Sonic hedgehog in the pharyngeal endoderm controls arch pattern via regulation of Fgf8 in head ectoderm

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Abstract

Fgf8 signalling is known to play an important role during patterning of the first pharyngeal arch, setting up the oral region of the head and then defining the rostral and proximal domains of the arch. The mechanisms that regulate the restricted expression of Fgf8 in the ectoderm of the developing first arch, however, are not well understood. It has become apparent that pharyngeal endoderm plays an important role in regulating craniofacial morphogenesis. Endoderm ablation in the developing chick embryo results in a loss of Fgf8 expression in presumptive first pharyngeal arch ectoderm. Shh is locally expressed in pharyngeal endoderm, adjacent to the Fgf8-expressing ectoderm, and is thus a candidate signal regulating ectodermal Fgf8 expression. We show that in cultured explants of presumptive first pharyngeal arch, loss of Shh signalling results in loss of Fgf8 expression, both at early stages before formation of the first arch, and during arch formation. Moreover, following removal of the endoderm, Shh protein can replace this tissue and restore Fgf8 expression. Overexpression of Shh in the non-oral ectoderm leads to an expansion of Fgf8, affecting the rostral–caudal axis of the developing first arch, and resulting in the formation of ectopic cartilage. Shh from the pharyngeal endoderm thus regulates Fgf8 in the ectoderm and the role of the endoderm in pharyngeal arch patterning may thus be indirectly mediated by the ectoderm.

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Introduction

The first pharyngeal arch gives rise to the maxillary and mandibular prominences and subsequently to skeletal structures of the upper and lower jaws. Both jaws are ultimately derived from tissues of various embryonic origins; ectoderm covers the outer and oral surfaces of the first pharyngeal arch, whilst endoderm is continuous with the foregut internally. Cells of the cranial neural crest form the bulk of the first pharyngeal arch along with cells of mesoderm origin which are located in the core and give rise to the musculature (Francis-West et al., 1998).

The growth and expansion of these cell populations are strictly co-ordinated resulting in a set pattern of muscles and skeletal components (Graham and Smith, 2001).

In the mandible, this patterning is largely achieved by the specific spatial and temporal expression of homeobox-containing transcription factors within the neural crest-derived mesenchyme (Sharpe, 1995; Cobourne and Sharpe, 2003). Epithelial removal and protein bead implantation experiments in both Aves and mice have shown that the expression of homeobox genes in the mesenchyme is induced by instructive signals from the oral ectoderm. Fgf8 (fibroblast growth factor 8) and Bmp4 (bone morphogenetic factor 4) are signalling molecules expressed in the first pharyngeal arch at the critical point when patterning of the mandibular mesenchyme occurs. Fgf8 is expressed in the proximal oral ectoderm of the maxilla and mandible and has been shown to induce expression of the
homeobox genes Lhx6, 7, Dlx1, -2 and Barx1 (Grigoriou et al., 1998; Ferguson et al., 2000; Tucker et al., 1998a). In contrast, Bmp4, is expressed in the distal region of the mandible at E10 in the mouse and has been shown to induce expression of both Msn1 and Msx2 in the underlying distal mesenchyme and repress expression of Barx1 (Chen et al., 1996; Tucker et al., 1998b). If the expression of Fgf8 is specifically lost in the first arch, the arch is severely reduced in size and the expression of many homeobox genes lost (Trumpp et al., 1999). A complete loss of the arch can be observed after inhibition of Fgf signalling using pharmacological blockers (Wilson and Tucker, 2004). Fgf8 is therefore vital for both the survival and pattern of the first arch. Although much is known about how neural crest cells are patterned as a result of signals from the oral ectoderm, relatively little is known about how the domains of signalling molecules are established in the early ectoderm.

In the chicken embryo, Fgf8 is expressed in the lateral head ectoderm at HH stage 11, prior to the formation of a distinctive first pharyngeal arch. Fate mapping studies indicate that Fgf8-expressing cells at HH 11 are fated to occupy ectoderm of the first pharyngeal arch (Haworth et al., 2004; Shigetani et al., 2000). Additional studies also indicate that cells occupying the oral ectoderm are fated to do so prior to the onset of Fgf8 expression (Haworth et al., 2004). How Fgf8 expression is regulated in the oral ectoderm of the presumptive maxilla and mandible is not well understood, but evidence exists for a possible role of endoderm in patterning of the embryonic head (Withington et al., 2001). Transplantation and rotation of the pharyngeal endoderm at the level of the presumptive first pharyngeal arch in the developing chick have been shown to re-pattern skeletal structures of the jaw, suggesting that instructive signalling potential resides in the foregut endoderm (Couly et al., 2002). In addition, removal of endoderm underlying the presumptive first pharyngeal arch region at HH stage 9 results in a lack of Fgf8 expression in the ectoderm (Haworth et al., 2004). We have previously shown with chicken explants in vitro that a lack of neural crest does not affect the ability of the ectoderm to express Fgf8 (Haworth et al., 2004), which supports the findings of previous studies (Veitch et al., 1999). Taken together, the current data suggest that anterior endoderm signalling regulates the spatial expression of signals such as Fgf8 in the ectoderm. The reported effects of endoderm on craniofacial skeletal patterning might therefore result from an indirect role, with the endoderm controlling the development of the neural crest derived skeleton via the ectoderm.

