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Negative effect of Hox gene expression on the development of the neural crest-derived facial skeleton

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SUMMARY

Diencephalic, mesencephalic and metencephalic neural crest cells are skeletogenic and derive from neural folds that do not express Hox genes. In order to examine the influence of Hox gene expression on skull morphogenesis, expression of *Hoxa2*, *Hoxa3* and *Hoxb4* in conjunction with that of the green fluorescent protein has been selectively targeted to the Hox-negative neural folds of the avian embryo prior to the onset of crest cell emigration. *Hoxa2* expression precludes the development of the entire facial skeleton. Transgenic *Hoxa2* embryos such as those from which the Hox-negative domain of the cephalic neural crest has been removed have no upper or lower jaws and no frontonasal structures. Embryos subjected to the forced expression of *Hoxa3* and *Hoxb4* show severe defects in the facial skeleton but not a complete absence of facial cartilage. *Hoxa3* prevents the formation of the skeleton derived from the first branchial arch, but allows the development (albeit reduced) of the nasal septum. *Hoxb4*, by contrast, hampers the formation of the nasal bud-derived skeleton, while allowing that of a proximal (but not distal) segment of the lower jaw. The combined effect of *Hoxa3* and *Hoxb4* prevents the formation of facial skeletal structures, comparable with *Hoxa2*. None of these genes impairs the formation of neural derivatives of the crest. These results suggest that over the course of evolution, the absence of Hox gene expression in the anterior part of the chordate embryo was crucial in the vertebrate phylum for the development of a face, jaws and brain case, and, hence, also for that of the forebrain.

Key words: Neural crest, Facial skeleton, Hox, Electroporation, Chimera, Chick, Quail

INTRODUCTION

Cell lineage studies carried out in the avian embryo have established the triple origin of the cells from which the head skeleton is built (Couly et al., 1993; Le Douarin and Kalcheim, 1999). The posterior part of the skull (the occipital bone) originates from the first five somite pairs, while the cephalic paraxial mesoderm forms most of the bones associated with the otic and orbital capsules in addition to the supraoccipital and the basi-postsphenoid. By contrast, the squamosal, parietal and frontal, as well as the basipresphenoid bones, the nasal capsule and the facial skeleton (maxillary process and lower jaw together with the hyoid cartilage) are neural crest derived. The migration routes taken by the skeletogenic cephalic neural crest have been determined by the quail-chick chimera system. It appears that the skeletogenic cephalic neural crest cells arise from two distinct domains of the brain; an anterior one (including the posterior diencephalon, mesencephalon and anterior rhombencephalon (r) 1 and 2), in which the neural crest cells do not express Hox genes [the homologs of the homeotic gene clusters (HOM-C) of *Drosophila*], and a posterior rhombencephalic domain (including r4 to r8) that, as well as the neural crest cells that it yields, expresses Hox genes of the first four paralogous groups. Hox-negative and Hox-positive domains can thus be distinguished in the cephalic skeletogenic neural crest (Fig. 1A-C) (Hunt et al., 1991; Prince and Lumsden, 1994; Grapin-Botton et al., 1995; Couly et al., 1996; Köntges and Lumsden, 1996). The head and facial skeleton are entirely formed by Hox-negative neural crest (Fig. 1D-G). The hyoid cartilage, by contrast, is mostly formed (except for the tongue skeleton: the entoglossum and the rostral part of the basihyal) by Hox-positive cells (Fig. 1C-G). Excision of the entire Hox-negative domain of the cephalic neural crest (from mid-diencephalon down to the r2/r3 limit) results in the lack of facial structures (Couly et al., 2002) (Fig. 1H,I).

Heterotopic transplantations of neural fold fragments along the anteroposterior (AP) axis have revealed that the neural crest cells retain their original Hox code, even when they migrate to a different AP position (Guthrie et al., 1992; Prince and Lumsden, 1994; Couly et al., 1996; Couly et al., 1998). Moreover, their fate is affected by the fact that they are led to develop in a different environment. We have previously shown that non-Hox-expressing neural crest cells, transplanted posteriorly to the rhombencephalic level (r6-r8), yield normal hyoid cartilages even though they develop in a Hox-expressing environment. By contrast, Hox-expressing neural crest cells (r4-r8) grafted to the mesencephalic domain are unable to form

the first branchial arch skeletal elements. They merely form small unpatterned nodules of cartilage, attesting that, in these circumstances, Hox-expressing ectomesenchymal cells do not respond to the cues responsible for patterning facial bones. By contrast, they do yield the neural derivatives corresponding to the AP level to which they were transplanted (Couly et al., 1998). Similar results have been obtained in the chick embryo by Grammatopoulos et al. (Grammatopoulos et al., 2000) in gain-of-function experiments where *Hoxa2* gene was selectively targeted to the neural crest.

Recently, we showed that the Hox-negative domain of the neural crest does not possess the regional information for facial skeleton patterning: (1) fragments of neural folds from the different levels of the fore-, mid- and anterior hindbrain can be substituted to one another without perturbing the normal facial pattern; (2) this Hox-negative domain of the neural crest is endowed of a high capacity for regeneration as only the posterior diencephalic neural crest (or mesencephalic, or r1, r2) can generate a normal face if they are left in situ after removal of the whole Hox-negative skeletogenic domain (Couly et al., 2002) (Fig. 1J,K). In fact, we demonstrated that the patterning information of the skeleton is provided to neural crest skeletogenic ectomesenchyme by the foregut ventral endoderm, which is also devoid of Hox gene expression.

The negative influence of Hox genes on the development of first branchial arch derivatives is also attested by the fact that the null mutation of *Hoxa2* results in the duplication of first branchial arch structures (the inco-malleus and Meckel's cartilages) at the expense of the neural crest cells that colonize the second branchial arch (Rijli et al., 1993; Gendron-Maguire et al., 1993; Kanzler et al., 1998). *Hoxa2*, the most rostrally expressed Hox gene, is activated in the r4-derived neural crest cells that are at the origin of the second branchial arch ectomesenchyme, which participates in the formation of the hyoid cartilages (Couly et al., 1996). Interestingly, gain-of-function experiments, in which *Hoxa2* was overexpressed not only in the neural crest cells but also in the other tissue components of the first branchial arch, resulted, in chick (Grammatopoulos et al., 2000) and in *Xenopus* (Pasqualetti et al., 2000), in the homeotic transformation of the first to the second branchial arch skeleton.

Gans and Northcutt (Gans and Northcutt, 1983) have underlined that the large increase of forebrain size, as well as the development of the sense organs (smell, eyes) and jaws that characterize the evolution of the vertebrate phylum, has been accompanied (and perhaps made possible) by the expansion of the anteriormost cephalic neural crest derivatives. The results mentioned above (Rijli et al., 1993; Gendron-Maguire et al., 1993; Couly et al., 1998) show that these structures have evolved from a domain of the neuraxis where the Hox genes that pattern the body plan are not activated. They also suggest that the expression of Hox genes may have an inhibitory effect on the development of the facial crest-derived skeletal structures (Kanzler et al., 1998). In order to further investigate how Hox gene products may negatively interfere with the regulatory genetic network that control the patterning of the facial skeleton, we have, in the present work, experimentally targeted expression of Hox genes of the first paralogous groups (*Hoxa2*, *Hoxa3*, *Hoxb4*) selectively to the neural crest of the non-Hox-expressing domain. The effects of Hox gene expression in the migratory to differentiation period of neural crest cell ontogeny turns out to preclude the development of the facial skeleton.

MATERIALS AND METHODS

Microsurgery

Experiments were carried out on quail and chick embryos at 5- to 6-somite stage [5-6 ss, i.e. 30 hours of incubation at 38°C; HH9 (Hamburger and Hamilton, 1951)]. Chick embryos were prepared for in ovo surgery according to the technique described by Teillet et al. (Teillet et al., 1999). Stage-matched quail or chick embryos were harvested and isolated in sterile phosphate-buffered saline (PBS) and defined fragments of the neural folds were removed and grafted into chick hosts that had been subjected to the ablation of the Hox-negative domain of their skeletogenic neural folds. The fate map of the cephalic neural primordium established by Grapin-Botton et al. (Grapin-Botton et al., 1995) served as reference to determine the presumptive level of the various encephalic structures: anterior and posterior diencephalon, mesencephalon and r1 to 8 (Fig. 1A).

Chick embryos were subjected to the bilateral surgical ablation of the neural folds from the mid-diencephalon down to the r2/r3 limit (Fig. 1H). Under these circumstances, no facial skeleton develops (Fig. 1I). When the posterior diencephalic or the anterior or posterior mesencephalic neural folds are either left in situ or backgrafted into these anterior neural fold ablated embryos, a normal face develops; a quarter of the Hox-negative domain of the crest is hence sufficient to generate a complete facial skeleton (Couly et al., 2002). In one set of experiments, fragments of the neural fold from quail or chick were back-transplanted into the chick at the posterior diencephalic level (Fig. 3A, Fig. 4A). Chimeric embryos were allowed to grow up to embryonic day (E) 2.5 to E8, depending on the experimental series.

In ovo electroporation

Ectopic Hox gene expression was obtained in the anterior neural fold by electroporation of Hox gene constructs in 5-6 ss quail or chick embryos. Depending on the experimental series, we used two types of constructs, one using the retroviral vector RCAS (BP)B for *Hoxa2*, active in chick but not in quail cells, and another using RCAS (BP)A for *Hoxa3* and *Hoxb4*, active in both quail and chick cells. The capacity of the retroviral construct to be active or not in the quail determined the choice of the electroporation protocol.

Hoxa2

The ectopic induction of *Hoxa2* in the anterior neural fold was achieved using retroviral vector RCAS (BP)B (Grammatopoulos et al., 2000).

