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Original Article

Effects of hepatocyte growth factor anti-sense oligodeoxynucleotides or *met* D/D genotype on mouse molar crown morphogenesis

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ABSTRACT Hepatocyte growth factor (HGF) is considered to be one of the mediators of epithelio-mesenchymal interactions during early organogenesis and to be also involved in the development of murine molars. In the developing tooth, HGF is expressed in the cells of the dental papillae, and c-Met, its receptor, in the cells of dental epithelia. In order to study the functional role played by HGF in tooth development, we tested the effects of HGF translation arrest by anti-sense phosphorothioate oligodeoxynucleotides on E-14 molars cultured *in vitro*. We also analyzed the histo-morphogenesis and crown cytodifferentiation of transgenic *met* E-14 molars cultured *in vitro*. 3D reconstructions revealed perturbations of the cusp pattern. However, histo-morphogenesis and crown cytodifferentiation were normal at the histological level.

KEY WORDS: *Mouse molar, hepatocyte growth factor, c-Met, oligodeoxynucleotides, in vitro.*

Introduction

The hepatocyte growth factor (HGF), a member of the family of neurotrophic factors, is a heterodimer with a 69 kDa α-chain and a 34 kDa β-chain, bound together by a single disulfide bond (Zarnegar and Michalopoulos, 1989). A single receptor for HGF has been identified as the product of the proto-oncogene c-Met, and consists of a transmembrane protein containing a tyrosine kinase (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a,b). The c-Met/HGF receptor is a heterodimeric molecule composed of an extracellular 50 kDa α-chain disulfide linked to a transmembranous 145 kDa β-chain. The cytoplasmic portion of the β-chain contains the catalytic domain and critical sites for the regulation of its kinase activity (Park *et al.*, 1987; Vigna *et al.*, 1994). The biological effects of HGF are mediated by autophosphorylation of the c-Met tyrosine kinase on two carboxyl-terminal tyrosines (Ponzetto *et al.*, 1996). Mutation of both tyrosine residues in the mouse genome (*met* D/D) is lethal and causes defects identical to the phenotype of c-*met* null mutants (Maina *et al.*, 1996). Inactivation of the *hgf* or *met* genes in the mouse causes embryonal lethality between E12.5 and E15.5 (Schmidt *et al.*, 1995; Uehara *et al.*, 1995).

During embryogenesis, *met* is expressed in the epithelial component of various organs, while *hgf* is expressed in the adjacent mesenchyme (Sonnenberg *et al.*, 1993). Extensive studies have established that HGF mediates epithelio-mesenchymal interactions. HGF is a paracrine mediator in these interactions (Rubin *et al.*, 1991; Galimi *et al.*, 1993; Matsumoto and Nakamura, 1996a, b, 1997). HGF has the ability to elicit a variety of responses in cultured cells, especially cells of epithelial origin. These responses include mitogenesis (Nakamura *et al.*, 1987; Defrances *et al.*, 1992; Balkovetz and Lipschutz, 1999) motogenesis (Stoker *et al.*, 1987; Weidner *et al.*, 1990, 1991; Lee *et al.*, 1999) and morphogenesis (Stern *et al.*, 1990; Tabata *et al.*, 1996).

Tooth development, from the earliest stage of initiation up to terminal differentiation, is dependent upon inductive interactions between the epithelium and the adjacent mesenchyme (Ruch, 1995; Sharpe, 1995; Lesot *et al.*, 1996, 1998, 1999; Slavkin and Diekwisch, 1996; Thesleff *et al.*, 1996; Maas and Bei, 1997; Peterkova *et al.*, 1997; Bei and Maas, 1998; Fausser *et al.*, 1998; Tucker *et al.*, 1998; Weiss *et al.*, 1998; Yoshiba *et al.*, 1998). HGF simultaneously promotes the proliferation and scattering of ameloblast-lineage cells (Matsumura *et al.*, 1998). Tabata *et al.* (1996)

Abbreviations used in this paper: HGF, hepatocyte growth factor; ODNs, oligodeoxynucleotides; IDE, inner dental epithelium; ODE, outer dental epithelium; M1, first lower molar; M2, second lower molar; 3D, three dimensional.

