Neural crest cell plasticity and its limits

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Summary

The neural crest (NC) yields pluripotent cells endowed with migratory properties. They give rise to neurons, glia, melanocytes and endocrine cells, and to diverse 'mesenchymal' derivatives. Experiments in avian embryos have revealed that the differentiation of the NC 'neural' precursors is strongly influenced by environmental cues. The reversibility of differentiated cells (such as melanocytes or glia) to a pluripotent precursor state can even be induced in vitro by a cytokine, endothelin 3. The fate of 'mesenchymal' NC precursors is strongly restricted by Hox gene expression. In this context, however, facial skeleton morphogenesis is under the control of a multistep crosstalk between the epithelia (endoderm and ectoderm) and NC cells.

Introduction

Owing to the invasiveness of its component cells, the neural crest (NC) is a unique structure in the vertebrate embryo. There is virtually not a single organ or tissue in the vertebrate body to which cells from the NC do not contribute. Cells from this transitory pluripotent structure fulfil three main roles. First, they coordinate various visceral functions through the peripheral nervous system (PNS) and enteric nervous system (ENS), while linking these two branches of the nervous system to the brain and spinal cord. The sympathetic and parasympathetic branches of the PNS, the preganglionic neurons (which are situated in the hindbrain and spinal cord), control bowel movements and heart beat rhythm, and accompany the vascular tree down to its smallest ramifications. In this way, the NC provides the body with an efficient tool with which to adjust to environmental changes. This capacity for coping with external conditions is reinforced by hormone-producing cells of NC origin: the adrenomedullary cells, which mediate rapid reactions; and calcitonin-producing cells, which mediate longer term reactions to changes in environmental ionic composition. Second, the NC participates in protecting the body from external conditions (i.e. UV radiation), by providing the skin and its appendages with pigment cells that synthesise melanin. Finally, the NC plays a key role in building the vertebrate head. This contribution is so crucial that the acquisition of the NC by protocordate ancestors is considered to be a turning point in the evolution of the vertebrates (Gans and Northcutt, 1983). Cell lineage studies carried out in avian, mammalian and amphibian embryos over the past few decades (reviewed by Le Douarin and Kalcheim, 1999) have supported this hypothesis.

The NC arises from the lateral margins of the neural primordium. An epithelio-mesenchymal transition individualises the NC cells (NCCs) and makes them ready to migrate within embryonic tissues, the extracellular matrix of which is permissive for cell migration. Changes in environmental conditions that inhibit their movement, as well as changes to the NCCs themselves, result in their homing to specific sites in the embryo where they aggregate and differentiate.

The multiple roles of the NC and the ubiquitous character of its derivatives co-exist with a striking level of plasticity of the NCCs, both during development and even after NC-derived structures have fully differentiated. However, this plasticity (i.e. the ability of NCCs to adjust to environmental conditions during development) is not equally distributed along the neural axis.

Here, we review the migration pathways that are followed by NCCs and the fate that they adopt during normal development. The experimental evidence for the plasticity exhibited by NCCs in the embryo in vivo and for the presence of quiescent precursors in NC derivatives until late in development will be provided. We review the major contribution that NCCs make to vertebrate head development and its complex morphogenesis. Finally, we discuss in vitro studies that have provided insights into the environmental cues that influence NCC fates and that have given rise to a model of cell lineage segregation during NC ontogeny.

Plasticity of PNS and ENS NC precursors

The quail-chick chimera system (see Box 1) has been used over the years to establish a fate map of the NC along the neural axis. These studies have shown that melanocytes arise from the entire length of the NC in higher vertebrates, whereas mesectodermal derivatives originate only from the cephalic NC region. NC-derived cells that contribute to the PNS and ENS arise only from some areas of the neural axis (Fig. 1).

Using the quail-chick system, well-defined areas of the NC have been exchanged to assess NCC plasticity (see Fig. 1). For example, in one study, the vagal region of the NC (which is located between somites 1 and 7, and gives rise to the enteric ganglia) was exchanged with NC from between somites 18 and 24 (which gives rise to the adrenal medulla and sympathetic ganglia) (Le Douarin and Teillet, 1974). This swap resulted

Box 1. The quail-chick chimera system

The quail-chick chimera system was first used to establish a fate map of neural crest (NC) derivatives along the anteroposterior neural axis (see Fig. 1). This system was devised by Nicole Le Douarin, who noticed that the interphase nuclei of all embryonic and adult cells in the Japanese quail (*Coturnix coturnix japonica*) contained a large amount of heterochromatin (Le Douarin, 1969; Le Douarin, 1973a; Le Douarin, 1973b). This is rare, as heterochromatin is usually evenly distributed within the nucleoplasm of animal cells, particularly in the chick. Thus, this feature of quail cells allowed them to be distinguished from chick embryonic cells in tissues grafts performed in ovo.

This system was used to determine the origin of NC derivatives, first by ablating a particular region of the neural tube or neural fold before the onset of NC cell migration in a chick (or quail) embryo. The region was then replaced by the equivalent region from a stage-matched quail (or chick) embryo. Quail cells were identified by Feulgen reaction or by species-specific monoclonal antibodies (see Le Douarin and Kalcheim, 1999).

in the normal colonisation of the suprarenal gland and sympathetic ganglia by NCCs fated to colonise the gut. However, although the adrenomedullary trunk NCCs invaded the pre-umbilical gut wall and differentiated into normal enteric plexuses, they failed to reach the post-umbilical bowel (Le Douarin et al., 1975).

This experimental system has since been used together with various molecular markers, such as the Schwann cell myelin protein (SMP), which is present on Schwann cells but not on other PNS and ENS glial cells, to allow a more refined analysis of NCC plasticity. These studies have shown that NCC differentiation into a specific type of glia depends upon the environment in which they develop (Dulac et al., 1988; Dulac and Le Douarin, 1991; Cameron-Curry et al., 1993). Similarly, the differentiation of the various types of autonomic neurons varies according to the milieu in which they differentiate (for reviews, see Le Douarin, 1982; Le Douarin and Kalcheim, 1999).

The conclusion of these heterotopic grafting experiments was that the fate of the NCCs that form the PNS and ENS is not fully determined before these cells migrate, but instead remains plastic until they receive differentiation signals at the end of, and possibly during, their migration. This finding raised the issue of whether all the precursors of PNS ganglion cells became fully differentiated and/or committed soon after reaching their sites of arrest, or whether some remained as quiescent undifferentiated cells. This was explored in the experiments discussed in the following section.

Undifferentiated precursors in PNS ganglia

To investigate the developmental potentials of PNS ganglion cells, fragments of sensory and autonomic ganglia from quail embryos, taken from embryonic day (E) 4 up to the end of the incubation period, were implanted into NCC migration pathways of E2 chick hosts when their own NCCs were migrating. The grafted neurons themselves died (probably because the necessary survival factors are not present in the younger host). However, the non-neuronal cells of implanted ganglia migrated and homed to host sensory and autonomic ganglia, where they differentiated into the types of neurons and glia corresponding to their novel environment (Ayer-Le Lièvre and Le Douarin, 1982; Schweizer et al., 1983; Dupin, 1984; Fontaine-Pérus et al., 1988).

These results show that, after completion of gangliogenesis, the non-neuronal cell population of PNS ganglia contains undifferentiated pluripotent cells that can be triggered to proliferate, re-migrate and differentiate by the novel environment of a younger host. These cells may be considered as putative NC stem cells, an interpretation that has since been confirmed by in vitro culture experiments (see below).

Mesectodermal NCC potentialities and patterning cues

The fate map of the NC revealed that the developmental potentials of the cephalic NCCs were greater than those of the trunk NC, as these cells provided the head with mesenchymal cells. These cells were designated as ‘mesectoderm’ or ‘ectomesenchyme’ by Julia Platt in 1893, in order to distinguish them from the mesenchyme that is derived from the mesodermal germ layer. The mesectoderm, which has turned out to play a crucial role in vertebrate head development, has many unique developmental characteristics.

Derivatives of the mesectoderm

The replacement of the cephalic NC by its quail counterpart in chick embryos showed that the facial and visceral skeleton, including the hyoid cartilages, as well as the frontal, parietal and squamosal bones, are NC derived; only the occipital and otic (partly) regions of the skull are of mesodermal origin (Le Lièvre, 1974; Le Lièvre and Le Douarin, 1974; Le Lièvre and Le Douarin, 1975; Johnston et al., 1974; Noden, 1975; Noden, 1978; Couly et al., 1993; Couly et al., 1996; Köntges and Lumsden, 1996) (Fig. 2A,B). Moreover, much of the dermis, all of the connective components of facial musculature (such as the tendons) and the wall (except endothelium) of the blood vessels that irrigate the face and forebrain are NC derived (Etchevers et al., 1999) (Fig. 2C,D; Box 2). The cephalic NCCs also yield the meninges of the forebrain and participate in the conotruncal structures of the heart (Le Lièvre and Le Douarin, 1975; Etchevers et al., 2001) (Fig. 2C-E). The contribution of the NC to the heart has been studied in detail by Margaret Kirby and co-workers, who designated the NC from the last rhombomeres as being ‘cardiac’ NC (Kirby et al., 1983; Kirby et al., 1985; Kirby and Waldo, 1995).