Electroporation in the endogenous posterior diencephalic neural fold of 5-6 ss chick embryos

Two platinum anodes (CUY 611; Tr Tech, Japan) were placed on the vitelline membrane flanking the head. The DNA construct was injected into the lumen of the presumptive prosencephalon of 5-6 ss chick embryos. A single needle-shaped cathode made of tungsten was then applied at the midline, on the vitelline membrane overlaying the prosencephalic territory (Fig. 2A). In order to determine the early fate of transfected neural crest cells, the plasmid carrying the gene of interest together with the expression vector of the green fluorescent protein (GFP), pCAGGS-GFP (Momose et al., 1999), were co-electroporated. Both constructs were used at a concentration of 1 μg/μl. Bilateral transfection of the neural folds was obtained by successive pulses (5×25 V) generated by the BTX T830 square electroporator (Appligene). The capacity of the diencephalic crest to regenerate the facial skeleton after *Hoxa2* ectopic expression was also tested. In these experiments, the neural fold extending from anterior mesencephalon down to the r2/r3 limit was bilaterally excised. Then,

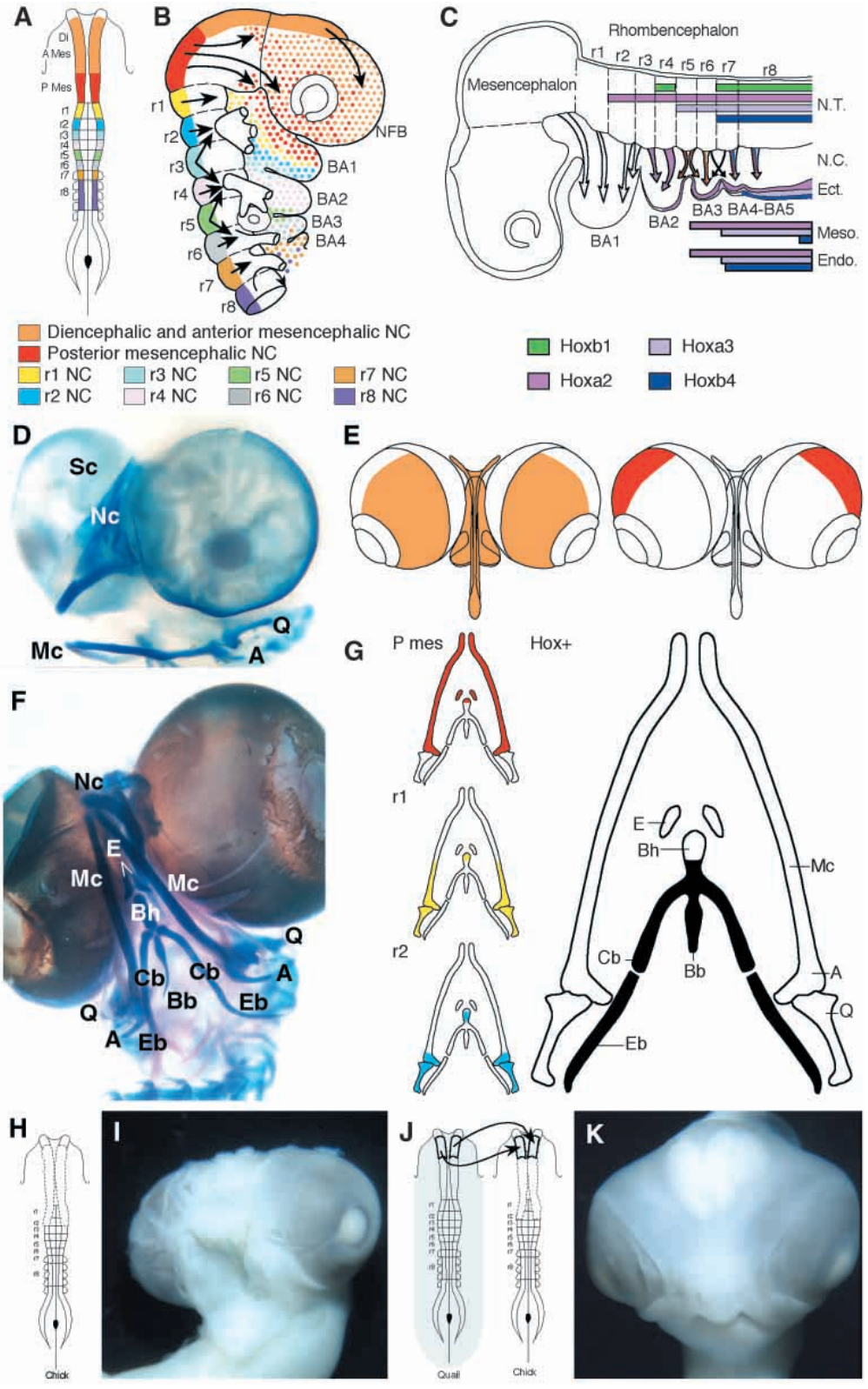

Fig. 1. The cephalic neural crest: fate map and Hox gene expression. (A) Presumptive diencephalic, mesencephalic and rhombencephalic territories of the neural fold in the avian embryo at 5 ss, as established by Grapin-Botton et al. (Grapin-Botton et al., 1995). (B) Migration map of cephalic neural crest cells in the avian embryo. The origin of neural crest cells found in the nasofrontal and periocular regions and in the branchial arches is color-coded as in A. Neural crest cells arising from the posterior diencephalon and mesencephalon populate the nasofrontal and periocular region. Posterior mesencephalon also participates in these structures, but in addition populates the anterodistal part of the first branchial arch. The complementary portion of the first branchial arch derives from r1/r2, together with a small contribution from r3. The major contribution to the second branchial arch comes from r4. Neural crest cells arising from r3 and r5 split into strains participating to two adjacent arches: r3 cells migrate to the first and second branchial arches; r5 cells migrate to the second and third branchial arches. r6 cells migrate to the third and fourth branchial arches, r7- and r8-derived cells migrate to the third and to the more caudal branchial arches. (C) Hox gene expression in the chick and quail embryo at E3 when the branchial arches are being colonized by neural crest cells originating from the posterior half of the mesencephalon and the rhombomeres (r1-r8). The arrows indicate the AP origin of the neural crest cells migrating to each branchial arch. Expression of Hox genes is also indicated in the superficial ectoderm, the endoderm and mesoderm. (D,E) Cartilages forming the upper face of a chick embryo at E8 and the contribution of the crest-derived cells according to the level from which they originate. (F,G) Lower jaw skeleton of E8.5 chick embryo: the participation of the crest derived cells is color coded as in A. Skeletal components of hyoid cartilages, which are formed by Hox-expressing crest cells (Hox +), are shown in black. (H) Bilateral surgical ablation of the Hox-negative domain of the skeletogenic neural fold, extending from the mid-diencephalon down to r2 included in 5 ss chick embryo. (I) Frontal view of E8 chick embryo subjected to the bilateral extirpation of the Hox-negative skeletogenic neural folds. In these embryos, which have virtually no face, structures anterior to the second branchial arch fail to develop: the nasal process, mandibular and maxillary buds are rudimentary. (J,K) In this context, the bilateral graft of the posterior diencephalic neural folds can regenerate the excised territory and form a normal face. BA, branchial arch; Ect., ectoderm; Endo., endoderm; Meso., mesoderm; NC, neural crest; NFB, nasofrontal bud; r, rhombomere. A, articular; Bb, basibranchial; Bh, basihyal; Cb, ceratobranchial; E, entoglossum; Eb, epibranchial; Mc, Meckel's cartilage; Nc, nasal capsule; Q, quadrate; Sc, sclerotic.

only the posterior diencephalic neural fold electroporated with *Hoxa2* was left in situ (Fig. 2A). One problem in these transfection experiments is that the electroporation is most effective at the transverse level where the electrodes are placed that is where the electric field is maximal. Thus, the number of transfected cells decreases anteriorly and posteriorly to this level. Owing to their vigorous regeneration potential, neural crest cells that are not expressing the transgene may therefore strongly bias the results of the experiment. To circumvent this difficulty, neural fold was resected posteriorly to the diencephalon-mesencephalon presumptive boundary and the regenerating capacity of the *Hoxa2*-expressing posterior diencephalon was tested (Fig. 2A).

Concurrently, a control series of experiments were performed to test the innocuity of RCAS transfection for neural crest cell behavior and head morphogenesis. For this purpose, electroporations of retroviral vector RCAS (BP)B not containing an exogenous DNA sequence were performed according to the protocol described above.

Electroporation of chick Hox-negative neural folds transplanted into chick embryos

In another series of experiments, the aim was to target *Hoxa2* to a larger segment of the Hox-negative neural folds. This was achieved by placing the anode on one side of the head of the embryo and the cathode on the opposite side. With this protocol, electroporation was successful unilaterally, on the side where the anode was placed. Unilateral transfections of neural folds were alternatively carried out on each side in two different chick embryos. The unilaterally transfected chick neural folds, extending from the diencephalon down to r2, were then bilaterally implanted at the homotopic level of a chick host (Fig. 3A). The contralateral untransfected neural folds of the chick embryos that were subjected to electroporation were bilaterally grafted into chick hosts that served as control embryos.

Hoxa3 or/and *Hoxb4*

Electroporation in 5-6 ss quail neural folds transplanted into chick embryos

Exogenous sequences of chick *Hoxa3* and *Hoxb4* genes were transfected in quail embryos using retroviral vectors RCAS (BP)A. This method allows the fate of the crest cells expressing the transgene to be selectively followed by means of the identification of quail cells in the host.

