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Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head

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SUMMARY

The vertebrate face contains bones that differentiate from mesenchymal cells of neural crest origin, which colonize the median nasofrontal bud and the first branchial arches. The patterning of individual facial bones and their relative positions occurs through mechanisms that remained elusive. During the early stages of head morphogenesis, an endodermal cul-de-sac, destined to become Sessel's pouch, underlies the nasofrontal bud. Reiterative outpocketings of the foregut then form the branchial pouches. We have tested the capacity of endoderm of the avian neurula to specify the facial skeleton by performing ablations or grafts of defined endodermal regions. Neural crest cells that do not express Hox genes respond to patterning cues produced regionally in the anterior endoderm to yield distinct skeletal components of the upper face and jaws. However, Hox-expressing neural crest cells do not respond to these cues. Bone orientation is likewise dependent on the position of the endoderm relative to the embryonic axes. Our findings thus indicate that the endoderm instructs neural crest cells as to the size, shape and position of all the facial skeletal elements, whether they are cartilage or membrane bones.

Key words: Foregut endoderm, Neural crest, Facial skeleton, Hox genes, Quail-chick chimeras

INTRODUCTION

Most of the head skeleton is formed by membrane and cartilage bones of neural crest origin. The paraxial mesoderm that contributes to the posteriormost region of the skull (including the otic capsule, the occipital, part of the sphenoid and post-orbital bones) covers only the midbrain and hindbrain. Cell lineage studies carried out in the avian embryo have revealed that, from an early developmental stage, the prosencephalon becomes covered by neural crest cells emigrating from the posterior diencephalon, mesencephalon and the first rhombencephalic segments. These cells are at the origin of the frontal, parietal, squamosal and basi-presphenoid bones, which belong to the neurocranium. The nasal bud, the first branchial arch (BA1), from which the nasal capsule, maxillary bone, lower jaw and tongue (entoglossum) skeleton develop, are populated by cells arising from the mesencephalic neural fold and from the two first rhombomeres (r1 and r2). The rest of the viscerocranium (i.e. the hyoid cartilage) originates from the crest of more caudal rhombomeres that colonizes branchial arches 2, 3, 4 (BA2, BA3, BA4) (Fig. 1) (Couly et al., 1996; Köntges and Lumsden, 1996).

One of the crucial issues related to head morphogenesis concerns the developmental mechanisms that underlie the patterning of various skeletal structures of the face, namely the bone shapes and sizes that exhibit a considerable variability in the vertebrate series.

Segmentation of the branchial arches close to the hindbrain into rhombomeres constitutes the first visible step in the patterning process of the facial and hypobranchial structures. The rhombencephalon becomes metamerized into eight rhombomeres (Vaage, 1969; Lumsden and Keynes, 1989) and an iterated series of endodermal pouches form in the pharynx. Specific Hox genes, as well as others, such as *Krox20* (Wilkinson et al., 1989) and *valentino* (Moens et al., 1996), are known to be involved in hindbrain segmentation. The Hox genes expressed in the hindbrain belong to the four first paralogue groups, and the anteriormost limit of Hox gene expression corresponds to the boundary between r1 and r2 (Prince and Lumsden, 1994) (Fig. 1A). As a general rule, the rhombomeres and the neural crest cells that they produce express the same set of Hox genes, forming a 'Hox code'; this rule holds for the crest cells that colonize BA2, BA3 and BA4 (Hunt et al., 1991). Thus, two domains can be distinguished in the neural crest cells that are at the origin of the facial and visceral skeleton: a rostral Hox gene non expressing domain (Hox negative), which gives rise to the membrane bones of the neurocranium, the nasal capsule, the maxillary bone and the lower jaw; and a Hox-positive domain, which yields the hyoid cartilages (except the entoglossum). It has been shown (Couly et al., 1998) that Hox-expressing neural crest cells transposed anteriorly to the Hox gene non-expressing domain fail to differentiate into cartilage and bone. By contrast, the neural crest cells of the Hox-negative domain transplanted posteriorly

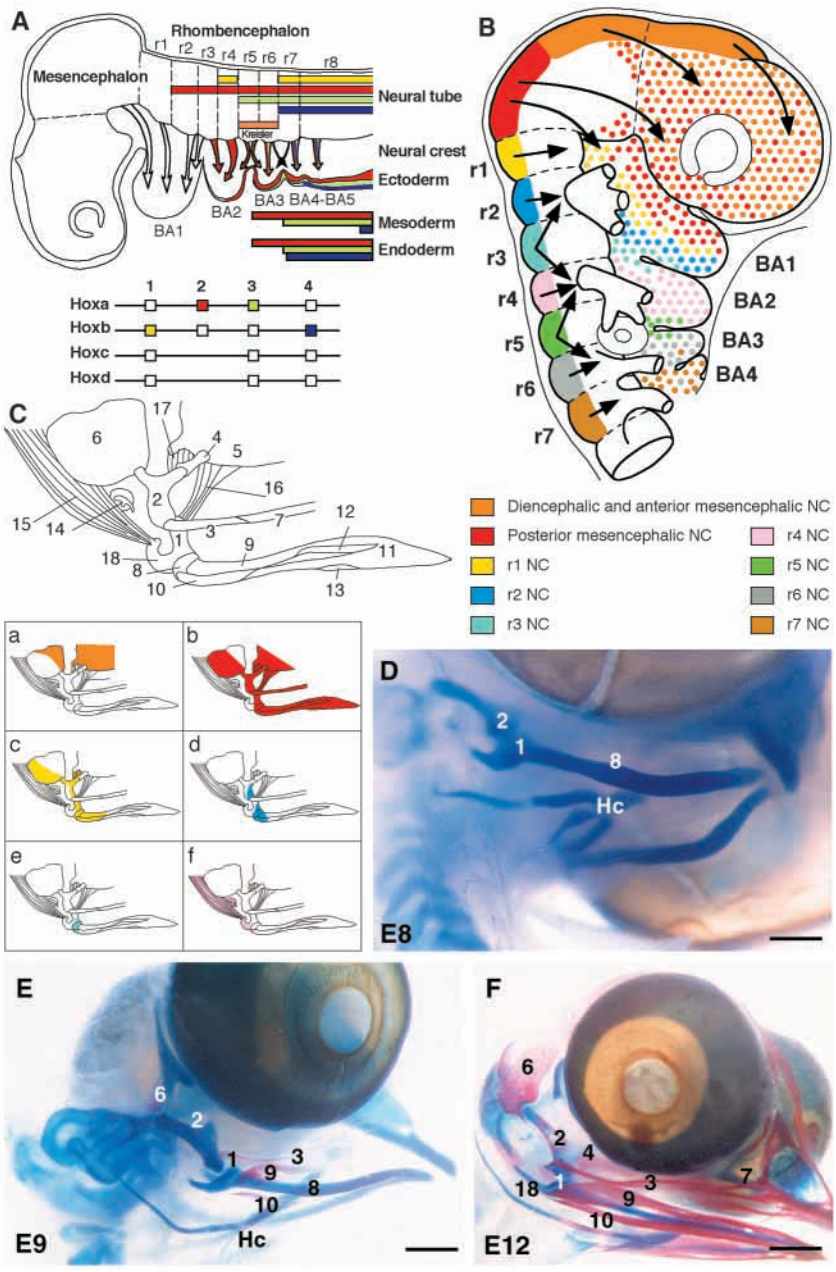

Fig. 1. (A) Hox gene expression in the chick and quail embryo at E3 when the branchial arches (BA) are being colonized by neural crest cells originating from the posterior half of the mesencephalon and the rhombomeres (r1-r8). The arrows indicate the AP origin of the neural crest cells migrating to each BA. Expression of Hox genes is also indicated in the superficial ectoderm, the endoderm and mesoderm. (B) Migration map of cephalic neural crest cells in the avian embryo. The origin of neural crest cells found in the nasofrontal and periocular mass and in the branchial arches is color coded. Anterior mesencephalon contributes to the nasofrontal and periocular mass. Posterior mesencephalon also participates in these structures, but in addition populates the anterodistal part of BA1. The complementary portion of BA1 derives from r1/r2 together with a small contribution from r3. The major contribution to BA2 comes from r4. Neural crest cells arising from r3 and r5 split into strains participating, respectively, in two adjacent arches: r3 cells migrate to BA1 and BA2; r5 cells migrate to BA2 and BA3. r6- and r7-derived cells migrate to BA3 and BA4. (C, a-f) Color-coded (see B) fate map of the neural crest issued from the prosencephalon, mesencephalon and rhombomeres 1-4. The bones, cartilages and muscles of the jaw are numbered in the upper panel. 1, articular; 2, quadrate; 3, quadratojugal; 4, pterygoid; 5, palatine; 6, squamosal; 7, jugal; 8, Meckel's cartilage; 9, supra-angular; 10, angular; 11, dentary; 12 opercular; 13, splenial; 14, columella; 15, depressor mandibulae; 16, pterygoideus; 17, M.pterygoquadrate; 18, retroarticular process. (D-F) The chick facial skeleton at E8, E9.5 and E12 (Hc, hyoid cartilage). Scale bar: 0.8 mm in D; 14 mm in E; 22 mm in F.

respond normally to the local cues and participate in the formation of normal hyoid cartilage while maintaining their Hox-negative status.

Evidence that Hox gene expression is involved in patterning the visceral skeleton has come from experiments carried out in the mouse, where some genes of the anterior paralogue groups were either mutated or misexpressed. Thus, in *Hoxa2*-null mutant mice, the neural crest cells of BA2 behave like their Hox-negative counterpart that populate BA1 and form pieces of the lower jaw skeleton (Rijli et al., 1993; Gendron-Maguire et al., 1993). Further studies have indicated that in normal development, *Hoxa2* acts by inhibiting chondrogenesis in the rostral part of BA2 where no other Hox gene is expressed (Kanzler et al., 1998). This is in line with the deficit in chondrogenesis observed when Hox-positive neural crest cells are transplanted to a Hox-negative domain (Couly et al., 1998). These and other results (Schilling and Kimmel, 1994; Sechrist et al., 1994) support the notion already put forward on the basis of earlier transplantation experiments (Noden, 1983) that the neural crest cells themselves possess the intrinsic information required for determining the identity of the various bones and cartilages that form the facial skeleton.