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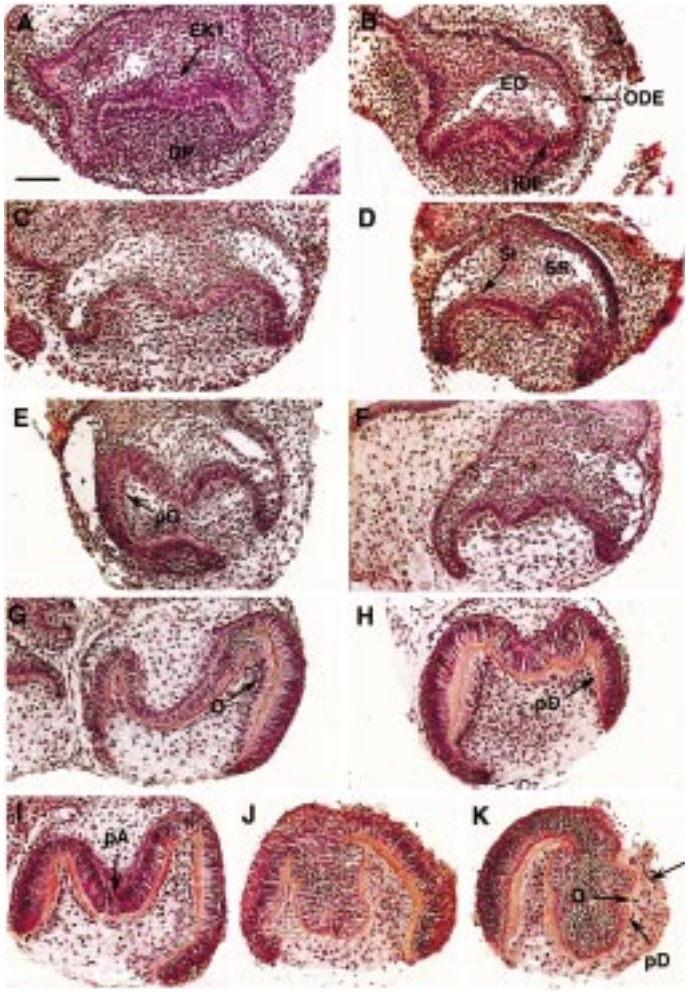

Fig. 1. Histological sections of molars cultured in the presence of sense (A, C, E, G, I) and anti-sense (B, D, F, H, J, K) phosphorothioate oligodeoxynucleotides (ODNs). *The E-14 lower molars were cultured for 2 (A-B), 4 (C-D), 6 (E-F), 8 (G-H) or 10 (I-K) days.* **(A-F)** *Tooth histomorphogenesis and crown cytodifferentiation occurred normally. All the compartments of the enamel organ (inner dental epithelium, outer dental epithelium, stellate reticulum and stratum intermedium) and the dental papillae developed.* **(G-I)** *Functional odontoblasts and polarizing ameloblasts are present.* **(J-K)** *Consecutive, non-adjacent sections of an E-14 molar treated for 10 days with anti-sense ODNs.* **(J)** *Normal crown histomorphology is apparent.* **(K)** *The lateral cusp (arrow) appears to be devoid of the IDE. DP, dental papillae; EO, enamel organ; IDE, inner dental epithelium; ODE, outer dental epithelium; SR, stellate reticulum; SI, stratum intermedium; EK1, primary enamel knot; pA, polarizing ameloblast; pO, polarizing odontoblast; O, odontoblast; pD, predentin. Bar, 100 μm.*

described the temporospatial distribution of HGF and c-Met during tooth germ development. These authors showed that HGF translation arrest with anti-sense treatment on mouse tooth germs cultured in chemically-defined and serum-free medium produced an abnormal histostructure in which the enamel organ was surrounded by a thin layer of dentin and the dental papilla appeared "inside-out" compared to control and sense-treated explants.

