

The role of Sonic hedgehog in normal and abnormal craniofacial morphogenesis

Diane Hu and Jill A. Helms*

Department of Orthopaedic Surgery, School of Medicine, U-453, University of California at San Francisco, 533 Parnassus Avenue, San Francisco, CA 94143-0514, USA

*Author for correspondence (e-mail: helms@cgl.ucsf.edu)

Accepted 15 August; published on WWW 6 October 1999

SUMMARY

There is growing evidence that implicates a role for Sonic hedgehog (SHH) in morphogenesis of the craniofacial complex. Mutations in human and murine SHH cause midline patterning defects that are manifested in the head as holoprosencephaly and cyclopia. In addition, teratogens such as jervine, which inhibit the response of tissues to SHH, also produce cyclopia. Thus, the loss of SHH signaling during early stages of neural plate patterning has a profound influence of craniofacial morphogenesis. However, the severity of these defects precludes analyses of SHH function during later stages of craniofacial development. We have used an embryonic chick system to study the role of SHH during these later stages of craniofacial development. Using a combination of surgical and molecular experiments, we show here that SHH is essential for morphogenesis of the frontonasal and maxillary processes (FNP and MXPs), which give rise to

the mid- and upper face. Transient loss of SHH signaling in the embryonic face inhibits growth of the primordia and results in defects analogous to hypotelorism and cleft lip/palate, characteristics of the mild forms of holoprosencephaly. In contrast, excess SHH leads to a mediolateral widening of the FNP and a widening between the eyes, a condition known as hypertelorism. In severe cases, this widening is accompanied by facial duplications. Collectively, these experiments demonstrate that SHH has multiple and profound effects on the entire spectrum of craniofacial development, and perturbations in SHH signaling are likely to underlie a number of human craniofacial anomalies.

Key words: Patterning, Craniofacial, Cleft lip/palate, Neural crest, Hypotelorism, Hypertelorism, Holoprosencephaly

INTRODUCTION

The face has fascinated man since the beginning of recorded history. From the cyclops Polyphemus, who plagued Homer's odyssey (850 B.C.), to Janus, whose duplicated face saw into the past and future, mythical creatures have reflected the spectrum of human craniofacial defects. Recently, clues about the regulation of craniofacial morphogenesis have come from analyses of Sonic hedgehog (SHH). In the head, *shh* is first expressed in the axial mesendoderm (Shimamura et al., 1995). Mutations in human (Belloni et al., 1996; Roessler et al., 1996) and mouse (Chiang et al., 1996) SHH lead to abnormal patterning of the neural plate and result in holoprosencephaly and cyclopia (Cohen and Sulik, 1992; Hammerschmidt et al., 1996). Later in facial development, *shh* is expressed in the ectoderm of the frontonasal and maxillary processes (FNP and MXPs, respectively; (Helms et al., 1997; Wall and Hogan, 1995). Grafts of *shh*-expressing facial ectoderm induce digit duplications in a limb bud assay system, suggesting that discrete domains of craniofacial epithelia may act as organizing centers (Helms et al., 1997); that is, they can instruct or program the developmental fate of host tissues.

However, these studies do not indicate the role that SHH plays in craniofacial development.

It has been assumed that the primary source of patterning information in the face was the cranial neural crest (Couly and Douarin, 1990; Hall, 1987; Noden, 1988, 1991; Osumi-Yamashita et al., 1994; Tyler and Hall, 1977). Analogies have been made between limb and visceral arch development (Wedden et al., 1988; Wolpert, 1988; Schneider et al., 1999), yet attempts to identify a signaling center in the face, similar to the zone of polarizing activity (ZPA) in the limb (Saunders and Gasseling, 1968), have not met with success (Richman and Tickle, 1989). Since SHH is likely to mediate the organizing activity of the ZPA in the limb (López-Martínez et al., 1995; Riddle et al., 1993), we postulated that it might play a similar role in instructing craniofacial development. Here, we present data to support a role for SHH in the program of facial development. Transient loss of SHH signaling during later stages of craniofacial morphogenesis produces a collapse of the facial midline and thus a condition known as hypotelorism, where the space between the eyes is reduced. Disrupting SHH signaling in the FNP or MXP leads to an interruption in their outgrowth, resulting in clefting between the primordia. These

avian defects are analogous to cleft lip/palate in humans. Conversely, excess SHH-N leads to hypertelorism. In severe cases, excess SHH-N in the midline of the developing face results in the formation of ectopic upper beak-like structures. Taken together, these data indicate that SHH plays critical, previously unrecognized roles in patterning the mediolateral axis of the mid- and upper face and suggest that perturbations in SHH, or its target genes, are likely to underlie a range of craniofacial malformations.

MATERIALS AND METHODS

Surgical excision of facial ectoderm

Embryos were incubated at 37°C to the appropriate Hamburger Hamilton (Hamburger and Hamilton, 1951) stage, then were cooled at room temperature for 30 minutes to slow heart rate. Embryos were positioned to gain access to the facial primordia; the ectoderm was removed using sharpened tungsten needles.

Dil labeling

Cells on the periphery of the wound were injected with 3.0 mg/ml 1,1'-dihexadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) dissolved in DMSO using glass micropipettes; embryos were collected after 48 hours and cells were visualized using fluorescence microscopy.

Anti-SHH antibody treatment

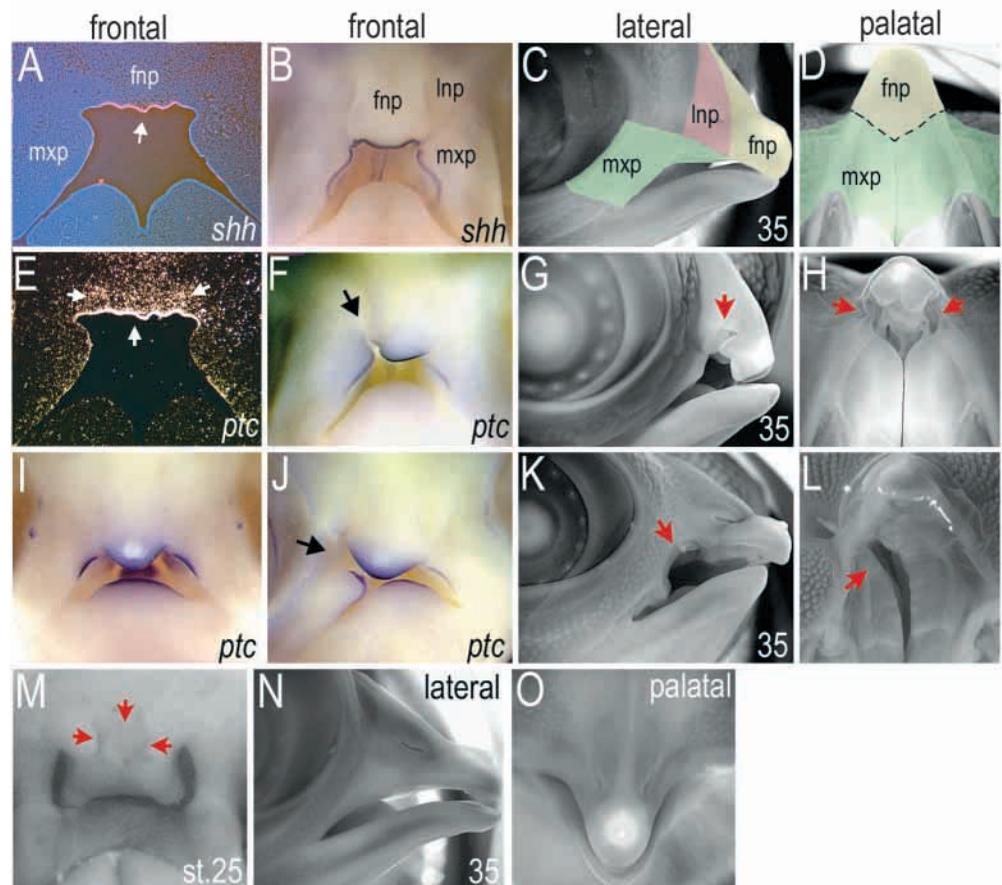
Affigel blue beads (Biorad) were shaken in 100 ng/ml 5E-1 (Ericson et al., 1996) for 1 hour, 37°C; control beads were soaked in preimmune serum, NS-1 (Ericson et al., 1996). Beads were implanted into the FNP or MXP mesenchyme at stage 20-21, or in the presumptive limb region at stage 16; bead position was confirmed 1 hour later.

Ectopic expression of SHH

RCAS-*shh*- or RCAS-*AP*-infected fibroblasts were injected into the FNP or MXP between stage 20 and 21, or into the anterior mesenchyme of a stage 20 limb as a control, and protein production was determined as described (Morgan and Fekete, 1996).

Fig. 1. Removing SHH ectoderm causes facial defects analogous to cleft palate/lip in humans.

