

Fgf-8 determines rostral-caudal polarity in the first branchial arch

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SUMMARY

In mammals, rostral ectomesenchyme cells of the mandibular arch give rise to odontogenic cells, while more caudal cells form the distal skeletal elements of the lower jaw. Signals from the epithelium are required for the development of odontogenic and skeletogenic mesenchyme cells. We show that rostral-caudal polarity is first established in mandibular branchial arch ectomesenchymal cells by a signal, Fgf-8, from the rostral epithelium. All neural crest-derived ectomesenchymal cells are equicompetent to respond to Fgf-8. The restriction into rostral (*Lhx-7*-expressing) and caudal (*Gsc*-expressing)

domains is achieved by cells responding differently according to their proximity to the source of the signal. Once established, spatial expression domains and cell fates are fixed and maintained by Fgf-8 in conjunction with another epithelial signal, endothelin-1, and by positional changes in ectomesenchymal cell competence to respond to the signal.

Key words: Patterning, Mandible, Lim homeobox, Fgf-8, Goosecoid, Mouse, Cell signalling

INTRODUCTION

The establishment of pattern during embryogenesis involves the cellular and molecular division of cells to provide 'positional information'. Branchial arches give rise to a wide range of structures specific to each arch. Thus each arch develops according to a unique programme of spatial and temporal patterning. Classic recombination experiments in avian and amphibian embryos have indicated that some aspects of skeletal development in the branchial arches are predetermined by the rostral-caudal origin of the cranial neural crest cells that populate the arches and which form the skeletal tissues (Noden, 1983; Hörstadius and Sellman, 1946; Wagner et al., 1949). However, the existence of such prepatterning is now under debate (Hunt et al., 1998a,b). Mutations in Hox genes such as *Hoxa-2* have been shown to produce changes in branchial arch skeletal development indicative of these genes playing roles in patterning of the arches caudal to the first branchial arch (Gendron-Maguire et al., 1993; Rijli et al., 1993). The first branchial arch does not express Hox genes, and has thus been interpreted as representing a branchial arch 'ground state' (Rijli et al., 1993).

In mammals the ectomesenchymal cells of the mandibular arch (first branchial arch) originate from the rostral hindbrain and caudal midbrain (Serbedzija et al., 1992; Osumi-Yamashita et al., 1994; Lee et al., 1995; Imai et al., 1996). Recombination experiments have shown that neural crest-derived mesenchyme from sources outside the mandibular arch is capable of forming

teeth but only when recombined with early rostral (oral) epithelium from the first branchial arch (Lumsden, 1988). In addition, non-neural crest-derived mesenchymal cells are incapable of forming teeth even when recombined with oral epithelium (Mina and Koller, 1987). These experiments argue that all ectomesenchymal cells have odontogenic capacity regardless of the axial origins of the cranial neural crest cells and thus suggest that, with respect to odontogenic capacity at least, ectomesenchymal cells are not prespecified.

Teeth develop from the rostrally located ectomesenchymal cells of the mandibular arch via interactions with the overlying oral epithelial cells. This implies that the requirement for oral epithelium in tooth development involves instructive signals from the epithelium that act on underlying ectomesenchymal cells to determine their fate as odontogenic (Lumsden, 1988). More caudal ectomesenchymal cells do not form teeth in vivo but give rise to the skeletal tissues of the mandible, most notably Meckel's cartilage. Thus early in embryogenesis, the ectomesenchymal cells of the mandibular arch must become determined into rostral and caudal populations which will form odontogenic and skeletogenic cells respectively. In the paper, we discuss possible genes involved in this process, and investigate the signalling pathways involved in setting up this rostral-caudal axis.

Fgf-8 has been identified as one of the key signalling molecules from oral epithelium capable of inducing and/or maintaining expression of several different mesenchymal genes in the mandibular arch (Neubüser et al., 1997; Kettunen and

Thesleff, 1998; Ferguson et al., 1998). Of these mesenchymal genes, the Lim-domain homeobox genes *Lhx-6* and *Lhx-7* have previously been identified as molecular markers of rostral (oral) mesenchyme of the mandibular and maxillary arches (Grigoriou et al., 1998) (Fig. 1B). *Lhx-7* is also thought to be similar or the same as *Lhx-8* (*L3*) (Wanaka et al., 1997; Kitanaka et al., 1998). *Fgf-8* has been identified as a strong candidate inducer of these Lim genes by virtue of its localised expression in oral epithelium, and the ability of exogenous *Fgf-8* applied on beads to maintain Lhx gene expression in mandibular arch mesenchyme (Grigoriou et al., 1998) (Fig. 1A). In this paper, the experiments will refer to *Lhx-7* for simplicity, although identical results were obtained for *Lhx-6*.

Goosecoid (*Gsc*) is another homeobox-containing gene expressed in mandibular mesenchyme, which can be used as a molecular marker of the caudal region of the mandibular portion of the first branchial arch (Gaunt et al., 1993) (Fig. 1C). *Gsc* was first cloned from a *Xenopus* dorsal lip cDNA library (Blumberg et al., 1991). It is expressed in the dorsal blastopore lip and is thought to have roles in organiser function (Cho et al., 1991; De Robertis et al., 1992). In mice, *Gsc* is expressed transiently at the rostral end of the primitive streak at the gastrula stage then reappears at E10.5 in the branchial arches, limb and ventral ribs (Gaunt et al., 1993). Targeted mutations

in *Gsc* have been described independently by two groups as affecting skeletal formation in the head and rib cage (Yamada et al., 1995; Rivera-Perez et al., 1995). In the head, these defects consist of aplastic nasal cavities, hypoplasia of the mandible, malformed Meckel's cartilage in which a novel groove appears, tongue defects, absence of the tympanic ring bone, and a reduced malleus in the middle ear. Lack of a defect in axial patterning may be explained by the existence of at least three goosecoid genes in vertebrates which may be able to functionally compensate for each other (Zhu et al., 1998). No tooth defects are present in the mutants. The lack of a tooth phenotype and presence of mandible skeletal defects in *Gsc* mutants was thus consistent with the observed restricted expression in the caudal half of the mandibular arch.

Gsc expression is lost when endothelin-1 signalling from mandibular arch epithelium is disrupted (Clouthier et al., 1998). Endothelin-1 (*Et-1*) is expressed throughout the mandibular arch epithelium and Et-A receptors are found in the ectomesenchyme. It has been suggested that Et-1 may promote growth and/or differentiation of cranial neural crest cells in culture (Kurihara et al., 1998). Targeted mutations in *endothelin-1* (*Et-1*), its receptor *endothelin A* (*Et-A*) and *endothelin converting enzyme 1* (*ECE-1*) produce mandible skeletal phenotypes resembling those of *Gsc*^{-/-} embryos, affecting mandible skeletal patterning but not tooth development (Kurihara et al., 1994; Clouthier et al., 1998; Yanagisawa et al., 1998). The *Gsc* mutation is less severe than the *endothelin 1* (and related) mutations, but affects similar populations of cells.

In this paper we investigate the relationship between these epithelially located signalling molecules and their mesenchymally located homeobox gene targets, and suggest that they have interacting roles in determining mandibular arch polarity.

We show that ectomesenchymal rostral-caudal polarity is established early in the development of the mandibular arch by specific signals produced from the epithelium. Early in development (E10.0) all the ectomesenchymal cells of the mandibular arch are equally responsive to epithelial signals and have the ability to form teeth. We identify *Fgf-8* as a signal from oral epithelium which induces expression of rostral ectomesenchyme genes, such as *Lhx-6/-7*, which in turn repress expression of caudal mesenchyme genes, such as *Gsc* (Grigoriou et al., 1998). Once established, this rostral-caudal polarity is maintained by *Fgf-8* and another epithelial signal, endothelin-1. This occurs together with changes in the competence of ectomesenchymal cells to respond to the signals, such that by E10.5-11.0, caudal cells lose their ability to respond to *Fgf-8* and express rostral genes, whereas rostral cells retain this ability and continue to respond to the signal. These changes in competence are reflected in the loss of plasticity of cells such that caudal cells are no longer capable of forming teeth. These results show that it is the localised expression of signals in the epithelium and not any apparent prespecification of cranial neural crest cells that directs rostral/caudal patterning.

