the area of the forming root, a pattern corresponding to that of *MK* gene expression.

MK mRNA Expression and MK Protein Distribution in Developing Tooth Are Regulated by Interactions between Epithelium and Mesenchyme

The *in vivo* patterns of *MK* gene and protein expression in developing tooth appeared to be associated with tissue interactions. As these patterns were not identical, we analyzed separately the expression of mRNA and protein in recombination cultures of mandibular arch epithelia and mesenchymes from the tooth region. Because *MK* gene expression in mandibular and maxillary mesenchyme became progressively restricted to dental mesenchyme after E12, we analyzed the effect of E11-E12 mandibular epithelium from the tooth region on the expression of *MK* in recombined mandibular mesenchyme from the tooth region after 24 h of culture. *MK* transcripts were detected by whole mount *in situ* hybridization throughout the mesenchyme, but the signal was stronger in the mesenchyme adjacent to the epithelium (Fig. 4 *b*).

The expression of the *MK* protein, as observed by whole mount immunostaining in the recombinants, was dependent on the developmental stage of the tissues. Staining was observed on the surfaces of mesenchymal cells adjacent to the epithelium in recombinants of E11.5-E12 mandibular tissues from the tooth region cultured for 24 h (Figs. 4 *e* and 5 *a*). In contrast, in the E11 recombinants cultured for 24 h, as well as in the E11.5 recombinants cultured for a shorter period (16 h), only a weak staining was detected in mesenchyme and was mainly associated with the forming basement membrane. These patterns of *MK* protein distribution in recombinants corresponded to those observed *in vivo* (E12-E13).

The distribution of *MK* protein in heterotypic recombinants was also studied. Tissue recombination data suggested that the early dental epithelium has the capacity to regulate tooth morphogenesis when cultured with non-odontogenic mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). To study the potential of dental epithelium to regulate *MK* expression in a mesenchyme already positive for *MK*, E11.5 limb mesenchyme was recombined with E11.5 mandibular epithelium from the tooth region and cultured for 24 h. *MK* staining was found in the forming basement membrane separating the two tissues, whereas in the mesenchyme adjacent to epithelium the staining was completely suppressed (Fig. 5 *b*).

Previous data from tissue recombination studies have shown that the odontogenic potential shifts from the dental epithelium to mesenchyme by E13 and resides in this tissue until E17 (Kollar and Baird, 1969; Ruch, 1987). Between E15 and E17 *in vivo*, the *MK* protein was abundant in inner dental epithelium but not present in other dental or oral epithelia. We asked if dental mesenchyme could regulate the expression of *MK* protein in an epithelium otherwise negative for *MK*. E13 *MK*-negative oral epithelium from the diastemal region was cultured with E13 dental mesenchyme for 3–4 d *in vitro*. After culture, the epithelium had invaded

the dental mesenchyme and it had acquired a cap-like configuration. *MK* immunoreactivity was found on the surfaces of epithelial cells which were in close contact with the mesenchyme, thus resembling the *in vivo* distribution of *MK* protein in the tooth germ at E15 (Fig. 5 *c*). In recombinants of oral epithelium and mesenchyme from the diastemal region we did not observe a cap-like configuration, and the *MK* staining was absent from these explants (data not shown).

Retinoic Acid Induces MK mRNA and Protein Expression in Early Dental Mesenchyme

RA regulates the expression of genes responsible for pattern formation and morphogenesis (Tabin, 1991). We studied whether RA could regulate the expression of *MK* in cultured E11-E12 mandibular mesenchyme from the tooth region. Resin and agarose beads soaked in 5–100 ng/ μ l RA were placed on mesenchyme and cultured for 24–36 h, and expression of *MK* mRNA and protein were analyzed by whole-mount *in situ* hybridization and immunohistochemistry, respectively. The hybridization signal was moderate throughout the explant, but RA increased the expression of *MK* transcripts in mesenchyme adjacent to the beads (Fig. 4 *a*). Similarly RA induced *MK* protein expression in mesenchymal cells adjacent to the beads (Fig. 4, *d* and *e*). Control beads soaked in DMSO had no effect on *MK* gene expression (Fig. 4 *b*), and explants hybridized with the sense riboprobe were completely negative (Fig. 4 *c*).

The observed shifts in the expression of *MK* between epithelium and mesenchyme in the forming tooth suggest that *MK* might have autoregulatory functions. This hypothesis was tested by application of *MK* protein in beads on E12 mandibular arch mesenchyme from the tooth region and by analyzing the expression of *MK* mRNA after 24 h of culture. The expression of *MK* transcripts was increased in mesenchyme adjacent to the beads (Fig. 4 *f*). Similarly, *MK* staining was detected on the surface of cells surrounding heparin acrylic *MK* beads (Fig. 4 *g*).

