

# Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH

Richard A. Schneider<sup>1,\*</sup>, Diane Hu<sup>1,\*</sup>, John L. R. Rubenstein<sup>2</sup>, Malcolm Maden<sup>3</sup> and Jill A. Helms<sup>1,‡</sup>

<sup>1</sup>Department of Orthopaedic Surgery, 533 Parnassus Avenue, Suite U-453, University of California at San Francisco, San Francisco, CA 94143-0514, USA

<sup>2</sup>Department of Psychiatry, Nina Ireland Lab of Developmental Neurobiology, University of California at San Francisco, San Francisco, CA 94143, USA

<sup>3</sup>MRC Centre for Developmental Neurobiology, King's College London, Guy's Campus, London Bridge, London SE1 9RT, UK

\*These authors contributed equally to this work

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

Accepted 2 April 2001

## SUMMARY

Correlations between facial anomalies and brain defects are well characterized throughout the clinical literature, yet a developmental basis for this association has not been identified. We demonstrate that the frontonasal process, which gives rise to the mid- and upper face, and the forebrain are linked early in their morphogenesis by a local retinoid signaling event that maintains the expression of key regulatory molecules. First, we show that aldehyde dehydrogenase 6, which synthesizes the ligand, retinoic acid, is localized to the ventral epithelium of the presumptive frontonasal process of chick embryos. At least two retinoid receptors are expressed in adjacent populations of mesenchyme. Second, using synthetic pan-specific retinoid antagonists, we transiently inhibit the ability of retinoid receptors to bind retinoic acid in the rostral head and we generate embryos with a hypoplastic forebrain, fused eyes, and no frontonasal process-derived

structures such as the upper beak. These defects are not due to eliminating mesenchymal progenitors, as neural crest cells still migrate into the frontonasal process, despite disruptions to retinoid signaling. Rather, these malformations result from loss of *fibroblast growth factor 8* and *sonic hedgehog* expression, which leads to increased programmed cell death and decreased proliferation in the forebrain and frontonasal process. Most significantly, we can rescue the morphological defects by re-introducing retinoic acid, or fibroblast growth factor and sonic hedgehog proteins into antagonist-treated embryos. We propose that the local source of retinoic acid in the rostral head initiates a regulatory cascade that coordinates forebrain and frontonasal process morphogenesis.

Key words: Craniofacial, Forebrain, Face, *ALDH6*, *FGF8*, *SHH*, Retinoic acid, Chick

## INTRODUCTION

Signaling by the secreted proteins, fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH), is indispensable for vertebrate development. A complete loss of *FGF8* and *SHH* gene function causes early embryonic lethality, or severe and widespread morphological defects (Chiang et al., 1996; Heisenberg et al., 1999; Sun et al., 1999). Genetic and biochemical disruptions to *FGF8* or *SHH* in a tissue- or stage-specific manner demonstrate that these molecules play essential roles in development of the limbs, neural tube, teeth and facial primordia (Arman et al., 1999; Helms et al., 1997; Hu and Helms, 1999; Laufer et al., 1994; Meyers et al., 1998; Tucker et al., 1999). The mechanisms by which *FGF8* and *SHH* are regulated are not entirely clear. Some clues come from studies in the limb, where *FGF8* and *SHH* expression depends upon retinoid signaling (Helms et al., 1996; Stratford et al., 1999). We test whether retinoids play a similar role in regulating FGF8 and SHH signaling during

morphogenesis of the forebrain and frontonasal process (FNP).

If retinoid signaling mediates *FGF8* and *SHH* in the forebrain and FNP, then components of the retinoid signaling pathway, including enzymes, ligands and receptors must be spatially and temporally localized in the same region of the rostral head. Members of the aldehyde dehydrogenase (ALDH) family are required for synthesis of retinoic acid (RA), a derivative of vitamin A (Duyster, 2000). At least two ALDHs have been detected in the rostral head of mice. *RALDH2* protein is localized to ventral portions of the optic vesicle and adjacent FNP tissues (Haselbeck et al., 1999), and null mutations in *RALDH2* result in a truncated FNP and other craniofacial malformations (Niederreither et al., 1999). *RALDH3* is localized to epithelia of the developing eye, the neuroepithelium of the telencephalon, and the olfactory placode (Li et al., 2000; Mic et al., 2000). Collectively, these data suggest that the ligand RA is synthesized in restricted regions within the rostral head.

All-*trans* RA binds to two classes of receptors, the retinoic acid receptors (RARs) and retinoid-X-receptors (RXRs). These receptors form heterodimers and act as ligand-dependent transcription factors (Chambon, 1996; Mangelsdorf et al., 1994; Sucov and Evans, 1995). In chicks, *RAR $\alpha$* , *RXR $\beta$* , and *RXR $\gamma$*  are detected in the neural crest mesenchyme that migrates out of the rostral neural tube and into the facial primordia (Hoover and Glover, 1998; Rowe and Brickell, 1995; Rowe et al., 1991; Rowe et al., 1992). In mice, *RAR $\alpha$* , *RAR $\beta$* , and *RAR $\gamma$*  are abundant in anterior facial mesenchyme (Dolle et al., 1990; Ruberte et al., 1991). Double null mutations in *RAR $\alpha$*  and *RAR $\gamma$*  result in severe craniofacial malformations, particularly an absence of FNP derivatives such as the nasal capsule and surrounding skeletal elements (Lohnes et al., 1994; see Smith and Schneider, 1998 for discussion).

To understand more precisely the role retinoids play during development of the forebrain and FNP, we use a biochemical approach to block retinoid signaling in a localized and transient manner (Johnson et al., 1995; Lala et al., 1996). Our results reveal that during a discrete developmental window, retinoid signaling maintains *FGF8* and *SHH* expression in the rostral head, and in so doing, synchronizes development of the forebrain and face. In the absence of an intact retinoid signaling pathway, *FGF8* and *SHH* expression is lost, cells fail to proliferate and undergo programmed cell death, and the forebrain and FNP cease their morphogenesis. Re-introduction of RA, or of FGF and SHH proteins into antagonist-treated embryos restores gene expression, enables cell survival, and 'rescues' the morphological defects. We propose a model in which local synthesis of RA in the rostral head is the first step in a series of epithelial-mesenchymal signaling interactions that enable patterned outgrowth of the forebrain and FNP.

## MATERIALS AND METHODS

### Preparation of embryos and disruptions to retinoid signaling

Fertilized chick eggs were prepared as described (Schneider, 1999). Ion exchange beads (AG1-X2, 100-200 mesh, and 106-180  $\mu$ m diameter; BioRad) were soaked in equimolar concentrations of RAR (LG100815) and RXR (LG100849) antagonists (Ligand Pharmaceuticals) dissolved in dimethylsulfoxide (DMSO; Sigma). Beads prepared in this manner provide a local diffusion-controlled release for approximately 24 hours (Eichele et al., 1984; Helms et al., 1996). The retinoid receptor antagonists are synthetic and pan-specific, and they compete in the nanomolar range with endogenous RA for binding to RARs and RXRs (Johnson et al., 1995; Lala et al., 1996). Based on similar synthetic retinoid analogs, these antagonists are metabolically stable and have a half-life of approximately 80 minutes, compared to 20 minutes for all-*trans* RA (Eichele et al., 1985). Two beads were positioned along the rostral margin of the forebrain. In an initial dose-response study, we determined that a 100  $\mu$ g/ml soaking concentration of the RAR and RXR antagonists consistently elicited the most severe phenotype (data not shown). Control beads were soaked in DMSO. Antagonist treatments were administered between HH stage 9 and stage 20 (Hamburger and Hamilton, 1951), a developmental period during which the neural tube closes, the brain vesicles are subdivided, the neural crest migrates into the facial primordia, and the forebrain and FNP undergo morphogenesis.

Ion exchange beads were soaked in a solution containing all-*trans* RA (25  $\mu$ g/ml; Sigma) and RAR/RXR antagonists (100  $\mu$ g/ml final concentration). Control embryos were treated with beads soaked in RA alone (25  $\mu$ g/ml). Embryos were also treated with the pan-specific RAR and RXR antagonists separately.

Bio-beads (SM2, approximately 150  $\mu$ m diameter, BioRad) were soaked in citral (*cis+trans*; Fluka) for 10 minutes. One bead was positioned along the rostral margin of the forebrain and then was removed after 6 hours.

### Histology

Embryos were sacrificed at stage 36 and their heads were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, dehydrated, and embedded in paraffin. Heads were cut into 10  $\mu$ m sagittal sections, which were deparaffinized, stained with Milligan's Trichrome (Presnell and Schreiber, 1997), and imaged using brightfield optics.

### Whole-mount and sectioned in situ hybridization

In situ hybridization was performed on whole embryos and paraffin sections as described (Albrecht et al., 1997). Subclones of *ALDH6*, *RAR $\beta$* , *RXR $\gamma$* , *SHH*, *FGF8*, *PAX6*, *OTX2*, *DLX2*, *NKX2.1*, *NKX2.2*, and *BF1* were linearized to transcribe either <sup>35</sup>S-labeled or digoxigenin-labeled antisense riboprobes. For <sup>35</sup>S-labeled riboprobes, images are Photoshop pseudo-colored superimpositions of the in situ hybridization signal and a blue nuclear stain (Hoechst Stain; Sigma).

### Programmed cell death

Immunohistochemical detection of DNA strand breaks was performed. Embryos were treated with RAR/RXR antagonists at stage 10, incubated for 4, 6, 12, or 24 hours, collected from the egg, rinsed in phosphate-buffered saline (PBS), fixed in 4% PFA for 2 hours at room temperature, dehydrated, paraffin embedded, and cut into 6  $\mu$ m sagittal sections. Sections were deparaffinized, incubated with proteinase K (10  $\mu$ g/ml in 10 mM TRIS/HCl pH 7.4), washed twice in PBS, incubated with TUNEL (Roche) reagent (conjugated to fluorescein) for 1 hour at 37°C, and imaged using epifluorescence optics.

### Cell proliferation

A bromodeoxyuridine (BrdU) assay (Zymed) was used. Following treatment with RAR/RXR antagonists at stage 10, embryos were incubated for 12 or 24 hours, at which time 1.0  $\mu$ l of the BrdU reagent was injected into the vitelline artery. Embryos were incubated for an additional 20 minutes at 37°C, then sacrificed, fixed in 4% PFA, dehydrated, and paraffin embedded. Each head was cut into 6  $\mu$ m sagittal sections and mounted on microscope slides. Sections were deparaffinized, processed according to the manufacturer's protocol, reacted with diaminobenzidine (DAB; Sigma), and imaged using brightfield optics.

