Chapter 6

The Contribution of the Neural Crest to the Vertebrate Body

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Abstract

As a transitory structure providing adult tissues of the vertebrates with very diverse cell types, the neural crest (NC) has attracted for long the interest of developmental biologists and is still the subject of ongoing research in a variety of animal models. Here we review a number of data from in vivo cell tracing and in vitro single cell culture experiments, which gained new insights on the mechanisms of cell migration, proliferation and differentiation during NC ontogeny. We put emphasis on the role of *Hox* genes, morphogens and interactions with neighbouring tissues in specifying and patterning the skeletogenic NC cells in the head. We also include advances made towards characterizing multipotent stem cells in the early NC as well as in various NC derivatives in embryos and even in adult.

Introduction

The neural crest (NC) is a transient structure of the vertebrate embryo which was discovered in 1868 by the Swiss embryologist Wilhelm His.[1] He described it in the chick embryo as a band of cells lying between the neural tube and the future epidermal ectoderm. Since the neural crest cells (NCCs) leaving the dorsal aspect of the closing neural tube, can be seen aggregating laterally into the spinal ganglia, it used often to be called *'ganglion crest'*.

Following its first description, the NC was found in selachians, teleosts, amphibians and all forms of vertebrates (for a review see ref. 2).

Because its component cells are endowed with migratory properties and become widely distributed over the whole embryo, the NC has readily attracted much interest. The work carried out on the NC during the first half of the 20th century and which concerned essentially lower vertebrates, was reviewed in a well-acknowledged monograph by Sven Hörstadius.[3]

A prerequisite for studying the behaviour and fate of NCCs is the availability of markers providing experimental long-term lineage labelling, which allow this cell type to be specifically identified throughout ontogeny.

The quail-chick marker system based on the construction of embryonic chimeras between two species of birds whose cells can be identified, either by the structure of their nucleus or by species-specific monoclonal antibodies, was instrumental in deciphering the contribution of the NC to the various anatomical and histological structures in birds and, by extrapolation, in mammals[4,5] (for a review see refs. 2,6).

Recently, molecular methods have been devised to label the NCCs in mammals by constructing transgenic mice in which expression of either β-galactosidase or of the green

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fluorescent protein (GFP) is driven by promoters of genes that are expressed in the NCCs. The genes that have so far been used for this purpose are essentially *Wnt1*,[7-9] *Sox10*[10] and *tissue plasminogen activator (tPA)*.[11] In the mouse and in other vertebrates, *Wnt1* is transiently expressed during embryogenesis in the dorsal neural primordium,[12-17] except in the first rhombomere (r1) (see ref. 10 online supplement) and in the early migrating NCCs (see ref. 18 and references therein). Early migratory cephalic and trunk NCCs and their derivatives can also be identified by genetic labelling using the gene encoding tPA,[11] a proteolytic enzyme synthesized by NCCs during the epithelio-mesenchymal transition.[19] *Sox10* transcription factor gene is expressed transiently in the early NC and later remains activated only in neural and melanocytic NC lineages while it is never detected in mesenchymal cells, whether derived from mesectoderm or from mesoderm (see ref. 10 and references therein).

Sox10 has been used to permanently label post-otic NCCs and trace their fate in the neck and shoulder in transgenic mice.[10] Permanent single cell labelling was obtained in double transgenic mice in which the production of the Cre-recombinase is conditioned upon expression of either *Wnt1* or *Sox10* genes. The *Wnt1-Cre* and *Sox10-Cre* founder mice were crossed to Cre-conditional *R26R-LacZ* and *-GFP* reporter lines.[20,21] Neither LacZ nor GFP carry a nuclear localization sequence, thus blue staining or fluorescence is mainly in the cytoplasm. After Cre-mediated excision of the floxed cassettes, F1 offspring expresses *LacZ* (or *GFP*) permanently in cells that have expressed *Wnt1* or *Sox10*, even if the endogenous *Wnt1* and *Sox10* genes ceased to be activated during development.[10] This elegant technique is thus a way to get single cell resolution and long-term labelling for NCC fate mapping in mammals. Recombinase-based fate mapping using *Gdf7* gene encoding a Bone morphogenetic protein (BMP) family member, has recently led to characterize a subset of late-emigrating NCCs present in the roof plate, which yield sensory-restricted progenitors.[22]

The Derivatives of the NC

The NCCs are remarkably invasive. There is virtually no tissue in the embryonic and adult body that does not possess cells originating from this highly pluripotent structure.

Investigations carried out in the avian embryo, using the quail-chick chimera system, have allowed the fate map of the NC to be established (Fig. 1).

The NC is at the origin of all the neurones and glial cells of the peripheral nervous system (PNS) except for the neurones of certain cranial nerve sensory ganglia.[6]

The PNS includes the sensory ganglia, the sympathetic chains and plexuses, the parasympathetic ganglia and the enteric nervous system (ENS) often called the *'second brain'*[23] because it includes sensory, intermediate and motor neurones and can function independently from the central nervous system (CNS) which modulates its activity through sympathetic and parasympathetic innervations.

The NC is also at the origin of sensory receptor cells (e.g., Merkel cells and Grandry corpuscules) associated with sensory nerve endings in the skin.[18,24,25]

The NC provides all the peripheral nerves with Schwann cells and the peripheral ganglia with their glial component. The pigment cells of the body (except those of the pigmented retina) derive from the NC, which is also at the origin of endocrine cells: the adrenal medulla and the calcitonin-producing cells (C cells). The latter develop in the ultimobranchial bodies (UB) in all vertebrates except in mammals, where the UB join the thyroid gland in which the C cells form the population of *'clear cells'* (also called *'parafollicular cells'*) located between the thyroid follicles.[6]

Another derivative of the NC is the *mesectoderm* i.e., mesenchymal cells which are derived from the ectodermal germ layer, in contrast to the other mesenchymal cells of the body, which are of mesodermal origin.

