The role of effectors of the activin signalling pathway, activin receptors IIA and IIB, and Smad2, in patterning of tooth development

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SUMMARY

The gene for activin βA is expressed in the early odontogenic mesenchyme of all murine teeth but mutant mice show a patterning defect where incisors and mandibular molars fail to develop but maxillary molars develop normally. In order to understand why maxillary molar tooth development can proceed in the absence of activin, we have explored the role of mediators of activin signalling in tooth development. Analysis of tooth development in activin receptor II and Smad2 mutants shows that a similar tooth phenotype to activin βA mutants can be observed. In addition, we identify a novel downstream target of activin signalling, the Iroquois-related homeobox gene, Irx1, and show that its expression in activin βA mutant embryos is lost in all tooth germs, including the maxillary molars. These results strongly suggest that other transforming growth factor β molecules are not stimulating the activin signalling pathway in the absence of activin. This was confirmed by a non-genetic approach using exogenous soluble receptors to inhibit all activin signalling in tooth development, which reproduced the genetic phenotypes. Activin, thus, has an essential role in early development of incisor and mandibular molar teeth but this pathway is not required for development of maxillary molars.

Key words: Activin, Activin receptors, Smad2, Irx1, Tooth development, Mouse

INTRODUCTION

Activin is a member of the transforming growth factor (TGF) β superfamily of extracellular signalling proteins whose effects are transduced intracellularly by Smads. Activin proteins are produced from two gene products, activin βA and activin βB that dimerise to form activin A (βA:βA), activin B (βB:βB) and activin AB (βA:βB) (Vale et al., 1990; Roberts et al., 1991; Roberts and Barth, 1994). Activins signal via cell surface serine-threonine kinase receptors that include type II ligand-binding receptors and type I signalling receptors. Once ligand is bound, the type II receptor heterodimerises with and phosphorylates a type I receptor (Attisano et al., 1992; Zimmerman and Mathews, 1996). This activates the type I receptor, which leads to phosphorylation of specific cytoplasmic Smad proteins (Massague and Chen, 2000). These Smads translocate to the nucleus where they are recognised by specific transcription factors.

Despite a wealth of evidence implicating activins in mesoderm formation during gastrulation, targeted inactivation of either the activin βA or βB genes or both in mice does not affect mesoderm formation but does have profound effects on craniofacial development (Matzuk et al., 1995b). The most striking aspect of the craniofacial phenotype is in the activin βA mutant mice, where development of teeth is differentially affected (Ferguson et al., 1998). Incisors and mandibular molar teeth fail to develop beyond a rudimentary bud in activin βA mutant mice, whereas maxillary molar teeth develop normally. Expression of activin βA is first detected at E10.5 in the pre-odontogenic mesenchyme at the sites of future tooth formation. It subsequently becomes expressed in condensing mesenchyme at the bud stage. The expression of activin βA during early tooth development is the same in all tooth types, yet maxillary molar teeth are unaffected in the absence of activin. Activin is required before E11.5 for incisors and mandibular molar teeth to progress beyond bud formation, as was shown by the ability of exogenous activin protein to rescue tooth development in mutant tooth explants (Ferguson et al., 1998).

There are two possibilities that could account for the activin βA tooth phenotype: either another TGFβ molecule is acting...
to activate signalling via activin receptors or downstream effectors such as Smads, or completely different signalling pathways are responsible for maxillary molar development. We have investigated the possibility that an alternative TGFβ signalling cascade is used redundantly to allow for maxillary molar development in these mutants. We also report a newly identified ‘downstream’ target gene revealed by in situ hybridisation analysis of gene expression patterns in mutant tooth buds.

MATERIALS AND METHODS

Generation of mutant mouse embryos

Activin βA mutants were generated using a heterozygote colony, the maintenance, matings and genotypings of which were carried out as described previously (Ferguson et al., 1998). Compound activin receptor IIA and IIB heterozygote/homozygote mice were generated by interbreeding of IIA+/– (Acvr2b+/– – Mouse Genome Informatics) and IIB+/– (Acvr2b+/– – Mouse Genome Informatics) mice as described previously (Song et al., 1999). Smad2 (Madh2 – Mouse Genome Informatics) heterozygotes (Smad2+/+/-) were generated and genotyped as described previously (Nomura and Li, 1998). For histology, embryos were fixed in 4% paraformaldehyde, wax embedded and serially sectioned. The heads of newborn mice were decalcified in 0.5 M EDTA (pH 7.6) after fixation, then double embedded, serially sectioned and stained using Alcian Blue/chlorontine Fast Red.