One possible candidate molecule present in the foregut endoderm and responsible for signalling to the ectoderm is Sonic Hedgehog (Shh). Shh is a developmentally regulated signalling molecule important in the development of numerous body systems (Ingham and McMahon, 2001). In the chick, Shh is expressed in the anterior pharyngeal endoderm from HH stage 9 (Marcucio et al., 2005). Expression of Shh in the endoderm therefore precedes expression of Fgf8 in the over-lying ectoderm. A reduction in Shh signalling in mice, humans and chick can result in a spectrum of craniofacial abnormalities manifesting as holoprosencephy and cyclopia; other more mild abnormalities include atrophy of the first pharyngeal arch (Cordero et al., 2004; Lazarro et al., 2004; Nagase et al., 2005). In the Shh mutant the first arch forms but is reduced in size. In keeping with this phenotype, expression of Fgf8 is dramatically reduced or lost in the first pharyngeal arch, although expression is still observed in the more posterior pouches (Aoto et al., 2002; Moore-Scott and Manley, 2005; Yamagishi et al., 2006).

Loss of Shh results in cell death in the mesenchyme and migrating neural crest cells (Ahlgren and Bronner-Fraser, 1999; Moore-Scott and Manley, 2005; Yamagishi et al., 2006). The defect in early arch development might therefore be due to Shh’s role in neural crest development. When the Shh pathway is knocked out specifically in the neural crest, however, the pharyngeal arches initially form normally (Jeong et al., 2004). The early defect in first arch development in the full Shh knockout is therefore likely to be independent of the role of Shh signalling in the neural crest, pointing to a role in the epithelium. After E10, in the conditional Shh knockout, the arches show a growth deficiency, resulting in mice with dramatically truncated faces. Expressions of many homeobox genes that are induced by Fgf8 in the oral epithelium, such as Spry1 and Dlx5, are induced as normal in these mutants, indirectly indicating that expression of Fgf8 is unaffected.

Gli proteins appear to play a role in mediation and interpretation of hedgehog signalling, and Gli3 has been shown to repress Shh signalling (Litingtung and Chiang, 2000). In Gli3−/− embryos, Fgf8 expression was expanded in the facial primordial, leading to expansion of gene expression of homeobox gene targets in the mesenchyme (such as Dlx2) (Aoto et al., 2002). Compound null Gli3/Shh mice, however, have a relatively normal first pharyngeal arch and apparently normal expression of Fgf8.

In the chick, inhibition of Shh signalling has been studied in vivo using cyclopamine. By altering the time and dose of this drug, the whole spectrum of holoprosencephy phenotypes can be observed (Cordero et al., 2004). In the normal development of the frontal nasal process a domain of Shh is induced in the ventral ectoderm. Adjacent to this is located a domain of Fgf8 expression. The two signalling molecules together mark a region of the frontal nasal process known as the frontal nasal ectodermal zone (FEZ), which controls the outgrowth of the upper beak (Hu et al., 2003). If cyclopamine is administered before this domain of Shh is induced, Shh fails to come on in the ventral ectoderm and the expression of Fgf8 extends into the ventral region. In this region of the embryos, therefore, Shh appears to be acting to restrict the expression of Fgf8 to the dorsal ectoderm of the upper beak. The expression domain of Shh in the pharyngeal endoderm was unaffected by treatment with cyclopamine at all stages investigated, indicating that once an expression domain of Shh is set up it is no longer susceptible to loss of Shh (Cordero et al., 2004). The effect of reduced Shh signalling on Fgf8 in the lower jaw was not investigated.

Overexpression studies of Shh in the chick have also shown that Shh is essential for the morphogenesis of the frontal nasal and maxillary processes (Hu and Helms, 1999). The role of Shh in patterning of the lower jaw, however, and its relationship with Fgf8 in this tissue have not been investigated.
We have previously shown that removal of the endoderm from explants of the presumptive first pharyngeal arch tissue results in loss of \( Fgf8 \) expression. In order to investigate the role of Shh in regulating \( Fgf8 \) expression, we blocked Shh signalling in cultured chick-derived presumptive first pharyngeal arch explants from HH stage 9 to stage 16. In addition, following removal of the endoderm we investigated whether Shh beads can rescue \( Fgf8 \) expression. We then overexpressed Shh in specific regions of the pharyngeal arch ectoderm, to see whether Shh is both necessary and sufficient to induce the expression of \( Fgf8 \) in oral ectoderm.

Materials and methods

Preparation of whole chicken embryos

Fertilised chicken eggs were incubated at 38.5°C for up to 10 days in a humidified incubator. Embryonic staging was determined according to the chick stage series of Hamburger and Hamilton (1951). Embryos were dissected from the eggs and the extra-embryonic membranes removed. Embryos were fixed at 4°C in 4% paraformaldehyde (PFA) overnight and then transferred to 1% PFA for storage.

Preparation of chicken embryo explants

Fertilised chicken eggs were incubated at 38.5°C for 30–60 h in a humidified incubator until the embryos had reached HH stage 8 and 16 (Hamburger and Hamilton, 1951). Embryos were dissected from their extra-embryonic membranes in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% foetal calf serum (Gibco-BRL). For the early cultures (HH 8–11) coronal head slices containing at the dorsal surface, the midbrain–hindbrain boundary and at the ventral surface the presumptive mandible ectoderm were dissected using fine tungsten needles. The position of the presumptive mandible was predicted from the fate map and dissections were increase the chance of hitting the pharyngeal arches multiple beads were added to the explants.