Mesectodermal cells exit from the cephalic and trunk NC in lower vertebrates (e.g., fish in which they yield the dorsal fin mesenchyme; see ref. 6 and references therein). In amniotes they are mainly restricted to the cephalic level of the neural axis (Fig. 1). However it was recently shown in the mouse that the trunk NC is able to give rise to endoneurial fibroblasts.[26]

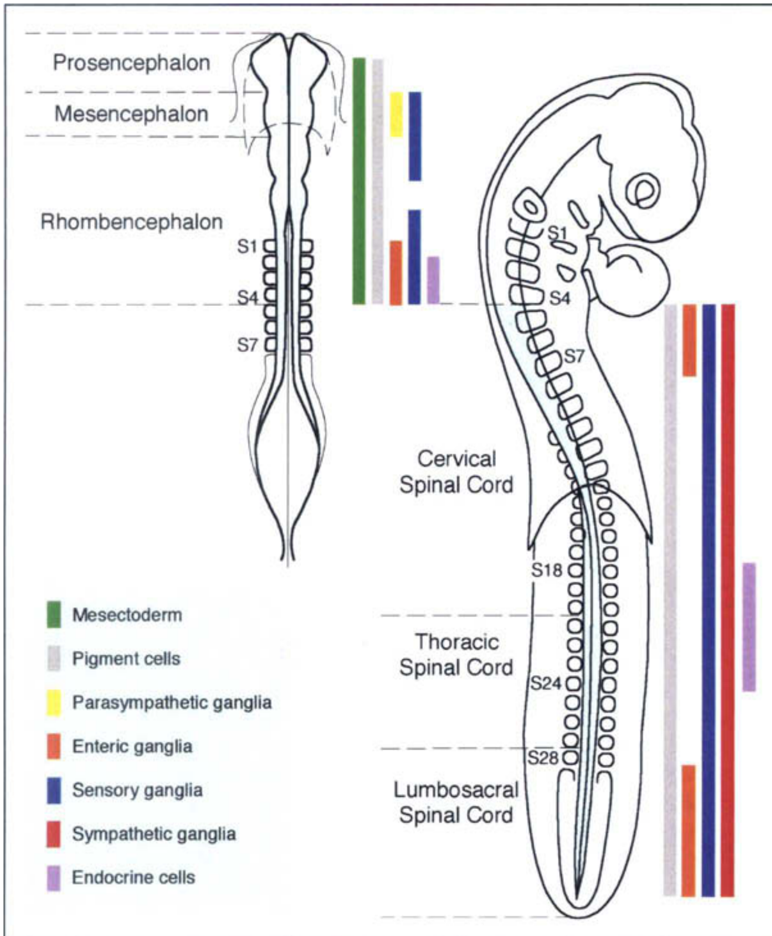

Figure 1. Fate map of NC derivatives. As established in the avian embryo,[38] the different phenotypes (see box) yielded by the cephalic (left panel) and the trunk (right panel) NC along the NCC-producing neural fold (shaded in light blue) are represented in 7- and 28 somite-stages embryos. S, somite.

Pathways and Molecular Control of NCC Migration

The early specification of the NC from the neural plate depends on the function of both soluble growth factors and regulatory genes, the relative influences of which have been reviewed elsewhere.[27-30] Soon after NC formation, NCCs undergo an epithelium to mesenchyme transition and acquire the ability to navigate along complex paths.[6] A number of molecules are known to participate in NCC delamination and migration such as cadherins, Rho GTPases, Noggin and molecules of the extra-cellular matrix (ECM).[31-34]

The routes through which NCCs navigate, have been mostly characterized in the avian embryo.[6] In the trunk region, NCCs follow two main patterns of migration, which differ spatio-temporally. First, NCCs of the dorso-ventral migratory wave travel between the neural tube and the somites, through the anterior half of each somite as well as between consecutive somites. These cells will give rise to most of the PNS by coalescing to form the dorsal root ganglia (DRG) and, more ventrally, the sympathetic ganglion chains and the adrenal medulla.

Second, a late-emigrating population of NCCs stays in the so-called *'migration staging area'*[35] circumscribed by the neural tube, the superficial ectoderm and the dorso-medial somite. When the dermatome becomes individualized, these NCCs take a medio-lateral route, penetrating the dermomyotome beneath the superficial ectoderm. These cells, which become committed to the melanocyte lineage, are fated to give rise to the pigment cells of the skin and feathers.[6]

In the cephalic region, the migration pathways and target sites of NCCs have been mapped along the antero-posterior axis[36,37] (see refs. 6,38 for additional references). Although the formation and emigration of NCCs is basically unsegmented along the dorsal hindbrain as it is in the trunk, NCCs adopt a stereotypic migratory pattern along three main streams.[39-42]

Therefore the NCCs that migrate from the various levels of the neural axis, are rapidly patterned by their environment. At the trunk level, the migratory pattern of NCCs is closely correlated with somitic development. The migration of NCCs can take place only in the rostral half of each somite, whereas its caudal half is inhibitory to the penetration of both NCCs and growth cones from the motoneurones. The segmental origin of NC progenitors of the DRG was described in an elegant series of experiments where small fragments of the quail neural primordium were grafted orthotopically in chick embryos.[43] These experiments showed that the NCCs facing two consecutive somites participate in the formation of each DRG (Fig. 2A). Moreover, the NCC migration and DRG formation are perturbed subsequently to replacement of contiguous somites by either multiple rostral or multiple caudal somitic halves. If a succession of rostral somitic halves is created, a continuous and giant ganglion forms. In contrast, following grafting of multiple caudal somitic halves, the NCCs give rise to very small, dorsally located ganglia with irregular segmentation.[44] Therefore, the normal morphogenesis and segmentation of the DRG depend upon the integrity of the rostro-caudal succession of somitic regions (Fig. 2B).

The mechanisms that direct NCCs to the appropriate migration pathways and how NCCs interprete external signals into different locomotion behaviours start to be understood. Time-lapse confocal imaging in intact chicken embryos has allowed the cell-cell interactions between individual migratory NCCs and their motile behaviour at trunk and cephalic levels to be visualized.[45-48]

NCCs whose individualization results from proteolytic activity,[19] are guided by both inhibitory and permissive cues that they encounter en route. These cues modify the expression and activity of integrins and cadherins responsible for intercellular detachment, cytoskeletal changes and cell motility.[49-51]

Signals permissive for NCC migration are provided by basal lamina and several ECM components, such as fibronectin,[52] laminin, collagens, thrombospondin and hyaluronic acid (reviewed by Perris and Perissinotto).[32] Inhibitory molecules expressed in particular regions of the embryo impair invasion by NCCs, such as chondroitin sulfate proteoglycans in the peri-notocordal mesenchyme. In the caudal somite, F-spondin[53] and lectin-binding glycoproteins[54] can function as a barrier for NCCs and thus participate in segmentation of the NC migratory streams in the trunk.