### Neural crest transplantations

Fate maps of presumptive FNP neural crest were generated as described (Schneider, 1999). Briefly, stage 9 to stage 10<sup>-</sup> quail donor neural crest cells from the caudal forebrain and rostral midbrain were grafted orthotopically into stage-matched chick hosts. The heads of these chimeric embryos were removed at stage 36, fixed in Serra's, paraffin embedded, cut into 10  $\mu$ m sagittal sections, immunostained with the quail-specific Q $\alpha$ PN monoclonal antibody (Developmental Studies Hybridoma Bank), reacted with DAB, counterstained with Fast Green FCF (Fisher), and imaged using brightfield optics.

Some chimeric embryos were treated with RAR/RXR antagonists at stage 10, incubated for 24 hours, processed as described above (without a counterstain), and imaged using Nomarski optics. Control chimeric embryos were treated with beads soaked in DMSO.

### DiI labeling of FNP neural crest

Approximately 0.15  $\mu$ l of DiI (Molecular Probes; 0.5% in 100% ethanol) were injected into the lumen and along the dorsal surface of the neural tube at the forebrain/midbrain junction of stage 10 embryos. Immediately afterwards, these embryos were treated with RAR/RXR antagonists, incubated for 24 hours, collected, rinsed in PBS, and imaged using epifluorescence optics. Control DiI-injected embryos were treated with beads soaked in DMSO.

### Rescue experiments

Ion exchange beads (100-200 mesh and 106-180  $\mu$ m diameter; BioRad) were soaked in all-*trans* RA (25  $\mu$ g/ml) as described (Helms et al., 1996). RAR/RXR antagonist beads were placed at stage 10 and after embryos reached stage 12 (8-10 hours), the antagonist beads were removed and a bead soaked in RA was positioned along the rostral margin of the forebrain. Control embryos had RAR/RXR antagonist beads placed at stage 10, and removed and replaced with DMSO-soaked beads at stage 12.

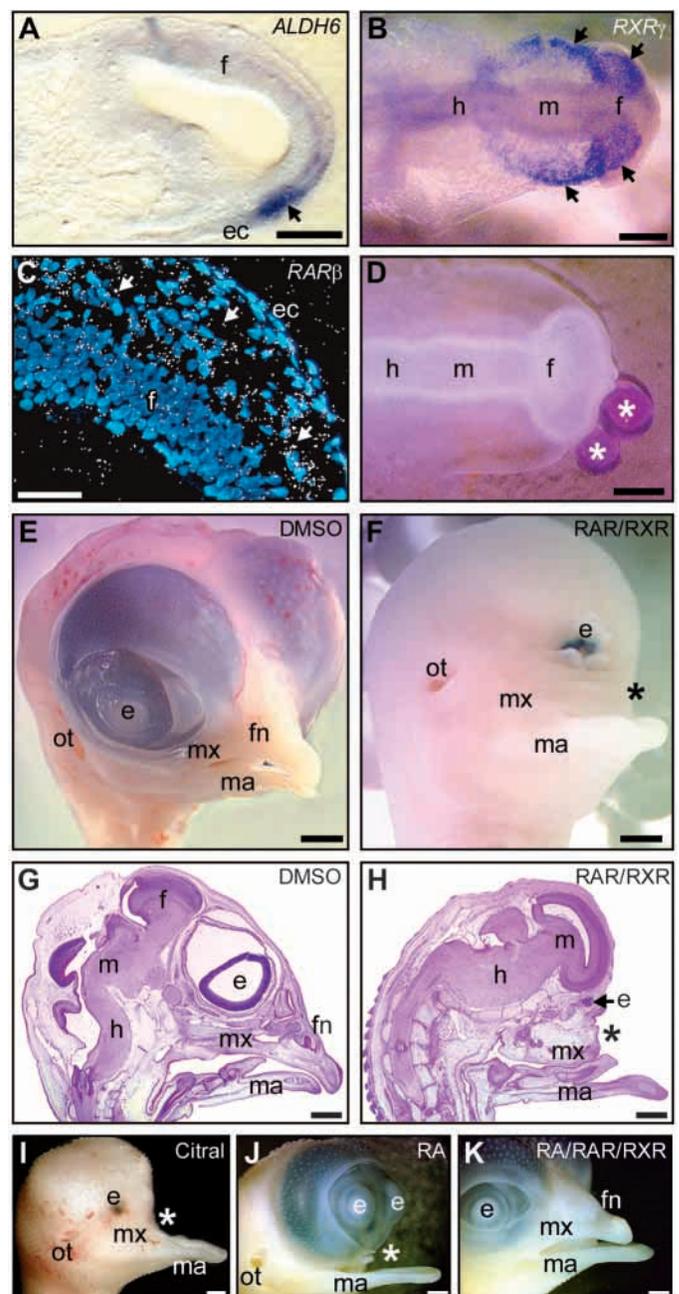
Affi-Gel Blue beads (50-100 mesh, 200-250  $\mu$ m diameter; BioRad) were soaked in a solution containing FGF2 protein (R & D Systems) and recombinant SHH-N protein (Ontogeny) at 37°C. Each protein was at a concentration of 400  $\mu$ g/ml in PBS with 0.1% bovine serum albumin (BSA). RAR/RXR antagonist beads were placed at stage 10 and after the embryos reached stage 12, the antagonist beads were removed and a protein-soaked bead was positioned along the rostral margin of the forebrain. Control beads were soaked in PBS with 0.1% BSA. Control embryos had their RAR/RXR antagonist beads removed and replaced with beads soaked in PBS with 0.1% BSA.

**Fig. 1.** Retinoid signaling is required for forebrain and FNP morphogenesis. (A) *ALDH6*, which synthesizes RA, is detected in ventral ectoderm (ec; arrow) of the presumptive FNP adjacent to the forebrain (f) of chick embryos at stage 10 (sagittal section, rostral towards right, dorsal on top). (B) *RXR $\gamma$*  is expressed in neural crest cells (arrows) that migrate out of the rostral neural tube of stage 10 embryos (dorsal view). (C) *RAR $\beta$*  is expressed in neural crest cells (arrows) that migrate between forebrain (f) neuroepithelium and overlying ectoderm (ec) of stage 10 embryos (sagittal section, rostral towards right, dorsal on top). Relative to cell density (blue nuclear stain), the neural crest shows higher expression levels (white dots) when compared with the neuroepithelium. (D) Ion exchange beads (asterisks) were soaked in RAR and RXR antagonists (100  $\mu$ g/ml) and placed along the rostral margins of the forebrain (f) of stage 10 embryos. Midbrain (m), hindbrain (h). (E) DMSO control; (F) RAR/RXR antagonist-treated embryos at stage 36 showing the effects of disrupting retinoid signaling. Antagonist-treated embryos lack forebrain tissues, fail to form an FNP (fn), and have fused eyes ( $n=18$ , i.e. number of embryos examined with this phenotype). These results are further illustrated by sagittal histological sections of control embryos (G) and treated embryos (H). In treated embryos, the maxillary (mx) and mandibular (ma) processes are unaffected whereas the FNP (asterisk) and forebrain are absent. Eye (e), ear (ot), midbrain (m) and hindbrain (h). Two additional controls confirm that these defects are due to disruptions in retinoid signaling specifically. First, we treated embryos at stage 10 with beads soaked citral (I), which is an inhibitor of RA biosynthesis. These embryos also lack forebrain tissues and an FNP (asterisk) while the maxillary (mx) and mandibular (ma) processes are unaffected ( $n=22$ ). Second, we used beads soaked concomitantly in all-*trans* RA and RAR/RXR antagonists (K). (J) Control embryos treated with RA alone have severe forebrain and FNP hypoplasia (asterisk;  $n=7$ ), whereas embryos exposed to RA and RAR/RXR antagonists simultaneously (K) are relatively normal with a slightly shortened FNP ( $n=10$ ). Scale bars: 100  $\mu$ m in A; 200  $\mu$ m in B,D; 30  $\mu$ m in C; 1 mm in E-H; 3 mm in I; 2 mm in J,K.

## RESULTS

### RA and its receptors co-localize to the same region of the presumptive FNP

In situ hybridization analyses reveal that a new member of the aldehyde dehydrogenase (ALDH) family, *ALDH6*, is expressed in a restricted region of ventral ectoderm covering the FNP of chick embryos (Fig. 1A). Expression of *ALDH6* in epithelial cells of the presumptive FNP begins precisely at stage 10 and ends at stage 12. At stage 10, we also detect two retinoid receptors, *RAR $\beta$*  and *RXR $\gamma$* , in neural crest mesenchyme, which migrates out of the rostral neural tube and eventually accumulates around the eyes, in the FNP, and throughout the other facial primordia (Fig. 1B,C; see also Hoover and Glover, 1998; Rowe and Brickell, 1995; Rowe et al., 1992).



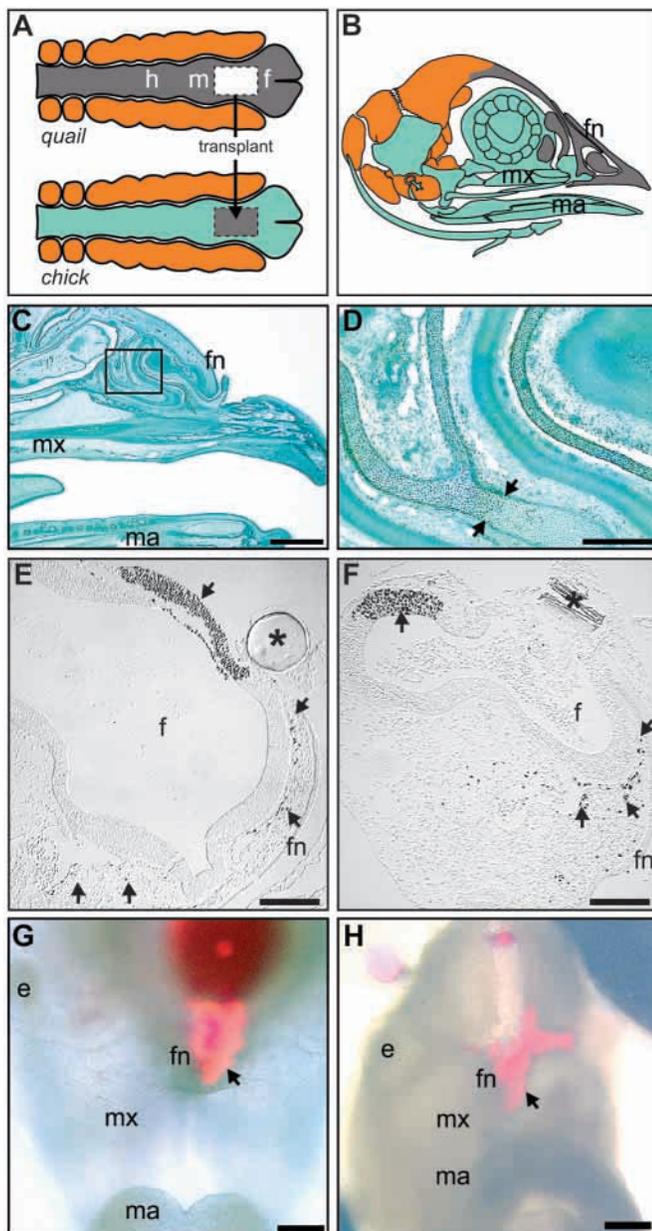
### Retinoid signaling coordinates forebrain and FNP morphogenesis

To understand the role that retinoids play during development of the rostral head, we used pan-specific receptor antagonists to inhibit retinoid signaling in a localized and transient manner. Embryos treated at stage 10 with RAR/RXR antagonists are the most severely affected. In 98% of these cases, the telencephalon, most of the diencephalon, and the entire FNP fail to develop (Fig. 1E-H; Table 1). Embryos lack structures such as the nasal capsule and upper beak, and also exhibit hypotelorism, occasionally (10% of the cases) to the extent where the eyes are completely fused (Fig. 1F). After stage 10, the dependency of the developing forebrain and FNP on retinoid signaling is progressively diminished (Table 1). Fifty percent of embryos treated with RAR/RXR antagonists at stage 12 have a severely hypoplastic FNP, but their forebrain defects are predominantly limited to telencephalic dysplasias.

