Novel skeletogenic patterning roles for the olfactory pit

Heather L. Szabo-Rogers*, Poongodi Geetha-Loganathan, Cheryl J. Whiting, Suresh Nimmagadda, Katherine Fu and Joy M. Richman†

The position of the olfactory placodes suggests that these epithelial thickenings might provide morphogenetic information to the adjacent facial mesenchyme. To test this, we performed in ovo manipulations of the nasal placode in the avian embryo. Extirpation of placodal epithelium or placement of barriers on the lateral side of the placode revealed that the main influence is on the lateral nasal, not the frontonasal, mesenchyme. These early effects were consistent with the subsequent deletion of lateral nasal skeletal derivatives. We then showed in rescue experiments that FGFs are required for nasal capsule morphogenesis. The instructive capacity of the nasal pit epithelium was tested in a series of grafts to the face and trunk. Here, we showed for the first time that nasal pits are capable of inducing bone, cartilage and ectopic PAX7 expression, but these effects were only observed in the facial grafts. Facial mesenchyme also supported the initial projection of the olfactory nerve and differentiation of the olfactory epithelium. Thus, the nasal placode has two roles: as a signaling center for the lateral nasal skeleton and as a source of olfactory neurons and sensory epithelium.

KEY WORDS: Chicken embryo, Placode, Nasal capsule, FGF8, Craniofacial, TuJ1, PAX7, Lateral nasal prominence

INTRODUCTION

The cranial placodes are focal thickenings of the ectoderm and include the olfactory, lens, otic, trigeminal, hypophyseal, lateral line and epibranchial placodes. Placodes are induced within the non-neural ectoderm outside of the neural plate (Coulby and Le Douarin, 1985), within a pre-placodal domain (Bailey et al., 2006; Bhattacharyya et al., 2004; Schlosser, 2005; Schlosser and Ahrens, 2004) where the olfactory and lens precursors are intermingled (Bhattacharyya et al., 2004; Streit, 2002). FGFs are required for the earliest steps of olfactory placode specification (Bailey et al., 2006; Kawauchi et al., 2005) and then later for formation of the nasal passages (Kawauchi et al., 2005).

In the chicken, the olfactory placode is first visible at the end of cranial neural crest cell migration, coincident with the appearance of the pharyngeal arches (stage 15 (Hamburger and Hamilton, 1951)). Twenty-four hours later (stage 20), the nasal pit has invaginated and, simultaneously, gonadotrophin-releasing neurons (GnRH) begin migrating to the telencephalon (Drapkin and Silverman, 1999). Later, the nasal pit deepens to form a nasal slit and ultimately the nasal passages that are lined by respiratory and olfactory epithelia (Croucher and Tickle, 1989). The olfactory placode gives rise to the olfactory nerve, which is composed of several types of neurons, olfactory ensheathing cells and glia.

The timing of their formation and their position suggest that the olfactory placodes might exert patterning influences on the adjacent facial mesenchyme, a possibility that had not previously been addressed. The mesenchyme of the frontonasal mass is medial to the placodes and gives rise to the midline skeletal elements (prenasal cartilage and premaxillary bone), whereas lateral nasal mesenchyme is lateral to the placodes and gives rise to the nasal capsule (nasal conchae and nasal bone). Both mesenchymes are derived from the same regions of neural crest and share similar migration pathways over the eye primordia (Creuzet et al., 2005). Cartilages from both frontonasal and lateral nasal mesenchymes are initially patterned as a group by signals from the foregut endoderm (Benouaiche et al., 2008). However, later, when the placodes appear, there may be a separation of the signals that pattern the nasal capsule from those that pattern the prenasal cartilage and interorbital septum. We have shown, for example, that Noggin and retinoic acid can induce the midline elements (interorbital septum and prenasal cartilage) but not the nasal capsule in pharyngula stage embryos (Lee et al., 2001).

Several secreted signaling molecules are expressed around the olfactory placode and pit in chicken and mouse embryos. The lateral edge expresses RALDH2, a retinoic acid synthesizing enzyme (Blentic et al., 2003; LaMantia et al., 2000), and the expression of FGF8 defines the medial edge of the nasal pit, whereas expression of BMP4 and SHH is caudal to the nasal pit (LaMantia et al., 2000; Song et al., 2004). Previous work in murine (LaMantia et al., 2000) and chicken (Firnberg and Neubuser, 2002) organ cultures has shown that frontonasal epithelia, including the nasal pits, maintain gene expression in frontonasal and lateral nasal mesenchyme.

Epithelial-mesenchymal recombination experiments have shown that by stage 20, frontonasal, maxillary and mandibular mesenchymes contain all the necessary information for generating prominence-specific skeletal patterns (Richman and Tickle, 1989). However, prior to this stage, the nasal placode may direct some aspects of skeletogenesis. Up until now, much of the work on nasal pit development has focused on the interactions of the olfactory bulbs and the olfactory pit, as well as on neuronal differentiation of the olfactory epithelium (Kawauchi et al., 2005; Lutz et al., 1994; Wang et al., 2001).