(A) Frontal section of a stage 25 embryo; the Hoescht nuclear stain (blue) shows tissue outline. The hybridization signal for *shh* is pseudocolored red. *shh* is restricted to the medial ectoderm of the frontonasal and process. (B) Whole-mount in situ hybridization shows the equivalent expression pattern for *shh* in both the frontonasal and maxillary processes (fnp and mxp, respectively). (C) A lateral view shows that the frontonasal process contributes to the upper beak and midline of the face and primary palate (fnp shaded yellow; equivalent in humans to the forehead, midline of the nose, middle of the upper lip and primary palate). The lateral nasal process (lnp, shaded red) forms the alae of the nose, and the maxillary processes (mxp, shaded green) contribute to the cheeks and (D) the secondary palate. (E) Between stages 23 and 26, *ptc* is primarily detected in the ectoderm although low to moderate levels of expression are also detected in the mesenchyme of the FNP (arrows). (F) Removing *shh*-expressing FNP ectoderm at stage 25 results in a loss of *ptc* expression by stage 30 (arrow) and asymmetrical FNP growth. (G) By stage 35, a cleft is evident between the FNP and the LNP/MXPs (arrow). (H) Although excision of the ectoderm was unilateral, bilateral clefting of the primary palate is frequently observed due to early asymmetrical growth. The MXP-derived palatal shelves were unaffected in all cases (in avians, unlike humans, the palatal shelves approximate but do not fuse). (I,J) A similar type of clefting is observed when *shh*-expressing ectoderm from the MXP is excised. Following removal of *shh*-positive MXP ectoderm, *ptc* expression is lost (arrow) and MXP growth is arrested. (K) As a consequence of the growth arrest, the MXP fails to fuse with the LNP and FNP (arrow). (L) Palatal view: this defect is analogous to unilateral cleft palate in humans. (M) Removing FNP ectoderm that does not express *shh* has no discernable effect on facial morphogenesis. The wound site is shown in a stage 25 embryo immediately after excision (arrows denote wound periphery). (N,O) By stage 35, this extirpation has not affected the symmetrical growth or subsequent fusion of any facial primordia (recall that clefts can develop as a result of early asymmetric growth, e.g., Fig. 1H).



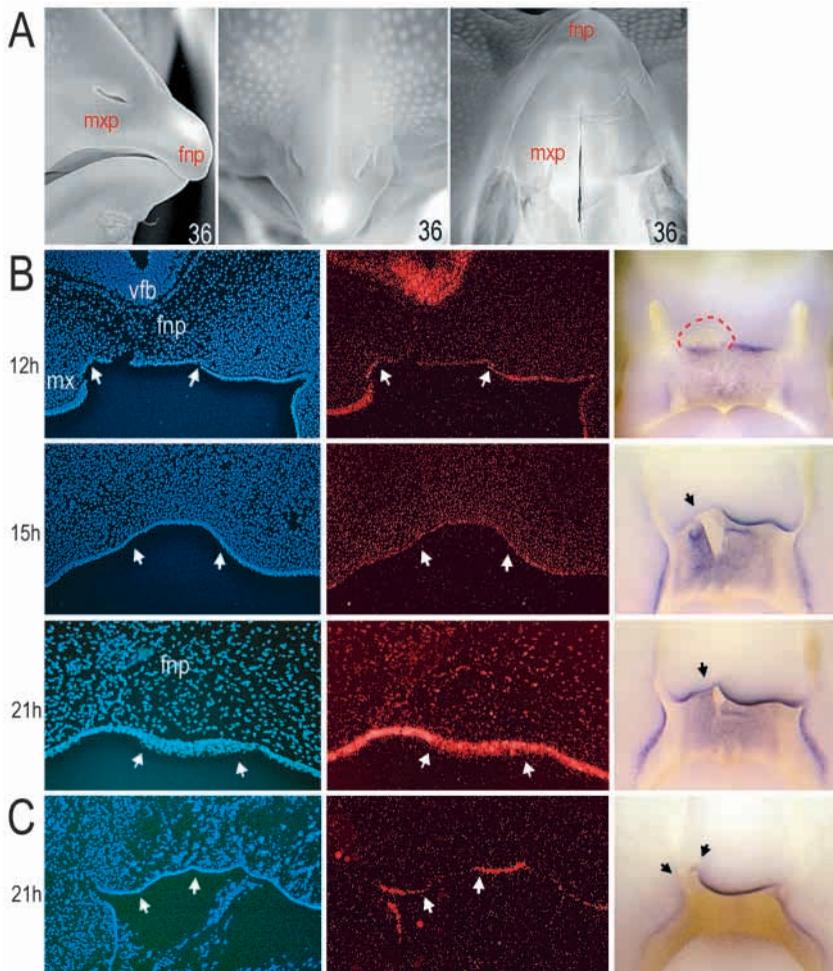


Fig. 2. Ectodermal SHH is essential for craniofacial morphogenesis. (A) Unlike the defects arising from excising stage 25-26 *shh*-positive ectoderm, removing *shh* ectoderm at stage 20-21 has no discernable morphological effect on facial development (lateral, superior and palatal views). (B) To ascertain why this surgical extirpation did not affect facial form, embryos were analyzed at multiple time points for wound re-epithelialization and *shh* re-induction. Within 12 hours of extirpation, the wound site (the space between the two arrows) is just beginning to re-epithelialize (the blue color is a Hoescht nuclear dye; the overlying expression patterns are shown as separate images where the hybridization signal is pseudocolored red). Although *shh* is abundantly expressed in the undisturbed FNP ectoderm, transcripts are not detected in the few cells of the newly regenerating ectoderm. Adjacent to this is an example of an embryo that had undergone the same surgery, and has been analyzed by whole-mount in situ hybridization for *shh* expression. The red dotted line indicates the site of the surgical excision. Note the loss of *shh* transcripts on the medial edge of the FNP; however, *shh* is expressed in the undisturbed ectoderm and in the roof of the stomadeum. 15 hours after surgery, the ectoderm has clearly regenerated (blue Hoescht stain), but *shh* expression is undetectable in the wound site. Whole-mount in situ hybridization shows that the surgical site (arrow) does not express *shh*, but *shh* is strongly expressed in adjacent FNP and MXP ectoderm. 21 hours after surgery, the wound site is re-epithelialized (arrows), and *shh* transcripts are detected in this regenerated ectoderm (arrows). A whole-mount embryo shows the same results (arrow). (C) The same surgical excision of FNP

ectoderm was performed on stage 25-26 embryos, which were analyzed in an identical manner for wound re-epithelialization and *shh* re-induction. The time course of wound re-epithelialization is the same in stage 25-26 embryos as in younger embryos (Hoescht stain). However, *shh* transcripts are not detected in the regenerated ectoderm of older embryos (arrows). Whole-mount analysis of these older embryos shows that *shh* is not re-expressed in the regenerated ectoderm (compare B, 21 hours with C, 21 hours). (vfb, ventral forebrain; fnp, frontonasal process; mxp, maxillary processes).

SHH-N beads

Affi-gel blue beads were soaked in 400-600 $\mu\text{g/ml}$ SHH-N for 1 hour at 37°C; beads were implanted underneath the FNP of stage 20-21 embryos.

BrdU labeling

Embryos were treated with beads soaked in SHH-N protein as described. 20 minutes prior to killing, 1 μl of BrdU-labeling reagent (Zymed, South San Francisco, CA) was injected into a blood vessel near the heart. Embryos were collected at 12 hours and 20 hours after bead implantation, and fixed in 4% PFA as described above. BrdU-labeled cells were visualized following manufacturer's instructions. Quantification of BrdU labeling was performed using Adobe Photoshop software to capture images of control and SHH-N-treated embryos, and Scion image to calculate statistical significance.

RESULTS

Loss of SHH function in the facial primordia

The ectoderm is required for the growth and development of

the facial primordia (Moury and Schoenwolf, 1995; Pinto and Hall, 1991; Richman and Tickle, 1989; Thorogood, 1988; Thorogood et al., 1986). However, the precise role that the ectoderm plays in mediating development of specific primordia is not well understood. Some data suggest that all facial epithelia are interchangeable and that facial ectoderm does not differ from limb bud ectoderm in its ability to support mesenchymal cell outgrowth and differentiation (Richman and Tickle, 1992). Other data argue that regions of the facial epithelia may serve different functions from limb ectoderm. One example is the ability of certain regions of facial ectoderm to act as organizers in a limb bud assay system (Helms et al., 1997), a quality that is found in the limb mesenchyme but not ectoderm (Saunders and Gasseling, 1968). Therefore, we set out to investigate in more detail the role of the ectoderm in facial development. We asked two questions. First, we investigated whether the ability of ectoderm to support the patterned outgrowth of the facial processes was a location-dependent property of the tissue. Second, we tested whether the capacity of the ectoderm to

maintain facial morphogenesis was dependent upon the developmental stage of the embryo.