In embryos treated at stage 14, the FNP is affected with mild hypoplasia and clefting defects in 49% of the cases, yet the forebrain appears normal. Treatments after stage 18 affect neither the forebrain nor FNP. At these later stages, even a fourfold increase in the dose of RAR/RXR antagonists fails to result in dysmorphologies (data not shown).

Unlike RARs, RXRs form heterodimers with proteins such as the peroxisome proliferator activated receptor and the vitamin D receptor, as well as the chicken ovalbumin upstream promoter transcription factor (reviewed in Mangelsdorf and Evans, 1995). Some of these proteins are found in the developing neural tube (Brubaker et al., 1996; Qiu et al., 1994). To determine whether the morphological defects that arise after RAR/RXR antagonist treatments are due to disruptions in other pathways that use RXRs, we treated embryos with RAR and RXR antagonists separately. Eighty-eight percent of embryos exposed to the RAR antagonist have severe hypoplasia in the forebrain and FNP, while the use of the RXR antagonist alone has no effect on 72% of the embryos treated. The remaining 28% of these cases treated with the RXR antagonist alone have only mild FNP defects such as a slightly shortened upper beak (data not shown).

To test further that the morphological defects are exclusively due to disruptions in retinoid signaling, we performed two additional experiments. First, we treated stage 10 embryos with citral, a selective competitive inhibitor of RA biosynthesis (Kikonyogo et al., 1999). Ninety-five percent of citral treated embryos lack an FNP and forebrain tissues, and have fused or absent eyes, whereas the maxillary and mandibular processes are unaffected (Fig. 1I). Second, we treated embryos at stage 10 with beads soaked concomitantly in all-*trans* RA (25 µg/ml) and RAR/RXR antagonists (100 µg/ml). If RAR/RXR antagonists disrupt craniofacial development by specifically binding to retinoid receptors, their teratogenic effects should be mitigated by the concurrent addition of RA, as this would



**Fig. 2.** Neural crest cells migrate into the presumptive FNP despite exposure to RAR/RXR antagonists. (A) Quail donor neural crest cells from the forebrain (f) and midbrain (m), when transplanted orthotopically to chick host embryos between stage 9 and stage 10<sup>-</sup>, give rise to components of the FNP (fn), as shown schematically (B) in a lateral view of the avian head skeleton (based on similar drawings by Noden, 1987). (C) In sagittal sections of chimeric embryos at stage 36, quail donor neural crest cells are found throughout chick host FNP-derived tissues, but not in the maxillary (mx) or mandibular (ma) processes ( $n=6$ ). In an area of nasal capsule cartilage (boxed, shown at higher magnification in D), quail neural crest-derived cells appear black and are completely integrated into the host structures. Cartilage of mixed chick host and quail donor origin can be observed (arrows). Quail donor neural crest cells were transplanted into chick host embryos between stage 9 and stage 10<sup>-</sup>, and then exposed 2-4 hours later at stage 10 to DMSO control beads (E, asterisk) or RAR/RXR antagonist beads (F, asterisk). After 24 hours, transplanted cells are integrated into the neuroepithelium above the lumen of the forebrain (f) and also have migrated into the FNP (fn; arrows) as shown in sagittal sections ( $n=7$ ). Embryos were also exposed to RAR/RXR antagonists at stage 10, and immediately thereafter neural crest cells from the forebrain (f) and midbrain (m) were labeled with DiI. Twenty-four hours later, we observe DiI labeled cells in the presumptive FNP of control (G) and treated (H) embryos, which demonstrates that the neural crest still migrates into the region despite treatment with RAR/RXR antagonists ( $n=7$ ). Scale bars: 1 mm in C; 100 µm in D; 200 µm in E-H.

**Table 1. Response of chick embryos to treatment with RAR and RXR antagonists\***

	Embryonic stages					
	9‡	10	12-13	14-15	16-17	18-20
Total treated	65	413	160	88	78	42
Telencephalon						
Absent	–	98%	19%	0%	0%	0%
Hypoplastic	–	0%	63%	0%	0%	0%
Unaffected	–	2%	18%	100%	100%	100%
Diencephalon						
Absent	–	16%	0%	0%	0%	0%
Hypoplastic	–	82%	25%	0%	0%	0%
Unaffected	–	2%	75%	100%	100%	100%
Eyes						
Fused	–	10%	0%	0%	0%	0%
Hypoteloric	–	88%	73%	6%	0%	0%
Unaffected	–	2%	27%	94%	100%	100%
Frontonasal process						
Absent	–	98%	14%	0%	0%	0%
Hypoplastic	–	0%	36%	9%	0%	0%
Cleft or Asymmetric	–	0%	32%	40%	15%	0%
Unaffected	–	2%	18%	51%	85%	100%

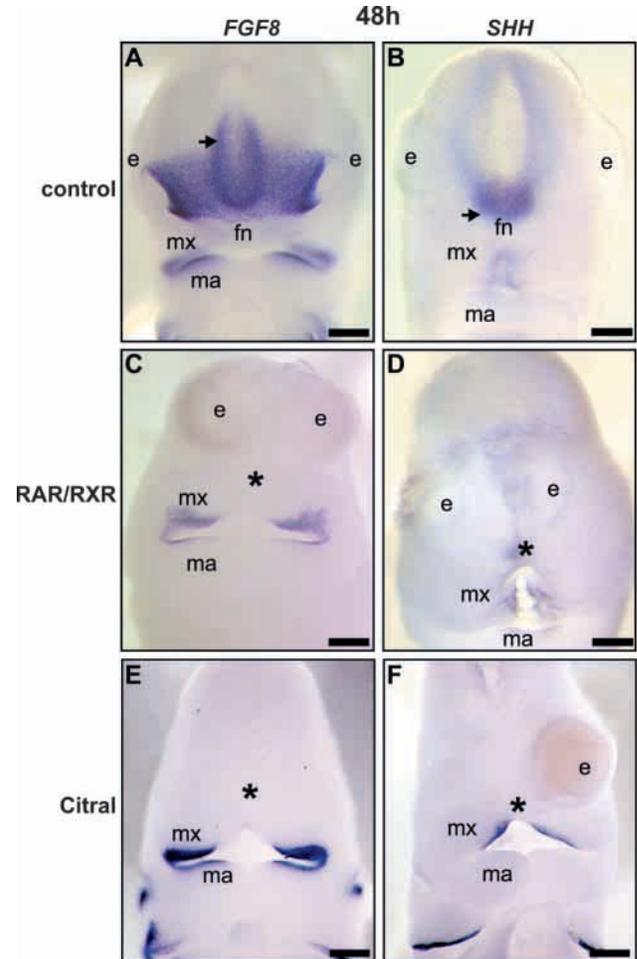
\*Administered in equimolar concentrations (100 µg/ml soaking concentration).

‡After treatment, all embryos at stage 9 and younger failed to be vascularized appropriately and died within the first 24 hours; we are currently investigating the basis for this embryonic lethality.

introduce competition between an activating ligand and one that blocks signal transduction. As a control, embryos were treated with RA alone. Eighty-nine percent of control RA-treated embryos are hypoteloric and have severe hypoplasia in the forebrain and FNP (Fig. 1J), whereas 90% of embryos simultaneously exposed to RA and RAR/RXR antagonists appear relatively normal with only slightly shortened upper beaks (Fig. 1K).

### Neural crest cells arrive in the FNP despite RAR/RXR antagonist treatments

A majority of craniofacial neural crest cells emigrate from the rostral neural tube between stage 9<sup>-</sup> and stage 10 (Tosney, 1982). The surgical removal of this population results in massive cell death within the forebrain neuroepithelium, cyclopia, and a loss of the FNP (Etchevers et al., 1999). Thus, we used two methods (quail-chick chimeras and vital dye tracing) to determine the fate of rostral neural crest cells in embryos treated with RAR/RXR antagonists. By transplanting quail neural crest into chick hosts, we confirmed that much of the mesenchyme in the FNP is of neural crest origin, and is derived from dorsal aspects of the rostral mesencephalon and caudal prosencephalon (Fig. 2A-D; see also Couly et al., 1993; Noden, 1978). Then, we transplanted presumptive FNP neural crest from quail donors between stage 9 and stage 10<sup>-</sup> into stage-matched chick hosts, and exposed these resulting chimeric embryos to RAR/RXR antagonists. Twenty-four hours after exposure to RAR/RXR antagonists, we detect the presence of neural crest cells in the FNP (Fig. 2E,F). We corroborated these results by labeling the same population of neural crest with DiI and exposing the embryos to RAR/RXR antagonists. Twenty-four hours later, we observe DiI-labeled cells in the presumptive FNP (Fig. 2G,H).



**Fig. 3.** Inhibition of retinoid signaling with RAR/RXR antagonists causes a loss of *FGF8* and *SHH* in the forebrain and FNP. Whole-mount in situ hybridization 48 hours after bead implantation at stage 10 demonstrates that control embryos (A) express *FGF8* in the rostromedial forebrain, which appears as a midline horseshoe-shape (arrow), and in the ectoderm covering the FNP (fn), as well as in the maxillary (mx) and mandibular (ma) processes. (C) Treated embryos lose *FGF8* in the forebrain and FNP ectoderm (asterisk;  $n=32$ ). Note normal expression of *FGF8* in the maxillary and mandibular processes. (B) Control embryos express *SHH* in the ectoderm of the FNP (fn; arrow) and forebrain (f), whereas treated embryos (D) show a downregulation of *SHH* (asterisk;  $n=35$ ). Even at this early stage, the facial midline of treated embryos is hypoplastic, causing the eyes (e) to approximate one another. The lack of a forebrain is evidenced by a depression in the upper face. (E,F) Embryos treated with citral, which is an inhibitor of RA biosynthesis, lack *FGF8* in the forebrain and ectoderm covering the FNP (E, asterisk;  $n=4$ ) and *SHH* in the ectoderm of the FNP (F, asterisk) and forebrain ( $n=5$ ). This result demonstrates that inhibiting retinoid signaling either at the point of RA biosynthesis or at the level of the receptors has similar downstream molecular and morphological consequences in the forebrain and FNP. Scale bars: 200 µm.