MK and RA Modify the Response of Mesenchyme to Epithelium

When E11-E12 mandibular arch epithelium from the tooth region was cultured with mandibular mesenchyme from the tooth region and limb mesenchyme for 24 h as recombinants, a translucent zone appeared in mesenchyme adjacent to the epithelium (Figs. 6 and 7, *a-c*). The formation of this zone has been reported in earlier studies and is believed to be an indicator of induction of mesenchyme by signals derived from the presumptive dental epithelium (Vainio et al., 1993). It has been shown that proliferative events are not always related with the formation of a translucent zone (Vainio et al., 1993). Agarose or heparin acrylic beads soaked in different concentrations of *MK* protein (5–150 ng/ μ l/5 μ l/100 beads) and resin beads soaked in RA (5–100 ng/ μ l/500 μ l/50 beads) never induced the appearance of a translucent zone in E11-E12 mandibular mesenchyme from the tooth area and limb mesenchyme during 24 h of culture (Figs. 6 and 7, *a-c*). In contrast, local application of BMP-2 protein (250 ng/ μ l/5 μ l/100 beads) induced a translucent zone in E11-E12 mandibular mesenchyme from the tooth area (Figs. 6 and 7 *d*),

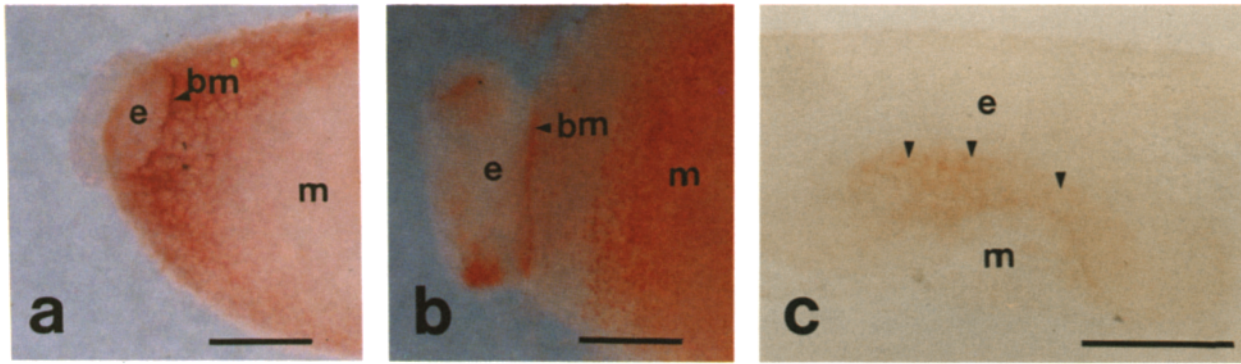
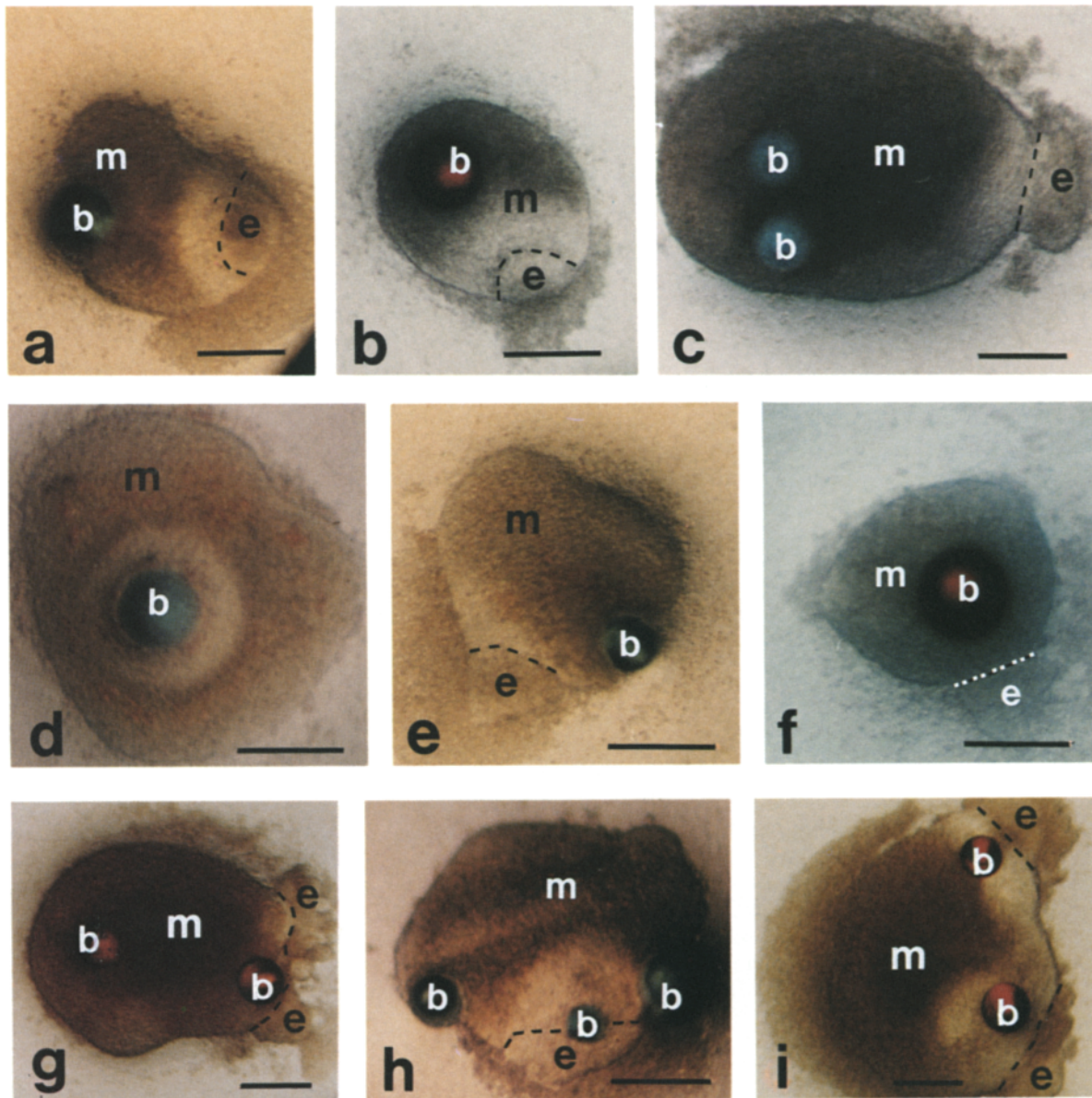


