

Sonic hedgehog signalling from foregut endoderm patterns the avian nasal capsule

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Morphogenesis of the facial skeleton depends on inductive interactions between cephalic neural crest cells and cephalic epithelia, including the foregut endoderm. We show that *Shh* expression in the most rostral zone of the endoderm, endoderm zone I (EZ-I), is necessary to induce the formation of the ventral component of the avian nasal capsule: the mesethmoid cartilage. Surgical removal of EZ-I specifically prevented mesethmoid formation, whereas grafting a supernumerary EZ-I resulted in an ectopic mesethmoid. EZ-I ablation was rescued by *Shh*-loaded beads, whereas inhibition of *Shh* signalling suppressed mesethmoid formation. This interaction between the endoderm and cephalic neural crest cells was reproduced in vitro, as evidenced by *Gli1* induction. Our work bolsters the hypothesis that early endodermal regionalisation provides the blueprint for facial morphogenesis and that its disruption might cause foetal craniofacial defects, including those of the nasal region.

KEY WORDS: Sonic hedgehog, Cephalic neural crest cells, Endoderm, Foregut, Mesethmoid, Nasal capsule, Chick

INTRODUCTION

The nasal capsule is dorsoventrally divided into two parts: the upper part, the ectethmoid, serves olfaction and is composed of the lamina cribrosa, the crista galli apophysis and the conchae. The lower part, the mesethmoid, is a thick cartilage bar extending from the corpus sphenoidalis to the rostral extremity of the nose (Fig. 1A–B'). In the avian embryo, the mesethmoid constitutes the cartilage primordium of the upper beak.

Lineage experiments have shown that Hox-negative cephalic neural crest cells (CNCCs) emigrating from the prosencephalic and the anterior mesencephalic neural folds give rise to the nasal capsule and to first pharyngeal arch (PA) structures (Couly et al., 1993; Creuzet et al., 2002; Kontges and Lumsden, 1996; Noden, 1992; Trainor and Tam, 1995). By contrast, Hox-positive NCCs, which are only found posteriorly to rhombomere 2, do not contribute to the facial skeleton, but generate more-posterior structures of the embryo (Couly et al., 1996; Kontges and Lumsden, 1996; Santagati and Rijli, 2003). Premigratory, Hox-negative CNCCs behave as an equivalence group and lack the topographic information needed to give rise to the different structures of the craniofacial skeleton (Couly et al., 2002). We have shown by surgical deletion and grafting of different parts of the foregut endoderm that this epithelium harbours the instructive signals (Couly et al., 2002; Noden, 1992; Ruhin et al., 2003), although their molecular nature remains to be determined. Among candidate cues, sonic hedgehog (*Shh*) is expressed in the most anterior part of the endoderm, endoderm zone I (EZ-I), of the early chick embryo (Brito et al., 2006). Later, incoming CNCCs express the *Shh* receptor patched 1, indicating a potentially active *Shh* signalling (Jeong et al., 2004). The importance of *Shh* signalling in the control of different aspects of craniofacial development has been demonstrated in several

models including zebrafish (Wada et al., 2005), mouse (Chiang et al., 1996; Jeong et al., 2004) and chicken (Brito et al., 2006; Cordero et al., 2004; Helms et al., 1997; Hu et al., 2003). Furthermore, human syndromes with nasal malformations, such as foetal alcohol syndrome, Smith-Lemli-Opitz syndrome and holoprosencephaly, have been associated with defective SHH signalling (Herman, 2003; Traiffort et al., 2004; Yamada et al., 2005).

MATERIALS AND METHODS

Avian embryos

Fertilised eggs were obtained from Morizeau Farms, France (chicken, *Gallus gallus*) or Cailles de Chanteloup Farms, France (quail, *Coturnix coturnix japonica*) and incubated at 38°C in a humidified atmosphere for approximately 32 hours to reach the 5-somite stage HH8+ (Hamburger and Hamilton, 1992; Teillet et al., 1998). Embryos were collected and dissected at room temperature in phosphate-buffered saline (PBS).

