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REVIEW

Patterning the neural crest derivatives during development of the vertebrate head: insights from avian studies

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Abstract

Studies carried out in the avian embryo and based on the construction of quail–chick chimeras have shown that most of the skull and all the facial and visceral skeleton are derived from the cephalic neural crest (NC). Contribution of the mesoderm is limited to its occipital and (partly) to its otic domains. NC cells (NCCs) participating in membrane bones and cartilages of the vertebrate head arise from the diencephalon (posterior half only), the mesencephalon and the rhombencephalon. They can be divided into an anterior domain (extending down to r2 included) in which genes of the *Hox* clusters are not expressed (Hox-negative skeletogenic NC) and a posterior domain including r4 to r8 in which *Hox* genes of the four first paraloguous groups are expressed. The NCCs that form the facial skeleton belong exclusively to the anterior *Hox*-negative domain and develop from the first branchial arch (BA1). This rostral domain of the crest is designated as FSNC for facial skeletogenic neural crest. Rhombomere 3 (r3) participates modestly to both BA1 and BA2. Forced expression of *Hox* genes (*Hoxa2*, *Hoxa3* and *Hoxb4*) in the neural fold of the anterior domain inhibits facial skeleton development. Similarly, surgical excision of these anterior *Hox*-negative NCCs results in the absence of facial skeleton, showing that *Hox*-positive NCCs cannot replace the *Hox*-negative domain for facial skeletogenesis. We also show that excision of the FSNC results in dramatic down-regulation of *Fgf8* expression in the head, namely in ventral forebrain and in BA1 ectoderm. We have further demonstrated that exogenous FGF8 applied to the presumptive BA1 territory at the 5–6-somite stage (5–6ss) restores to a large extent facial skeleton development. The source of the cells responsible for this regeneration was shown to be r3, which is at the limit between the *Hox*-positive and *Hox*-negative domain. NCCs that respond to FGF8 by survival and proliferation are in turn necessary for the expression/maintenance of *Fgf8* expression in the ectoderm. These results strongly support the emerging picture according to which the processes underlying morphogenesis of the craniofacial skeleton are regulated by epithelial–mesenchymal bidirectional crosstalk.

Key words facial and neural ectoderm; *Fgf8*; *Hox* genes; pharyngeal endoderm; sectoderm; skeletogenesis.

Contribution of the neural crest to the vertebrate head structures

The cephalic neural crest (NC) is at the origin of most of the mesenchymal components of the vertebrate head, whereas the contribution of the cephalic mesoderm is limited to the striated masticatory and extra-ocular muscles and to the endothelium of all the head blood vessels.

The connective tissues associated with the head muscles as well as the tendons which join muscles to bones are of NC origin. Such is the case also for the dermis (including the adipose tissue associated with the skin), for the musculo-connective component of head blood vessels and for most of the skull and all the facial skeleton (see Le Douarin & Kalcheim, 1999 for a review, and Le Douarin et al. 2004). The contribution of the

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mesoderm to the head skeleton is limited to the occipital and otic (partly) regions of the skull (Couly et al. 1993). These ideas have been gained through investigations carried out in the avian embryo by constructing quail–chick chimeras. They are in agreement with the results of recent investigations carried out in amphibians (Gross & Hanken, 2004, 2005). By contrast, data based on NC cell (NCC) labelling through β-galactosidase expression driven by the *Wnt1* promoter indicate a mesodermal origin for the parietal in the mouse (Jiang et al. 2002).

The problem was then raised of the molecular mechanisms controlling the patterning of the complex and elaborated structures of the vertebrate face.

One previously held view was that the NCCs themselves were the source of patterning information. This was based on an experiment in which the first branchial arch (BA1) NC was transplanted posteriorly at the level at which the second branchial arch (BA2) normally forms in the chick embryo. Such a transposition led to the duplication of the lower jaw at the expense of the hyoid cartilage, suggesting that the mesencephalic NCCs, which normally colonize BA1, are responsible for patterning the corresponding skeleton, whatever the environment in which they migrate (Noden, 1983; see also Le Douarin & Kalcheim, 1999).

This experiment was repeated by Couly et al. (1998). Grafts from quail into chick embryos were made in order for the exact fate of the grafted cells be assessed. Moreover, transplantation involved only the neural fold (NF) without including the adjacent neural tube (Fig. 1). In

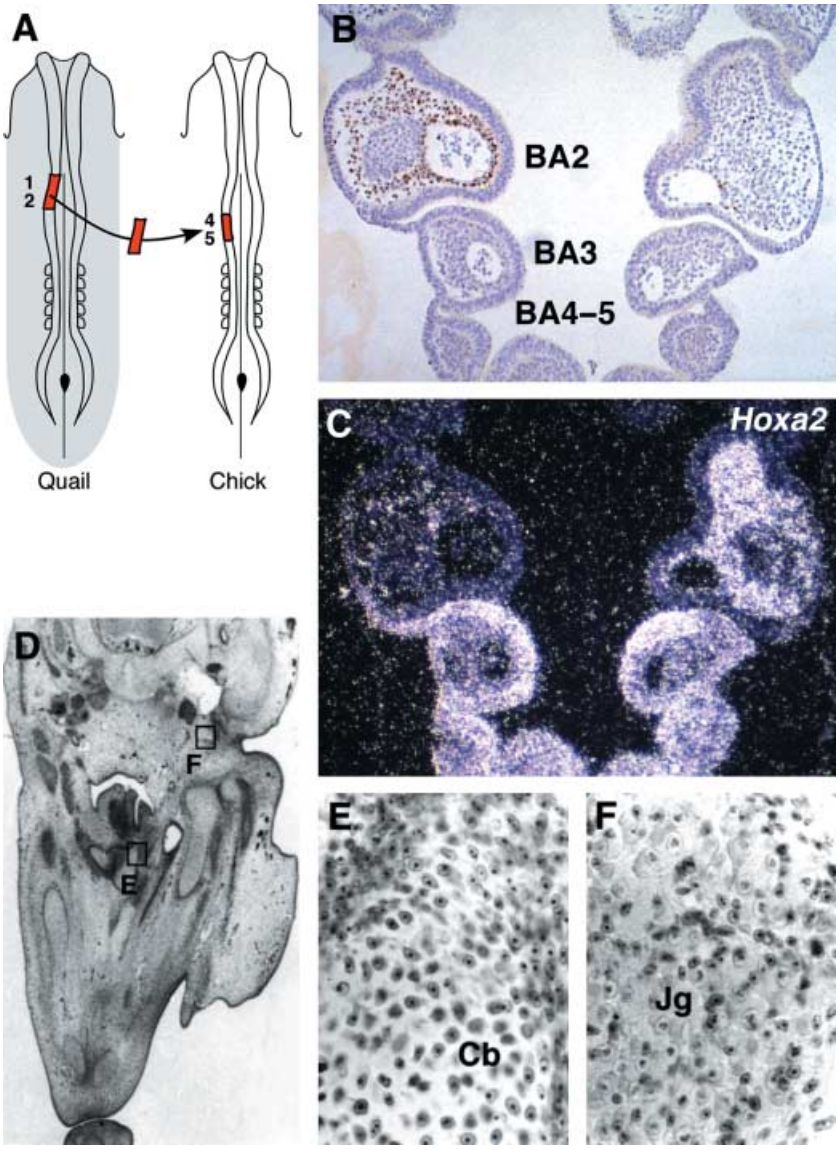

Fig. 1 Translocation of isthmic neural crest. (A) Replacement of chick neural fold at the mid-rhombencephalic r4–r5 level in a 5ss embryo by the anterior r1–r2 neural fold fragment taken from a stage-matched quail embryo. (B) At E3, NCCs emanating from the graft have invaded BA2 as evidenced by the QCPN monoclonal antibody. (C) These graft-derived cells maintain the Hoxa2-negative status in the ectopic environment. (D) Quail isthmic NCCs caudally translocated to populate BA2 in chick host, form the ceratobranchial (Cb; E) and participate in the jugular ganglion at E9 (Jg; F).

such a case, the NCCs fated to colonize BA1 were found to participate in the formation of the hyoid cartilage which normally develops from BA2.