### Forebrain and FNP defects are preceded by changes in gene expression, programmed cell death, and proliferation

We investigated whether the forebrain and FNP defects that arise following RAR/RXR antagonist treatments are correlated

with abnormal expression of *FGF8* and *SHH*, which, given data from studies in the limb (Helms et al., 1996; Stratford et al., 1999), are candidate downstream targets of retinoid signaling. Forty-eight hours after embryos are exposed to RAR/RXR antagonists we observe a loss of *FGF8* and *SHH* expression in the forebrain and ectoderm covering the FNP (Fig. 3A-D). Coincident with these changes in gene expression, embryos display hypotelorism and midfacial hypoplasia. To confirm that the loss of *FGF8* and *SHH* expression is due to disruptions in retinoid signaling exclusively, we also treated embryos at stage 10 with beads soaked in citral. Resulting embryos lack *FGF8* and *SHH* expression in the forebrain and ectoderm covering the FNP (Fig. 3E,F).

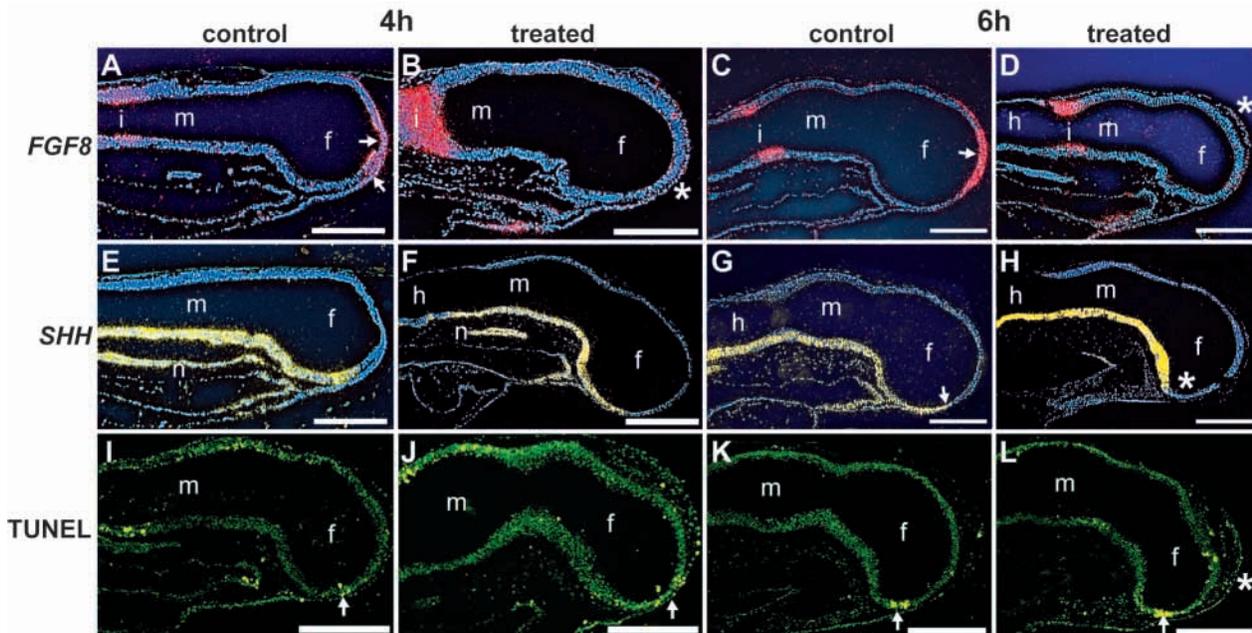
To determine whether the reduced expression of *FGF8* and *SHH* precedes the accompanying morphological defects, we examined RAR/RXR antagonist-treated embryos at 4, 6, 12, and 24 hours after bead implantation at stage 10. Our results reveal that alterations in gene expression occur gradually and are detectable prior to cellular and morphological abnormalities. *FGF8* begins to be downregulated in the rostralmost neural and FNP epithelia between 4 and 6 hours after antagonist treatment (Fig. 4A-D). *SHH* is unaffected at 4 hours, but by 6 hours, the rostralmost expression domain in the ventral forebrain is absent (Fig. 4E-H). In the first 4 hours after inhibition of retinoid signaling, there is no visible difference in programmed cell death based on a TUNEL assay (Fig. 4I,J).

Thus, changes in gene expression are detected before an observed increase in programmed cell death. By 6 hours there is a slight indication of programmed cell death within the neural crest mesenchyme (Fig. 4K,L). The few dying mesenchymal cells are adjacent to the region where *FGF8* expression in the neural and FNP epithelia is lost 2 hours earlier.

Twelve hours after RAR/RXR antagonist treatment, *FGF8* (Fig. 5A,B) and *SHH* (Fig. 5E,F) are lost in the forebrain and FNP. Additionally, there is a massive amount of programmed cell death in the FNP mesenchyme (Fig. 5I,J) and reduced proliferation in the FNP and forebrain based on decreased BrdU incorporation (Fig. 5M,N). The dying and non-proliferating mesenchymal cells lie adjacent to those epithelial domains of *FGF8* and *SHH* expression that had been lost at earlier time points. Similar molecular (Fig. 5C,D,G,H) and cellular defects (Fig. 5K,L,O,P) are found at 24 hours. These defects are also observed at 48 hours (data not shown). At 72 hours, *FGF8* and *SHH* are lost and the expression of other markers in the rostral head such as *BF1*, *NKX2.1*, *NKX6.1*, and *DLX2* is also downregulated (Fig. 6). Quite strikingly, expression of *OTX2* is maintained and *PAX6* is expanded in forebrain domains where *FGF8* and *SHH* are lost (Fig. 6H,M).

#### RAR/RXR antagonist-induced defects can be rescued

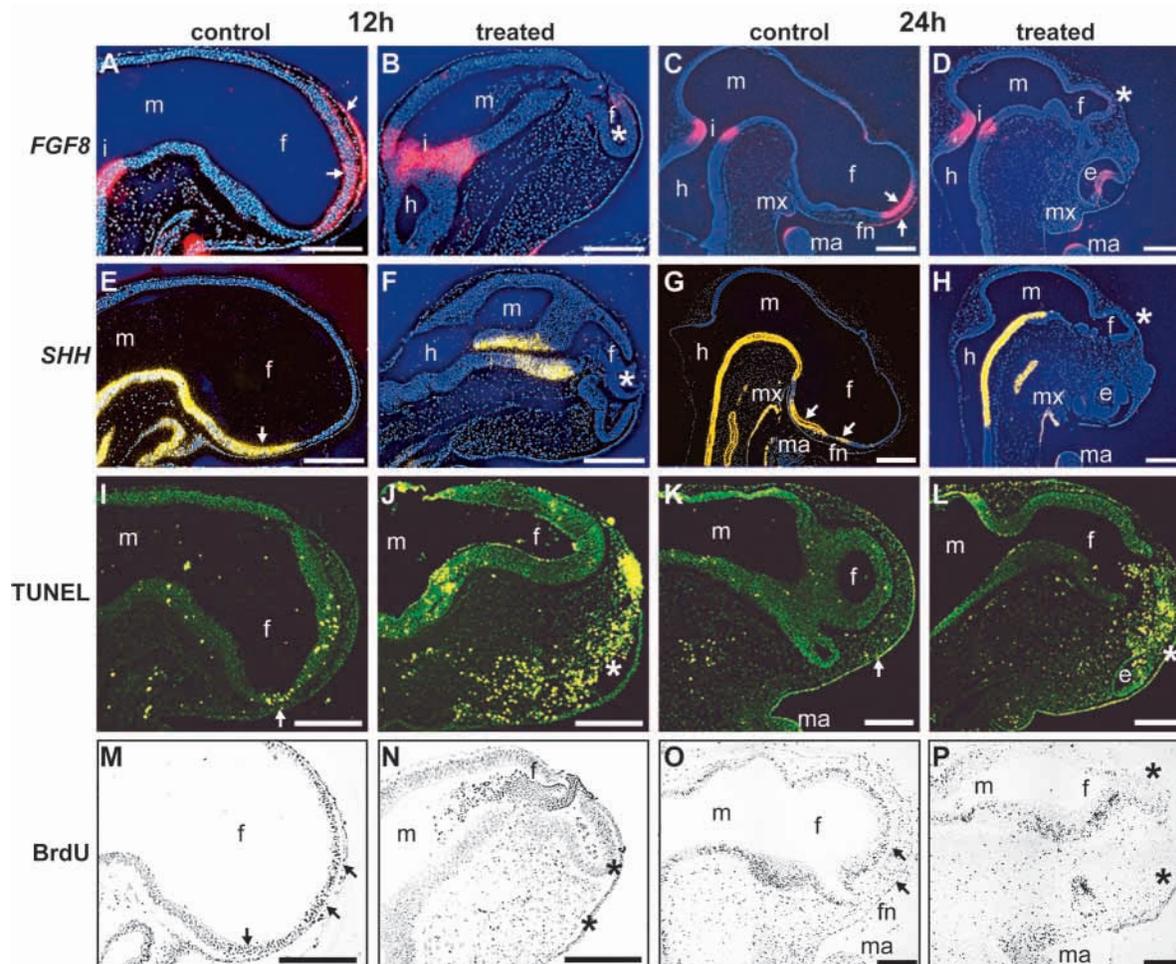
To test the possibility that re-introduction of RA could restore