Recent studies have uncovered the role in directing NCC migration of secreted proteins originally characterized for their function in axonal guidance such as Ephrin, class III Semaphorin, Slit and Netrin families of ligands, which transduce repulsive or attractive signals through interactions with the cognate receptors expressed by NCCs.

Ephrins-B ligands expressed in the caudal somite act as repulsive signals to restrict trunk NCC migration to the anterior somite and the dorso-ventral pathway.[55-57] However, at a later stage, NCCs switch their response to Ephrins-B, which then act as positive cues required for the lateral migration of presumptive melanocytes.[57] Other inhibitory elements supplied by the epithelial dermatome include secreted Slit proteins, which interact with Robo receptors expressed by early NCCs to confine them to the dorso-ventral pathway.[58] Slit 2 produced by the splanchnic mesenchyme repels trunk but not vagal migratory NCCs, inhibiting the former cell population to invade the gut.[59]

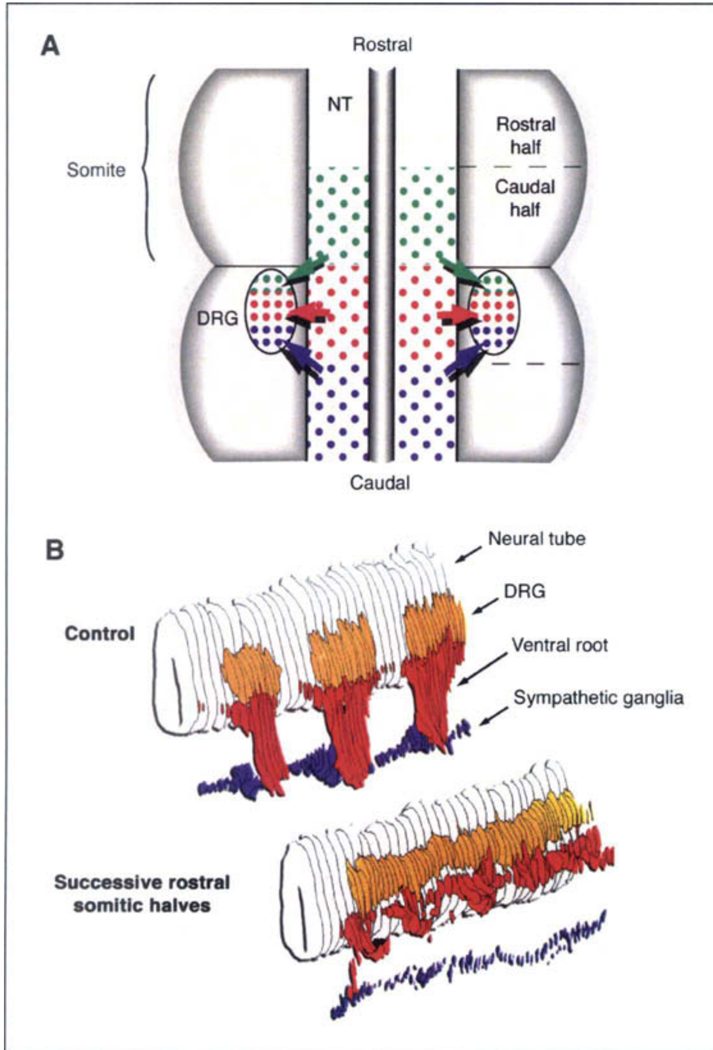

Figure 2. Segmental pattern of peripheral ganglia: influence of the somite. A) Each dorsal root ganglion (DRG) forms opposite the rostral part of the somite and is colonized by NCCs migrating from the part of the neural tube (NT) facing the same somite (80% of DRG cells). NCCs corresponding to the caudal half of the preceding somite populate the rostral 20% of the DRG (adapted from ref. 43). B) Three-dimensional reconstruction of the neural tube and peripheral nervous structures in E4.5 embryos.[6] In controls, the DRG, ventral roots and primary sympathetic ganglia are segmentally patterned. In embryos implanted with successive rostral somitic halves, NCCs generate a continuous dorsal polyganglion and yield unsegmented nerves and sympathetic ganglia.

Recent grafting experiments to perturb the spatio-temporal relationships between the NCC medio-lateral migration and dermis formation have suggested that the emerging dermatome supplies a long-range diffusible attractant to stimulate NCC entry into the dorsal path.[60] In other regions as well, NCCs may display chemotactic responses to growth factors such as Fibroblast growth factor 2 (FGF2) in the mesencephalon.[61] Glial cell line-derived neurotrophic

factor (GDNF) peptide is chemoattractant to NC enteric precursors that express Ret tyrosine kinase receptor during colonization of the gut.[62,63] At later stages of ENS development, Netrins have been reported to attract the enteric nerve cells expressing the Netrin receptor DCC (for '*deleted in colorectal cancer*'), thus promoting the secondary formation of sub-mucosal and pancreatic ganglia.[64]

Semaphorin 3A, a secreted chemorepellent of class III Semaphorins, which signals via Neuropilin 1 receptor, plays a role not only in the patterning of the sympathetic innervation but is also required for the accumulation of neuronal precursors that form the sympathetic ganglia.[65] In addition, Semaphorin 3A reduces the migration area of trunk NCCs in vitro.[66] Semaphorin 3/Neuropilin 2 signalling has been suggested recently to play a crucial role in the segregation of hindbrain NCCs into migration streams in the chick[67] and zebrafish.[68] Semaphorins 3 however function in concert with other inhibitory signals to create crest-free zones in the mesenchyme adjacent to r3 and r5. Abnormal NCC invasion of these zones occurs in mice mutated for genes encoding Twist transcription factor[69] and ErbB4 receptor of Neuregulins.[70,71] This phenotype has been suggested to result from alterations of Ephrin-B/Eph receptors signalling. In particular, conditional mutation of *Ephrin-B1* in mouse NC has recently shown that Ephrin-B1 regulates NCC directional migration to craniofacial target tissues.[72]