Specific regions of facial ectoderm support patterned outgrowth of the primordia

Shh expression is limited to the ectoderm of the FNP and MXP (Fig. 1A,B) which gives rise to the upper beak and primary palate, the side of the face and the secondary palate (Fig. 1C,D). Since SHH regulates the patterned outgrowth of multiple tissues (Riddle et al., 1993; Helms et al., 1997; Chiang et al., 1996; reviewed in Hammerschmidt et al., 1997; Weed et al., 1997), we wondered whether *shh*-expressing ectoderm differed from other facial ectoderm in its capacity to maintain FNP and MXP growth. We excised small regions of *shh*-positive ectoderm from the FNP of stage 25-26 chick embryos, taking care to leave the underlying mesenchyme intact. 24 hours after the surgery, *patched* (*ptc*), a target of SHH (Goodrich et al., 1996; Marigo et al., 1996; Stone and Hynes, 1996), which is normally expressed in both the ectoderm and mesenchyme at this stage (Fig. 1E, arrows), was no longer detectable in the mesenchyme at the surgical site (Fig. 1F; $n=44$). Subsequently, we noted that the symmetrical growth of the FNP was inhibited (Fig. 1F). This growth inhibition eventually led to a failure of the FNP to fuse with the adjacent MXP (Fig. 1G,H), creating a defect in the chick embryos analogous to bilateral cleft lip and palate seen in humans (Tamarin et al., 1984). Similarly, when *shh*-expressing ectoderm from the MXP was removed, *ptc* expression was again lost in the mesenchyme (compare control embryo Fig. 1I, with Fig. 1J; $n=44$). As before, the MXP failed to grow symmetrically and did not fuse with the other primordia. As a consequence, a defect analogous to unilateral cleft palate/lip developed (Fig. 1K,L).

To test whether all epithelia supported the outgrowth of the FNP equally, we excised regions of *shh*-negative FNP ectoderm that were adjacent to the *shh* domain (Fig. 1M-O). Although these excisions were performed in areas of high cell proliferation (McGonnell et al., 1998), removal of the ectoderm had no discernable effect on the symmetry of the FNP, or on subsequent facial morphology (Fig. 1N,O and data not shown). From these extirpation experiments, we draw two conclusions. First, that *shh*-expressing ectoderm is required to support the outgrowth of the FNP. This is in agreement with previous reports that the FNP ectoderm is required for normal development of the facial primordia. Second, our results show that *shh*-positive ectoderm supported facial outgrowth differently than some other regions of ectoderm. Whereas the removal of *shh*-expressing ectoderm led to target gene downregulation and asymmetrical growth, removing adjacent FNP ectoderm, even in areas of high cell proliferation, did not cause discernable asymmetry in the primordia.

The role of the facial ectoderm changes during development

We next sought to determine whether the maintenance of craniofacial outgrowth was a stage-dependent property of the ectoderm. We were surprised to find that when we performed identical ectodermal extirpation experiments on the FNP of younger (stage 20-21) embryos, they developed normally (Fig. 2A; $n=60$). To ensure that we had removed a sufficient amount

of FNP ectoderm, we enlarged the region of surgical excision and examined embryos at frequent intervals for alterations in *shh* expression and their wound healing potential. To facilitate comparisons, we performed most of the experiments on one side of the FNP and left the other side undisturbed. 3 hours after ectoderm excision at stage 21, *shh* expression was not detected in the ectoderm in the operated site, indicating that ectoderm removal was complete ($n=10$; data not shown). 12 hours following excision, the ectoderm had begun to regenerate across the wound site (Fig. 2B, arrows); *shh* expression was undetectable in the regenerated ectoderm ($n=10$; Fig. 2B, arrows, dotted line). By 15 hours, the wound site had completely re-epithelialized, although *shh* expression was still undetectable ($n=14$; Fig. 2B). However, within 21 hours of the surgery, *shh* expression was clearly detectable in the regenerated ectoderm (Fig. 2B, $n=12$). By 40 hours postsurgery, *shh* transcripts were detected in a continuous band in the ectoderm, and only a slight asymmetry in the FNP was observed (data not shown; $n=32$). The regenerated *shh* expression pattern was limited to the ectoderm and no transcripts were detected in the underlying mesenchyme (Fig. 2B and data not shown). These data demonstrate that ectodermal wounds created in stage 20-21 embryos heal by re-epithelialization within 15-18 hours. In addition, the results indicate that *shh* is re-induced in the regenerated FNP ectoderm of these younger embryos.

We were intrigued by the fact that removing large regions of *shh*-expressing ectoderm from stage 20-21 embryos had no discernable effect on facial development (Fig. 2A), whereas removing even small regions of *shh*-expressing ectoderm from stage 25-26 embryos produced severe malformations (Fig. 1). We postulated that this might be related to the capacity of older embryos to re-epithelialize the wound site. To evaluate this possibility, we excised the FNP ectoderm from stage 25-26 embryos and evaluated them at frequent intervals. Within 12 hours of extirpation, there was no evidence of epithelium covering the wound site (data not shown). After 15 hours, a single layer of epithelial cells was generally evident at the wound site ($n=14$; data not shown). Within 21 hours of extirpation, the FNP surgical site had fully re-epithelialized. However, *shh* was not detected in the new ectoderm at this or subsequent stages (Fig. 2C, $n=10$). Taken together, these data indicate that facial ectoderm is not uniformly equivalent, either in regards to its location or stage. Whereas removing *shh*-expressing ectoderm at stage 25-26 results in facial clefts, removing *shh*-expressing ectoderm from a similar position at stage 20-21 does not result in facial deformity. This difference is not attributable to the ability of the wound site to re-epithelialize, but rather appears to be dependent upon the ability of the ectoderm to re-express *shh*. Furthermore, these data suggest that, even at a single stage of development, not all ectodermal domains are equivalent.

Regulation of *shh* re-induction in the FNP

Our next experiments were directed towards understanding what regulated the expression of *shh* in the regenerated ectoderm. Since lesions heal by the proliferation of epithelial cells from the wound periphery, one possibility was that *shh*-expressing cells on the edges of the wound preferentially populated the site. To test this, we labeled cells at multiple locations around the wound periphery using DiI. Some of the

injection sites were in regions that corresponded to *shh* expression domains (Fig. 3A, hatched region corresponds to site of *shh* expression), whereas other injections were performed in regions that did not express *shh* (Fig. 3A). Cells from the entire wound periphery contributed to the re-epithelialized site (Fig. 3B,C; $n=16$). DiI-labeled cells were detected in regions coincident with the site of *shh* re-induction in the ectoderm (Fig. 3D). These data suggest that the FNP wound healed by proliferation of epithelial cells from the entire circumference of the wound, rather than by the selective proliferation of *shh*-expressing cells.

We next investigated whether the underlying mesenchyme played a role in re-inducing *shh* in the regenerated ectoderm. To test this, FNP mesenchyme from stage 20-21 donor embryos was transplanted underneath the FNP ectoderm of stage 20-23 host embryos. None of these mesenchyme grafts induced the expression of *shh* in the overlying ectoderm (Fig. 3F,G; $n=16$). Although not conclusive, these results suggest that the FNP mesenchyme by itself was not sufficient to induce *shh*. However, other tissues, in combination with the mesenchyme, might be required for the induction of *shh* in the facial ectoderm. One potential source of inductive signals is the ventral forebrain (Rubenstein and Beachy, 1998), which is situated immediately adjacent to the FNP ectoderm at stage 20-21 (Fig. 3G,H). *Shh* is abundantly expressed in the basal telencephalon at this stage (Fig. 3H), and there are other examples where SHH in one tissue (e.g., the notochord) induces *shh* in an adjacent tissue (the floorplate; Ericson et al., 1996; Martí et al., 1995). We are currently exploring whether the neuroepithelial domain of SHH may regulate the expression of *shh* in the facial ectoderm.

Shh signaling is required for FNP and MXP development

Thus far, our experimental results demonstrate that localized, stage-dependent ectodermal signals are required for craniofacial morphogenesis. However, they do not separate the contribution of SHH signaling from other molecules produced by these regions of facial ectoderm. To isolate the effects of SHH signaling from other ectodermally expressed molecules, we decreased SHH signaling transiently in the FNP using a function-blocking antibody (Ericson et al., 1996). The antibody was effective in reducing SHH signaling in a limb assay, where it caused the downregulation of *ptc* and the subsequent loss of anteroposterior digit patterning ($n=3$; Fig. 4A). To reduce SHH signaling in the face, anti-SHH antibody beads were implanted into the facial primordia beginning at stage 20. Beads soaked in preimmune serum affected neither *ptc* expression nor craniofacial morphology (Fig. 4A, ctrl bead soaked in PBS indicated with an asterisk; $n=6$). In contrast, anti-SHH antibody beads decreased *ptc* expression (Fig. 4A, anti-SHH), which resulted in a failure of fusion between the primordia (Fig. 4B; $n=30$). These studies demonstrate that the ectoderm and specifically, SHH signaling in this tissue, is required for the growth and subsequent fusion of the mid- and upper face primordia.

From our loss-of-function data, we draw three conclusions. First, we have confirmed previous findings that the ectoderm is required for facial morphogenesis. We have extended these observations to show that all regions of the facial ectoderm are not equivalent. Removing those regions expressing *shh* caused

asymmetrical growth that led to clefting, whereas removing other ectoderm did not produce this same asymmetry. Second, we have shown that facial ectoderm regenerates following extirpation, regardless of the developmental stage of the embryo. An important difference, however, is that *shh* is re-induced in the new ectoderm of young (stage 20-21) embryos whereas it is not re-expressed in the new ectoderm of older (stage 25-26) embryos. The mechanism responsible for the ability of younger embryos to generate a new *shh* expression domain is unclear.