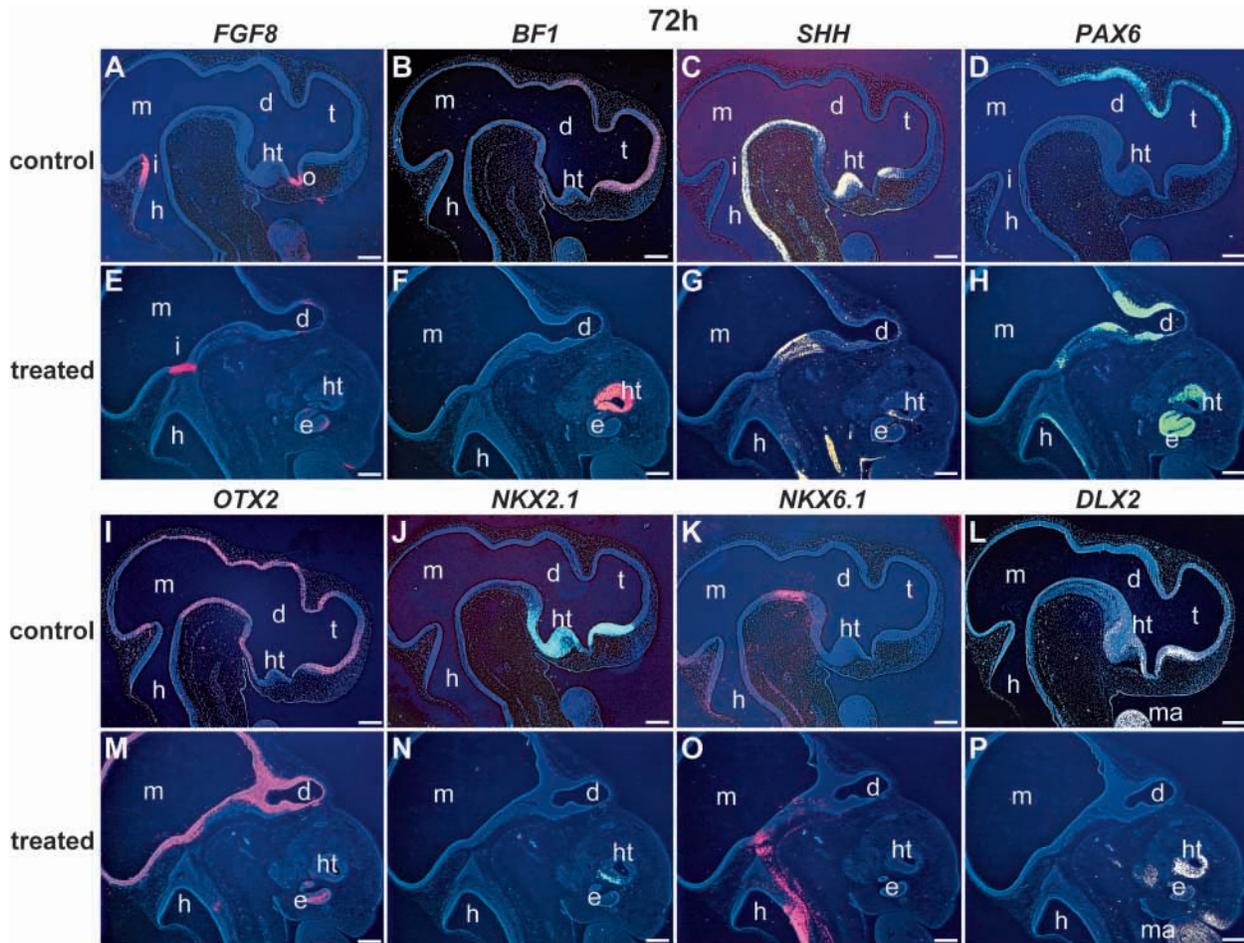
**Fig. 4.** Retinoid signaling is required for maintenance of *FGF8* and *SHH* in the forebrain and FNP ectoderm. In situ hybridization of midline sagittal sections 4 hours after bead implantation at stage 10 show that control embryos (A) express *FGF8* (red) in the rostradorsal forebrain (f) and ectoderm of the presumptive FNP (arrows). (E) *SHH* (yellow) is strongly expressed in the ventral forebrain (f) and notochord (n). (B) Exposure to RAR/RXR antagonists results in a downregulation of *FGF8* in the forebrain (asterisk), but not in the isthmus (i), where expression is normal ( $n=15$ ). (F) At this early stage, *SHH* expression does not appear to be downregulated ( $n=15$ ). The change in *FGF8* expression precedes any evidence of an increase in programmed cell death. Four hours after bead implantation at stage 10, the amount of programmed cell death (bright green) is equivalent in control (I) and RAR/RXR antagonist-treated (J) embryos (arrows), as determined with a TUNEL assay ( $n=10$ ). Sections 6 hours after bead implantation at stage 10 show that control embryos continue to express *FGF8* (C) and *SHH* (G) in the ventral forebrain (arrow). (D) RAR/RXR antagonist-treated embryos lack *FGF8* in the forebrain and FNP ectoderm (asterisk;  $n=15$ ). (H) *SHH* is downregulated along the ventral forebrain (asterisk;  $n=15$ ). Six hours after bead implantation at stage 10, the amount of programmed cell death is nominally increased in the mesenchyme (asterisk) of RAR/RXR antagonist-treated embryos (L,  $n=7$ ), compared with that found in controls (K,  $n=6$ ). Other labeled structures are the hindbrain (h) and midbrain (m). Scale bars: 200  $\mu\text{m}$ .

gene expression, enable neural crest cell survival, and thereby rescue the antagonist-induced morphological defects, we exposed embryos to RAR/RXR antagonists at stage 10. Then, after 8-10 hours, we removed the antagonist-containing beads and replaced them with beads containing all-*trans* RA. For controls, embryos were exposed to RAR/RXR antagonists at stage 10. Then, after 8-10 hours, the antagonist-containing beads were removed to ensure that the absence of the antagonists themselves would not be sufficient to rescue the embryos (Fig. 7B). Control embryos have severe hypoplasia in the forebrain and FNP, demonstrating that the RAR/RXR antagonists elicit their effects within the first 8-10 hours of

treatment. Administration of RA reversed the effects of the RAR/RXR antagonist treatments in 74% of the cases. RA-rescued embryos have a relatively normal forebrain and upper beak, and their eyes are not conjoined, although frequently their eyes are smaller and closer together (compare Fig. 7G with Fig. 1G). When we administered RA at 18 and 24 hours after antagonist treatment at stage 10, the forebrain was not rescued, but the defects previously observed in the FNP were mitigated (data not shown). We also examined RAR/RXR antagonist-treated embryos for changes in gene expression after introducing RA. Compared with the antagonist-treated embryos, which lose *SHH* and *FGF8* expression, RA-rescued



**Fig. 5.** A loss of retinoid signaling causes a downregulation of *FGF8* and *SHH* in the forebrain and FNP ectoderm, as well as an increase in programmed cell death and a decrease in cell proliferation in the FNP mesenchyme. In situ hybridization of midline sagittal sections 12 hours after bead implantation at stage 10 show that control embryos (A) express *FGF8* (red) in the forebrain (f), FNP ectoderm and isthmus (i); (E) *SHH* (yellow) is strongly expressed in the ventral forebrain (f). (B) RAR/RXR antagonist-treated embryos lose *FGF8* in the remnant of the forebrain (f; asterisk), but not in the isthmus (i;  $n=25$ ). (F) *SHH* is lost in the remaining forebrain tissue (asterisk) but is present in the floorplate of the midbrain ( $n=25$ ). (I,J) Twelve hours after bead implantation, the amount of programmed cell death (bright green) found in the FNP mesenchyme (arrows) is much greater in RAR/RXR antagonist-treated embryos (J, asterisk;  $n=5$ ), compared with controls (I,  $n=6$ ), as determined by a TUNEL assay. (M,N) The amount of cell proliferation is reduced in the forebrain and FNP mesenchyme of RAR/RXR antagonist-treated embryos (N, asterisks;  $n=23$ ), compared with controls (M,  $n=6$ ), as determined with BrdU labeling (black cells). (C,G) Sections 24 hours after bead implantation at stage 10 show that control embryos (C,G) express *FGF8* and *SHH*. (D) RAR/RXR antagonist-treated embryos lack *FGF8* in the forebrain and FNP ectoderm (asterisk) but still maintain expression in the mandible (ma) and eye (e;  $n=25$ ). (H) *SHH* is lost along the forebrain floor (asterisk;  $n=25$ ). (K,L) Twenty-four hours after bead implantation at stage 10, the amount of programmed cell death found in the FNP mesenchyme is much greater in RAR/RXR antagonist-treated embryos (L, asterisk;  $n=6$ ), compared with controls (K, arrow;  $n=4$ ). (P,O) Cell proliferation is much less in the forebrain and FNP mesenchyme of RAR/RXR antagonist-treated embryos (P, asterisks;  $n=35$ ), compared with controls (O,  $n=6$ ). Scale bar: 200  $\mu$ m.



**Fig. 6.** Inhibition of retinoid signaling alters expression of regulatory genes in the forebrain and FNP as shown by in situ hybridization on parasagittal sections 72 hours after bead implantation at stage 10. (A) *FGF8* is normally expressed in the rostral neural tube, including the optic recess (o) and isthmus (i). (E) In antagonist-treated embryos, *FGF8* expression is lost in the rostral neural tube, although low levels can be detected in the truncated diencephalon (d). (B) Normal *BF1* expression marks most of the telencephalon (t) and hypothalamus (ht). (F) In antagonist-treated embryos, expression of *BF1* is not detected in the remnant of the forebrain except some expression remains in the malformed hypothalamus. (C) *SHH* is normally expressed in ventral neural tissues, including the hypothalamus and basal telencephalon. (G) In antagonist-treated embryos, *SHH* expression is lost in the diencephalic remnant. (D) Normal *PAX6* expression marks dorsal forebrain structures up to the midbrain (m) and the eye (not shown). (H) In antagonist-treated embryos, *PAX6* expression is expanded into the remaining diencephalon (d) and can also be detected in the eye (e) and hypothalamic remnant. (I) Normally, *OTX2* is broadly expressed in the forebrain and midbrain. (M) In antagonist-treated embryos, *OTX2* expression is maintained in these domains. The expression of *PAX6* and *OTX2* serves as an important control, demonstrating that loss of expression is due to RAR/RXR antagonist-induced misregulation of target genes, rather than a general inability of remaining tissues to synthesize mRNA transcripts. (J) Normally, *NKX2.1* marks most of the hypothalamus and basal telencephalon. (N) In antagonist-treated embryos, residual *NKX2.1* expression remains in the hypothalamic remnant. (K) Normally, *NKX6.1* marks the basal plate. (O) In antagonist-treated embryos, *NKX6.1* expression persists in the basal plate of the midbrain but not in the residual forebrain. (L) Normally, *DLX2* expression marks part of the hypothalamus, all of the basal telencephalon, and mandibular arch (ma) neural crest. (P) In antagonist-treated embryos, *DLX2* expression persists in the hypothalamic remnant and in the mandibular arch. Scale bars: 100  $\mu$ m.

embryos re-express *SHH* and *FGF8* in the forebrain and FNP (Fig. 7A-F).

In another set of rescue experiments, we tested whether candidate downstream targets of retinoid signaling could also reverse the forebrain and FNP defects. Embryos were exposed to RAR/RXR antagonists at stage 10, and then, after 8-10 hours, the antagonist-containing beads were removed and replaced with beads containing FGF2 and SHH (we used FGF2 rather than FGF8 because FGFs can substitute for one another (Sun et al., 2000), and they elicit comparable effects when administered at these doses (Cohn et al., 1995)). Again, for controls, embryos

were exposed to RAR/RXR antagonists at stage 10; after 8-10 hours, these beads were removed. FGF2 and SHH treatments reversed the RAR/RXR antagonist-induced forebrain and FNP defects in 86% of the cases (Fig. 7H).