MATERIALS AND METHODS

In situ hybridisation

³⁵S in situ hybridisation procedures were carried out as described by Wilkinson (1992), with the following modifications: embryonic

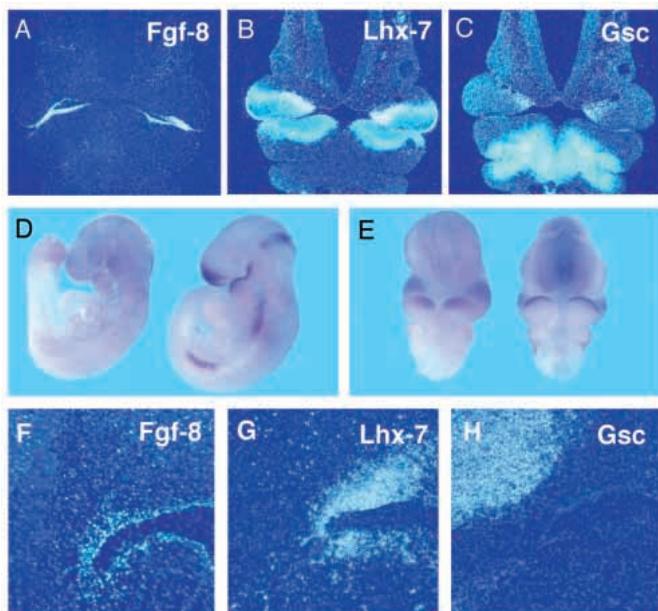


Fig. 1. Expression patterns of *Fgf-8*, *Lhx-7* and *Gsc*. (A-C,F-H) Comparison of *Fgf-8* (A,F), *Lhx-7* (B,G) and *Gsc* (C,H) in serial frontal sections of E10.5 heads by ³⁵S in situ hybridisation. (A) *Fgf-8* is expressed in the oral epithelium. (B) *Lhx-7* is expressed in the mesenchyme directly underlying the *Fgf-8* expression. (C) *Gsc* is expressed in the first and second branchial arches but in the first its expression is restricted to the caudal (aboral) mesenchyme. (D,E) Comparison of *Fgf-8* and *Lhx-7* in whole mounts at E9.0 (D) and E9.5 (E) by DIG in situ hybridisation. *Lhx-7* is shown on the left hand side in each case. Note *Fgf-8* is strongly expressed at E9.0 while *Lhx-7* is very faint. (F-H) Close up of the pharyngeal pouch at the base of the second branchial arch. (F) *Fgf-8* is expressed in the arch endoderm. (G) *Lhx-7* is expressed in the mesenchyme surrounding the *Fgf-8* expression domain. (H) *Gsc* is expressed at a distance from the *Fgf-8* expression domain, and does not overlap with the *Lhx-7* expression domain.

mouse heads were sectioned at 8 μm and floated onto TESPA (3-aminopropyltriethoxysilane) coated slides. The slides were pre-treated with 5 $\mu\text{g}/\text{ml}$ Proteinase K and 0.25% (v/v) acetic anhydride to reduce background. Hybridisation was carried out overnight in a humidified chamber at 55°C. The slides were then washed twice at high stringency (20 minutes at 65°C in 2 \times SSC, 50% formamide, 10 mM DTT) and treated with 40 $\mu\text{g}/\text{ml}$ RNase A for 30 minutes at 37°C to remove any non-specifically bound probe. The high stringency washes (at 65°C in 2 \times SSC, 50% formamide, 10 mM DTT) were repeated, followed by a further wash at 65°C in 0.1 \times SSC, 10 mM DTT. The sections were then washed in 0.1 \times SSC at room temperature, and dehydrated through 300 mM ammonium acetate in 70% ethanol, 95% ethanol and absolute ethanol. The slides were air dried and dipped in Ilford K.5 photographic emulsion. Autoradiography was performed by exposing the sections in a light tight box at 4°C for 10-14 days. Slides were developed using Kodak D19, fixed in Kodak UNIFIX and counter stained with malachite green. Digoxigenin whole-mount in situ hybridisation was carried out as described by Pownall et al. (1996). The radioactive antisense probes used were generated from mouse cDNA clones. *Lhx-6* and *-7* were linearised with *NotI*, *Fgf-8* was linearised with *HindIII*, and *Gsc* was linearised with *XbaI*. All three were transcribed with T3.

Cultures

Cultures were carried out using tissue from mouse CD1 embryos aged between E10.0 and E11.5. Noon of the day on which the plugs were detected was considered as E0.5. For more precise staging, somite number was counted from the hindlimb (Kaufman, 1994). Mandibular arches, limb buds and 2nd branchial arches were dissected out in D-MEM. For many of the experiments the mandibles were dissected into rostral (oral) and caudal (aboral) halves as indicated in Fig. 4A. The epithelium, and endoderm in the case of the 2nd branchial arches, was removed using Dispase in calcium- and magnesium-free PBS at 2 units per ml. The explants were incubated for 9-13 minutes at 37°C, depending on age and size. After incubation the tissues were washed in D-MEM with 10% fetal calf serum (FCS), and the epithelium was dissected using fine tungsten needles. The mesenchyme was placed on membrane filters supported by metal grids according to the Trowel technique as modified by Saxén (Trowel, 1959; Saxén, 1966). It is important that the mandible mesenchyme is placed so that the position of the oral/aboral surface is noted. Beads were then placed on top of the mesenchyme. For the BSA application (control) and Et-1 protein application Affi-Gel-blue beads (Bio Rad) were used. These were washed and dried out before being placed in the protein for 1 hour at 37°C. Et-1 was used at a concentration of 100 ng/ μl (Sigma). For the Fgf8 protein heparin acrylic beads (Sigma) were used. Heparin acrylic beads were washed several times in PBS, then incubated overnight at 4°C in Fgf8b at concentrations ranging from 50 $\mu\text{g}/\text{ml}$ to 1 mg/ml (R&D Systems). Protein-soaked beads were stored at 4°C for up to 3 weeks. The explants were cultured for 24 hours in D-MEM with 10% FCS. A standard incubator was used at 37°C with an atmosphere of 5% CO₂ in air and 100% humidity. All solutions used contain penicillin and streptomycin at 20 IU/ml. After the period of culture, cultures were washed in ice-cold methanol for 1 minute then fixed in fresh 4% paraformaldehyde for 1 hour at RT. Cultures were then prepared for whole-mount digoxigenin or radioactive ³⁵S in situ hybridisation.

Epithelial-mesenchymal tissue recombinations

Recombinations were carried out at E10.0 and E11.0. Mandibles were dissected into rostral (oral) and caudal (aboral) halves (Fig. 4A). The epithelium and mesenchyme were isolated as above. The epithelium and mesenchyme were re-aligned in the correct orientation on top of transparent Nucleopore membrane filters (0.1 μm pore diameter; Costar). The recombinations were cultured for 48 hours in D-MEM with 10% foetal calf serum, after which they were either fixed and processed for radioactive in situ hybridisation, or transplanted under

the kidney capsule of male adult mice and cultured for a further 10 days to allow for full development of teeth. Before implantation into the kidney capsule the recombinations were bisected so that the pieces could fit under the capsule. The resulting teeth and cysts were dissected from the kidneys, photographed then fixed for several days in Bouin's, before embedding for sectioning. Sections were stained with a trichrome stain.

Goosecoid mutants

Mutant mice were generated as described by Yamada et al. (1995).