Gain of SHH function in the FNP

Midline patterning of the neural plate depends on SHH signaling (Chiang et al., 1996). Therefore, we reasoned that ectopic SHH might lead to an expansion of midline structures. We used two approaches to deliver SHH-N ectopically to the craniofacial primordia. First, retrovirally infected fibroblasts expressing the amino terminal portion of *shh* (RCAS-*shh*) or control fibroblasts expressing alkaline phosphatase (RCAS-AP) were injected into facial primordia between stages 20 and 21. Fibroblasts infected with RCAS-AP neither affected endogenous *shh* expression (Fig. 5A), nor altered subsequent craniofacial morphology in a discernable way (Fig. 5B). In contrast, RCAS-*shh* fibroblasts produced a localized area of cell proliferation in the FNP (Fig. 5C,D). These cells expressed *shh* (Fig. 5C,D). In the majority of cases (65%; $n=26$), the mediolateral (nose-to-ear) width of the FNP was expanded by almost 40%, and the intercanthal distance by 30% (Fig. 6C). In the remaining 35% of the cases ($n=16$), the expansion along the mediolateral axis was also accompanied by the formation of ectopic structures in the soft and hard tissues (Fig. 5E-J'). In some cases, the ectopic structures were located more proximally on the upper beak, along the superoinferior axis (Fig. 5E-F''). In other cases, the ectopic structures were located along the mediolateral axis (Fig. 5H-J'). In all cases, the ectopic tissues were vascularized, had a hyperkeratotic epithelial thickening that resembled the egg tooth on their superior surfaces (Fig. 5, asterisks) and contained a cartilaginous rod resembling the primordium of the nasal septum (Fig. 5, arrows). These ectopic elements did not appear to form as a result of a bifurcation of the autochthonous nasal septum, since its shape was unaffected (Fig. 5E',F'').

In a second gain-of-function approach, we used the amino-terminal portion of SHH (Porter et al., 1995) to test the effects of the protein on craniofacial patterning. Beads soaked in SHH-N were implanted into the FNP between stages 20 and 21 and embryos were incubated for varying times, then examined for alterations in cell proliferation and gene expression. To simulate the location-specific endogenous SHH signal, the bead was positioned immediately ventral to the surface ectoderm and in direct contact with the underlying mesenchyme. The bead remained in contact with the mesenchyme for a minimum of 12 hours (Fig. 6A). In comparison to control embryos treated with PBS beads, those treated with SHH-N showed increased cell proliferation in the FNP within 20 hours of bead implantation (Fig. 6A; $n=5$, control embryos; $n=8$, treated embryos at 12 hours; $n=8$ at 20 hours). The average number of BrdU-labeled cells in the FNP of SHH-N-treated embryos was 420.4 ± 72.6 as compared to 159.3 ± 19.7 in control embryos from the same stage (Fig. 7). The cell proliferation also appeared to be asymmetrical, as

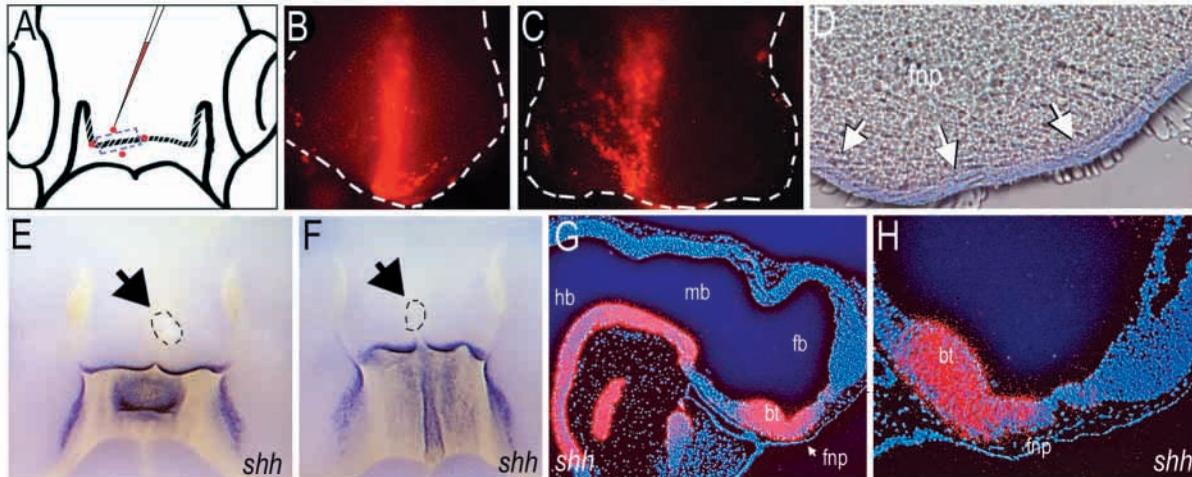
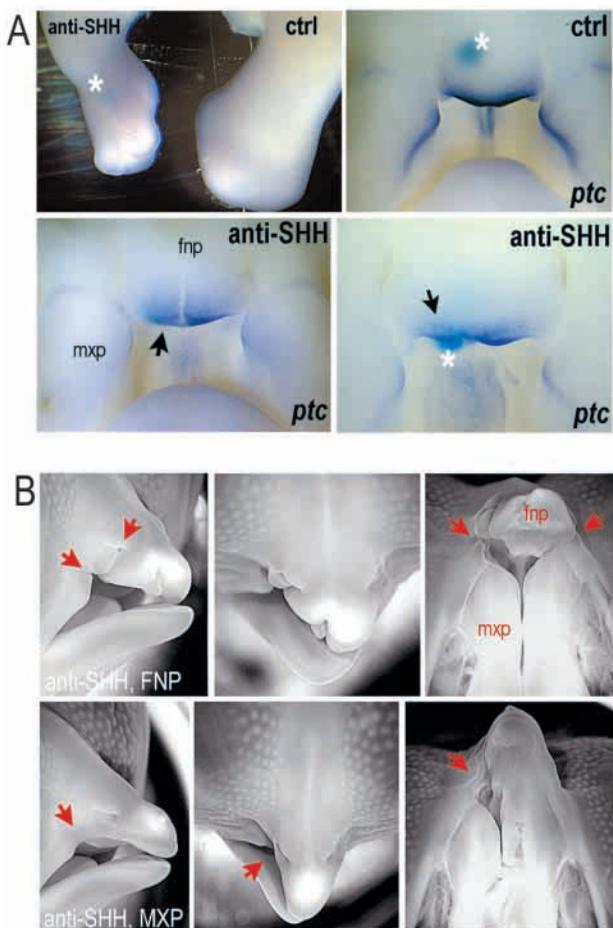


Fig. 3. Contribution of mesenchyme and forebrain tissues to the re-induction of *shh* in the FNP ectoderm. (A) FNP ectoderm was excised at stage 20-21, following which embryos received single DiI injections in one of four different locations (indicated by red dots on the FNP of a stage 21 embryo). Cells from superior regions did not express *shh* whereas cells from lateral and inferior positions (hatched region of FNP) expressed *shh* at the time of labeling. Embryos were incubated for 24 hours, then examined under fluorescence for the position of DiI-labeled cells. (B,C) Two examples of DiI-labeled cells in the FNP all resulted from injections in the superior site (indicated by the position of the glass needle in A). The white dashed line indicates the margins of the FNP in frontal view of the treated embryos. (D) Embryos treated as in B were subjected to whole-mount in situ hybridization and sectioned; *shh* transcripts were detected exclusively in the regenerated FNP ectoderm and were excluded from the underlying mesenchyme (arrows). (E,F) Transplantation of stage 20-23 mesenchyme underlying the *shh* domains did not lead to ectopic sites of *shh* in the FNP (arrows, dotted lines indicate site of grafts). (G) The close proximity between the forebrain domain of *shh* and the FNP ectoderm is seen in sagittal sections of stage 21+ embryos. (H) A higher magnification shows that *shh* in the neuroepithelium lies within a few cell diameters of the FNP ectoderm. fb, forebrain; bt, basal telencephalon; mb, midbrain; hb, hindbrain. Arrow indicates FNP.