Embryo processing

Immunoperoxidase detection was performed as previously described (Couly et al., 2002). Quail nuclei were detected with the quail-specific monoclonal antibody QCPN at 1/500 (obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Chick and quail *Gli1* proteins were detected using rabbit polyclonal antibody #2553S (Cell Signaling Technologies) at 1/200. *Shh* transcripts were detected by in situ hybridisation as previously described (Couly et al., 2002). Whole-mount skeletons were visualised according to standard staining protocols using Alcian Blue for cartilage and Alizarin Red for bone (Couly et al., 2002).

Ablation of EZ-I

Experiments were carried out in ovo on windowed chick embryos at the 5-somite stage HH8+ (Couly et al., 2002). Two bilateral excisions were first performed on the superficial ectoderm on each side of the neural tube at the level of the prosencephalon and down to the mesencephalon (see Fig. S1 in the supplementary material). A very fine curved tungsten microknife was then passed under the neural tube and the notochord, resulting in their separation from the dorsal foregut endoderm. A transversal incision was then performed on the neural plate at the level of the posterior mesencephalon. The neural epithelium was reclined rostrally in order to gain access to the endoderm. The ventral endoderm was separated from the ventral ectoderm by passing a very fine curved tungsten microknife between the two tissues. The rostral-most attachment between the ventral endoderm and the anterior neural fold was then cut. At this point, the ventrolateral endoderm was free of any attachment and we could then easily remove the EZ-I by performing

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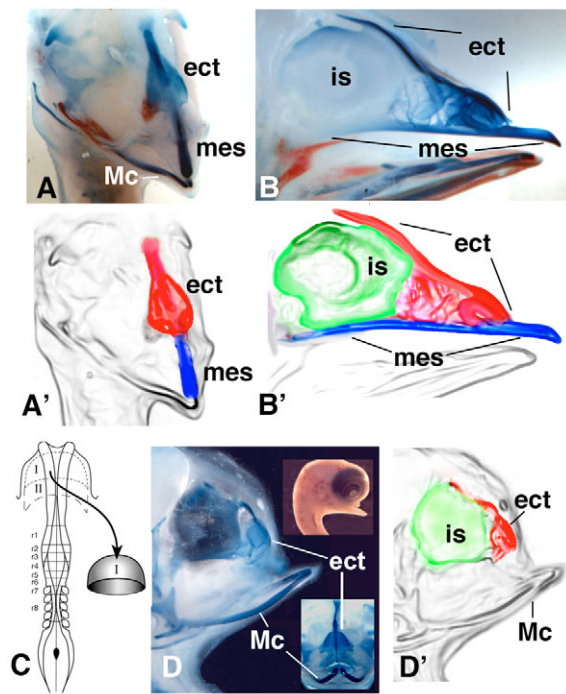


Fig. 1. Endoderm zone I ablation prevents mesethmoid formation. (A–B') Frontal (A) and lateral (B) views of the cephalic skeleton of a chick embryo at 9 days (HH35) with corresponding drawings (A', B'), highlighting the elements of the nasal capsule: blue, mesethmoid (mes); red, ectethmoid (ect); green, infraorbital septum (is). Mc, Meckel's cartilage. (C) Schematic of the surgical procedure of endoderm zone I (EZ-I) ablation. I and II, endoderm zones I and II; r1–r8, rhombomeres 1 to 8. (D) Lateral view of the chondrocranium of a representative HH35 embryo in which EZ-I has been ablated at the 5-somite stage. (Upper inset) The same embryo before skeletal preparation. (Lower inset) Frontal view of the same embryo showing the normal size and shape of the ectethmoid. (D') Drawing corresponding to D, using the same colour code as above. Note the absence of the mesethmoid, whereas the ectethmoid and the infraorbital septum are still present.

a transversal incision at a distance of ~150 μ m from its rostral end, and ~100 μ m in front of the anterior intestinal portal vein. At this stage, the caudal domain is EZ-II (Couly et al., 2002). The neural tube and the ectoderm were then delicately placed back in their initial position, where they rapidly reconnected with the rest of the embryonic tissues, resuming their development with no obvious defects. In a series of experiments, before closing the embryo, we placed a heparin acrylic bead (Sigma) in the vacant region where the EZ-I had been ablated. Embryos were reincubated until stage HH35 for morphological and chondrocranial analysis.