By contrast, if the neuroepithelium of the isthmus was associated with the NF of the posterior mesencephalon and of rhombomeres 1 and 2 (r1, r2), an extra Meckel's cartilage developed in the recipient's BA2. An explanation for this observation was later proposed by Trainor et al. (2002), who claimed that the isthmus exerts its effect through the production of the morphogen fibroblast growth factor 8 (FGF8). According to these authors, FGF8 was able to inhibit *Hoxa2* expression in the NCCs arising from the isthmic region, thus allowing facial-type structures to develop. In addition, *Hoxa2* was shown to inhibit the activity of *Sox9*, a master gene in cartilage differentiation, and to be able to block dermal ossification (Kanzler et al. 1998). Moreover, Bobola et al. (2003) have demonstrated that, in the context of BA1, *Hoxa2* activation inhibts the expression of *Pitx1*, a transcription factor involved in mandibular development (Lanctôt et al. 1997; Couly et al. 2002).

Hox genes and head morphogenesis

An important discovery was that the homeotic genes of the *Hox* gene family play a critical role in the genetic control of head morphogenesis through patterning the hindbrain and the NC derivatives that originate from its mid- and posterior segments (i.e. r4 to r8).

By contrast, the forebrain, midbrain and anterior hindbrain (r1 to r2) and the NCCs arising from these encephalic vesicles fail to express any *Hox* gene (Hunt et al. 1991a,b; Prince & Lumsden, 1994; Couly et al. 1996). Two different domains are thus defined by the expression of the homeotic *Hox* genes within the NF which yields the mesectodermal derivatives of the NC: a rostral domain (from mid-diencephalon down to r3) in which the NCCs are Hox-negative and a caudal domain (from r3 to r8) in which NCCs express *Hox* genes of the four first paralogous groups (and thus are Hox-positive; Fig. 2A,B). Note that r3 participates in the two domains, as it provides a few NCCs to both BA1 and BA2. The cells migrating rostrally lose their *Hoxa2* expression as they reach BA1, while those migrating caudally into BA2 maintain it (Fig. 2C–E).

Experiments based on the construction of quail–chick chimeras have shown that the NCCs arising from the rostral (*Hox*-negative) domain of the crest are at the origin of the entire facial skeleton. This domain will be further designated, for this reason, FSNC (for facial skeletogenic neural crest). The NCCs arising from the posterior domain contribute to the hyoid cartilage (except for the entoglossum and the anterior part of the basihyal, which both belong to the lower jaw) (Couly et al. 1996; Köntges & Lumsden, 1996; Fig. 3). Interestingly, the skeletogenic capacities of the NCCs of the Hox-negative and Hox-positive domains are different: both yield cartilage whereas only the anterior one yields membrane bones (Fig. 2A,B). Moreover, these two domains are not interchangeable.

The *Hox*-positive domain of the head neural crest is unable to generate the facial skeleton

The experimental transposition of the NF from the *Hox*-positive posterior domain to the anterior *Hox*-negative domain results in the failure of facial structure formation (Couly et al. 1998, 2002; Fig. 4A,B). The *Hox*-positive NCCs transposed anteriorly are, however, able to form the neural and glial derivatives of the NCCs at this level. Moreover, complete excision of the FSNC results in a complete absence of face in the operated embryos together with dramatic malformations of the brain (Fig. 4C,D). It is interesting to note that a similar phenotype was obtained in mouse embryos by the inactivation of the *β-catenin* gene by *Wnt1-Cre*-mediated deletion. Although the *β-catenin* mutation did not hamper the migratory potential of cranial NCCs, the observed effect was correlated with increased apoptosis in the frontonasal ectomesenchyme and in the proximal portion of the first two BAs (Brault et al. 2001).

In strong contrast, any fragment of the *Hox*-negative NC, whether it belongs to the diencephalon, the mesencephalon or to r1–r2, is able to regenerate a complete facial skeleton (Fig. 4E,F).

The FSNC thus behaves as an 'equivalence group' because any part of it has the same developmental capacities and behaves like the whole domain itself as far as the building of a face is concerned (Couly et al. 2002). In the reverse experiment, as stated above, a fragment of *Hox*-negative NC transplanted at the r4–r6 level participates in the formation of the hyoid cartilage. It appeared therefore that *Hox* gene expression had an inhibitory effect on the differentiation of facial cartilages and bones. This notion was further confirmed by the experiments detailed below.