RESULTS

Temporal and spatial expression of *Fgf-8*, *Lhx-7* and *Gsc* in the branchial arches

Fgf-8 is expressed in the oral epithelium of the forming first branchial arch from E8.75-E9.0 (Crossley and Martin, 1995). When the onset of *Lhx-7* and *Fgf-8* expression were compared in E8.75 to E9.5 embryos *Fgf-8* appeared several hours earlier (Fig. 1D,E). This early expression of *Fgf-8* suggests that it is the primary signal for inducing the expression of *Lhx-7* and setting up the A-P axis of the first branchial arch, which is in agreement with previous results (Grigoriou et al., 1998).

Fgf-8 is also expressed at the extreme lateral regions of each pharyngeal pouch and the overlying surface ectoderm of the pharyngeal groove (Mahmood et al., 1995; Crossley and Martin, 1995). *Lhx-7* is expressed in restricted regions surrounding these areas at the base of the branchial arches, further linking the expression of *Fgf-8* with *Lhx-7* (Fig. 1F,G). There is, however, no expression of *Fgf-8* or *Lhx-7* in the main bulk of the second branchial arches, as can be seen in Fig. 1A,B.

Expression of *Lhx-6/-7* was compared with other genes expressed in branchial arch mesenchyme by using radioactive in situ hybridisation on serial frontal sections of E10.5 mouse embryo heads. Expression of the murine homeobox gene, goosecoid *Gsc*, was found to be localised in caudal mesenchyme in a pattern that complemented that of the *Lhx* genes (Fig. 1C). *Gsc* expression is not restricted to the mesenchyme of the first branchial arch derivatives but is also expressed in the 2nd arch. The highest level of expression was found at the junction between the 1st and 2nd branchial arch in the region that will go on to form the auditory meatus. *Gsc* expression was also associated with the mesenchyme around the pharyngeal pouches. However, it was expressed at a distance from the *Fgf-8* expression domain and seemed to be excluded from the *Lhx-7* expression domain, in a similar pattern to that seen for the first branchial arch (Fig. 1H). *Lhx-7* is highly expressed in the 1st branchial arch from E9.5, and thus its expression pattern precedes that of *Gsc* by at least 24 hours.

Induction of *Lhx* gene expression in second branchial arch mesenchyme

The ability of *Fgf-8* to induce de novo *Lhx* gene expression was first tested by implanting *Fgf-8* beads into the distal regions of the mesenchyme of the second branchial arch from which the epithelium had been removed. This region of second branchial arch is far removed from the *Fgf-8*-expressing pharyngeal pouch and the endogenous *Lhx-7* domain. Following 24 hours of explant culture, *Fgf-8* beads

had induced expression of *Lhx* genes in the second arch mesenchyme (Fig. 2A). Other Fgfs were also able to induce expression. Fgf-9, which is also expressed in oral epithelium (Kettunen and Thesleff, 1998), induced high levels of *Lhx-7* (Fig. 2B), but Fgf-1 beads only gave a weak induction (Fig. 2C). Control BSA beads, and beads loaded with other signalling molecules such as Bmp-4 and Shh, did not induce expression (Fig. 2D and data not shown). The ability of Fgf-8 to induce *Lhx* gene expression in second branchial arch mesenchyme suggests that it may have an inductive as well as maintenance role in regulating expression in the mandibular arch in vivo.

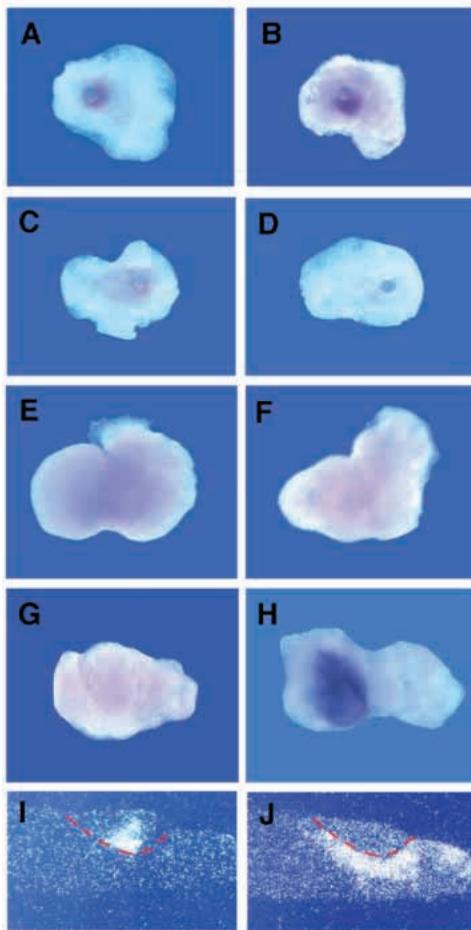


Fig. 2. *Lhx-7* induction is restricted to neural crest-derived mesenchyme. (A-H) *Lhx-7* DIG whole-mount in situ hybridisation. (I,J) ^{35}S in situ hybridisation of sections. (A-D) Second branchial arch mesenchyme minus epithelium. (A) Fgf-8-loaded bead induces *Lhx-7*. (B) Fgf-9-loaded bead induces *Lhx-7*. (C) Fgf-1-loaded bead causes very weak induction of *Lhx-7*. (D) BSA-loaded bead has no effect on induction of *Lhx-7*. (E,F) Limb mesenchyme. (E) E10.5 limb plus epithelium showing no endogenous expression of *Lhx-7*. (F) E10.5 limb minus epithelium plus a Fgf-8 loaded bead. No induction of *Lhx-7* is seen. (G-H) E10.5 mandible cultures minus epithelium. (G) Mandible minus epithelium, showing loss of *Lhx-7* expression. (H) Mandible minus epithelium plus limb epithelium, showing expression of *Lhx-7*. (I) Section of a similar culture showing expression of Fgf-8 in the limb epithelium. (J) Serial section showing re-induction of *Lhx-7* in the mandible mesenchyme underlying the Fgf-8-expressing limb epithelium. Red dashed lines outline the epithelium. All explants were cultured for 24 hours. $n=12$ for each case.

Fgf-8 cannot induce expression of *Lhx-7* in limb mesenchymal cells

Fgf-8 expression is not restricted to the branchial arches but is also highly expressed in other regions of the embryo where *Lhx-6/-7* are not expressed, most notably in the apical ectodermal ridge (AER) of developing limb buds (Crossley and Martin, 1995; Crossley et al., 1996) (Fig. 2E). The relative abilities of Fgf-8 to induce *Lhx* expression in limb bud mesenchyme and limb bud epithelium to induce *Lhx* expression in branchial arch mesenchyme were investigated. Fgf-8 beads cultured with limb bud mesenchyme from E10.5 embryos did not induce *Lhx* gene expression (Fig. 2F). Limb bud epithelium recombined with E10.5 branchial arch mesenchyme did induce *Lhx* gene expression (Fig. 2H), the induction corresponding to mesenchyme immediately underlying *Fgf-8*-expressing epithelium (Fig. 2I,J).

Fgf-8 produced from oral epithelium, limb bud epithelium or as a purified source is therefore capable of inducing *Lhx* gene expression in neural crest-derived mesenchymal cells but not in limb mesenchyme. In this respect, the requirements for induction of *Lhx* gene expression correlate with the tissue requirements for tooth development as defined by recombination experiments (Mina and Kollar, 1987; Lumsden, 1988).

Competence changes in mandibular mesenchyme

Only the rostral (oral) half of the mandible expresses *Lhx-6/-7* in vivo. To determine whether this is due to an inability of the caudal (aboral) mesenchyme to respond to an Fgf-8 signal, mandibles were dissected into rostral and caudal halves at E10.0 and Fgf-8 beads were implanted into each half (Fig. 3A). Expression of *Lhx-7* was detected around the beads implanted in both the rostral and caudal mesenchyme explants with no expression being detected in controls (Fig. 3B-E). The ability of caudal mesenchyme to respond to Fgf-8 and express *Lhx* genes showed that all the mesenchyme of the mandibular arch at E10.0 is competent to respond.