Targeting pharyngeal arch ectoderm

XexcShh16 and Beta actin-GFP (Green fluorescent protein) DNA constructs were co-electroporated into HH stages 9–10 and HH stage 14 leghorn chick embryos in ovo, at a concentration of 2 μg/μl xexcShh16 and 1 μg/μl GFP DNA. At HH stages 9–10 the ectoderm of the pharyngeal arches was targeted by making a hole in the vitelline membrane and then bending the head of the embryo back and injecting the DNA constructs underneath. The head was then folded back and a pulse was sent between two silver electrodes, the cathode on top of the neural tube at the level of the midbrain and anode below the embryo at the same A–P level, 8 pulses were passed at 15 V, 20-ms duration. At HH stage 14 the ectoderm of the pharyngeal arches was targeted by injecting the DNA constructs on top of the arches below the developing extra-embryonic membrane. A pulse was sent between two silver electrodes, the cathode on top of the arches and anode below the embryo at the level of the arches, 8 pulses were passed at 15 V, 20-ms duration. The embryos were allowed to develop for 4–20 h and the protein produced by the co-electroporated GFP was detected under a fluorescence microscope. Each embryo was photographed at this stage so that the initial overexpressed pattern could be compared to the final morphology of the embryo. All the HH stage 11 electroporated embryos were fixed at this early stage for in situ hybridisation. Of the HH stage 14 electroporated embryos, some were taken out at this stage and the remainder allowed to develop to E4, E6 or E10. After approximately E7, the GFP could no longer be visualised using fluorescence microscopy. After electroporation at HH stage 14, the survival to E6 was approximately 70% with 50% of embryos.
surviving to E10. For controls, embryos were electroporated with Beta actin: GFP only. No effect was seen in these embryos, thus indicating no non-specific defect caused by the electroporation technique.

Whole mount in situ hybridisation

Whole mount in situ hybridisation was performed as described (Mootoo-samy and Dietrich, 2002). Shh was linearised with SalI and transcribed with Sp6; Patched was linearised with SalI and transcribed with T3; Fgf8 was linearised with BamH1 and transcribed with T7; Lhx6 was linearised with SnaI and transcribed with T7; Barx1 was linearised with EcoR1 and transcribed with T7; Claudin-1 was linearised with Not1 and transcribed with T3.

Radioactive in situ hybridisation

Embryos were fixed in 4% PFA, dehydrated through a graded series of ethanol and embedded in paraffin wax. Radioactive in situ hybridisation was carried out on 8-μm sections as described (Wilkinson, 1982).

Vibratome sectioning

Embryos were embedded in 20% gelatin diluted into PBS. Gelatin blocks were submerged in 4% PFA for a minimum of 48 h and 40-μm-thick vibratome sections cut, with sections mounted under a glass coverslip in Vector Shield mounting solution (Vector Laboratories, UK).
Cartilage staining

E10 heads were dissected and the eyes removed, fixed in 4% paraformaldehyde, then washed and stained overnight in 100 mg/l of Alcian Blue 8GX (Ingrain Blue 1, EM Science), in 70% EtOH, 30% acetic acid. Heads were then destained in 95% EtOH and slowly rehydrated. Once in H2O, heads were cleared in 1% KOH and photographed.

Immunohistochemistry

GFP staining was performed on E3 and E6 paraffin wax sections. Sections on slide were blocked in 0.9% H2O2 for 20 min at room temperature to inhibit endogenous peroxidases and a polyclonal anti-GFP rabbit antibody (Molecular Probes) was used at a concentration of 1/200 in PBS containing 5% NBCS and 1% Triton x-100 overnight at 4°C. Secondary anti-rabbit horse radish peroxide antibody (Amersham) was used at a concentration of 1/50 at room temperature for 2 h. GFP localisation was then visualised by using DAB (3′,3′-diaminobenzidine) colour reaction (Sigma). Slides were counter stained with eosin and cover slipped with DePeX (BDH). Type II collagen staining was performed using the II-II6B3 antibody (Developmental Studies hybridoma bank) on E6 paraffin wax sections. To enhance the signal the slides were microwaved in 0.01 M citrate buffer (Shi et al., 1991), and treated with chondroitinase ABC (0.25 U/ml) and hyaluronidase (1.45 U/ml) at 37°C for 45 min (Sigma). The collagen antibody supernatant was used at a dilution of 1/100.

Photography

Photographs of radioactive in situ hybridisation were taken under darkfield illumination using a Zeiss compound microscope. The images were captured using Axiosvision (Zeiss) software and converted into Photoshop (Adobe) format. Whole mount in situ photographs were taken on a 2% agarose support using a Leica MZFLIII microscope.

Results

Expression of Shh and Fgf8 in the developing chick head

Whole mount in situ hybridisation at HH stage 10 showed that Shh is present in the forebrain adjacent to the Fgf8 expression domain (Fig. 1A), and vibratome sectioning confirmed that it is also present in the foregut endoderm underlying the presumptive first arch ectoderm (Fig. 1C), which at this stage is negative for Fgf8. A few hours later at HH stage 11, Fgf8 is expressed in two lateral patches of head ectoderm, a region fated to rise to the first pharyngeal arch ectoderm (Haworth et al., 2004; Shigetani et al., 2000), which overlie the foregut endoderm (Figs. 1B, D). Expression studies therefore place Shh in the correct region of the embryo during the right developmental stage to be a candidate for inducing expression of Fgf8 in presumptive first arch ectoderm (schematically represented in Fig. 1E).

As the arches form, Shh continues to be expressed in the endoderm and its expression appears as a boundary between the endoderm and the ectoderm at the buccal–pharyngeal membrane. At stage 14, Fgf8 is expressed in the first arch ectoderm on the oral surface and its expression is observed up to the border with Shh (Figs. 1F, G). Expressions of Shh and Fgf8 are complementary and non-overlapping (Figs. 1H–J). In contrast, in the second pharyngeal arch at the same stage, Shh and Fgf8 are co-expressed in the endoderm, along with other signalling molecules such as Bmp7 and Wnt7a, leading to the proposal that this region is an arch signalling centre (Wall and Hogan, 1995). The close relationship between the expression of Shh in
the endoderm and Fgf8 in the ectoderm thus continues throughout arch development.