To address the patterning abilities of the nasal epithelium on facial mesenchyme, we carried out a series of in ovo extirpation experiments and grafts of supernumerary nasal pits. Our data show a specific requirement for the olfactory placode in the patterning and differentiation of the lateral nasal skeleton. Grafts demonstrated skeletogenic capacity and the ability to form nasal passages and neuronal outgrowths when the nasal pit was placed in a competent, HOX-negative environment.

---

Accepted 31 October 2008

Department of Oral Health Sciences, Life Sciences Institute, The University of British Columbia, Vancouver BC, V6T 1Z3, Canada.

*Present address: King’s College London, Department of Craniofacial Development, Floor 27, Guy’s Tower, London SE1 9RT, UK

†Author for correspondence (e-mail: richman@interchange.ubc.ca)
MATERIALS AND METHODS

Embryos

Fertile White Leghorn chicken eggs (Gallus gallus; University of Alberta) and Japanese quail eggs (Coturnix coturnix japonica; Oregon State University, Corvalis) were used. Quail embryos were incubated ~12 hours after chicken embryos so that they reached the appropriate developmental stage (Schneider and Helms, 2003). Embryo work was approved by the UBC Animal Care Committee.

Extractions of nasal ectoderm, foil barrier placement and bead implants

Nile Blue sulfate (0.1% in phosphate-buffered saline) was painted on the nasal placode of stage 15-16 (25-28 somites) embryos or on nasal pits of stage 20 embryos. The epithelium was removed with a tungsten needle. Control embryos received Nile Blue and the epithelium was left intact. Embryos were collected at 0, 6, 16 and 24 hours following surgery and gene expression was analyzed. Other embryos were collected at stage 38 for analysis of skeletal phenotypes. A third set of extripated embryos had either an all-trans-retinoic acid-soaked bead (0.01 mg/ml; AG1X2 beads, BioRad) or an FGF8-soaked bead (1 mg/ml, Peprotech; Affigel beads, Biorad) stapled onto the exposed mesenchyme. For barrier experiments, aluminium foil was inserted on the medial or lateral side of the nasal placode of stage 15 embryos.

Grafting experiments

Donor tissue preparation

Stage 20 or 26 donor (quail or chicken) frontonasal mass and lateral nasal prominences were collected in Hank’s Balanced Salt Solution with Ca2+ and Mg2+ (HBSS). Frontonasal mass epithelium including the nasal pits was separated with 2% trypsin (see Fig. 1A) (Richman and Tickle, 1989). The surrounding surface epithelia were trimmed away from the nasal pit and 0.5% Neutral Red was added to the dissection medium to help visualize the donor epithelium in the host.

Host embryo preparation

Stage 15 or 20 host embryos were stained with Neutral Red. A tungsten needle was used to remove epithelium and expose the underlying mesenchyme. The donor epithelium was placed on the basement membrane side in contact with the graft-site mesenchyme and pinned in place with platinum staples (Fig. 1B,C). Staples were removed the following day to permit sectioning.

Bone and cartilage staining

To study bone and cartilage morphology, stage 37-39 embryos were fixed in 100% ethanol, permeabilized with acetone and then stained with Alcian Blue and Alizarin Red (Plant et al., 2000). For bone and cartilage staining on sections, Picrosirius Red and Alcian Blue were used (Ashique et al., 2002).

Whole-mount in situ hybridization

Whole-mount in situ hybridization (WISH) was performed in the Intavis InstituPro Robot, with DIG-labeled antisense probes using previously published protocols (Song et al., 2004). A subset of embryos was embedded in 20% agarose and Vibratome sectioned at 50 μm. The following individuals provided avian probes for in situ analyses: K. Patel, PAX7; A. Kispert, TBX22; C. Tabin, TBX2; M. Kessel, DLX5; G. Eichele, HOXB1; A. Streit, HOXB9; and O. Pourquie, exonic FGF8. We cloned a 920 bp fragment of SP8 (bp 1776 to 2696) into pCRRII-TOPO (Invitrogen). We assayed the mesenchymal response to extirpation of the nasal placode by looking at the effects on Tbx2 and Tbx22, two T-box transcription factors expressed in the frontonasal mass and 1997). Horseradish peroxidase-conjugated secondary antibody was used (1:100; Jackson Labs) and sections were counterstained in 0.1% Methyl Green.

RESULTS

Previous studies detailing tissue interactions in the embryonic face have focused on the surface epithelium of the frontonasal mass (Hu et al., 2003; Richman and Tickle, 1989), maxillary and mandibular prominences (Richman and Tickle, 1989; Richman et al., 1997; MacDonald et al., 2004). However, no one had studied the interactions between the nasal placode epithelium and the adjacent facial protrusions, which comprise the nasal placode and the frontonasal mass. We first carried out loss-of-function experiments to determine which parts of the beak skeleton were dependent on the nasal placode.