The competence of mandibular arch mesenchyme to respond to Fgf-8 was investigated further by repeating the rostral-caudal experiments with mesenchyme from embryos 12-36 hours older at E10.5-E11.5. When E10.5 and E11.5 mesenchyme was used, a different result was obtained to that at E10.0. Fgf-8 was still capable of re-inducing *Lhx* expression in rostral mesenchyme, but no induction of expression was detectable in caudal mesenchyme (Fig. 3F-K). The caudal mesenchyme, however, did form a translucent zone around the bead and induction of the homeobox gene *Msx-1* was detected (data not shown). Therefore, the failure of Fgf-8 to induce *Lhx-7* expression at this stage is due to a specific competence change of the mesenchyme to express *Lhx-7*. Interestingly, in a few cases where some of the rostral mesenchyme was still present at the edges of the caudal cultures, these regions expressed *Lhx-6/-7* despite being far from the beads and separated by a region of non-expressing mesenchyme (Fig. 3K). The Fgf-8 signal is therefore able to pass across areas of non-competent mesenchyme.

Rostral expression of *Lhx-6/7* is not restricted by *Gsc*

Since *Gsc*-expressing cells are restricted to caudal mesenchyme in the mandibular arch, it seemed possible that

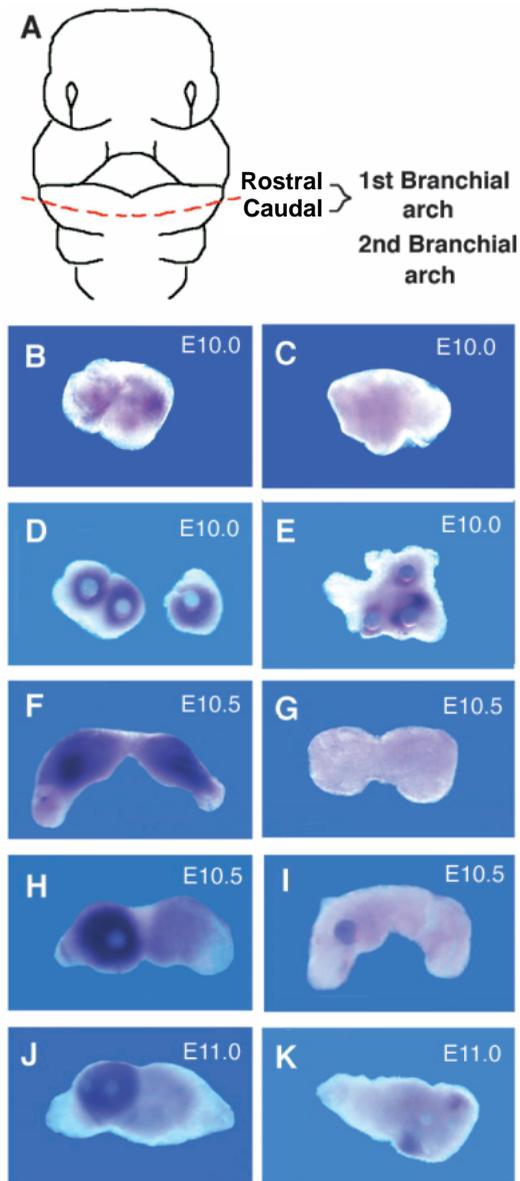


Fig. 3. Competence changes in the first arch mesenchyme. Whole-mount in situ hybridisation of *Lhx-7*. Explants all cultured for 24 hours. (A) Diagram showing dissection of the mandible into rostral (oral) and caudal (aboral) mesenchyme. (B,F) Rostral mesenchyme with epithelium showing endogenous expression of *Lhx-7*. (C,G) Caudal mesenchyme with epithelium showing no endogenous expression. (D,H,J) Rostral mesenchyme minus epithelium plus a Fgf-8 bead. (E,I,K) Caudal mesenchyme minus epithelium. Note lack of expression around the bead in I in comparison to E. $n=15$ for each case.

the caudal limit of expression of *Lhx-6/-7* genes in rostral mesenchyme might result from repression of Lhx gene expression in *Gsc*-positive cells. In order to test this possibility, the expression of *Lhx-6/-7* was investigated in *Gsc*^{-/-} embryos. Frontal sections of E11.5 embryos were used to examine *Lhx-6/-7* gene expression by in situ hybridisation. No difference in the caudal extent of the *Lhx-6/-7* expression domains between wild-type and *Gsc*^{-/-} embryos was observed (Fig. 4A,B). Thus the loss of *Gsc* from caudal cells did not

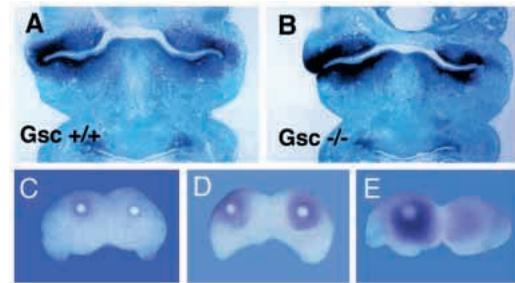


Fig. 4. Restriction of *Lhx-7* to the rostral mesenchyme. (A,B) ³⁵S in situ hybridisation of frontal sections at E11.5 showing expression of *Lhx-7* in the *Gsc* mutant (B) and wild type (A). Note rostrocaudal boundary in the mutant mandible is identical to that seen in the wild type. (C-E) Whole-mount DIG in situ hybridisation of E10.5 mandible explants minus epithelium cultured for 24 hours with beads loaded with Fgf-8 at various concentrations: (C) 50 µg/ml; (D) 500 µg/ml; (E) 1 mg/ml. Note the graded response of *Lhx-7*. $n=8$ for each case.

result in induction of *Lhx-6/-7* expression in more caudal cells, suggesting that *Gsc* does not repress Lhx expression and is not responsible for creating the caudal boundary of expression. This is consistent with the fact that *Lhx-7* is expressed prior to *Gsc* in the mandibular arch.

Concentration effects of Fgf-8 on *Lhx-7* expression

A possible explanation for the restriction of *Lhx-6/7* to the rostral mesenchyme of the first branchial arch, given the fact that early on the caudal mesenchyme is competent to respond, is that *Lhx-6/7* can only be induced at high concentrations of Fgf-8. Thus, *Lhx-7* induction is restricted to the mesenchyme directly surrounding the Fgf source. To test this, the level of induction of *Lhx-7* was compared at three different Fgf-8 concentrations. Concentration-dependent responses to Fgf-8 were observed, with Fgf-8 at 1 mg/ml being able to induce *Lhx-7* more strongly than at 500 µg/ml and 50 µg/ml (Fig. 4C-E). This indicates that in vivo Fgf-8 is at too low a concentration in the caudal mesenchyme to induce *Lhx-6/7* expression.