Mesectodermal potentialities are expressed by the trunk NC of lower vertebrates, which, for example, generates the dorsal fin of teleosts (Raven, 1931; Raven, 1936; Smith et al., 1994). However, this is not the case in amniotes. Thus, when the quail trunk NC is orthotopically implanted in the chick, no quail mesenchymal cells are ever present in the host. Moreover, if the NC cephalic domain is entirely replaced by trunk NC, no mesectodermal cells develop from the graft. But when trunk NCCs are grafted to just one side of the cephalic region, donor NCCs migrate together with host skeletogenic cephalic NCCs and differentiate into myofibroblasts and connective tissue cells. However, they never participate in skeletogenesis (Nakamura and Le Lièvre, 1982). Therefore, it seems that the capacity to yield mesenchymal cells has not completely disappeared during vertebrate evolution.

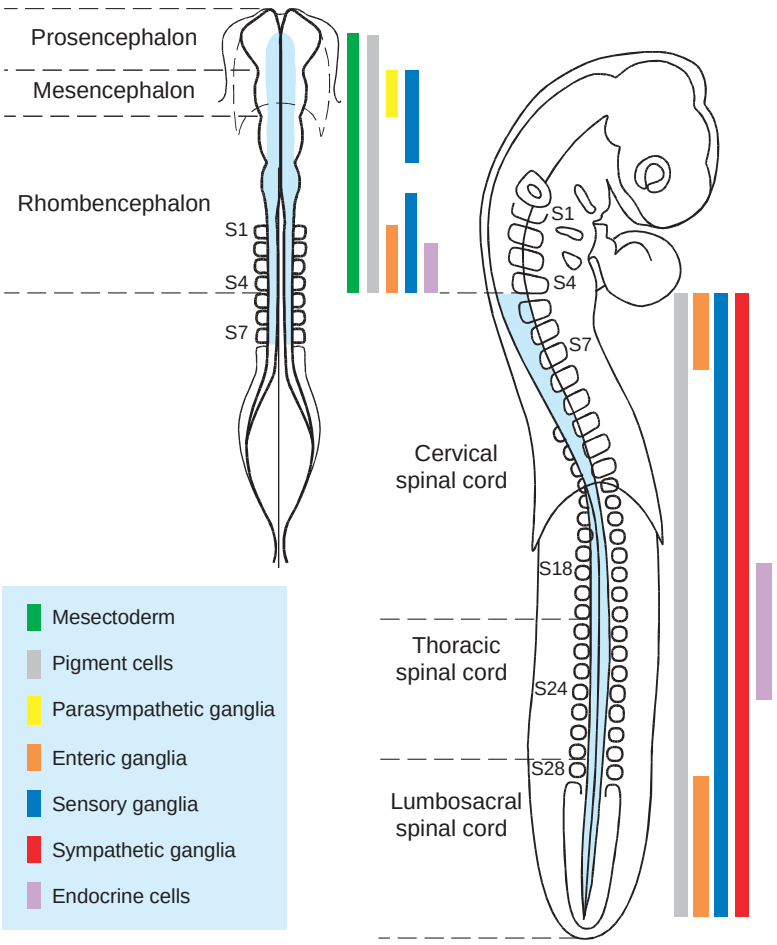

Fig. 1. Fate map of the neural crest-derived phenotypes along the neural axis. The various cell phenotypes yielded by neural crest (NC) cells at different anteroposterior levels of the neural fold (light blue) are shown in chick embryos of 7 (left) and 28 (right) somites (S). Left, tissues that arise from the cephalic NC; right, tissues that arise from the trunk NC in cervical, thoracic and lumbosacral regions of the spinal cord. The region that gives rise to mesectoderm (green) extends from the level of mid-diencephalon down to rhombomere (r) 8 (corresponding to S4). Melanocytes (grey) are produced along the entire length of the neural axis. The parasympathetic ciliary ganglion (yellow) derives from the mesencephalic NC. Enteric ganglia (orange) arise from both vagal (S1-S7) and lumbosacral (posterior to S28) NC. Caudal to S4, the trunk NC yields PNS sympathetic ganglia (red), whereas the sensory ganglia (dark blue) are generated by the mesmetencephalic NC and by the NC from posterior rhombencephalic to lumbosacral levels. Endocrine cells (violet) originate from the NC of S2-S4 and S18-S24 levels.

Recent experiments have shown that long-term in vitro culture of avian trunk NCCs can trigger their differentiation into cartilage (McGonnell and Graham, 2002; Abzhanov et al., 2003). Moreover, mouse trunk NC explants yield dentine and bone when recombined with branchial (pharyngeal) arch 1 (BA1) epithelium in intraocular grafts (Lumsden, 1988). Therefore, although in normal development, the ability of the NC to form mesectoderm is restricted to the cephalic part of the neural axis in higher vertebrates, a hidden capacity of trunk NC to yield mesenchymal cells can be revealed by appropriate environmental cues. In support of this notion, clonogenic cells from trunk NC generate myofibroblasts and neural-melanocytic cell types in vitro (Shah et al., 1996; Trentin et al., 2004).

Interestingly, the ability to form skeletal tissue is not uniformly distributed within the cephalic NC (Fig. 3A,B). Its rostral area, which extends from the mid-diencephalic level down to rhombomere 2 (r2), is the only part that participates in forming facial skeleton and the skull. More caudally, the NC from r4 to r8 yields medial and posterior parts of the hyoid bone and no membrane bone. The hinge between these two domains lies in r3, which gives rise to a relatively small number of NCCs that become distributed to both BA1 and BA2 (Birgbauer et al., 1995; Couly et al., 1996; Köntges and Lumsden, 1996).

The prosencephalic and anterior diencephalic neural fold does not undergo epithelio-mesenchymal transition, and yields epithelial, glandular (adenohypophysis) and neural (olfactory placode) structures (Couly and Le Douarin, 1987).

Hox genes and development of NC-derived skeleton

It is striking that the rostral and caudal cephalic NC domains defined above differ in their expression of the Hox genes. As first established in the mouse (Hunt et al., 1991), and later confirmed in the chick (Prince and Lumsden, 1994; Couly et al., 1996), the caudal domain of the cephalic NC expresses Hox genes of the four first paralogous groups, whereas in the rostral domain, which yields the facial skeleton, these Hox genes are not expressed (Fig. 3A,B). Membrane bones arise only from Hox-negative skeletogenic NCCs, whereas cartilage originates from both Hox-positive and Hox-negative NC.

Accordingly, two interesting features have been revealed concerning the role of Hox genes in patterning head NC derivatives. First, it has been shown that, if expression of *Hoxa2* is experimentally induced in all BA1 tissues (i.e. in the ectoderm, NC, mesoderm and endoderm of BA1), partial homeotic transformation of BA1 into BA2 is observed in the chick (Grammatopoulos et al., 2000) and in *Xenopus* (Pasqualetti et al., 2000). By contrast, if *Hoxa2*, *Hoxa3* or *Hoxb4* are individually transfected into the rostral domain of the cephalic NC, the ability of NCCs to differentiate into skeletal structures is abolished (completely for *Hoxa2*, and partly for *Hoxa3* and *Hoxb4*) (Creuzet et al., 2002). Therefore,

the environment in which these NCCs develop is crucial for specifying their fate, and Hox genes play a role in this respect.

In quail-chick combinations, Hox-positive NCCs surrounded by a Hox-negative environment are able to yield neural and melanocytic derivatives only, and do not develop into skeletal tissue of any kind (cartilage or membrane bone). These cells cannot, therefore, substitute for Hox-negative NCCs in facial skeletogenesis (Couly et al., 1998; Couly et al., 2002) (Fig. 3C-F). By contrast, Hox-negative NCCs that are transplanted caudally can replace the Hox-positive cells and yield normal hyoid bone (Couly et al., 1998). Within the Hox-negative NC rostral domain, a great deal of regulation can occur: a fragment as small as one-third of the neural fold is able to build up a complete facial skeleton (Couly et al., 2002) (Fig. 3G,H). The Hox-negative NC rostral domain (or FSNC, for facial skeletogenic NC) thus behaves as an 'equivalence group' (as far as its ability to construct the facial skeleton is concerned), as each of its parts appears to have similar developmental potentialities.