Explants cultured with soluble activin receptors

Mice of the CD-1 strain were used. Timed matings were set up such that noon of the day on which vaginal plugs were detected was considered to be E0.5. Mandibles and maxillae from embryos at E10.5 were dissected in D-MEM with glutamax 1 (Gibco BRL; see Fig. 4). To accurately assess the age of embryos, somite pairs were counted by another TGFb ligand

RESULTS

Irx1 expression is downstream of activin signalling

In order to determine the possible role of other TGFβ molecules in compensating for activin A function in maxillary molar development, we needed to identify downstream targets of activin signalling. An in situ hybridisation screen of activin βA mutant embryos was carried out with known developmentally regulated genes. Irx1, a member of the Iroquois family of homeobox genes was identified as being downregulated in activin βA mutant embryo tooth germs. In wild-type embryos, Irx1 is reported to be expressed in the epithelium of the first and second branchial arches at E9.5 (Bosse et al., 1997). However, a careful reanalysis of Irx1 expression within the branchial arch region at E9.5 (Fig. 1A-C) revealed that Irx1 expression was found in the epithelium of the branchial clefts between arches 1 and 2, and it was excluded from the oral epithelium of the 1st branchial arch. By E10.5-11, Irx1 was strongly expressed in the oral epithelium (Fig. 1D,E) which coincides with the stage at which dental epithelial thickening becomes prominent and when activin βA expression is detectable (Fig. 1F-I). Expression was found in the dental epithelium of all developing teeth and persisted at least until E14.5 (Fig. 1J-M). Irx1 expression was found to be absent in the dental epithelium in activin βA mutant embryos (Fig. 2), indicating that the activin signalling pathway is required for expression. Significantly, Irx1 expression was also lost in maxillary molar dental epithelium in the mutants (Fig. 2F). Loss of Irx1 was specific to the dental epithelium: in wild-type embryos, Irx1 was also expressed in the cephalic mesenchyme around the optic vesicle and in the superficial dermis of the head. Expression in these tissues in the mutants is not affected (compare Fig. 2B,F with 2A,E; arrows).

In common with activin βA, expression of an activin signalling target, Irx1, is not required for development of maxillary molar teeth. The loss of Irx1 expression in maxillary molar dental epithelium in activin βA mutants implies that the activin signalling pathway is not being activated in this tissue by another TGFβ molecule.

Tooth development in activin receptor and Smad2 mutants

To genetically test the possibility that another TGFβ ligand compensates for activin in maxillary molar development by activating the activin signalling pathway, we examined tooth development in activin receptor and Smad2 mutant mice.

Targeted mutations in individual activin receptor (ActR) genes have been produced (Matzuk et al., 1995a; Oh, 1997; Gu et al., 1998; Gu et al., 1999). ActRIB−/− mice, which have a more severe head phenotype than ActRIIB−/− mice, show anterior head defects such as a hypoplastic mandible (22%
penetrance), which can result in secondary defects such as a lack of incisors (Matzuk et al., 1995a). ActRIIB−/− mice have no mandibular or tooth defects but exhibit cleft palate at low penetrance, which varies according to the genetic background (Oh, 1997). Comparison of these phenotypes with that of activin βA mutants indicates that there is functional compensation between the two type II receptors.

Because the activin signal is transduced via dimers of ActRI with ActRIIA or ActRIIB, a knockout of both type II receptor genes should be sufficient to disrupt signalling by activin as well as by any potential compensatory TGFβ molecules binding to these receptors. We have recently generated mice carrying mutations of both ActRIIA and ActRIIB genes (Song et al., 1999). The compound mutants, ActR[IIA+/−IIB−/−] and ActR[IIA−/−IIB+/−] all die in utero before E10.5, before the onset of activin βA expression in tooth development. However, ActR[IIA+/−IIB−/−] mice survive until birth and tooth development could thus be analysed in these animals.