## DISCUSSION

### Retinoid signaling is an essential feature of rostral head development

Contrary to previous reports (Maden et al., 1998), our results

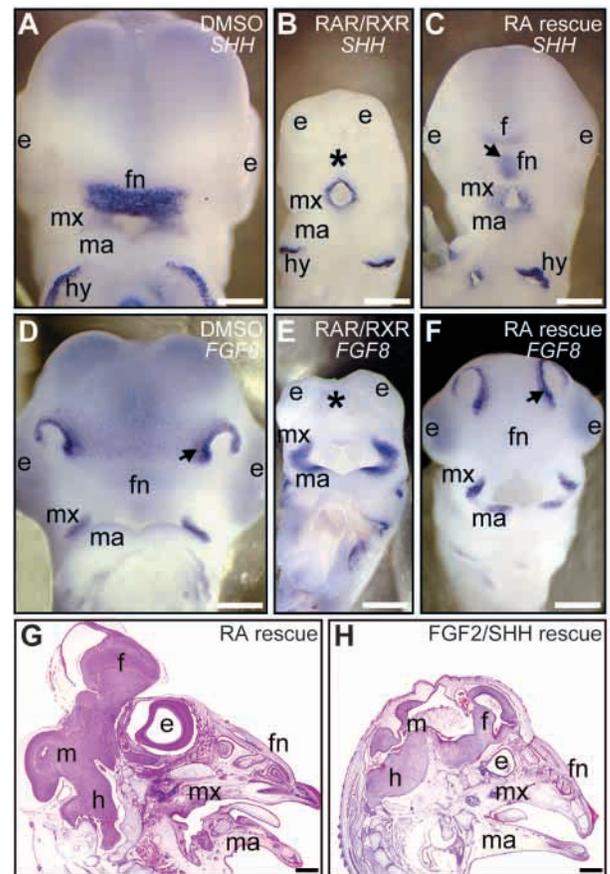
demonstrate that retinoid signaling is a key component of forebrain and FNP morphogenesis. First, we show that chick *ALDH6*, which can synthesize RA in vitro (Grün et al., 2000), is expressed in epithelial cells of the presumptive FNP (Fig. 1A). Second, at least two retinoid receptors (*RARβ* and *RXRγ*) are present in adjacent populations of neural crest mesenchyme (Fig. 1B,C). The proximity of cells that produce RA to cells that express the receptors suggests that a retinoid pathway is an essential feature of local epithelial-mesenchymal signaling interactions in the rostral head.

Although multiple retinoid receptors are expressed in the neural crest mesenchyme throughout craniofacial morphogenesis (Hoover and Glover, 1998; Rowe and Brickell, 1995; Rowe et al., 1991; Rowe et al., 1992), synthesis of the ligand in the epithelium of the presumptive FNP, as determined by *ALDH6* expression, appears to be more temporally restricted. Limiting the production of RA to a discrete developmental window (between stage 10 and 12) may be a mechanism by which retinoid-dependent signaling events are regulated in a tissue-specific manner. One caveat, however, is that two additional *ALDHs* have been identified in mice (Haselbeck et al., 1999; Li et al., 2000; Mic et al., 2000), suggesting that other local sources of RA synthesis may be present in the rostral head.

### Treatments specifically disrupt retinoid signaling

The ability of RXRs to heterodimerize with RARs, as well as other members of the steroid/thyroid hormone receptor superfamily, raises the possibility that RA-independent pathways are also disrupted by our antagonist treatments. If this is the case, then the molecular and morphological defects that we observe may not be exclusively due to inhibition of retinoid signaling and, instead, may be a consequence of perturbing a diverse array of pathways that are also mediated by steroidal molecules.

Three independent lines of evidence demonstrate that the molecular and morphological defects we observe are exclusively due to disruptions in retinoid signaling. First, the synthetic retinoids used in this study function as high affinity, pan-specific antagonists, as established through in vitro binding assays (Johnson et al., 1995; Lala et al., 1996). Second, the ligand RA and the RAR/RXR antagonists compete for binding to the same retinoid receptors, as simultaneous addition of all-*trans* RA and the RAR/RXR antagonists results in a near normal phenotype (Fig. 1K). If the receptor antagonists inadvertently blocked activation of other nuclear receptors, the addition of RA would be insufficient to rescue the dysmorphic phenotype. Third, treating embryos with the RAR antagonist alone induces the same dysmorphic phenotype as treating with the RAR/RXR antagonists together, whereas treating embryos with only the RXR antagonist elicits a near-normal phenotype. Thus, the craniofacial malformations we observe are not due to disruptions of different pathways that also require RXRs, rather, the defects arise from perturbations to RAR-dependent signaling. This result is consistent with studies demonstrating that specific teratogenic processes can be mediated by individual members of the RXR and RAR families in other tissues such as the limb (Sucov et al., 1995). These experimental approaches demonstrate that the effects reported are only attributable to perturbations in retinoid signaling.



**Fig. 7.** Forebrain and FNP defects can be 'rescued' by local application of either all-*trans* RA, or recombinant FGF2 and SHH proteins. Embryos were exposed to RAR/RXR antagonists at stage 10 and subsequently treated 8-10 hours later with either RA, or with FGF2 and SHH. Control embryos were treated at stage 10 with DMSO-soaked beads or RAR/RXR antagonist-soaked beads, which were removed after 8-10 hours. (A,B) By stage 19, DMSO-treated control embryos (A) exhibit normal *SHH* expression in the FNP ectoderm (fn), maxillary process (mx), and hyoid arch (hy), whereas RAR/RXR antagonist-treated embryos (B) lose *SHH* expression in the FNP ectoderm (asterisk), fail to develop a forebrain, and have eyes (e) that approximate one another. *SHH* expression remains unaffected in the maxillary process (mx) and hyoid (hy) arch. This control demonstrates that RAR/RXR antagonists elicit their effects between stage 10 and stage 12, as the RAR/RXR antagonist-soaked beads had been removed after 8-10 hours. Embryos exposed to RAR/RXR antagonists at stage 10 had their beads removed after 8-10 hours and then were treated with either RA, or with FGF2 and SHH. (C) By stage 19, expression of *SHH* is restored in the forebrain (f) and FNP (fn; arrow;  $n=15$ ). *SHH* expression in the maxillary process (mx) and hyoid arch (hy) is unaffected. (D) DMSO-treated control embryos exhibit normal *FGF8* expression in FNP ectoderm adjacent to the nasal pits (arrow) whereas RAR/RXR antagonist-treated embryos (E) lose this domain of *FGF8* expression and show a collapse of the facial midline (asterisk). *FGF8* expression remains unaffected in the maxillary (mx) and mandibular (ma) processes. (F) Embryos exposed to RAR/RXR antagonists at stage 10 had their beads removed after 8-10 hours and then were treated with either RA, or with FGF2 and SHH. Expression of *FGF8* is restored adjacent to the nasal pits (arrow;  $n=11$ ). *FGF8* expression in the maxillary (mx) and mandibular processes (ma) is unaffected. By stage 36, embryos rescued with RA (G) or with FGF2 and SHH (H) have a well-developed FNP and forebrain tissues ( $n=27$ ). Scale bars: 2 mm in A-F; 1 mm in G,H.

Our experiments also show that a high dose of RA leads to defects that resemble those induced by RAR/RXR antagonists (Fig. 1J). This is not unexpected, given previous reports where both excesses and deficiencies of RA produce similar abnormal phenotypes (Griffith and Zile, 2000). Some preliminary data suggest that these RA-induced defects arise via disruption to the same downstream pathways affected by RAR/RXR antagonists. Such results suggest that biologically available levels of RA must be precisely regulated in order to signal appropriately through retinoid receptors, and provide additional evidence that the molecular and morphological defects we observe after RAR/RXR antagonist treatments are a direct and specific consequence of disruptions to retinoid signaling.

Likely targets of retinoids are sensitive to perturbations in retinoid signaling. We employ complementary approaches that disrupt retinoid signaling either downstream at the level of receptor activation or upstream at the level of ligand production. The use of pan-specific retinoid receptor antagonists, or citral, which is a selective competitive inhibitor of RA biosynthesis (Kikonyogo et al., 1999), generates comparable phenotypes. Embryos lose *FGF8* and *SHH* expression domains in the rostral head, and the forebrain and FNP fail to undergo morphogenesis. Moreover, the period in which these genes and tissues are most sensitive to retinoid signaling disruptions correlates precisely with the time during which RA is synthesized (based on *ALDH6* expression from stage 10 to stage 12) in epithelial cells of the presumptive FNP. These data provide strong evidence that retinoid signaling is required during initial stages of forebrain and FNP morphogenesis.

### The forebrain and FNP defects arise through significant molecular and cellular changes

In this study, we provide a molecular and cellular dissection of the downstream consequences of disrupting a localized retinoid signaling event. We show that retinoids mediate expression of both *FGF8* and *SHH* in the forebrain and FNP. In the absence of these molecules, and most probably additional downstream effectors, there is an increase in programmed cell death and a reduction in cell proliferation. These cellular alterations are consistent with previous reports, which indicate that *FGF8* and *SHH* act as survival factors in the brain and other facial primordia (Ahlgren and Bronner-Fraser, 1999; Hu and Helms, 1999; Lee et al., 1997; Martinez et al., 1999; Rowitch et al., 1999; Shamim et al., 1999; Trumpp et al., 1999; Wechsler-Reya and Scott, 1999). Consistent with these data, our experiments demonstrate that *FGF8* and *SHH* act as survival factors for the FNP neural crest, and also show that the expression of these molecules depends upon retinoid signaling.

By analyzing the effects of antagonist treatments at early time points, we show that the loss of *FGF8* and *SHH* expression precedes detectable cellular and morphological abnormalities and, therefore, reflects actual decreases in mRNA levels rather than a loss of epithelial cells via programmed cell death. Moreover, we find that expression of *OTX2* is maintained, and that of *PAX6* is expanded throughout the dysmorphic tissues at 72 hours (Fig. 6H,M). This result serves as an important control demonstrating that the loss of expression of *FGF8*, *SHH*, and other genes is a consequence of RAR/RXR antagonist-induced misregulation, rather than a

general inability of remaining tissues to synthesize mRNA transcripts.

One potential consequence of the RAR/RXR antagonist treatments, which could account for the morphological defects, is that neural crest cells fail to migrate into the FNP. We have ruled out this possibility by using two independent techniques, quail-chick transplants and Dil labeling, which show that neural crest cells arrive in the FNP after retinoid perturbation. Thus, the forebrain and FNP dysmorphologies are not a consequence of a failure in neural crest cells to be generated and migrate into the FNP. Our results are consistent with a previous report demonstrating that inhibition of retinoid signaling does not reduce numbers of neural crest cells, although the routes these cells take to their final destinations may be altered (Wendling et al., 2000).