Grafts of quail EZ-I in chick embryos

Grafts of EZ-I were obtained from 5-somite quail embryos following the same dissection procedure described for EZ-I ablation in the chick. EZ-I grafts were transplanted into chick embryos at the 5-somite stage in ovo after performing a unilateral ectodermal incision at the appropriate axial level. The graft was placed either in the anteroventral mesenchyme (below the level of the prosencephalon) or into the presumptive mesenchyme of the first branchial arch. Some embryos were fixed and sectioned 24 hours after operation (HH14) to analyse *Shh* expression, to detect quail nuclei using the QCPN antibody, and to examine *Gli1* expression. In some cases, a simultaneous graft of both EZ-I and anterior neural crest was used to reveal, by QCPN immunostaining, the relative position of migrating CNCCs and of the grafted endoderm.

Effects of *Shh*- or cyclopamine-loaded beads

Experiments were carried out in ovo on windowed chick embryos at the 5-somite stage (Couly et al., 2002). For rescue experiments using *Shh*, heparin beads (120 μ m diameter; Sigma, St Louis, MO) were soaked in 100 μ g/ml mouse recombinant *Shh* (R&D systems, Minneapolis, MN) in PBS for 1 hour at 37°C, then rinsed three times in PBS immediately prior to use. Beads soaked in 0.1% BSA were used as control. EZ-I was ablated as previously described and then one bead was placed in the vacated territory. For experiments with cyclopamine, heparin beads were soaked in crystalline 11-deoxyjervine (Toronto Research Chemicals, Toronto, Canada) at 4 mg/ml in 95% ethanol, and then rinsed three times in PBS prior to use (Watkins et al., 2003). Control beads were soaked in PBS.

Culture and analysis of endoderm and CNCC explants

Microdissected tissue fragments from transverse domains of the ventrolateral endoderm or bilateral neural plate apical ridges were collected as illustrated in Fig. 4A (Couly et al., 2002; Ruhin et al., 2003). Endodermal stripes and neural crest fragments were washed in PBS, then either deposited onto 0.4 μ m porosity Millipore nylon inserts (Gitton et al., 1999) or onto glass Lab-Tek multichambered slides (Nunc) for up to 48 hours and cultured in Dulbecco's Modified Eagle Medium (Gibco, France). This defined medium was supplemented with a 1:1 mix of serum replacement medium (Sigma) and B27 solution (Gibco), a 1:1 mix of penicillin and streptomycin antibiotic mix (25 μ g/ml and 25 U/ml) and L-glutamine (2 mM, Gibco) as previously described (Dahmane et al., 2001). Pharmacological treatments included 20 μ M cyclopamine (R&D Systems) or 10 μ M *Shh* [recombinant mouse N-terminal fragment (*Shh*-N), R&D Systems] diluted in the culture medium. Significant cell emigration was observed around neural crest explants as early as 12 hours after incubation.

At the end of the culture period, the explants and surrounding cells were gently washed in culture medium, scraped and detached from the support and collected for total RNA extraction using the RNeasy Microkit (Qiagen). To obtain equivalent amounts of extracted material, equivalent numbers of tissue fragments were used, i.e. two EZ-I or two bilateral CNCC fragments were equated with each co-culture of EZ-I and CNCCs. For random hexamer-primed reverse transcription into total cDNA, we used the RT-PCR First-Strand Synthesis System (Invitrogen) including DNaseI treatment. Negative controls that omitted the Superscript II reverse transcriptase demonstrated that all samples were free of contaminant nucleic material (data not shown). Total cDNAs were analysed by PCR in the linear amplification range (30 cycles). Expression of chicken *Gapdh*, *Shh* and *Gli1* was monitored by PCR; primers and conditions are available upon request.

RESULTS AND DISCUSSION

To determine the function of EZ-I in the control of craniofacial development, we first ablated this endodermal territory in ovo from 5-somite chick embryos, well before CNCC migration (Fig. 1C; see Fig. S1 in the supplementary material) and analysed their skeletal morphology at Hamburger Hamilton stage 35 (HH35). In all surviving embryos (8/19), the mesethmoid cartilage was absent or severely reduced, whereas the ectethmoid and the infraorbital septum were always present and of relatively normal size and shape (Fig. 1D, D'). The adjacent ectoderm, neural tube and first arch CNCC derivatives developed normally.