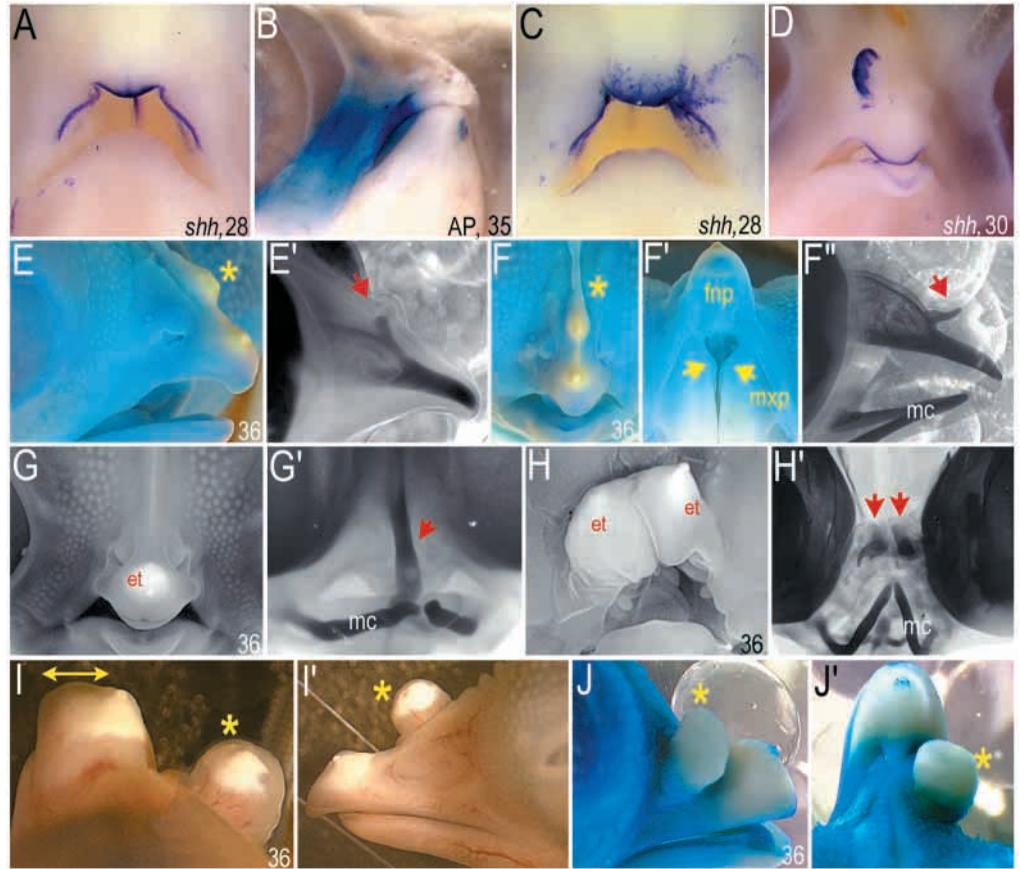
most of the BrdU-labeled cells were located distally to the bead (Fig. 6A, arrow).

Embryos were also examined for the induction of genes in response to SHH-N at multiple time points after SHH-N bead implantation. SHH-N did not induce ectopic *shh* expression in the FNP after 12 hours (Fig. 6B) or after 20 hours (Fig. 6B, $n=11$). However, in comparison to control embryos, SHH-N did induce an expansion of the ectodermal domains of *bmp2* at 12 hours (Fig. 6B, *bmp2* arrow, $n=11$). By 20 hours, an upregulation of *ptc*, *bmp2*, and *gli1* was detected in the mesenchyme surrounding the bead as compared to control embryos (Fig. 6B, 20 hours SHH-N bead panel). Although *ptc* and *gli1* were induced symmetrically around the bead, *bmp2* was upregulated primarily in cells distal to the bead (Fig. 6B), corresponding to the area of increased cell proliferation (Fig. 6A).

3 days after SHH-N bead implantation, SHH-N-treated

Fig. 4. Transient inhibition in SHH signaling arrests the growth of the facial primordia. (A) Treating limb buds with anti-SHH antibody results in a loss of anteroposterior polarity, demonstrating the efficacy of the antibody to reduce SHH signaling in ovo (asterisk indicates the placement of the bead). When the anti-SHH antibody beads are placed into the FNP, they have a similar effect on *ptc* expression. While control (ctrl) beads soaked in preimmune serum have no discernable effect on *ptc* expression or on craniofacial morphology, anti-SHH beads reduced *ptc* expression on the treated side of the FNP (arrows). (B) Reducing SHH signaling produces clefting between the FNP and the LNP/MXPs (arrows). Superior views show truncation of the FNP, and palatal views illustrate the clefting between the FNP and MXP. Similar results are obtained when the MXP is treated with anti-SHH beads.

Fig. 5. Gain of SHH results in hypertelorism and ectopic skeletal structures. (A) Injections of control fibroblasts infected with RCAS-AP do not alter endogenous *shh* expression (shown in frontal view at stage 28). (B) These control embryos develop normal upper beaks (frontal view shown at stage 35) despite the fact that the fibroblasts produced abundant alkaline phosphatase, as indicated by blue staining (in this case the RCAS-AP fibroblasts were injected into the MXP). (C) Injections of RCAS-*shh* fibroblasts into the FNP cause an expansion of *shh* expression by stage 28. (D) By stage 30, most embryos exhibit an ectopic site of *shh* expression. (E) By stage 36, RCAS-*shh* fibroblasts induce the formation of ectopic egg teeth (asterisk); (E') underlying this egg tooth is an ectopic cartilaginous rod (arrow) extending from the nasal septum as shown by Alcian blue staining (embryo in lateral view). (F) Another treated embryo, as viewed from a superior position, exhibits an ectopic egg tooth (asterisk); (F') a palatal view of this same embryo shows the widening between the palatal shelves caused by the hypertelorism. (F'') A lateral view shows the ectopic cartilage (arrow) associated with the egg tooth seen in F (embryo stained with Alcian blue; mc, Meckel's cartilage). (G) Frontal view of a control embryo at stage 36 (et, egg tooth); (G') The same embryo stained with Alcian blue to show the midline cartilaginous nasal septum (arrow). (H) Another example of an embryo treated with RCAS-*shh* fibroblasts; in this case, the phenotype is more severe. Instead of a single, median egg tooth, two egg teeth are present, as well as (H') an apparent duplication of the nasal septum (arrows). (I, superior and I', lateral views) An example of another embryo treated with RCAS-*shh* shows the large ectopic structure resembling an upper beak (asterisk). The autochthonous upper beak is widened considerably as compared to that in control embryos (the arrow illustrates the average width of an upper beak at stage 36). (J, lateral and J', superior) Another example of an embryo with ectopic structures that resemble a duplicated upper beak; asterisk indicates the ectopic egg tooth.



embryos exhibited a substantial widening of the mid-face, as compared to stage-matched control embryos treated with PBS beads (Fig. 6C). The average increase in width of the FNP was 48%, with a 28% increase in the intercanthal distance ($n=26$). Despite this gross morphological alteration, the ectodermal expression of *shh* and *ptc* at this stage were relatively unaffected (Fig. 6C). When embryos reached stage 36, their palatal shelves failed to abut, resulting in a gap (Fig. 6D; $n=20$). This defect is analogous to secondary palatal clefting in humans, which frequently accompanies hypertelorism and expansion of the ventral forebrain (Gorlin et al., 1990). Taken together, these gain-of-function studies demonstrate that excess SHH-N induces cell proliferation, the ectopic expression of *ptc*, *gli1* and *bmp2* in the FNP, and leads to an expansion of the mediolateral width of the face and related brain structures. Embryos also exhibited clefting of their secondary palate, most likely a consequence of increasing midfacial width. In the most severely affected cases, ectopic SHH-N induced the formation of ectopic structures that resembled upper beaks.

DISCUSSION

Craniofacial morphogenesis is a complex process that requires the coordinated growth and fusion of distinct primordia, each of which may have their own organizing centers and elaborate interactions between multiple tissue layers. The ectoderm and its associated placodes, the brain and axial mesendoderm, and the cranial neural crest all contribute both inductive and positional information to the face (Couly et al., 1993; Köntges and Lumsden, 1996; Noden, 1988, 1991). Here, we have specifically focused on the role of SHH in mediating the later stages of craniofacial morphogenesis.

Loss of SHH function

Four lines of evidence demonstrate that SHH signaling is required at specific stages during facial morphogenesis, and that disruptions in SHH signaling lead to varying degrees of facial dysmorphologies. First, mutations in human and murine SHH perturb mediolateral patterning of the neural plate, resulting in holoprosencephaly and cyclopia (Belloni et al.,

1996; Hammerschmidt et al., 1997; Roessler et al., 1996). Second, pharmacologic doses of retinoids (Lammer et al., 1985) and cholesterol analogs (Cooper et al., 1998; Incardona et al., 1998; Keeler, 1975; Porter et al., 1996a,b; Sim et al., 1983) induce facial dysmorphologies in part through their misregulation of SHH signaling (Helms et al., 1997). Third, other teratogens, such as the plant alkaloid jervine, are potent inducers of holoprosencephaly and clefting in sheep and other animals (Gaffield and Keeler, 1996) and act by inhibiting the response of target tissues to SHH (Cooper et al., 1998; Porter et al., 1996a,b). Fourth, humans with cholesterol metabolism disorders, such as Smith-Lemli-Optiz syndrome (Cunniff et al., 1997), exhibit holoprosencephalic and microcephalic characteristics, which may result from an inability of target tissues to respond appropriately to SHH (Cooper et al., 1998; Porter et al., 1996a,b). The vast majority of these genetically and epigenetically induced defects result from perturbations during the earliest stages of embryonic development. Since SHH is abundantly expressed in the facial primordia long after these early patterning events have occurred, we were interested what role the protein might be playing at subsequent stages of development. The data presented here indicate that SHH, and proteins in the SHH signaling pathway such as *Gli1*, *Bmp2* and *Ptc*, play key roles in regulating the patterned outgrowth of the FNP and MXPs and specifying the mediolateral axis of the face. We show that transiently inhibiting SHH signaling results in a truncation in the primordia and subsequently leads to facial clefting. Conversely, ectopic SHH signaling leads to an expansion in the mediolateral width of the FNP and, in some cases, the apparent duplication of midline structures such as the nasal bone.