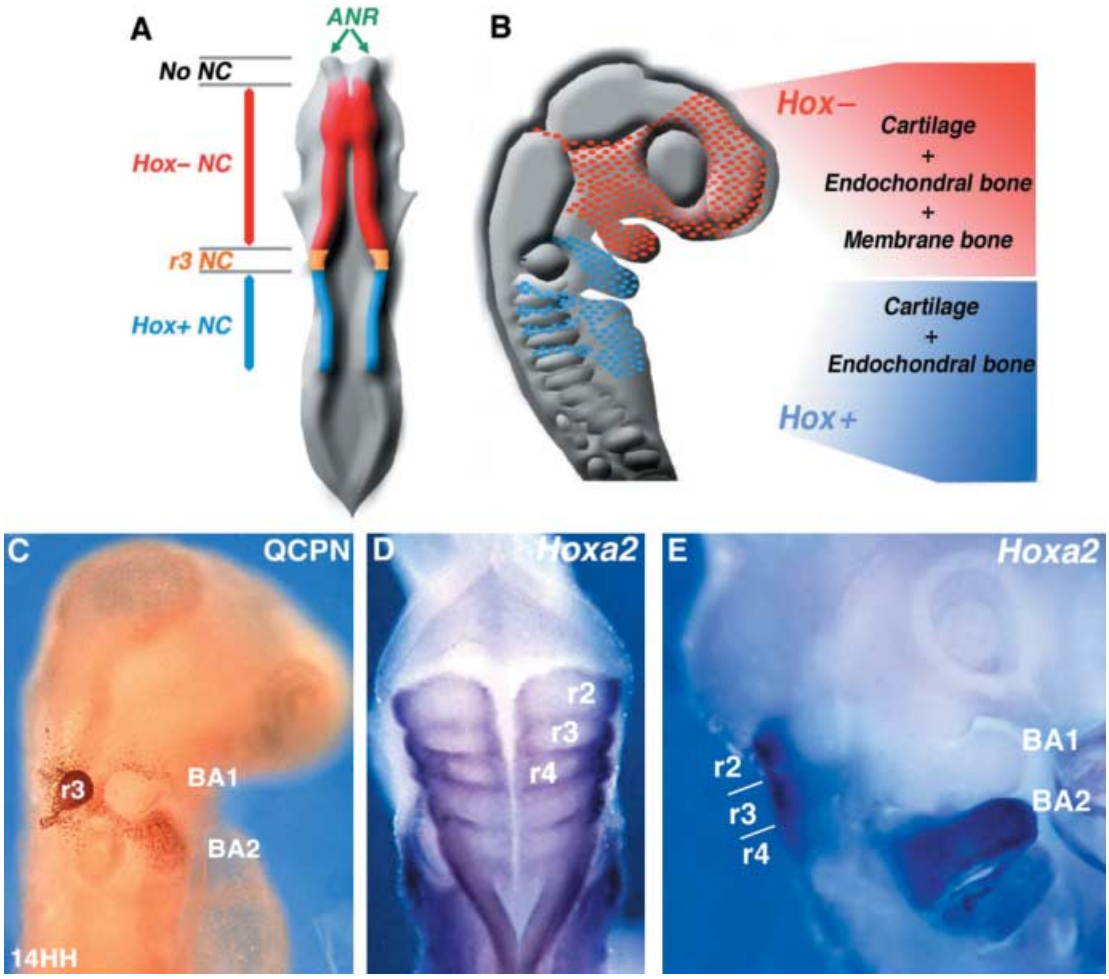

Fig. 2 Cephalic neural crest: Hox gene expression and skeletogenic properties. (A) In a 5ss chick embryo, the cephalic NC is divided into an anterior Hox-negative domain (in red) and a posterior, Hox-positive domain (in blue). The transition between these two domains corresponds to r3 (in orange). The neural fold (NF) rostral to the mid-diencephalon does not produce NCCs. (B) Hox-negative NCCs (in red) yield cartilages as well as endochondral and dermal bones of the entire upper face and jaws. By contrast, skeletogenic properties of Hox-negative NCCs (in blue) are limited to chondrogenesis and endochondral ossification in the hyoid structure. (C) At the edge of the Hox-negative and Hox-positive domain of NC, r3-NCCs (here replaced by their quail counterparts and evidenced by the QCPN monoclonal antibody) migrate in BA1 and BA2. (D) Whereas the neuroepithelium at this level is *Hoxa2*-positive, r3-derived NCCs have a dual molecular profile according to the environment the cells pervade (E). These cells are *Hoxa2*-negative in BA1 and *Hoxa2*-positive in BA2. ANR, anterior neural ridge.

Gain-of-function of *Hoxa2*, *Hoxa3* and *Hoxb4* genes in the FSNC

The avian embryo is easily amenable for functional approaches by targeting gene expression to limited and well-defined embryonic territories at elected times in development.

Forced expression of *Hoxa2* (together with green fluorescent protein, GFP) was elicited by electroporation of retroviral constructs in the FSNC cells before the onset of their migration. This led *Hoxa2*-expressing NCCs to migrate and differentiate within a *Hox*-negative environment, a situation different from that produced by Pasqualetti et al. (2000) in which all the tissues of BA1 had been transfected with *Hoxa2*. Pasqualetti et al.'s experiments produced a homeotic transformation of BA1 into BA2 structures.

In our experiments, expression of this gene abolished the capacity of the FSNC to form the facial skeleton (Fig. 4G–J; Creuzet et al. 2002). Experiments similar in principle, using *Hoxa3* and *Hoxb4* as transgenes, prevented partly but severely the development of the facial skeleton. A combination of the two constructs