Nasal placode extirpation decreases the expression of nasal pit and lateral nasal genes

We initially removed placodal epithelium and examined genes known to be expressed in the epithelium itself and in the mesenchyme. Epithelial markers were used to see whether the placode could be removed with mechanical techniques and whether it would regenerate. FGF8 is expressed on the medial side of the placode and in the frontonasal mass ectoderm from stage 15-22 (Song et al., 2004). The transcription factor, DLX5, is expressed in the olfactory placode and pit (Bhattacharyya et al., 2004; Brown et al., 2005; McLaren et al., 2003). SP8 is an FGF8-responsive, button-head-like transcription factor (Kawakami et al., 2004; Sahara et al., 2007). SP8 is also localized to the early placode epithelium (see Fig. S1A’; A’ in the supplementary material).

Chicken embryos fixed immediately after extirpation had lost the thin band of FGF8 expression that normally marks the medial side of the nasal pit (6/6 had no expression, data not shown). Expression did not return at 6 hours (4/4) (Fig. 2A-A”). By stage 20, 24 hours later, there is typically strong FGF8 expression in the frontonasal mass ectoderm between the nasal pits. In extripated embryos, FGF8 extended laterally only as far as the presumptive location of the nasal pit (Fig. 2B”, asterisk), illustrating that in the frontonasal mass, FGF8 was able to upregulate in the normal spatiotemporal manner without signals from the nasal pit.

DLX5 expression was also lost immediately following the surgery (0 hour, 6/6, data not shown; 6 hours, 5/5 (Fig. 2C-C”). A similar loss of expression was seen with SP8 (6 hours, 8/10) (see Fig. S1A’-A” in the supplementary material). However, by 24 hours, most of the specimens had some expression in what appeared to be a remnant of the medial edge of the nasal pit (DLX5, 12/19, Fig. 2D”; SP8, 4/8). The remaining specimens had almost no expression (DLX5, 7/19, Fig. 2E-E”; SP8, 4/8, see Fig. S1B-B” in the supplementary material). In order to determine whether residual nasal pit was present in the 24-hour specimens, we sectioned the hybridized embryos. In the cohort of those embryos with some residual expression, DLX5 transcripts were found in medial lip epithelium of the remaining nasal pit (Fig. 2D”). The most likely explanation is that there was some residual nasal pit, rather than regeneration. The mesenchyme also appeared thinner on the treated side and the olfactory nerve was missing.

We assayed the mesenchymal response to extirpation of the nasal placode by looking at the effects on Tbx2 and Tbx22, two T-box transcription factors expressed in the frontonasal mass and
lateral nasal mesenchyme (Fig. 3A-B”) (Firnberg and Neubuser, 2002; Gibbons-Brown et al., 1998; Haenig et al., 2002). We also examined PAX7, a specific marker of lateral nasal mesenchyme (Firnberg and Neubuser, 2002; Kawakami et al., 1997; Otto et al., 2006).

In contrast to the epithelial markers we examined, extirpation of the nasal placode did not affect TBX2 or TBX22 expression (24 hours, 21/21) (Fig. 3A-B”). Thus, mesenchyme was still present after removal of the nasal placode and there was no evidence of a disruption of patterning of either the frontonasal mass or lateral nasal prominence. However, PAX7 expression was lost in all specimens (16 hours, 6/6) (Fig. 3C-C”). These data suggest that the placodal epithelium is providing signals essential for lateral nasal patterning.

To determine whether diffusible signaling molecules were being released into the mesenchyme, we placed impermeable barriers lateral to the placode. Such barriers did not impair the invagination of the nasal pit; however, PAX7 expression was reduced or absent (16 hours, 14/14) (Fig. 3D-D”). Implantation of the foil barrier medially in the frontonasal mass did not affect the lateral nasal expression of PAX7 (6/6) (Fig. 3E-E”).

**Extirpation leads to loss of nasal capsule and these defects are rescued by FGF8**

The effects of epithelial extirpation on PAX7 and TBX2/22 expression were contradictory. Therefore, it was not clear whether the morphology of the skull and jaw bones would be affected. In order to address this, we examined the effects on the beak skeleton.

Extirpation caused significant loss of lateral nasal prominence-derived structures (Fig. 1D) in the majority of embryos, including complete deletion of the nasal bone (25/39) and nasal conchae (28/39) (Fig. 4B,B”). Unlike the lateral nasal derivates, the skeleton derived from the frontonasal mass was conspicuously unaffected, except for a secondary overgrowth of the nasal process of the maxilla: the larger bony process was encroaching into the space left by the missing nasal conchae (23/39) (Fig. 4B,B”). These data allow us to conclude that: (1) healing of the surface ectoderm or any residual olfactory placode is insufficient to allow normal skeletal patterning; (2) general outgrowth of the upper beak does not depend on the nasal placode; and (3) the stage 15 olfactory placode is not required for lip fusion. The effects of nasal placode ablations on the nasal cartilage agree with those observed by others on slightly older,
stage 16 embryos (Wang et al., 2001). Although the skeleton was not the focus of their study, an examination of their figures reveals a unilateral absence of nasal cartilage on the ablated side.