Figure 5. Localization of MK protein expression by whole mount immunohistochemistry in explants of recombined mandibular arch epithelium and mesenchyme from the tooth region (*a*), mandibular arch epithelium from the dental area and limb mesenchyme (*b*), oral (distal) epithelium and dental mesenchyme (*c*). The explants were cultured for 24 h (*a* and *b*) and 3 d (*c*). (*a*) MK protein is localized on the surfaces of mesenchymal cells adjacent to the epithelium and in basement membrane in the E11.5 recombinants. MK staining is absent from other mesenchymal cells. (*b*) MK staining is found in the forming basement membrane separating the two recombined tissues (E11.5). The punctuated MK staining observed in the limb mesenchyme is suppressed in the area adjacent to epithelium. (*c*) MK protein is localized on the surfaces of oral epithelial cells (E13) that are in close contact with the recombined dental mesenchyme (E13; *arrowheads*). *bm*, basement membrane; *e*, epithelium; *m*, mesenchyme. Bars, 100 μ m.



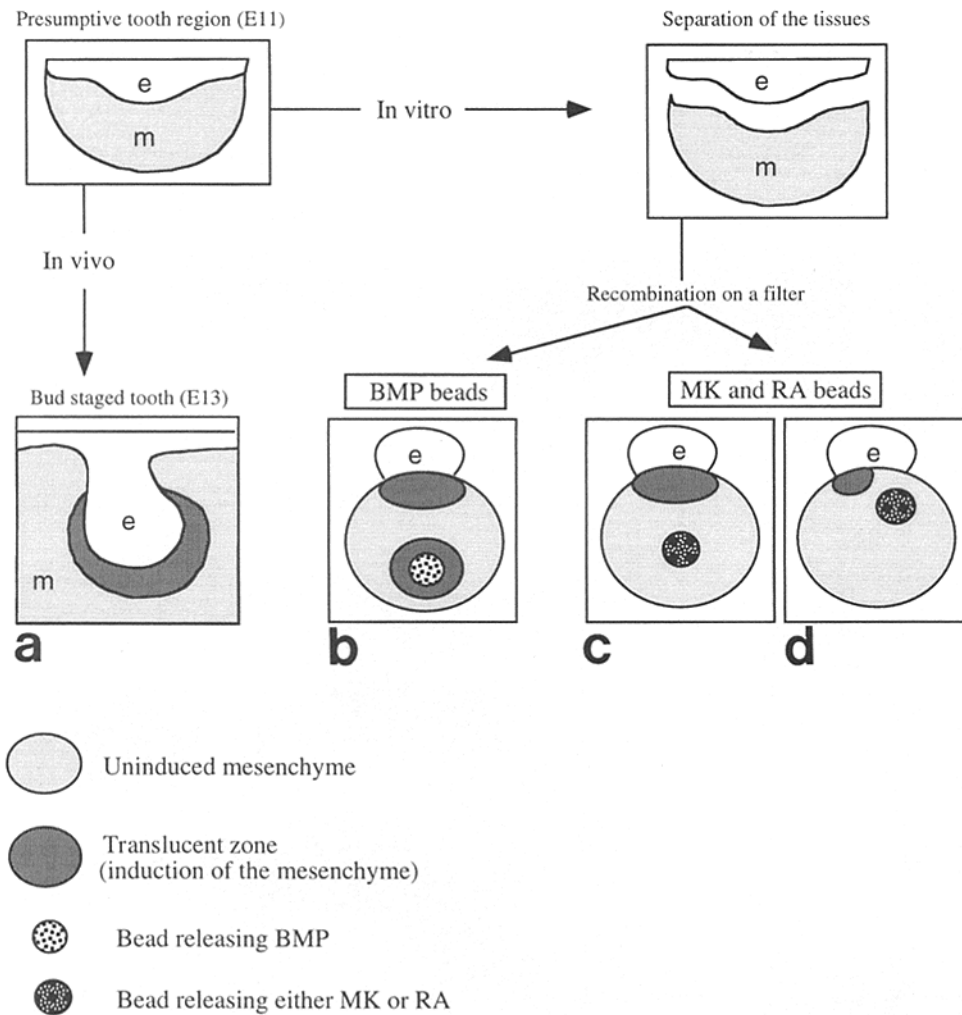


Figure 6. The design of experiments used to analyze tissue interactions during early tooth development and the signaling roles of MK, RA, and BMP. (a) In vivo several genes are activated in the dental mesenchyme which condense around the growing epithelium (Thesleff et al., 1995). (b) In vitro the epithelium and mesenchyme are separated and cultured in recombination on a filter. The epithelium induces in the adjacent mesenchyme a translucent zone (Vainio et al., 1993), in which the same genes are expressed as in the condensed mesenchyme in vivo. Agarose beads releasing BMP also induce a translucent zone. (c) Agarose or heparin acrylic beads releasing MK and resin beads releasing RA do not induce the appearance of a translucent zone. (d) Beads releasing MK or RA cause a narrowing or complete suppression of the translucent zone when placed on mesenchyme adjacent to epithelium. e, epithelium; m, mesenchyme.

as earlier reported (Vainio et al., 1993). In recombinants, both MK and RA beads caused a narrowing or complete suppression of the translucent zone when placed on mesenchyme adjacent to the mandibular arch epithelium from the tooth region (Figs. 6 and 7, e-g). Control beads incubated in BSA and DMSO had no effect on this zone (Fig. 7, h and i). These results suggest that MK may act as a competence modifier controlling the response of mesenchyme to epithelial induction.