**Fgf8 expression is downregulated in explants cultured in the presence of Shh pathway inhibitors**

In order to determine whether inhibition of Shh signalling results in either reduced or absent Fgf8 expression, a chick head explant system was established. Chick head explants containing tissue of the presumptive midbrain–hindbrain boundary and the presumptive first pharyngeal arch were dissected between HH stages 8–11 (Fig. 2A) and cultured as described previously (Haworth et al., 2004). This ranges from several hours prior to the onset of Fgf8 expression up to the initiation of Fgf8 expression in the presumptive first pharyngeal arch.

To begin, we verified that development of the dissected explants cultured in vitro was comparable to development of normal tissue in vivo. Gene expression patterns in cultured explants were compared with the expression in the normal developing embryo. In situ hybridisation using probes for Fgf8, Barx1 and Lhx6 was performed on explants cultured between 3 and 4 days and on normal chick embryos between HH stages 17–21. In the explants, Fgf8 was expressed in a stripe corresponding to the isthmus and in two lateral patches corresponding to the pharyngeal oral ectoderm (Fig. 2B). Despite the changes in morphology that occur during culture, expression was comparable to that seen in the whole mount in situ hybridisation of a HH stage 20 chick (Fig. 2C). Barx1 and Lhx6 (Figs. 2D, F) were both expressed in two lateral patches in the explants. These regions corresponded to the normal neural crest-derived mesenchymal expression domains of these genes (Figs. 2E, G) in cells underlying the Fgf8 domain. Expression studies therefore indicate that following in vitro culture, the explants recapitulated the normal embryonic expression of genes in the first pharyngeal arch as far as these genes are concerned.

In order to determine whether Shh signalling was necessary for early expression of Fgf8 in the presumptive first pharyngeal arch ectoderm, chick embryonic explants were cultured in the presence of Shh pathway inhibitors for 1 to 4 days (Fig. 3A). Cyclopamine inhibits the Shh pathway by direct interaction with the Smoothened (Smo) transmembrane domain protein (Chen et al., 2002). In situ hybridisation showed that Fgf8 expression was either completely lost or dramatically reduced in all treated explants (N=8/8 expression reduced or lost) compared to control explants (N=7/8 expression present) at all stages examined (Figs. 3B, C). Explants were also cultured in the presence of either 5E1, an antibody that binds the active signalling region of Shh or Forskolin, a PKA activator and generic inhibitor of HH signalling. When explants were cultured with either of these inhibitors Fgf8 expression was either dramatically reduced or lost (SE1 2/3; Forskolin 3/3) (data not shown). Together, these data show that inhibition of Shh signalling results in downregulation of Fgf8 expression in the presumptive first pharyngeal arch ectoderm and suggests that Shh is an endogenous inducer of Fgf8 expression. Following incubation it was apparent that the experimental explants were considerably smaller than controls, even after 24 h in culture. This may reflect the role of Shh in proliferation and cell death survival (Hu and Helms, 1999; Britto et al., 2000). In order to ascertain that the loss of Fgf8 expression was due to loss of Shh transduction and not due to non-specific cell death in the explant, the expression of Shh was analysed in cultured explants. Shh expression in both the cyclopamine-treated and the DMF control was observed (Figs. 3D, E). To rule
out that the loss of Fgf8 was due to loss of the pharyngeal ectoderm we also investigated the expression of Claudin-1. Claudin-1 is a marker of epithelial cells and is expressed at high levels in the pharyngeal ectoderm (Simard et al., 2005). Claudin-1 expression was unaffected by the treatment with cyclopamine (Figs. 3F, G).

As indicated from their expression patterns, Shh and Fgf8 remain closely associated throughout development of the first pharyngeal arch. To investigate whether Shh remains essential for expression of Fgf8 once the arch has formed, we repeated the culture experiments at later stages. First pharyngeal arches dissected at HH stage 14 and stage 16 showed complete loss of Ptc after treatment with cyclopamine in the culture medium (Figs. 4A, B and data not shown). Ptc induction provides a reliable transcriptional indication of Shh signalling activity (Pearse et al., 2001). Expressions of Shh and claudin-1 were again unaffected in the explant cultures (Figs. 4C, D and data not shown). Cultures dissected at HH stage 14 showed almost complete loss of Fgf8 expression in the mandible (N=4/4) (Figs. 4E, F). A similar result was obtained with Forskolin (N=3/3, data not shown). Cultures dissected out at HH stage 16, however, showed no obvious change in the expression of Fgf8 in the mandible, despite the loss of Ptc, indicating that at this stage the expression of Fgf8 is no longer dependent on Shh signalling (Figs. 4G, H). Expression of Fgf8 in the mandible was similarly lost or severely reduced after treatment of embryos with cyclopamine in ovo at HH stage 14 (N=3/5) (Figs. 4I, J). The variation in Fgf8 expression after treatment in ovo is likely to be due to dilution of the inhibitor in the egg. Whilst expression of Fgf8 was lost in the mandible, in the frontal nasal process the expression of Fgf8 was observed to extend ventrally into the normal Shh expressing domain, as has previously been reported (Cordero et al., 2004).