We also removed nasal pits from stage 20 embryos and found that the nasal capsule and nasal bone were reduced in most specimens (9/14) (see Fig. S2 in the supplementary material). In general, elements were more likely to be reduced in size rather than completely deleted and unlike stage 15 extirpations, there was no effect on the premaxilla (see Fig. S2A in the supplementary material). The milder phenotypes suggest that the lateral nasal mesenchyme at stage 20 is less dependent on the nasal pit for patterning cues.

We then asked which signals the nasal pit was providing to promote differentiation of the nasal capsule. Work in mouse and chicken has shown that retinoids are important in lateral nasal development (Bhasin et al., 2003; Dupe et al., 2003; LaMantia et al., 2000; Song et al., 2004). FGFs are also needed for olfactory epithelium differentiation and nasal capsule morphogenesis (Kawauchi et al., 2005). Furthermore, we have recently shown that FGFs are released from the medial edge of the nasal slit to pattern the frontonasal mass mesenchyme (Szabo-Rogers et al., 2008). It is likely that the lateral side of the nasal slit is also a source of FGFs because several FGF genes are expressed in this vicinity (Karabagli et al., 2002). We therefore attempted to replace the extirpated nasal placode with a bead soaked in either FGF8 or retinoic acid (RA). RA exacerbated the phenotype and caused reduction of the premaxilla and maxillary bone in addition to the lateral nasal derivatives (10/11, data not shown). RA beads placed in non-extirpated embryos had minimal effects on development (3/12 had mild nasal bone defects, data not shown). Therefore, in the absence of the placode, RA has complex actions that affect lateral nasal, frontonasal and maxillary morphogenesis. Rescue of lateral nasal development might not be possible owing to the effects on these other regions.

We found that FGF8 almost completely restored the nasal bone and nasal conchae on the treated side in the majority of specimens (18/21) (Fig. 4C/C′,H11032; 200 μm in A′′,B′′,C′′,D′′,E′′). To understand the mechanism underlying the FGF8 rescue, we analyzed cell death and proliferation. FGF8 increased cell proliferation in the mesenchyme
compared with the contralateral side or non-rescued embryos (3/3) (Fig. 4D). As expected, we observed increased cell death in the extirpated specimens (3/3) and FGF8 did not prevent apoptosis at this time point (3/3) (Fig. 4D). FGF8 therefore expands the progenitor population of mesenchyme cells that will give rise to the lateral nasal skeleton.

Facial mesenchyme is able to respond to instructive cues from the nasal pit

Next, we tested the instructive abilities of the nasal placode, nasal pit and nasal slit on the mesenchyme. We grafted donor epithelia into HOX-negative and HOX-positive mesenchyme to test whether there was a difference in the mesenchymal response. We selected the maxillary region as our HOX-negative region (Fig. 1) because the maxillary prominence forms only bony elements (Lee et al., 2004; Richman and Tickle, 1989) and any induced ectopic cartilages will be obvious. The temporal constraints of this experiment were determined by our previous work, which had shown that the fate of the maxillary mesenchyme can be changed by exogenous factors at stage 15 (Lee et al., 2001). By stage 20, the maxillary mesenchyme is determined to give rise to maxillary bones (Richman et al., 1989) but still requires epithelial signals for outgrowth. Facial mesenchyme at stage 26 no longer requires epithelium for outgrowth or patterning (MacDonald et al., 2004). We tested both stage 15 and stage 20 host embryos (Fig. 1B,D). The interlimb lateral plate mesoderm of the stage 15 embryos was used as the HOX-positive region because this region forms ectopic limbs in response to FGFs (Cohn et al., 1995; Vogel et al., 1996), and we had shown that the lateral nasal skeleton relies on FGFs for patterning. Furthermore, grafts of the nasal region to the flank can induce ectopic outgrowths in chicken embryos (Street, 1937).

Stage 15 host embryos did not respond to grafts of stage 15 placodes, even though the placode is at the right stage to be inducing the nasal skeleton, as shown by our extirpation experiments (20/20, data not shown). By contrast, stage 20 nasal pits were more stage 15 and stage 20 host embryos (Fig. 1B,D). The interlimb lateral plate mesoderm of the stage 15 embryos was used as the HOX-positive region because this region forms ectopic limbs in response to FGFs (Cohn et al., 1995; Vogel et al., 1996), and we had shown that the lateral nasal skeleton relies on FGFs for patterning. Furthermore, grafts of the nasal region to the flank can induce ectopic outgrowths in chicken embryos (Street, 1937).