The electroporation was performed according to the protocol 2 described above, in quail embryos: unilateral electroporations of the neural fold were performed at the diencephalic level in two sets of 5-6 ss quail embryos. The diencephalic transfected neural folds were removed and homotopically transplanted into stage-matched chick which had been subjected to the ablation of the entire cephalic neural folds (Fig. 4A).

In another group of experiments, both *Hoxa3* and *Hoxb4* DNA constructs were co-electroporated according to the same experimental design. As for the former experiments, constructs were used at the concentration of 1 μl/μg each. This resulted in doubling the amount of exogenous Hox gene sequences targeted to the transfected neural folds.

Heterotopic transplantation of rhombencephalic neural folds

We also analyzed the fate of neural crest cells from rhombencephalic level r4-r8, which display an endogenous expression of *Hoxa2*, *Hoxa3* and *Hoxb4*, transplanted rostrally to the Hox-negative domain and tested their effect on head morphogenesis. Rhombencephalic r4-r8 neural folds from stage-matched quail embryos were bilaterally implanted at the level of the excised territory into 5-6 ss chick embryos subjected to the ablation of their Hox-negative neural folds (from diencephalon down to r2 included) (Fig. 9A).

Analysis of chimeras

Preparation of embryos and tissues

For whole-mount preparations, the grafted embryos were harvested and immersed in sterile PBS supplemented with 0.1 M EGTA then fixed in 4% formaldehyde. Embryos subjected to histological analysis were fixed in 60% ethanol, 30% formaldehyde, 10% acetic acid, dehydrated and embedded in paraffin wax. Sections (6 μm) were collected on Superfrost-Plus slides, and processed for either immunocytochemistry or transcript detection.

Immunocytochemistry

In series involving the transfection of the *Hoxa2* exogenous sequence in chick neural folds, the identification of glial derivatives and pericytes was achieved using HNK1 (Developmental Studies Hybridoma Bank) and 1A4 (Sigma) antibodies (Ab), respectively. In series involving the transfection of either *Hoxa3* or *Hoxb4* exogenous sequences in quail neural folds, the fate of neural crest derived cells was determined using the monoclonal antibody (mAb) QCPN (Developmental Studies Hybridoma Bank), which targets a nuclear antigenic determinant that is specific to quail cells. Using an Horseradish peroxidase (HRP)-linked Ab (Southern Biotechnology Associates) as the secondary Ab, the specific immunolabeling was evidenced in 3-3′diaminobenzidine tetra-hydrochloride (DAB) and hydrogen peroxide.

Whole-mount immunochemistry was carried out by blocking the endogenous peroxidase activity with PBS, Triton 0.5%, H_2O_2 0.5%, and preincubating the embryos for 3 hours in a mixture of PBS, Triton 0.5%, BSA 0.2% and goat serum (GS) 5%. Incubation with QCPN supernatant diluted 1:5 in PBS, BSA 0.1%, GS 1%, was performed overnight at 4°C. After extensive washes for 6 hours in PBS, Triton 0.5%, BSA 0.2%, the embryos were preincubated in PBS, Triton 0.5%, BSA 0.2%, GS 5% and then incubated overnight with HRP-conjugated Ab diluted 1:100 in PBS, BSA 0.1%, GS 1%. Embryos were rinsed for 6 hours and treated for 10 minutes with PBS, Triton 0.5%, DAB 0.05%. Finally, quail cells were visualized after the addition of H_2O_2 0.1%.

For sections, the endogenous peroxidase activity of tissue preparations was blocked with H_2O_2 0.03% solution in PBS. Primary Ab and the secondary Ab were applied overnight or for 2 hours, respectively, at room temperature. The quail antigen was revealed by a reaction using DAB 0.01%, H_2O_2 0.03% in PBS. Finally, sections were counterstained with Gill's Hematoxylin and mounted in Entellan.

In situ hybridization

The probe of *Hoxa2* was prepared from a partial cDNA construct, including the coding sequence 3′ to the *Eco*RI site within the homeobox (Prince and Lumsden, 1994). The *Hoxa3* probe represents a 900 bp *Kpn-Eco*RI fragment of the chicken cDNA as described by Grapin-Botton et al. (Grapin-Botton et al., 1995). The *Hoxb4* probe represents the full-length RNA (Sasaki and Kuroiwa, 1990). RNA probes were synthesized using Riboprobe Combination System kit (Promega) and labeled with UTP-Digoxygenin (Boehringer).

Whole-mount in situ hybridization was performed according to the method of Henrique et al. (Henrique et al., 1995). Probes deposited on paraffin wax sections were allowed to hybridize with embryonic transcripts as described by Etchevers et al. (Etchevers et al., 2001).

Cartilage staining

To reveal cartilaginous structures from E6 to E8, embryos were fixed in 80% ethanol, 20% acetic acid and 0.015% Alcian Blue 8GX. The duration of fixation was adapted to the stage of the embryos. After extensive washes in 100% ethanol, skeletal structures were cleared in 1% KOH and 20% glycerol.

RESULTS

Forced expression of *Hoxa2* in the diencephalic neural crest abolishes its capacity to generate a facial skeleton

Ectopic *Hoxa2* gene expression was targeted to the anterior neural fold of 5-6 ss chick embryos by electroporation of a *Hoxa2* retroviral construct containing *Hoxa2* cDNA that is active in chick and not in quail cells (Grammatopoulos et al., 2000). It was co-electroporated with a GFP DNA construct (Momose et al., 1999), in order to feature the early migration of the transfected neural crest cells (Fig. 2A). Concurrently, similar operations that served as controls were performed using a retroviral vector exempted of exogenous DNA insert (Fig. 2B).

In a first series of experiments, both diencephalic and mesencephalic neural folds were bilaterally electroporated. Then, the mesencephalic and anterior rhombencephalic folds were removed in order to prevent regeneration from neural crest cells located at the margin or out of the limits of the electrical field that could have escaped transfection (Fig. 2A,B).

Twenty-four hours after electroporation, at E3, GFP label showed that the transfected neural crest cells were not affected in their migration by the presence of *Hoxa2* cDNA. As in normal embryos, they spread forward from the diencephalic neural crest left in situ, covered the prosencephalon and populated the nasofrontal primordium (not shown; *n*=12). As in embryos with diencephalic neural crest cells not transfected (Fig. 4D) or electroporated with an empty RCAS (BP)B construct (Fig. 2B), *Hoxa2*-transfected neural crest cells moved down around the optic vesicles and filled the developing first branchial arch. In E3 embryos that were co-transfected with both the GFP and retroviral control or *Hoxa2* constructs, the detection of *Hoxa2* transcript revealed that the endogenous pattern of this gene is not affected by the operation (Fig. 2C,D). Additionally, *Hoxa2* was transcribed ectopically at the diencephalic level (*n*=26). At E6 (*n*=2) and E7 (*n*=2), the embryos in which the diencephalic neural crest had been electroporated with *Hoxa2* cDNA, did not develop facial structures (Fig. 2G,H) similar to those where the total cephalic neural folds (including the posterior diencephalon) (Couly et al., 2002) had been removed (Fig. 1I). By contrast, electroporation of a control construct, followed by removal of the mesencephalic and anterior rhombencephalic neural folds as in experimental embryos, had no effect on facial development: the embryos from the latter series exhibited normal nasofrontal and mandibular buds, and later on, a normal face (Fig. 2B,E,F).

In a second step, we targeted the *Hoxa2* construct to the entire Hox-negative territory of the neural fold in 5-6 ss chick embryos (extending from the diencephalic level down to r2 included). The transfected fragments of cephalic neural folds were subsequently implanted bilaterally in stage-matched chick embryos (Fig. 3A). In E3 embryos in which endogenous neural folds of the Hox-negative domain have been replaced by their homotopic counterparts carrying both *Hoxa2* and GFP constructs (Fig. 3A), the migratory behavior of the cells arising from transplants was visualized by the GFP labeling (*n*=8) (Fig. 3B). These cells migrated rostrally from transplants over the prosencephalon, and bilaterally colonized the presumptive