Next we transplanted a supplementary EZ-I from 5-somite quail embryos into the presumptive nasal capsule territory of stage-matched chick embryos and analysed skeletal morphology at HH35. All survivors (12/21) displayed one ectopic, supernumerary mesethmoid-like cartilage (Fig. 2A, B, B') next to the normal and complete nasal capsule. Heterotopic quail EZ-I grafts, implanted within the 5-somite chick presumptive first PA territory, induced a supernumerary mesethmoid-like element in 10/12 survivors (Fig. 2C, D). Dermatocranial elements [putatively premaxillary bones (Couly et al., 1993)] formed in close proximity to the supernumerary cartilage. All other first and

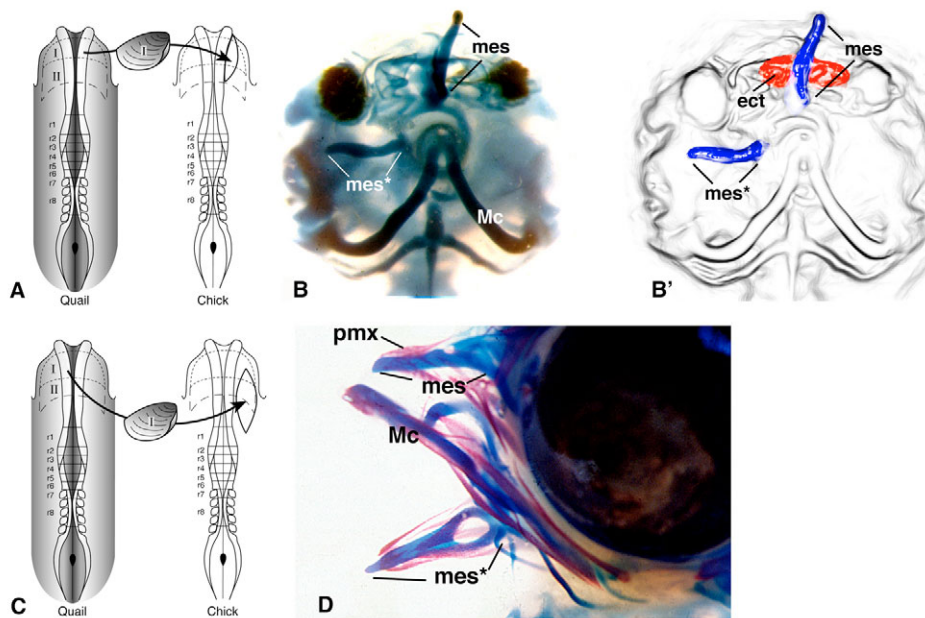


Fig. 2. Supernumerary EZ-I graft in the cephalic region induces an ectopic mesethmoid. (A) Dorsal schematic view of 5-somite quail and chick embryos showing the dissection of EZ-I from a quail embryo and its transplantation into the lateral anterior mesenchyme of a chick embryo. (B) Ventral view at HH35 of the chondrocranium of an operated embryo. A supernumerary mesethmoid develops within the nasofrontal region perpendicularly to the host nasal capsule, which develops normally. (B') Drawing corresponding to B, with the same colour code as in Fig. 1. (C) Transplantation of a quail EZ-I into the mesenchyme of the presumptive first PA of a recipient chick embryo. (D) Lateral view of a skeletal preparation from a 12-day (HH38) operated embryo. A supernumerary mesethmoid can be seen developing within the maxillary arch under the native Meckel's cartilage. ect, ectethmoid; I and II, endoderm zones I and II; Mc, Meckel's cartilage; mes, mesethmoid; mes*, supernumerary mesethmoid.

second PA derivatives developed normally. Both homotopic and heterotopic grafts suggest that EZ-I is necessary and sufficient to induce the differentiation of any Hox-negative CNCC contingent into a mesethmoid cartilage. Ectopic cartilage elements consistently failed to develop when we grafted EZ-I into the Hox-positive neural crest domain (data not shown).