Stage 15 host embryos did not respond to grafts of stage 15 placodes, even though the placode is at the right stage to be inducing the nasal skeleton, as shown by our extirpation experiments (20/20, data not shown). By contrast, stage 20 nasal pits were more stage 15 and stage 20 host embryos (Fig. 1B,D). The interlimb lateral plate mesoderm of the stage 15 embryos was used as the HOX-positive region because this region forms ectopic limbs in response to FGFs (Cohn et al., 1995; Vogel et al., 1996), and we had shown that the lateral nasal skeleton relies on FGFs for patterning. Furthermore, grafts of the nasal region to the flank can induce ectopic outgrowths in chicken embryos (Street, 1937).
Fig. 4. Effects of nasal placode extirpation and rescue of skeletal elements with FGF8 beads. Lateral (A-C) and frontal (A’-C’) views of chicken embryo skulls fixed 11-12 days after manipulations carried out at stage 15. (A,A’) Normal anatomy in a control embryo that had Nile Blue sulfate applied but no epithelium removed. (B,B’) Nasal placode extirpation caused complete loss of the right nasal bone and nasal conchae (asterisk). The nasal process of the right premaxillary bone has expanded (white arrow). (C,C’) FGF8-soaked bead applied at the time of extirpation rescues the right nasal bone and nasal conchae (white arrow, see adjacent camera lucida drawing). Inset shows the staple used to hold the bead. The nasal process of premaxillary bone is also normal on the treated side. The right nasal bone and nasal conchae are smaller than in the normal, untreated side. (D) Frontal section of FGF8-treated extirpated specimen fixed 24 hours after the manipulation showing increased BrdU labeling on the treated side (arrowheads). (D’) Adjacent section showing TUNEL-positive cells (arrowheads) in the same area indicating high-level proliferation.

L, left; R, right; b, bead; f, frontal bone; mxb, maxillary bone; nb, nasal bone; nc, nasal conchae; npp, nasal process of premaxillary bone; pmx, premaxilla. Scale bars: 5 mm in A-C’; 500 μm in C inset; 200 μm in D, D’; 100 μm in D’ inset.

derived from chicken (9/9 in whole-mount and 5/5 in sections) (Fig. 5A,B,D”). Six days after grafting there was an obvious outgrowth near the auditory meatus that, when sectioned, containing a lumen lined by thickened epithelium; however, no cartilage formed around the lumen (Fig. 5C; Fig. 7A,B,D).

To test whether the grafted nasal pit epithelium had retained its identity in the ectopic location, we examined SP8 and DLX5 expression. Neither gene was expressed in the grafted epithelium 24 or 48 hours later (14/14) (see Fig. S3A-B’ in the supplementary material; data not shown). Thus, to maintain the expression of olfactory-specific transcription factors, it is necessary for the nasal pit to be situated adjacent to the lateral nasal mesenchyme and telencephalon.

By the time host embryos had reached stage 38, ectopic outgrowths proximal to the eye were often found (stage 20 nasal pit, 6/17, Fig. 6A,B; stage 26 nasal pit, 12/22, Fig. 6D). There were significant alterations in skeletal morphology. The stage 20 nasal pit grafts induced skeletal changes mainly localized to the jaw joint region. The results can be divided into two categories: one in which grafts consisted of a collection of skeletal elements in close association with each other (compound grafts, 4/14) (Fig. 6A’,A”,C; Table 1A); and a second that includes ectopic, supernumerary elements (10/14) (Fig. 6B’,B”; Table 1A). The compound elements consisted of ~4-6 intramembranous bones that articulated with each other (Fig. 6A”,C). In the second, more common category, 1-2 isolated bones were formed at the proximal end of the quadratojugal bone (7/10). The nasal pits also induced small pieces of cartilage (6/10) (Fig. 6C) and these were sometimes closely associated with membranous bones (Fig. 6C, black arrowhead). In general, few of the skeletal elements formed had recognizable features, with one exception. The normal quadratojugal articulates with the quadratojugal bone, and at the point of articulation a characteristic secondary cartilage forms. We observed a rod of intramembranous bone with a similar secondary cartilage in all of the compound grafts (4/4) (Fig. 6C). The remaining intramembranous bones could have been either maxillary (jugal, maxillary or palatine) or lateral nasal (nasal bone).

Stage 26 nasal pits also induced compound (5/15) or isolated supernumerary (10/15) skeletal elements, but here cartilages were much larger and resembled the nasal conchae observed in grafts of supernumerary (10/15) skeletal elements (Fig. 6A’,A”,C; Table 1A). The compound elements consisted of 2-3 intramembranous bones that articulated with each other (Fig. 6A”,C). In the second, more common category, 1-2 isolated bones were formed at the proximal end of the quadratojugal bone (7/10). The nasal pits also induced small pieces of cartilage (6/10) (Fig. 6C) and these were sometimes closely associated with membranous bones (Fig. 6C, black arrowhead). In general, few of the skeletal elements formed had recognizable features, with one exception. The normal quadratojugal articulates with the quadratojugal bone, and at the point of articulation a characteristic secondary cartilage forms. We observed a rod of intramembranous bone with a similar secondary cartilage in all of the compound grafts (4/4) (Fig. 6C). The remaining intramembranous bones could have been either maxillary (jugal, maxillary or palatine) or lateral nasal (nasal bone).