This idea is at odds with the proposed interpretation of an experiment carried out in 1983 by Drew Noden. In this study, the heterotopic transplantation of NC that is normally fated to colonise BA1 to the mid-rhombencephalic level (roughly r4-r5), resulted in the partial duplication of the lower jaw skeleton, together with an additional lower beak rudiment (Noden, 1983). According to the author, this indicated that NCCs themselves possess the information for patterning the facial skeleton. However, when similar experiments were carried out, in which only neural fold tissue was transplanted (without the neural tube attached to it), the transposed NCCs were found to participate in formation of the hyoid bone, and no jaw duplication ever occurred (Couly et al., 1998). Noden's result could be reproduced, however, when dorsal neural tube from the posterior mesencephalon region was included in the graft, together with the corresponding NC.

These findings led Trainor et al. (Trainor et al., 2002) to propose that a signalling molecule, fibroblast growth factor 8 (FGF8), which is produced by the neural tube at this level (at the midbrain-hindbrain junction), could be responsible for inducing this jaw duplication.

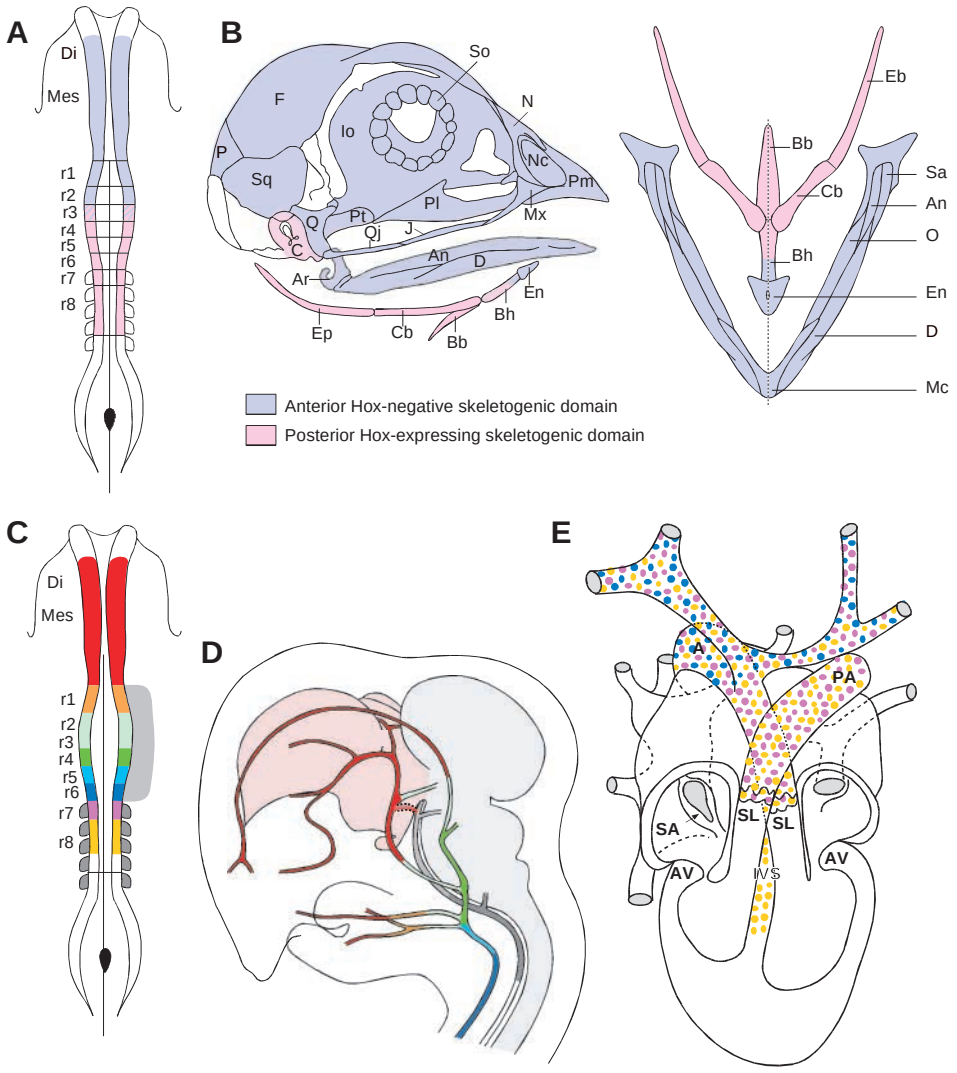

Fig. 2. Cephalic neural crest contribution to head skeleton, vasculature and to conotruncal structures of the heart. (A) Fate map of the cephalic neural crest (NC) in five-somite stage (ss) chick embryo. The anterior neural fold domain extending from mid-diencephalon down to rhombomere (r) 2 (in light blue) yields Hox-negative NC cells (NCCs) only, while the posterior one (in pink), generates Hox-positive NCCs [reproduced, with permission, from Couly et al. (Couly et al., 1996)]. Both are present in r3. (B) Respective contribution of Hox-negative and Hox-positive NC domains to the craniofacial and hypobranchial skeleton. (C) Refined colour-coded map of the cephalic NC levels at 5 ss and (D) their contribution to the musculo-connective wall of the head vascular tree. Prosencephalic meninges (pink) derive from diencephalic-mesencephalic (Di-Mes) NCCs, whereas meninges in the mesencephalon and more caudal CNS (light grey) originate from the mesoderm (light grey in C). (E) Relative contribution to the conotruncal structures of the heart of r6 to r8 cardiac NCCs [reproduced, with permission, from Etchevers et al. (Etchevers et al., 1999; Etchevers et al., 2001)]. A, aorta; An, angular; Ar, articular; AV, atrioventricular valve; Bb, basibranchial; Bh, basihyal; C, columella; Cb, ceratobranchial; D, dentary; Di, diencephalon; Eb, epibranchial; En, entoglossum; F, frontal; Io, interorbital septum; IVS, intraventricular septum; J, jugal; Mc, Meckel's cartilage; Mes, anterior mesencephalon; Mx, maxillary; N, nasal; Nc, nasal capsule; O, opercular; P, parietal; PA, pulmonary artery; Pl, palate; Pm, premaxilla; Pt, pterygoid; Q, quadrate; Qj, quadratojugal; r, rhombomere; SA, sinoatrial valve; Sa, supra-angular; SL, semilunar valve; So, sclerotic ossicles; Sq, squamosal.

It thus appears that the developmental potentials of the NCCs are restricted by Hox gene expression. This concurs with the fact that targeted mutation of *Hoxa2* in mice leads to partial duplication of the lower jaw at the expense of the normal BA2 skeleton (the hyoid cartilage) (Gendron-Maguire et al., 1993; Rijli et al., 1993; Kanzler et al., 1998; Ohnemus et al., 2001).

In the lamprey (*Lampetra fluviatilis*), a jawless vertebrate, the forepole of the embryo does not express *Hox3* (Murakami et al., 2004), whereas, as observed by Cohn (Cohn, 2002), the *HoxL6* gene (the lamprey homologue of vertebrate *Hoxb6*) does not obey the colinearity rule (between Hox gene organisation in the chromosome and the anterior limit of their expression in the embryo). The *HoxL6* expression domain reaches the rostral-most part of the embryo. It is thus tempting to interpret this molecular feature as being responsible for the absence of the jaw in the agnathes. However, this is unlikely because, in another species of lamprey (*Lethenteron japonicum*), although no jaw develops, *HoxL6* expression does not reach such a rostral domain (Takio et al., 2004).

FGF8: a key signalling molecule in facial skeletogenesis

The role of FGF8 in facial skeleton development has recently been demonstrated in the chick. As mentioned above, removal of FSNC at the 5- to 6-somite stage (ss) results in the failure of facial skeleton development. This is accompanied by a striking decrease of *Fgf8* expression in the prosencephalon and BA ectoderm as early as 24 hours after surgery (Fig. 4A-C). If these operated embryos are treated with exogenous FGF8 on heparin-acrylic beads placed on the surface of the presumptive BA1 ectoderm, much of the facial skeleton, including the lower jaw, regenerates: NCCs derived from r3 (r3-NCCs) are the unique source of regenerating cells (Creuzet et al., 2004) (Fig. 4D-G). During normal development, r3-NCCs participate very little in the formation of the lower jaw. Thus, in the experiments already described, FSNC removal eliminates the apoptotic effect that is normally exerted by r2 on the r3 neural fold (Graham et al., 1993; Graham et al., 1994; Ellies et al., 2000), but this is not sufficient to promote lower jaw regeneration by r3-NCCs. By contrast, if exogenous FGF8 is added, r3-NCCs exhibit enhanced survival and proliferation, and can provide enough cells to BA1 to regenerate a complete jaw skeleton.