**Shh beads can rescue loss of Fgf8 expression caused by endoderm removal**

Loss of the pharyngeal endoderm results in loss of Fgf8 expression in the ectoderm (Haworth et al., 2004). In order to investigate whether a loss of Shh signalling from the pharyngeal endoderm was responsible for this loss, the endoderm was dissected from explants at HH stages 8+ to 9. These explants were cultured for 3–4 days with either control BSA beads or Shh protein beads. In explants without endoderm and with control beads, expression of Fgf8 was observed in the isthmus, but expression in the pharyngeal ectoderm was either dramatically reduced or lost (Figs. 5B, E), compared with those cultures were the endoderm was left intact (Figs. 5A, D) (N=18/20). When explants were incubated with Shh beads, Fgf8 expression was restored in the pharyngeal ectoderm (Figs. 5C, F) (N=14/22). It was noted, however, that Fgf8 expression did not correspond to the exact position of the beads,

Fig. 4. Shh is important for maintenance of Fgf8 expression during arch formation. (A, B, G, H) Cultures explanted at HH stage 16 and grown for 24 h in culture. (C–F) Cultures explanted at stage 14 and grown for 24 h in culture. In each case the first arch is positioned facing downwards. (A) Control treated explant showing Ptc expression in the brain and oral region (arrow). (B) Similar explant treated with cyclopamine, showing complete loss of Ptc expression. (C, D) No effect of cyclopamine treatment at stage 14 on expression of Claudin-1 in the ectoderm. (E) Expression of Fgf8 in control culture from stage 14 in oral region (arrow). (F) Loss of expression of Fgf8 in the oral region after treatment with cyclopamine at stage 14. (G) Expression of Fgf8 in control culture from stage 14 in oral region (arrow). (H) No effect on the expression of Fgf8 in the oral region after treatment with cyclopamine at stage 16. (I, J) Embryos injected in ovo and left to develop for 24 h. (I) Control injected embryos. Arrows indicate expression of Fgf8 in the first arch ectoderm and the dorsal part of the frontal nasal process. (J) Cyclopamine treated embryo with no Fgf8 expression in the mandible (arrow) and an extension of Fgf8 expression into the ventral region of the frontal nasal process (arrow).
rather to the position of endogenous \textit{Fgf8} in the ectoderm. Thus, Shh was not able to induce new sites of \textit{Fgf8}, but was able to restore expression. These data suggest that Shh signalling is necessary for initial expression of \textit{Fgf8} in the first pharyngeal arch ectoderm, but other factors limit and regulate the spatial position.

\textit{Overexpression of Shh in the arch ectoderm expands the endogenous domain of Fgf8}

Having shown that Shh was necessary for \textit{Fgf8} expression from initiation to HH stage 14 we decided to investigate whether Shh was sufficient to induce ectopic expression of \textit{Fgf8} in the arch ectoderm. To do this we electroporated \textit{Shh} in the ectoderm surrounding the first and second pharyngeal arches, a region normally devoid of \textit{Shh} expression. Beta actin:GFP and XexcShh16 constructs were co-electroporated into the ectoderm at HH stages 9–10 and stage 14, and the GFP was visualised after 4 to 20 h to indicate the location of the ectopic Shh. Co-electroporation of two plasmids has been shown to result in virtually identical expression patterns (Momose et al., 1999). The results for HH stage 14 only are presented for simplicity, but identical changes in gene expression were observed after electroporations at HH stages 9–10 (data not shown). At HH stage 14, the arches are formed and it is possible to accurately target specific areas. In this manner we could target the mandible, maxilla, or second pharyngeal arch and look at the effect of overexpression on these regions in isolation (Figs. 6A, B, C). Only those embryos with specific expression patterns of GFP in the arches 20 h after electroporation were kept for further study (\(N=54/220\)). As predicted after 20 h in culture, the ectopic expression of Shh closely matched that of the GFP, as shown in serial sections by anti-GFP immunocytochemistry (Figs. 6D, E). Ectopic Shh induced its receptor \textit{Ptc} in the ectoderm and adjacent mesenchyme, confirming that the construct was functional (Fig. 6F).

Having assessed that the electroporated Shh was functional we then investigated its effect on \textit{Fgf8} expression. In the
mandible there was a clear upregulation of Fgf8 in the pharyngeal arch ectoderm on the electroporated side after 20 h (Figs. 7A, B). This was observed as a spreading of the normally oral expression domain of Fgf8 into the lateral regions of the arch. In order to correlate upregulation of Fgf8 with ectopic expression of Shh we analysed the electroporated embryos using serial sections, comparing expression on the operated (Figs. 7C, E, G, I) and control side (Figs. 7D, F, H, J) of the same embryo. Ectopic expression of Shh and GFP was used to assess the sites of overexpression (Figs. 7C–F). In sections it was clear that the regions of ectopic Fgf8 (as indicated by the asterisk) corresponded to those of ectopic Shh in the lateral ectoderm of the mandible (Figs. 7G, H).

Overexpression of Shh was not, however, able to induce expression of Fgf8 in regions far from the endogenous expression domain such as on the aboral side of the arch (Figs. 7G, H). Ptc expression was upregulated in the mesenchyme under both rostral and lateral sites of overexpression (data not shown). In total an expansion of Fgf8 was observed in 5/8 cases. In the cases where no upregulation was observed the ectopic Shh was found at a distance from the endogenous Fgf8 domain. Thus Shh overexpression in the mandible is only able to extend the existing domain of Fgf8. This agrees with the fact that after removal of the endoderm, Shh beads were only able to rescue Fgf8 expression in the normal Fgf8 expression domain and not at ectopic sites (Figs. 5C, F). Electroporated GFP alone had no affect on Fgf8 expression (data not shown).

In the maxilla overexpression of Shh also led to induction of Fgf8, however, the relationship between the two genes was different. In the mandible overexpression of Shh led to co-expression of Shh and Fgf8. In the maxilla, however, ectopic Shh induced Fgf8 in adjacent regions of the ectoderm and was able to induce a completely ectopic expression domain of Fgf8 (Figs. 8A–E) (N=3/3). During normal development of the upper beak, Shh and Fgf8 are expressed in the ectoderm in adjacent zones of the frontal nasal process (FNP) from HH stage 17 onwards (Cordero et al., 2004; Hu et al., 2003). The differing results with overexpression in the mandible and maxilla may reflect the different relationship of these two genes in different parts of the face during normal development.