The capacity to re-express *shh* in the facial ectoderm is a stage-dependent property

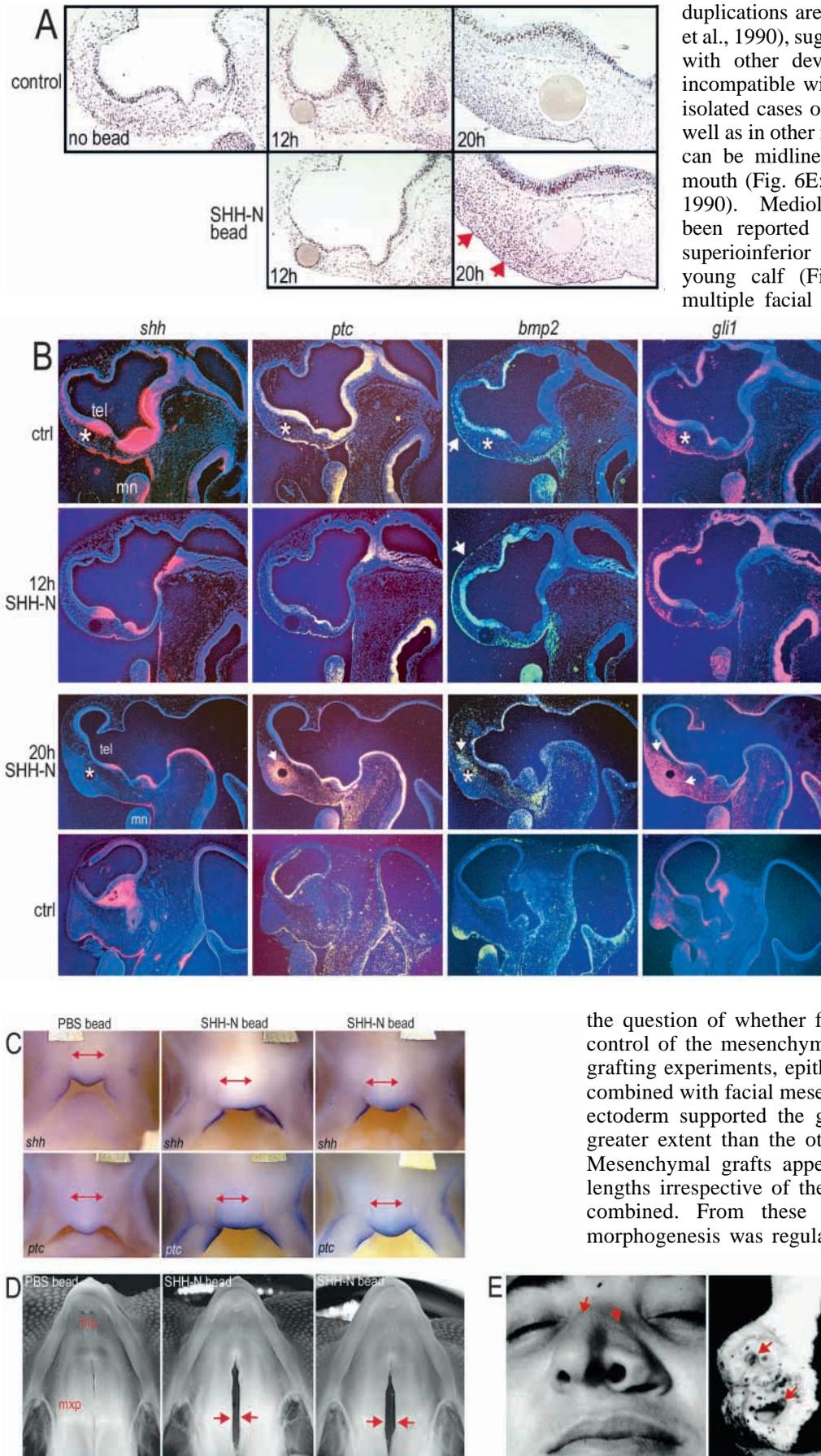
Our results, and those of others (Tamarin et al., 1984), indicate that excising FNP or MXP ectoderm produces clefts in the primary palate. Therefore we were surprised to find that younger embryos treated in an identical manner did not develop clefts. A detailed temporal and spatial analysis revealed an inherent difference between younger and older embryos treated in this manner: although the wound sites in both younger and older embryos re-epithelialized, only in younger embryos were transcripts of *shh* detected in the regenerated ectoderm. Presumably, the lack of SHH in the regenerated ectoderm of older embryos led to a loss of cell proliferation whereas, in younger embryos, the regenerated SHH domain maintained proliferation of mesenchymal cells and thus promoted further growth of the FNP.

We next asked whether the underlying mesenchyme was responsible for this re-induction of *shh*. Our results indicated that, by itself, the FNP mesenchyme was not sufficient to induce ectopic *shh* in the face. However, the experiments did not eliminate the possibility that the mesenchyme could be acting in combination with other tissues, such as the ventral forebrain, to induce or maintain *shh* expression. Alternatively, planar inductive events may account for the regenerated *shh* ectodermal domain. At earlier stages of development, *shh* is expressed in the roof of the stomadeum and this epithelium contributes to the regenerated wound site (Fig. 3), suggesting that at least some of the *shh*-positive cells in the new *shh* domain could have originated from this region.

A gain of SHH function causes hypertelorism and the formation of ectopic midline structures

Increasing SHH in the FNP promotes mediolateral expansion of the face, a condition referred to as hypertelorism. In humans, hypertelorism can be accompanied by an expansion of the ventral forebrain. In our experimental system, embryos with the most severe cases of hypertelorism also exhibited apparent duplications of midline facial structures. True facial

Fig. 6. Excess SHH-N induces hypertelorism and secondary palatal clefting. (A) Beads soaked in SHH-N, or control beads soaked in PBS, were implanted into the FNP of stage 20-21 embryos. Embryos were incubated for 12 and 20 hours, and received an injection of BrdU 20 minutes prior to killing. Untreated embryos, and those that received PBS beads are shown in the top panel. By 12 hours, stage-matched embryos treated with SHH-N show a slight increase in BrdU labeling over that seen in control embryos; see Fig. 7. By 20 hours, a moderate increase in BrdU labeling is observed in SHH-N embryos; most labeled cells are distal to the bead (arrows). There also appeared to be an increase in BrdU labeling in the neuroepithelium of the forebrain. (B) SHH-N induces ectopic target gene expression. Sagittal sections through the head region of control and treated embryos were stained with Hoescht nuclear dye (blue); hybridization signals were collected as white light and pseudocolored to differentiate among the mRNAs. The embryos were sectioned in their entirety, and representative sections are shown. To facilitate direct comparisons between expression domains in a single embryo, near-adjacent sections are used. PBS beads were used as controls and show that the experimental manipulations did not alter endogenous gene expression. An asterisk marks the bead holes. 12 hours after bead implantation, endogenous *shh* expression is seen in the midbrain, basal telencephalon (tel) and in the FNP ectoderm (12h PBS bead). SHH-N neither induces nor expands the endogenous *shh* domain at 12 or 20 hours (compare ctrl embryos with 12h SHH-N bead, 20h SHH-N bead). In the same embryo, SHH-N did not cause an increase in *ptc* expression after 12h; however, after 20h, *ptc* was strongly upregulated in the mesenchyme surrounding the bead (compare ctrl embryos with 12h and 20h SHH-N). SHH-N causes an expansion in the ectodermal domain of *bmp2* at 12 hours (arrows) and predominantly induces *bmp2* anterior to the bead (asterisk marks bead hole, arrow indicates expression domain). *Gli1* is most notably induced in the mesenchyme surrounding the bead hole after 20h (arrows). Although some changes were seen, no consistent alterations in forebrain and midbrain expression patterns for *shh*, *ptc*, *bmp2* and *gli1* were observed. (C) Compared to stage-matched controls treated with PBS-soaked beads, embryos treated with SHH-N show considerable widening of the FNP by stage 28. This widening of the FNP causes a change in the shape of the beak, but does not significantly alter the expression pattern of *shh* (top panel) or *ptc* (bottom panel) in the facial primordia at this stage. In all figures, the arrow is same length. The metal bar – top right of the pictures – was used to show equivalent magnifications and to determine absolute width of the FNP. (D) By stage 36, SHH-N-treated embryos have developed gaps between the palatal shelves (arrows), which are not evident in control embryos. This clefting is a secondary consequence of mediolateral expansion of the midline. (E) Spontaneously occurring facial duplications. A 19 year-old man exhibits a duplication of the external nares (photograph with permission from [Craniofacial Malformations](#), M. Striker, editor). Duplications of the nose of a calf; note four external nares (arrows; photograph courtesy of D. Noden). The most complete duplications are seen in this pig ('Ditto'), who has two complete snouts, two tongues, two esophagi, three eyes (asterisk denotes middle eye) with three optic stalks. The maxilla contains four rows of teeth. The pig was donated to J. A. H. by Pigs without Partners, courtesy of R.T.