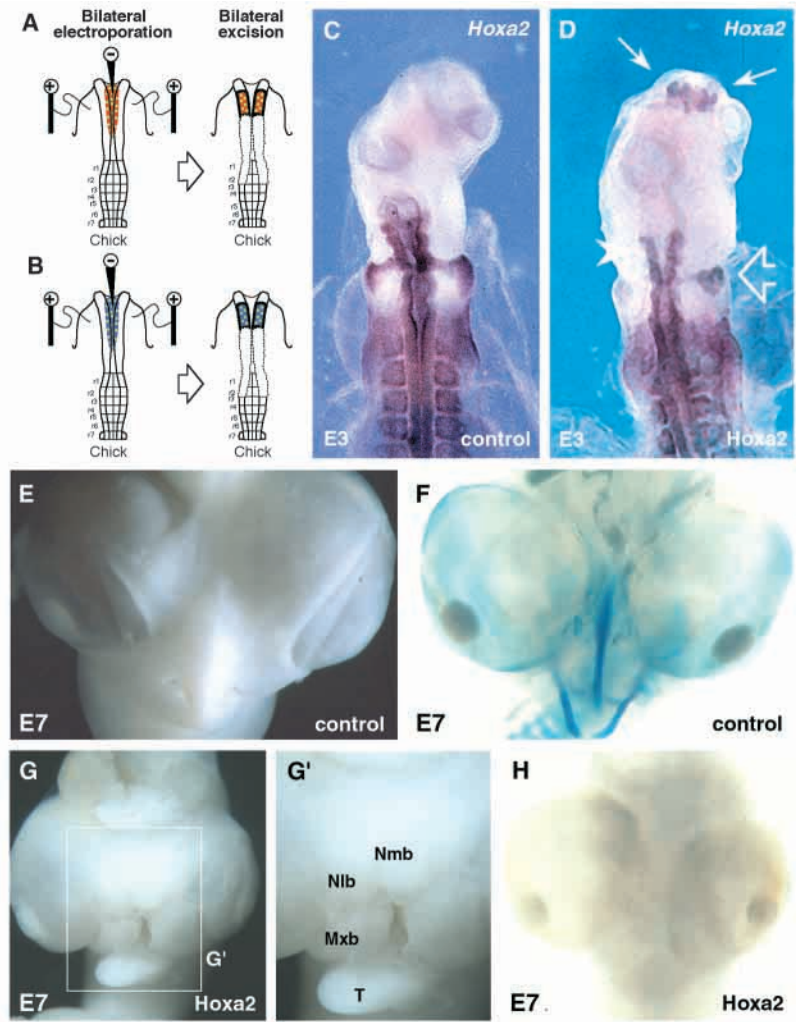

Fig. 2. Forced expression of *Hoxa2* in the endogenous posterior diencephalic neural fold and the facial phenotype. (A,B) Bilateral co-electroporation of *Hoxa2* RCAS (BP)B (red) or control RCAS (BP)B (blue), and GFP constructs (green) in the anterior neural fold of a 5 ss chick embryo. Bilateral extirpation (broken lines) of the cephalic neural fold extending from mid-mesencephalic level down to r2/r3 boundary in the electroporated chick embryo. (C,D) In E3 control and *Hoxa2*-transfected embryos, in situ hybridization for *Hoxa2* shows the endogenous pattern of gene expression at the rhombencephalic level (arrowhead), and in the second branchial arch (open arrow). (D) In *Hoxa2*-transfected embryos, the ectopic expression is evidenced at the diencephalic level (arrows) where the neural crest cells have started to migrate over the forebrain. Facial morphology and skeleton of a control embryo (E,F) and *Hoxa2*-transfected embryo (G,G′,H) at E7. The latter exhibits severe morphological defects: its face consists in underdeveloped nasal and maxillary buds (G,G′) in which no skeletal components of the upper face or lower jaw form as shown in H, representing an embryo treated with Alcian Blue (compare with F). Mxb, maxillary bud; Nlb, nasolateral bud; Nmb, nasomedial bud; T, tongue.

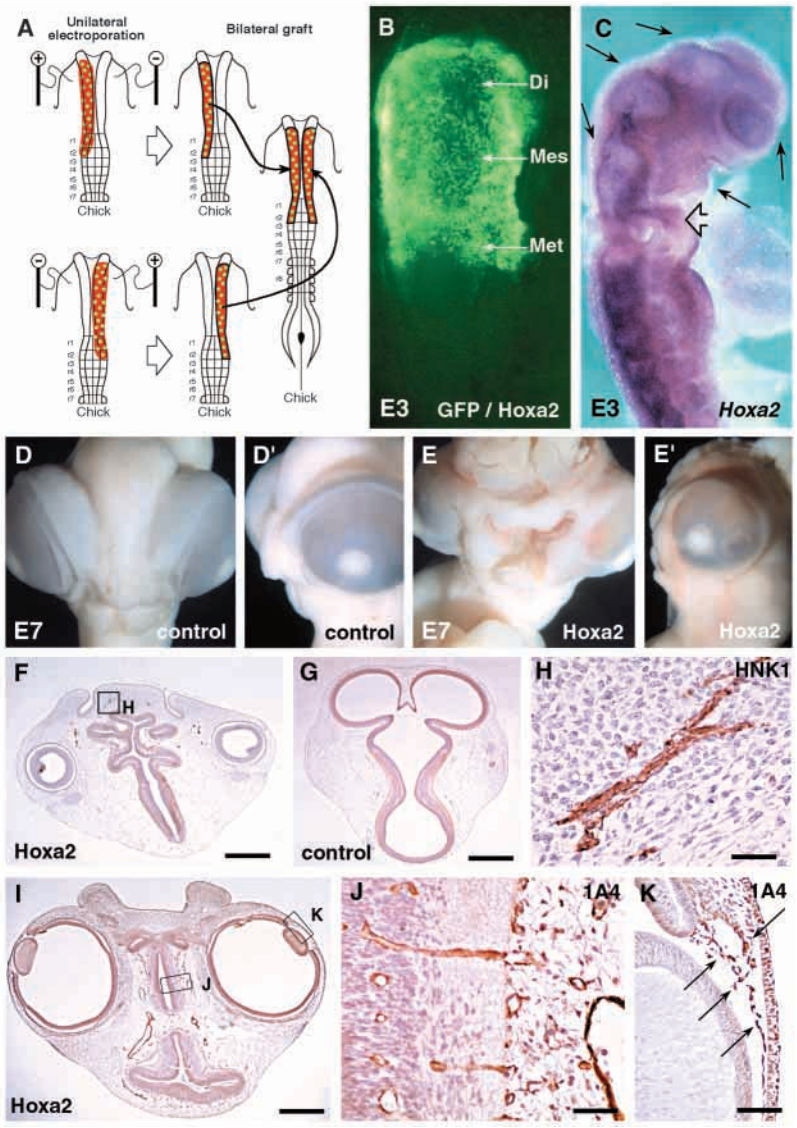

Fig. 3. Forced expression of *Hoxa2* in the entire Hox-negative neural fold and contribution of *Hoxa2* neural fold-derived cells to facial development. (A) The experimental procedure consists in the unilateral co-electroporation of *Hoxa2* RCAS (BP)B (red) and GFP (green) constructs in the Hox-negative neural fold, performed alternatively on each side of two different 5 ss chick embryos. The transfected neural folds are excised and bilaterally transposed to homotopic level into a stage-matched recipient chick embryo. Concurrently, the untransfected contralateral neural folds are bilaterally engrafted in a chick embryo that serves as a control. (B) Dorsal view of E3 embryo (HH11) showing the migration of GFP-labeled cells. (C) E3 embryo (HH14) after hybridization for *Hoxa2*, showing (1) endogenous expression in the second branchial arch (open arrow); and (2) ectopic expression in mesectodermal cells that both fill the first arch and nasofrontal bud and overlay the prosencephalic, mesencephalic and metencephalic vesicles (arrows). At E7, control embryos, in which the contralateral non transfected neural folds have been grafted, (D,D′) show a normal development of both upper and lower beaks. By contrast, embryos engrafted with *Hoxa2* transfected neural crest (E,E′) do not develop facial structures. At E4, embryos in which the diencephalic neural fold has been transfected with the *Hoxa2* construct (F), show a severe misdevelopment of the forebrain vesicles compared with control embryos (G). (H) *Hoxa2* neural fold-derived cells form glial cells in peripheral nerves, as observed after HNK1 immunolabeling. At diencephalic level (I), neural crest cells give rise to pericytes (that accumulate smooth α-actin, detected by 1A4 Ab) to the forebrain vasculature (J), and differentiate into cilliary muscles and corneal endothelium (arrows, K). Di, diencephalon; Mes, mesencephalon; Met, metencephalon. Scale bars: 350 μm in F; 400 μm in G; 50 μm in H; 600 μm in I; 60 μm in J; 75 μm in K.

territory from which mesectodermal cells normally differentiate into the skeletal derivatives of the upper face and lower jaw (Fig. 3B, Fig. 1). In these embryos, transcripts of *Hoxa2* featured the site of endogenous expression as well as the ectopic location that extended bilaterally from prosencephalic to metencephalic level towards the facial primordium (Fig. 3C). At E7, control embryos (n=2) in which a vector exempted of exogenous DNA insert was electroporated in similar condition displayed normal facial development, whereas embryos in which the neural crest cells were transfected with *Hoxa2* were devoid of facial skeleton (n=3) (Fig. 3D,E). Embryos in which the Hox-negative neural crest was transfected with *Hoxa2* did not survive beyond E7 (n=3 out of 22 embryos subjected to forced *Hoxa2* expression). This stage is, however sufficient to confirm that no facial skeleton develops in these birds.

Effect of forced expression of *Hoxa2* on the brain, on the neural derivatives of the neural crest and on the general growth and differentiation of the embryo

The neuroepithelium of the pro- and mesencephalon was not transfected by the *Hoxa2* gene in these experiments, as controlled by in situ hybridization with an *Hoxa2* probe (not shown). The development of the forebrain and midbrain was, however, severely perturbed. At E4, the anterior brain vesicles were strongly reduced in size (see Fig. 3E,F, and compare with Fig. 3D,G showing stage-matched controls). It is noticeable that the same brain morphology was regularly observed after surgical removal of the di-, mesen- and metencephalic neural fold (Fig. 1I). In contrast to the latter case, however, the development of the neural derivatives of the anterior cephalic neural crest was not abolished by *Hoxa2* forced expression (Fig. 3H). Mesenchymal cells of neural crest origin were present in the head of these animals and participated in the formation of the blood vessel wall as in normal birds (Fig. 3I,J). The corneal epithelium and the ciliary muscles which originate from the di- and mesencephalic neural crest were normally differentiated (Fig. 3K). A striking observation made on the embryos of these experimental series was their general growth retardation, which was also noticed in the embryos subjected to di-, mes- and metencephalic neural fold excision. In addition, the seven *Hoxa2*-treated (out of 58 operated) embryos that reached E6-7 were devoid of any Alcian Blue-positive cartilaginous tissues (e.g. they did not show vertebrae and limb cartilaginous condensations). This retardation of growth and differentiation does not imply that *Hoxa2* has essentially an