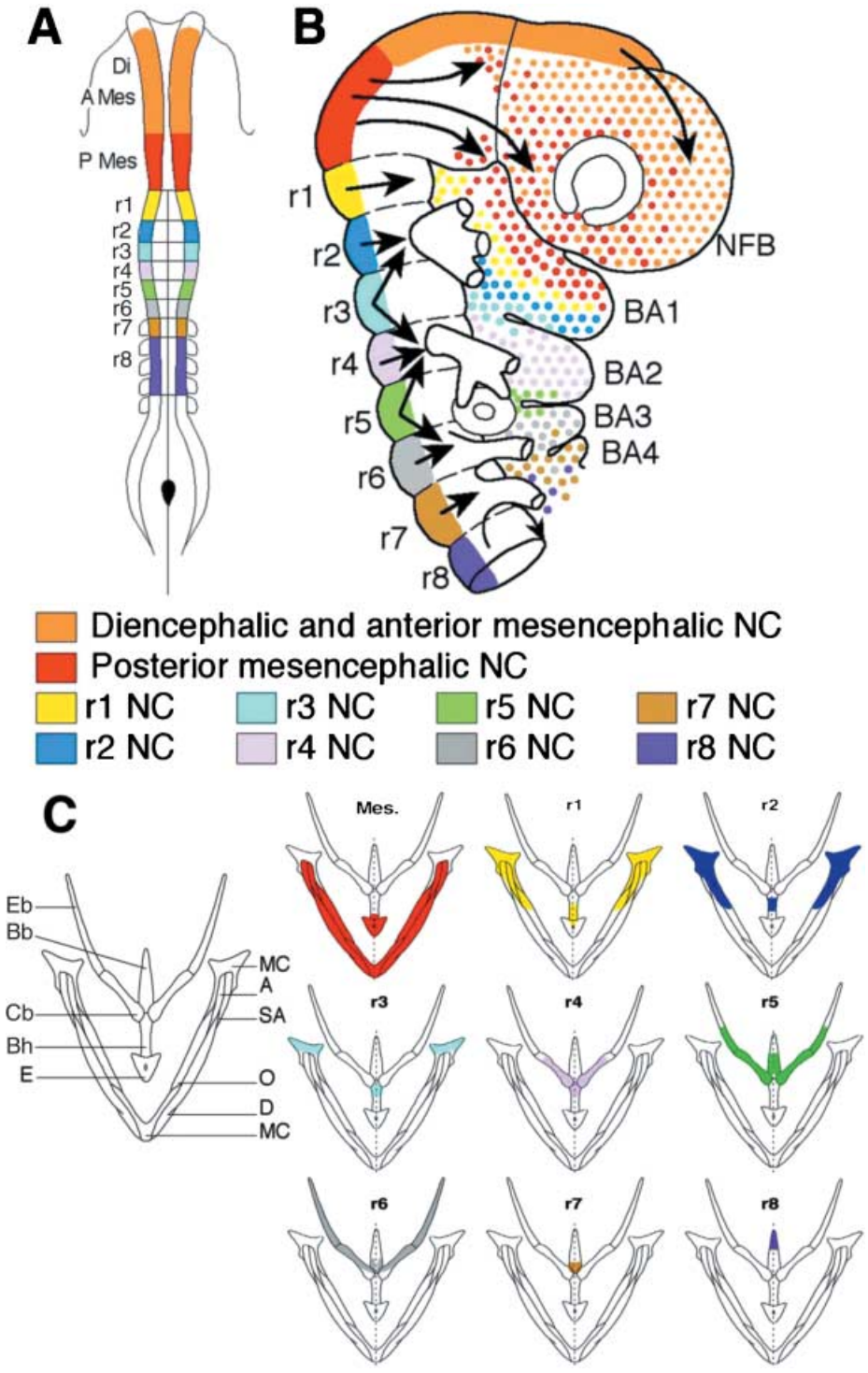

Fig. 3 The cephalic neural crest: cell migration streams and fate map in hyoid structure. (A) Presumptive diencephalic, mesencephalic and rhombencephalic territories of the NF in the avian embryo at 5ss as established by Grapin-Botton et al. (1995). (B) Migration map of cephalic NCCs in the avian embryo. The origin of NCCs found in the nasofrontal, periocular regions and in BAs is colour-coded as in A. NCCs arising from the posterior diencephalon and mesencephalon populate the nasofrontal and periocular region. Posterior mesencephalon also participates in BA1. NCCs from r1–r2 together with a small contribution of r3 complete the crest-derived mesenchyme in BA1. The major contribution to the 2nd branchial arch comes from r4. NCCs arising from r3 and r5 split to participate in the two adjacent arches: BA1 and BA2 for r3-NCCs; BA2 and BA3 for r5-NCCs, respectively. r6–r8-derived cells migrate to the more caudal BAs. (C) Skeletal components of hyoid cartilages: the participation of the crest-derived cells is colour-coded as in A. A, angular; Bb, basibranchial; Bh, basihyal; Cb, ceratobranchial; D, dentary; E, entoglossum; Eb, epibranchial; Mc, Meckel's cartilage.

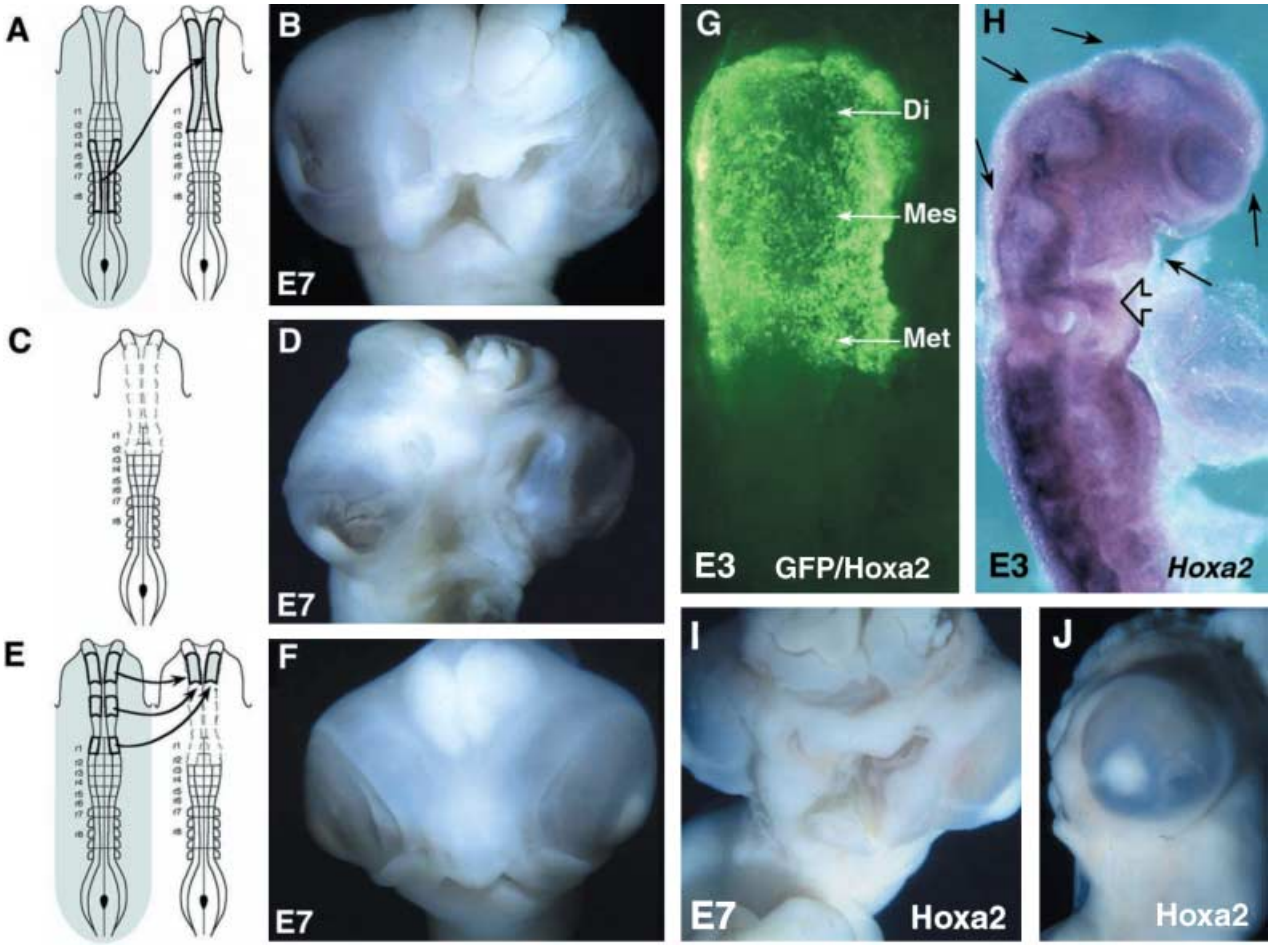

Fig. 4 Hox gene expression hampers facial development. (A) Replacement of FSNC in 5ss chick embryo by a fragment of Hox-positive NC taken from a stage-matched quail embryo severely hampers head morphogenesis at E7 (B). (C) Removal of the Hox-negative FSNC in early chick neurula (dotted lines) abolishes the development of facial structures and results in brain malformation in E7 operated embryo (D). (E) Following removal of whole FSNC (as in C), implantation of only a fragment of the FSNC (from either di-, mes- or anterior rhombencephalic level) restores normal development of face and forebrain at E7 (F). In ovo, coelectroporation of Hoxa2 retroviral and GFP constructs in the entire Hox-negative NF leads to the expression of the GFP reporter gene in the FSNC migrating tranfected cells (G) along with the forced expression of Hoxa2 in the forehead territory (arrows; H), rostrally to the normal pattern of expression in BA2 (arrowhead; H). (I) At E7, Hoxa2 transgenic embryo exhibits severe defects in facial development, ressembling those illustrated in B and D. (J) Left side of the same embryo as in I.

(*Hoxa3* + *Hoxb4*) yielded results similar to those obtained by transfecting *Hoxa2* alone. These results are in agreement with the fact that translocation of *Hox*-positive NCCs anteriorly inhibits the development of facial structures (Couly et al. 1998).

Excision of the FSNC in the chick embryo at 5–6ss resulted in multiple effects. In addition to the absence of facial skeleton differentiation, and the strong impairment of brain development, we observed the inhibition of *Fgf8* expression in several regions of the developing head. The experiments described below showed that FGF8 is a key factor in patterning the facial skeleton.