In this paper, we repeated HGF translation arrest experiments and also cultured the molars from mouse embryos with, *met* D/D *met* D/+ *met* +/+ genotypes. The transgenic *met* abrogation altered the cusp pattern and reduced the growth of the cervical loop. Terminal odontoblast and ameloblast differentiation was normal.

Results

Oligodeoxynucleotide (ODN) treatment of E-14 molars

Fifty-four molars were cultured in the presence of sense ODNs. After 2 days of culture, sense-treated explants showed the presence of the primary enamel knot (EK1) (Fig. 1A). The inner and outer dental epithelia (IDE, ODE) and the stratum intermedium developed. The stellate reticulum demonstrated the presence of some lacunae. After 4 and 6 days of culture, the cervical loop progressed in an apical direction and a tooth-specific bell developed (Fig. 1 C,E). After 6 days, polarizing odontoblasts were observed (Fig. 1E). After 8 and 10 days of culture, predentin-dentin was secreted and polarizing preameloblasts were present (Fig. 1 G,I).

Fifty-seven teeth were cultured *in vitro* in the presence of anti-sense ODNs. After 2, 4, 6 and 8 days of culture, no histological differences existed when compared to the sense ODNs treated teeth. Histogenesis of the enamel organ and the progression of the cervical loop had occurred (Fig. 1 B,D,F,H). After 8 days, functional odontoblasts secreted predentin-dentin (Fig. 1H) and polarizing ameloblasts were observed (Fig. 1 H,J,K). After 10 days of culture, one explant demonstrated the presence of functional odontoblasts in the absence of an IDE (Fig. 1K). Both the sense and anti-sense ODNs treatments led to limited growth of the teeth (compare Figs. 1 and 3).

In vitro culture of the E-14 transgenic molars

The genotyping of embryos born from the mating of *met* D/+ mice revealed the occurrence of the three anticipated genotypes *met* D/D, *met* D/+ and *met* +/+ (Fig. 2). The PCR procedure resulted in the amplification of fragments of different length visualized on agarose gel (Fig. 2). Three distinct genotypes existed: the 500 bp fragments corresponded to the homozygotes *met* D/D, the 350 bp

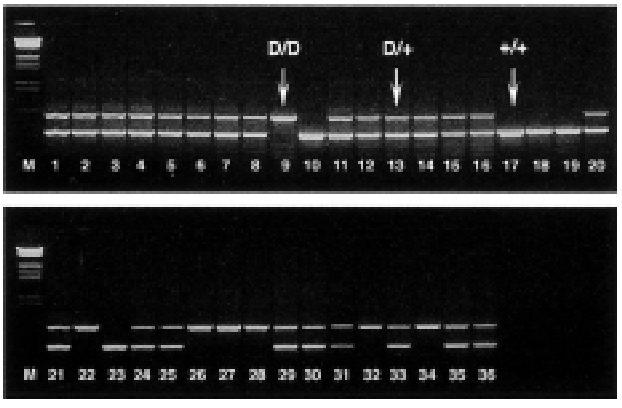

Fig. 2. Met expression in transgenic embryos revealed by polymerase chain reaction (PCR). Lanes 1-8, 11-16, 20-21, 24-25, 29-31, 33, 35-36, *correspond to Met D/+ embryos with 350 and 500 base pair PCR products.* **Lanes 20 and 36** *correspond to female and male adult Met D/+ respectively.* **Lanes 9, 22, 26-28, 32, 34** *correspond to Met D/D embryos with 500 base pair PCR products.* **Lanes 10, 17-19, 23** *correspond to Met +/+ embryos with 350 base pair PCR products. (M) molecular marker (DNA/EcoRI, Hind III. From the top to the bottom: 21226, 4268, 2027, 1904, 1548, 1375, 947, 831 and 546 bp).*

fragments to the homozygotes *met* +/+ and the 350 and 500 bp fragments to the heterozygotes *met* D/+. Seven *met* D/D, twenty-two *met* D/+ and five *met* +/+ embryos were obtained.