It has to be emphasized, however, that other tissue components, the endoderm, mesoderm and superficial ectoderm, participate in facial and visceral arch morphogenesis and may influence neural crest development. It has been shown that neural crest cells can differentiate into cartilage only in the presence of pharyngeal endoderm, whereas ectoderm is crucial for membrane bone differentiation from ectomesenchymal cells (Le Douarin, 1982; Takahashi et al., 1991). The paraxial mesoderm, in which a discrete segmentation into somitomeres has been described, exerts an effect on neural crest cell migration and differentiation (Trainor and Krumlauf, 2000). It has recently been shown that the segmental characteristics of the endodermal pharyngeal pouches develop independently of the presence of neural crest cells (Veicht et al., 1999). Moreover, endodermal segmentation depends upon the activity of specific genes. For example, in *vgo* null mutant zebrafish, larval hindbrain segmentation proceeds normally but the endodermal gill slits do not form and the distinct streams of

neural crest cells exiting from the rhombencephalon ventrally fuse and do not form the individual skeletal elements of the viscerocranium (Piotrowski and Nusslein-Volhard, 2000). This suggests that at least part of the information for patterning the face could originate from signals of endodermal origin.

We have further explored the origin of the developmental signals responsible for determining the shape of the individual facial bones, as well as their relative positions. We first established that the neural crest cells that form the bones and the cartilages do not themselves possess all the information necessary for patterning the facial skeleton. We then tested the capacity of the foregut endoderm to specify facial bone identity in the avian neurula by performing ablations or grafts of defined endodermal regions.

MATERIALS AND METHODS

Quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus*) eggs from commercial sources were used throughout this study. Microsurgery was performed on embryos at 5 to 6 somite stages [ss; HH9 (Hamburger and Hamilton, 1951)], corresponding to approximatively 32 hours of incubation in a humidified atmosphere at 38°C.

Excision and grafts of the neural fold in the *Hox*-non expressing domain of the neural crest

Chick embryos were prepared for in ovo surgery as described (Teillet et al., 1998). Concurrently, stage-matched quail embryos were harvested and isolated in sterile PBS. The chick embryos were subjected to the surgical ablation of the bilateral neural folds from the mid-diencephalon down to the presumptive r2/r3 limit (Fig. 2A). In certain experimental series, different fragments of the quail neural fold were transplanted into the chick at the posterior diencephalic level (Fig. 2B-D). The fate map of the cephalic neural primordium established by Grapin-Botton et al. (Grapin-Botton et al., 1995) (Fig. 1A) served as a reference to determine the presumptive level of the various encephalic structures: anterior and posterior diencephalon and mesencephalon and rhombomeres (r) 1 to 8 (Fig. 1). Chimeric embryos were allowed to grow from embryonic day (E) 4 to E8, depending on the experimental series. In another set of experiments, we ablated unilaterally the diencephalic neural fold, leaving the contralateral side unperturbed. The space resulting from the excision was filled with a fragment of equivalent length taken from the quail neural fold at the diencephalic, posterior mesencephalic or rhombencephalic (r4-r6) level (Fig. 2E-G).

Preparation of the endodermal stripes

The experiments were carried out on chick embryos at 5-6 ss in ovo. Superficial ectoderm was incised on the right side of the neural tube from the rostral tip to the prospective level of r3 included and reflected laterally to expose the endoderm and paraxial mesoderm (Fig. 2H-J). Four transverse stripes of endoderm were defined corresponding to the levels of the diencephalon (stripe I), anterior (stripe II) and posterior mesencephalon (stripe III) and metencephalon (stripe IV), according to previous fate maps of the neural primordium (Couly and Le Douarin, 1987; Grapin-Botton et al., 1995). Thus, the pharyngeal endodermal pocket was divided into approximately equal (150 μm width) regions from its rostral end (zone I) to the level underlying the caudal end of r3 (zone IV) (Fig. 2I,J).

Endodermal stripes were cleared of paraxial cephalic mesoderm as completely as possible. However, a few adherent mesodermal cells may be transplanted together with the endodermal layer. In previous experiments (Couly et al., 1992) (G. Couly, unpublished) transplantations or excisions of definite regions of cephalic mesoderm in 5-6 ss chick and quail embryos never perturbed morphogenesis of the facial skeleton. We, thus, assumed that the effects observed after foregut manipulations were attributable to the endoderm (or to the endoderm together with the few mesodermal cells that may still adhere to it). The endodermal stripes that are either excised or grafted in these experiments must include the endodermal floor of the foregut.

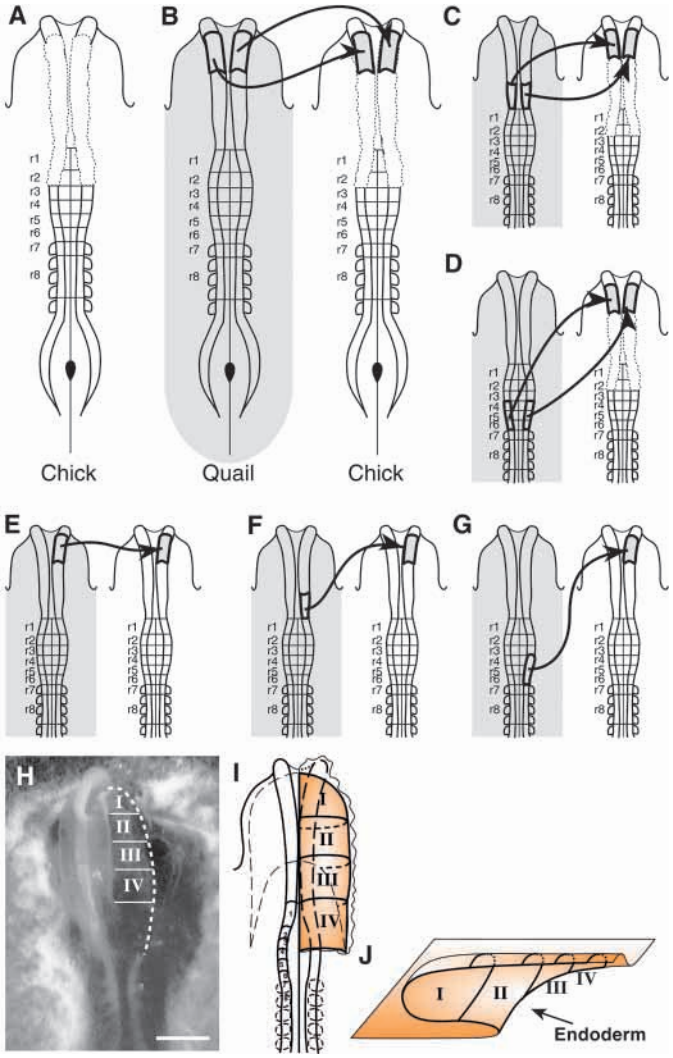

Fig. 2. Schematic representation of the operations performed on avian embryos at five-somite stage. (A) Bilateral extirpation of the cephalic neural crest extending from diencephalic level down to r2/r3 boundary in chick embryo (dotted line). Bilateral implantation of quail posterior diencephalic (B), posterior mesencephalic (C) or rhombencephalic r4-r6 (D) neural folds at the diencephalic level in chick host, after the excision of the endogenous neural fold segments as described above. (E) Unilateral replacement of the posterior diencephalic neural fold in chick by its quail counterpart. Unilateral substitution of the posterior diencephalic neural fold in chick embryo by the quail posterior mesencephalic (F) or rhombencephalic r4-r6 (G) neural folds. (H,I) Four endodermal stripes of equivalent widths, I, II, III and IV are defined for microsurgery at HH9: stripes I and II divide the pharyngeal pocket in two parts, underlying the prosencephalon and anterior mesencephalon, respectively. Stripe III underlies the posterior mesencephalon and IV underlies the first two rhombomeres (metencephalon). In J, a sagittal section of the foregut shows the respective position of the endodermal stripes I to IV. Scale bar: 0.13 mm in H.

Experiments involving only the dorsal aspect of the foregut had no effect on head morphogenesis.

Manipulations of the foregut endoderm

The endodermal stripes already defined were first subjected to unilateral ablation on the right side of the embryo (Fig. 5A,D, Fig. 6A). Then, the ectoderm was replaced and the embryos were further incubated until E6 to E9 and their skeleton was observed. In some cases, embryos were treated at E5 for in situ hybidization.

In a second series, similar stripes of endoderm were removed from 5-6 ss quail embryos and grafted underneath the cephalic ectoderm on one side or under the floor of the endogenous foregut of the stage-matched chick embryos in the migration pathway of the cephalic neural crest cells. Their dorsoventral and anteroposterior (AP) axes were either maintained or inverted (Fig. 7A, Fig. 8A,F,G, Fig. 9A, Fig. 10A,D,G,J). The AP position of the graft with respect to the foregut transverse level of the host varied according to the experimental series being considered (Fig. 11A,C,E). The endogenous chick foregut was untouched in all these experiments.

Analysis of chimeras

The embryos were sacrificed from 3 hours after grafting up to E6 for histology, and at E8, E9 and E12 for whole staining of the skeleton. Embryos were processed for immunocytochemistry with the quail-specific monoclonal antibody (Mab) QCPN (Developmental Studies Hybridoma Bank), according to the method described by Catala et al. (Catala et al., 1996), for whole skeletal stains (Simons and van Horn, 1971), or for whole-mount in situ hybridization with the Pitx1 probe at E5 as described (Henrique et al., 1995) and on sections with the chicken *Hoxa2* (Etchevers et al., 2001). Chick *Hoxa2* was linearized with *Hin*dIII and transcribed using T3 (Prince and Lumsden, 1994). Chick *PitxI* was linearized with *Eco*R1 and transcribed with T7 (Lanctôt et al., 1997).