Interestingly, these experiments indicate that reciprocal relationships exist between the NCCs and the ectodermal epithelial structures in which *Fgf8* is activated. Although NCCs need FGF8 to survive and proliferate, they, in turn, trigger the induction/maintenance of *Fgf8* expression in the forebrain neuroepithelium, and in the superficial ectoderm of the forebrain and BAs (Creuzet et al., 2004).

One can therefore conclude that the facial skeleton can form exclusively from the Hox-negative NC rostral domain. Moreover, within this domain, significant plasticity and regeneration capabilities exist, meaning that the cephalic NCCs do not possess the patterning information that is necessary to shape and position the various elements of the skeleton. This raises the issue of where such patterning information originates. Recent investigations have indicated the involvement of the pharyngeal endoderm and facial ectoderm, as discussed below.

Pharyngeal endoderm in facial skeleton morphogenesis

That the pharyngeal endoderm plays a role in facial skeleton development became apparent when defined regions of endoderm were surgically removed in 5-6 ss chick embryos, resulting in the absence of facial skeletal elements (Couly et al., 2002). Using this approach, defined areas of the endoderm were identified as being necessary for the development of the nasal septum, Meckel's cartilage, articular and quadrate cartilages, and the anterior part of the hyoid complex. These findings were confirmed when stripes of pharyngeal endoderm were grafted from stage-matched quail embryos into the migration pathway of cephalic chick NCCs, causing the duplication of the corresponding skeletal pieces (Fig. 5A). The extra cartilages that formed in contact with the quail endoderm were made up of chick cells, meaning that they resulted from the induction of the host NCCs by the grafted endoderm (Fig. 5B-F). Further experiments showed that, in addition to being essential for shaping cartilage rudiments, signals from the ventral foregut endoderm also dictate the position that is adopted by facial cartilages 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).

Transplanted NCCs keep species-specific characters

Notwithstanding the role of the pharyngeal endoderm in determining which bone will be formed at a given place in the face and how it will be orientated with respect to body axis, the NCCs themselves also possess a certain amount of

Box 2. Mesectodermal derivatives of the neural crest

The cephalic neural crest (NC) provides the head with diverse mesenchymal derivatives that form the so-called 'mesectoderm or ectomesenchyme', to distinguish them from mesenchymal cells derived from the mesoderm.

Cephalic neural crest (NC)
The cephalic NC gives rise to the cranial skeleton and other tissues of the head and neck (Fig. 2).

Skeleton
Dermatocranium: Frontal, Parietal, Squamosal, Sphenoid (basipre-), Otic capsule (partly), Nasal, Vomer, Maxilla, Jugal, Quadratojugal, Palatine, Pterygoid, Dentary, Opercular, Angular, Supraangular
Chondrocranium: Nasal capsule, Interorbital septum, Scleral ossicles, Meckel's cartilage, Quadrate, Articular, Hyoid, Columella, Entoglossum
Odontoblasts and tooth papillae

Other tissues
Dermis, smooth muscles, adipose tissue of the skin over the calvarium and in the face and ventral part of the neck
Musculo-connective wall of the conotroncus and all arteries derived from aortic arches (except endothelial cells)
Pericytes and musculo-connective wall of the forebrain blood vessels and all of the face and ventral neck region
Meninges of the forebrain
Connective component and tendons of ocular and masticatory muscles
Connective component of the pituitary, lacrymal, salivary, thyroid, parathyroid glands and thymus

Trunk NC
Dorsal fins in lower vertebrates

information that influences the shaping of the face, as recently demonstrated (Schneider and Helms, 2003; Tucker and Lumsden, 2004).

In one such study (Schneider and Helms, 2003), cephalic NCCs were exchanged orthotopically between quail and duck embryos at Hamburger and Hamilton stage 9.5 (HH9.5) (Hamburger and Hamilton, 1951), and the chimeras were compared with normal birds of the two species at HH37-39 (Fig. 6A-D). In the duck-to-quail (‘duail’) combination, and when the host cranial region was abundantly colonised by donor NCCs, the beak was enlarged (especially the upper beak), making the ‘duail’ beak look more like a duck beak than a quail beak (Fig. 6D). In the reverse combination (quail NCCs into duck), the chimeric ‘quck’ beak adopted a quail-like morphology (Fig. 6C). The incubation times differ between duck and quail experiments and are 28 and 17 days, respectively. The authors took advantage of molecular markers that are expressed at different developmental stages in the two species to examine the interactions between the ectoderm and NCCs in these grafts. When grafted into a foreign host, NCCs maintained their own temporal and spatial gene expression patterns and seemed to impose on the host ectoderm a donor- rather than a host-like gene expression pattern, such as that of *Shh* and *Pax6* (Schneider and Helms, 2003).

Quail-duck NC chimeras were also constructed by Tucker and Lumsden (Tucker and Lumsden, 2004). In these experiments, the levels of the exchanged neural folds and the stages at which the grafting operations were performed were precisely defined; the morphology of the quail cartilages that developed within the duck environment (and vice versa) was studied. The results showed 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, as a result of species-specific differences in growth rate (Fig. 6E,F). Therefore, once induced by the endoderm to develop into a particular cartilage, NCCs follow a species-specific genetic program involving a particular growth 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. Indeed, further studies showed that where *Shh* and *Fgf8* expression domains abut in the frontonasal ectoderm, a signalling centre for positioning and refining the shape of NC-derived skeletal pieces forms, called the frontonasal ectodermal zone (FEZ) (Hu et al., 2003). The FEZ is required for the outgrowth of the underlying mesenchyme. Heterotopic FEZ transplantations cause the duplication of beak distal elements, the polarity of which is controlled by the position of the rotated or supernumerary FEZ.