We then examined whether *Shh* expression by EZ-I (Fig. 3A,B) (Brito et al., 2006) could be responsible for its capacity to induce mesethmoid formation. First, we doubly transplanted EZ-I and CNCCs from 5-somite quail into stage-matched chicken embryos. In all cases, the grafted EZ-I was strongly *Shh*-positive and was in close contact with grafted post-migratory CNCCs (Fig. 3A-C). We

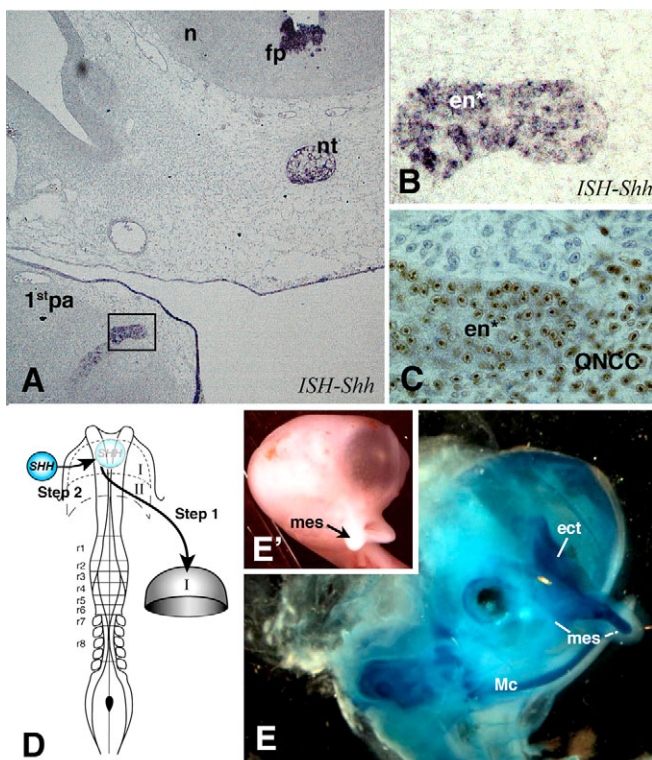


Fig. 3. *Shh* is expressed in EZ-I and can rescue the mesethmoid loss induced by EZ-I ablation. (A-C) This HH14 chick embryo simultaneously received two quail grafts at the 5-somite stage. One was a homotopic substitution of the anterior neural crest and the other a supernumerary EZ-I graft in the mesenchyme of the presumptive first PA. (A,B) Frontal section analysed by in situ hybridisation with a *Shh* probe, showing strong expression in the floor plate, in the notochord and in the grafted EZ-I (boxed). (C) Immunodetection of quail nuclei confirms the quail origin of the grafted endoderm and of the neighbouring CNCCs. (D) Dorsal schematic view of a 5-somite chick embryo showing the strategy of *Shh* rescue. After EZ-I ablation (Step 1), a *Shh*-loaded bead was implanted in the vacant region (Step 2). (E) Representative rescued embryo (HH35) showing almost normal development of the mesethmoid. (E') The same embryo before skeletal preparation showing the development of an upper beak. 1stpa, first pharyngeal arch; I and II, endoderm zones I and II; ect, ectethmoid; en*, supernumerary graft of EZ-I; fp, floor plate; Mc, Meckel's cartilage; mes, mesethmoid; n, neural tube; nt, notochord; QNCC, quail neural crest cells; r1-r8, rhombomeres 1-8.