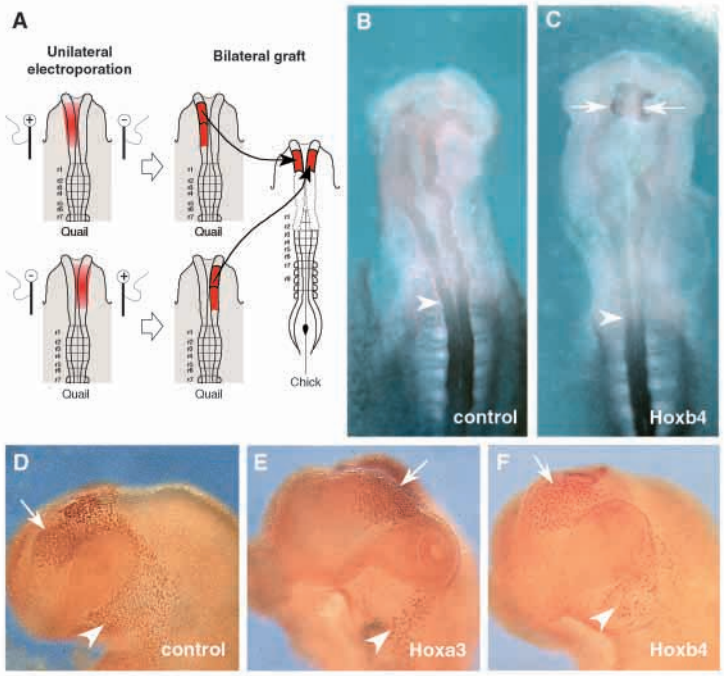

Fig. 4. Forced expression of *Hoxa3* or *Hoxb4* and the early steps of neural crest cell migration. (A) To target *Hoxa3* or/and *Hoxb4* expression in posterior diencephalic neural crest, unilateral electroporations of anterior cephalic neural fold were alternatively carried out on each side in two 5 ss quail embryos. Then, transfected neural folds were bilaterally implanted in a stage-matched chick embryo, which had been subjected to the bilateral ablation of the Hox-negative domain of its skeletogenic neural folds (broken lines). Recipient chick embryos that are bilaterally engrafted with the untransfected contralateral quail neural folds are referred to as quail-grafted controls. In situ hybridization with *Hoxb4* probe in control embryo (B) and embryo that has received *Hoxb4* transfected neural folds (C): the endogenous expression of *Hoxb4* at posterior rhombencephalic level was unperturbed (arrowheads). In experimental embryos engrafted with *Hoxb4* neural folds, *Hoxb4* transcript expression also features the exogenous neural crest cells that start to spread from the transplant (arrows). Twenty hours after the operation (HH14), quail cell detection show that the migratory behavior of implanted neural crest cells in control embryos (D) is equivalent to that observed in embryos carrying *Hoxa3* (E) or *Hoxb4* (F) transfected neural fold-derived cells. In all these embryos, quail cells move rostrally to populate the nasofrontal bud (arrows) as well as laterally to colonize the presumptive first branchial arch (arrowheads).

unspecific effect on facial structure development. In fact at E3-4, the size of the treated and untreated embryos was the same. However, the former showed severe hypotrophy of their facial processes, thus showing a specific effect of *Hoxa2* expression on the growth of these structures.

How the perturbations brought about in the head by the *Hoxa2* forced expression did affect the general development of the embryo has not been elucidated but may be related to abnormalities in head and blood vessel development resulting in alterations in blood supply to the embryonic tissues.

Forced expression of *Hoxa3* and *Hoxb4* in the cells of the anterior cephalic neural crest hampers their ability to form the facial skeleton

Recipient 5-6 ss chick embryos were first subjected to the bilateral excision of the neural fold domain responsible for generating the facial skeleton as in the previous experimental series. The diencephalic neural folds, from stage-matched quails, were electroporated with retroviral vectors (RCAS (BP) A, active in chick and quail) carrying the coding sequence of *Hoxa3*, or of *Hoxb4*, or were co-electroporated with both, and grafted into the chick as shown in Fig. 4A. Because only one neural fold was transfected in electroporated embryos, the contralateral folds were engrafted on chick hosts that served as controls.

The early steps of migration of electroporated and control neural crest cells were visualized 4 hours (n=2), 8 hours (n=6), 12 hours (n=4) and 20-24 hours (n=52) after the operation, by using either the QCPN mAb to reveal quail cells or by in situ hybridization with the appropriate probe (either *Hoxa3* or *Hoxb4*). The accumulation of transcripts corresponding to the electroporated DNA could be seen in the neural crest cells that dispersed from the graft (Fig. 4B,C). Twenty hours after grafting, the quail cells were dispersed similarly in the electroporated and control neural crest cells (Fig. 4D-F). The cells migrating from the graft moved rostrally over the forebrain and colonized the nasofrontal bud. They also dispersed laterally along the optic vesicles to invade the forming first branchial arch. The effect of transfected Hox genes on the development of the facial skeleton was evaluated at E6, E7 and E8.

Effect of *Hoxa3* transgene expression

At E6 (n=4), the embryos engrafted with diencephalic neural folds expressing the *Hoxa3* transgene had a reduced nasofrontal bud and the first branchial arch was completely absent (Fig. 5A,B,D,E). At E8 (n=3), the nasal capsule was present but poorly developed. The lower jaw skeleton (Meckel's cartilage, articular, quadrate) and the entoglossum were absent (Fig. 5C,F). The hyoid cartilage was developed except for its anterior region, which is normally made up from Hox-negative neural crest cells (Fig. 5A) (Couly et al., 1996).

The use of quail neural folds for Hox gene electroporation allowed the fate of transfected cells to be determined. As shown in Fig. 4E, during the first 24 hours after the implantation, neural crest cells migrating from transplants expressing the *Hoxa3* gene colonized the facial primordium and overlaid the telencephalic vesicles in a normal fashion. At E7 (n=3), myoepithelial pericytes of quail origin were found lining the endothelium of capillaries in the meninges and in the vessels that colonized the neuroepithelium of the cerebral hemispheres as in normal development (Etchevers et al., 2001) (Fig. 5G,H). At the diencephalic level, quail cells were present at the frontal midline, rostral to the adenohypophysis rudiment. Later on, they formed only a small part of what should have been the basipresphenoid as well as contiguous cartilaginous structures, forming a rudimentary nasal septum and nasal capsule (Fig. 5I,J). Around the optic vesicles, the sclerotic cartilage did not develop. In the absence of sclerotic cartilage, no extraocular muscles were found, probably because of the lack of skeletal insertion. Quail-derived Schwann cells were identified in the trigeminal nerve, located laterally to the mesencephalic vesicle. Ciliary muscles and the internal corneal epithelium were of quail origin (Fig. 5K). Some quail cells identified as pericytes

were sparsely observed in the choroid membrane associated with small vessels. By contrast, arteries or veins that displayed a considerable expansion in the size of their lumen, looking more like blood lacunae than blood vessels, were lined by endothelial cells but lacked neural crest-derived pericytes (Fig. 5I).

Effect of *Hoxb4* transgene expression

At E6 (*n*=3), the embryos carrying grafts of *Hoxb4*-transfected neural folds had virtually no face and, in this respect, they looked like embryos in which the Hox-negative neural crest had been transfected with *Hoxa2* (Fig. 6A,D). Later on, at E8 (*n*=3), the total absence of the nasofrontal bud and upper beak was confirmed (Fig. 6B,E). As far as the first branchial arch is concerned, rudimentary skeletal components of the lower jaw developed (Fig. 6B,C,E,F): Meckel's cartilage was restricted to its proximal region associated with the quadrate and articular cartilages. This evolution of the first branchial arch is not seen in either *Hoxa2* or *Hoxa3* transgenic embryos. The sclerotic cartilages were absent.

These embryos were examined at E6 (*n*=3) on sections treated with QCPN mAb in order to determine the respective contributions of donor and host cells to the remaining head structures (Fig. 7A). The quail cells arising from the *Hoxb4*-expressing neural fold transplants became Schwann cells in peripheral cranial nerves, pericytes in the choroid membrane and around the endothelial wall of the blood vessels, as well as loose mesenchyme in the head (Fig. 7B,D). Their participation in cartilage was investigated in E8 chimeras (*n*=3): in the diencephalic region, the sella turcica, normally formed by the juxtaposition of the basi-postsphenoid (of mesodermal origin) (Couly et al., 1993) and the basi-presphenoid (of neural crest origin) (Couly et al., 1993), was the most rostral skeletal structure formed in these embryos (Fig. 6E, Fig. 7E). The basi-postsphenoid was made up of chick host cells (Fig. 7F). By contrast, the basi-presphenoid was of quail graft origin (Fig. 7G). In the first branchial arch, the quadrate cartilage was of mixed quail and chick origin, whereas the articular and Meckel's cartilages were exclusively of donor type (Fig. 7H,J). The cells of host origin may be derived from a small flux of migration from r3. Experiments are in progress to test this possibility.

Effect of *Hoxa3* and *Hoxb4* co-transfection

Additionally, we tested the effects of *Hoxa3* and *Hoxb4* co-transfection. At E7 (*n*=4), embryos engrafted with diencephalic neural fold co-electroporated with both *Hoxa3*- and *Hoxb4*-coding sequences failed to develop the skeletal structures of both the upper and lower beaks. The general aspect of their face consisted in the presence of underdeveloped nasofrontal, maxillary and mandibular buds, which remained as independent masses surrounding the stomodeal cavity; no cartilage developed in them whatsoever (Fig. 8A).