Histological observations

After 6 or 7 days *in vitro*, all the cultured first lower molars (*met* +/+ n=10 teeth, *met* D/+ n=42 teeth, *met* D/D n=12 teeth) demonstrated the presence of well-defined dental papillae and enamel organs. Normal gradients of odontoblast terminal differentiation were observed and polarizing ameloblasts were present (Fig. 3 A-F). *met* D/+ and *met* D/D molars demonstrated short cervical loops (Fig. 3F). One of twelve *met* D/D explants demonstrated fused M1 and M2 (Fig. 3 E,F).

3D reconstructions

Four randomly selected right or left *met* +/+ molars cultured for 6 or 7 days were analyzed by means of 3D reconstructions. Two anterior, two median and two posterior cusps were visible (Fig. 4 A-D). Cusp 4 appeared in the posterior part of three teeth and was separated from the cusps L3 and B3 in front by a third posterior transverse cleft (Fig. 4 B-D). A curved, nearly closed, crest linked the four anterior and median cusps (Fig. 4 A-D). The teeth were subdivided by a deep median transverse fissure into a larger anterior portion including four cusps (L1, B1, B2, L2) and a smaller posterior one including cusps L3, B3 and 4 (Fig. 4 B-D).

Four randomly selected left or right *met* D/+ molars cultured for 6 or 7 days were reconstructed. Crowns of the left and right molars comprised six (Fig. 4G) or seven (Fig. 4 E,F,H) cusps: two series of three buccal and three lingual cusps, essentially of a paired nature and eventually one single posterior cusp. One left molar demonstrated a supernumerary ridge linking elongated L2 and L3 (Fig. 4G).

Four randomly selected left or right *met* D/D molars cultured for 6 or 7 days were reconstructed. The left and right crowns demonstrated variable cusp morphology. One left tooth cultured for 7 days demonstrated a normal cusp pattern with seven cusps. A normal curved, nearly closed, crest linked the four anterior and median cusps (Fig. 4I). For the three other teeth cultured for 6 days, B1 was small and was often fused with L1 (Fig. 4 J-L). In some teeth, cusp B3 also was rudimentary (Fig. 4 J,K). The crest linking the four anterior and median cusps had a variable configuration: either the crest just linked the median cusps (Fig. 4 J,K); or an abnormal crest linked L2, L1, B1 and B2 but not L2 and B2 (Fig. 4L); or the crest that normally joined B1, L1 and L2 was nearly absent (Fig. 4 J,K).

Discussion

During embryogenesis, HGF supports organogenesis and morphogenesis of various organs including liver, kidney, lung, gut, mammary gland and skeletal system (Matsumoto and Nakamura, 1996a, b). HGF appears also to be involved in tooth morphogenesis. Tabata *et al.* (1996) documented the expression of HGF by dental mesenchyme from early cap to mid bell-stage of tooth development, and the expression of c-Met, the cognate receptor, by dental epithelial cells from cap to advanced bell-stage. These authors also analyzed the effects of HGF translation arrest by means of anti-sense oligodeoxynucleotides, and suggested that the imbalance between the proliferation activities of the IDE and dental mesenchyme caused by HGF translation arrest could lead to abnormal tooth morphogenesis. Abnormal tooth structures in