MK Affects Cell Proliferation but Not Syndecan-1 Expression in Dental Mesenchyme

The effects of MK on cell proliferation were analyzed by placing agarose or heparin acrylic beads soaked in MK (5-150 ng/ μ l) on E11-E14 mandibular mesenchyme from the

tooth region and by labeling the explants with BrdU after 24 h of culture. In E11-E12 recombinants, the mandibular arch epithelium from the tooth area stimulated cell proliferation in adjacent mesenchyme, whereas MK and resin RA (50-100 ng/ μ l) beads had no detectable effects (Fig. 8, a, d, and e). This is in line with our earlier observations on effects of MK on E11-E12 mandibular and limb mesenchyme (Mitsiadis et al., 1995). In the recombinants of E13-E14 dental tissues, BrdU-positive mesenchymal cells were markedly more abundant than in those of E11-E12 explants. When MK beads (30-90 ng/ μ l) were applied to E13-E14 dental mesenchyme, the proliferation of cells was inhibited in a large zone around the beads (Fig. 8, b and c). However, cell proliferation induced by epithelium was not decreased by the application of MK beads in mesenchyme adjacent to the epithelium (data not shown).

Figure 7. Effect of MK, RA and BMP-2 beads on dental and limb mesenchyme. Mandibular mesenchyme from the tooth region (a, b, d-f, and h) and limb mesenchyme (c, g, and i) was cultured in recombination with mandibular arch epithelium from the dental region (E11-E12) and with beads soaked in different concentrations of MK (5-150 ng/ μ l) (a, c, e, and g), RA (5-100 ng/ μ l) (b and f), and BMP-2 (250 ng/ μ l) (d) for 16-32 h. MK and RA beads have not induced a translucent zone like the epithelium (a-c) and BMP-2 (d). In contrast, MK and RA beads placed on mandibular mesenchyme from the tooth region (e and f) and limb mesenchyme (g) adjacent to epithelium have caused a reduction in the extent of the translucent zone. The translucent zone is not affected by control beads (BSA and DMSO) placed near the epithelium (h and i). b, bead; e, epithelium; m, mesenchyme. Bars, 200 μ m.