Maintenance of *Gsc* expression by Endothelin-1

The restriction of *Gsc* expression to caudal mesenchyme of the mandibular arch was investigated following the same lines as the Lhx experiments. Removal of mandibular arch epithelium at E10.5 resulted in loss of *Gsc* expression within 3 hours as seen for the *Lhx-6/-7* genes (Fig. 5A,B). A possible candidate molecule for the inducer of *Gsc* in the mesenchyme is *Endothelin-1* (*Et-1*), which is expressed in the epithelium surrounding the branchial arches (Clouthier et al., 1998). *Gsc* expression is lost in mice deficient in the receptor for *Et-1*, *Et-A* (Clouthier et al., 1998), and this could explain why *Gsc* expression is lost after removal of the epithelium. To test this, *Et-1* beads were placed in E10.5 mandible mesenchyme cultures which had their epithelium removed. *Gsc* and *Lhx-7* were not expressed in the explants, which looked identical to control cultures with BSA beads (data not shown) (Fig. 5C,D). To assess whether *Et-1* beads could maintain expression of *Gsc* or *Lhx-7*, mandibular arches were taken a day later at E11.5 and the epithelium was removed. At this stage, low levels of *Gsc* and *Lhx-7* are maintained in the cultures, showing that by

E11.5 the mesenchymal expression is partially independent of a signal from the epithelium (data not shown). At this stage Et-1 beads upregulated the expression of *Gsc* but had no effect on the normal expression pattern of *Lhx-7* (Fig. 5E,F). Et-1 would thus seem to have a role in maintaining the expression of *Gsc*.

Role of Fgf-8 in *Gsc* expression

Since *Gsc* is expressed at a distance from the source of Fgf-8, in the mandible and pharyngeal pouches, we investigated whether Fgf-8 had any effect on *Gsc* expression. This was tested by adding Fgf-8 beads to intact mandibular arch explants (i.e. explants with epithelium), at E10.0-E10.5 and culturing for 24 hours. Radioactive in situ hybridisation was used, with adjacent sections being hybridised with *Gsc* and *Lhx-7* probes, to allow comparison of the effects on these two genes. Implantation of a Fgf-8 bead into one side of the mandibular explant in the *Gsc* expression domain resulted in a loss of *Gsc* expression compared with the other, non-treated side (Fig. 6A). Adjacent sections showed that the bead produced the expected increase in *Lhx-6/-7* gene expression (Fig. 6B). Fgf-8 was therefore capable of repressing endogenous *Gsc* expression. This repression was seen at all concentrations where *Lhx-7* was induced by an Fgf-8 bead (data not shown). Surprisingly, however, when this experiment was repeated with the epithelium removed, so that the endogenous expression of *Gsc* and *Lhx-7* were lost, Fgf-8 beads appeared to be able to induce *Gsc* expression. This induced expression of *Gsc* was seen only at the edge of cultures, with no expression being found around the bead (Fig. 6C). From reciprocal sections, *Lhx-7* could be seen to be induced in the complementary area around the bead (Fig. 6D). This was seen reproducibly in 15 out of 16 cultures. Fgf-8 thus appeared to have opposing effects on *Gsc* depending on the distance from the source. Clearly a signal from the Fgf-

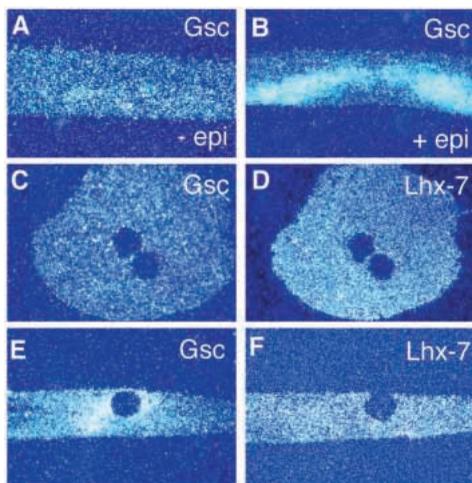


Fig. 5. Maintenance of *Gsc* expression by Endothelin-1. ³⁵S in situ hybridisation of sections. (A-D) E10.5 mandibles cultured for 24 hours. (A) Mandible minus epithelium, showing loss of *Gsc* expression. (B) Mandible plus epithelium showing endogenous expression of *Gsc*. (C,D) E10.5 mandible minus epithelium plus Et-1-loaded bead, cultured for 24 hours. No expression of *Gsc* (C) or *Lhx-7* (D) induced by bead. (E,F) E11.5 mandible minus epithelium plus Et-1-loaded bead, cultured for 24 hours. (E) Upregulation of *Gsc* expression around the bead. (F) Corresponding serial section showing no effect on *Lhx-7* expression. $n=12-20$ for each case.

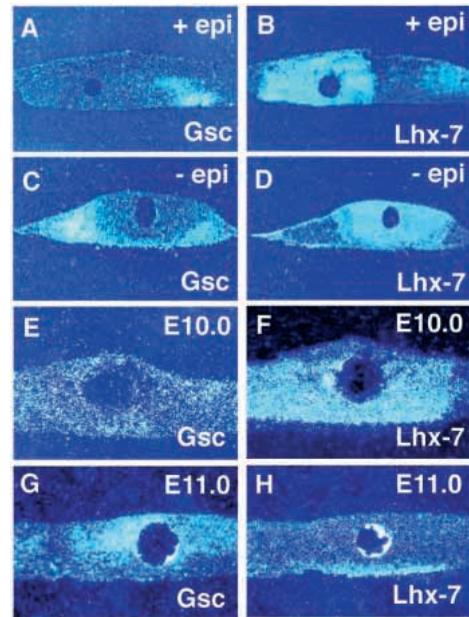


Fig. 6. Regulation of *Gsc* expression by Fgf-8. (A-D) E10.5 mandible explants cultured for 24 hours. (A,B) Mandible plus epithelium and Fgf-8-loaded bead on one side of culture. (A) Loss of *Gsc* expression around the bead. (B) Serial section showing corresponding upregulation of *Lhx-7* around the bead. (C,D) Mandible minus epithelium plus Fgf-8-loaded bead. (C) No *Gsc* expression around the bead but upregulated expression at far margins. (D) Serial section showing corresponding upregulation of *Lhx-7* around the bead. (E-H) Caudal mandibular arch mesenchyme and epithelium plus an Fgf-8 bead cultured for 48 hours. (E) E10.0 mandible showing inhibition of *Gsc* around the bead. (F) Serial section showing induction of *Lhx-7* around the bead. (G) E11.0 mandible showing induction of *Gsc* around the bead on the aboral side. (H) Serial section showing no induction of *Lhx-7* around the bead. Note the reversal of gene induction of *Lhx-7* and *Gsc* between these two stages. In H induction of *Lhx-7* can be seen at the oral margin of the explant, and *Gsc* expression is still repressed in this region. $n=12-20$ for (A-D) and $n=8$ for (E-H).

8 bead is being transmitted to the outer edges of the explants since there was no *Gsc* expression in the explants without a bead. An explanation for the observed dual role of Fgf-8 on *Gsc* expression could be that low concentrations of Fgf-8 induce expression whereas higher concentrations repress. Alternatively, Fgf-8 may induce *Gsc* expression but high levels of *Lhx-7*, which is also induced by Fgf-8, may repress *Gsc*.

To investigate these two possibilities Fgf-8 beads were implanted into caudal (aboral) halves of mandibles at E10.0 and E11.0 and cultured for 24 hours. If the *Lhx-6/-7* genes act to repress *Gsc* expression it was expected that *Gsc* would be induced around the bead in E11.0 caudal mesenchyme, which was not competent to express *Lhx-6/-7* genes, but not in E10.0 caudal mesenchyme, which was competent to express *Lhx* genes. At E10.0 the caudal mesenchyme was able to activate *Lhx-7* expression around the bead, as shown in Fig. 3, while *Gsc* expression was excluded from this region (Fig. 6E,F). Twenty-four hours later at E11.0 the caudal mesenchyme was no longer competent to activate *Lhx* gene expression in response to Fgf-8 and instead high levels of *Gsc* were seen