Embryos exposed to RAR/RXR antagonist beads for as little as 8–10 hours exhibit severe forebrain and FNP hypoplasia (Fig. 7B,E). In other words, the alterations in gene expression and the resulting craniofacial dysmorphologies do not arise from continual exposure to the antagonists, but rather are achieved by perturbations to retinoid signaling within a narrow developmental window. Despite the loss of retinoid signaling, neural crest cells still arrive in the FNP. Once there, however, they fail to receive the appropriate molecular signals. This raises the possibility that re-introduction of either the ligand or downstream targets can restore gene expression, enable neural crest cell survival and thus rescue the morphological defects. We use two separate strategies to reverse the antagonist-induced phenotype. We re-introduce either all-*trans* RA, or *FGF2*/*SHH* proteins, into antagonist-treated embryos. Quite strikingly, both the ligand and downstream targets are sufficient to restore gene expression and reverse the RAR/RXR antagonist-induced defects. As the alterations in gene expression and the resulting craniofacial dysmorphologies do not resolve simply by dissipation of the antagonists after removal of the beads, we conclude that the rescue is a consequence of reinitiating retinoid-mediated signaling pathways that are required for proper morphogenesis of the forebrain and FNP.

### A model for retinoid-mediated craniofacial morphogenesis

Traditionally, the forebrain has been viewed as a type of scaffold upon which the face develops, and this observation has led to the notion that forebrain defects are always accompanied by facial defects due primarily to mechanical influences of one tissue on the other (DeMyer, 1964). Although the brain must clearly play a substantial physical role in shaping the face, our results demonstrate that the forebrain and FNP are also intimately linked because both structures depend upon the same local retinoid signaling event to mediate their early morphogenesis. Previous reports have shown that secreted factors such as *FGF8* and *SHH* play important roles before and during patterning of the neural plate (Chiang et al., 1996; Sun et al., 1999; Ye et al., 1998). We demonstrate that these *FGF8* and *SHH* signaling pathways are also required during initial stages of forebrain and FNP morphogenesis. Moreover, we show that as in the limb, the expression of these molecules depends upon retinoid signaling, which supports the observation that there is remarkable conservation of signaling pathways mediated by these morphogens across multiple organ

systems (Schneider et al., 1999). Specifically, our results indicate that FGF8 and SHH are downstream targets of retinoid signaling in the rostral head, as they are sufficient to rescue the defects caused by RAR/RXR antagonists. What our results do not address, however, is how the *FGF8* and *SHH* expression domains are initially established in the forebrain and FNP. These genes may be induced independently as *FGF8* can still be detected in mice that lack *SHH* (Chiang et al., 1996). Once *FGF8* and *SHH* are induced, however, they function through reciprocal interactions (Crossley et al., 1996; Grieshammer et al., 1996; Sun et al., 2000; Ye et al., 1998). For this reason, we use FGF and SHH together to rescue the RAR/RXR antagonist-induced phenotype, but in principle, each factor alone may be sufficient. This might be especially true given that a loss of function in either *FGF8* or *SHH* generates a phenotype similar to that in RAR/RXR antagonist-treated embryos (Chiang et al., 1996; Trumpp et al., 1999). Unraveling the precise roles of these molecules will be critical to understanding rostral head development.

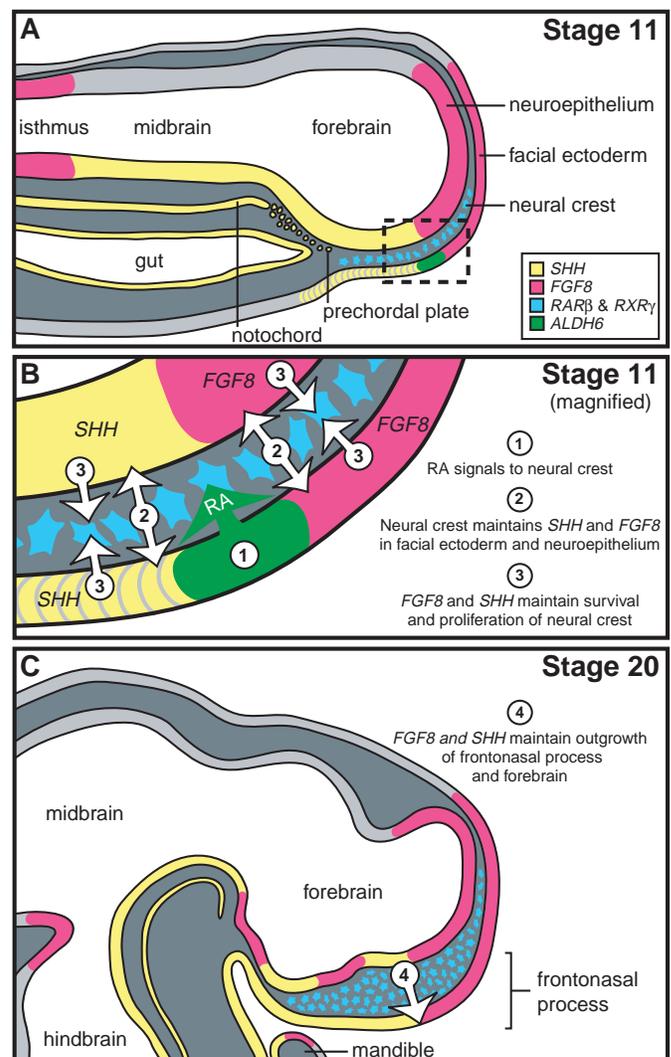
We propose that morphogenesis of the forebrain and FNP depends upon local synthesis of RA in the rostral head. This retinoid signaling event initiates a regulatory cascade that coordinates forebrain and FNP morphogenesis (Fig. 8). Migrating neural crest cells, which interpose themselves between the epithelia of the forebrain and FNP, and which express several retinoid receptors including *RARβ* and *RXRγ*, are probably targets of RA signaling. We hypothesize that a retinoid-dependent signal (currently unidentified) emanates from the neural crest mesenchyme and signals to the forebrain and FNP epithelia, maintaining their expression of *FGF8* and *SHH*. Alternatively, if retinoid receptors other than the ones we examined are present in the forebrain and FNP epithelia, then RA might also signal through them and maintain expression of

*FGF8* and *SHH*. For example, *RARα* and *RARγ* are detected in the rostral head of mice (Dolle et al., 1990; Ruberte et al., 1991) and a double null mutation in these receptors (Lohnes et al., 1994) produces craniofacial defects similar to those that result from RAR/RXR antagonist treatments. Regardless of which retinoid receptors are functioning in the rostral head, the use of pan-specific antagonists blocks all of them and causes a loss of *FGF8* and *SHH* expression. The maintenance of *FGF8* and *SHH* expression is required for survival of the neural crest mesenchyme. A loss of gene expression at this step leads to increased programmed cell death and decreased proliferation in the neural crest. The continued expression of *FGF8* and *SHH* enables the forebrain and FNP to undergo their patterned outgrowth.

### Disruptions to retinoid signaling may account for a range of craniofacial malformations

Superficially, the defects that arise after RAR/RXR antagonist treatments resemble craniofacial malformations associated with holoprosencephaly (HPE). HPE is a condition that includes a failure of formation and/or bilateralization of the rostral end of the neural tube, as well as cyclopia (Wallis and Muenke, 1999). A fundamental aspect of HPE pathogenesis is that the defect originates very early in development during

**Fig. 8.** A proposed model for the molecular regulation of forebrain and FNP morphogenesis via epithelial-mesenchymal signaling interactions. (A) Spatial relations of tissues and expression domains in the rostral head are shown in a schematic sagittal section through a stage 11 embryo. *FGF8* (pink) and *SHH* (yellow) are expressed in the neuroepithelium and FNP ectoderm. *RARβ* and *RXRγ* (blue) are detected in neural crest mesenchyme, and *ALDH6* (green) is localized to ventral FNP ectoderm. The black dashed box indicates area drawn at higher magnification in B, where we propose the retinoid-mediated signaling events occur. (Step 1) Between stage 10 and stage 12, RA is synthesized in FNP ectoderm (based on expression of *ALDH6*), and signals through receptors in neural crest cells that populate the FNP (based on expression of *RARβ* and *RXRγ*). Blocking this step between stage 10 and stage 12 either by citral inhibition of RA biosynthesis or by antagonizing the receptors, has similar downstream consequences. (Step 2) We hypothesize that a retinoid-dependent signal (currently unidentified) emanates from the neural crest mesenchyme and signals to the forebrain and FNP epithelia, maintaining expression of *FGF8* and *SHH*. Alternatively, if retinoid receptors other than the ones we examined are present in the forebrain and FNP epithelia, then RA might also signal through them and maintain expression of *FGF8* and *SHH*. (Step 3) The maintenance of *FGF8* and *SHH* expression is required for survival of the neural crest mesenchyme. A loss of gene expression at this step leads to increased programmed cell death and decreased proliferation in the neural crest. (C) Similar patterns of gene expression are observed in embryos through stage 20. (Step 4) The continued expression of *FGF8* and *SHH* enables the forebrain and FNP to undergo their patterned outgrowth.



gastrulation (Roessler and Muenke, 1999). Our treatments, however, are administered at later stages following neurulation, and after bilateral subdivision of the forebrain and eye field. Therefore, we interpret the RAR/RXR antagonist-induced morphological defects to be severe forebrain hypoplasia and a subsequent fusion of the optic vesicles due to a collapse of the midline, and not HPE.

Collectively, our results reveal how a single signaling event can serve as a common morphogenetic switch that synchronizes and enables the formation of structures as distinct as the brain and FNP. The coordinated growth of the forebrain and face has been observed in the clinical study of human malformations. Forebrain and facial dysmorphologies frequently co-segregate (Gorlin et al., 1990) and our results indicate that coincident defects in the brain and FNP can, in fact, arise from disruptions to a single pathway. Furthermore, these experiments demonstrate that there is a critical period in which morphogenesis of the forebrain and FNP is most dependent upon retinoid signaling. This discrete developmental window correlates precisely with the timing of RA production in the FNP ectoderm (based on *ALDH6* expression) and the presence of at least two retinoid receptors (*RAR $\beta$*  and *RXR $\gamma$* ) in adjacent populations of neural crest mesenchyme. Forebrain and FNP-derived tissues are sensitive to disruptions in retinoid signaling during their early development (from stage 10-12), but they become surprisingly insensitive by stage 14 (Table 1). Although the treatments may be less effective at later stages, owing to an increase in the number of cells that express retinoid receptors, we believe this is unlikely, as doses of antagonists four times greater than those used at stage 10 fail to elicit a morphological defect. Furthermore, the insensitivity to inhibitions in retinoid signaling does not appear to be due to a loss of retinoid receptor expression (Hoover and Glover, 1998; Rowe and Brickell, 1995; Rowe et al., 1991; Rowe et al., 1992), an inability of the tissue to synthesize RA from its biological precursor, or an absence of endogenous RA in the FNP (Helms et al., 1997). Rather, we suspect that as development proceeds, morphogenesis of the forebrain and FNP relies less on retinoid-mediated signaling and more on pathways that are, or become, retinoid independent. We are currently exploring this hypothesis.