FGF8 plays a key role in the development of the facial skeleton

In normal embryos at this stage, *Fgf8* is expressed (1) in the neuroepithelium and the superficial ectoderm of the anterior neural ridge (ANR); (2) in the ectoderm lining BA1 and in the ectoderm lateral to the stomodeum; and (3) in the isthmus.

Ablation of the FSNC at 5–6ss is followed by a dramatic decrease of *Fgf8* expression in the ANR and BA1 ectoderm, as early as 24 h after surgery (Fig. 5A–C).

If exogenous recombinant FGF8 is administered to the operated embryo after FSNC excision (by applying

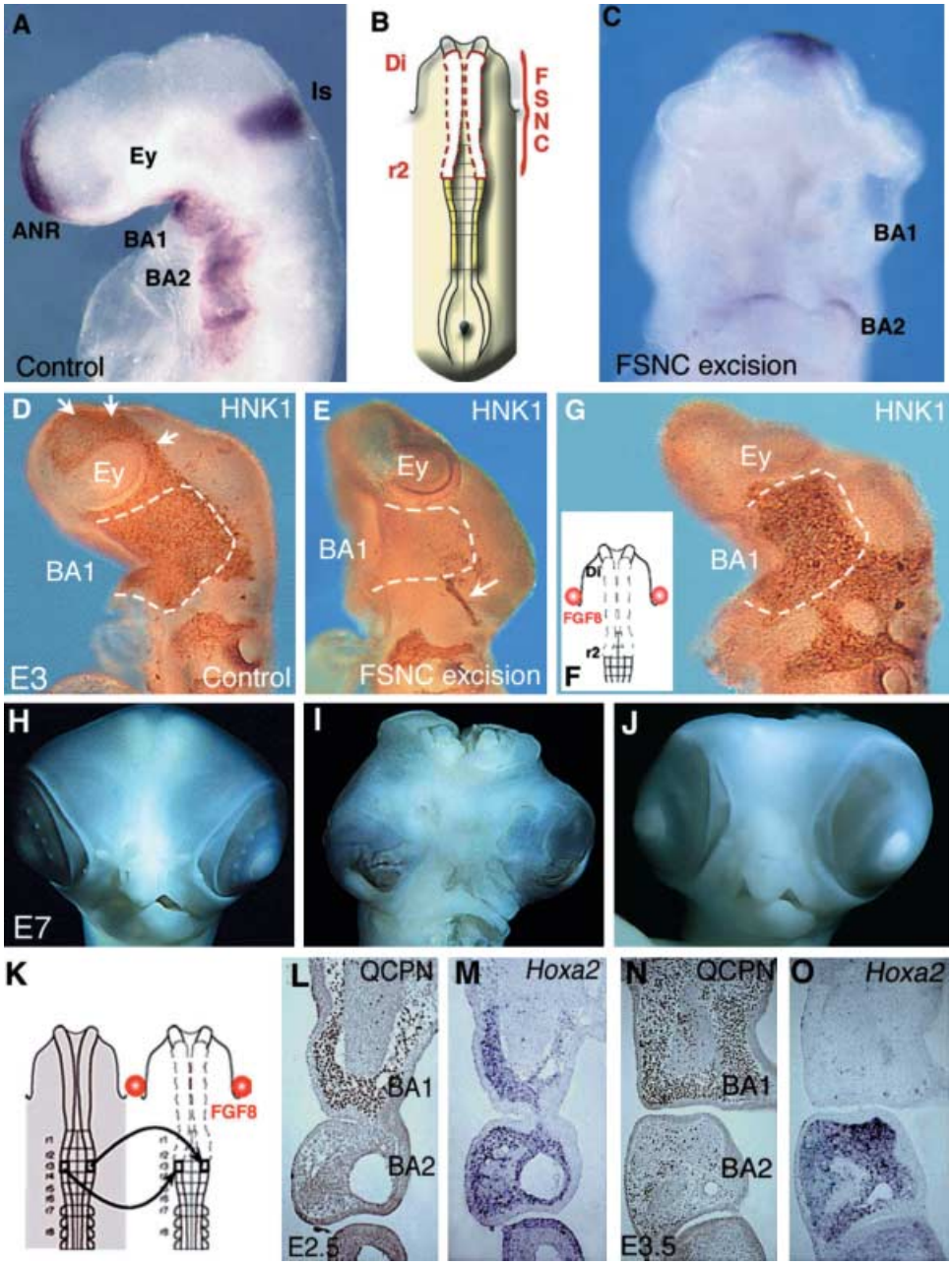

Fig. 5 Restoration of facial morphogenesis by exogenous FGF8. (A) At E3, in normal development, Fgf8 is expressed in the neuroepithelium and the superficial ectoderm of the anterior neural ridge (ANR), in the ectoderm lining BAs and in the isthmus (Is). (B) Removal of FSNC [from the posterior diencephalon (Di) down to r2] at 5ss. (C) Ventral side of E2.5 operated embryo showing the dramatic loss of Fgf8 expression in ANR and BA1. Comparison of NC migration (D–G; HNK1 monoclonal antibody staining at E3) and E7 facial morphology (H–J) in control (D, H), FSNC-excised (E, I) and FSNC-excised embryos subjected to exogenous FGF8 through beads placed at the presumptive level of BA1 ectoderm (F–G, J). In E3 control embryos, cephalic NCCs massively populate the forming BA1 (dotted line) and are migrating rostrally to colonize the nasofrontal bud (arrows). After FSNC excision, rare HNK1-labelled cells are present in BA1 (E; compare with control in D). Note the labelling of the trigeminal ganglion (arrow; E) that attests to the contribution of mid-rhombencephalic NCCs to the cranial peripheral nervous system. Treatment of FSNC-excised embryos with FGF8-soaked beads implanted at the level of the presumptive BA1 ectoderm (F) rescues the colonization of BA1 by r3-derived cells (G). At E7, these embryos display a partial restoration of their facial morphology with the development of maxillary and mandibular components of the upper and lower beaks, respectively (J). (K) Following bilateral excision of the FSNC, the r3 NF of the host embryo was bilaterally replaced by its quail counterpart and FGF8 beads were supplied as before. At E2.5, the r3-derived NCCs (evidenced by QCPN monoclonal antibody) that are in the process of populating BA1 (L) express Hoxa2 (M). Onwards, at E3.5, they turn to an Hox-negative status (N–O), though those that are in BA2 exhibit the accumulation of the *Hoxa2* transcript.

FGF8-soaked heparin acrylic beads on presumptive BA1 ectoderm), a spectacular rescue of facial structures ensues (Fig. 5D–J). Moreover the encephalic vesicles, which remain open, close on the dorsal midline and the development of the brain tends to normalize (Creuzet et al. 2004; our unpublished results).