The Mesectoderm Is at the Origin of Most Head Structures

The crest-derived mesenchyme, which arises from the posterior diencephalic level, is destined to populate the upper facial primordia corresponding to the naso-frontal and naso-lateral buds (Fig. 3A). Mesencephalic and anterior rhombencephalic (r1-r2) NCCs fill up the branchial arch 1 (BA1). In the more caudal BAs, migration streams are delimited by several factors (see above) including by apoptotic foci of the cells in r3 and r5, which restrict the NCC scattering to a segmented pattern:[36,37,39-41] while r4-derived cells colonize BA2, NCCs that originate from r6 to r8 migrate into caudalmost BAs (BA3, BA4-6). Moreover, the final fate of these mesectodermal cells could be revealed thanks to the stability of the labelling provided by the chimeric quail-chick combination. The BA1 NCCs form the maxillo-mandibular skeleton while mesectodermal cells from the most posterior arches differentiate into the ventral part of the neck where they give rise to the hyoid bone[6] (Fig. 3B). In mammals, the NC is at the origin of mesenchymal cells in the dentine and the pulp of the teeth.

As a whole, the investigations carried out so far in the avian embryo have allowed the delimitation of the mesectodermal and mesodermal components of the skull (Fig. 4A). Ventrally, this limit corresponds to the rostral end of the notocord between the basipost- and the basipre-sphenoid, both forming the *Sella turcica* on which the pituitary gland sits (Fig. 4B). Dorsally, the posterior mesectodermal limit was placed at the transition between the parietal and the occipital bones, the latter being mesoderm-derived.[73] The parietal itself differentiates late during embryogenesis and was primitively thought not to belong to the NC-derived skeleton.[73,74] An approach based on the expression of *Wnt1* in the neural fold of the mouse, has excluded the parietal bone from the NC-derived territory.[9] These results could however be due to the fact that the r1 NC, the main source of mesectoderm for building the parietal bone, does not express *Wnt1* (see ref. 10, online complementary information and our own unpublished observations). More investigations are required to ascertain the origin of the parietal bone in mammals.

Long-term fate maps established in the avian embryo have revealed differences in the ability of the NCCs to form the various types of skeletal tissues: yielding cartilage and endochondral bone appears as a ground property of the entire skeletogenic NC domain. In contrast, the ability to form membrane bones is restricted to its most rostral part, corresponding to NC derivatives of the naso-frontal and maxillo-mandibular processes. Striking is the fact that, as discussed below, this partition matches different molecular traits involving *Hox* gene expression.

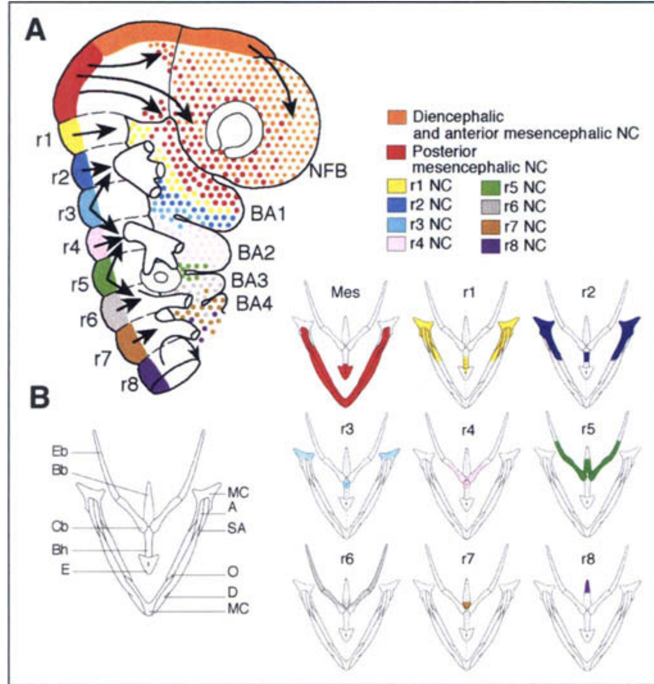

Figure 3. Cephalic NCC migration and contribution to visceroskeleton. A) Migration streams of cephalic NCCs to the naso-frontal bud (NFB) and branchial arches (BA), according to their level of origin. B) Composite origin of the maxillo-mandibular and hypobranchial skeleton (the level of NCC origin is colour-coded as in (A). A: angular; Bb: basibranchial; Bh: basihyal; Cb: ceratobranchial; D: dentary; E: entoglossum; Eb: epibranchial; MC: Meckel's cartilage; Mes: mesencephalic level; O: opercular; r: rhombomere; SA: supra-angular.

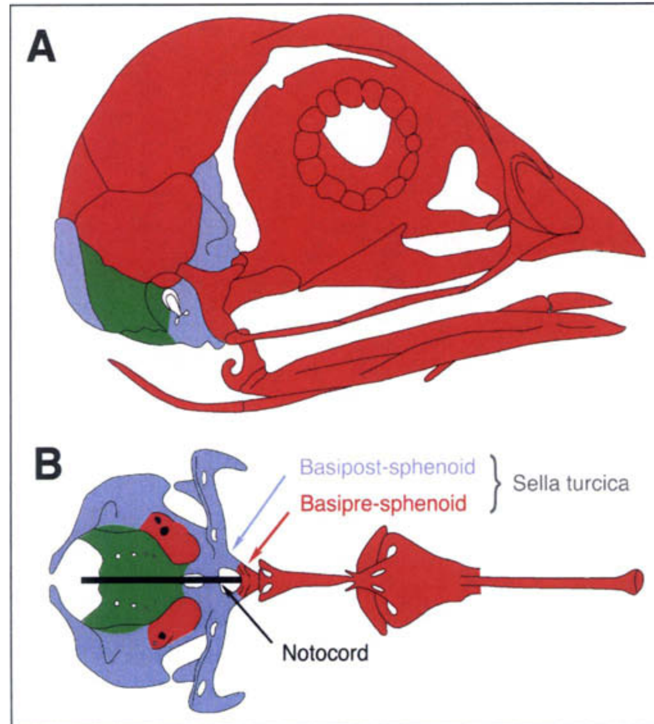

Figure 4. Triple origin of the head skeleton. A) Contribution of the cephalic NC, cephalic paraxial mesoderm, and somitic mesoderm to the craniofacial skeleton at E14. B) Ventral view of E10 chondrocranium showing the transition between the NC- and the mesoderm-derived skeleton.