RESULTS

The Hox-negative neural crest possesses the exclusive capacity to generate the facial skeleton but does not carry the information to pattern it

Previous experiments based on the construction of quail/chick chimeras have shown that the facial skeleton is derived from neural crest cells originating from the posterior diencephalic, mesencephalic and anterior rhombencephalic (r1, r2) neural folds (Couly et al., 1996; Köntges and Lumsden, 1996) (Fig. 1). Moreover, ablation of fragments of the neural fold in the avian neurula was shown to be followed by a regulation originating from the anterior and posterior neural crest cells left in situ (Couly et al., 1996). We have explored the capacity of the rhombencephalic neural folds from the Hox gene expressing domain of the neural axis to replace cells of the Hox-negative domain and to generate facial bones. For this purpose, the bilateral extirpation of the neural folds from the presumptive level of the mid-diencephalon down to the r2/r3 limit was performed in 5-6 ss chick embryos: this operation was followed by a high level of mortality. The twelve (out of 104 operated) embryos that survived until E8, were smaller

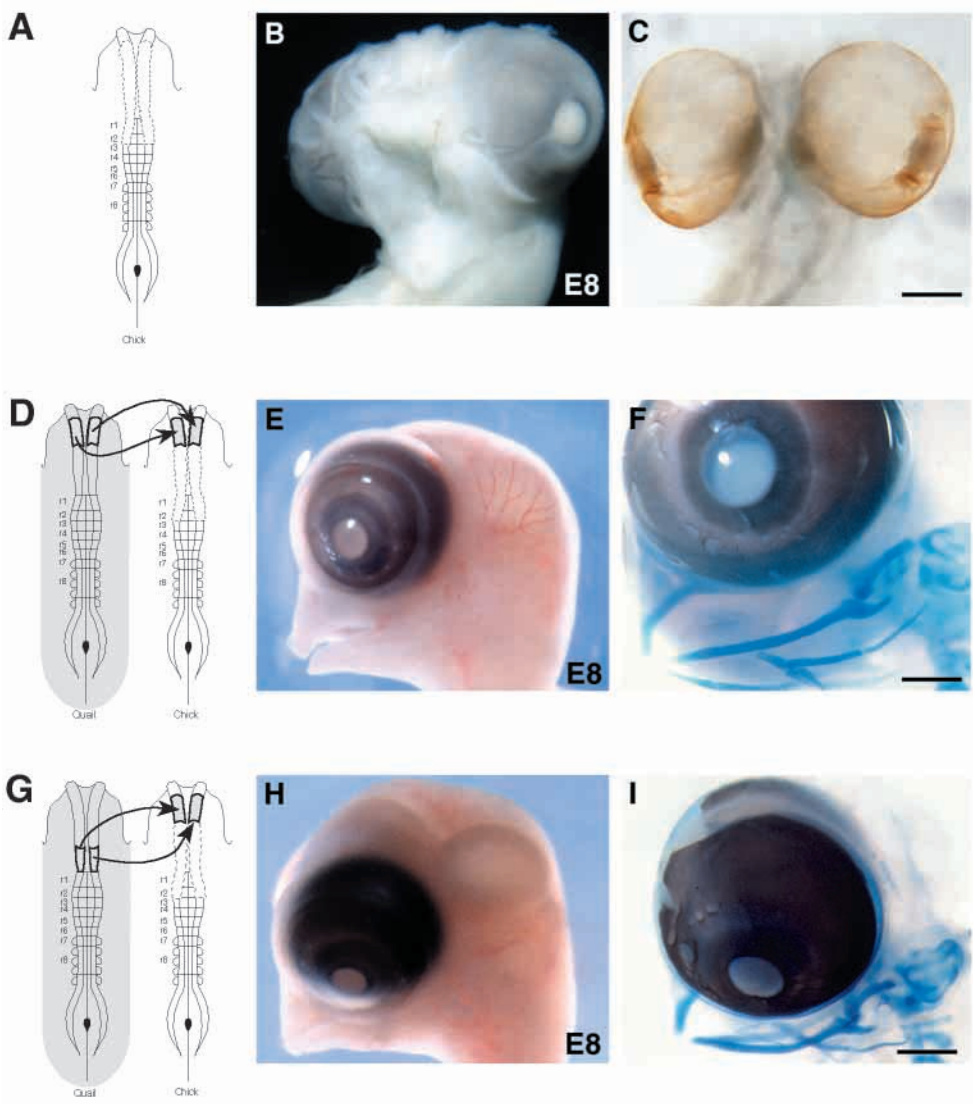

Fig. 3. Capacity of the Hox-negative neural crest to generate the facial skeleton. The bilateral extirpation of the neural folds from presumptive level of the mid-diencephalon down to the r2/r3 limit (dotted line) performed in five-somite stage chick embryos (A) leads to the absence of nasofrontal and maxillo-mandibular buds (B). These embryos fail to develop facial and mandibular skeleton (C). In embryos that have been subjected to the complete cephalic neural fold excision, the orthotopic bilateral graft of the posterior diencephalic neural fold from a stage-matched quail embryo (D) restores the normal development of the head (E) and generates a complete skeleton of the upper face and lower jaw (F). According to the same paradigm, the bilateral transposition of the posterior mesencephalic neural folds to diencephalic level (G) ensures normal development of the face (H) and of its skeletal structures (I). Scale bar: 1.5 mm in C; 1.1 mm in F; 1.5 mm in I.

than the unoperated controls and lacked the facial structures normally visible at that stage, i.e. the nasofrontal and maxillo-mandibular buds (Fig. 3A-C).

In the next series of experiments, excision of the same neural fold segments was followed by the orthotopic bilateral graft of the posterior diencephalic neural fold from a stage-matched quail embryo. The chimeric embryos examined at E8 (n=4) exhibited normal development of their skeletal structures and facial morphology (Fig. 3D-F). Histological examination of the tissues after immunostaining with QCPN Mab revealed that the neural crest derived structures were exclusively made up of quail cells that had migrated from the implanted diencephalic neural folds (not shown). Thus, the diencephalic neural folds alone can generate enough neural crest cells to form the complete facial skeleton and head connective tissue normally derived from the diencephalic, mesencephalic and anterior rhombencephalic (r1, r2) crest. The same result was obtained when the posterior mesencephalic neural folds were implanted at the diencephalic level after complete removal of the crest down to r3 (Fig. 3G-I). In previous experiments (Couly et al., 1998), the mesencephalic neural folds grafted to r4-r6 level were also found to be able to replace the excised Hox-negative domain of the neural crest, thus showing that the level of the neural axis at which the competent neural fold fragments (of about 150 μm length) are implanted is not crucial [Fig. 5F,G by Couly et al. (Couly et al., 1998)]. By contrast, in the same experiments r4-r6 neural folds failed to generate a facial skeleton [Fig. 4G by Couly et al. (Couly et al., 1998)]. Therefore, the capacity of a 150 μm fragment of the neural fold from the non Hox-expressing domain to generate a complete facial skeleton depends neither upon its level of origin along the AP axis nor its position after grafting.

The fate of neural crest cells from either the diencephalic or mesencephalic levels was strikingly different when the quail neural folds were implanted at the diencephalic level into a chick embryo that had not been subjected to the complete cephalic neural fold excision as before. After orthotopic substitution of the posterior diencephalic neural fold by its quail counterpart, quail cells could be seen around the optic vesicles, and participating in the ciliary muscles, the corneal endothelium, the sclerotic and the cartilages of the nasal septum (Fig. 4A-D). They were also found as pericytes