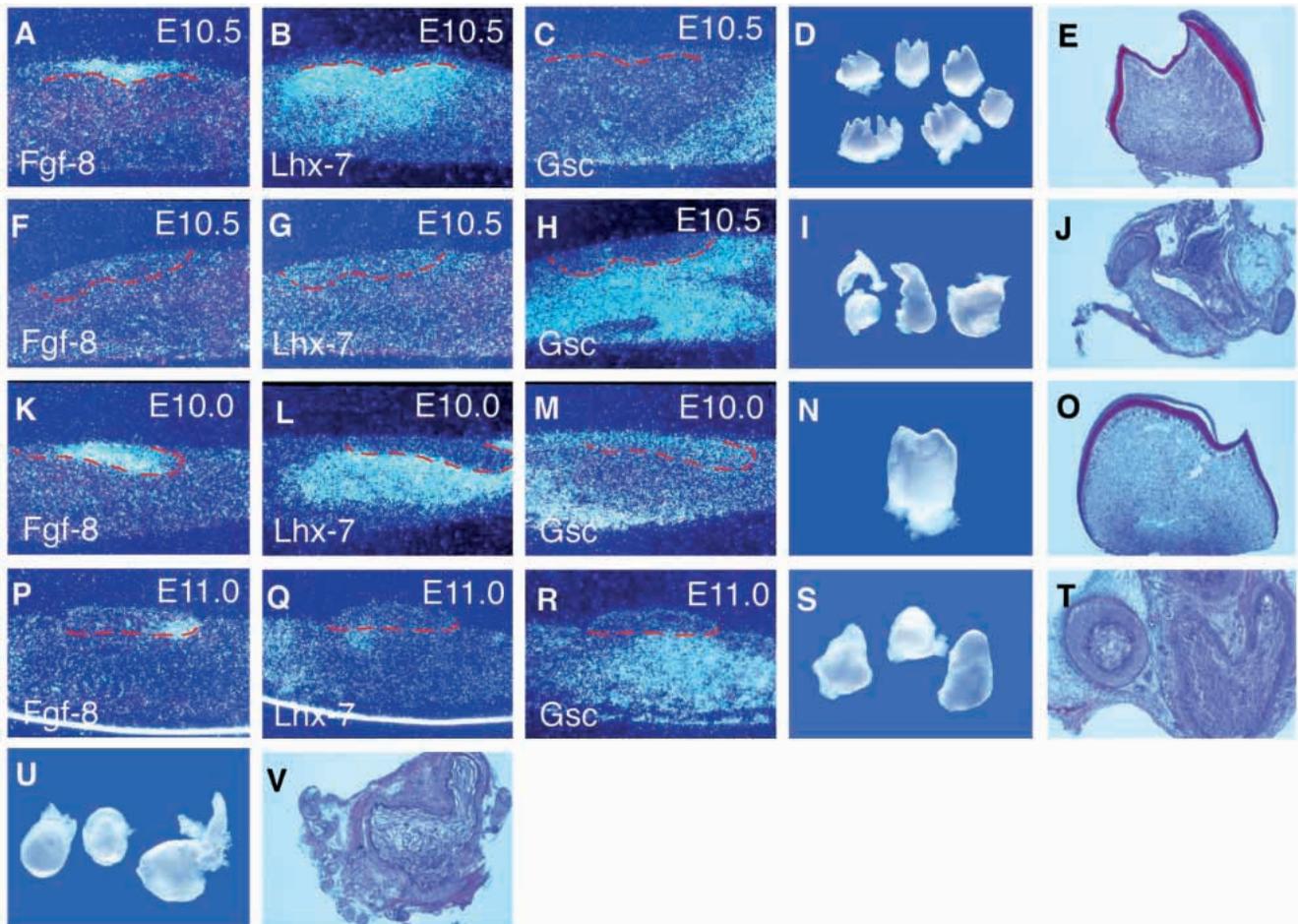


Fig. 7. *Lhx-7* expression corresponds to tooth development. (A-C,F-H,K-M,P-R) ^{35}S in situ hybridisation. Mandible explants cultured for 48 hours. (A,F,K,P) *Fgf-8* expression. (B,G,L,Q) *Lhx-7* expression. (C,H,M,P) *Gsc* expression. (D,E,I,J,N,O,S,T,U,V) Explants cultured under a kidney capsule for 12 days after culturing in vitro for 48 hours, whole and sectioned. (A-E) Rostral mesenchyme and epithelium at E10.5, showing high expression of *Fgf-8* in the epithelium (A), high expression of *Lhx-7* in the mesenchyme (B) and low *Gsc* expression in the mesenchyme (C), resulting in tooth formation (D,E). (F-J) Caudal mesenchyme and epithelium at E10.5, showing no expression of *Fgf-8* in the epithelium (F), low expression of *Lhx-7* in the mesenchyme (G), and high expression of *Gsc* in the mesenchyme (H), resulting in cartilage and cyst formation (I,J). (K-T) Caudal mesenchyme and rostral epithelium. (K-O) E10.0 recombinations, showing high expression of *Fgf-8* in the epithelium (K), high expression of *Lhx-7* induced in the mesenchyme (L) and low *Gsc* expression in the mesenchyme (M) resulting in tooth formation (N,O). (P-T) E11.0 recombinations, showing expression of *Fgf-8* in the epithelium (P), failure to induce expression of *Lhx-7* in the mesenchyme (Q), and high expression of *Gsc* in the mesenchyme (R), resulting in cartilage and cyst formation (S,T). Note the reversal of gene induction of *Lhx-7* and *Gsc* by the *Fgf-8*-expressing epithelium between these two stages. In R high levels of *Gsc* expression can be seen near to the expression domain of *Fgf-8* in the epithelium. (U,V) Caudal mesenchyme and epithelium plus a *Fgf-8* bead implanted at E10.0. Cysts and cartilage form despite induction of high levels of *Lhx-7* and repression of *Gsc* (see Fig. 6E,F). Red dashed lines outline the epithelium.

around the bead (Fig. 6G,H). Thus it would seem that the repression of *Gsc* by *Fgf-8* can only occur in conjunction with induction of *Lhx-6/-7*.

***Lhx* expression correlates with but is not sufficient for odontogenesis**

The highly restricted expression of *Lhx-6/-7* genes to presumptive odontogenic mesenchyme suggested they have an important role in determining the fate of mesenchymal cells of the first arch as odontogenic. If this is the case then the induction of *Lhx-6/-7* gene expression by an epithelial source of *Fgf-8* should correlate with acquisition of odontogenic capacity.

Recombinations between E9.0 mandibular arch rostral and

caudal epithelium and mesenchyme were previously carried out by Lumsden (1988). We wished to determine whether induction of *Lhx-6/-7* gene expression correlated with the capacity of the resulting recombined explant to form teeth. Explants from E10.0 to E11.0 embryos were manipulated and after culture for 48 hours were either sectioned for in situ hybridisation or implanted under the kidney capsules of adult mice to provide suitable conditions for development of teeth (Kratochwil et al., 1996). When rostral (oral) epithelium was recombined with rostral mesenchyme at E10.5, *Lhx-6/-7* gene expression was induced in the mesenchyme underlying the *Fgf-8*-expressing epithelium and the expression of *Gsc* was limited to the borders (Fig. 7A-C). In this recombination teeth formed with high efficiency in kidney capsules (20 teeth out

of 16 half mandibles implanted) as previously demonstrated by Lumsden with E9.0 explants (Lumsden, 1988; Fig. 7D,E). When caudal epithelium was recombined with caudal mesenchyme at E10.5, *Lhx-6/-7* expression was not induced and high levels of *Gsc* were observed (Fig. 7F-H), resulting in the formation of cysts and arches of cartilage and bone after culture under kidney capsules (0 teeth out of 11 half mandibles implanted) (Fig. 7I,J). In contrast to the experiment shown in Fig. 5C, where Et-1 was unable to maintain *Gsc* expression at E10.5, addition of caudal epithelium to caudal mesenchyme did result in *Gsc* expression being maintained (Fig. 7H).