The Posterior Limit of the NC-Derived Skeleton in Mammals

Labelling of the mammalian post-otic NC by taking advantage of the transgenic mice approach mentioned above, has provided novel information on the participation of the NCCs to the neck and shoulder skeleton.[10]

The neck region has been subjected to major changes during evolution from fish to terrestrial vertebrates, and the respective boundaries of NC and mesoderm contribution in the neck transition zone had not been previously investigated. According to the classical view,[75,76] the dermal bones of the forelimb girdles are of NC origin while their endochondral components are considered mesodermal. This so-called *"ossification model"* to account for either a NC or a mesoderm origin of the skeleton had however not been experimentally tested in any vertebrate.

As noticed by G. Koentges' group, there are variations in the mode of ossification (either endochondral or dermal) of the shoulder bones in various organisms, whereas the pattern of neck muscle attachments on these bones is very conserved throughout evolution.[10] In fact, the origin of the muscle connective component and the attachment points of the muscles on the skeleton were shown to be similar in both cranial and trunk regions throughout the vertebrate phylum.[37,77] On this ground, Matsuoka et al[10] have proposed a competing model according to which morphogenesis of the bones in various forms of vertebrates has obeyed constraints imposed by a strict muscle attachment scaffold. This idea is based upon the homologies that can be drawn concerning the relationships that exist between the muscles and bones in the head and neck regions of different vertebrates. It was found that the cellular distributions of NC and mesoderm correspond to the muscle attachment scaffold rather than to the type of ossification of the bones: if the muscle connective tissue is of NC origin, its attachment site on a given bone will be made up of NC-derived cells, irrespective of whether the bone is of dermal or endochondral type.[10]

Thus in mouse, the post-otic NC extends to the anterior lining of the shoulder girdle at the sites where the trapezius muscle is fixed, whose connective component is of NC origin. The post-otic NCCs undergo dermal ossification of the anterior clavicle and form endochondral bone at the insertion of branchial muscles (i.e., sternocleidomastoid and the fascia of omohyoid).[10] Generally speaking, the branchial muscles that have a NC connective component extend their attachment site to NC-derived bone in all skeletal structures of the neck and shoulder regions. However, in the cervical vertebra whose body and neural arches are somitic in origin,[73] the NC contribution is cryptic since it is limited to spots in the spinal process, which correspond to muscle attachment sites.

Similar results had been obtained for the attachment of the cleidohyoid muscle on the medial part of the clavicle of birds by McGonnell and coworkers[78] through the use of several NCC labelling procedures. It is noticeable that the scapula was found to be entirely mesoderm-derived in birds.[79]

Contribution of the Mesectoderm to the Cardiovascular and Periocular Structures

As evoked above, the cephalic NC scaffolds the fascicular organization of the myogenic primordia derived from cephalic paraxial and somitic mesoderm; regarding the splanchnopleural mesoderm, NCCs play a similar role. The first insights of NCC contribution to conotroncal structures in vertebrates were gained from experiments using quail-chick chimeras. They demonstrated that mesectodermal cells nested in BAs 3 to 6 were lining the endothelial cells of the large aortic arteries (from brachiocephalic down to pulmonary arteries).[80] These results prompted Margaret Kirby and colleagues to further investigate the migration and fate of NCCs into the heart anlage and to question their functional role. It turned out that NCCs form the aorticopulmonary septum and the sigmoid valves.[80-83] This NCC distribution was later on generalized to mammals by transgenic approaches.[81-85] The large-scale defects in cardiac outflow tract septation recorded in the absence of the posterior rhombencephalic mesectoderm corresponding to the r6-r8 area of the neural fold, led Kirby and coworkers to put forward the notion of *'cardiac neural crest'*. This territory was shown to play an important role not only in its

contribution to the heart anlage but also in regulating various aspects of the proliferation, differentiation and physiology of the cardiomyocytes.[86,87]

Long-term cell tracing investigations revealed for the first time the composite origin of the cephalic blood vessels.[80] Two notions came out from these observations. First, the endothelium was found to be of mesoderm origin all over the body. Later on, it was shown that the angiogenic lineage shares a common *'hemangioblastic'* precursor with hematopoietic cells.[88] These precursor cells can be early evidenced by expression of VEGFR2 (for *'vascular endothelial growth factor receptor 2'*).[88-90] Second, cephalic blood vessels from the large arteries arising from the heart up to the very thin ramifications of cerebral capillaries of the vascular tree in the face and the anterior brain, are surrounded by cells of mesectodermal origin (Fig. 5A-C). NC provides the craniofacial and