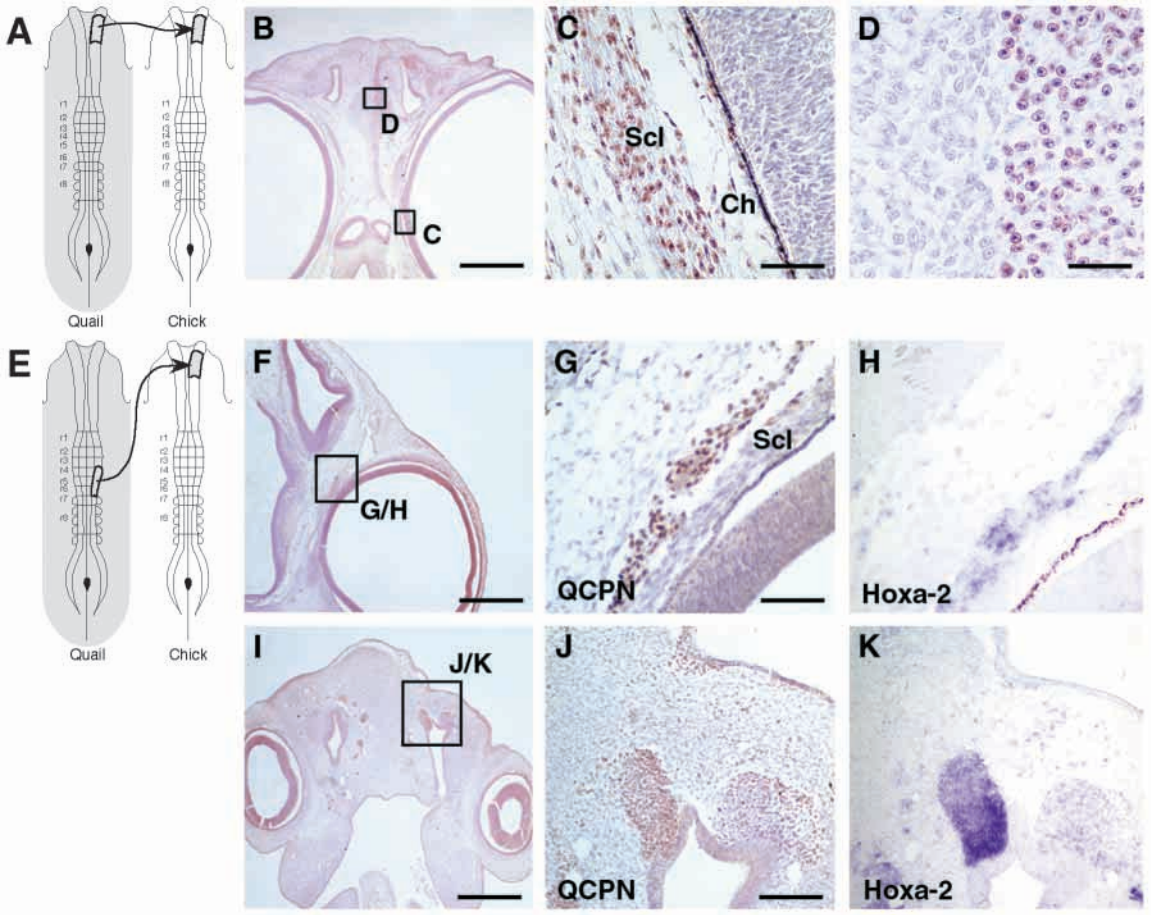

Fig. 4. The fate of posterior diencephalic and rhombencephalic neural crest cells transposed into the diencephalic level. Diencephalic crest cells of quail origin orthotopically grafted in chick (A) spread into the perioptic area (B,C) where they contribute to the sclerotic cartilage (Scl) and to the choroid membrane (Ch). (D) In the nasal region, they form the ipsilateral half of the nasal septum (quail cells are recognizable by their immunostaining with the QCPN MAb). The contralateral half is made up of chick host chondrocytes. (E) Quail rhombencephalic r4-r6 neural fold unilaterally substituted to the host diencephalic neural fold yields crest cells that invade the nasofrontal area. In the perioptic region of the chimeric embryos (F), quail cells fail to take part into the sclerotic (Scl) which is made up of endogenous chick cells (G). In the nasal region (I), quail cells do not contribute to the nasal septum but form aggregates distributed around the olfactory placode (J). On adjacent sections, quail cells contain *Hoxa2* transcript (H,K). Scale bar: 840 μm in B; 80 μm in C; 75 μm in D; 675 μm in F; 120 μm in G; 690 μm in I; 130 μm in J.

associated with blood vessels in the prosencephalic meninges and in the choroid membrane. This wide distribution of quail cells was regularly observed in all the embryos (*n*=6) examined at E6 and E8. It reflects the normal fate of the diencephalic mesectodermal neural crest cells.

When the same experiment was performed, using quail mesencephalic neural folds transplanted to the diencephalic level, the grafted neural crest cells contributed to the same structures as the diencephalic derived crest cells, as observed at E4 (*n*=3), E5 (*n*=2), E7 (*n*=2) and E8 (*n*=3).

The posterior rhombencephalic (r4-r6) neural fold from 5-6 ss quail implanted at the same diencephalic level into stage-matched chicks yielded neural crest cells that colonized the anterior cephalic area where they contributed to the prosencephalic meninges and pericytes. However, they did not participate in the sclerotic that was exclusively composed of chick host cells originating from the mesencephalon (Fig. 4E-H). These embryos were observed at E4 (*n*=3) and E5 (*n*=2). At later stages (E6, *n*=5; E8, *n*=8) quail cells were found lining peripheral nerves and forming the connective component of ocular muscles; these quail cells contained *Hoxa2* transcripts, showing that they had maintained their original molecular identity while migrating within an environment where Hox genes are not activated (Fig. 4H,K). In the nasal bud, some quail cells formed abnormally dense aggregates but did not participate in the cartilage of the nasal septum that developed, by compensation, from endogenous neural crest of more posterior origin (Fig. 4I-K).

In conclusion, the cartilages and bones of the face originate from the anterior domain of the neural crest in which the genes of the Hox-clusters are not activated. After complete ablation of this domain, the facial skeleton fails to develop, owing to the incapability of the neural crest cells of more posterior origin to replace them. The neural folds of the anterior Hox-negative domain possess, by contrast, a high capacity of regulation: a 150 μm long fragment of the neural fold (corresponding to about a quarter of the total length of the neural folds that normally generate the facial skeleton) is sufficient to ensure the normal development of the head. Under the experimental conditions just described, the territory extending from the diencephalon down to r3 can be considered as an ‘equivalence group’ in which each fragment of the neural fold is endowed with a virtually similar development potential.

These results indicate that the information for the specific morphogenesis of the facial bones is not carried by the neural crest cells themselves, as previously suggested (Noden, 1983). We then investigated the possible role of the foregut endoderm in this process. In a first step, transverse stripes of foregut endoderm were removed and the consequences of this operation on the facial skeleton were recorded.

Ablation of endodermal stripes of foregut endoderm

Stripe I

Ablation of stripe I was performed on the right side of 14 embryos, of which eight could be recovered at E8. Four were anatomically normal. Four had an abnormal nasal capsule in which the nasal septum was deflected to the left side (not shown). In the next series, stripe I was removed on both sides. This resulted in the strong reduction (or absence) of the nasal bud and later on of the nasal capsule, nasal septum and upper beak (*n*=5) (Fig. 5A-C).

Stripe II

Ablation of stripe II was performed on 70 embryos (Fig. 5D); 12 survived until E8-E9, in all of which Meckel’s cartilage was missing in the first branchial arch on the operated side. In one out of 12, part of the articular cartilage was also missing (Fig. 5E,F).

One control embryo was sacrificed at E5 for whole-mount in situ hybridization with the *Pitx1* probe. *Pitx1* is strongly expressed by the stomodeal ectoderm and the mesenchymal

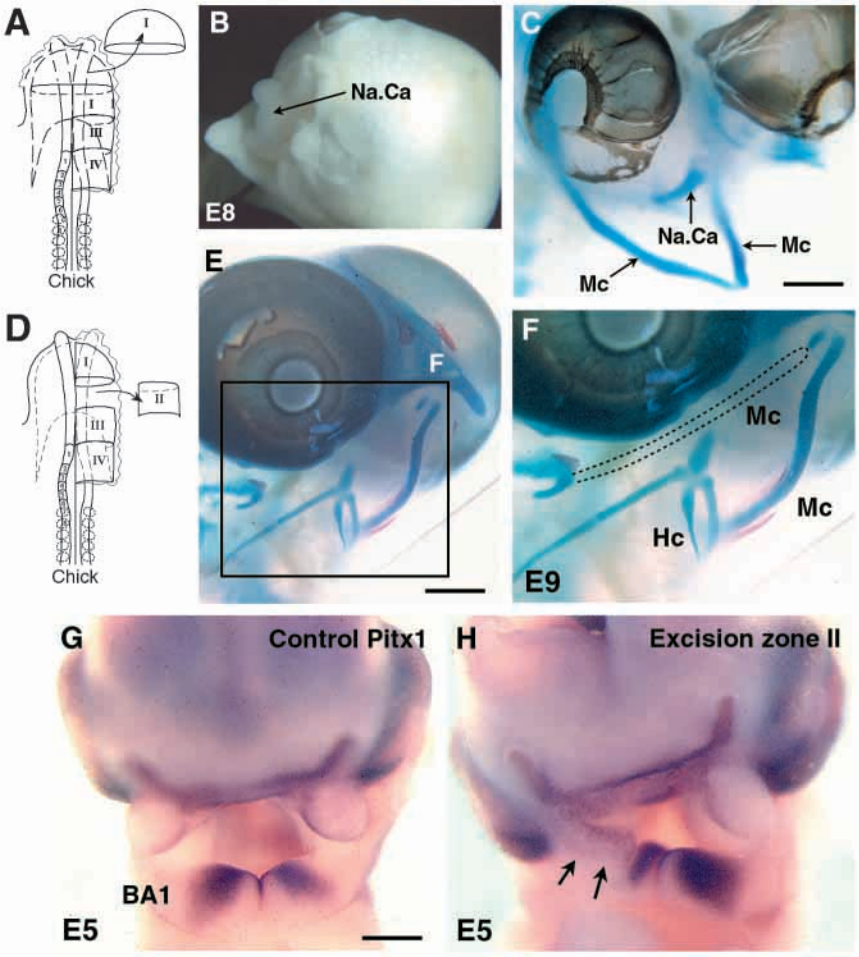

Fig. 5. Removal of foregut endoderm prevents facial bone development. (A-C) Bilateral removal of stripe I generates the near-complete absence of the cartilaginous nasal capsule (Na. Ca) and associated membrane bones at E8. Unilateral ablation of stripe II (D) leads to the absence of Meckel’s cartilage (Mc) and associated membrane bones (E,F), on the side of the operation at E9. Hyoid cartilage (Hc) is not affected. At E5 the normal gene expression of *Pitx1* in BA1 (G) is affected after excision of zone II on the right side (arrows) (H). Scale bar: 1.5 mm in C; 2.8 mm in E; 0.4 mm in G.

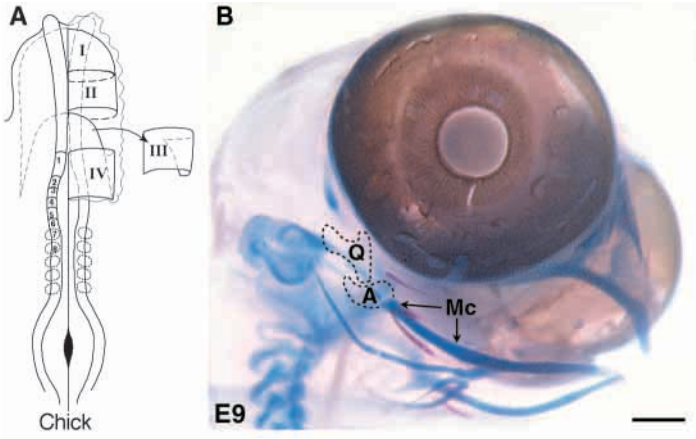

Fig. 6. Unilateral ablation of stripe III (A). This operation leads to the absence of the articular (A) and quadrate (Q) bones and associated membrane bones at E9 (B). Scale bar: 1.1 mm in B.

cells of the proximal part of the BA1 at E5 (Fig. 5G). In the embryo subjected to removal of stripe II endoderm on the right side, *Pitx1* was normally expressed in the left branchial arch. By contrast, *Pitx1* expression was strongly reduced on the operated side while the corresponding BA1 was underdeveloped (Fig. 5H). The remaining small distal zone of *Pitx1* expression may correspond to the presence of a distal short piece of Meckel's cartilage observed in most of the Alcian Blue stained embryos that were subjected to this operation (see Fig. 5E,F).