A total of seven ActR[IIA+/−IIB−/−] newborn mice were examined by serial sectioning of the heads. We found that two out of the seven had an obvious tooth phenotype where incisors and mandibular molars were absent and maxillary molars were present (Fig. 3A,D), while the other five had apparently normal molars. Six of the seven had cleft palates. This tooth phenotype was thus the same as that observed in activin βA mutants.

Activin signalling is transduced intracellularly by the phosphorylation of Smads, and Smad2 has been identified as the specific Smad involved in the activin pathway (Graff et al., 1996; Macias-Silva et al., 1996; Baker and Harland, 1996; Zhang et al., 1996). It can therefore be argued that any induction of activin signalling by other TGFβ molecules in the absence of activin would be expected to require Smad2. Moreover if Smad2 does transduce the activin signal, we might expect that tooth development in Smad2 mutants would be affected in a similar way to that in activin βA and activin receptor mutants. We generated a null mutation in Smad2 locus and found that homozygous embryos died at E10 (Nomura and Li, 1998). Interestingly Smad2+/− embryos exhibited a range of

Fig. 1. Expression patterns of Irx1 and activin βA in wild-type heads as seen by DIG whole-mount (A-E) and radioactive section (F-M) in situ hybridisation analyses. (B,C) Frontal vibratome sections through the branchial arches of the embryo in A. (E) Frontal vibratome section through the branchial arches of the embryo in D. At E9.5, DIG-labelled Irx1 probe localises to the branchial arch region (A), labelling the epithelium of the branchial clefts and more posterior arches (C). It is excluded from the oral epithelium of the first branchial arch (B). By E10.5-E11, Irx1 is strongly expressed in the oral epithelium of the first branchial arch (white arrowhead, D: E). Radioactive in situ hybridisation on wax sections was used to analyse the Irx1 expression in the developing teeth at E11 (F,G), E12.5 (J), E13.5 (L) and E14.5 (M). Irx1 expression can be found in the dental epithelia of the incisors (J) and molars (F,G,L,M). Analysis of consecutive serial sections using a 35S-labelled activin βA probe shows that the expression of these two genes is juxtaposed in the developing teeth. Activin βA is expressed in the odontogenic mesenchyme underlying the Irx1-positive dental epithelium at E11 (white arrowheads, H) and E12.5 (K). (F,I) Corresponding brightfield illumination of the sections in G,H, respectively, shows the histological detail. ba1, first branchial arch (mandibular process); bc1, first branchial cleft; bc2, second branchial cleft; oe, oral epithelium; mand, mandible; max, maxilla; tg, tongue.
phenotypes with different severity: some exhibited gastrulation defects, a phenotype reminiscent of the homozygotes, whereas in embryos that gastrulated normally, 10% had craniofacial defects. Smad2 thus acts in a dose-dependent manner. We analysed a total of eleven Smad2+/− pups all of which had some craniofacial abnormalities and three had obvious tooth defects. The tooth phenotype in the three different mutant pups was identical, with no evidence of any incisors or mandibular molars but normal maxillary molars (Fig. 3C,F). Moreover, the obvious abnormal oral cavity in these three pups was identical to that in the ActR[IIA+/−IIIB−/−] animals (Fig. 3D).

**Soluble activin receptors block activin signalling and phenocopy the activin A mutants**

The fact that ActR[IIA+/−IIIB−/−] and Smad2+/− tooth phenotypes are the same as the activin βA−/− phenotype, is consistent with the idea that the pathway is not active in maxillary molar tooth development. However, because in both these mutants the tooth phenotype was variably penetrant and there was always one functional allele present in the animals, there is the possibility that another TGFβ molecule might be activating the pathway. It has been shown for example, that bone morphogenetic proteins (BMPs) can bind to activin receptors (Yamashita et al., 1995) and that truncated activin receptors can block BMP signalling in *Xenopus* embryos (Chang et al., 1997; New et al., 1997). In order to eliminate this possibility entirely, we used soluble forms of activin RIIA and RIIB receptors to sequester all ligands present in early tooth germs that might bind to these endogenous receptors and activate the pathway.