Recombinations of rostral (oral) epithelium with caudal (aboral) mesenchyme at E10.0 resulted in induction of *Lhx-6/-7* gene expression in the mesenchyme underlying the *Fgf-8*-expressing epithelium and low levels of *Gsc* at the borders of the culture furthest away from the epithelium (Fig. 7K-M). In this recombination teeth developed after culture under kidney capsules (4 teeth out of 5 half mandibles implanted; Fig. 7N,O) again confirming the data of Lumsden (1988) for E9.0 explants. However, when the same recombination was carried out just one day later at E11.0 *Lhx-7* was no longer induced in the caudal (aboral) mesenchyme by the *Fgf-8* expressed in the epithelium, and in its place high levels of *Gsc* expression were observed, particularly under the *Fgf-8* expression domain (Fig. 7P-R). In this case no teeth formed and cysts developed (0 teeth out of 10 half mandibles implanted) (Fig. 7S,T). Thus there is a dynamic shift of gene expression induced by oral epithelium in caudal (aboral) mesenchyme during just 12 hours of development, with this shift in expression having a profound effect on tooth forming ability. Induction of *Lhx-6/-7* gene expression thus correlates with the ability of the mesenchyme to support tooth development.

High *Lhx-7* expression and low *Gsc* expression, which is required for tooth formation, were also seen in intact caudal (aboral) cultures at E10.0 when an *Fgf-8* bead was added (see Fig. 6E,F). In this case, however, no teeth developed when cultured under kidney capsules and only cysts were seen (0 teeth out of 7 half mandibles implanted; Fig. 7U,V). Similarly recombinations between limb bud epithelium and mandibular mesenchyme which induce *Lhx-6/-7* gene expression were shown not to support tooth development (data not shown). Thus although *Lhx* gene expression correlates with the ability of mesenchymal cells to support tooth development, it is not sufficient for odontogenesis.

DISCUSSION

It is well established that teeth develop via interactions between oral epithelium and underlying ectomesenchyme cells (reviewed by Thesleff et al., 1995; Thesleff and Sharpe, 1997). Recombination experiments have determined that the initiation of tooth development requires oral epithelium and that other embryonic epithelium will not support tooth

development. However the source of ectomesenchymal cells is less specific and teeth can develop from neural crest-derived cells of non-first branchial arch origins (Mina and Kollar, 1987; Lumsden, 1988). This indicates that odontogenic capacity is present in ectomesenchymal cells of all branchial arches and that the signals to initiate odontogenesis are localised in oral epithelium. Prespecification of cranial neural crest cells as odontogenic can therefore not be assumed. Since there is a demarcation between the rostral/caudal locations of ectomesenchymal cells in the mandibular arch and their subsequent development into either odontogenic (rostral) or skeletogenic (caudal), we investigated the molecular mechanisms that determine these two populations.

We confirmed previous results showing that oral epithelium is the source of signals that initiate tooth development (Lumsden, 1988) and in addition show that at E10.0, all

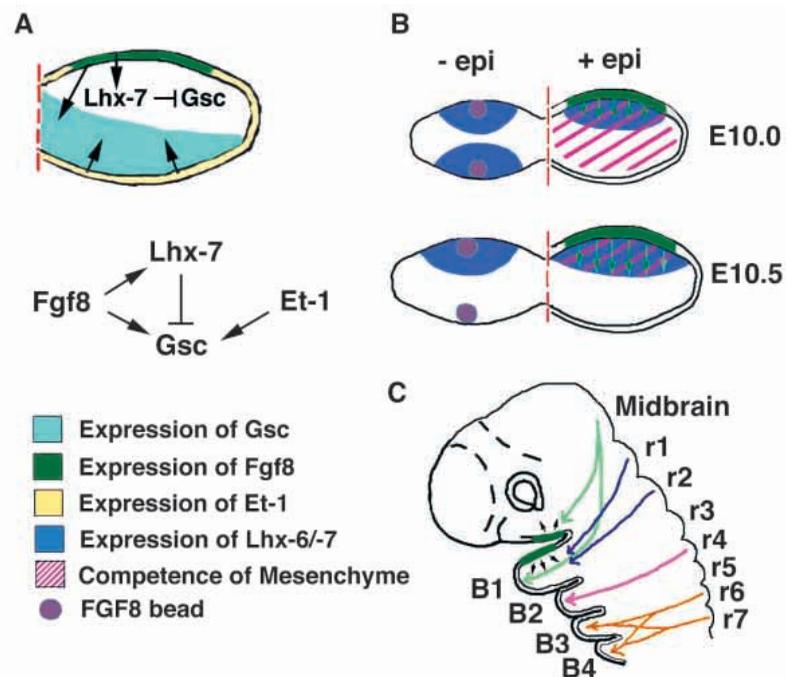


Fig. 8. Schematic diagram of signalling molecules acting within the first branchial arch and neural crest migration. Key refers to A and B. (A) Pathways involved in restricting the expression pattern of *Gsc* to caudal (aboral) mesenchyme. *Fgf-8* in the rostral epithelium induces *Lhx-7* expression in the rostral mesenchyme. In the rostral mesenchyme *Lhx-7* inhibits *Gsc* expression, limiting its expression to the most caudal mesenchyme. The expression of *Lhx-7* is maintained by *Fgf-8*, while the expression of *Gsc* is maintained by Et-1. (B) Competence changes in the mesenchyme between E10.0 and E10.5. Left side shows experimental results involving the removal of the epithelium and induction of *Lhx-6/-7* by *Fgf-8* beads. Right side shows changes in competence of the mesenchyme to respond to the *Fgf-8* signal (shown as green arrows). At E10.0 all the mandible cells are equicompetent to express *Lhx-7*, indicating that the ectomesenchyme is not prespecified. 12 hours later at E10.5 only the rostral cells are now able to respond. (C) Migration pathways of cranial neural crest, indicated by coloured arrows. The neural crest cells of the first branchial arch are made up of two populations. The most distal cells are derived from midbrain neural crest which are patterned in situ by *Fgf-8* from the oral epithelium. The most proximal cells are derived from rhombomeres 1 and 2 and may be prepatterned by *Hoxa-2* expression. The different migration paths of the distal and most proximal neural crest cells of the first branchial arch may indicate the different evolutionary origins of these regions.

ectomesenchymal cells of the mandibular arch are competent to respond to signals from the oral epithelium and form teeth (Figs 3, 7). We have identified Fgf-8 as the molecule expressed in oral epithelium which is likely to be responsible for establishing the rostral restricted expression of *Lhx-6/7* and may also be responsible for the caudal restricted expression of *Gsc* (Figs 6, 7). *Fgf-8* and *Fgf-9* are co-expressed in oral epithelium and limb bud epithelium (Kettunen and Thesleff, 1998; Martin, 1998) and in all our assays Fgf-9 behaved exactly as Fgf-8. *Fgf-9*, however, is expressed slightly later than *Fgf-8* and thus is not such a strong candidate for the endogenous inducer of *Lhx-6/7* (data not shown). Other Fgfs, such as *Fgf-4*, are not expressed in the oral epithelium at these early stages (Kettunen and Thesleff, 1998).

At E10.0 caudal ectomesenchyme cells are able to respond to Fgf-8 and activate ectopic *Lhx-6/7* expression in vitro. Since in vivo, these cells do not express *Lhx-6/7*, there must be some mechanism which is acting to restrict their expression to the rostral (oral) cells of the mandible. Fgf receptors are widely distributed throughout the developing mandibular arch, so their expression patterns will have little effect on defining the expression patterns of Fgf inducible genes (Orr-Urtreger et al., 1991, 1993; Peters et al., 1992, 1993; Yamaguchi et al., 1992). One possibility is that *Gsc* expression, which is restricted to the caudal region, might inhibit *Lhx-6/7* expression, and thus prevent the caudal cells from expressing these genes. This seems unlikely from the expression patterns of the genes, since *Lhx-6/7* are expressed at least one day earlier than *Gsc*. Furthermore, an analysis of *Lhx* expression in the *Gsc* mutant showed that lack of *Gsc* does not affect the expression pattern of *Lhx-7* although the possible roles of other *Gsc*-like genes cannot be excluded (Fig. 4A,B). Another possibility is that the Fgf-8 signal can only induce the *Lhx* genes at high concentrations, close to the source, and we have shown that the expression of *Lhx-7* does indeed respond in a graded fashion to different concentrations of Fgf-8 (Fig. 4C-E). Thus in caudal (aboral) regions the concentration of Fgf-8 would be too low to cause induction in vivo. Such concentration effects of the Fgfs have previously been noted in *Xenopus* animal cap assays (Green et al., 1991). Fgfs, however, may not be able to diffuse over such a large range and this mechanism of apparent concentration dependence might be occurring through some form of signal relay. An alternative explanation is that Fgf inhibitors such as Sprouty may be present in restricted areas of mandibular arch mesenchyme (Chi et al., 1998).