Stripe III

Ablation of stripe III was performed on 20 embryos. Five embryos were observed at E9. All were devoid of the articular, the quadrate and the proximal part of Meckel's cartilages (Fig. 6A,B). Ablation of stripes II and III together (n=3) resulted in the total absence of the entire skeleton of the first branchial arch (not shown).

Stripe IV

Ablation of stripe IV was performed on 12 embryos; five were observed at E8 and showed a strong reduction of the articular and quadrate cartilages (not shown).

In conclusion, removal of transverse stripes of endoderm from the extreme tip of the foregut (corresponding to the level of the prosencephalon) did not perturb the development of the jaw but affected that of the nasal capsule and upper beak. Ablation of the endoderm of zone II, III and IV, corresponding (respectively) to the levels of the mesencephalon (II, III) and of r1-2 (IV) prevented the morphogenesis of Meckel's (zone II and III) and of articular and quadrate cartilages (zone III-IV). Moreover, when cartilaginous bones failed to develop, nearby membrane bones, which normally ossify later directly from the neural crest mesenchyme of the nasofrontal bud or of BA1, did not form either. Thus, in the absence of the nasal septum, the other bones of the nasal capsule are missing. Without the quadrate, articular and Meckel's cartilages, the squamosal, pterygoid, quadratojugal, angular, supra-angular, opercular and dentary bones of the jaw did not appear.

The absence of skeletal structures after ablation of defined regions of the endoderm could have been due either to mechanical prevention of neural crest cell migration or to the absence of a specifically localized patterning effect of the endoderm on the ectomesenchyme. To clarify this, we implanted defined regions of 5 ss quail foregut endoderm into the head of stage-matched chicken embryos. In such chimeras, donor cells can be identified immunohistologically with the quail-specific QCPN Mab. In these chimeras, migrating cephalic neural crest cells, exiting from the Hox-negative domain of the encephalic vesicles, are subjected to the influence of ectopic pieces of endoderm, even though the host endoderm is left intact.

Ectopic grafts of defined foregut endodermal areas in the migration pathway of cephalic neural crest cells induce the formation of specific cartilages and bones

Grafts of stripe II quail endoderm were made laterally to the mesencephalon of chicken hosts, thus overlying their endogenous counterpart (Fig. 7A). At E3, the grafts formed vesicles embedded within the host neural crest-derived mesenchyme of BA1 (Fig. 7B). At E5, in situ hybridization showed that *Pitx1*, usually expressed only within the stomodeal ectoderm and mesenchyme of BA1 (Lanctôt et al., 1997), was reproducibly induced by the stripe II endodermal grafts in an ectopic, subocular position (Fig. 7C, n=3). Consistent with the assumption that BA1 identity is specified in the mesenchyme surrounding the endodermal graft, a supernumerary Meckel's cartilage formed near the eye, as seen at E8 (n=2), E9 (n=3)

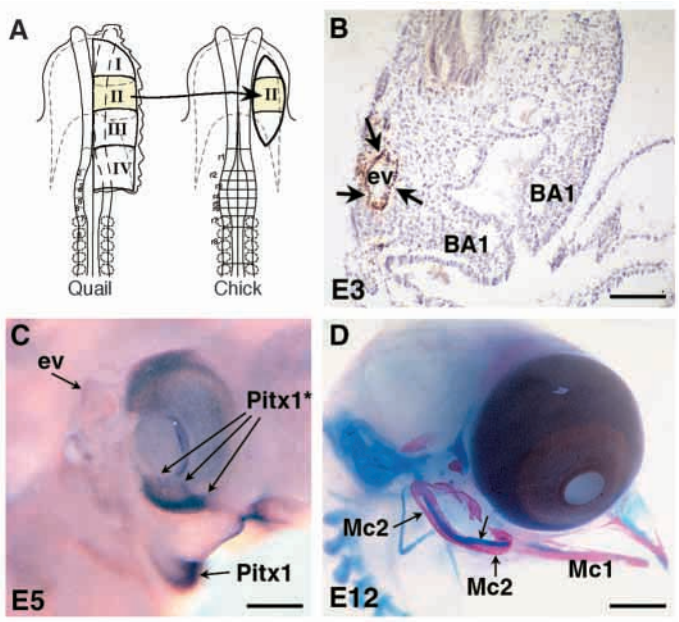

Fig. 7. Grafts of quail foregut endoderm in the head induce supernumerary skeletal elements in the chicken embryo. Stripe II quail endoderm grafted into a stage-matched HH9 chicken embryo (A) is visible at E3 (B) as a vesicle (ev, arrows) lateral to BA1. (C) *Pitx*1 is expressed at E5 in subocular mesenchyme (asterisk) above its endogenous expression in distal BA1 and oral ectoderm. (D) At E12, a supernumerary Meckel's cartilage (Mc2) and corresponding membrane bones (red) have developed laterally to the endogenous lower jaw skeleton (Mc1). Scale bar: 115 μm in B; 0.3 mm in C; 2.2 mm in D.

and E12 (*n*=1). These ectopic cartilages, examined at E6 in two chimeras, developed from host neural crest cells (not shown). The corresponding supernumerary membrane bones of the lower jaw also formed (Fig. 7D).

A bilateral stripe II of quail endoderm was implanted underneath and around endogenous stripes II (Fig. 8A-C) (*n*=5). An ectopic lower beak was induced in the host (Fig. 8D). It contained not only the two Meckel's cartilages but extra lower jaw membrane bones that differentiated in contact to the supernumerary cartilages (Fig. 8E). If the stripe II endodermal fragments were implanted lateral to the endogenous foregut and neural tube [as represented in Fig. 8F,G (*n*=1)], an extra lower beak with the corresponding skeleton and the two part of entoglossum developed hanging lateral to the lower beak of the host (Fig. 8H,I).

Likewise, a graft of ectopic stripe III endoderm in addition to its host counterpart (Fig. 9A) led to the formation of vesicles of graft origin lateral to the mesencephalon by E3 and the condensation of chick host-origin quadrate cartilage around the graft at E6 (Fig. 9B,C). A supernumerary membranous squamosal bone was associated with the ectopic quadrate at E9 (Fig. 9D,E). Thus, precise regions of the pharyngeal endoderm instruct neural crest cells in BA1 to become the cartilaginous bones of the jaws, and the patterning of adjacent membranous bones. These cartilages are necessary for the subsequent patterning of adjacent membranous bones. The instruction given by ectopic endodermal grafts, placed in the migratory path of BA1 neural crest cells, supersedes that of other local signals for differentiation and patterning.