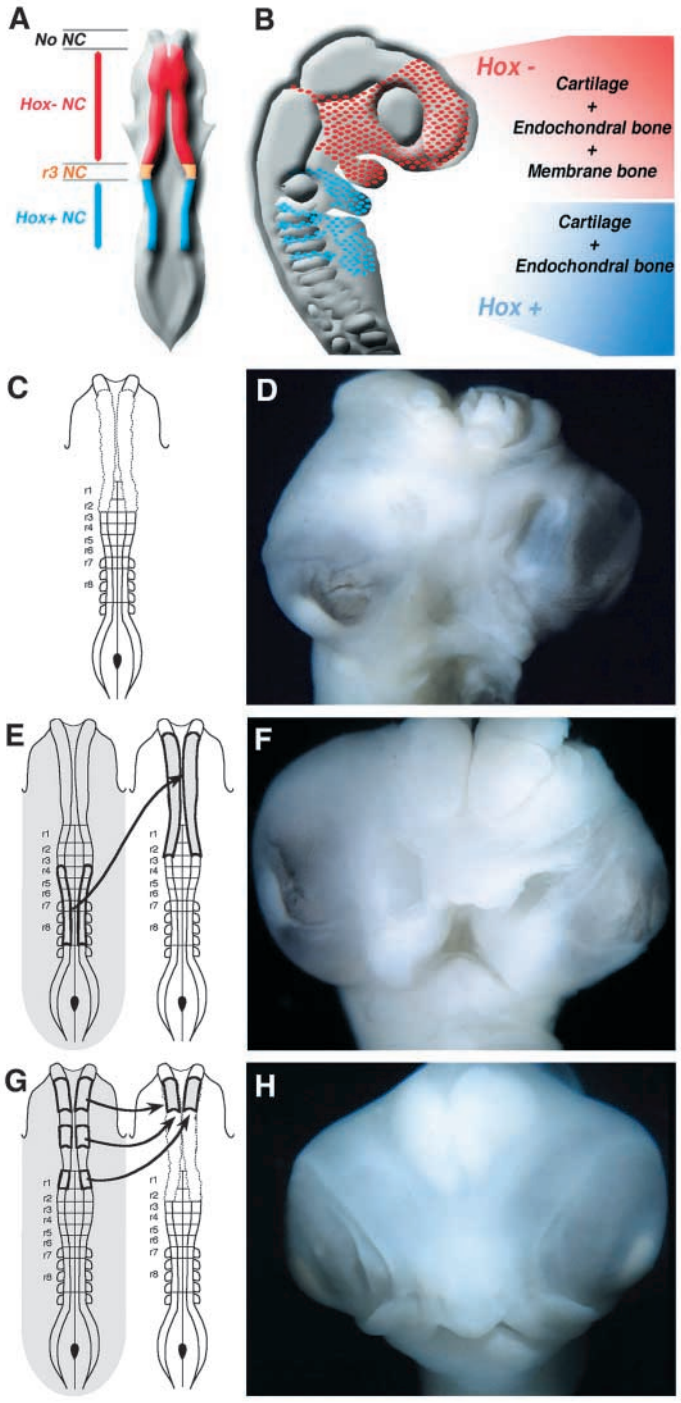

Fig. 3. Hox gene expression restricts skeletogenic properties of the cephalic neural crest. (A) In a 5-somite stage (ss) chick embryo, the cephalic neural crest (NC) is divided into an anterior Hox-negative (Hox$^-$) domain (red) and a posterior Hox-positive (*Hox*$^+$) domain (blue). The transition between these two domains corresponds to rhombomere (r) 3 (orange). The neural fold rostral to the mid-diencephalon does not produce NC cells (NCCs). (B) Postmigratory Hox$^-$ NCCs (red) yield cartilages, as well as endochondral and membrane bones of the entire upper face and jaws. By contrast, skeletogenic functions of Hox$^+$ NCCs (blue) are limited to chondrogenesis and endochondral ossification in the hyoid structure. (C-H) Facial development at embryonic day (E) 7 after resection and/or exchange of cephalic NC domains in 5 ss chick embryo. The removal of Hox$^-$ FSNC (facial skeletogenic neural crest; broken lines) (C) abolishes head development (D). Replacement of FSNC by Hox$^+$ neural fold (E) severely hampers head morphogenesis (F). Following removal of whole FSNC (as in E) (G), implantation of only a fragment of the FSNC (from either di-, mes- or metencephalic level) restores normal development of complete face and forebrain (H). Reproduced, with permission, from Couly et al. (Couly et al., 2002).

These data imply that a subset of the NCC population, which can be recruited for skeletogenesis by local ectoderm, is pre-patterned while retaining some degree of plasticity.

In vitro analysis of NCC potentialities

Over the past few years, in vivo experiments have been carried out to address the issue of NC pre-patterning versus its plasticity. By changing the fate of NCCs through transplantation or by modifying their gene expression patterns, these studies have looked at the behaviour of NCC populations. How individual NCCs integrate patterning signals to account for differentiation and morphogenesis could not be revealed by these studies. Systems for culturing single NCCs and early phenotype-specific markers were developed in order to determine whether diversified NCC types arise from a differentiation choice by multilineage progenitors or through the selection of early committed precursors.

Pluripotent stem cells in trunk NC

During recent decades, assays of avian NCCs, which have been clonally propagated from single cells isolated as they migrate from explanted trunk neural primordium, have been instrumental in revealing the existence of a variety of pluripotent NC progenitors (Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989; Sieber-Blum, 1991; Dupin and Le Douarin, 1995; Lahav et al., 1998). Altogether, these studies have shown that the trunk NC contains progenitors that can give rise both to pigment cells, glial cells and several types of PNS neurons, thus recapitulating the repertoire of trunk NC derivatives.

These assays have also been carried out on rat and mouse trunk NC, where similar pluripotent progenitors have been identified (Stemple and Anderson, 1992; Ito et al., 1993; Rao and Anderson, 1997; Paratore et al., 2001). By studying those progenitors that give rise to glia, autonomic neurons and myofibroblasts, Stemple and Anderson showed, for the first time, that these NCCs self-renew, a unique characteristic of ‘stem cells’ (Stemple and Anderson, 1992).

True stem cell properties have also been demonstrated in avian species. Bipotent precursors with the ability to generate glia and melanocytes (GM) or glia and myofibroblasts (GF) have been isolated that can self-renew in vitro through successive rounds of subcloning (Trentin et al., 2004).

Common mesectodermal and neural-melanocytic lineage progenitors in cephalic NC

The clonal analysis of quail NCCs grown on feeder layers of 3T3 fibroblasts has also been instrumental in revealing the developmental potential of cephalic NCCs. In addition to tissues that arise from both trunk and cephalic NC, mesencephalic-rhombencephalic NCCs in culture also give rise to mesectodermal derivatives, such as cartilaginous cells and myofibroblasts (Baroffio et al., 1988; Baroffio et al., 1991; Dupin et al., 1990; Trentin et al., 2004). Cells with the potential to develop into mesenchymal, as well as neuronal, glial and melanocytic, cells co-exist in some subsets of clonogenic cells that are identified as pluripotent and bipotent progenitors (Fig. 7). Such progenitors can give rise both to neural-melanocytic and to mesenchymal derivatives, but constitute a relatively small proportion (7%) of the clonogenic migratory NCCs (see Table S1 in the supplementary material). Thus, it is possible that some precursors are restricted to one or the other of these fates (i.e. neural-melanocytic or mesectodermal) prior to cephalic NCC emigration.

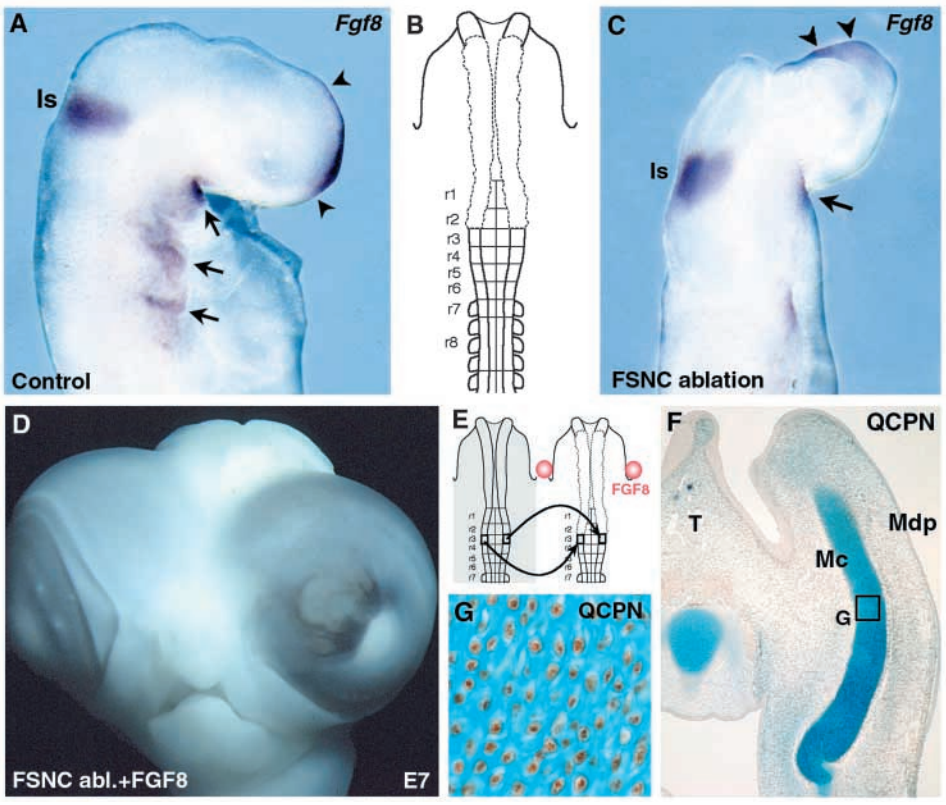

Fig. 4. FGF8 promotes facial regeneration. (A) In a normal chick, at embryonic day 2 (E2), *Fgf8* is expressed in the branchial arches (BAs, arrows) and nasofrontal (arrowheads) ectoderm, as well as in the neuroectoderm of the isthmus (Is) and prosencephalon. (B,C) After ablation of the FSNC (facial skeletogenic neural crest) at the 5-somite stage (ss) (B), *Fgf8* expression is dramatically reduced both in BA1 (arrow) and forehead territories (arrowheads) (C). (D) Implantation of FGF8-soaked beads at the presumptive level of BA1 ectoderm following ablation of FSNC (D) induces regeneration of facial and cephalic structures. (E-G) Role of the rhombomere (r) 3-derived neural crest (NC) in regenerating the jaws. (E) Replacement of the r3-NC by its quail counterpart in a 5 ss chick embryo and FGF8-soaked bead implantation. (F) Skeletogenic cells in Meckel’s cartilage (Mc) are exclusively quail derived, as shown in G, which shows a higher magnification of quail cell-specific antibody against a perinuclear antigen (QCPN) staining. Mdp, mandibular process; T, tongue.