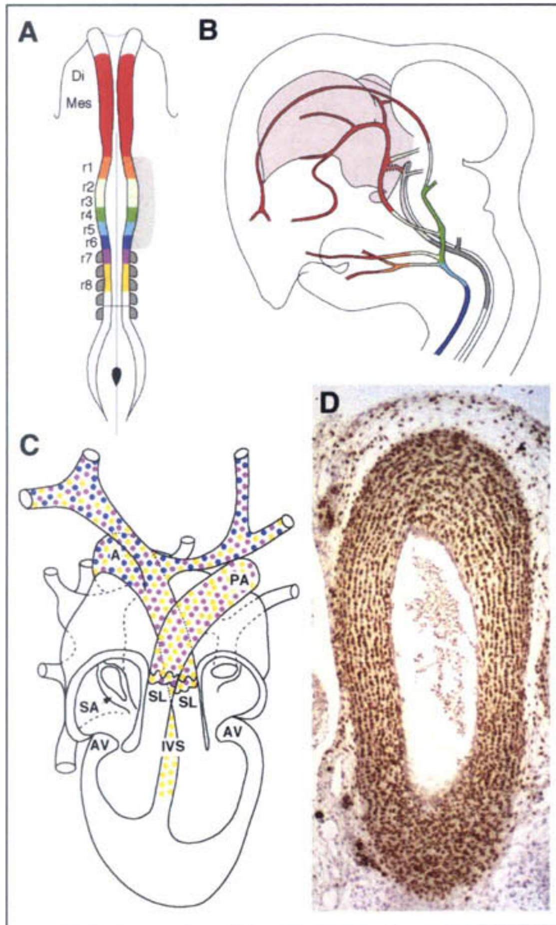

Figure 5. Cephalic NCC contribution to the cardiovascular system. A) Refined colour-coded cephalic neural fold of a 5 somite-stage chick embryo and NCC contribution to the musculo-connective wall of artery tree in the head. B) Prosencephalic meninges (in pink) derive from the diencephalic and mesencephalic NCCs; from mesencephalon down to more caudal cephalic structures, meninges (in grey) are of mesoderm origin. C) Contribution of the posteriormost rhombencephalic NCCs to the conotruncal structures of the heart. D) NCCs form the musculo-connective wall of chimeric cephalic artery (QCPN immunoreactivity of quail rhombencephalic NCCs grafted into chick host embryo). A: aorta; AV: atrioventricular valve; IVS: intraventricular septum; PA: pulmonary artery; SA: sinoatrial valve; SL: semilunar valve.

Table 1. Origin of the avian ocular and periocular structures

Ectoderm	Neural Crest	Mesoderm
Corneal epithelium	Corneal stroma and endothelium	Blood vessel endothelium
Lens	Iridial stroma and muscles	
Iridial epithelium and lamella	Ciliary muscles	Myofibers of extra-ocular muscles
	Mesenchyme of ciliary corpus and process	
Pigmented and neural retina	Wall of Schlemm's canal	
	Sclero-corneal limbus	
Eyelid epithelia	Sclerotic ossicles	
	Choroid membrane	
	Sclerotic cartilage	
	Muscles and mesenchyme of eyelids and nictitating membrane	
	Mesenchyme of lacrimal gland	
	Connective tissue of extra-ocular muscles	
	Pericytes of ocular blood vessels	
	Frontal, lacrimal, ethmoïd, maxilla, zygomatic bones	

prosencephalic blood vessels with their media, meaning that these cells are locally induced to acquire pericyte and smooth muscle cell phenotypes[80,91] (Fig. 5D). In this respect, the ocular and periocular region offers a unique site where the NC gives rise not only to the smooth muscles of the ciliary bodies but also to the striated muscle components of the iris.[92] Altogether, the variety of NC derivatives identified in the developing eye concurs in maintaining homeostasis of the anterior optic chamber and in endowing the vertebrate eye with refracting media (Table 1).

Hox Gene Expression in the Cephalic NCCs and Their Derivatives

As first established in the mouse[93] and later confirmed in the chick,[94,36] the caudal domain of the cephalic NC (from r4 down to r8) expresses *Hox* genes of the first four paralogous groups, whereas in the rostral domain (from diencephalon down to r2), these *Hox* genes are not expressed. At the edge of both, NCCs from r3 exhibit a versatile *Hox*-status according to the environment into which they migrate. If r3 NCCs invade *Hox*-negative BA1, they lose *Hoxa2* expression while, if they migrate into *Hox*-positive BA2, they maintain it.

The evidence that *Hox* gene expression exerts a crucial influence on BA identity has been provided by seminal experiments based on gene targeting in mice: the null-mutation of *Hoxa2* generates the duplication of BA1-type skeleton (mandible) at the expense of BA2 hyoid bone structures.[95,96] By contrast, if *Hoxa2* expression is targeted to all BA1 tissues, a partial homeotic transformation of BA1 into BA2 is observed in the chick[97] and in *Xenopus*.[98]

The phenotype resulting from surgical extirpation of the anterior *Hox*-negative NC is the absence of the facial skeleton together with severe defects of forebrain and midbrain development[99] (Fig. 6A,B). If the *Hox*-expressing neural fold from r4 to r8 is heterotopically transplanted into the *Hox*-negative forehead environment, no regeneration of the skull and facial skeleton takes place. Despite their capacity to migrate and invade facial processes, *Hox*-expressing NCCs fail to form either cartilage or bone, and therefore are unable to substitute for *Hox*-negative NCCs in facial skeletogenesis[100,101] (Fig. 6C,D). A strikingly different result is obtained when the *Hox*-negative anterior domain of the NC (from mid-diencephalon down to r3 excluded) is replaced by small fragment of NC from either the posterior diencephalon, the mesencephalon or r1-r2 levels coming from a stage-matched quail. As shown in Figure 6E,F, the morphogenesis of the head and brain is rescued and all the NC-derived structures of the face are made up of quail cells.