The results of these FGF8-rescue experiments raised the problem of the origin of the NCCs that regenerate the face. Replacing r3 of the operated chick embryos by their quail counterpart after FSNC removal showed that regeneration of the lower jaw induced by FGF8-soaked beads is due to the strong stimulation of r3-derived NCCs. The latter, which massively invade BA1, transiently express *Hoxa2* (Fig. 5K–M) and become *Hox*-negative after about 24 h (Fig. 5K,N,O; Creuzet et al. 2004). These experiments demonstrate the strong regeneration capacities of r3-derived NCCs and the role of FGF8 in the regulation of NCC proliferation and migration. In addition, it has been proposed that, in mice, *Fgf8* expression from the superficial ectoderm restricts the skeletogenic properties of the mandibular crest-derived mesenchyme and refines the position of the developing jaw joint (Tucker et al. 1999; Wilson & Tucker, 2004).

The r3- and r5-derived NF have previously been described as crest-cell-depleted territories, thus accounting for the defined streams of crest migration into pharyngeal arches (Lumsden et al. 1991). The depletion of NCCs in r3 and r5 has been attributed to a large-scale apoptosis induced by BMP4 upon a signal regulated early by the neighbouring even-numbered rhombomeres (Graham et al. 1994, 1996; Ellies et al. 2000). However, the early migration of a small number of NCCs from both r3 and r5 has been demonstrated by *in vivo* studies (Birbauer et al. 1995; see also the review by Kulesa et al. 2004). In our experiments, the resection of either r2 or r4 is likely to release r3 NCCs from the apoptotic activities of these even-rhombomeres, thus turning this territory into a source of suriviving cells.

The discrepancies in fate maps performed respecively by Couly and co-workers and Köntges and Lumsden in the quail–chick chimera system can be accounted for by differences in the stage elicited for the interspecific exchanges (Couly et al. 1996; Köntges & Lumsden, 1996). When performed at 8–9ss, no skeletal contribution of r3 to the hypobranchial structures was recorded (Köntges & Lumsden, 1996). By contrast, when replacement of the r3 territory in the chick host by its quail counterpart was performed before exit of the few cells from r3, the latter were seen to supply a minor skeletogenic contribution to the articular and basihyal cartilages, as found by Couly et al. (1996). Following the extirpation of FSNC and the administration of FGF8, the cellular potential of the r3 crest is preserved and cells exiting this territory are able to migrate massively rostralward. This shows that r3-derived cells are able to respond to a local skeletogenic organizing centre, the ventral foregut endoderm, responsible for the patterning cues that form the primary scaffold of the facial and hypobranchial skeleton (Couly et al. 2002; Ruhin et al. 2003). Hence, in the regenerating lower jaw, r3 NCCs exhibit expanded capacities of differentiation because they are the sole source of chondroblasts and osteoblasts regenerating the entire set of mandibular components. At the same time, the r3-derived NCCs participate in the hyoid cartilage in BA2 according to their normal pattern (Couly et al. 1996). This therefore demonstrates the high degree of cranial crest plasticity and emphasizes that, in the early neurula, mandibular skeleton patterning is not crest-inbuilt.

That r3-exiting cells are globally Hoxa2-positive at embryonic day (E)2 but turn to a Hox-negative status from E3.5, once they have settled in BA1, also means that the genetic ground of r3-crest is not maintained in the cells migrating in BA1 as a consequence of tissue interactions with the pharyngeal environment (Trainor & Krumlauf, 2000). In that respect, the molecular plasticity of r3-NCCs relates to a similar trait of another crest-depleted domain, r5, in which Hoxa3 expression is versatile and obeys subtle constraints that are dictated by the environment (Saldivar et al. 1997). This astonishing ability of the r3 crest to adapt its molecular profile can be interpreted as a high degree of plasticity that helps such a pivotal crest domain to establish the boundary between the Hox-negative and Hox-positive mesenchyme.

Experiments in which the expression of *Fgf8* is repressed by RNA-interference further confirmed the strong effect of this signalling molecule on FSNC

Double-stranded RNA of *Fgf8* was electroporated into the presumptive BA1 ectoderm in 5–6ss embryos. Observation of NCC migration by using the HNK1 monoclonal antibody showed a strong deficit in NCCs in BA1 on the operated side as compared with the opposite control side.

This was interpreted as an effect of FGF8 on both survival/proliferation and migration of NCCs during the process of branchial arch formation (Creuzet et al. 2004).

FGF8 acting on *Hox*-negative cells placed in the context of BA2 produce BA1 skeletal structures

FGF8 was proposed by Trainor et al. (2002) to be able to inhibit *Hoxa2* expression in the NCCs (see above). We decided to test this hypothesis further by trying to induce BA1-like structures in BA2 after providing BA2 NCCs with an exogenous source of this morphogen. The test was the formation of Meckel's cartilage in BA2 accompanied by the differentiation of membrane bones, which normally never form in the posterior *Hox*-positive domain of the neural crest.

As shown previously (Couly et al. 1998), translocation of the *Hox*-negative NF from the mesencephalon to the level of r4 to r6 (which normally yields the NCCs colonizing BA2) results in the formation of a normal hyoid cartilage, which is partly derived from the grafted *Hox*-negative quail NCCs.

If an FGF8-soaked bead is placed on the grafted NF, as shown in Fig. 6, the outcome is very different.

The recipient embryos, which are allowed to develop until E9–E10, presented a bulge at the ventral side of the neck, inconsistently associated with a beak-like structure pointing out from the neck (n = 5/7; Fig. 6A–C). Extra pieces of cartilage had developed that could be assimilated to a quadrate and a Meckel's cartilage rod. In addition, membrane bones were also present (n = 7; Fig. 6D–H), a situation which is never observed in normal BA2.

Interestingly, an FGF8-soaked bead placed upon the *Hox*-positive endogenous r4–r6 neural fold of unoperated embryos did not perturb the normal development of the hyoid cartilage and no extra skeletal pieces were ever seen in these embryos (n = 5). These results show that FGF8 acting on *Hox*-negative NCCs in the context of BA2 is able to induce the formation of BA1-type structures, including membrane bones, but it cannot do so on endogenous *Hox*-positive NCCs.

We have previously shown that in the absence of the FSNC, r3 NCCs, which were thus no longer subjected to the inhibitory influence of the even-numbered rhombomeres such as r2 (Graham et al. 1993, 1994; Ellies et al. 2000), were able to regenerate a lower jaw when stimulated by an exogenous source of FGF8. We then tested the capacity of r3-derived NCCs to provide a larger contribution than normal to BA2 in the absence of the regular source of BA2 NCCs (i.e. r4, r5, r6). If the NF of these three rhombomeres is removed and if a bead of FGF8 is placed on top of r3 (which has been replaced in a 5–6ss chick embryo by its quail counterpart), development of the hypobranchial skeletal structures of BA2 is perturbed. At E9–E10, the embryos have an abnormal protrusion on the ventral side of their neck resulting from the development of extra skeletal elements that had expanded in relationship to the hyoid cartilages (n = 9; Fig. 6I–L). Owing to their shape and position, these extra cartilaginous elements were interpreted as being bilateral duplicated quadrates that normally develop from BA1 crest mesenchyme. They were made up of quail cells and thus derived from r3 (n = 6; Fig. 6M–Q). In the absence of exogenous FGF8 and following the mere excision of r4–r6 NF, the development of the hyoid cartilages was normal and mostly derived from the remaining posterior rhombomeres r6–r8 (Fig. 6R–T).