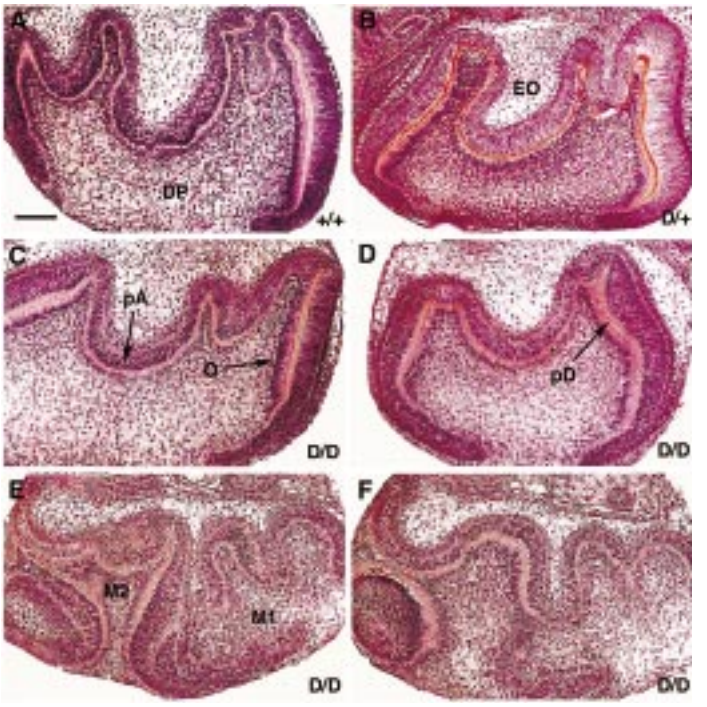

Fig. 3. Histological sections of *in vitro* **cultured E-14 molars of transgenic embryos. (A)** *met +/+,* **(B)** *met D/+ and* **(C-F)** *met D/D .* **(A-D)** *After 6 (A-B) or 7 (C-D) days of culture, the sagittal sections reveal functional odontoblasts and polarizing ameloblasts.* **(E-F)** *Consecutive, non-adjacent, sections of a specimen illustrating fusion of M1 and M2. Such sections can lead to histological misinterpretations. DP, dental papillae; EO, enamel organ; pA, polarizing ameloblast; O, odontoblast; pD, predentin; M1, first lower molar; M2, second lower molar. Bar, 100 μm.*

which the enamel organs appeared surrounded by a thin layer of dentin were observed for E-14 ICR mice molars cultured *in vitro* for 14 days.

Using the same phosphorothioate sense and anti-sense oligodeoxynucleotides as Tabata *et al.* (1996), but extending only slightly the culture and treatment period to the period of time during which *in vitro* cultured E-14 mouse molars normally achieve their histo-morphogenesis (Schmitt *et al.*, 1999), we observed normal crown morphogenesis and cytodifferentiation at the histological level. In only one of the specimens cultured and treated for 10 days with anti-sense oligodeoxynucleotides, we observed the presence of functional odontoblasts (secreting predentin-dentin), no longer in contact with IDE cells. The constant physiological coexistence of normally distributed preodontoblasts-odontoblasts and preameloblasts (IDE cells) during the shorter culture periods and the role of the IDE in odontoblasts differentiation (Ruch, 1998) leads us to assume that in the molar cultured for 10 days, the IDE disappeared as a secondary event. Both the sense and anti-sense oligodeoxynucleotides probably had slight cytotoxic effects leading to reduced growth. The anti-sense treatment strategy implemented by Tabata *et al.* (1996) is not completely identical with ours: we introduced the ODN's in agar solidified medium and cannot exclude a possible, albeit unlike, limitation of the distribution of the ODN's leading eventually to restricted concentrations at cellular level and incomplete HGF translation arrest. Complete loss of the targeted protein should be documented.