**Overexpression of Shh leads to altered the rostral–caudal axis of the arch**

Fgf8 has been shown to be important for setting up both the rostral–caudal (oral–aboral) and proximo-distal axes of the mandible (Tucker et al., 1998a, 1999). By inducing Fgf8 expression on the lateral side of the arch we would predict that...
Fig. 8. (A–E) Serial frontal sections through the maxilla region of an embryo at E3. (A, D) Shh expression. (B) Ptc expression (bright field image so expression shows up at black grains). Ptc is upregulated in the mesenchyme underlying the ectopic Shh expression. (C, E) Ectopic Fgf8 expression in the maxilla on the operated side. (D, E) High power views of panels A and C.

Fig. 9. Alterations in shape of the developing cartilage condensations. (A–D) Embryo at E6, showing GFP surrounding ectopic bulges in face (arrow) on electroporated side. (B–D) Frontal sections through same embryo. (B) Immuno for type II collagen, showing a change in shape and polarity of the forming Meckel’s cartilage on the operated side. (C) High power of the ectopic bulge showing GFP expressing cells restricted to the ectoderm around the bulge (arrows). (D) Immuno for type II collagen, showing altered direction of development of the quadrate on the operated side.
the normal pattern of the arch would be disrupted. To assess this the expression of Lhx6 was investigated in serial sections. Lhx6 expression is restricted to the oral mesenchyme and is induced by Fgf8 (Grigoriou et al., 1998). In those regions where Fgf8 was induced by ectopic Shh, ectopic expression of Lhx6 was observed, confirming a disruption of the rostral–caudal axis of the arch (Figs. 7I, J).

**Overexpression of Shh induces ectopic cartilage**

Since overexpression of Shh led to a change in the rostral–caudal axis of the arch, as indicated by the change in Lhx6 expression, we examined its effect on the developing cartilages. Electroporation at HH stages 9–10, compared to HH stage 14, resulted in a high mortality rate and few embryos survived beyond 4 days of culture. This is possibility due to the difficulty of accessing the presumptive oral ectoderm at these early stages. Therefore in order to observe changes in cartilage pattern only those embryos electroporated at stage 14 were used (N=30/44 survived to E6). At E6, the cartilages are starting to differentiate and can be viewed by type II collagen immunocytochemistry. From the mandible, Meckel’s cartilage develops running through the arch, ending proximally in the articular which articulates with the quadrate forming the main jaw articulation between the upper and lower jaws (Wilson and Tucker, 2004). Overexpression of Shh caused an ectopic bulge to appear in the facial primordial on the lateral part of the arch in 50% of cases, and in the ectoderm covering this bulge GFP cells were observed (Fig. 9A). Those embryos that did not display a bulge had low GFP in the arch region (data not shown). When sectioned the GFP expressing cells were localised to the ectoderm covered the bulge in the mandible and in the maxilla (Fig. 9C shows section through mandible). There was an increase in the mesenchymal tissue adjacent to the labelled ectoderm on the electroporated side, indicating an increase in proliferation. To investigate whether overexpression of Shh did indeed lead to an increase in proliferation, electroporated embryos were injected in ovo with BrdU. Slightly higher levels of proliferation were observed 24–48 h after electroporation in the mandibular mesenchyme underlying the Shh expressing ectoderm compared to the control side in the same embryo (Supplementary Fig. 1). The effect was fairly subtle and may be masked by the relatively high proliferation observed normally in this tissue at this stage. Fgf8 was still expressed ectopically in the ectoderm at this stage, despite the fact that the ectopic Shh was no longer detectable by in situ hybridisation (data not shown). Using a type II collagen antibody we then investigated the effect of earlier Shh overexpression on the formation of the cartilages using concurrent sections. The developing Meckel’s cartilage on the operated side had its polarity and size altered, it was now orientated in a rostral–caudal direction when compared to the control side (Fig. 9B). In a more proximal section the developing quadrate on the operated side was observed pointing laterally compared to the control side, where the quadrate forms directly above the articular part of Meckel’s (Fig. 9D). Changes in early arch patterning have therefore resulted in the cartilages condensing in different orientations within the arch.

By day 10 of development the cartilages have clearly developed in the embryo and each of these elements has a unique shape specific to their location (Kontges and Lumsden, 1996). At E10, the surviving embryos (N=22) were stained with Alcian blue. Overexpression of Shh in the mandible lead to a bifurcation of Meckel’s cartilage on the operated side of the head in 12 of the 22 embryos investigated. The ectopic cartilage was seen to develop at an angle of approximately 45° to the main cartilage rod, whilst Meckel’s cartilage on the control side remained straight (Figs. 10A, B). Normally the quadrate develops as a triangle, with the otic process extending pro-

![Fig. 10. Duplications and altered polarity of cartilage elements of the first branchial arch. (A–H) Skeletal preparations showing cartilages of electroporated embryos E10. (A, C, E) Operated side. (B, D, F) Control side. (A) Bifurcation of Meckel’s cartilage in the lower jaw. (B) Single straight cartilage rod on control side. (C) Star-shaped quadrate. (D, F) Control quadrates showing normal triangular structure. (E) Quadrade developing in the wrong direction. Processes face back towards rostral part of Meckel’s. Arrows (A–F) indicate direction of growth. (G) Ectopic nasal cartilage, showing ectopic egg tooth development at tip (arrows). (H) No defect in second arch cartilages of the tongue. The basihyoid and ceratobranchial cartilages (arrows) are unaffected.](image-url)
ximally, the optic (pterygoid) process extending rostrally and the articular process extending caudally to articulate with the most proximal part of Meckel’s cartilage to form the jaw joint (Figs. 10D, F). After overexpression of Shh, the quadrate was observed to bifurcate in a number of cases with the two otic processes extending in different directions (N=6/22) (Fig. 10C). In other cases, the quadrate was observed to bend back on itself so that the otic process was now pointing distally (Fig. 10E).