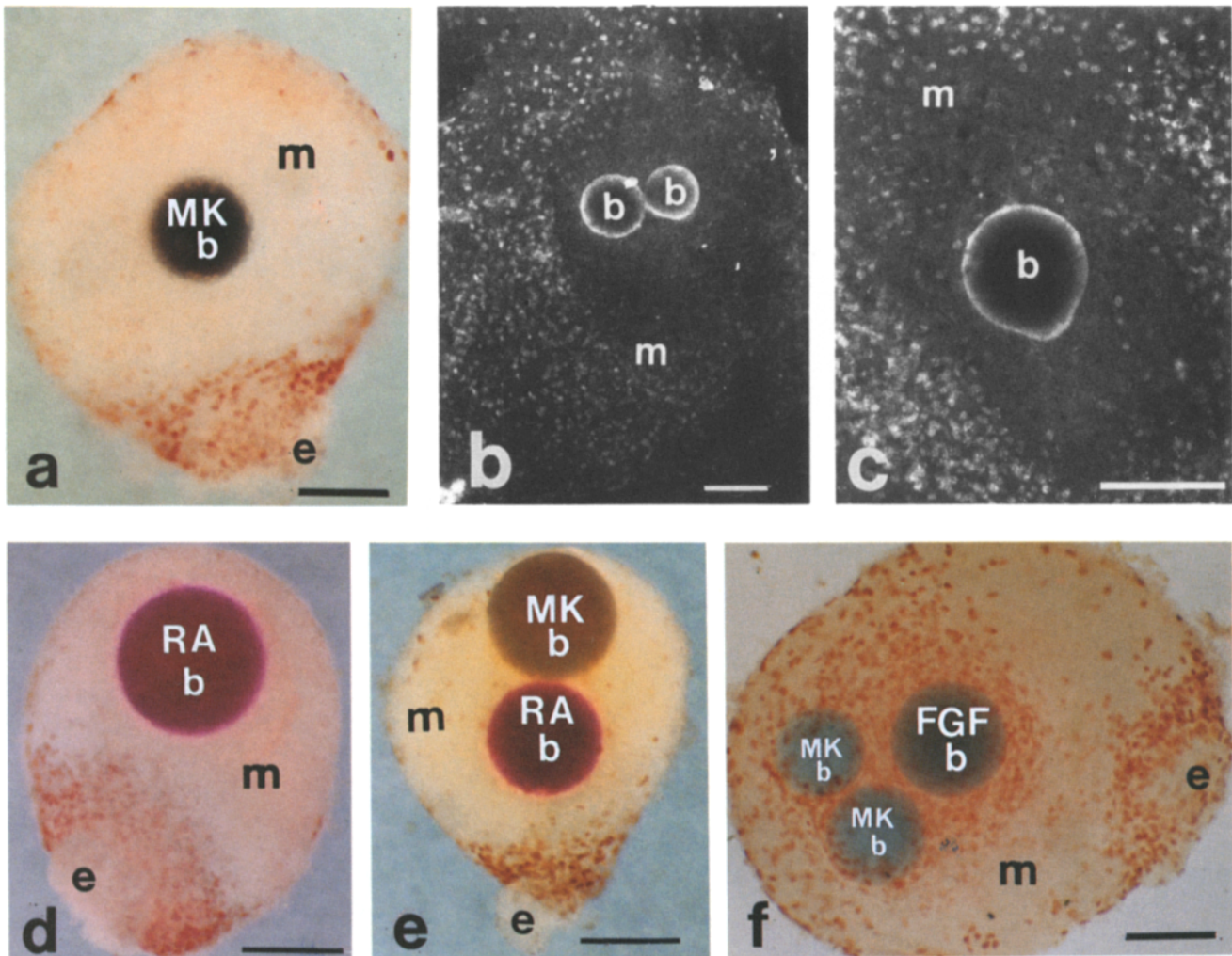


Figure 8. Effects of MK, RA, and FGF-2 on cell proliferation in mandibular mesenchyme from the tooth region. Cells in S phase were labeled by BrdU, and the proliferative cells were detected by immunostaining of the explants as whole mounts. (a) The mesenchyme (E11) was cultured for 24 h with recombined mandibular arch epithelium from the dental area and with agarose beads soaked in MK (5–150 ng/ μ l). The epithelium has induced cell proliferation in mesenchyme, but no effect can be seen around the beads. (b) MK (90 ng/ μ l) has inhibited the proliferation of cells in the E13 dental mesenchyme. (c) Inhibition of cell proliferation by MK (100 ng/ μ l) in the E14 dental mesenchyme. (d) RA (5–100 ng/ μ l) has not affected cell proliferation in E11 mesenchyme. (e) Heparin acrylic beads soaked in MK (5–150 ng/ μ l) and resin beads soaked in RA (50 ng/ μ l) have not affected cell proliferation in E12 mesenchyme. (f) FGF-2 (100–250 ng/ μ l) has induced cell division in dental mesenchyme (E12–E13). Cell proliferation can be seen also around MK beads, which were placed near FGF-2 beads. *b*, bead; *e*, epithelium; *m*, mesenchyme. Bars, 100 μ m.

Because of the controversial effects of MK beads on proliferation, we analyzed the possibility that other growth factors may modify the response. Increased cell division was evident in mesenchyme in a large area around beads releasing FGF-2 (100–250 ng/ μ l). Cell proliferation was increased also around beads releasing MK when they were placed near FGF-2 beads (Fig. 8 *f*). Control beads soaked in BSA had no influence on cell proliferation (data not shown).

Syndecan-1 expression in E11–E12 mandibular mesenchyme from the tooth region was not stimulated by MK protein released from agarose beads. The mandibular arch epithelium from the dental area induced the expression of syndecan-1 in the contacting mesenchyme (data not shown), as earlier reported (Vainio et al., 1989). Similar observations were earlier made in recombinants of mandibular and limb epithelium and mesenchyme (Mitsiadis et al., 1995).

Neutralizing Antibodies to MK Inhibit Tooth Morphogenesis and Differentiation

At the onset of culture, the mandibular first molars of E13 and E16 mouse embryos were at the bud and early bell stage, respectively. After culture for several days (7 d for the E13 tooth germs and 4–5 d for the E16 tooth germs) in control medium they had undergone cuspal morphogenesis. Histological examination indicated that polarized odontoblasts had appeared in the cusps (Fig. 9, *a* and *c*). Addition of neutralizing antibodies to MK (40 μ g/ml) caused the inhibition of morphogenesis and cytodifferentiation of the E16 tooth germs (Fig. 9, *b* and *d*). A more dramatic effect of the anti-MK antibodies was observed in several of the cultured E13 tooth germs, which lost completely their dental morphology (data not shown).