In older, stage 20 host embryos, ectopic bones and cartilages were not as frequently observed as in younger hosts. The most common phenotype induced by the stage 20 or stage 26 epithelium was the induction of ectopic processes on the quadratojugal or squamosal bone (29/36) (Table 1B; see Fig. S4A,A”,C”,C, E in the supplementary material). Out of the remaining cases, most had formed small pieces of ectopic bone (7/36) (Table 1B; see Fig. S4B,D,D in the supplementary material). In general, cartilage was rarely observed in these grafts from stage 20 donors (3/12) (Table 1B), whereas the stage 26 donors induced large, lobular cartilages similar those seen in stage 15 hosts (10/24, data not shown). Controls in which only graft sites were prepared, or in which central frontonasal mass epithelium was placed, had normal morphology (Table 1B).

Therefore, we have shown that the stage 15 mesenchyme is able to respond to ectopic nasal pits, and that the inducing capacity increases with increased age of the epithelium. In general, there were no
replacements or deletions of the normally occurring skeletal elements showing that there are no repressive effects on skeletal development. Instead, the nasal pits induced supernumerary structures.

Since we had shown that FGF8 was one of the signals being provided by the nasal placode in the rescue experiments, we also implanted FGF8-soaked beads into the maxillary mesenchyme in the same position as the grafts. None of the embryos had abnormalities in the skeleton (11/11, data not shown). Therefore, diffusible signals in addition to FGF8 are being provided by the nasal epithelium. Similar to the effects of the maxillary bead implants, very few ectopic elements were induced in the flank grafts (3/100). Therefore, the patterning signals from the nasal pit are sufficient to pattern HOX-negative facial mesenchyme, whereas the HOX-positive trunk mesenchyme is not competent to respond. The nasal pit epithelium is clearly not sufficient to induce ectopic structures in the same way that an FGF-soaked bead can (Cohn et al., 1995; Vogel et al., 1996). We tested whether the nasal pit was able to locally change HOX gene expression in the flank. We looked at the expression of several HOX genes and many had to be excluded because they were downregulated in the lateral plate mesoderm at stage 20. On this basis, two members of the HOXB cluster were selected, HOXB1 and HOXB9. HOXB9 is responsive to FGFs and its expression is altered in embryos at sites where ectopic limbs are induced (Cohn et al., 1997), but HOXB1 has not been examined in this context. We found no change in either gene 16 hours after grafting (HOXB1, 4/4, see Fig. S5A,B in the supplementary material; HOXB9, 3/3, see Fig. S5C,D in the supplementary material), nor were SP8 and DLX5 present in the graft (see Fig. S5A-D in the supplementary material). Thus, a lack of change in gene expression correlated with the absence of ectopic limbs.

**Nasal pits induce PAX7 and differentiate autonomously in facial mesenchyme**

Since grafted nasal pits lacked SP8 and DLX5 expression there was a possibility that the epithelium had lost the ability to differentiate into olfactory epithelium. Furthermore, the grafting data were suggestive of ectopic nasal structures but were not definitive. We therefore investigated whether nasal passages lined with olfactory epithelium had formed and whether these were adjacent to PAX7-expressing mesenchyme.

Initially after grafts were placed, we were unable to detect PAX7 in the surrounding mesenchyme (22/22 for 24-72 hours) (see Fig. S3C-D’ in the supplementary material; data not shown). To determine whether there was a delay in the response of the host, we collected embryos 5-6 days after grafting. Indeed, after 5 days of growth we found that in all specimens (6/6) there was PAX7 antibody staining adjacent to the grafted epithelium in the mesenchyme (Fig. 7C-D’). The expression of PAX7 suggested that there was a partial conversion to a lateral nasal identity, while the remainder of the mesenchyme retained maxillary identity. This is consistent with the complex skeletal elements that were formed in fully differentiated grafts. We also examined sections to see whether the grafts were able to form neurons in an ectopic location. Differentiated neurons, as recognized by the TuJ1 (βIII tubulin) antibody, were present within and adjacent