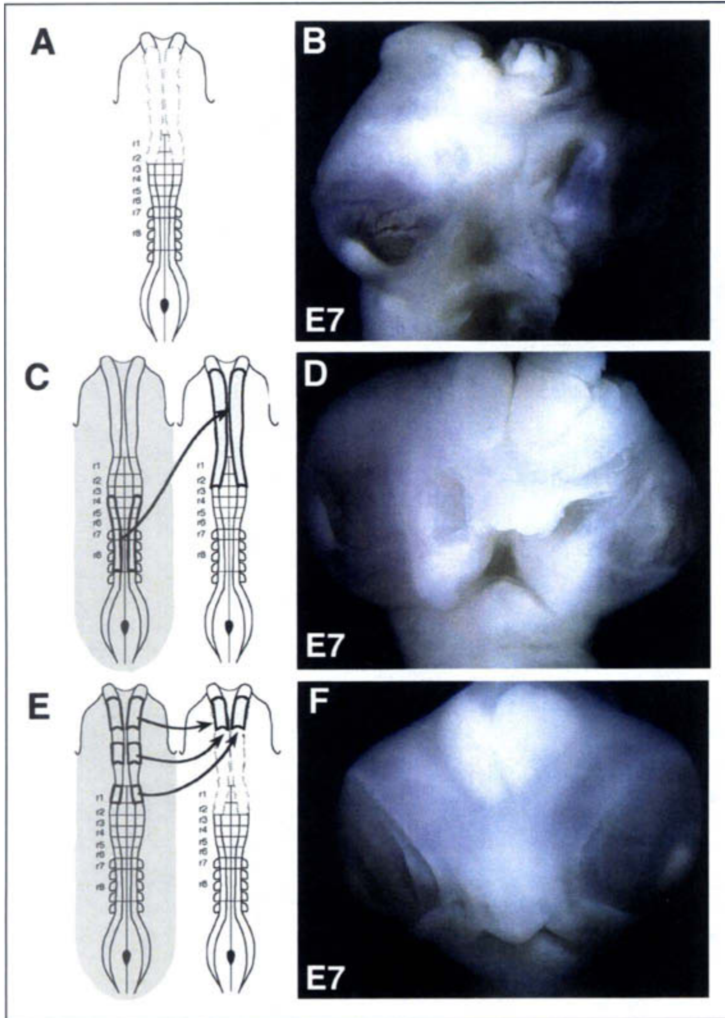

Figure 6. Facial development relies on the *Hox*-negative NC. A) Removal of the *Hox*-negative NC in early chick neurula (dotted lines) results in absence of facial structures and exencephaly in E7 operated embryos (B). C) Replacement of the anterior *Hox*-negative NC by an exogenous fragment of *Hox*-positive NC severely hampers head morphogenesis at E7 (D). E) Following removal of the anterior *Hox*-negative NC (as in A), the implantation of only a fragment from either di-, mes- or anterior rhombencephalic level (i. e., *Hox*-negative NC) restores normal development of the face and forebrain at E7 (F).

Therefore, the *Hox*-positive NC cannot replace the *Hox*-negative domain to construct a facial skeleton. In addition, *Hox*-positive NCCs transplanted rostrally yield the PNS derivatives corresponding to the level of their implantation but do not form any facial skeletal tissues. We have shown that the anterior *Hox*-negative domain is more plastic: when posteriorly transplanted, anterior *Hox*-negative NCCs are able to participate in the formation of the hyoid bone, thus adopting a fate corresponding to their new location.[100,101] Moreover, these NCCs are necessary for brain morphogenesis.

These experiments have demonstrated that the NC territory extending from the mid-diencephalon down to r2 (included) shows equivalent developmental potential whatever the

level considered and thus its component cells behave as an *'equivalence group'*. This implies that the anterior domain of the NC relies on environmental cues for patterning the facial skeleton.

Shifting Rostrally the Limit of *Hox* Gene Expression

As previously mentioned, the NC ability to form skeletal tissues is not uniformly distributed along the cephalic neural axis since the *Hox*-expressing rhombencephalic NC is unable to yield facial skeleton.[100,101] That this trait could essentially rely on *Hox* gene expression was further investigated by forcing *Hox* gene activation in the anterior *Hox*-negative neural fold fated to yield facial skeleton.[102] If gain-of-function for *Hoxa2* is restricted to mesectodermal cells, *Hoxa2*-transfected embryos are utterly deprived of facial skeleton. On the other hand, forced expression of *Hoxa3* and *Hoxb4* promotes different phenotypes. While neither *Hoxa3* nor *Hoxb4* transfection affects NCC migratory properties, it strongly reduces their skeletogenic potential. In these embryos that have virtually no face, some rudimentary skeletal elements still develop in either the nasal bud (*Hoxa3*) or the mandibular bud (*Hoxb4*). According to the same paradigm, cotransfection of *Hoxa3* and *Hoxb4* completely abolishes the development of the facial skeleton.[102] This experiment therefore led to phenocopy of the facial defects resulting from the replacement of the endogenous *Hox*-negative NC by more posterior *Hox*-positive neural fold or from the ectopic activation of *Hoxa2* in the anterior neural fold.

Aside from skeletal defects, forced expression of *Hoxa3* was also detrimental to the differentiation of transfected mesectodermal cells into pericytes, causing lethal haemorrhages at the head level.[102] Together with the effects of *Hoxa3* null mutation in mice, these data show that *Hoxa3* plays an essential role in patterning the cardiovascular system.[103-105]

Altogether, these experiments show that *Hox* gene gain-of-function by the rostral NCCs prevents the formation of the skeletal (and, for *Hoxa3*, the vascular) apparatus. It thus appears that the *Hox*-free rostral domain of the neural fold is the site of striking diversity of NC-derived structures.

Patterning the Facial Skeleton by the Pharyngeal Endoderm

In lower vertebrates, tissue associations in organotypic culture have indicated that intimate contact between the NCCs and the pharyngeal endoderm is required for cartilage differentiation.[106,107] More recently, it has been put forward that skeletal morphogenesis relies on the pharyngeal segmentation. The absence of NC did not significantly perturb the early patterning of the pharyngeal endoderm.[108] Conversely, mutations affecting the endoderm resulted in aberrant organization of the BAs in zebrafish.[109] Recent studies have enlightened the synergistic effect of *Fgf3* and *Fgf8* genes in this process, by directing lateral progression of endodermal cells in order to sculpt the pharyngeal pouches.[110] Hence the segregation of NCC migratory flows into the BAs tightly depends on the early segmentation of the pharyngeal endoderm.[109]

Experiments carried out in the avian embryo have established the capacity of defined regions of the pharyngeal endoderm to convey instructive patterning cues to the NCCs during facial skeleton morphogenesis.[101,111] Extirpation of endodermal stripes at neurula stage results in the disruption of pieces of the facial skeleton, which vary according to the level of the ablation (Fig. 7A-C). Reciprocally, grafting similar endodermal stripes into otherwise intact host embryos along the migration pathway of cephalic NCCs, induced predicted duplications of facial skeletal components (Fig. 7D-F). Moreover, changing the orientation of the grafted stripes accounted for modifying the polarity of the induced supernumerary skeletal pieces.[101] Therefore, the *Hox*-negative NC behaves as a naïve entity to which the ventral foregut endoderm confers patterning cues in order to specify the shape and spatial orientation of the maxillo-mandibular skeleton.

Later on, these results were extended to the formation of the hyoid bone that arises from both *Hox*-negative and *Hox*-positive NCCs.[111] Thus, the patterning ability of the more caudal levels of the pharyngeal endoderm (facing the mid- and posterior rhombencephalic structures in the neurula) to design the pharyngeal skeleton was demonstrated.