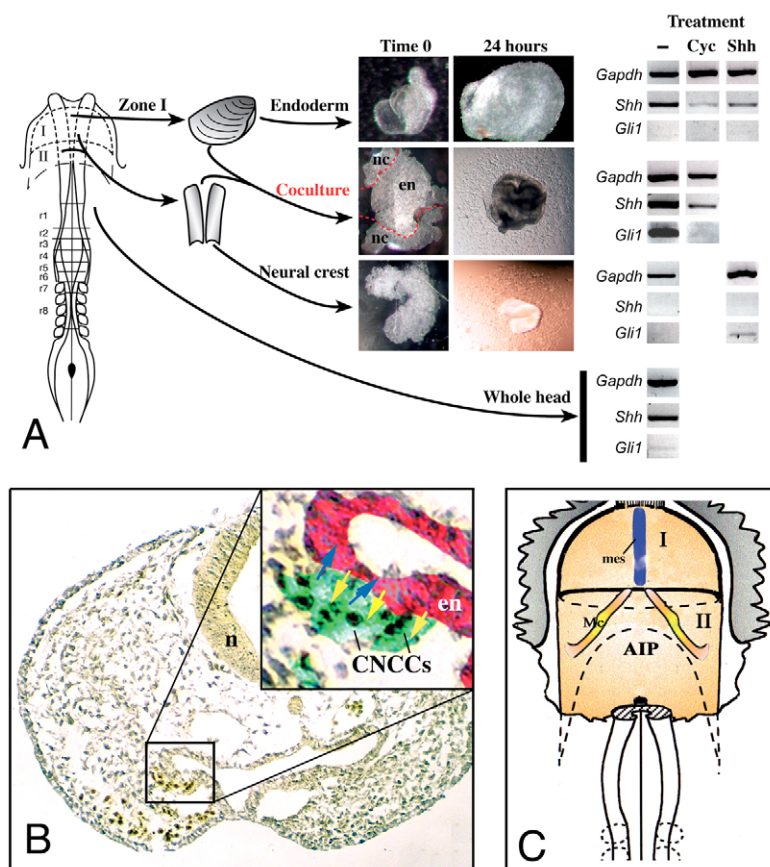


Fig. 4. The Shh-*Gli1* pathway mediates the interaction between EZ-I and CNCCs.

(A) Explant assay. Fragments were dissected from EZ-I and anterior apical neural crest ridges (see also Fig. S1 in the supplementary material) and were either cultured alone or co-cultured. (Right panel) RT-PCR for *Gapdh*, *Shh* and *Gli1* in untreated explants (–), after treatment with 10 µM cyclopamine (Cyc) or 10 µM Shh-N (Shh). The forehead of a 13-somite embryo was used as a control (whole head). Note the strong, cyclopamine-sensitive induction of *Gli1* expression occurring only in the co-cultures. **(B)** CNCC-endoderm interaction. Frontal section of a 13-somite chicken head grafted with 5-somite quail CNCCs and processed for QCPN immunodetection (dark nuclei). (Inset) Post-migratory donor CNCCs (green) contact the ventral aspect of the host endoderm (red) triggering a mutual exchange of inductive signals (arrows). **(C)** Endodermal fate map indicating the induction by EZ-I of the mesethmoid cartilage (blue). The 150 µm EZ-I domain is adjacent to the EZ-II domain, which drives Meckel's cartilage morphogenesis (Couly et al., 2002). I and II, endoderm zones I and II; AIP, anterior intestinal portal; en, endoderm; Mc, Meckel's cartilage; mes, mesethmoid; n, neural tube; nc, neural crest; r1-r8, rhombomeres 1-8.

then transplanted EZ-I into the presumptive first PA close to the ocular region and observed a strong ectopic induction of *Gli1*, a *Shh* target (Dahmane et al., 2001) not normally expressed in this area (see Fig. S2 in the supplementary material). To determine whether *Shh* expression from EZ-I directly contributed to mesethmoid induction, we ablated EZ-I from 5-somite embryos and implanted a *Shh*-loaded bead in the vacant territory (Fig. 3D). In all surviving embryos (18/36), the nasal capsule presented a mesethmoid-like cartilage correctly located under a normal ectethmoid (Fig. 3E,E'). Control BSA-loaded beads did not rescue the lack of mesethmoid resulting from EZ-I ablation ($n=6$).

To assess whether Shh-*Gli1* signalling was necessary to generate a mesethmoid cartilage, we then implanted a cyclopamine-loaded bead in contact with EZ-I at the 5-somite stage. In all survivors (14/36), the mesethmoid was absent, whereas the ectethmoid could be recognised (see Fig. S3 in the supplementary material).