Two sets of experiments were performed using different sources of soluble receptors. In the first set, a cell supernatant from RIIB-expressing COS cells was used containing an unknown concentration of soluble receptors (Table 1). In the second set of experiments, commercially available (R&D Systems), purified RIIA and RIIB of known concentration were used (Table 2).

**Fig. 2.** *Irx1* expression is absent from tooth germs in activin βA mutants. Radioactive in situ hybridisation was used to compare *Irx1* expression in frontal sections of wild-type (+/+, A,E) and mutant (−/−, B,D) heads. *Irx1* expression is lost specifically from the epithelia of all incisor (B) and molar tooth buds (F) in mutants. Expression in non-odontogenic tissues such as the superficial dermis of the skin is, however, normal (white arrows, A,B,E,F). The position of the incisor and molar tooth buds in the mutant tissue is indicated by broken orange lines on the darkfield photomicrographs (B,F), corresponding brightfield (bf) photomicrographs (C,G, black arrowheads) and mesenchymal Pax9 expression in consecutive serial sections (D,H), which underlies the oral epithelium and is unaffected in the activin βA mutants. Note that bud stage tooth germs of wild-type (E13.5) and mutant heads (E14.5) are compared here.

**Fig. 3.** Tooth phenotype in newborn heads of ActR[IIA+/−IIIB−/−] (A,D) and Smad2+/− (C,F) pups. Corresponding frontal sections of wild-type littermates are shown in B,E. Both maxillary (A,C) and mandibular incisors (data not shown) are missing from the heterozygotes. Mandibular molars are lost from the heterozygotes in D,F, whereas maxillary molars develop normally. mol, molar; nc, nasal cartilages; ns, nasal septum; oc, oral cavity; tg, tongue. Note that the oral cavity in the heterozygotes is grossly mis-shapen. Furthermore, remnants of bone (arrowheads, asterisk) and muscle masses take the place of the mandibular bone.
From previous studies we have identified that activin is required early, before E11.5, in order to get normal tooth development (Ferguson et al., 1998). The soluble receptor(s) was added to maxillary and mandibular explants of E10.5 mice in culture, the stage when odontogenesis is initiated and just before activin βA expression is first detected (Fig. 4). After 2 days in culture, molar tooth germs were dissected and transferred to the renal capsules of host mice where they were grown for 12 days to allow fully mineralised teeth to develop. Table 1 and Table 2

### Table 1. Numbers of molar teeth retrieved from kidney capsule grafts after treatment with supernatant containing Activin RIIIB

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<thead>
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<th></th>
<th>Controls</th>
<th>+ Activin RIIIB</th>
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<tr>
<td>Maxillary molar explant</td>
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<td>15/14</td>
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<tr>
<td>Mandibular molar explant</td>
<td>11/13</td>
<td>3/16</td>
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### Table 2. Numbers of molar teeth retrieved from kidney capsule grafts after treatment with a combination of purified Activin RIIA and RIIIB at various concentrations

<table>
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<th>12.5µg/ml</th>
<th>35µg/ml</th>
<th>RIIA+RIIB</th>
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</thead>
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<tr>
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<td>7/6</td>
<td>7/6</td>
<td>5/6</td>
<td>12/12</td>
</tr>
<tr>
<td>Mandibular molar explant</td>
<td>7/6</td>
<td>3/6</td>
<td>3/8</td>
<td>6/14</td>
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Fig. 4. The procedure followed for treatment of maxillary and mandibular explants with soluble activin receptors. A frontal view of an E10.5 embryo is shown. Representative molars and a cyst retrieved from the kidney capsule grafts are shown in section stained with Alcian Blue/chlorontine Fast Red. The molars have multiple cusps and a characteristic red-stained dentine layer, which is secreted by the underlying layer of odontoblasts. The amorphous cysts lack cusps and are epithelial, keratinising in nature rather than having cytodifferentiated odontoblasts and ameloblasts. ba2, second branchial arch; fnm, frontonasal mass; mand, mandibular process; max, maxillary process; np, nasal process.
show the numbers of molars retrieved from the kidney capsule grafts. Both sets of experiments produced similar results. All of the maxilla explants treated with soluble receptors developed fully mineralised teeth, as did the majority of control explants (85-100%). Mandibular explants treated with the soluble receptors produced large numbers of keratinised cysts. The combined results from Table 1 and Table 2 show that teeth were only observed in less than 30% of cases. Soluble activin receptors were therefore able to mimic the genetic phenotype and block mandibular molar tooth development while having no effect on maxillary molar development.