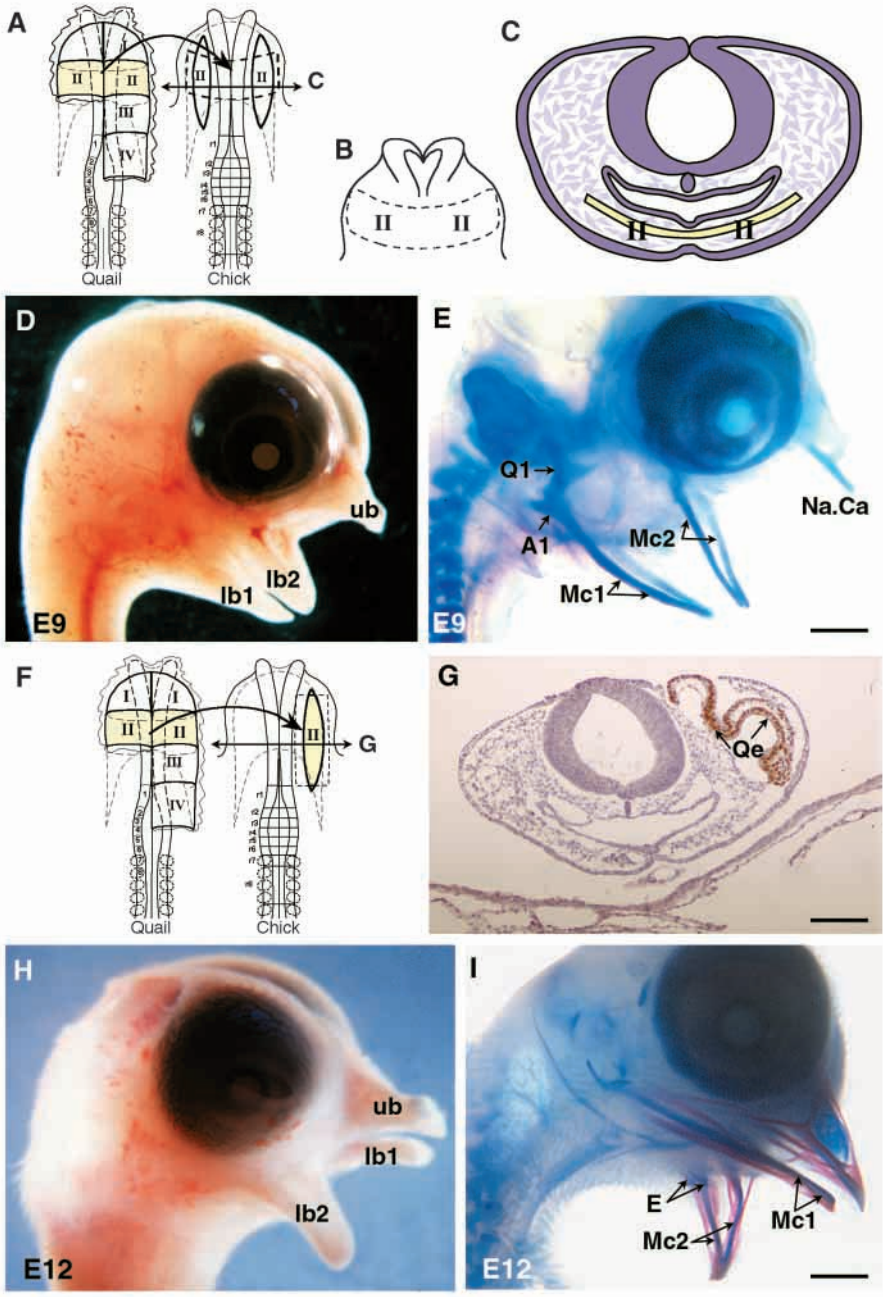

Fig. 8. Grafts of bilateral quail foregut stripes duplicate lower jaw skeleton. Two stripes of zone II quail ventral endoderm are positioned as represented in A (dorsal view), B (ventral view) and C (transverse section). (D) This graft leads to the formation of a supernumerary lower beak (lb2) at E9 between the host upper (ub) and lower beaks (lb2). (E) The two supernumerary Meckel's cartilages (Mc2) of lb2 at E9 are positioned between the nasal capsule (Na. Ca) and the host BA1 skeleton (Mc1, Meckel's cartilage; A1, articular; Q1, quadrate). (F) A similar double graft of stripes II is positioned laterally under the ectoderm (Qe, quail endoderm; arrows) 6 hours after the operation (G). This operation leads to the appearance of a supernumerary lower beak (lb2 in H at E12) with two Meckel's cartilages (Mc2 in I) and corresponding membrane bones; the two entoglossum (E) are present under and lateral to the host Meckel's cartilages (Mc1). Scale bar: 1.6 mm in E; 125 μm in G; 2 mm in I.

Facial bone orientation relative to the embryonic axes also depends on an endodermally derived information

When a piece of quail endoderm, including both stripes II and III was implanted above the chicken stripes II and III in its normal AP and mediolateral (ML) orientation (Fig. 10A), a supernumerary lower jaw developed above that of the host, as expected (Fig. 10B,C, Fig. 8). Rostrocaudal inversion of the graft (Fig. 10D) gave rise to a duplicate lower jaw developing toward the back of the head (Fig. 10E,F). Meckel's cartilage resulting from a 90º rotation of the endodermal graft to the left (anterior becoming medial, posterior becoming lateral, Fig. 10G), pointed toward the top of the head, at the expense of the ipsilateral eye (Fig. 10H,I). After a 90º rotation of the graft to the right (Fig. 10J), the induced jaw was oriented caudally (Fig. 10K,L). The rostrocaudal position of the early foregut endoderm underlying BA1 therefore translated into the orientation of the distoproximal axis of the jaw.

The foregut endoderm patterning cues influence only the non-*Hox*-expressing cephalic neural crest cells

As mentioned before, two distinct populations of neural crest cells differentiate into the components of the facial and visceral skull. A rostral, Hox-negative population gives rise to all of the membrane bones of the brain case, face and jaws, as well as the cartilaginous bones of the face; a caudal, Hox-positive population yields the hyoid cartilage (except the entoglossum) (Couly et al., 1996). To test whether the endoderm involved in patterning the Hox-negative neural crest can influence the Hox-expressing, chondrogenic rhombencephalic crest cells, foregut stripes II (Fig. 11A) or III from quail embryo were implanted adjacent to r7-r8 of 5 ss chick recipients. Small nodules of cartilage of chick origin formed around the grafts (n=5) in the host cervical region by E9 (Fig. 11B). However, when the quail anterior mesencephalic neural fold accompanied the endodermal graft (Fig. 11C,E), recognizable cartilages and bones rudiments could be observed at E9. Although reduced in size if compared with their normal endogenous counterpart, Meckel's, articular and quadrate cartilages, and some associated membrane bones could be identified at the level of the upper neck (Fig. 11D,F,G; n=3). Note that the shape and size of the skeletal pieces that form in these ectopic locations are as close to their normal counterpart as when the grafts are placed in the facial region (c.f. the experiments described above).

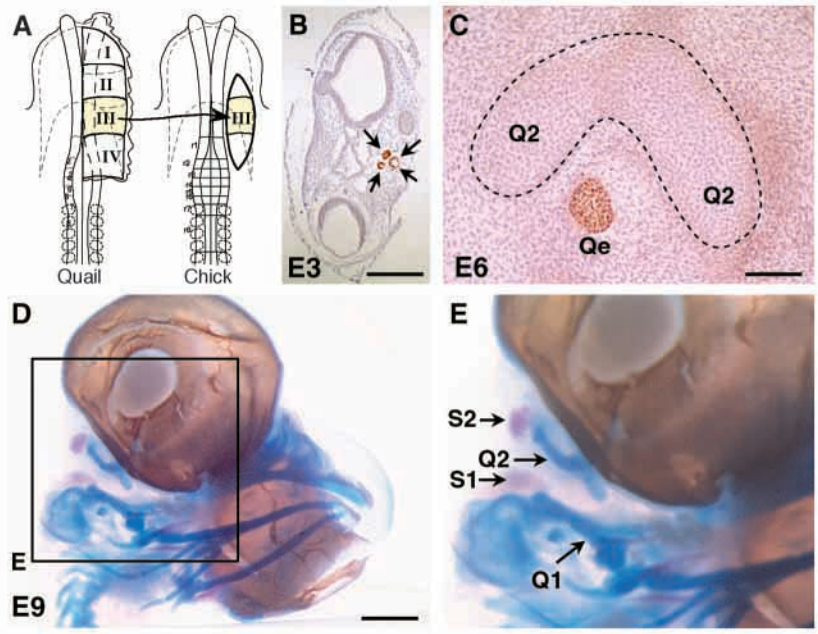

Fig. 9. Graft of stripe III endoderm. (A) A graft of quail stripe III endoderm to the chick head results in (B) the presence of vesicles of grafted endoderm at E3 (arrows). (C) Supernumerary quadrate cartilage (Q2) has formed around the grafted foregut quail vesicle (Qe) by E6. (D,E) At E9, a supernumerary quadrate cartilage (Q2) and squamosal membrane bone (S2) form in a subocular position close to their endogenous counterparts (S1,Q1). Scale bar: 300 μm in B; 125 μm in C; 1.28 mm in D.

Interestingly, supernumerary bones were strictly of quail origin (not shown) and therefore constructed by the grafted non-Hox-expressing neural crest cells. Supernumerary stripe III endoderm, grafted with a posterior mesencephalic neural fold from the same quail donor, induced quadrate (and squamosal) formation in the neck from donor neural crest cells (Fig. 11E-G). Localized information for constructing specific facial skeletal components hence resides in the anterior pharyngeal endoderm. However, only the 'equivalence group' of cephalic neural crest cells that do not express Hox genes can interpret this information.

DISCUSSION

The foregut endoderm exerts a patterning activity on skeletogenic neural crest cells of the anterior *Hox*-negative domain

The experiments reported above demonstrate that the foregut endoderm of the avian neurula displays a regional activity essential in specifying the identity and orientation of the neural crest-derived bones forming the vertebrate facial skeleton.

The anteriormost region of the endoderm, although fated to form Sessel's pouch and thus to degenerate, is crucial for specification of the nasal septum and later the nasal capsule, the ethmoid bone, and the upper beak. Stripes of endoderm corresponding to the transverse level of the mesencephalon (stripe II and III) and metencephalon (stripe IV) are necessary for the morphogenesis of the bones derived from the maxillary and mandibular buds forming the first branchial arch (Table 1). The foregut endoderm patterning capacities lie in the lateroventral aspect of the foregut endodermal layer. Thus, the size, shape and orientation of the cartilages forming in the facial anlage of the vertebrate embryo seem to be prefeatured in the endoderm of the early head primordium. Although much progress has been made in the understanding of the mechanisms that underlie cell differentiation, the questions raised by morphogenesis have remained largely unaddressed and the fact, disclosed here, that a definite region of foregut endoderm precisely specifies the shape and orientation (with respect to body coordinates) of every given bone of the face is striking.

Another key point resulting from the present work is that, whatever the nature of the endoderm-derived signals, they trigger a response only from the anterior domain of the neural crest in which no genes of the Hox clusters are expressed. More

Table 1. Effect of removal of endodermal stripes on the development of the facial skeleton

	Level on AP axis	
Absence of facial cartilages	Foregut endoderm	Encephalic primordium
Nasal capsule Upper beak	Zone 1	Prosencephalon
Lower jaw		
Meckel's cartilage →	Zone 2	Mesencephalon
Articular →	Zone 3	Mesencephalon
Quadrate ⇢	Zone 4	Rhombomeres 1 and 2