On sections at E7 (*n*=3), quail cells that migrated from the transplant, populated the telencephalic region, and gave rise to pericytes, which are tightly associated with the endothelium of capillaries in the prosencephalic neuroepithelium (Fig. 8B,C). Many quail cells also became pericytes in the choroid membrane, but choroid vessels failed to develop normally, forming large blood lacunae around the optic vesicles (Fig. 8D). Laterally, ciliary muscles and corneal endothelium were of quail origin (Fig. 8E,F). In the nasal region, quail-derived mesectodermal cells filled the nasolateral and nasomedial buds, forming a loose

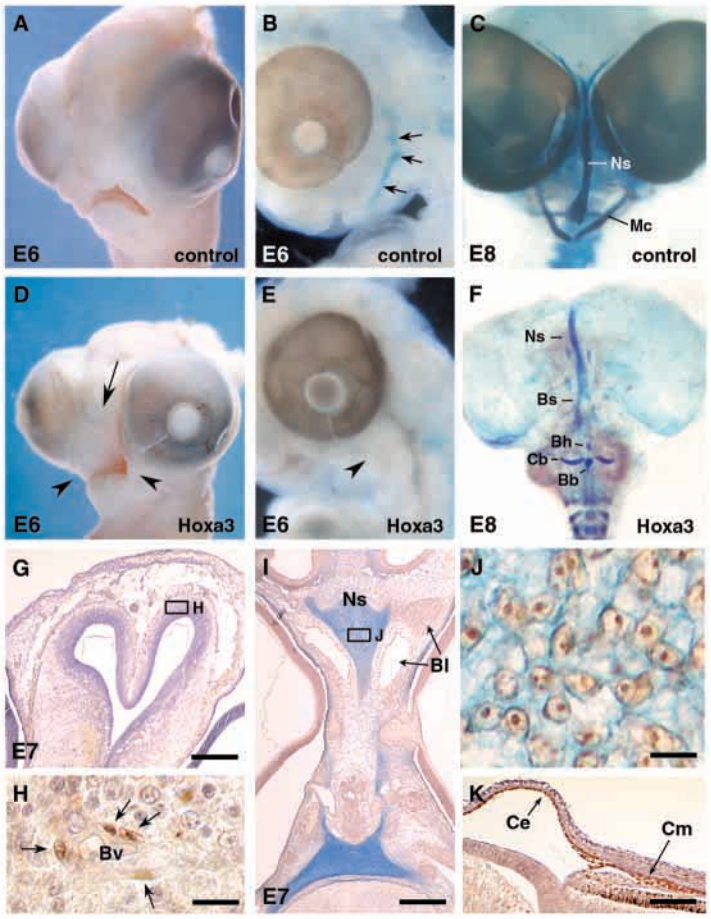

Fig. 5. Effect of ectopic *Hoxa3* expression on facial development. At E6, quail-grafted control embryos develop a normal face (A) and show skeletal elements in the first branchial arch (B), consisting of Meckel's, articular and quadrate cartilages (arrows). At E8, neural crest cells arising from untransfected diencephalic neural folds yield the entire facial and mandibular skeleton (C). By contrast, E6 embryos that have been engrafted with *Hoxa3* transfected neural folds (D) show a reduced nasal bud (arrow) and fail to develop the first branchial arch (arrowheads). All the skeletal components of the mandibular bud are missing (E, arrowhead). At E8 (F), the nasal septum has developed rostral to the basipresphenoid and towards the top of the head. Although the posterior part of hyoid structure forms, in which the basihyal (Bh), ceratobranchial (Cb) and basibranchial (Bb) cartilages are recognizible, the lower jaw skeleton is completely absent. (G) At E7, in the forehead territory, telencephalic hemispheres are evidenced in which quail cells are identified as pericytes (H, arrows) of the neuroepithelium capillaries. At diencephalic level (I), the nasal septum that forms at the midline is of quail origin (Alcian Blue and QCPN mAb staining)(J). (K) Quail cells give rise to ciliary muscles and to the corneal endothelium. Bl, blood lacunae; Bs, basipresphenoid; Bv, blood vessel; Ce, corneal endothelium; Cm, ciliary muscle; Mc, Meckel's cartilage; Ns, nasal septum. B,C,E,F are Alcian Blue stained embryos. Scale bars: 600 μm in G; 15 μm in H; 350 μm in I; 10 μm in J; 60 μm in K.

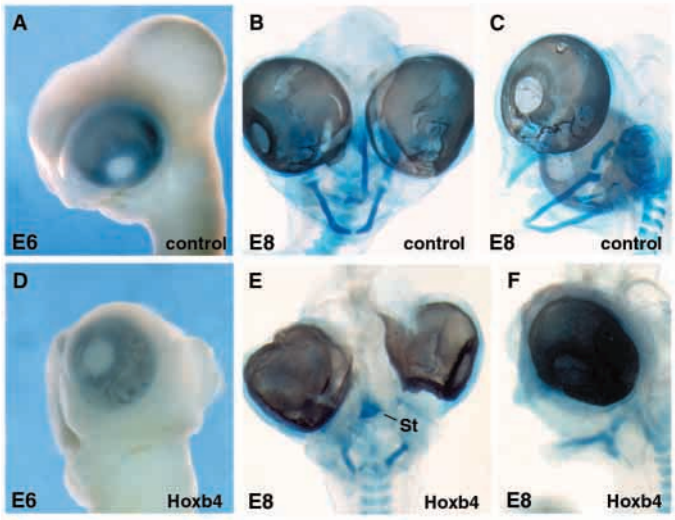

Fig. 6. Effect of the ectopic *Hoxb4* expression on head morphogenesis. At E6, when compared with quail-grafted-control embryos (A), which exhibit a normal face, the embryos that develop with *Hoxb4*-expressing diencephalic crest cells (D) have no nasal bud and a reduced first branchial arch. (B,C) At E8, untransfected neural crest cells grafted in control embryos ensure the normal development of the facial and mandibular skeleton. (E,F) In embryos carrying *Hoxb4*-transfected neural folds, the sella turcica is the most rostral skeletal structure, as the upper beak skeleton is missing. In the first branchial arch, the skeleton is reduced to its proximal elements: quadrate, articular and the proximal region of Meckel's cartilage. St, sella turcica.

mesenchyme underneath the olfactory epithelium of the nasal pits (Fig. 8E,G).

As the rhombencephalic neural crest cells arising from r4 to r8 level display combined expression of *Hoxa2*, *Hoxa3* and *Hoxb4*, we decided to substitute it for Hox-negative neural crest to compare with the *Hoxa2*, or *Hoxa3* and *Hoxb4* overexpression experiments. These r4 to r8 neural crest cells are endowed with skeletogenic potential as they give rise to the mesectodermal derivatives from which hyoid cartilages (except the tongue skeleton) develop.

In chick embryos from which the Hox-negative neural folds were ablated, quail r4-r8 neural folds were bilaterally engrafted (Fig. 9A). At E3 (n=4) and E4 (n=3), quail neural crest cells spread from the transplant and colonized the nasofrontal mass and the first and second branchial arches. In addition, graft-derived cells also colonized the third branchial arch. At E7 (n=7), the embryos showed severe defects in their facial development: their face consisted of rudimentary nasofrontal, and maxillary buds, while the mandibular prominences were indistinguishable (Fig. 9B). On sections at E7, the localization of quail donor cells showed that the rhombencephalic crest cells failed to generate any skeletal structures in the head (Fig. 9C). By contrast, quail cells were found to become Schwann cells lining peripheral cranial nerves (Fig. 9D). Others were sparsely present as pericytes around blood vessels and in the choroid membrane (Fig. 9E,F). The contribution of graft-derived cells to the corneal epithelium or ciliary muscles was rare, while normally these structures are made up of neural crest cells (Fig. 9G). These results confirm both previous observations (Couly et al., 1998)

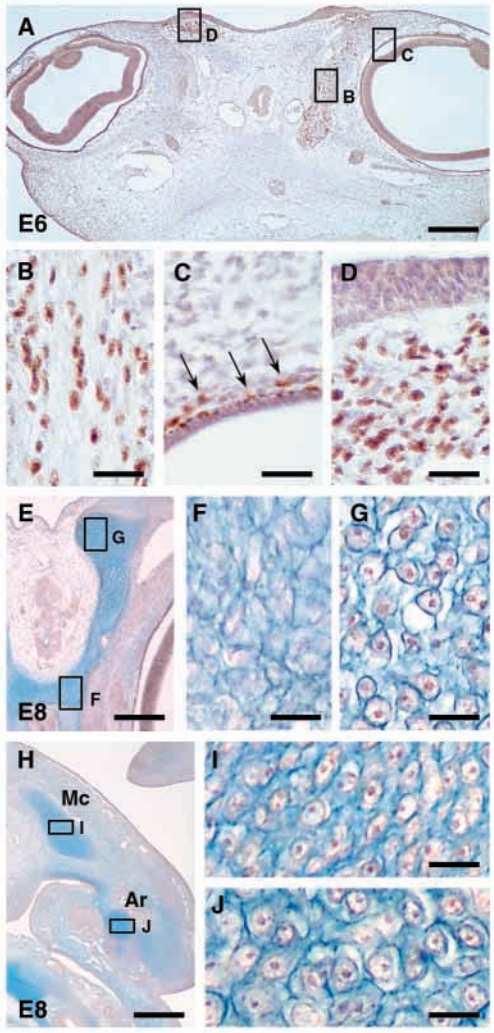

Fig. 7. Contribution of *Hoxb4* neural fold-derived cells to facial development. At E6, in chimeric embryos engrafted with *Hoxb4* transfected neural folds, quail cells that populate the nasofrontal area (A) contribute to nerve glia (B), become pericyte (arrows) in choroid membrane (C), and form a loose mesenchyme under the olfactory epithelium (D). At E8, in such embryos, the sella turcica remains the most rostral skeletal structure (E) in which the basipostsphenoid is of host mesodermal origin (F) and the basipresphenoid of quail mesectodermal origin (G) (same staining procedure as in Fig. 5I,J). In the first branchial arch (H), quail cells generate both Meckel's (I) and articular (J) cartilages. Mc, Meckel's cartilage; Ar, articular. Scale bar: 230 μm in A; 30 μm in B; 30 μm in C; 30 μm in D; 375 μm in E; 10 μm in F; 10 μm in G; 600 μm in H; 12 μm in I; 12 μm in J.

and the results described above obtained in gain-of-function experiments (Table 1).