In the second set of experiments, radioactive in situ hybridisation analysis was carried out on one third of the explants treated for 2 days with commercially purified soluble receptors (Fig. 5, Fig. 6). Each explant was treated with a combination of soluble RIIA and RIIB at a concentration of either 0, 12.5 or 25 μg/ml. For each sample, adjacent sections through tooth germs were analysed for the expression of different genes: follistatin (Fst) (Ferguson et al., 1998) and Irx1 expression were analysed to assess the effects of this treatment on known downstream targets of activin signalling; Msx1 expression, a homeobox gene that is expressed in the oral mesenchyme around the epithelial tooth buds (Vainio et al., 1993) was used to show viability of the explants after 2 days treatment, and also to indicate the position of the tooth germs within the explants (Fig. 5B,F,I, Fig. 6B,G,L). Msx1 expression is unaffected in the activin βA mutants (Ferguson et al., 1998).

The expression of Fst and Irx1 was found to be downregulated in the dental epithelium of the treated explants, consistent with inhibition of activin signalling by the soluble receptors; however, this downregulation appeared to be dose dependent (Fig. 5, Fig. 6).

The lack of tooth development in treated mandibles was associated with the following gene expression profiles: on the addition of 12.5 μg/ml of each receptor, Fst was detectable but downregulated in tooth epithelium (compare Fig. 5H with 5D). At 25 μg/ml and higher concentrations of soluble receptors, Fst was not detectable in the dental epithelium of the explants (Fig. 5L). Irx1 expression, was also considerably reduced in dental epithelium after addition of soluble receptors at 12.5 and 25 μg/ml (Fig. 5C,G,K) but was not completely lost until concentrations of 35 μg/ml were used (data not shown). In maxillary explants, the expression of Fst and Irx1 showed similar downregulation after addition of soluble receptors (Fig. 6). The resulting tooth buds in all explants exhibited the expected morphology: the treated tooth buds in maxillary explants grew to the same size as controls, compared with the mandibular tooth buds in treated mandibles that do not seem to develop beyond the epithelial thickening stage after 2 days in culture.

In order to test the possibility that maxillary molar development may require TGFβ signalling independent of the activin pathway, we treated mandible and maxilla explants with higher concentrations of soluble receptors to encourage
nonspecific binding of ligands to the receptors. Explants treated with 35 µg/ml of soluble receptors produced five maxillary molars out of six transferred, whereas only three mandibular molars were recovered from eight transferred (Table 2). At 45 µg/ml of soluble receptors there was some evidence of toxicity, as the numbers of teeth recovered were low, with one maxillary tooth from four transferred and no mandibular molars from six transferred (data not shown). After 2 days in culture, explants treated with 45 µg/ml soluble receptors appeared smaller than controls, suggesting that may be slightly toxic and possibly responsible for the diminished survival of the renal capsule grafts. A proportion of the explants were kept aside for analysis by in situ hybridisation. In those treated explants that expressed Msx1, Irx1 expression was found to be abolished, as expected (data not shown). Thus, the explants that survived treatment with 45 µg/ml of soluble receptors resulted in the development of maxillary and not mandibular molar teeth.

**DISCUSSION**

**Irx1 is downregulated in activin βA mutants**

In an ongoing search for downstream targets of the activin signalling pathway, we have used radioactive in situ hybridisation to analyse gene expression in activin βA mutant heads. Analyses have revealed downregulation of expression of Irx1, an Iroquois-related homeobox gene, specifically in the dental epithelia of tooth germs in the mutants. As in the case of Fst, a previously identified target of activin signalling (Ferguson et al., 1998), Irx1 is downregulated in all tooth buds including maxillary molars.