duplications are extremely rare in humans (Gorlin et al., 1990), suggesting that they may be associated with other developmental disturbances that are incompatible with life. There are a few reports of isolated cases of facial duplications in humans, as well as in other mammals. The duplicated elements can be midline structures such as the nose and mouth (Fig. 6E; Gorlin et al., 1990; Striker et al., 1990). Mediolateral nasal duplications have been reported in humans, and an example of superioinferior nasal duplication was seen in a young calf (Fig. 6E). A striking example of multiple facial duplication is illustrated in a pig

with two complete snouts, two tongues, a bifurcated esophagi, four sets of maxillary teeth and three eyes with their associated optic stalks (Fig. 6E). All of these duplications are likely to have originated at earlier developmental stages than the ectopic structures elicited in our experiments, due to the presence of ectopic external nares. However, the mere fact that such anomalies exist suggests that facial patterning may be a continuum that ranges from a collapse of the midline, seen in cyclopia and hypotelorism, to an expansion of the midline as observed in cases of hypertelorism. Whether facial duplications actually represent an extreme example of midline expansion remains an intriguing possibility.

Epithelial-mesenchymal interactions in facial development

Previous studies have explored the question of whether facial morphogenesis is under the control of the mesenchyme or the ectoderm. In a series of grafting experiments, epithelia from the face and limb were combined with facial mesenchyme to determine whether one ectoderm supported the growth of the grafted tissue to a greater extent than the other (Richman and Tickle, 1989). Mesenchymal grafts appeared to grow in vitro to similar lengths irrespective of the ectoderm with which they were combined. From these results, it was concluded that morphogenesis was regulated by the mesenchyme and that

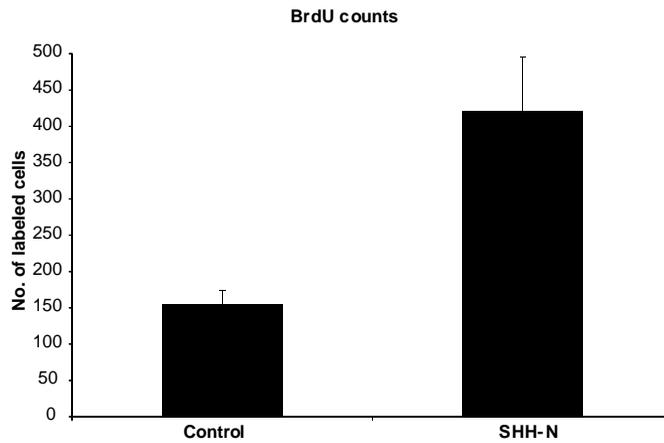


Fig. 7. Cell proliferation resulting from SHH-N treatment of FNP tissues. Embryos were treated with SHH-N as described, then labeled with BrdU for 20 minutes prior to killing. BrdU staining was performed as described by the manufacturer (Zymed). Histomorphometric analyses were performed to quantify the amount of BrdU. To do this, images stained for BrdU are captured on a digital camera, imported into Adobe Photoshop and the total number of stained cells is counted using Scion image. Microsoft Excel is used to determine statistical significance (Student's T-test).

facial and limb ectoderm were equivalent in their ability to support the growth of the graft. Conversely, our *in vivo* results suggest that specific regions of the facial ectoderm are essential for the patterned outgrowth of the FNP and MXP, and that morphogenesis is controlled by interactions between these ectodermal domains and the underlying tissues. It is difficult to compare the results from these studies for a number of reasons, the most obvious being that the previous study was carried out in the limb bud and the current study was performed in the facial primordia. Although there are some similarities between the visceral arches and the limb buds, their differences are of considerable importance (reviewed in Schneider and Helms, 1999). The face is derived from the cranial neural crest, an ectomesenchymal population of cells that differs considerably from limb mesenchyme. The developmental potential of the host tissues and their influence on the behavior of grafted tissues is therefore inherently different. Perhaps a more fundamental question is the nature of morphogenesis in the context of facial development. The extent of growth exhibited by a graft of facial mesenchyme was the primary criteria used to describe morphogenesis in the previous study, whereas we focused on the regulation of target genes and cell proliferation in response to ectodermal signals, and the resulting morphology of FNP-derived tissues. In other organ systems, such as the tooth, reciprocal signaling between the ectoderm and mesenchyme is required for the elaboration of tooth shape and position. Considerable debate has surrounded the question of whether the ectoderm or the mesenchyme controlled odontogenic patterning; recent data indicate that this control switches between the ectoderm and mesenchyme in a stage-dependent fashion (P. Sharpe, personal communication). Initially, localized regions of the ectoderm influence the apparently naïve mesenchyme; subsequently, the mesenchyme becomes regionally committed to either

adopting a skeletal or dental fate and ectodermal signals are no longer required. Therefore, depending upon the time of the assay, odontogenic patterning may be controlled by either the ectoderm or the mesenchyme. We propose that a similar situation exists in the FNP and MXP, where localized regions of the ectoderm initially influence the patterned outgrowth of the underlying mesenchyme. By default, neural crest-derived mesenchyme will form cartilage; we and others propose that ectodermal cues are then required to further specify the shape of the resulting skeletal elements (reviewed in Hall and Miyake, 1992).

Conclusions

The results from these studies have important implications for our understanding of the etiologies of craniofacial birth defects. The fact that birth defects of the face and limbs frequently co-exist is fully consistent with the observed role of SHH, and other related signaling systems (*e.g.*, Bmps, Fgfs, Wnts and retinoids) that influence development of both tissues (reviewed in (Osumi-Yamashita et al., 1997). For instance, application of Bmp2 and Bmp4 beads to the mandibular arch induce *msx1* expression in the tissue surrounding the bead and induce the formation of ectopic cartilages continuous with Meckel's cartilage (Barlow and Francis-West, 1997). These results are reminiscent of the effects elicited by Bmps when they are applied to other regions of the skeleton (reviewed in Reddi, 1994; Hogan, 1996). Close examination of mouse and chick mutants with well-characterized limb defects, such as the Talpid chicken (Cole, 1942; Schneider et al., 1999), and the *extra-toes/Gli3* mouse (Hui and Joyner, 1993), has also revealed that these animals have FNP and MXP developmental defects. These limb and face malformations are consistent with a model of conserved signaling pathways in both tissues (reviewed in Schneider and Helms, 1999).

One of the most intriguing questions raised by these experiments is how craniofacial organizers first get established. Some clues come from our observations that younger embryos apparently retain the capacity to re-express *shh* in the face. These results bear a striking resemblance to the regeneration of Hensen's node following removal of 40% of the primitive streak (Psychoyos and Stern, 1996). In both cases, the ability to restore a functional organizer is a stage-dependent property that relies on surrounding tissues for inductive influences. The forebrain is one possible source of signals that induce *shh* in the FNP. Retinoids, which are required for the induction of *shh* in the limb bud (Helms et al., 1996), may also play a role in SHH induction in the craniofacial primordia. The answers to such questions are bound to shed light on the molecular basis for retinoid-induced embryopathies as well as other birth defects associated with perturbations in SHH signaling.

This manuscript is dedicated to the memory of Peter Thorogood, whose seminal contributions to the field of craniofacial biology and whose genuine friendship will not soon be forgotten. The authors gratefully recognize the intellectual contributions of Zena Werb, John Rubenstein and Richard Schneider. In addition, we thank Claire Ferrari for discussions regarding craniofacial defects Sangwei Lu, who produced the retroviral constructs, and to D. E. G. for his support. This work was supported by grants from the March of Dimes, NIDR and NICHD to J. A. H.