We thank I. Banga, J. Chou, K. Lee, and K. Ngo for help in preparation of specimens, and Z. Werb, D. Noden, and P. Sharpe for helpful discussions. B. Eames, C. Ogilvie, R. Marcucio, and L. de la Fuente aided in editing the manuscript. The Q $\alpha$ PN monoclonal antibody, developed by B. Carlson and J. Carlson, was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa under Contract NO1-HD-7-3263 from the NICHD. The *ALDH6* cDNA was obtained from G. Duester. R. A. S. is supported by NIDCR DE07236. J. L. R. R. is supported by Nina Ireland, March of Dimes and NIMH K02 MH01046. J. A. H. is supported by March of Dimes, NICHD KO8 HD01079 and NIDCR R29 DE12462-03.

## REFERENCES

- Ahlgren, S. C. and Bronner-Fraser, M. (1999). Inhibition of sonic hedgehog signaling in vivo results in craniofacial neural crest cell death. *Curr. Biol.* **9**, 1304-1314.
- Albrecht, U. E. G., Helms, J. A. and Lin, H. (1997). Visualization of gene

- expression patterns by in situ hybridization. In *Molecular and Cellular Methods in Developmental Toxicology* (ed. G. P. Daston), pp. 23-48. Boca Raton, FL: CRC Press.
- Arman, E., Haffner-Krausz, R., Gorivodsky, M. and Lonai, P. (1999). *Fgfr2* is required for limb outgrowth and lung-branching morphogenesis. *Proc. Natl. Acad. Sci. USA* **96**, 11895-11899.
- Brubaker, K., McMillan, M., Neuman, T. and Nornes, H. O. (1996). All-trans retinoic acid affects the expression of orphan receptors COUP-TF I and COUP-TF II in the developing neural tube. *Dev. Brain Res.* **93**, 198-202.
- Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940-954.
- 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.
- Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-746.
- 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.
- Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84**, 127-136.
- DeMyer, W. (1964). The face predicts the brain: diagnostic significance of median facial anomalies for holoprosencephaly (arhinencephaly). *Pediatrics* **August**, 256-263.
- Dolle, P., Ruberte, E., Leroy, P., Morriss-Kay, G. and Chambon, P. (1990). Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110**, 1133-1151.
- Duester, G. (2000). Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. *Eur. J. Biochem.* **267**, 4315-4324.
- Eichele, G., Tickle, C. and Alberts, B. M. (1984). Microcontrolled release of biologically active compounds in chick embryos: beads of 200-microns diameter for the local release of retinoids. *Anal. Biochem.* **142**, 542-555.
- Eichele, G., Tickle, C. and Alberts, B. M. (1985). Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: temporal and spatial aspects. *J. Cell Biol.* **101**, 1913-1920.
- Etchevers, H. C., Couly, G., Vincent, C. and Le Douarin, N. M. (1999). Anterior cephalic neural crest is required for forebrain viability. *Development* **126**, 3533-3543.
- Gorlin, R. J., Cohen, M. M. and Levin, L. S. (1990). *Syndromes of the Head and Neck*. New York: Oxford University Press.
- Grieshammer, U., Minowada, G., Pisenti, J. M., Abbott, U. K. and Martin, G. R. (1996). The chick limbless mutation causes abnormalities in limb bud dorsal-ventral patterning: implications for the mechanism of apical ridge formation. *Development* **122**, 3851-3861.
- Griffith, M. and Zile, M. H. (2000). Retinoic acid, midline, and defects of secondary neurulation. *Teratology* **62**, 123-133.
- Grün, F., Hirose, Y., Kawachi, S., Ogura, T. and Umesono, K. (2000). *ALDH6*, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J. Biol. Chem.* **275**, 41210-41218.
- Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
- Haselbeck, R. J., Hoffmann, I. and Duester, G. (1999). Distinct functions for *Aldh1* and *Aldh2* in the control of ligand production for embryonic retinoid signaling pathways. *Dev. Genet.* **25**, 353-364.
- Heisenberg, C. P., Brennan, C. and Wilson, S. W. (1999). Zebrafish aussicht mutant embryos exhibit widespread overexpression of *ace* (*fgf8*) and coincident defects in CNS development. *Development* **126**, 2129-2140.
- 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.
- Hoover, F. and Glover, J. C. (1998). Regional pattern of retinoid X receptor-alpha gene expression in the central nervous system of the chicken embryo and its up-regulation by exposure to 9-cis retinoic acid. *J. Comp. Neurol.* **398**, 575-586.
- Hu, D. and Helms, J. A. (1999). The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development* **126**, 4873-4884.

- Johnson, A. T., Klein, E. S., Gillett, S. J., Wang, L., Song, T. K., Pino, M. E. and Chandraratna, R. A. (1995). Synthesis and characterization of a highly potent and effective antagonist of retinoic acid receptors. *J. Med. Chem.* **38**, 4764-4767.
- Kikonyogo, A., Abriola, D. P., Dryjanski, M. and Pietruszko, R. (1999). Mechanism of inhibition of aldehyde dehydrogenase by citral, a retinoid antagonist. *Eur. J. Biochem.* **262**, 704-712.
- Lala, D. S., Mukherjee, R., Schulman, I. G., Koch, S. S., Dardashti, L. J., Nadzan, A. M., Croston, G. E., Evans, R. M. and Heyman, R. A. (1996). Activation of specific RXR heterodimers by an antagonist of RXR homodimers. *Nature* **383**, 450-453.
- Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.
- Lee, S. M., Danielian, P. S., Fritsch, B. and McMahon, A. P. (1997). Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959-969.
- Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A. and Drager, U. (2000). A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech. Dev.* **95**, 283-289.
- Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, D., Gorry, P., Gansmuller, A. and Chambon, P. (1994). Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120**, 2723-2748.
- Maden, M., Sonneveld, E., van der Saag, P. T. and Gale, E. (1998). The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. *Development* **125**, 4133-4144.
- Mangelsdorf, D. F., Umesono, K. and Evans, R. M. (1994). The retinoid receptors. In *The Retinoids: Biology, Chemistry, and Medicine*, (ed. M. B. Sporn, A. B. Roberts and D. S. Goodman), pp. 319-349. New York: Raven Press.
- Mangelsdorf, D. J. and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83**, 841-850.
- Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmus organizer and isthmo-cerebellar development via a repressive effect on Otx2 expression. *Development* **126**, 1189-1200.
- Meyers, E. N., Lewandoski, M. and Martin, G. R. (1998). An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat. Genet.* **18**, 136-141.
- Mic, F. A., Molotkov, A., Fan, X., Cuenca, A. E. and Duester, G. (2000). RALDH3, a retinaldehyde dehydrogenase that generates retinoic acid, is expressed in the ventral retina, otic vesicle and olfactory pit during mouse development. *Mech. Dev.* **97**, 227-230.
- Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.
- Noden, D. M. (1978). The control of avian cephalic neural crest cytodifferentiation. I. Skeletal and connective tissues. *Dev. Biol.* **67**, 296-312.
- Noden, D. M. (1987). Interactions between cephalic neural crest and mesodermal populations. In *Developmental and Evolutionary Aspects of the Neural Crest* (ed. P. F. A. Maderson), pp. 89-119. New York: John Wiley & Sons.
- Presnell, J. K. and Schreibman, M. P. (1997). *Humason's Animal Tissue Techniques*. Baltimore: The Johns Hopkins University Press.
- Qiu, Y., Cooney, A. J., Kuratani, S., DeMayo, F. J., Tsai, S. Y. and Tsai, M. J. (1994). Spatiotemporal expression patterns of chicken ovalbumin upstream promoter-transcription factors in the developing mouse central nervous system: evidence for a role in segmental patterning of the diencephalon. *Proc. Natl. Acad. Sci. USA* **91**, 4451-4455.
- Roessler, E. and Muenke, M. (1999). The molecular genetics of holoprosencephaly: a model of brain development for the next century. *Childs Nervous System* **15**, 646-651.
- Rowe, A. and Brickell, P. M. (1995). Expression of the chicken retinoid X receptor-gamma gene in migrating cranial neural crest cells. *Anat. Embryol.* **192**, 1-8.
- Rowe, A., Eager, N. S. and Brickell, P. M. (1991). A member of the RXR nuclear receptor family is expressed in neural-crest-derived cells of the developing chick peripheral nervous system. *Development* **111**, 771-778.
- Rowe, A., Richman, J. M. and Brickell, P. M. (1992). Development of the spatial pattern of retinoic acid receptor-beta transcripts in embryonic chick facial primordia. *Development* **114**, 805-813.
- Rowitch, D. H., Jacques, B. S., Lee, S. M., Flax, J. D., Snyder, E. Y. and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci.* **19**, 8954-8965.
- Ruberte, E., Dolle, P., Chambon, P. and Morriss-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111**, 45-60.
- Schneider, R. A. (1999). Neural crest can form cartilages normally derived from mesoderm during development of the avian head skeleton. *Dev. Biol.* **208**, 441-455.
- Schneider, R. A., Hu, D. and Helms, J. A. (1999). From head to toe: conservation of molecular signals regulating limb and craniofacial morphogenesis. *Cell Tiss. Res.* **296**, 103-109.
- Shamim, H., Mahmood, R., Logan, C., Doherty, P., Lumsden, A. and Mason, I. (1999). Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. *Development* **126**, 945-959.
- Smith, K. K. and Schneider, R. A. (1998). Have gene knockouts caused evolutionary reversals in the mammalian first arch? *BioEssays* **20**, 245-255.
- Stratford, T., Logan, C., Zile, M. and Maden, M. (1999). Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. *Mech. Dev.* **81**, 115-125.
- Sucov, H. M. and Evans, R. M. (1995). Retinoic acid and retinoic acid receptors in development. *Mol. Neurobiol.* **10**, 169-84.
- Sucov, H. M., Izpisua-Belmonte, J. C., Gañan, Y. and Evans, R. M. (1995). Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. *Development* **121**, 3997-4003.
- Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev.* **13**, 1834-1846.
- Sun, X., Lewandoski, M., Meyers, E. N., Liu, Y. H., Maxson, R. E., Jr and Martin, G. R. (2000). Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. *Nat. Genet.* **25**, 83-86.
- Tosney, K. W. (1982). The segregation and early migration of cranial neural crest cells in the avian embryo. *Dev. Biol.* **89**, 13-24.
- Trumpp, A., Depew, M. J., Rubenstein, J. L., Bishop, J. M. and Martin, G. R. (1999). Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev.* **13**, 3136-3148.
- Tucker, A. S., Yamada, G., Grigoriou, M., Pachnis, V. and Sharpe, P. T. (1999). Fgf-8 determines rostral-caudal polarity in the first branchial arch. *Development* **126**, 51-61.
- Wallis, D. E. and Muenke, M. (1999). Molecular mechanisms of holoprosencephaly. *Mol. Genet. Metab.* **68**, 126-138.
- Wechsler-Reya, R. J. and Scott, M. P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* **22**, 103-114.
- Wendling, O., Dennefeld, C., Chambon, P. and Mark, M. (2000). Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. *Development* **127**, 1553-1562.
- Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.