In vertebrates, Iroquois-related homeobox genes are expressed in the distinct patterns in the developing nervous system and heart and are thought to be involved in pattern formation and tissue specification in these systems (Bellefroid et al., 1998; Bosse et al., 2000; Bosse et al., 1997; Christoffiels et al., 2000; Cohen et al., 2000; Gomez-Skarmeta et al., 1998; Goriely et al., 1999; Tan et al., 1999). Six murine Iroquois-related homeobox genes have been identified, Irx1 to Irx6 (Bosse et al., 2000; Bosse et al., 1997; Bruneau et al., 2000; Cohen et al., 2000), of which Irx1 to Irx3 and Irx6 are expressed in the branchial arch epithelium. However, no knockouts of these genes have been reported to date, and thus the role of Irx genes in mammalian development is not understood.

Odontogenesis involves a series of epithelial-mesenchymal interactions. Activin has been implicated as an essential early mesenchymal signal that we believe, through data collected from recombination experiments, has a crucial role in activating other mesenchymally expressed genes (Ferguson et al., 1998). To date, however, no mesenchymal targets have been identified. The fact that the epithelially expressed targets Fst and Irx1 are missing from maxillary molars suggests that their role is to modify the levels of activin signalling, rather than have a direct role in odontogenesis per se. A modulatory role has been demonstrated for follistatin, which has been reported as a specific antagonist of activin in other systems (Michel et al., 1993; de Winter et al., 1996). Moreover, we showed previously that Fst, which is expressed in the dental epithelium immediately overlying activin-expressing mesenchyme, can be induced directly by activin, and possibly acts to set up a sink to regulate the levels of activin protein/site of activin activity in the developing tooth germ (Ferguson et al., 1998). In the absence of activin βA it would not be required in maxillary molars. In the experiments where we treated explants with
soluble activin type II receptors, the effects of concentration on gene expression patterns showed that Fst expression was more sensitive than Irx1 to levels of activin signalling, as it was downregulated at a lower concentration of receptors than that required for loss of Irx1. This suggests that unlike Fst, Irx1 may not be a direct target of activin signalling. Furthermore, explant cultures of mandibular epithelium that was separated from mesenchyme at E10.5-E11, did not result in significant loss of Irx1 expression, suggesting that once established, Irx1 expression rapidly becomes independent of mesenchymal signals, including activin A (data not shown).

**Activin βA, activin receptor and Smad2 mutants have a common tooth phenotype**

We have shown that ActR[IIA+/--;IIB−/−] and Smad2−/− mice exhibit the activin βA−/− tooth phenotype, in which the incisors and mandibular molars are missing but the maxillary molars develop normally. Although this phenotype occurs with low penetrance, it is consistent with previous reports that these receptors and Smad2 do indeed mediate activin signalling (Mathews and Vale, 1991; Attisano et al., 1992; Graff et al., 1996; Macias-Silva et al., 1996; Baker and Harland, 1996; Zhang et al., 1996). Furthermore, that the maxillary molars are not affected, it is consistent with the idea that there is no redundancy between activin A and another TGFβ-like molecule that may use this pathway and allow development of these molars. Despite the consistency of the tooth phenotype in the three mutants, the presence of one functional allele in these receptor and Smad2 mutants, may be sufficient to allow the activin signalling pathway to be activated. As stated previously, early embryonic lethality of homozygous mutants prevents analysis of tooth formation in the absence of these two activins for maxillary molar development. Moreover, from the fact that they are involved in odontogenesis, is that they share an upstream activator: FGF8 has been shown to be upstream of Dlx and activin βA genes, in that these genes can be induced in mesenchyme surrounding an FGF8-soaked bead (Ferguson et al., 1998; Ferguson et al., 2000; Thomas et al., 2000). Quite why development of what are grossly very similar structures, molar teeth, is controlled by different genetic mechanisms on the upper and lower jaws remains to be addressed. One obvious possibility, however, is that this may reflect the different responses to FGF8 of the cranial neural crest cells that populate the mandibular and maxillary arches (Ferguson et al., 2000).

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