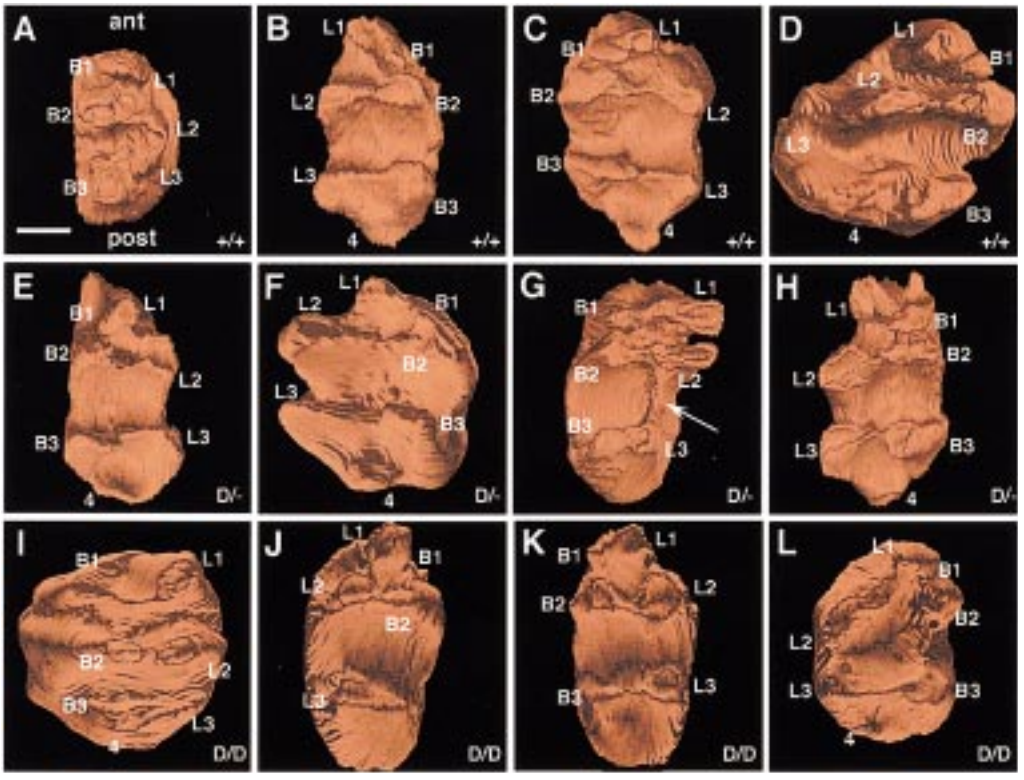

Fig. 4. 3D reconstructions of the dental papillae of E-14 transgenic molars cultured *in vitro* **for 6 or 7 days.** *The cusps are named according to the terminology of Gaunt (1955, 1961).* **(A-D)** *met +/+ molars: a biserial arrangement of three buccal (B1, B2, B3) and three lingual cusps (L1, L2, L3) exists. Cusps L1, B1, B2 and L2 express a "trefoil"-like pattern. Cusp 4 has a median and posterior localization.* **(B-D)** *The crest connecting L1, B1, B2 and L2 has a lingual discontinuity.* **(E-H)** *met D/+ molars: the physiological cusp patterns of right and left handed M1 are evident.* **(G)** *A supernumerary crest linking L2 and L3 is visible.* **(I-L)** *met D/D molars: the ridges are not deep, some cusps and crests are missing. ant, anterior; post, posterior. Bar, 100 μm.*

To avoid the possibility of such technical limitation and get more information on the putative role of HGF during tooth histo-morphogenesis, we cultured *in vitro* E-14 molars from transgenic *met* D/D embryonic mice. These mice express a severe loss of function phenotype which results in embryonic lethality and recapitulates the defects of *met* and *hgf* null mutants (Maina *et al.*, 1996). The c-Met receptor is the only known receptor for HGF (Park *et al.*, 1987; Bottaro *et al.*, 1991; Naldini *et al.*, 1991a, b; Vigna *et al.*, 1994; Ponzetto *et al.*, 1996; Matsumura *et al.*, 1998; Kajihira *et al.*, 1999). The cap-stage, E-14 molars, from *met* +/+, *met* D/+, *met* D/D embryos had similar morphological features as observed under the stereomicroscope, and most of the isolated M1 tissues included the primordium of M2.