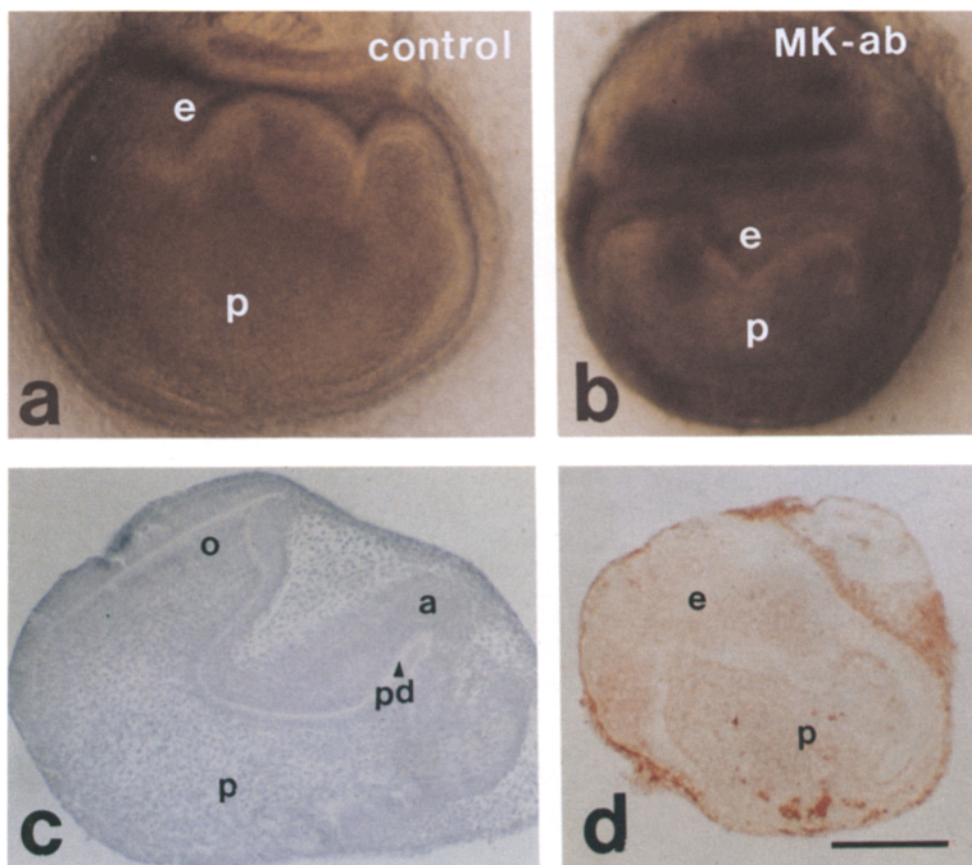


Figure 9. Inhibitory effects of the neutralizing antibodies against the MK protein on tooth morphogenesis and differentiation. Molar germs from E16 mouse embryos were cultured for 4–5 d. (a) In the control medium morphogenesis of the lower first molar has advanced and the cuspal pattern is evident. (b) In the medium supplemented with 40 µg/ml of anti-MK antibody morphogenesis has been inhibited. (c) Histological picture of a molar cultured in the control medium indicates that it has reached the late bell stage, and the mesenchymal cells have differentiated into odontoblasts. No MK antibodies are detected with the secondary anti-rabbit antibodies. (d) Section of a molar grown in the presence of anti-MK antibody. Tooth morphogenesis and odontoblast differentiation have been inhibited. MK antibodies are localized on the surfaces of epithelial and mesenchymal cells indicating penetration of antibodies into the explant. *a*, ameloblasts; *e*, enamel epithelium; *o*, odontoblasts; *p*, dental papilla mesenchyme; *pd*, pre-dentin. Bar, 200 µm.

The penetration of the antibodies into the cultured tooth germs was examined by their immunohistochemical detection by secondary antibodies. Staining was observed on the surfaces of mesenchymal cells of the dental papilla and follicle, as well as in epithelial cells (Fig. 9 *d*), indicating that the MK antibodies had reached the central parts of the explants. Tooth germs cultured in control medium were negative (Fig. 9 *c*).

The development of cultured salivary glands and kidney rudiments from E12 embryos occurred normally in the presence of neutralizing antibodies to MK (data not shown).

Discussion

Differential Expression Patterns of MK Gene and Protein in Developing Tooth

Our *in situ* hybridization and immunohistochemical studies indicated differences between the patterns of MK mRNA and protein expression in the developing tooth of the mouse. Discrepancies were observed in the mandibular and maxillary processes at the stage of tooth initiation and in cap staged teeth. In both cases transcripts were strongly expressed in the mesenchyme, whereas the protein was more abundant in the epithelium. Similar differences between mRNA and protein expression appear to concern HB-GAM which is an

other member of the MK family. Previous studies have shown that *HB-GAM* gene is solely expressed in mesenchyme of the developing tooth (Vanderwinden et al., 1992), whereas the protein is localized in both epithelium and mesenchyme (Mitsiadis et al., 1995; our unpublished results). “Appositional” expression patterns are also reported for *TGFβ* mRNA and protein during development of several epithelial–mesenchymal organs, including salivary glands, whisker follicles, and teeth. In these organs, *TGFβ1* transcripts are detected in epithelium (Lehnert and Akhurst, 1988), whereas the protein is localized in mesenchyme (Heine et al., 1987).

The distribution of the protein on the surfaces of epithelial cells which expressed very small or non-detectable amounts of mRNA suggests that MK may act by a paracrine mechanism during several stages of tooth development. For example, during the late cap stage the MK gene was intensely expressed in the mesenchyme, whereas the protein was localized to the adjacent epithelium and the basement membrane (Figs. 3 and 10). MK protein, expressed in mesenchyme may diffuse rapidly to the epithelium passing through the basement membrane. Alternatively, cells of the inner dental epithelium expressing the receptors could capture the molecule from the basement membrane, which may serve as a site of storage for the MK protein (Fig. 10). The expression of several growth factors and their respective receptors in ad-