DISCUSSION

This work is part of an ongoing study of craniofacial development which started in the 1970s with the demonstration that the entire facial and visceral skeleton as well as a large part of the brain case of the avian embryo are derived from the

neural crest mesenchyme, also designated as mesectoderm (for a review, see Le Douarin and Kalcheim, 1999). This being established, the problem of how patterning of these skeletal structures is controlled at the genetic level appeared crucial for deciphering the complex morphogenetic processes that are involved in vertebrate head development. Among the numerous genes encoding transcription factors and secreted proteins that are expressed during head development, Hox genes, the vertebrate homologs of the homeotic genes (HOM-C) of *Drosophila*, have attracted much attention (Krumlauf, 1993; Krumlauf, 1994). Hox genes of the first four paralogous groups are expressed in the neural crest from early developmental stages and through late stages of pharyngeal arch morphogenesis (Barrow and Capecchi, 1999; Kanzler et al., 1998; Prince and Lumdsen, 1994).

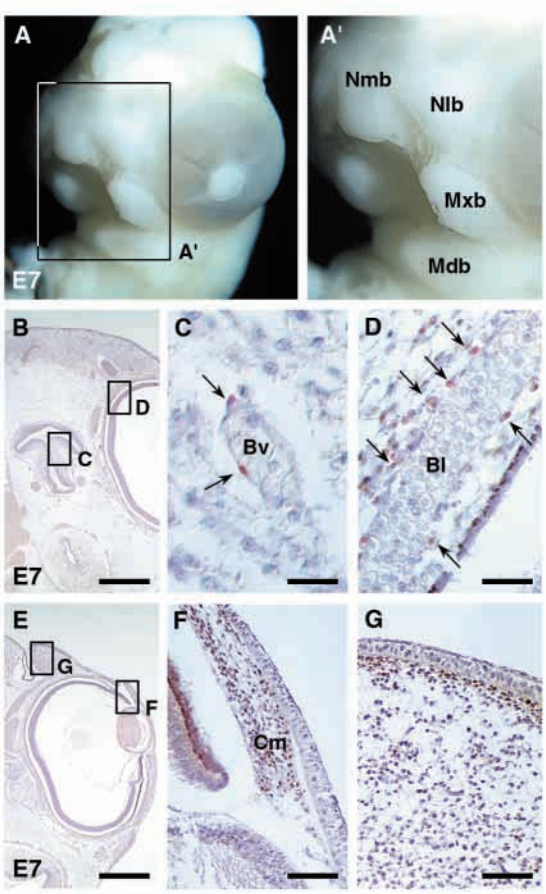

Fig. 8. Effect of the combined ectopic expression of *Hoxa3 and Hoxb4* on facial development. (A) At E7, embryos carrying *Hoxa3* and *Hoxb4* co-electroporated neural folds are devoid of facial structures. (A′) Higher magnification showing the underdeveloped nasal, maxillary and mandibular buds. (B) In the forehead of chimeric embryos, graft-derived cells differentiate as pericytes (arrows) for the telencephalic capillaries (C) and for the choroid membrane (D), though in the latter, large blood lacunae (Bl) form. Note the blood vessels (Bv) and blood lacunae are filled with nucleated erythrocytes. (E) In the nasal region, quail cells contribute to ciliary muscles and corneal endothelium (F) and give rise to mesenchyme in the nasal buds (G). Cm, ciliary muscle; Mdb, mandibular bud; Mxb, maxillary bud; Nlb, nasolateral bud; Nmb, nasomedial bud. Scale bar: 360 μm in B; 15 μm in C; 25 μm in D; 700 μm in E; 50 μm in F; 60 μm in G.

Hox gene expression defines two ‘non-equivalent’ domains in the skeletogenic neural crest

Mutational analyses in the mouse have revealed that Hox genes of the first paralogous group are involved in the development of various neural crest-derived structures. Such is the case for *Hoxa2*, of which the null mutation strongly affects the development of the second branchial arch skeleton. The *Hoxa3* gene, which is expressed in the neural crest cells that colonize the third and the fourth to fifth branchial arches [the ‘cardiac neural crest’, according to Kirby et al. (Kirby et al., 1983; Kirby et al., 1985)] is crucial for the development of the hyoid bone, the cardiovascular system and pharyngeal glands such as the thymus whose mesenchymal component is derived from the mesectoderm (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995).

As shown by Couly et al. (Couly et al., 1996) and Köntges and Lumsden (Köntges and Lumsden, 1996) in the avian embryo, the neural crest arising from the posteriormost rhombomeres (r4 to r8) and expressing genes of the first four

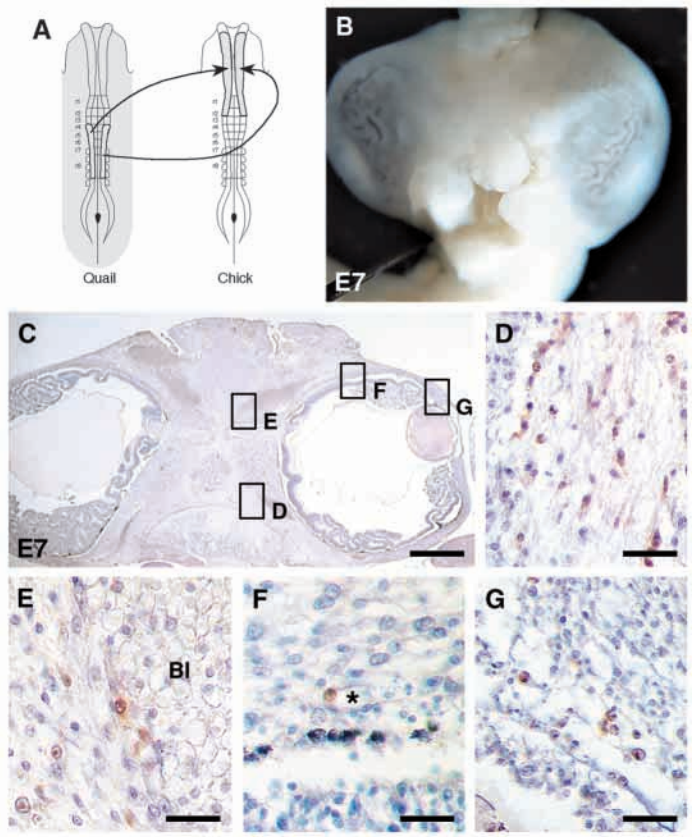

Fig. 9. Fate of rhombencephalic r4-r8 neural crest cells rostrally transposed into the Hox-negative domain. (A) Bilateral substitution of the quail rhombencephalic r4-r8 neural fold to the host Hox-negative neural fold (extending from diencephalon down to r2). (B) E7. Frontal view of a chimeric embryo showing the complete absence of upper face and lower jaw. (C) Rhombencephalic quail cells that have colonized the facial primordium contribute Schwann cells to the trigeminal nerve (D). Some rare graft-derived cells are identified as pericytes in vascular wall of blood lacunae (E) and into the choroid membrane capillaries (asterisk, F). (G) Quail cells are poorly present in ciliary muscles and absent from the cornea. Scale bar: 250 μm in C; 50 μm in D; 30 μm in E; 20 μm in F; 30 μm in G.

Table 1. Effect of ectopic Hox gene expression on facial morphogenesis

Genes ectopically expressed in the normally Hox-negative neural crest	Abnormalities recorded			
	Skeletal			
	Nasofrontal bud derivatives	BA1 derivatives	Vascular	Neural
Hoxa2	Absent	Absent	Pericytes of cephalic capillaries	Schwann cells present
Hoxa3	Rudimentary (medial bones)	Absent	Pericytes of cephalic capillaries, absent in veins or arteries	Schwann cells present
Hoxb4	Absent	Rudimentary (proximal bones)	Pericytes present in cephalic capillaries, pericytes of veins or arteries	Schwann cells present
Hoxa3 and *Hoxb4*	Absent	Absent	Pericytes in cephalic capillaries, extremely rare in veins or arteries	Schwann cells present

paralogous groups (including *Hoxa2*, *Hoxa3* and *Hoxb4*), contribute to a large part of the hyoid bone. The skeletal derivatives of the neural crest are in this respect divided into an anterior region, including the nasal capsule and the lower jaw, in which none of the Hox genes are expressed, and a posterior domain, in which genes of the first four paralogous groups are activated.

In vivo transplantation experiments carried out in the avian embryo have shown that the anterior transposition of Hox-positive neural crest to the Hox-negative domain is not compatible with the differentiation of skeletal elements, whether they are of cartilaginous or of the membranous bone type. By contrast, when Hox-negative neural cells are transposed to a Hox-positive domain, the former participate in that part of the hyoid cartilages corresponding to the site of transplantation (Couly et al., 1998).