The expression of *Gsc* in the caudal mandible is lost after removal of the epithelium at E10.5 (Fig. 5A,B). Thus there must be an inducer of *Gsc* present in the epithelium. This inducer was found not to be Endothelin-1 (Et-1), despite loss of *Gsc* expression in *Et-A* receptor knockout mice (Clouthier et al., 1998) (Fig. 5C). Et-1, however, was shown to be able to maintain *Gsc* expression from E11.5 (Fig. 5E). In comparison, caudal epithelium was able to maintain expression of *Gsc* at E10.5 (Fig. 7H), suggesting that another factor is produced by caudal epithelium at E10.5 that acts with Et-1 to maintain *Gsc* expression. In vivo, *Fgf-8* and *Gsc* are expressed at a distance from each other in the first branchial arch and around the pharyngeal pouches (Gaunt et al., 1993; Crossley and Martin, 1995) (Fig. 1). These expression patterns imply a role for Fgf-8 in defining the expression pattern of *Gsc*. Supporting this we have shown that Fgf-8 is able to repress the expression of *Gsc*

in intact mandibular cultures (Fig. 6). In the region of *Gsc* repression *Lhx-7* is induced, implying that the repression of *Gsc* by Fgf-8 may be acting indirectly via induction of *Lhx-7*. This again is supported by the expression patterns of the three genes.

When the epithelium was removed from mandibular cultures, Fgf-8 appeared to be able to induce *Gsc* expression at a distance from the bead (Fig. 6C). The ability of Fgf-8 to induce *Gsc* expression was only seen when *Lhx-7* was not expressed. Thus, induction of *Gsc* around an Fgf-8 bead at E10.0, in caudal mesenchyme cultured for 24 hours, is prevented by the prior induction of *Lhx-7*, while induction of *Gsc* can occur around an Fgf-8 bead in E11.0 caudal cultures since at this stage no *Lhx-7* is induced (Fig. 6E-H). Since *Gsc* is only repressed by Fgf-8 in the presence of *Lhx-7*, this supports the view that *Lhx-7*, not Fgf-8, is the direct repressor of *Gsc* expression in vivo. *Lhx-7*, thus, acts to restrict *Gsc* expression to caudal regions of the mandible. This is detailed schematically in Fig. 8A.

Competence of caudal ectomesenchymal cells to respond to Fgf-8 and express *Lhx-6/7* is lost by E10.5 (Fig. 3). Thus within a period of 12 hours there is a dramatic change in the properties of the caudal mandibular arch mesenchymal cells. The changes in competence of the first arch mesenchyme to respond to Fgf-8 are indicated schematically in Fig. 8B. This loss in competence coincides with the activation of *Gsc* expression in the caudal half of the mandible. Thus although *Gsc* does not act to define the initial rostral-caudal border of expression of the *Lhx-6/7* genes it may well play a role in altering the competence of caudal mesenchyme to respond to an Fgf-8 signal at a later time.

Recombination experiments had previously indicated that tooth development could be supported in vitro by neural crest-derived cells of different axial origins (Lumsden, 1988). Our results confirm this by showing that up to E10.0 all mesenchymal cells of the mandibular arch are equally responsive to instructive epithelial signals such as Fgf-8 and can develop into teeth (Fig. 7). The origin of neural crest cells that populate the distal mandibular arch has been mapped to the midbrain (Imai et al., 1996). Thus the fate of the midbrain neural crest cells is not prespecified but instead is dependent upon the signals received from the branchial arch epithelium. This agrees with grafting experiments involving early and late mesencephalic neural crest in the chick (Baker et al., 1997). Fate mapping studies have shown that the most proximal cells of the mandibular arch have a different neural crest origin and migrate from rhombomeres 1 and 2 (Köntges and Lumsden, 1996). This population of neural crest cells forms the skeletal elements of the jaw articulation and inner ear. It is this most proximal region of the first arch which is duplicated in *Hoxa-2* mutants and in the grafting experiments of Noden (Rijli et al., 1993; Gendron-Maguire et al., 1993; Noden, 1983). In both these experiments the distal elements were not affected thus suggesting that the populations of neural crest cells that populate the distal and proximal mandibular arch may have different characteristics which may be related to their axial origins (Fig. 8C).

After E10.0 we show that the caudal mesenchyme is no longer able to form teeth when combined with oral epithelium. The change in odontogenic competence coincides with changes in expression of *Gsc* and *Lhx-7*. Upregulation of *Lhx-*

7 and loss of *Gsc* alone, however, is not able to trigger tooth development. Thus, although *Lhx-7* expression correlates with odontogenesis it is not sufficient for it (Fig. 7).

The role of other oral/dental epithelial signals

Fgf-8 is not the only signalling molecule present in oral epithelium. *Bmp-4* and *Shh* are both expressed early in oral epithelium in regions which overlap those of *Fgf-8* (Aberg et al., 1997; Iseki et al., 1996; Grigoriou et al., 1998). *Bmp-4* has been shown to be involved in upregulating the expression of several different ectomesenchymal genes including *Msx-1* (Vainio et al., 1993; Chen et al., 1996; Tucker et al. 1998). *Bmp-4* has been suggested to act antagonistically with *Fgf-8* to produce localised sites of ectomesenchyme which express *Pax-9* and specify where teeth will develop (Neubüser et al., 1997). *Bmp-4* does not induce or repress expression of either *Lhx-6/-7* or *Gsc*, indicating that the role of *Bmp-4* is in positioning of tooth development and not in rostral-caudal patterning (data not shown).

Shh is expressed in oral epithelium and subsequently localised to very small sites which correspond to presumptive dental epithelium (Bitgood and McMahon 1995, Iseki et al., 1996; Hardcastle et al., 1998). Genes in the *Shh* pathway such as *Ptc* and *Gli* are expressed in underlying ectomesenchyme and also in dental epithelium and *Shh* is capable of inducing *Ptc* and *Gli-1* expression in mesenchyme cells (Hardcastle et al., 1998). *Shh* is not able to induce expression of other ectomesenchymal genes such as *Msx*, *Dlx*, *Barx-1*, *Lhx*, *Gsc* etc, its regulatory effects seem to be confined to genes recognised to be immediately downstream in the pathway (P. Sharpe, unpublished). Ectopic application of *Shh* protein to non-dental oral epithelium results in induction of bud formation suggesting *Shh* has a role in the physical formation of epithelial tooth buds and it has been suggested that this signalling pathways regulates epithelial cell proliferation in normal tissue and tumours (Bellusci et al., 1997; Dahmane et al., 1997; Hardcastle et al., 1998).

Given that different signals are present in oral epithelium it is not surprising that a single signal, *Fgf-8*, is unable to convert non-odontogenic tissue, such as caudal (aboral) mesenchyme to an odontogenic fate, and that for this oral epithelium is necessary. Thus the explanation for why *Lhx-6/-7* expression correlates with odontogenic capacity but is not sufficient is that other epithelial signals regulating other mesenchymal genes are also required.

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