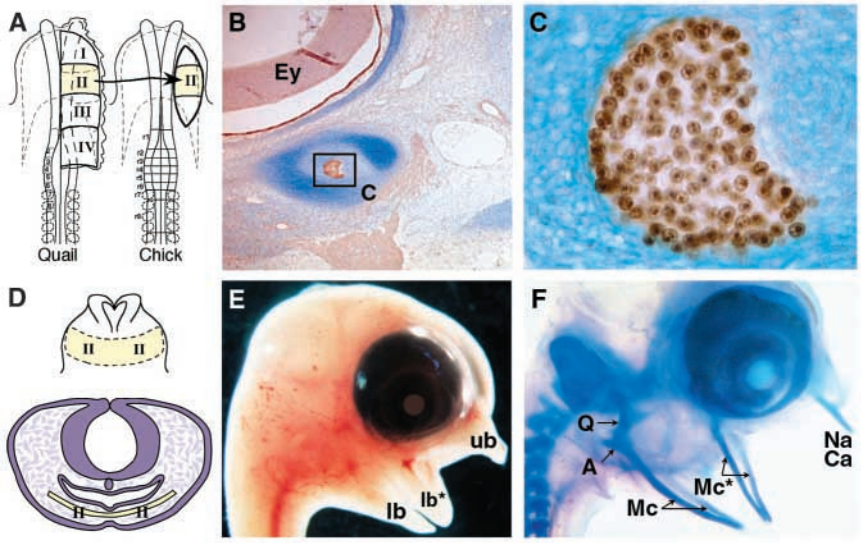

Fig. 5. The foregut endoderm patterns the neural crest-derived skeleton. (A) Transplantation of endodermal zone II from quail into stage-matched chick neurula. (B,C) Sections of the operated embryonic day (E) 6 host after detection of quail cells [quail cell-specific antibody against a perinuclear antigen (QCPN), brown] and cartilage staining (Alcian Blue). (B) Laterally engrafted endodermal cells have induced ectopic differentiation of chondrogenic cells, which all derive from host mesectoderm (see enlargement in C). (D) Bilateral grafting of quail endodermal stripe (II) implanted ventrally into a stage-matched chick embryo. (E) At E9, the host shows an additional lower beak apparatus (lb*) interposed between the endogenous upper (ub) and lower (lb) components of the host bill. (F) This supernumerary structure is accompanied by an additional Meckel's cartilage (Mc*) (Alcian Blue staining). Reproduced, with permission, from Couly et al. (Couly et al., 2002). A, articular; Ey, eye; NaCa, nasal capsule; Q, quadrate.

Similar in vitro experiments performed using different culture conditions and sets of lineage markers have revealed the existence of common progenitors for neurons, pigment cells, and myofibroblasts and chondrocytes in the cardiac NC of quail (Ito and Sieber-Blum, 1991) and mouse (Youn et al., 2003). Even at later stages of development, and after they have colonised BA3 and BA4, quail cardiac NC-derived cells retain progenitors that can generate both serotonergic neurons and myofibroblasts and/or chondrocytes in vitro (Ito and Sieber-Blum, 1993).

These results demonstrate that mesectodermal lineages are not completely segregated from the other 'trunk-like' lineages in the cephalic NC, even at migratory stages. Moreover, they argue against the contention that the mesectoderm is derived from a lineage that is totally separated from 'authentic' NC because it arises not from the neural fold itself, but through the early delamination of the cephalic ectoderm (Weston et al., 2004).

Therefore, in both mammals and birds, the developmental potential of the mesectoderm is a true property of head neurectodermal NCCs. As documented above, this capacity to yield mesenchymal cells is shared by a subset of pluripotent progenitors able to differentiate along some or all kinds of other NC-derived lineages. In addition to pluripotent cells, the trunk and cephalic NC of the quail has also been shown to give rise to partially restricted precursors and precursors already specified to a single phenotype. These data, which are summarised in Fig. 7, also suggest that progressive restrictions in the ability of NCCs to differentiate into different cell types underlie the segregation of cell lineages during NC ontogeny. Another striking result is that all the intermediate, including bipotent, precursors recorded were able to yield glial cells, indicating that the gliogenic differentiation potential of NCCs might constitute a general NCC 'marker'.

Restrictions of NCC developmental potentials

As reviewed above, populations of clonogenic NCCs display heterogeneous proliferative and developmental potentials. The fact that single NCCs grown under the same environmental conditions (whatever these conditions might be) behave either as multipotent, bipotent or unipotent progenitors implies that lineage restrictions operate at early migratory stages. A similar conclusion was drawn from in vivo lineage-tracing studies of individual NCCs in avian and zebrafish embryos (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Raible and Eisen, 1994).

To investigate the sequential restrictions that might be imposed on trunk NCCs, single quail NCCs were labelled at various times after their migration from neural primordium explanted in vitro (Henion and Weston, 1997). Under these conditions, which do not necessarily mimic the normal time-course of NCC lineage segregation, 44% of clonogenic cells appeared to be already specified to yield a single derivative as rapidly as a few hours after they had left the neural primordium. However, bipotent NCCs that generate both neurons and glia, or glia and melanocytes, were still present in the cultures up to 30 hours after NCC migration had begun (the latest time point examined). The completion of the segregation of neurogenic precursors occurred later than did the production of melanocytic fate-restricted cells. In the same experimental system, the specification of NCCs to produce melanocytes correlated only with the surface expression of the Kit receptor, whereas another subset of NCCs, which were able to differentiate into neurogenic but not melanogenic cell types, was identified as expressing the tyrosine kinase receptor, TrkC (Luo et al., 2003).

A growing body of data supports the early restriction of sensory ganglion cells among the derivatives of the NC that populate the PNS. In vitro and in vivo studies in birds first suggested that the sensory PNS lineage is segregated earlier than the autonomic one (Ziller et al., 1983; Ziller et al., 1987; Le Douarin, 1986). Although common precursors for both classes of PNS neurons have been identified in vitro and in vivo in the trunk NC (Sieber-Blum, 1989; Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989), another NCC subset has been identified that is apparently restricted to a sensory neuronal fate (Sieber-Blum, 1989), and is unable to respond to signals that promote autonomic differentiation (Greenwood et al., 1999). The basic helix-loop-helix transcription factors, neurogenins (*Ngn1* and *Ngn2*), have been implicated in the early specification of the sensory lineage (Ma et al., 1996; Ma et al., 1998; Ma et al., 1999; Fode et al., 1998; Greenwood et al., 1999;