It is thus very striking to see that the r3-derived NCCs can give rise to maxillo-mandibular bones when they are subjected to a source of FGF8 whether they are placed in the context of BA1 or BA2 (our unpublished observations). Rhombomere 3 can be considered as the true marginal zone between the anterior cephalic vesicles and the upper trunkal region represented by the posterior rhombomeres.

Another component in the patterning of the facial skeleton is obviously the pharyngeal endoderm as it is closely associated with the NCCs that colonize the BAs.

The pharyngeal endoderm as a source of patterning information for the skeletogenic NCC

Excision of transverse stripes of pharyngeal endoderm in the 5–6ss chick embryo results in the absence of definite pieces of facial skeleton (Fig. 7A–C). Moreover, addition of these transverse endodermal stripes from quail embryos within another stage-matched intact chick host was performed so that the grafted endoderm was superposed onto the endogenous one within the cephalic NCC migration pathway. The operated chicken showed a duplication of the cartilages and bones which normally develop at this level of the face (Fig. 7D–G). Further experiments showed that, in addition

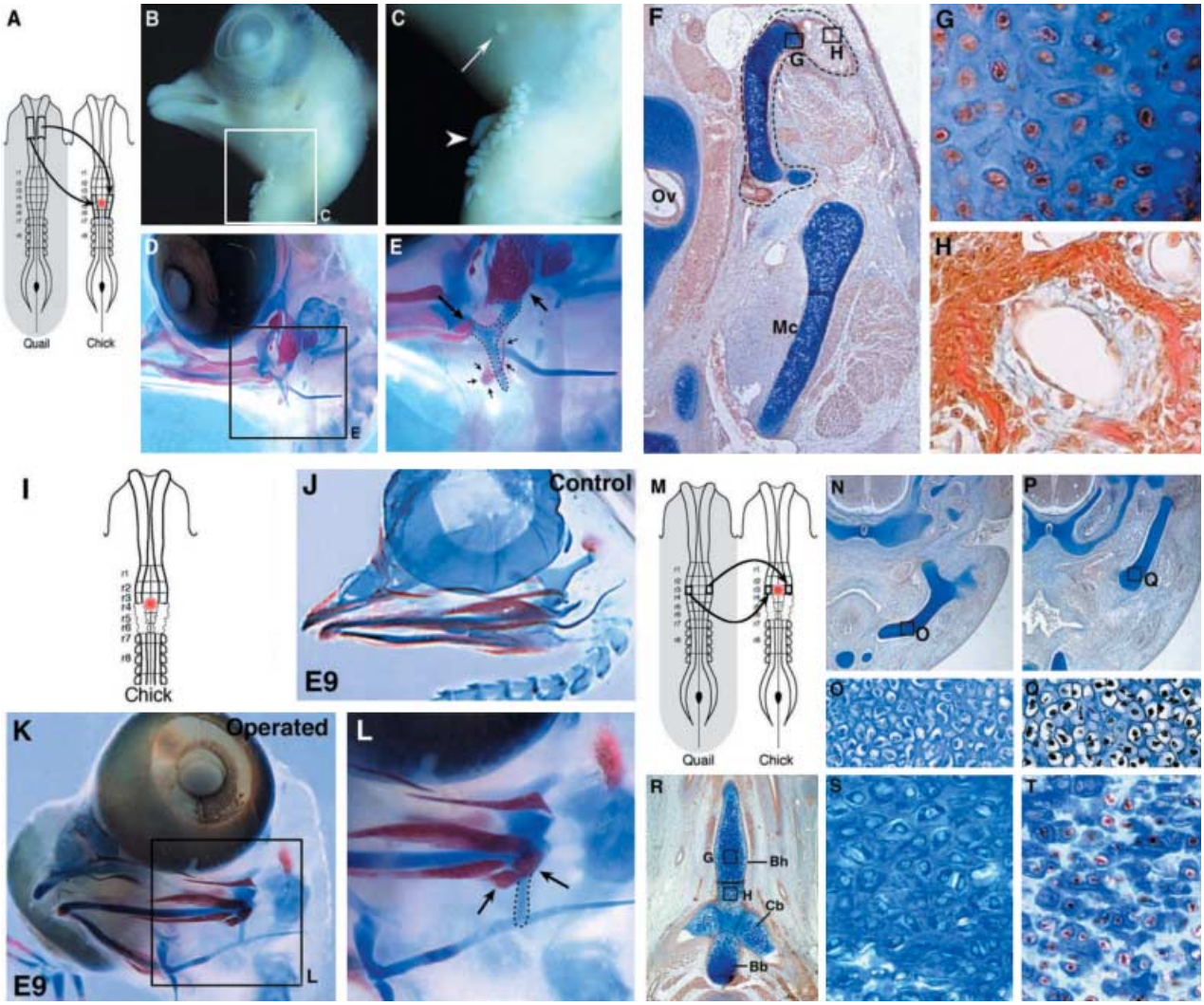

Fig. 6 FGF8 promotes formation of mandibular-like structure and differentiation of associated dermal ossification in BA2. (A) The anterior mesencephalic NC taken from a 5ss quail embryo is bilaterally engrafted at the level of r4–r6 in a stage-matched chick together with an FGF8-soaked bead. (B, C) At E10, the recipient embryo presents an abnormal bulging of its throat (arrow) along with a bilateral beak-like structure pointing out from the neck (arrowhead). (D, E) Whole-mount skeletal preparation shows that a mandibular-like cartilage (stained with Alcian blue; dotted line; E) has developed in the vicinity of the ceratobranchial; this extra-structure coincides with the differentiation of superficial ectopic membrane bones (stained with Alizarin red; arrows; E). (F–H) At E9, histological detection of quail cells (using the QCPN monoclonal antibody) combined with Alcian blue–Alizarin red staining of skeletal tissues. (F) The mandibular-like structure (dotted line) which forms a mirror-duplication of the endogenous Meckel's cartilage (Mc) is derived from the grafted cells as its cartilaginous (G) and bony (H) components are entirely made up of quail cells (in brown). (I) Bilateral extirpation of the r4–r6 NF (dotted line) in 5–6ss chick embryo, followed by implantation of a single FGF8-soaked bead in contact with the edge of the excised territory. (J–L) Whole-mount staining of skeketal structures in control (J) and operated (K,L) embryos at E9. As a result of the operation, supernumerary skeletal pieces (K) have expanded towards the hyoid structures (L). Owing to their shape and position, these extra-cartilaginous elements are interpreted as being a duplicated quadrate (dotted line) associated with supernumerary membrane bones (arrows). (M) Replacement of r3 NF by its quail counterpart in the same experimental context as in I. (N–P) Histological preparation shows that NCCs emanating from the quail r3 territory do not contribute to the endogenous Meckel's cartilage (N), as evidenced at higher magnification (O), but are at the origin of the duplicated cartilage (P,Q). (R–T) Posteriorly, the contribution of the r3-derived cells in hyoid structure (R) is strictly restricted to its normal pattern (Bh; T). Bb, basibranchial; Cb, ceratobranchial.

to being essential for shaping cartilage rudiments, signals from the ventral foregut endoderm also dictate the position that is adopted by Meckel's cartilage with respect to the body axis (Couly et al. 2002). *Hox*-expressing NCCs are similarly responsive to endodermal cues arising from the more caudal part of the foregut endoderm (Ruhin et al. 2003). The nature of the signals involved in this process is still unresolved.