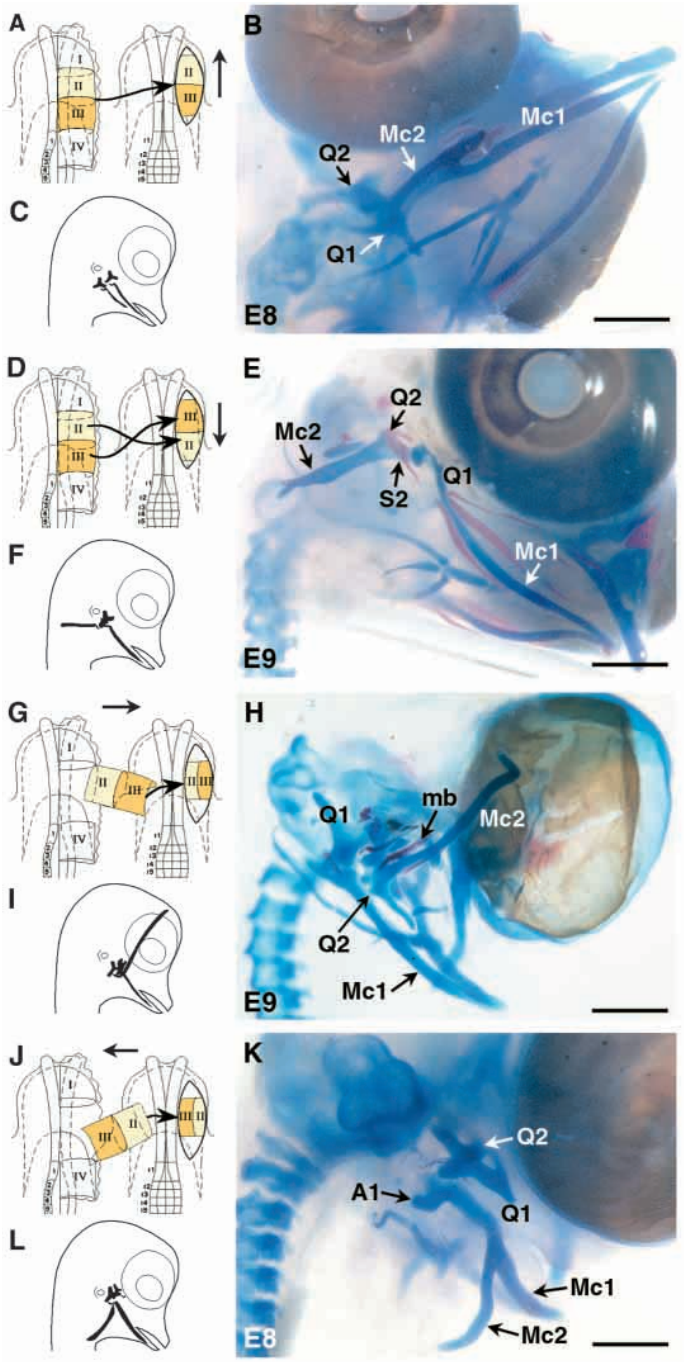

Fig. 10. Foregut endoderm determines facial bone orientation. Quail stripes of zones II and III endoderm are grafted together into the chick head in 4 orientations relative to their endogenous counterpart: (A) the normal AP and ML orientations, yielding (B) supernumerary BA1 skeletal components (Mc2, Q2) of which the distal end is appropriately directed as represented schematically in C. (D) The inverted AP and ML orientations, yielding (E) supernumerary cartilages and bones (Mc2, Q2, S2), as schematically represented in F. (G) After a 90° rotation from anterior to medial, yielding (H) the formation of a lower jaw skeleton (Mc2, Q2, mb: membrane bones) similarly orientated rostrally, as represented in I. After a 90° rotation from anterior to lateral, yielding (K) a lower jaw skeleton directed caudally (see L). Mc1, Meckel's cartilage; A1, articular; Q1, quadrate. Scale bar: 1.3 mm in B; 2.0 mm in F; 1.9 mm in H; 1.5 mm in K.

posterior Hox-expressing neural crest cells are unable to generate facial bones when they are transplanted into a Hox-negative environment. As shown before (Couly et al., 1998), fragments of neural fold heterotopically transplanted along the AP axis maintain their Hox code, while yielding crest cells that remain closely associated in large groups. Thus, when Hox-negative neural crest cells are transferred to the level of BA3-BA4, they participate in the formation of the hyoid cartilages without being induced to express genes of the Hox clusters. Inversely, posterior Hox-positive neural crest cells transplanted anteriorly follow the migration routes taken by the endogenous neural crest arising from the same level and continue to express their specific Hox code, although they have reached ectopic locations. However, in this position, they are unable to generate the skeletal structures specific to their novel environment. It is interesting to note that experiments of the same type performed in the mouse have revealed that maintenance of Hox code in transposed neural crest cells seem to depend upon a community effect. If posterior cells are transferred anteriorly in small number, they may lose their Hox gene expression when mixed up with the endogenous Hox-negative cells. The eventual fate of such cells (i.e. whether they die or differentiate according to their novel position) is not known, as the embryos could not be cultured long enough to be examined at the appropriate stage (Trainor and Krumlauf, 2000).

We have also investigated the possible patterning capabilities of the other – non neural crest and non endoderm-derived constituents of the branchial arches. By using a similar microsurgical approach, defined fragments of the ectoderm covering the cephalic region of the five-somite stage avian neurula, which correspond to previously defined 'segments' called ectomeres (Couly and Le Douarin, 1990) were either removed or ectopically transplanted. In no case did these experiments perturb the development of the facial skeleton (G. Couly, unpublished). Similar investigations carried out with the cephalic paraxial mesoderm showed that, at these early stages, the various AP regions of the cephalic mesoderm (Couly et al., 1992) are similarly interchangeable. Thus, in strong contrast to the foregut endoderm, neither the ectoderm nor the mesoderm of the early neurula display patterning properties on the skeletogenic process that leads to the formation of the facial bones (G. Couly, unpublished).

The cells of the *Hox*-negative domain of the cephalic neural crest behave as an equivalence group

In the Hox-negative domain, the cephalic neural crest cells exhibit a large range of plasticity:

(1) their proliferative capacity can be considerably increased in certain of the experimental situations described above. For example, if a large part (three quarters) of the neural fold that normally participates in head morphogenesis is removed, the remaining segment that is left in situ can regulate the deficiencies caused by the operation and construct the complete skeletal and connective tissue components of a normal head

(2) individual areas can be identified in the diencephalic, mesencephalic and metencephalic neural crest that are normally devoted to participate in a definite set of skeletal structures. Nevertheless this regionalization does not correspond to a strict determination of the cells to a given fate. Limited territories of the anterior Hox-negative neural fold have the capacity to regenerate the whole cephalic neural crest

and to yield the entire facial skeleton. Thus, although the anterior domain of the neural crest exhibits a certain degree of specificity in being the only one able to form the facial skeleton, it does not contain the information required to specify each of its constitutive bones and cartilages.

The fact that *Hoxa2*$^{-/-}$ mice crest cells originating from r4 and colonizing BA2 form first arch structures shows that, even in this posterior region, the cephalic neural crest can display the same developmental capabilities as their more anterior counterpart (provided they do not activate *Hoxa2*). This may also imply that the pharyngeal endoderm of the second branchial pouch is as able, like that of the first one, to specify bones of the lower jaw if it is in contact with *Hoxa2*-negative neural crest cells. The fate map of the foregut endoderm and the determination of the spatial areas participating in each branchial pouch in the pharynx has not yet been established. The exact contribution of zones II to IV to pharyngeal pouches at later stages, when facial morphogenesis is under way, is not yet known, but work is being carried out to clarify this.

The skeletogenic capacities of neural crest cells depend upon *Hox* gene expression not only in neural crest cells but also in the other cellular constituents of the branchial arches

The experiments described in this and other articles (Gendron-Maguire et al., 1993; Rijli et al., 1993; Couly et al., 1998; Kanzler et al., 1998; Pasqualetti et al., 2000) indicate that the effect of Hox-selector genes on the morphogenesis of neural crest derivatives involves cellular interactions between the neural crest cells and the environment within which they differentiate. This is particularly clear for the duplication of the lower jaw, as it can be obtained not only by the inactivation of *Hoxa2* in the mouse but also by the anteroposterior transfer of the mesencephalic neural crest to the BA2 level (Noden, 1983; Couly et al., 1998). By contrast, if Hox-negative neural crest cells are transferred to the levels of BA3 or BA4, formation of an ectopic lower jaw does not ensue. However, formation of BA1 specific cartilages can be obtained even in this posterior location, provided that *Hox*-negative neural crest cells are transferred together with this appropriate region of foregut endoderm (see Fig. 11). It is characteristic that BA2 endoderm does not express *Hoxa2* or any other Hox gene, whereas that of BA3 and BA4 do. It thus appears that the lower jaw skeleton can develop only if *Hoxa2* remains unactivated in both the neural crest cells and their immediate environment. By contrast, forced expression of *Hoxa2* in *Xenopus* embryo induces the homeotic transformation of BA1 into BA2 skeleton (Pasqualetti et al., 2000). In such a case, not only BA1 neural crest cells but also the surrounding tissues, including the endoderm, do express *Hoxa2* from a stage preceding crest cell emigration up to complete facial morphogenesis. This experiment supports the notion that *Hoxa2* gene expression is not compatible with facial skeleton development. Similarly, in the chick embryo, homeotic transformation of BA1 into BA2 structures (although at a more discrete level than in *Xenopus*) could be obtained when the constitutive tissues of BA1 were all subjected to *Hoxa2* overexpression. Thus, a bifurcation of tongue skeleton could be seen and was assumed to arise form modified BA1