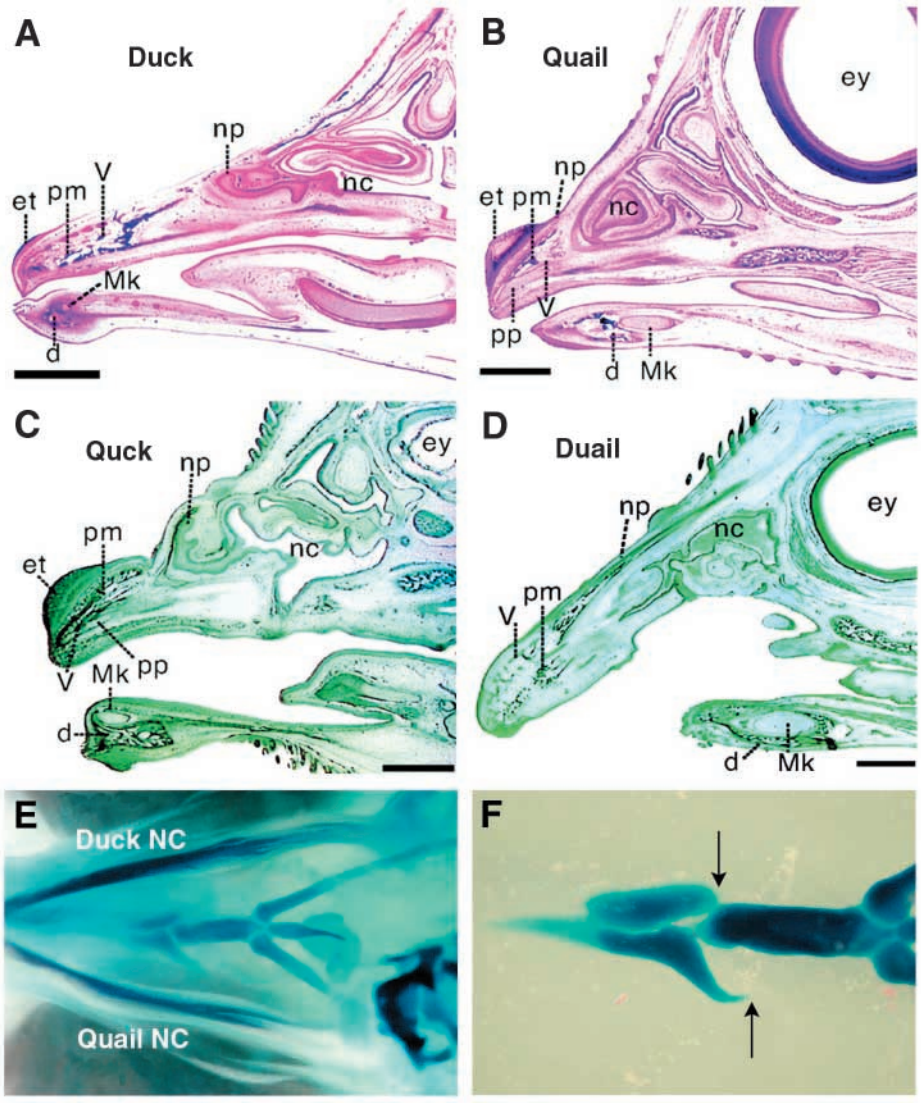

Fig. 6. Neural crest cells impart morphological features to skeletal and ectodermal structures. Sagittal sections of the beaks of control duck (A) and quail (B) embryos. (C,D) Beak sections of duck-quail chimeras after orthotopic replacement of quail neural crest (NC) into duck host ('Quck' chimera) (C), and reciprocal graft of duck NC into quail embryo ('Duail') (D), showing that the host upper bill morphology is modified according to the species origin of the NC [reproduced, with permission, from Schneider and Helms (Schneider and Helms, 2003)]. (E) Chimeric hyoid skeletal structures of a quail at embryonic day (E) 9, after unilateral replacement by duck NC (Alcian Blue staining). (F) Higher magnification of the entoglossum: on grafted side, the ipsilateral half has acquired a duck-shaped morphology [reproduced, with permission, from Tucker and Lumsden (Tucker and Lumsden, 2004)]. Arrows indicate the proximal limit of the cartilages on both sides. d, dentary; et, egg tooth; ey, eye; Mk, Meckel's cartilage; nc, nasal capsule; np, nasal passage; pm, premaxilla; pp, prenasal process; V, trigeminal sensory neurons.

Lo et al., 2002). Fate mapping studies using the Cre/*lox*P system in the mouse have revealed that NCCs that transiently express *Ngn2* are biased towards a sensory neuro-glial phenotype in vivo (Zirlinger et al., 2002). As *Ngn2* is activated in migratory (and also some premigratory) NCCs, the precise stage at which a subset of committed sensory precursors emerge from the trunk NC, remains to be defined.

Persistence of clonogenic stem cells in PNS ganglia and nerves

In vivo investigations have revealed that undifferentiated precursors that can differentiate into a variety of NCC types are present in the PNS ganglia. This has been more recently confirmed in vitro for autonomic and sensory ganglia (Duff et al., 1991; Sextier et al., 1992; Hagedorn et al., 1999; Hagedorn et al., 2000), peripheral nerves (Morrison et al., 1999; Nataf and Le Douarin, 2000; Bixby et al., 2002) and enteric plexuses (Sextier et al., 1994; Bixby et al., 2002; Kruger et al., 2002). Pluripotent progenitors were found in these sites until late in development, and even in postnatal and adult life (Kruger et al., 2002). Some of them are able to self-renew (Morrison et al., 1999; Bixby et al., 2002; Kruger et al., 2002), and therefore could be called 'stem cells'. Such cells might constitute a putative reservoir for ensuring the turnover of glia and neurons in the PNS. They may be the origin of several tumour types that affect NC-derived cells in various malignant neurocristopathies, such as neurofibromas or Schwannomas (Riccardi, 1981; Gutmann, 1994; Bolande, 1997; Ferner and O'Doherty, 2002).

Cytokines acting on NC progenitors

The results discussed above raised the question: what is the nature of the cues (intrinsic or extrinsic) that regulate the final choice of NCC differentiation? Since the in vivo studies (already described) had revealed the strong effect that the environment has on NCC fate, external cues could be considered as being crucial in this process.

Several cytokines that can act specifically on one or the other of the NC precursors have been identified (reviewed by Anderson, 1997; Le Douarin and Kalcheim, 1999; Sieber-Blum, 2000; Le Douarin and Dupin, 2003).

Mice bearing mutations that affect specific NC derivatives have been instrumental in documenting the role of various secreted proteins in NCC differentiation. For example, the

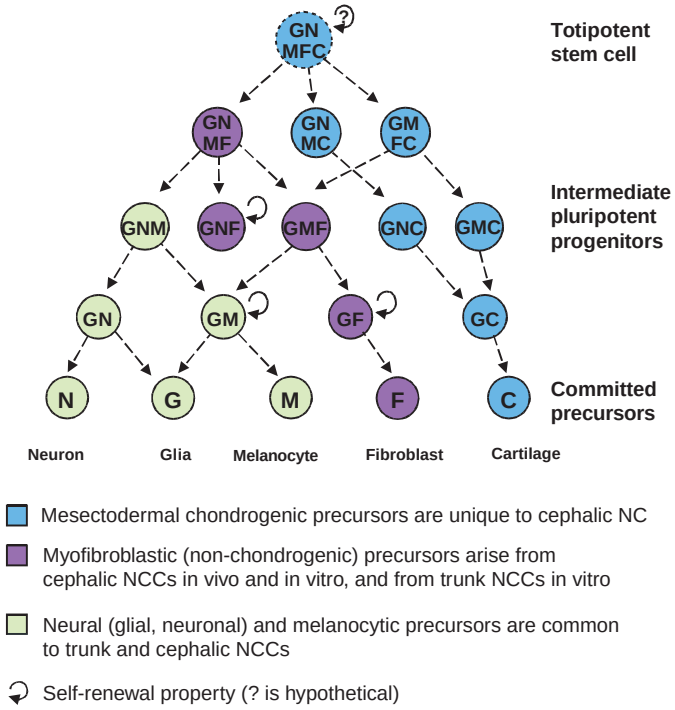

Fig. 7. Model for neural crest lineage segregation. Neural crest (NC) progenitors identified by in vitro clonal analysis are ordered according to their number of developmental potentials [data taken from elsewhere (Baroffio et al., 1991; Trentin et al., 2004)]. In the cephalic NC, neurons (N), glia (G), melanocytes (M) and mesectodermal derivatives – myofibroblasts (F) and cartilage (C) – arise from diverse 'intermediate' pluripotent and bipotent progenitors, which suggest that committed cells are generated through progressive restrictions in the potentialities of a putative 'totipotent-like' NC stem cell (broken circle). In the trunk NC, clonogenic cells endowed with chondrogenic potential (blue) are not recovered; however, various myofibroblastic (non-chondrogenic) progenitors (pink) are present as in the cephalic NC. Self-renewal was demonstrated for rat trunk GNF-like cells (Stemple and Anderson, 1992), and for quail trunk and cephalic GM and GF progenitors (Trentin et al., 2004).

mouse strains *dominant spotting* (*W*) and *steel* (*Sl*), which carry mutations of the Kit tyrosine kinase receptor and its ligand (the steel factor or stem cell factor – SCF), respectively, have revealed that SCF plays a crucial role in the survival and migration of early melanocyte precursors during their homing to the skin (reviewed by Yoshida et al., 2001). SCF is produced by the dermis in mammals (Matsui et al., 1990) and by the epidermis in birds (Lecoin et al., 1995), and activates Kit, which is expressed by NCCs that differentiate along the melanocyte pathway (Murphy et al., 1992; Lahav et al., 1994; Wehrle-Haller and Weston, 1995; Reid et al., 1995; Luo et al., 2003).

Another well-documented case is that of endothelin 3 (Edn3). The role of this peptide in the development of NCCs into melanocytic and enteric lineages was discovered during studies of the *lethal spotted* and *piebald lethal* mouse mutants, and of the targeted knocking out of *Edn3* and its receptor, *Ednrb* (endothelin receptor B), in mice (Baynash et al., 1994; Puffenberger et al., 1994; Hosoda et al., 1994; Shin et al., 1999; Lee et al., 2003). These mice display pigment cell defects and an absence of intrinsic innervation in the posterior gut, which reveals the abnormal development of melanocytes and enteric ganglia that are derived from the NC. Edn3 added at the appropriate concentration to quail NC primary or single cell cultures strongly promotes NCC proliferation (Lahav et al., 1996) and differentiation into glia and melanocytes, without significantly modifying the onset of differentiation of other lineages (Lahav et al., 1998). Interestingly, in avian species, Edn3 induces the switch from *EDNRB*, which is expressed by native NCCs (Nataf et al., 1996), to *EDNRB2*, which is exclusively active in melanocytic precursors and in differentiated pigment cells (Lecoin et al., 1998).