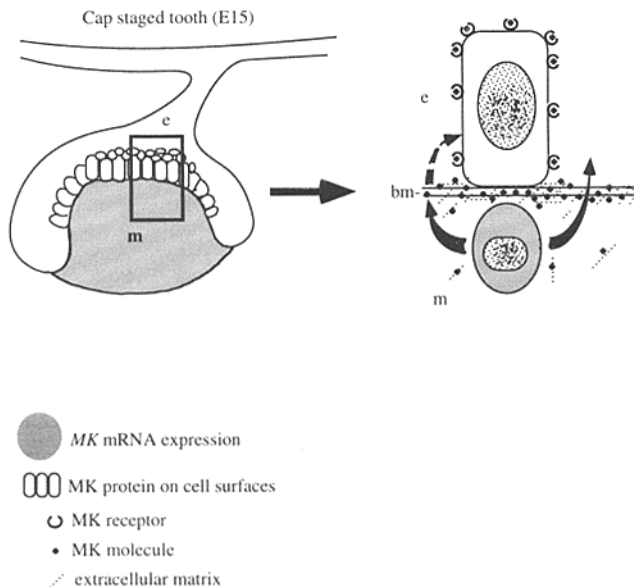


Figure 10. Schematic representation indicating the differences between the patterns of *MK* mRNA and protein expression in a cap staged mouse tooth (E15); a working model to explain this discrepancy. Cells of the dental papilla mesenchyme, which express the *MK* gene, synthesize and secrete the MK protein. These cells lacking the MK receptors are not able to capture the secreted protein, which could bind partly to the extracellular matrix and mainly to basement membrane, thus concentrating MK herein. Dental epithelium cells adjacent to mesenchyme expressing the MK receptors could capture the protein from the basement membrane. Alternatively, MK protein may diffuse rapidly through the basement membrane to the epithelium, where upon binding to its receptors could mediate biological responses. *bm*, basement membrane; *e*, epithelium; *m*, mesenchyme.

adjacent interacting tissues during organogenesis indicates signaling across cell layers, and suggests paracrine functions. This is the case for molecules of the PDGF (Orr-Urtreger and Lonai, 1992), stem cell growth factor (Keshet et al., 1991), and scatter factor/hepatocyte growth factor (SF/HGF) (Sonnenberg et al., 1993). Most notably, SF/HGF is expressed in dental mesenchyme, whereas its receptor, the *c-met* tyrosine kinase, is expressed in the epithelium of the developing tooth (Sonnenberg et al., 1993). In dental mesenchyme MK may have autocrine functions at stages when the cell surface localization of the protein corresponded with the expression of the gene. Interestingly, we observed nuclear localization of the MK molecule in E18-E19 teeth, at a time when mRNA and protein are coexpressed. Recently, nuclear translocation of FGF-2 was demonstrated in developing kidney and limb (Dono and Zeller, 1994), suggesting that this may be a common feature of heparin-binding growth factors.

The localization of MK protein on cell surfaces may imply the existence of an as yet unidentified cell-surface receptor for MK, and may indicate the sites where the molecule is active. Thus, the absence of staining on the surfaces of cells expressing the *MK* gene could be due to the lack of MK receptors, or, alternatively, their saturation by other heparin-binding factors (e.g., FGFs, heparin-binding epidermal growth factor, SF/HGF, PDGF) competing with MK for the binding

sites. We have shown previously that MK binds to the cell surface heparin sulfate proteoglycan syndecan-1 (Jalkanen et al., 1993; Mitsiadis et al., 1995). The expression of syndecan-1 shows a close correlation with MK protein distribution during the early stages of development in several organs (Mitsiadis et al., 1995). In tooth, both syndecan-1 and MK are preferentially expressed in mesenchyme during active morphogenesis (bud and cap stages) (Thesleff et al., 1988; Vainio et al., 1991). Syndecan-1 and MK protein appear to be downregulated very rapidly between E15 and E16 when the shape of the crown of the tooth becomes established. However, *MK* gene continues to be expressed in the mesenchyme until completion of crown morphogenesis. Thereafter, the expression of syndecan-1 as well as *MK* gene and protein persists only in the area of the forming root. Both the binding studies and distribution analysis are in line with molecular interactions between syndecan-1 and MK. One function of syndecan-1 may be to bind MK and to thereby regulate its local concentration and availability. The nature of the high-affinity receptors for MK is yet unknown but it is possible that binding of MK to syndecans is required for interactions of MK with a signaling receptor, as has been shown for FGF-2 (Klagsburn and Baird, 1991; Ruoslahti and Yamaguchi, 1991).

Association of *MK* Gene and Protein Expression with Epithelial-Mesenchymal Interactions During Early Organogenesis

Tooth organogenesis is a process regulated by epithelial-mesenchymal interactions and it is characterized by sequential activation of stage- and tissue-specific regulatory genes (Thesleff et al., 1990, 1995). The expression of *MK* gene in dental mesenchymal cells correlates with their determination to form the odontoblastic cell lineage. *MK* mRNA was expressed in mesenchyme of the mandibular and maxillary processes before tooth initiation, then restricted progressively to the dental mesenchyme, and finally the expression was downregulated in mesenchyme at the time of odontoblast terminal differentiation, suggesting that MK may be involved in cytodifferentiation events. However, it is conceivable that the biological functions of MK depend on the expression of receptors, which could be visualized by the cell-surface distribution of the protein.

The distribution of MK protein in dental cells correlates with epithelial-mesenchymal interactions. Tissue recombination experiments have shown that the potential to instruct tooth development resides in the presumptive dental epithelium (Mina and Kollar, 1987; Lumsden, 1988), where MK protein was first localized. The activities of the presumptive dental epithelium include the initiation of the morphogenetic movements and the induction and patterning of the underlying mesenchyme. The shift of the odontogenic potential to mesenchyme corresponds to the upregulation of MK protein in this tissue. Thereafter the mesenchyme signals back to the epithelium, pursuing the sequential and reciprocal nature of tissue interactions (Mina and Kollar, 1987; Ruch, 1987). Our tissue recombination analysis demonstrated that the expression of MK in mesenchyme is regulated by the mandibular arch epithelium from the tooth region during early tooth development. The induction took place at the level of both mRNA and protein. In contrast, in heterotypic recombinants

the mandibular epithelium from the dental area suppressed MK protein expression in the limb mesenchyme, suggesting that the mechanisms of MK regulation differ between tooth and limb morphogenesis.