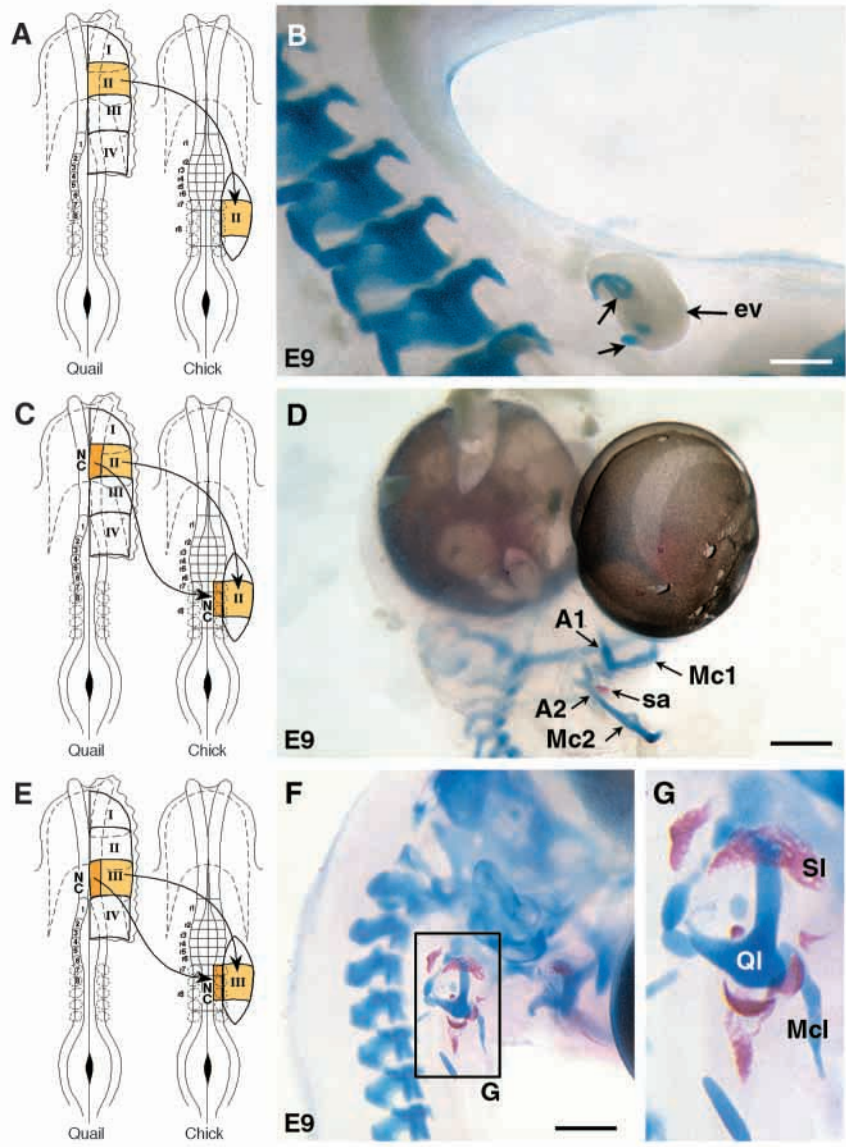

Fig. 11. Only Hox-negative chondrogenic neural crest is patterned by BA1 endoderm. (A) Graft of quail stripe II endoderm adjacent to rhombemeres 7/8 of HH9 chick leads to (B) formation of cartilaginous nodes (arrows) around the graft by E9. ev, endodermal vesicle; anterior towards top. (C) The same endodermal graft associated with a graft of quail mesencephalic neural fold yields (D) supernumerary Meckel's and articulate cartilages (Mc2, A2) and membrane bones in the cervical region (sa, supra angular), caudal to their endogenous counterparts (Mc1, A1) by E9. (E) Similarly, a graft of stripe III endoderm together with a piece of mesencephalic neural fold yields (F,G) ectopic structures that are identified as Meckel's cartilage-like (Mcl), quadrate-like (Ql) associated with a squamosal-like membrane bone (Sl) located in the neck by E9. Scale bar: 1 mm in B; 1.15 mm in D; 1.1 mm in F.

skeletogenic neural crest cells. Sometimes this extra bone was fused to the remains of Meckel's cartilage, thus further supporting the view that it arose as a result of homeosis. By contrast, this did not occur when only the neural crest was targeted before migration. In the latter case, first arch skeletal structures were strongly reduced or absent (Grammatopoulos et al., 2000). In the experiments that involved the anterior transposition of a Hox-positive (r4-r6) fragment of the neural fold, the neural crest cells were surrounded by an environment where no Hox gene was expressed. Although yielding normal neural derivatives, these neural crest cells were unable to form recognizable cartilage structures. Apoptotic figures observed in certain zones where the grafted neural crest cells were aggregated (S. Creuzet, unpublished) support the contention that, in the absence of an appropriate Hox-positive environment, these cells undergo apoptosis. The homeotic transformations of BA1 into BA2 cartilages, observed in the experiments carried out in *Xenopus* by Pasqualetti et al. (Pasqualetti et al., 2000) and on chick by Grammatopoulos et al. (Grammatopoulos et al., 2000), seem therefore to indicate that overexpression of *Hoxa2* exerts, in these circumstances, an antiapoptotic role on the crest-derived chondrogenic precursors.

About the nature and mode of action of the endodermal signal(s) on neural crest cells

The nature of the signal(s) arising from the endoderm is so far unknown but it is interesting to see that the presumptive first arch endoderm (zone II) triggers in the neural crest the expression of *Pitx1*, a gene normally activated in BA1 ectoderm and neural crest cells during the skeletogenic process leading to lower jaw formation. Moreover, the fact that not only the shape but also the proximodistal and anteroposterior polarity of the skeleton is dictated by the endoderm in our ectopic graft experiments, is in line with the observation of Veitch et al. (Veitch et al., 1999) that the polarity of the branchial pouch endoderm is established in the embryo in the absence of neural crest cells. This was deduced from the observation that the expression of genetic markers such as *Bmp7*, *Fgf8* and *Pax1* occurs in the same regions of the pouch endoderm whether neural crest cells immigrate or not. Our experiments show that these polarities are already determined within the foregut endoderm at the early neurula stage, i.e. well before the branchial pouches are formed.

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REFERENCES

Catala, M., Teillet, M. A., De Robertis, E. M. and Le Douarin, N. M. (1996). A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls. *Development* **122**, 2599-2610.

Couly, G. F. and Le Douarin, N. M. (1987). Mapping of the early neural primordium in quail-chick chimeras. II. The prosencephalic neural plate and neural folds: implications for the genesis of cephalic human congenital abnormalities. *Dev. Biol.* **120**, 198-214.

Couly, G. and Le Douarin, N. M. (1990). Head morphogenesis in embryonic avian chimeras: evidence for a segmental pattern in the ectoderm corresponding to the neuromeres. *Development* **108**, 543-558.

Couly, G. F., Coltey, P. M. and Le Douarin, N. M. (1992). The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development* **114**, 1-15.

Couly, G., Grapin-Botton, A., Coltey, P. and Le Douarin, N. M. (1996). The regeneration of the cephalic neural crest, a problem revisited: the regenerating cells originate from the contralateral or from the anterior and posterior neural fold. *Development* **122**, 3393-3407.

Couly, G., Grapin-Botton, A., Coltey, P., Ruhin, B. and Le Douarin, N. M. (1998). Determination of the identity of the derivatives of the cephalic neural crest: incompatibility between *Hox* gene expression and lower jaw development. *Development* **125**, 3445-3459.

Etchevers, H. C., Vincent, C., Le Douarin, N. M. and Couly, G. F. (2001). The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development* **128**, 1056-1068.

Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993). *Hoxa-2* mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75**, 1317-1331.

Grammatopoulos, G. A., Bell, E., Toole, L., Lumsden, A. and Tucker, A. S. (2000). Homeotic transformation of branchial arch identity after *Hoxa2* overexpression. *Development* **127**, 5355-5365.

Grapin-Botton, A., Bonnin, M. A., McNaughton, L. A., Krumlauf, R. and Le Douarin, N. M. (1995). Plasticity of transposed rhombomeres: *Hox* gene induction is correlated with phenotypic modifications. *Development* **121**, 2707-2721.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Hunt, P., Wilkinson, D. and Krumlauf, R. (1991). Patterning the vertebrate head: murine *Hox* 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest. *Development* **112**, 43-50.

Kanzler, B., Kuschert, S. J., Liu, Y. H. and Mallo, M. (1998). *Hoxa-2* restricts the chondrogenic domain and inhibits bone formation during development of the branchial area. *Development* **125**, 2587-2597.

Köntges, G. and Lumsden, A. (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.

Lanctôt, C., Lamolet, B. and Drouin, J. (1997). The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124**, 2807-2817.

Le Douarin, N. (1982). *The Neural Crest*. Cambridge University Press. Cambridge.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Moens, C. B., Yan, Y. L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). *valentino*: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.

Noden, D. M. (1983). The role of the neural crest in patterning of avian cranial skeletal, connecive, and muscle tissues. *Dev. Biol.* **96**, 144-165.

Pasqualetti, M., Ori, M., Nardi, I. and Rijli, F. M. (2000). Ectopic *Hoxa2* induction after neural crest migration results in homeosis of jaw elements in Xenopus. *Development* **127**, 5367-5378.

Piotrowski, T. and Nusslein-Volhard, C. (2000). The endoderm plays an important role in patterning the segmented pharyngeal region in zebrafish (Danio rerio). *Dev. Biol.* **225**, 339-356.

Prince, V. and Lumsden, A. (1994). *Hoxa-2* expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-23.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. *Cell* **75**, 1333-1349.

Schilling, T. F. and Kimmel, C. B. (1994). Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. *Development* **120**, 483-494.

Sechrist, J., Scherson, T. and Bronner-Fraser, M. (1994). Rhombomere

rotation reveals that multiple mechanisms contribute to the segmental pattern of hindbrain neural crest migration. *Development* **120**, 1777-1790.

Simons, E. V. and van Horn, J. R. (1971). A new procedure for whole-mount alcian blue staining of the cartilaginous skeleton of chicken embryos, adapted to the clearing procedure in potassium hydroxide. *Acta Morphol. Neerl. Scand.* **8**, 281-292.

Takahashi, Y., **Bontoux, M. and Le Douarin, N. M.** (1991). Epithelio-mesenchymal interactions are critical for *Quox 7* expression and membrane bone differentiation in the neural crest-derived mandibular mesenchyme. *EMBO J.* **10**, 2387-2393.

Teillet, M.-A., Ziller, C. and Le Douarin, N. (1998). Quail-chick chimeras. In *Methods in Molecular Biology: Methods and Protocols* (ed. Sharpe and Mason), pp. 305-317. Humana Press.

Trainor, P. and Krumlauf, R. (2000). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nat. Cell Biol.* **2**, 96-102.

Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (Gallus domesticus). A morphological, histochemical and autoradiographical investigation. *Ergeb. Anat. Entwicklungsgesch* **41**, 3-87.

Veitch, E., Begbie, J., Schilling, T. F., Smith, M. M. and Graham, A. (1999). Pharyngeal arch patterning in the absence of neural crest. *Curr. Biol.* **9**, 1481-1484.

Wilkinson, **D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charney, P.** (1989). Segment-specific expression of a zinc finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-464.