REFERENCES

- Barlow, A. J. and Francis-West, P. H. (1997). Ectopic application of recombinant BMP-2 and BMP-4 can change patterning of developing chick facial primordia. *Development* **124**, 391-398.
- Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H. F., Donis-Keller, H., Helms, C., Hing, A. V., Heng, H. H., Koop, B., Martindale, D., Rommens, J. M., Tsui, L. C. and Scherer, S. W. (1996). Identification of Sonic hedgehog as a candidate gene in holoprosencephaly. *Nature Genetics* **14**, 353-356.
- Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.
- Cohen, M. M. J. and Sulik, K. K. (1992). Perspectives on holoprosencephaly: Part II. Central nervous system, craniofacial anatomy, syndrome commentary, diagnostic approach, and experimental studies. *J. Craniofac. Genetics Dev. Biol.* **12**, 196-244.
- Cole, R. K. (1942). The 'talpid' lethal in the domestic fowl. *J. Heredity* **33**, 82-86.
- Cooper, M. K., Porter, J. A., Young, K. A., Kelley, R. L., and Beachy, P. A. (1998). Plant-derived and synthetic teratogens inhibit the ability of target tissues to respond to Sonic hedgehog signaling. *Science* **280**, 1603-1607.
- Couly, G. and Douarin, N. M. L. (1990). Head morphogenesis in embryonic avian chimeras: evidence for a segmental pattern in the ectoderm corresponding to the neuromeres. *Development* **108**, 543-558.
- Couly, G. F., Coltey, P. M. and Le Douarin, N. M. (1993). The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* **117**, 409-429.
- Cunniff, C., Kratz, L. E., Moser, A., Nowicz, M. R. and Kelley, R. I. (1997). Clinical and biochemical spectrum of patients with RSH/Smith-Lemli-Opitz syndrome and abnormal cholesterol metabolism. *Am. J. Med. Genet.* **68**, 263-269.
- Ericson, J. M., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.
- Gaffield, W. and Keeler, R. F. (1996). Steroidal alkaloid teratogens: molecular probes for investigation of craniofacial malformations. *J. Toxicology Toxin Reviews* **15**, 303-326.
- Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.
- Gorlin, R. J., Cohen, M. M. and Levin, L. S. (1990). *Syndromes of the Head and Neck*, 3rd Edition, Volume 1. New York: Oxford University Press.
- Hall, B. K. (1987). Tissue interactions in the development and evolution of the vertebrate head. In *Developmental and Evolutionary Aspects of the Neural Crest*. (ed. P. F. A. Maderson). pp. 215-260. New York: John Wiley & Sons.
- Hall, B. K. and Miyake, T. (1992). The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. *Anatomy and Embryology* **186**, 107-124.
- Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.
- Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Genes Dev.* **10**, 647-658.
- Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet.* **13**, 14-21.
- Helms, J. A., Kim, C. H., Eichele, G. and Thaller, C. (1996). Retinoic acid signaling is required during early chick limb development. *Development* **122**, 1385-1394.
- Helms, J. A., Kim, C. H., Hu, D., Minkoff, R., Thaller, C. and Eichele, G. (1997). Sonic hedgehog participates in craniofacial morphogenesis and is down-regulated by teratogenic doses of retinoic acid. *Dev. Biol.* **187**, 25-35.
- Hogan, B. L. (1996). Bone morphogenetic proteins in development. *Current Opin. Genet. Dev.* **6**, 432-438.
- Hui, C. C. and Joyner, A. L. (1993). A mouse model of greig cephalopolysyndactyly syndrome: the extra-toes1 mutation contains an intragenic deletion of the Gli3 gene. *Nat. Genet.* **3**, 241-246.
- Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.
- Keeler, R. F. (1975). Teratogenic effects of cyclopamine and jervine in rats, mice and hamsters. *Proc. Soc. Exp. Biol. Med.* **149**, 302-306.
- Kelley, R. L., Roessler, E., Hennekam, R. C., Feldman, G. L., Kosaki, K., Jones, M. C., Palumbos, J. C. and Muenke, M. (1996). Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: does abnormal cholesterol metabolism affect the function of Sonic Hedgehog. *Am. J. Med. Genet.* **66**, 478-484.
- Köntges, G. and Lumsden, A. (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.
- Lammer, E. J., Chen, D. T., Hoar, R. M., Agnish, N. D., Benke, P. J., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W. J. and Lott, I. T. (1985). Retinoic acid embryopathy. *New England J. Med.* **313**, 837-841.
- López-Martínez, A., Chang, D. T., Chiang, C., Porter, J. A., Ros, M. A., Simandl, B. K., Beachy, P. A. and Fallon, J. F. (1995). Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Current Biol.* **5**, 791-796.
- Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and C.J., T. (1996). Biochemical evidence that Patched is the Hedgehog receptor. *Nature* **384**, 176-179.
- Martí, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, A. P. (1995). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.
- McGonnell, I. M., Clarke, J. D. and Tickle, C. (1998). Fate map of the developing chick face: analysis of expansion of facial primordia and establishment of the primary palate. *Dev. Dyn.* **212**, 102-118.
- Morgan, B. A. and Fekete, D. M. (1996). *Manipulating Gene Expression with Replication-Competent Retroviruses*. (ed. M. Bronner-Fraser). New York: Academic Press.
- Moury, J. D. and Schoenwolf, G. C. (1995). Cooperative model of epithelial shaping and bending during avian neurulation: autonomous movements of the neural plate, autonomous movements of the epidermis, and interactions in the neural plate/epidermis transition zone. *Dev. Dyn.* **204**, 323-337.
- Noden, D. M. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103**, 121-140.
- Noden, D. M. (1991). Vertebrate craniofacial development: the relation between ontogenetic process and morphological outcome. *Brain, Behavior and Evolution* **38**, 190-225.
- Osumi-Yamashita, N., Ninomiya, Y., Doi, H. and Eto, K. (1994). The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. *Dev. Biol.* **164**, 409-419.
- Osumi-Yamashita, N., Ninomiya, Y. and Eto, K. (1997). Mammalian craniofacial embryology in vitro. *Int. J. Dev. Biol.* **41**, 187-94.
- Pinto, C. B. and Hall, B. K. (1991). Toward an understanding of the epithelial requirement for osteogenesis in scleral mesenchyme of the embryonic chick. *J. Exp. Zool.* **259**, 92-108.
- Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V. and Beachy, P. A. (1996a). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21-34.
- Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K. and Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374**, 363-6.
- Porter, J. A., Young, K. E. and Beachy, P. A. (1996b). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255-9.
- Psychoyos, D. and Stern, C. D. (1996). Restoration of the organizer after radical ablation of Hensen's node and the anterior primitive streak in the chick embryo. *Development* **122**, 3263-73.
- Reddi, A. H. (1994). Bone and cartilage differentiation. *Current Opinion in Genetics and Development* **4**, 737-744.
- Richman, J. M., and Tickle, C. (1989). Epithelia are interchangeable between facial primordia of chick embryos and morphogenesis is controlled by the mesenchyme. *Dev. Biol.* **136**, 201-210.
- Richman, J. M., and Tickle, C. (1992). Epithelial-mesenchymal interactions in the outgrowth of limb buds and facial primordia in chick embryos. *Dev. Biol.* **154**, 299-308.
- Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
- Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S. W., Tsui, L. C. and Muenke, M. (1996). Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nature Genetics* **14**, 357-360.
- Rubenstein, J. L. and Beachy, P. A. (1998). Patterning of the embryonic forebrain. *Current Opinion in Neurobiology* **8**, 18-26.
- Saunders, J. W. J. and Gasseling, M. T. (1968). Ectoderm-mesenchymal

- interactions in the origin of wing symmetry. In *Epithelial-Mesenchymal Interactions* (ed. R. Fleischmajer and R. E. Billingham). pp. 78-97. Baltimore: Williams and Wilkins.
- Schneider, R.A., Hu, D. and Helms, J.A.** (1999). From head to toe: conservation of molecular signals regulating limb and craniofacial morphogenesis. *Cell and Tissue Research* **296**, 103-109.
- Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L.** (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.
- Sim, F. R., Matsumoto, N., Goulding, E. H., Denny, K. H., Lamb, J., Keeler, R. F. and Pratt, R. M.** (1983). Specific craniofacial defects induced by jervine in the cultured rat embryo. *Teratogenesis, Carcinogenesis and Mutagenesis* **3**, 111-121.
- Stone, D. M. and Hynes, M. e. a.** (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog [see comments]. *Nature* **384**, 129-134.
- Stricker, M.** (1990). *Craniofacial Malformations* (ed. M. Stricker). Edinburgh; New York: Churchill Livingstone.
- Tamarin, A., Crawley, A., Lee, J. and Tickle, C.** (1984). Analysis of upper beak defects in chicken embryos following with retinoic acid. *J. Embryol. Exp. Morph.* **84**, 105-123.
- Thorogood, P.** (1988). The developmental specification of the vertebrate skull. *Development* **103**, 141-153.
- Thorogood, P., Bee, J. and Mark, K. v. d.** (1986). Transient expression of collagen type II at epitheliomesenchymal interfaces during morphogenesis of the cartilaginous neurocranium. *Dev. Biol.* **116**, 497-509.
- Tyler, M. S. and Hall, B. K.** (1977). Epithelial influences on skeletogenesis in the mandible of the embryonic chick. *Anat. Record* **188**,
- Wall, N. A. and Hogan, B. L.** (1995). Expression of bone morphogenetic protein-4 (BMP-4), bone morphogenetic protein-7 (BMP-7), fibroblast growth factor-8 (FGF-8) and sonic hedgehog (SHH) during branchial arch development in the chick. *Mech. Dev.* **53**, 383-392.
- Wedden, S. E., Ralphs, J. R. and Tickle, C.** (1988). Pattern formation in the facial primordia. *Development* **103 Supplement**, 31-40.
- Weed, M., Mundlos, S. and Olsen, B. R.** (1997). The role of sonic hedgehog in vertebrate development. *Matrix Biology* **16**, 53-58.
- Wolpert, L.** (1988). Craniofacial development: a summing up. *Development* **103 Supplement**, 245-249.