Competence is the ability of cells to respond to a particular inductive signal which will determine their developmental fate (Gurdon, 1992; Slack, 1994). Experiments in *Xenopus* embryos have provided evidence that several agents with little or no inductive ability may modify the cellular response to inductive signals. For example, exogenous RA has no ability to induce mesoderm in blastula animal cap explants. However, the addition of RA in the explants treated with activin, which initiated induction of dorsal mesoderm, modifies the response, so that the mesoderm differentiates with a ventral and posterior character (Ruiz i Altaba and Jessel, 1991). Similarly, the presence of Xwnt-8 dorsalizes the response of animal caps to both FGF and activin, suggesting that pattern may arise through localized expression of agents modifying the effects of uniformly distributed inductive factors (Christian et al., 1992). These agents have been named competence modifiers (reviewed by Jessel and Melton, 1992; Moon and Christian, 1992). During early tooth formation, MK may act as a competence modifier regulating the responsiveness of cells to inductive agents. MK protein released from the beads restricted or suppressed the translucent zone induced by epithelium in mesenchyme (Figs. 6 and 7). This zone is induced by diffusible signals, presumably growth factors (Vainio et al., 1993), and hence, MK may interfere with the growth factor/cell interactions. Furthermore, the findings that MK gene is expressed in the mandibular and maxillary processes of both the inducing (epithelium) and responding (mesenchyme) tissue before tooth initiation (Kadomatsu et al., 1990; Mitsiadis et al., 1995) suggest that mesenchyme may contain some elements of the final odontogenic pattern.

Our experimental studies suggest that MK may act synergistically with other growth factors, FGF-2 in particular. MK did not have a detectable effect on cell proliferation in mandibular mesenchyme from the tooth region of E11-E12 embryos. Similar observations for MK have been made earlier in mandibular and limb mesenchyme. During subsequent development (E13-E14) MK had an antiproliferative effect in dental mesenchyme. However, MK appeared to stimulate cell proliferation when it was applied with a bead next to another bead releasing FGF-2, suggesting that the effects of MK could be both positive and negative according to the presence of other growth factors in the microenvironment of the target cells. This experiment indicates that FGFs may modulate the actions of MK, and it may reflect more accurately the in vivo situation in the developing tooth. Similar effects have been observed in mesoderm of *Xenopus* where FGF modulated and potentiated the actions of activin (Green et al., 1992), and in the developing limb where BMP-2 reduces the stimulatory effect of FGF-4 on mesenchymal cell proliferation (Niswander and Martin, 1993). Thus, the reduction of the size of tooth cultured with anti-MK antibodies could be explained by hindrance of positive effects of MK on cell proliferation stimulated by endogenous growth factors. However, it is also possible that the antibodies prevent some important functions of MK in epithelial-mesenchymal interactions which would lead to inhibited developmental and secondarily also to reduction of growth.

Induction of MK Gene and Protein Expression by Retinoic Acid in Dental Mesenchyme

There is increasing evidence that the induction of dental mesenchyme, leading to morphological and molecular changes, is mediated by epithelial-derived diffusible signaling factors, such as BMPs (Vainio et al., 1993). These factors may either stimulate proliferation in predisposed populations of cells and act as survival factors, or change the fate of cells (Gurdon, 1992).

RA regulates embryonic development and it is thought that it is an endogenous morphogen (Tabin, 1991; Tickle, 1991). RA may be directly involved in the regulation of genes since it binds to specific nuclear receptors (RARs and RXRs; Tabin, 1991; Nagpal et al., 1993) acting as transcription factors. Developmentally regulated patterns of RARs have been detected in both dental epithelium and mesenchyme (Dollé et al., 1990; Ruberte et al., 1990). Moreover, the expression patterns of cellular retinoic acid-binding protein I (CRABP I) and cellular retinol-binding protein I (CRBP I) correlate with epithelial-mesenchymal interactions during initiation of several organs, including the tooth (Dollé et al., 1990; Mark et al., 1991; Gustafson et al., 1993). At stages of tooth initiation CRBP I expression and retinol accumulation was seen in epithelium, whereas mesenchymal cells expressed CRABP I, suggesting the involvement of RA in early tooth morphogenesis. Specific functions for RA and retinol have also been implicated in experimental studies at various stages of tooth development (Hurmerinta et al., 1980; Mark et al., 1992; Kronmiller et al., 1993). Furthermore, teratologic studies in vivo have demonstrated that RA causes aberrant tooth development (Geelen, 1979; Lammer et al., 1985).

We showed by bead experiments that RA stimulates the expression of MK gene and protein in dental mesenchyme at the time when the mesenchyme acquires the instructive capacity. In a recent study, Matsubara et al. (1994) have shown that RA-induced expression of MK gene is mediated by a RA-responsive enhancer. Taken together, these findings suggest that MK may be involved in the pathway whereby RA regulates tooth morphogenesis.

The progression of tooth morphogenesis is largely governed by epithelial-mesenchymal interactions and characterized by sequential changes in the expression of a number of structural and regulatory molecules (Thesleff et al., 1991, 1995). The preferential and unique localization of MK gene and protein in dental tissues during tooth initiation and morphogenesis, their regulation by RA, and the findings that morphogenesis and cytodifferentiation were inhibited in tooth germs cultured in the presence of anti-MK antibodies in vitro, suggest important functions for MK in the cascade of molecular events regulating tooth development.

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