To address whether direct CNCC–EZ-I interaction could trigger Shh-*Gli1* signalling, we examined organotypic cultures of endoderm and neural crest fragments from 5-somite embryos (Fig. 4A). We cultured the explants for 24 hours either alone or in combination. RT-PCR analysis of EZ-I explants alone indicated a weak constitutive *Shh* expression accompanied by a barely visible *Gli1* signal. Conversely, neither *Shh* nor *Gli1* transcription could be seen in CNCC explants. Co-cultures of EZ-I and CNCC fragments displayed substantially higher levels of *Gli1* transcripts, as compared with either component alone. *Shh* levels were also increased. Cyclopamine inhibition of Shh-*Gli1* transduction in the co-cultures prevented *Gli1* expression and reduced *Shh* levels (Fig. 4A). Furthermore, co-cultures of CNCC explants and endoderm zone II (EZ-II) failed to display *Gli1* expression, even after 48 hours in vitro (data not shown).

Consistent with the observation that endoderm-derived *Shh* activates *Gli1* in CNCCs, recombinant Shh-N protein induced *Gli1* in cultures of CNCCs alone, thereby reproducing the endoderm inductive effect. By contrast, exposure to either Shh-N or cyclopamine repressed *Shh* expression in cultures of endoderm explants.

Collectively, our in ovo and ex ovo data suggest a reciprocal, cyclopamine-sensitive interaction between EZ-I and uncommitted CNCCs. *Shh* expression increases in the rostral-most endoderm following contact with post-migratory CNCCs (Fig. 4B). *Shh*, in turn, strongly induces *Gli1* transcription in CNCCs. This inductive process leads to chondrogenesis, resulting in the formation of the mesethmoid cartilage (Fig. 4C), and later to the formation of the corresponding dermatocranial elements.

Our previous results suggested a very precise topographical engraving of endodermal information as the orientation of the endodermal grafts was precisely reflected in the shape and orientation of CNCC-derived ectopic cartilages (Couly et al., 2002).

Although the nature of these molecular clues is not yet elucidated, candidates include *Bmp4*, *Fgf8*, *Shh*, endothelin 1, noggin and retinoic acid (Foppiano et al., 2007; Hu et al., 2003; Lee et al., 2001; Song et al., 2004; Vieux-Rochas et al., 2007). The importance of Shh signalling for craniofacial morphogenesis (Brito et al., 2006; Haworth et al., 2007) and CNCC survival (e.g. Ahlgren and Bronner-Fraser, 1999; Brito et al., 2006; Charrier et al., 2001; Cordero et al., 2004) has been reported previously, but the topological and chronological significance of this pathway remains to be determined. In particular, it has been shown that the maintenance of a boundary between the territories of expression of *Fgf8* and *Shh* in the frontonasal process depends on reciprocal inhibitory interactions (Abzhanov et al., 2007; Abzhanov and Tabin, 2004; Haworth et al., 2007).

Our observations imply that endoderm-derived Shh signalling, which takes place already at the 5-somite stage, predates the ectodermal Shh contribution to craniofacial patterning (Hu and Helms, 2001; Hu et al., 2003). Globally, these results indicate that whereas early mesethmoid differentiation requires EZ-I *Shh* expression, ectodermal expression of Fgf8, Shh and Bmp4 controls the size and shape of the beak at later stages of development (Cordero et al., 2004; Hu et al., 2003; Wu et al., 2004). Shh is both a long-range diffusible morphogen and a short-range contact-dependent factor (Chuang and McMahon, 1999; Johnson and Tabin, 1995). Here, ablation of EZ-I, a topographically restricted source of Shh in the embryo, leads to a morphological lesion limited to the mesethmoid. This implies that, at least in this context, the action of Shh takes place in a localised paracrine, possibly contact-mediated, fashion. The human mesethmoid cartilage gives rise to the nasal septum and the vomer. It thus contributes to determining the proximodistal size of the nose and the position of the premaxillary bone. Our results could help to clarify the origin of a number of malformations of the nasofrontal bud (Couly, 1981) that are characterised by abnormal mesethmoid development, with normal ectethmoid. These pathologies include, for example, cases of hypo- and hyper-septoethmoidism such as a flat nose, short nose or very pronounced nose and a median nasal cleft without cerebral anomalies. Thus, although as yet we cannot answer Cyrano de Bergerac's question "*De quoi sert cette oblongue capsule?*" (What use this oblong capsule?), we may begin to understand where this oblong capsule comes from.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/13/2221/DC1>

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