Having cultured embryonic teeth in a panel of different culture media (Schmitt *et al.*, 1999 and references therein) we concluded that in our hands the most "physiological" crown morphology was expressed when molars were grown on agar solidified medium containing 20% of fetal calf serum. In these conditions, E-14 molars developed a normal cusp pattern, the cusps containing post-mitotic odontoblasts and ameloblasts after 6 days *in vitro*. We also observed that longer culture periods could eventually lead to histological alterations. For these reasons the molars of the transgenic mice were cultured on agar solidified medium for 6-7 days. Furthermore the 3D reconstructions provided an assessment of the entire tooth morphology allowing for avoidance of possible misinterpretation of single sections. The histo-morphological analysis of these molars revealed a more or less apparent shortening of the cervical loop for *met* D/+, and *met* D/D molars. The 3D reconstructions of cultured *met* +/+, *met* D/+ and *met* D/D molars revealed significant alterations of the cusp pattern and morphology for the *met* D/D molars, which could be related to discrete alterations in cell proliferation kinetics. Since HGF has the ability to elicit motogenesis (Stoker *et al.*, 1987; Weidner *et al.*, 1990; Balkovetz, 1998; Maffe and Comoglio, 1998; Lee *et al.*, 1999), the abrogation of HGF activity could also interfere with cell migration, which has been suggested to play an important role in cuspidogenesis. Indeed, Coin *et al.* (1999) have shown that non-cycling inner dental epithelium cells initially associated with the signaling primary enamel knot underwent sequential segregation and that this led to the formation of distinct groups of cells, each one corresponding to a particular developing cusp. Coin *et al.* (1999) suggested an essential role of these cells in cusp formation. Incomplete segregation of these cells could lead to the fusion of cusps and to abnormal crest formation. On the other hand, the exceptional fusion of M1 and M2 could be a culture artifact. The use of a genetically engineered mouse model, rather than our anti-sense strategy confirms that HGF is involved in the crown morphogenesis. Further challenge is to understand how and when HGF works knowing the signaling activity of the enamel knots, and the implication of cell migration, cell proliferation, cell death, cell-cell and cell-matrix interactions during cusp formation.

Materials and Methods

Tissues

Normal E-14 embryos were obtained by mating Swiss mice. The morning of the appearance of the vaginal plug was designated as day 0 of embryonic development. First right lower molars (M1) were dissected on day 14 of gestation (E-14). E-14 *met* +/+, *met* D/+and *met* D/D embryos were obtained by mating CD1 *met* D/+ mice. The morning of the appearance of the vaginal plug was designated as day 0 of embryonic development. First right and left lower molars (M1) were dissected.

Oligodeoxynucleotides

Phosphorothioate oligodeoxynucleotides (ODNs) for the cDNA sequence of mouse HGF, a 15 mer 5'CAT GCT TGC AGT TCG 3', were used according to Tabata *et al.* (1996). The corresponding control sense ODNs was 5'CGA ACT GCA AGC ATG 3'. All the ODNs were prepared by IGBMC (Strasbourg, France).

Organ culture

The molars of Swiss mouse were cultured for 2, 4, 6, 8 or 10 days on 100 µl of semi-solid medium per microwell (Nunclon delta SI, Nunc, Denmark). The medium consisted of BGJ-B (Gibco, Fitton Jakson modified) supplemented with ascorbic acid (0,18 mg/ml, Merck), L-Glutamine (2 mM, Seromed), kanamycin (0.1 mg/ml, Gibco) and Difco agar (0.5%). During the first two days culture, transferrin was added. Sense or anti-sense ODNs were added with culture medium at 30 µM and changed every other day together with the culture medium. The E-14 molars of *met* +/+, *met* D/+and *met* D/D embryos were cultured for 6 or 7 days on 2 ml of semi-solid medium per Petri dish (Nunc, Roskilde, Denmark; 35x10 mm). The medium consisted of BGJ-B (Gibco, Fitton Jakson modified) supplemented with ascorbic acid (0.18 mg/ml, Merck), L-Glutamine (2 mM, Seromed), fetal calf serum (20%, Boehringer Bioproducts), kanamycin (0.1 mg/ml, Gibco) and Difco agar (0.5%). The teeth were incubated and grown at 37°C in a humidified atmosphere of 5% CO2 in air and the medium was changed every two days.