Overexpression of Shh in the upper beak has previously been shown to result in a duplication of the upper beak cartilage (Hu and Helms, 1999), and this was also observed in us in those embryos where Shh was overexpressed near to the frontal nasal process (Fig. 10G). These ectopic upper beaks formed an egg tooth at the tip (arrow), identifying them as true upper beak cartilages. The 2nd arch neural crest cells differentiated into the retroarticular process, the columella, the upper beak cartilages. The 2nd arch neural crest cells differentiated into the retroarticular process, the columella, the basihyoid and the ceratobranchial of the tongue. These differentiate into the retroarticular process, the columella, the upper beak cartilages. The 2nd arch neural crest cells formed an egg tooth at the tip (arrow), identifying them as true upper beak cartilages (Hu and Helms, 1999), and this was also observed by been shown to result in a duplication of the upper beak frontal nasal process (Fig. 10G). These ectopic upper beaks been observed to bend back on itself so that the otic process was now pointing distally (Fig. 10E).

Discussion

Fgf8 is secreted from the oral ectoderm and is an important signalling molecule that regulates patterning of the primordial jaw mesenchyme in vertebrates (Trumpp et al., 1999; Tucker et al., 1998a). Many of the transcription factors that are temporally and spatially expressed in the early jaw mesenchyme are regulated by Fgf8. Conditional loss of Fgf8 expression from the oral ectoderm in mice results in grossly truncated jaws due to a requirement of this signalling molecule for the survival and proliferation of first pharyngeal arch mesenchymal cells (Trumpp et al., 1999). Bmp4 also plays a key role in regulating mesenchymal gene expression in the early jaw derivatives and the spatial relationship between Bmp4 in distal regions and Fgf8 proximally, forms the first crude pre-pattern of a jaw axis. These spatial domains of Bmp4 and Fgf8 expression in the oral ectoderm are established prior to pharyngeal arch formation and neural crest migration in the head ectoderm (Haworth et al., 2004). In the developing chick, Fgf8 is expressed from HH stage 11 in the ectoderm of the presumptive first pharyngeal arch (Shigetani et al., 2000). Here we demonstrate that HH stage 9 explants of the presumptive first arch, cultured with inhibitors of the Shh pathway, do not go on to express Fgf8. We have previously shown that removal of pharyngeal endoderm prior to the onset of Fgf8 expression prevents subsequent expression of Fgf8 in the presumptive first pharyngeal arch ectoderm (Haworth et al., 2004). Importantly, however if endoderm is replaced by Shh-coated beads, Fgf8 expression is restored. Shh signalling is clearly important for normal development of the first pharyngeal arch.

Shh−/− mice demonstrate a lack of development of jaw structures (Chiang et al., 1996), whilst conditional abrogation of Smo receptor activity in cranial neural crest results in a severe jaw phenotype (Jeong et al., 2004). In addition, human conditions affected by defects in cholesterol biosynthesis have been reported to have jaw anomalies (Dehart et al., 1997). Cholesterol is essential for normal Shh activity and micrognathia is present in both the human and murine forms of Lathosterolosis, an inherited disorder of cholesterol metabolism (Krakowiak et al., 2003).

The oral ectoderm is believed to pattern the underlying neural crest cells (Grigoriou et al., 1998; Ferguson et al., 2000; Tucker et al., 1998a,b) and there is increasing evidence to suggest that the facial ectoderm is patterned earlier in development, by signals originating in the foregut endoderm (Coulby et al., 2002; Haworth et al., 2004; Ruhin et al., 2003; Withington et al., 2001). Loss of endoderm has been shown to result in the absence of Fgf8 expression in both the presumptive first pharyngeal arch ectoderm (Haworth et al., 2004) and earlier in development in the developing forebrain (Withington et al., 2001). Shh is expressed in the most anterior part of the foregut endoderm (Cordero et al., 2004; Withington et al., 2001; Marcucio et al., 2005). At HH stage 11, when Fgf8 expression is first detected in ectoderm of the presumptive first pharyngeal arch by in situ hybridisation (Shigetani et al., 2000; Haworth et al., 2004) the region underlying this ectoderm has already been populated by neural crest cells. It has however been shown that following neural crest ablation, Fgf8 is still expressed (Haworth et al., 2004). It is therefore possible that the initial signal from the endoderm is transferred to the ectoderm prior to the influx and proliferation of neural crest cells. Later on in arch development, the expressions of Fgf8 and Shh are found to abut at the buccal pharyngeal membrane, where the ectoderm meets the endoderm. Loss of Shh signalling once the arch has formed (HH stage 14) still leads to loss of Fgf8 expression, indicating that the ectoderm is still dependent on the endoderm for maintenance of Fgf8 expression. At HH stage 16, however, loss of Shh signalling has no effect on the expression of Fgf8, indicating that at this stage in arch development Fgf8 expression has become independent of Shh. In the mouse, treatment with the Shh inhibitor Jervine, at E9.5 leads to a clear reduction in size of the lower jaw but no change was observed in the expression pattern of Fgf8 (ten Berge et al., 2001). This may be due to the timing of treatment since chick HH stage 14 is developmentally equivalent to E9.0 in the mouse. The skeletal defect observed in the mouse after treatment at E9.5 may be due to a reduction in Shh signalling to the underlying neural crest, similar to the situation observed in the conditional Shh knockout (Jeong et al., 2004).