---

**Fig. 5. Quail-chicken chimeras.** (A) Twenty-four hours after grafting, only the graft (inset) is stained with quail-specific Q¢PN antibody. The staple that secures the graft is visible. (B) The donor nasal pit has incorporated into the host mesenchyme and comprises quail epithelium. There are no contaminating quail mesenchymal cells. (C-D) An ectopic outgrowth is present on the side of the head under the eye. Alcian Blue stain shows cartilage is not present in the graft (C inset, arrowhead). The white line in the inset shows the plane of section in C and D. The graft is present within the outgrowth on the lateral side of the head (C,D, arrowhead). Differentiated neurons are observed near the graft (D’, arrowhead). A near-adjacent section, staining with Q¢PN antibody indicates that epithelium and neurons (arrowhead) are quail derived (D’). bc, basis cranium; e, eye; en, entoglossum; mb, mandibular branch of the trigeminal nerve; mc, Meckel’s cartilage; md, mandibular prominence; np, nasal pit; o, otic capsule; r, rhombencephalon; tg, trigeminal nerve body. Scale bars: 1 mm in A; 100 μm in A inset; 200 μm in C,D; 2 mm in C inset; 100 μm in B,D’,D’.
to the grafted epithelium (8/8) (Fig. 5D,D’; Fig. 7B’,B”D”,D”)). In addition, quail antibody staining showed that the neurons were graft in origin (Fig. 5D”; data not shown). In three cases, a distinct ectopic neural outgrowth close to the trigeminal ganglion was observed (Fig. 5D; Fig. 7B”), suggesting that the presence of the ganglion might have facilitated the ectopic outgrowth. There were regions of the grafted nasal pit epithelium that did not stain with the TuJ1 antibody and which we presume to be respiratory epithelium (Fig. 5D’; Fig. 7B’,B”D”,D”). Thus, the nasal pit invaginated and differentiated into neuron-containing epithelium in the ectopic location.

**DISCUSSION**

In this study, we have determined the roles of the nasal placode in patterning the craniofacial skeleton. The placodal ectoderm provides specific patterning cues to the lateral nasal skeleton, whereas removing large areas of maxillary ectoderm had no effect on skeletogenesis. We also found in gain-of-function experiments that nasal pit epithelium is capable of inducing the maxillary mesenchyme to express lateral nasal markers and ectopic skeletal elements. Both these approaches revealed the novel skeletogenic capacity of a structure mostly known for its role in olfactory neurogenesis.

**FGF8 is one of several signals required to make a nose**

We showed that replacing the olfactory placodal epithelium with an FGF8-soaked bead was sufficient to completely rescue the skeleton. We favor the explanation that FGF8 stimulated the proliferation of mesenchyme and restored some of the patterning cues provided by the nasal placode. A strong proliferative response in facial mesenchyme induced by FGF was also seen in another study from our laboratory (Szabo-Rogers et al., 2008). Similarly, FGF8 beads can promote proliferation of neural crest cells and these cells can
repopulate an extensive defect (Creuzet et al., 2004). When the placode ectoderm is removed, increased apoptosis is seen, depleting the progenitor cells. Interestingly, FGF8 was not sufficient to rescue apoptosis. Therefore, we might have removed additional, as yet unidentified cell survival signals.

Our gain- and loss-of-function experiments show that the nasal pit has instructive properties. However, it is clear whether unidentified signals in addition to FGFs are necessary for the elaboration of a complete nasal program. Although retinoids on their own are not sufficient to rescue the nasal

**Table 1. Skeletal changes induced in host embryos that received nasal pit grafts**

<table>
<thead>
<tr>
<th>A. Stage 15 host</th>
<th>Graft</th>
<th>Compound elements</th>
<th>Ectopic bone</th>
<th>Ectopic cartilage</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 20 nasal pit (n=14)</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Stage 26 nasal pit (n=15)</td>
<td>5</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stage 26 FNM epithelia (n=6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Control* (n=9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Stage 20 host</th>
<th>Graft</th>
<th>Ectopic processes on bones</th>
<th>Ectopic bone</th>
<th>Ectopic cartilage</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 20 nasal pit (n=12)</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stage 26 nasal pit (n=24)</td>
<td>22</td>
<td>9</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Stage 26 FNM epithelia (n=15)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Control* (n=15)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

FNM, frontonasal mass.
*Graft site preparation only.
skeleton, they might cooperate with FGFs and additional signals, such as BMPs (LaMantia et al., 2000), to pattern the nasal skeleton.

**The nasal placode can pattern skeletogenic mesenchyme but only in one direction**

The in ovo extirpation experiments in our study have shown that signals released from the placodal epithelium are secreted and are directional in nature. The normal expression of *TBX2/22* and *FGF8* and normal midline skeletal elements in extirpated embryos suggest that the nasal placode does not affect frontonasal patterning. These data differ from those of our previous study in which the medial side of the nasal slit epithelium was shown to provide diffusible FGF signals to the frontonasal mass mesenchyme (Szabo-Rogers et al., 2008). The difference in our two studies is that the present study focused on stage 15 embryos, whereas the previous study investigated patterning in stage 26 embryos. This shows that new functions are acquired for nasal slit epithelium as development proceeds. We also saw evidence of this in the induction of skeletal elements by differently staged nasal epithelia in the present study. The older nasal slit ectoderm was able to induce large cartilage elements, whereas younger epithelia could not.