Edn3 produced in the skin and gut wall is an important factor not only for melanocyte, but also for ENS, development. When *Edn3* or *Ednrb* are inactivated in mice, the posterior bowel is not colonised by NCCs. Such a failure of gangliogenesis in the distal gut is the most common cause of congenital intestinal obstruction in Hirschsprung's disease in humans (Gershon, 1999; McCallion and Chakravarti, 2001).

During ENS development, vagal NCCs, which initially form a small pool of cells, invade the entire length of the bowel, thus requiring strong proliferative and migration-promoting cues to ensure complete enteric gangliogenesis. Glial cell line-derived neurotrophic factor (GDNF), which is produced by the gut mesenchyme and acts on enteric NCCs expressing its receptor, Ret, is crucially required for this process (reviewed by Airaksinen and Saarma, 2002). Mice that lack functional *Gdnf*, *Ret* or the co-receptor *Gfra1* genes have aganglionic mid- and hindguts. Moreover, the GDNF/Ret signalling pathway has been found to regulate in vivo and in vitro the migration, proliferation and/or differentiation of enteric NCCs (Gershon, 1999; Young et al., 2001; Natarajan et al., 2002; Iwashita et al., 2003). ENS progenitor responses to GDNF are modulated by Edn3. It is still unclear how the two factors interact to coordinately control NCC development and progression in the gut wall (Wu et al., 1999; Kruger et al., 2003; Barlow et al., 2003).

Phenotypic plasticity of NC-derived glial and pigment cells

The plasticity displayed by NCCs is somehow retained by some differentiated NC-derived cells. This idea, of reprograming NC phenotypes, is supported by in vitro culture experiments that illustrate the ability of epidermal pigment cells and peripheral nerve Schwann cells isolated from quail embryos to undergo reciprocal transdifferentiation (Dupin et al., 2000; Dupin et al., 2003).

When stimulated to proliferate in vitro by Edn3, single pigment cells from quail embryos de-differentiate and activate glial-specific genes, giving rise to clonal progeny that contain glial cells and melanocytes (Dupin et al., 2000). The converse transition, from glia to melanocytes, also involves the production by Schwann cells of a mixed glial-melanocytic progeny upon in vitro clonal expansion by Edn3 (Dupin et al., 2003). In both cases, descendant cells exhibit a transitory state where they co-express glial- and melanocyte-specific proteins. Melanocytes and peripheral glia are thus able to reverse to their bipotent GM progenitor, which lies upstream in NC lineage hierarchy (Fig. 7). Such plasticity of glial and pigment cell phenotypes in vitro reflects the flexibility of NCC lineage commitment. Whether NC-derived cells display a similar potential for phenotypic plasticity in vivo, under pathological

conditions or during repair is largely unknown, but this idea is supported by the finding that adult mouse Schwann cells can generate pigment cells after severe peripheral nerve injury (Rizvi et al., 2002).

Therefore, differentiated NCCs may bypass lineage restrictions and adopt alternative phenotypes when they escape from their normal environmental context and become exposed to re-specifying signals. These results are consistent with previous reports of cell fate change in higher vertebrates (reviewed by Eguchi and Kodama, 1993; Tosh and Slack, 2002; Raff, 2003) and with recent studies of CNS and hematopoietic lineages, which demonstrate the reversal of restricted progenitors to pluripotent stem cells (e.g. Kondo et al., 2000; Kondo and Raff, 2000; Doetsch et al., 2002; Heyworth et al., 2002).

Conclusions

This survey of over 40 years of study of the ontogeny of the NC, its developmental capacities and the mechanisms that underlie the segregation of the multiple cell lineages that it produces, has significantly enriched our knowledge of this pluripotent structure. We highlight here the most striking ideas that have emerged from these studies.

NCC heterogeneity on migration from the neural primordium

NCCs just emigrating from the neural primordium have been shown to be predominantly pluripotent. Even when they have reached their sites of arrest in the body, a number of them remain undifferentiated, pluripotent and even endowed with the stem cell capacity of self-renewal. This pluripotentiality of NCCs is accompanied by some degree of plasticity, which has particularly been demonstrated for neurons, glia and melanocytes.

One property shared by all the pluripotent (including bipotent) NC progenitors that have been identified by in vitro clonal studies, is that they all have the potential to yield glial cells. Thus, down to the bipotent state, the ability to differentiate into glial cells appears to be a ‘marker’ of the NC lineage.

The plasticity displayed by NCCs makes them able to respond to environmental cues and particularly to various cytokines, which have been shown to play a crucial role in NCC differentiation and perhaps also in their migration and homing to specific sites in the embryo. At present, only a few of them are known. One of the best documented is Edn3, through its influence on melanocytes and glial cells, and (together with that of GDNF) on the NC precursors of the ENS. Several other growth factors have also been identified that have an effect on NCC differentiation, such as various neurotrophins and members of the TGFβ family (e.g. BMP2). Their effect on NCCs have not been discussed above but have been recently reviewed elsewhere (Anderson, 1997; Le Douarin and Kalcheim, 1999; Le Douarin and Dupin, 2003).

The avian GM precursor of the NC has been shown to respond to Edn3, which increases its proliferation rate and favours its differentiation into melanocytes. Moreover, Edn3 also induces the progeny of differentiated glial cells and melanocytes to reacquire the bipotent state of the original GM precursors from which they are derived. The GM and GF precursors are able to self-renew in culture. As the NC gives rise to many different cell types and contributes to a variety of tissues and organs in the body, such NC-derived stem cells might exist in these sites to ensure the turnover of their differentiated progeny, the lifespan of which is likely to be limited. Results from both in vivo and in vitro experiments in birds and mammals support this view. Thus, the persistence of ‘stem cells’ in various types of NC derivatives, even in adults, provides them with a regenerative and repair capacity, together with plasticity. These ‘stem cells’ might also be the origin of NC-derived tumours.

The NC: an important asset to vertebrate evolution

Cell tracing experiments in birds have revealed that the contribution of the NC to the formation of the vertebrate head is much broader than was originally believed from pioneer studies carried out in amphibian embryos (Hörstadius, 1950). 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).

The recent findings that mesenchymal cell types can arise from trunk NCCs even in amniotes, suggest that, when it appeared in the early vertebrates, the NC was the structure that provided the body not only with the PNS, but also with the most primitive skeletal elements of this phylum. The superficial skeleton of NC origin is absent in protocordates (such as Amphioxus) but was present in some early vertebrates (Smith and Hall, 1990). This exoskeleton has been maintained until now in the head and has played a major role in allowing the development of the brain, sense organs and their related functions (Gans and Northcutt, 1983).

Endogenous properties of the NC, such as Hox gene expression, limit the plasticity of 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 wandering NCCs cooperate 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 genetic networks and molecular pathways that are involved in the numerous cell-to-cell interactions that operate during NCC migration, homing and differentiation.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/19/4637/DC1

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Table S1. Progenitors with both mesectodermal and neural-melanocytic potentials in the cephalic neural crest

Types of mixed progeny (number from total clones)*									
GNMC	GNMF	GMFC	GNC	GMC	GNF	GMF	GC	GF	Reference
0/173	n.d.	n.d.	0/173	0/173	n.d.	n.d.	7/173	n.d.	Baroffio et al. (1988) †
0/165	n.d.	n.d.	0/165	0/165	n.d.	n.d.	3/165	n.d.	Dupin et al. (1990) †
1/305	n.d.	n.d.	1/305	0/305	n.d.	n.d.	6/305	n.d.	Baroffio et al. (1991) †
0/36	1/36	1/36	0/36	1/36	0/36	0/36	0/36	1/36	Trentin et al. (2004)‡
0/163	11/163	1/163	0/163	0/163	2/163	17/163	0/163	7/163	E.D. and N.M.L.D., unpublished‡

*Summary of the clone types derived from quail NCCs grown on feeder layers of 3T3 fibroblasts. Only those containing both neural-melanocytic and mesectodermal (cartilage and myofibroblasts) derivatives are considered here.

†Refers to clones derived from migratory NCC isolated from 9- to 12-somite stage (ss) embryos at the mesencephalic/anterior rhombencephalic level (the presence of myofibroblasts in these cultures was not determined; n.d.).

‡Refers to clones from NCCs obtained in primary cultures of mes-rhombencephalic neural primordium isolated at 4-6 ss.

In all experiments, single NCCs were aspirated from a diluted cell suspension and seeded in individual culture wells by micromanipulation to ensure culture clonality. After 9-15 days, the progeny was analyzed with cell type-specific markers to assess presence of melanocytes (M), glial cells (G) and PNS neurons (N). Mesectodermal cell types, chondrocytes (C) and myofibroblasts (F), were identified by differentiation of cartilage nodules and expression of α-smooth muscle actin, respectively.