Genotyping

Extraction of DNA

DNA samples were extracted from embryonic skin using the standard proteinase K digestion as described by Sambrook *et al.* (1989) with minor modifications. Briefly, *met* D/+ mice were mated and embryos collected. While molars from each embryos were cultured, small pieces of skin (~16 mm^2) were suspended in 700 µl of extraction buffer (Tris 50 mM pH 8.0; EDTA 100 mM; SDS 0.5%; proteinase K: 0.25 mg/ml), and incubated at 55°C overnight. The samples were then heated at 95°C for 10 min and mixed with an equal volume of absolute ethanol and 0.1 volume of sodium acetate 3M. After centrifugation (6 min at 8500 g) the pellets were washed in 700 µl of ethanol 70% and centrifuged 6 min at 8500 g. The pellets were then air dried and resuspended in sterile double-distilled water. The concentration of the DNA was measured by absorption at 260 nm.

Amplification conditions

The polymerase chain reaction (PCR) was employed to generate double-stranded DNA fragments. Each amplification was performed in the presence of 20 mM Tris HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$ (Gibco BRL), 200 µg BSA, 0.7 µM of primer 610 and 655, 1.4 µM of primer 611, 1.5 U of Taq DNA polymerase (Perkin Elmer Cetus), 200 µM of each dNTP (Boehringer Mannheim) and 100 ng of template DNA in a volume of 40 µl. The following set of primers was employed.

P610: 5'-AGGATTGATCATTGGTGCGGTC-3'
P611: 5'-CATCTCTGTAGTTGGACTTACAC-3'
and P655: 5'-CAGCTCATTCCTCCCACTC-3'.

Reactions were done in a Perkin Elmer Cetus DNA thermocycler 480 as follows: pre-denaturation (3 min at 94°C). Eight cycles of denaturation (1 min at 94°C), annealing (1 min at 60°C), extension (1 min at 72°C) and then, 35 cycles of: denaturation (1 min at 94°C), annealing (1 min at 54°C), extension (1 min at 72°C), followed by a final extension step (10 min at 72°C).

Amplification products were electrophoresed on 1.8 % agarose gels in TBE buffer (Tris base 87 mM, boric acid 89 mM, EDTA 2 mM, pH 8.0) in the presence of 1 µg/ml of ethidium bromide. Electrophoresis was performed at 200 mA for 4 h in TBE. Gels were than examined and photographed under UV light with a Polaroid system. Embryos were classified as follows: *met* +/+ corresponds to a 350 bp fragment; *met* D/D to a 500 bp fragment and *met* +/D to a 350 and 500 bp fragments.

Histology

Teeth were fixed in Bouin-Hollande fluid and embedded in paraffin wax. Five µm serial sections were stained with Mallory's stain.

3D reconstructions

Drawings of the contours of the mesenchyme and of the inner dental epithelium of the molars were made at 5 µm intervals from the serial histological sections using a Zeiss Jenaval microscope equipped with a drawing chamber. The digitalization of the serial drawings was achieved using a Hamamatsu C2400 camera connected to a digital imaging system. The digitalization of the serial drawings and correlation of successive images (Olivo *et al.*, 1993) have been previously described (Lesot *et al.*, 1996). Software packages allowing image acquisition and treatment were developed and adapted to this work. Three-dimensional images were generated using a volume rendering program (Sun Voxel, Sun Microsystems).

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