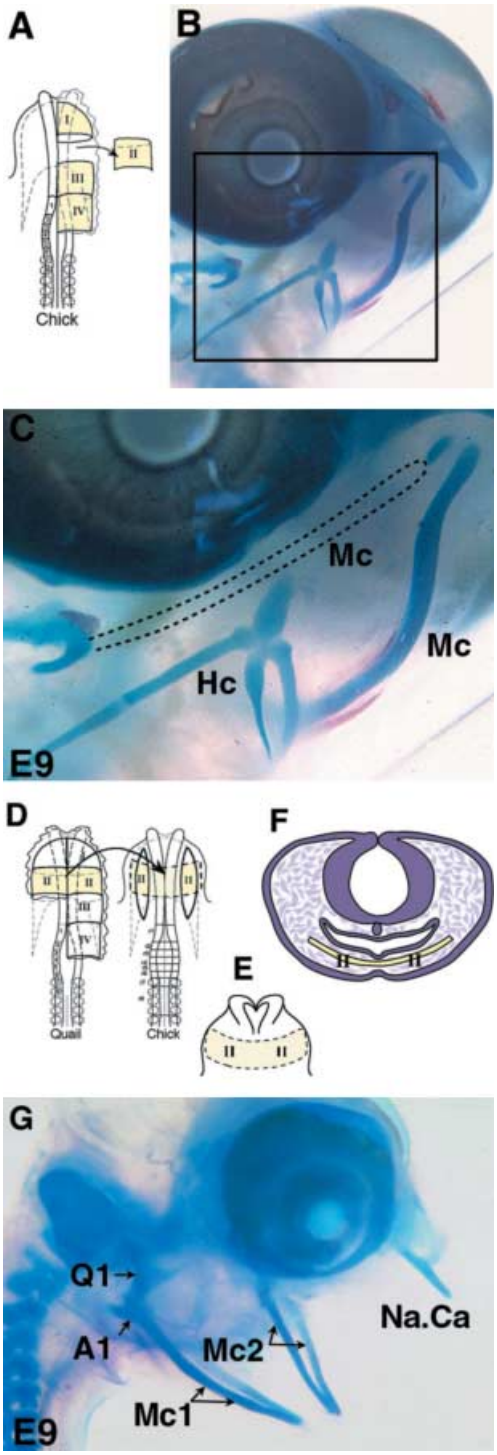

Fig. 7 The ventral foregut endoderm patterns the crest-derived skeleton. (A) Unilateral surgical extirpation of the zone II of the ventral foregut endoderm (shaded in yellow) facing the anterior mesencephalic anlage in 5ss chick embryo. (B) In E9 operated embryo, the ablation of the endodermal stripe leads to the ipsilateral absence of Meckel's cartilage, as evidenced at higher magnification (C; dotted line), without perturbing the rest of the visceroskeleton. (D–F) Bilateral transplantation of endodermal zone II (shaded in yellow) from quail into stage-matched chick neurula, implanted ventrally (E) to the endogenous ventral foregut of the host as schematically represented (F). (G) In E9 recipient embryo, the additional endodermal stripes have generated a supernumerary mandibule (Mc2) interprosed between the endogenous upper and lower jaw apparatus. A1, endogenous articular; Hc, hyoid cartilage; Mc1, endogenous Meckel's cartilage; Na.Ca, nasal capsule; Q1, endogenous quadrate.

Transplanted NCCs keep species-specific characters

The NCCs are not devoid of 'information' for designing the facial structures. This was demonstrated by striking experiments in which NCCs were orthotopically exchanged between quail and duck embryos at Hamburger and Hamilton stage 9.5 (HH 9.5; Hamburger & Hamilton, 1951). Moreover, this model, in which the two birds develop according to significantly different timing (incubation times: 17 days for the quail; 28 days for the duck), was able to reveal that the timing of expression of specific genes was genetically determined in NCCs. The latter seemed to impose a donor- rather than host-type gene expression pattern on the host ectoderm. This was particularly clear for *Shh* and *Pax6* expression timing (Schneider & Helms, 2003).

Quail–duck NC chimeras were also constructed by Tucker & Lumsden (2004). The levels of the exchanged NF and the stages at which the grafting operations were performed were precisely defined and the grafts involved specific regions of the developing brain and rhombomeres. The morphology of the quail cartilages that developed within the duck environment (and vice versa) was finely recorded. They found that the shape of the facial cartilages (the entoglossum and retroarticular process, which differ between the duck and quail) was always of NC donor type. Therefore, once induced by the endoderm to develop into a particular cartilage, NCCs follow a species-specific genetic program involving a particular growth and morphogenetic pattern. Another important finding from this study was that membrane bones that are associated with facial skeletal cartilages maintain their species-specific timing of differentiation.

Thus, during facial morphogenesis, a temporally regulated and multistep crosstalk occurs between the epithelia (endoderm and ectoderm) and the NCCs.

The ectoderm of the fronto-nasal process was further shown to play an important role in positioning and refining the shape of the upper beak. This effect is mediated by two major signalling molecules, SHH and

FGF8, the expression domains of which abut in the so-called FEZ (for frontonasal ectodermal zone), which plays a role in the growth of the underlying mesenchyme of NC origin (Hu et al. 2003; Wu et al. 2004).

Concluding remarks

In conclusion, significant progress has been made in deciphering the cellular and molecular mechanisms underlying the morphogenesis of the facial skeleton.

Among the most important findings made during the last 40 years was that the contribution of the NC to the vertebrate head is far more significant than was believed from the results of the classical and pioneering work carried out during the first half of the twentieth century on the amphibian embryo (for reviews see Hörstadius, 1950 and Le Douarin, 1982).

This knowledge has been provided by the cell-tracing experiments based on the construction of quail–chick chimeras. Moreover, recent experiments have revealed that the cephalic NC is required for the development of the forebrain and midbrain (Etchevers et al. 1999; Creuzet et al. 2004).

Although NCCs reveal a great deal of plasticity (Le Douarin & Dupin, 2003; Le Douarin et al. 2004), some endogenous properties of the NC, such as *Hox* gene expression, limit this character in mesectodermal NC derivatives. As reported in this review, NC-derived mesectoderm does not develop into facial skeleton when it expresses *Hox* genes of the first four paralogous groups. Moreover, the head membrane bones can develop only from *Hox*-negative cephalic NCCs. As a population, the cephalic NCCs exhibit a high level of plasticity as they behave as an 'equivalence group' that depends upon cues arising from the pharyngeal endoderm. These cues direct the shape and orientation of the various pieces of the facial skeleton. In addition, intrinsic species-specific properties of the NCCs help to refine the size and final shape of the facial elements.

The results described above offer new perspectives on the study of how the migrating NCCs co-operate with the cells that originate from the three germ layers in constructing tissues and organs. Further efforts will be directed at deciphering more precisely and completely the role of the genetic networks and molecular pathways involved in the numerous cell-to-cell interactions that operate during NCC migration, homing and differentiation.

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