Other data also support the specific competence of lateral nasal mesenchyme to respond to secreted signals, as reported in other studies (Firnberg and Neubuser, 2002; LaMantia et al., 2000). In organ cultures of stage 18 chicken frontonasal mass and lateral nasal prominences, replacement of epithelium by an FGF8-soaked bead maintained *PAX7* in the lateral nasal mesenchyme but did not ectopically induce expression in frontonasal mesenchyme (Firnberg and Neubuser, 2002). Thus, we conclude that lateral nasal mesenchyme differs from frontonasal mass in its sensitivity to patterning molecules.

**The identity of host mesenchyme is influenced by the ectopic nasal pit**

A directional patterning effect on mesenchyme was also demonstrated in the grafting experiments. Although the formation of cartilage is suggestive of lateral nasal-derived skeleton, the clearest evidence was provided by the induction of *PAX7* expression in the maxillary mesenchyme, a protein that is exclusively found in lateral nasal mesenchyme. Although a specific frontonasal mass marker awaits identification, we can nonetheless rule out the possibility that skeletal elements were frontonasal mass in character by their morphology. The frontonasal mass derivatives (premaxillo-nasal, premaxilla or egg tooth) can form in the stage 15 maxillary region when provided with the proper signals (Lee et al., 2001); however, here we have shown that nasal pit is incapable of inducing these structures even when placed into competent mesenchyme.

The nasal pit is almost able to induce an ectopic nose, with nasal passages and surrounding skeletal support, and this ability improves as development progresses. However, some organizational information is clearly lacking. It is possible that additional signals, perhaps from the adjacent tissues, would give rise to a more complete nose. This idea is supported by the effect of FGF4 beads placed in direct contact with nasal pits grafted onto frontonasal mass mesenchyme (Firnberg and Neubuser, 2002). This experiment results in a broad induction of FGF-responsive genes, an effect different to that of grafts of isolated nasal pits (no induction of expression) or placement of FGF beads without epithelium (local expression).

It is interesting to note that older studies on amphibians showed that the nasal region is able to organize the growth of an ectopic limb in the flank (Balinsky, 1933). By contrast, we rarely saw induction of skeletal elements in the flank, nor was there a change in HOX gene expression. We are unsure of the exact source of the donor tissue in the studies by Balinsky, nor are we certain whether mesenchyme was excluded. Perhaps tissue contamination or species differences between axolotl and chicken contributed to the difference in results. The difference we observed in the response of HOX-positive and HOX-negative mesenchyme is consistent with a lack of skeletogenic capacity in neural crest cells cultured from the trunk versus the head (Abzhanov et al., 2003), and with the inhibitory effect on facial skeletal formation if HOX genes are ectopically expressed (Creuzet et al., 2002).

**Autonomous differentiation of the nasal pit epithelium is supported in an ectopic location**

We have shown that the nasal pit is irreversibly determined by stage 20, coinciding with the onset of olfactory neuron production (Drapkin and Silverman, 1999; Wang et al., 2001). The differentiation of the epithelium continued as it would have in situ, with the formation of TuJ1-negative (presumptive respiratory) and TuJ1-positive (presumptive olfactory) domains. However, the nasal pit grafts were unable to maintain *SP8* or *DLX5* expression when grafted to an ectopic location in the face. This could mean that the mesenchyme lacks the signals to maintain olfactory epithelial gene expression, or that the maxillary mesenchyme suppresses the site-specific gene expression in the nasal epithelium. Nonetheless, even in the absence of stereotypic gene expression, the nasal pit can form nasal passages and neurons in other areas of the embryonic face.

The generation of neurons from our grafts is consistent with previous studies on frogs in which ectopic nasal placodes were grafted in locations close to the brain (Byrd and Burd, 1993; Koo and Graziadei, 1995; Stout and Graziadei, 1980). However, in these studies, not only did olfactory nerves form, but in the case of the midline grafts the ectopic nerve made the correct connection to the olfactory bulb (Byrd and Burd, 1993; Stout and Graziadei, 1980). These results are intriguing and in future studies we will use our established grafting paradigm to determine whether the olfactory nerve is capable of pathfinding and fully differentiating in facial mesenchyme that is distant from the central nervous system.

**Note added in proof**

A recent study by Bhattacharyya and Bronner-Fraser (Bhattacharyya and Bronner-Fraser, 2008) confirms our results that the olfactory placode is able to form thickened epithelium and neurons in ectopic locations. Our data extend their work by testing the competence of older mesenchyme. We show that stereotypical gene expression is not a prerequisite for placode-derived neurons to ingress into host mesenchyme.

We thank Sara Hooseini for help with photography, Nonhiza Hishagihori for preparation of Fig. 7, and Karen J. Liu and anonymous reviewers for helpful comments. H.L.S.-R. was supported by a J. Tonzetch fellowship. This work was supported by CIHR and MSFHR grants to J.M.R.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/219/DC1

**References**

Nasal pit effects on face morphogenesis