Moreover, the neural crest cells of the Hox-negative domain behave as an 'equivalence group', as substitution of any region of the cephalic neural fold from the mid-diencephalon down to r3 does not prevent the normal development of the facial skeleton. In addition, we showed that within this Hox-negative domain, the capacity for regeneration of the neural crest is remarkably developed: as little as a quarter of the whole Hox-negative skeletogenic neural fold can yield the complete set of facial cartilage and bones (Couly et al., 2002). By contrast, such a regeneration cannot take place from the Hox-positive neural folds. It appears therefore that, contrary to earlier assumptions (Noden, 1983), the information for patterning its own derivatives is not contained in the neural crest itself, but is imposed by extrinsic cues to its cells after they have started their migration. Noden's view was based on the fact that transposition of mesencephalic neural crest and tube to the mid-rhombencephalic level resulted in the duplication of the lower beak at the expense of the second branchial arch structures. This was confirmed by Couly et al. (Couly et al., 1998). An explanation of these results has been recently proposed involving the negative effect of FGF8 produced in the isthmus, on *Hoxa2* expression in the second branchial arch (Trainor et al., 2002).

Moreover, recent work from our laboratory has shown that at least some of these cues are provided to the neural crest cells by the ventral endoderm of the foregut (Couly et al., 2002).

The foregut endoderm is responsible for the specification of the cartilage and bones of the facial skeleton

Defined regions of the ventral foregut endoderm distributed along the rostrocaudal axis have been identified on the basis of their ability to specify not only the shape, but also the orientation with respect to the body coordinates, of each individual cartilage and bone of the facial and visceral skeleton (Couly et al., 2002).

The pharynx itself can be divided into three different regions with respect to Hox gene expression: (1) an anterior one, in which none of the tissue components expresses Hox genes (as is the case for the first branchial arch); (2) a posterior one, in which not only the neural crest cells but also the endoderm of the branchial pouch, the ectoderm lining the branchial arch and the mesoderm all express the same Hox code (as is the case for the third and fourth to fifth branchial arches); and (3) an intermediate domain in which the neural crest cells, but neither the endoderm nor the mesoderm (and only partly the ectoderm), express *Hoxa2* (as is the case for the second branchial arch) (Hunt et al., 1991; Couly et al., 1998). Inactivation of *Hoxa2* in the mouse leads the second branchial arch to acquire the same Hox gene-negative status as the first arch. This results in the duplication of the first branchial arch skeleton at the expense of normal second arch skeletal derivatives. This spectacular result prompted further experiments aimed at elucidating the mode of action of *Hoxa2* in branchial arch morphogenesis.

The impact of loss-of-function and gain-of-function of Hox genes on facial and visceral skeletogenesis

In light of the role played by homeotic genes in *Drosophila*, the most obvious explanation for the results of *Hoxa2* inactivation was to assume that it functions as a selector gene and provides the second branchial arch with its specific developmental program (Rijli et al., 1993; Gendron-Maguire et al., 1993). This conclusion was further supported by the fact that overexpression of *Hoxa2* in the first branchial arch in the chick embryo produced abnormalities in first arch skeleton that were interpreted as homeotic conversions of the first to the second arch skeleton (Grammatopoulos et al., 2000). Such conversions occurred when the transgene was administered to all of the first arch tissues. By contrast, when *Hoxa2* transgene

expression was selectively targeted to the neural crest cells, arch skeletal structures failed to develop. Experiments in which the Hox-positive neural crest was transplanted into the Hox-negative domain (Couly et al., 1998) are consistent with the results of Grammatopoulos et al. (Grammatopoulos et al., 2000), because they showed that Hox-expressing neural crest cells do not develop skeletal structures in a Hox-negative context.

Conversion of first arch into second arch skeletal elements was also obtained in *Xenopus* subjected to temporally controlled *Hoxa2* gain-of-function experiments (Pasqualetti et al., 2000). Segmentation phenotypes could be obtained by inducing the *Hoxa2* transgene activity at early stages but not after neural crest migration into the arch. In this case, as in the chick embryo experiments, the transgene was expressed in all the tissues that compose the branchial arches.

Hox gene gain-of-function experiments prevent the neural crest fated to yield the facial skeleton to differentiate into skeletal structures

The experiments carried out in the present work were aimed at further exploring the influence of Hox gene expression on the development of the facial skeleton. The paradigm used was designed to ensure that Hox gene function was selectively gained in neural crest cells, to the exclusion of the other tissues of the facial buds.

We observed that, whichever Hox gene was used, transfection did not perturb the dispersion of the neural crest cells that was similar to that of normal Hox-negative neural crest cells. However, the effect on facial morphogenesis was different according to the nature of the transfected gene.

Hoxa2 gain of function

In the E3 embryo, the cephalic neural crest cells carrying the *Hoxa2* construct invaded the rudiments of the nasal bud and of the mandibular arch. However, transfected neural crest cells failed to grow and the facial buds remained rudimentary or even regressed totally; and the head morphology at E6 was comparable with that of embryos subjected to the removal of the entire Hox-negative neural folds (compare Fig. 1I with Fig. 3E,E′ or Fig. 2E with 2G).

Treatment of these embryos with Alcian Blue failed to reveal any cartilaginous tissues which developed normally in the controls. Survival of these embryos was impaired; those able to reach E7 were smaller in size than normal.

Forced expression of Hoxa3 and Hoxb4 differently affect the development of the facial skeleton and blood vessels

The effect of either of these genes on facial morphogenesis was spectacular. *Hoxa3* gain-of-function in embryos at E6 had a truncated face in which the frontonasal bud was reduced and the mandibular buds were absent. At E8, the visceral skeleton was represented only by to an incomplete nasal septum and a truncated hyoid cartilage in which the derivatives of first branchial arch (entoglossum and basihyal) were missing. No cartilage corresponding to the lower jaw developed in these embryos. In *Hoxb4* gain-of-function E6-E8 embryos, the facial structures were missing. No nasofrontal bud or upper beak had formed in the six embryos that survived until this age. In contrast to *Hoxa2* or *Hoxa3* gain-of-function embryos, the proximal region of the lower jaw developed, showing recognizable quadrate, articular and the most proximal part of Meckel's cartilages.

Thus, while *Hoxa2* misexpression in the Hox-negative domain of the neural crest has a deleterious effect on the formation of the entire visceral skeleton, *Hoxa3* and *Hoxb4* affect selectively definite chondrogenic fields. *Hoxa3* abolishes chondrogenesis in the first branchial arch, whereas *Hoxb4* misexpression eliminates cartilage formation in the nasofrontal bud and the distal part of the mandibular arch.

The conjugated effect of these two genes results in the complete absence of facial skeleton and yields embryos that are comparable with those subjected to *Hoxa2* misexpression in the Hox-negative neural crest.

The use of quail neural fold transplants in the experiments involving *Hoxa3* and *Hoxb4* genes allowed the allocation and fate of transfected cells to be explored in E6 chimeras. As a general rule, misexpression of Hox genes in the Hox-negative domain of the neural crest did not prevent the development of its neural derivatives. Such an observation confirms our previous results obtained after transplantation of Hox-positive neural crest to the Hox-negative domain (Couly et al., 1998), as well as the experiments described here in which the anterior Hox-negative neural folds are replaced by that from r4-r8. Schwann cells of graft origin, lining peripheral nerves in the head were regularly observed in *Hoxa3* and *Hoxb4* overexpression experiments. An original observation was the effect of *Hoxa3* on the contribution of the neural crest to head blood vessels: in *Hoxa3*-treated embryos, head blood vessels were replaced by large blood lacunae. As shown in our laboratory, the pericytes and smooth muscle cells lining the endothelial wall, which are essential for blood vessel development, are of neural crest origin in the meninges of the forebrain and in the facial and ventral neck area down to the conotruncus of the heart (Le Douarin and Kalcheim, 1999; Etchevers et al., 1999; Etchevers et al., 2001). Involvement of *Hoxa3* in vasculogenesis has already been documented, as the *Hoxa3*-null mutants show extensive malformations in their cardiovascular system (Chisaka and Capecchi, 1991; Lufkin et al., 1991).

It seems therefore that, for vasculogenesis, as for the skeletal development, Hox gene products, which are involved in morphogenesis of neural crest cell derivatives arising from the mid- and posterior hindbrain, have an inhibitory effect on the anterior domain of the neural crest, which plays a major role in the formation of the vertebrate head.

Forced expression of Hox genes in the anterior domain of the neural crest corresponds to a 'posteriorization' of this domain along the AP axis. Similar 'posteriorization' effects are obtained by treating embryos with excess of vitamin A or its derivative retinoic acid (RA) with a reduction of the forebrain, midbrain and of their neural crest derivatives. This can be explain by the fact that Hox gene expression has been shown to be controlled by RA receptors acting as transcription factors via RARE (retinoic acid receptor response elements) sequences present in Hox gene promoters (for a review, see Le Douarin and Kalcheim, 1999).

General conclusions

From the results presented here, together with those of other authors, it appears that morphogenesis of the derivatives of the

cephalic neural crest depends on their expression or non-expression of Hox genes belonging to the four first paralogous groups. Development of the facial skeleton can occur only if neural crest cells do not express Hox genes. By contrast, the lack of Hox gene expression is compatible with morphogenesis of the hyoid cartilage.

The experiments reported in this article show that Hox genes of the four first paralogous groups have an inhibitory effect on the development of the facial skeleton. However, this effect is not identical for the genes of the different paralogous groups tested (the second, third and fourth): the most effective gene is the more anteriorly expressed *Hoxa2*.

A recent report concerning Hox gene expression in the rostral region of the lamprey has shown that the homolog of Hox genes of the sixth paralogous group does not obey the colinearity rule in this animal and that its expression which extends rostrally to that of more 3′ Hox genes, involves the first branchial arch (Cohn, 2002). Such an extended domain of Hox gene expression might be responsible for the jawless status of the lamprey. This observation together with the results presented here support the contention that the absence of Hox gene expression rostrally to r2 has been a critical factor for the development of the head and lower jaw in gnathostome vertebrates.

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