It is significant that the restoration of Fgf8 expression by ectopic application of Shh beads in pharyngeal explants devoid of endoderm does not correspond to the position of the beads but to the site of normal expression. This indicates that Shh is required for the expression of Fgf8 but does not necessarily regulate its exact location. It has been shown that the expression of Bmp4 surrounds and overlaps with the expression of Fgf8 at HH stage 13 and that misexpression of Bmp4 inhibits Fgf8
expression (Shigetani et al., 2000). It is therefore possible that the expression of Fgf8 in head ectoderm is limited by the expression of Bmp4. Noggin and Chordin are natural antagonists of Bmp4 and are expressed in the developing mouse mandible in various tissue layers. Mice with reduced Noggin and Chordin function have reduced Fgf8 expression in the mandible and truncation and agnathia are frequently observed (Stottmann et al., 2001). Therefore, it is likely that the Shh in the endoderm induces Fgf8 expression in the presumptive first arch ectoderm and a combination of Bmp4, Noggin and Chordin restricts the expression and defines the Fgf8 expression domain.

Overexpression of Shh in the mandibular arch ectoderm was able to expand the normal expression domain of Fgf8, but was unable to induce expression of Fgf8 far from its normal domain in the oral ectoderm. This may be due to the presence of inhibitors of Shh signalling present in different regions of the mandible. Overexpression of Shh, and therefore Fgf8, led to a change in the rostral–caudal axis of the arch, as indicated by downstream targets such as Lhx6. This in turn led to a change in position of the cartilage condensations of the lower jaw, which resulted in the formation of ectopic structures (quadrade and Meckel’s cartilage) that branched off from the normal cartilages, or led to a change in direction/polarity of the normal elements. A similar bifurcation of Meckel’s cartilage was reported after a graft of the FEZ (frontal nasal ectodermal zone) region of the frontal nasal process (FNP) was transplanted into a stage 25 mandible (Hu et al., 2003). The FEZ region expresses both Shh and Fgf8, and we believe that our ectopic Shh was having a similar affect to the implanted FEZ, in that it had the ability to re-program the molecular and cellular fate of the 1st arch mesenchyme.

Overexpression of Shh in the maxilla, in contrast to the mandible, resulted in ectopic expression of Fgf8 in adjacent cells to those expressing Shh. This implies both a positive inductive effect of Shh from a distance, and a negative effect at close range. This may explain the slightly counter intuitive findings that Fgf8 is lost in the first pharyngeal arch in Shh mutants, whilst Fgf8 expands in the frontal nasal process after loss of Shh in the chick (Cordero et al., 2004; Moore-Scott and Manley, 2005). Overexpression of Shh in the maxilla at stage 14 led to the formation of ectopic cartilages bifurcating off the upper beak. This is similar to previously reported findings that addition of Shh loaded beads in the frontal nasal process led to an increase in cell proliferation, and an expansion of the mediolateral width resulting in the formation of ectopic upper beak-like structures (Hu and Helms, 1999). This may be due to the induction of a second frontal nasal process signalling centre (FEZ) in the upper jaw (Hu et al., 2003). We were also able to show a subtle increase in proliferation in the mandible after electroporation of Shh.

Whilst ectopic cartilages were observed after overexpression of Shh in the first arch (mandible and maxilla), no change was observed in the second pharyngeal arch. The first pharyngeal arch and more anterior regions are Hox-free. The second pharyngeal arch, in contrast, expresses high levels of Hox genes, such as Hoxa2 (Prince and Lumsden, 1994). It is possible that this arch is less susceptible to overexpression of signalling molecules such as sonic hedgehog due to the presence of these Hox genes. In agreement with our results, FEZ grafts that contained a Shh and Fgf8 signal transplanted from the FNP into the 2nd arch, caused no ectopic soft or hard tissue to form (Hu et al., 2003). In these grafts, however, the expressions of Shh and Fgf8 were not maintained after grafting. Also in a similar vein, grafts of anterior foregut endoderm into the path of crest cells migrating to the second pharyngeal arch did not influence development of the second arch skeletal tissue (Coully et al., 2002).

Our results overexpressing Shh are rather different from those observed by Abzhanov and Tabin (2004). This group overexpressed first Fgf8 and then Shh independently and then together. Overexpression of Fgf8 using an RCAS viral vector led to induction of ectopic cartilage nodules but not ectopic outgrowths. Overexpression of Shh using a similar construct, led to small outgrowths in the upper and lower beaks, but these did not express cartilage markers. Co-overexpression of Shh and Fgf8 resulted in multiple small outgrowths of cartilage all over the head. These differences may reflect a difference in the way that the Shh was overexpressed, and a difference in timing of the overexpression due to the use of retroviruses.

In summary, we have identified Shh as a signal from a restricted region of the developing pharyngeal endoderm that acts to induce Fgf8 expression in adjacent ectoderm of the first pharyngeal arch. This signalling interaction may in part explain the dramatic facial regulatory affects of endoderm transplantations and suggests that the role of pharyngeal endoderm in facial patterning may be indirect. A similar indirect role of HH signalling on skeletal patterning has recently been demonstrated in zebrabish (Eberhard et al., 2006). Hedgehog signalling from the neural tube was shown to instruct the oral epithelium of the upper jaw and organise the skeletal development of the anterior neurocranium. In this paper we show that a HH signal also provides instructions to the lower jaw oral epithelium but that the source for the hedgehog signal is not the neural tube but the pharyngeal endoderm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.11.009.
References