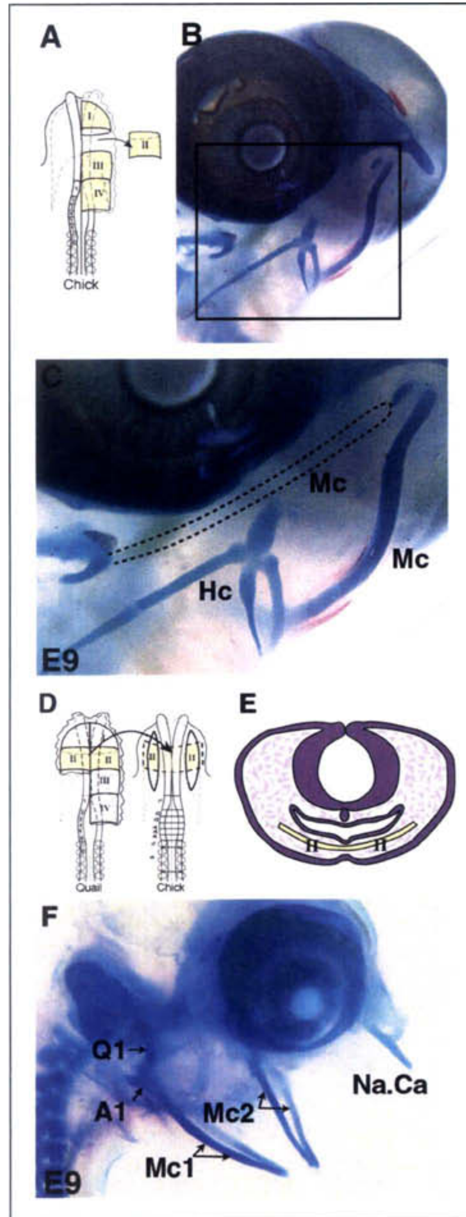

Figure 7. The ventral foregut endoderm patterns the NC-derived skeleton. A) Unilateral surgical extirpation of the zone II of the ventral foregut endoderm (shaded) facing the anterior mesencephalon in chick embryo. B) In E9 operated embryo, this extirpation leads to ipsilateral loss of Meckel's cartilage as evidenced on higher magnification (C; dotted line). Bilateral transplantation of endodermal zone II taken from a quail embryo (D) implanted ventrally to the endogenous ventral foregut of the host (E). F) In E9 recipient embryo, the additional endodermal stripe has induced a supernumerary mandible (Mc2) interposed between the endogenous upper and lower jaws. A1: endogenous articular; Hc: Hyoid cartilage; Mc1: endogenous Meckel's cartilage; Na.Ca: nasal capsule; Q1: endogenous quadrate.

Role of the Superficial Facial Ectoderm in Shaping the Face

In the mouse, *Fgf8* gene inactivation in the BA ectodermal component impairs lower jaw development.[112] Moreover, isthmus-derived FGF8 has been shown to be involved in the patterning of the visceral skeleton.[113]

One of the striking and early consequences of the surgical extirpation of the anterior cephalic NC which is responsible for building up the entire facial skeleton (i.e., NCCs from the di-mesencephalon and r1-r2), is the strong down-regulation of *Fgf8* expression in the anterior neural ridge (ANR, i.e., the prosencephalic superficial and neural ectoderm) (Fig. 8A,B).

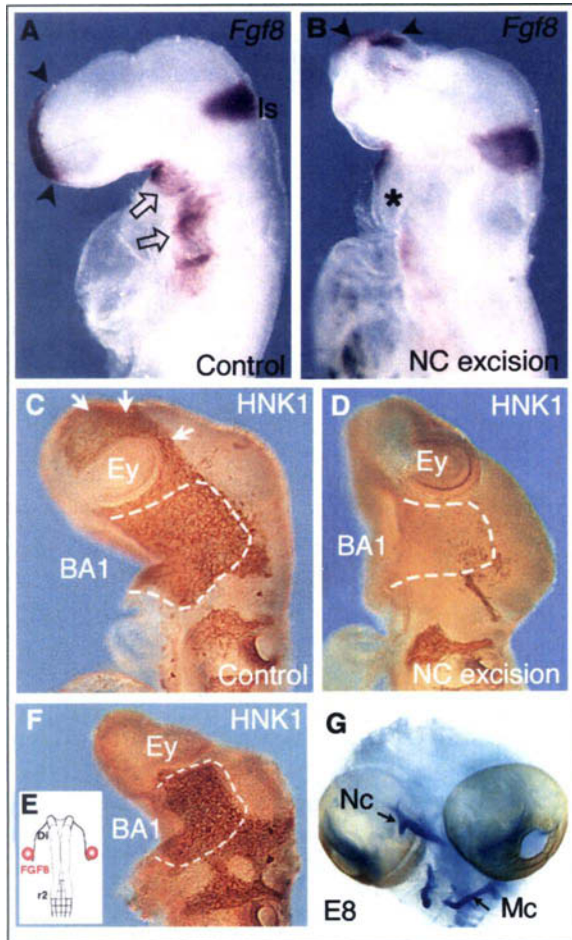

Figure 8. Reciprocal interplay between NCCs and *Fgf8* expression by the superficial and neural ectoderm. A) In E3-control embryos, *Fgf8* is expressed in the anterior neural ridge (ANR; i.e. the prosencephalic superficial and neural epithelium; arrowheads), in the ectoderm lining BA1 (open arrows), and in the isthmus (Is). B) Ablation of the *Hox*-negative NC entails a dramatic decrease of *Fgf8* expression in the ANR and BA1 ectoderm (*) 24 hours after surgery. C) In control embryos, the cephalic NCCs (HNK1 immunoreactivity) massively populate the forming BA1 (dotted line) and colonize the nasofrontal bud (arrows). D) After *Hox*-negative NC excision, only rare HNK1-labelled NCCs are present in BA1. E) Bilateral treatment of NC-excised embryos with FGF8 rescues the colonization of BA1 by r3-derived cells at E3 (F). G) At E8, these embryos have developed a maxillo-mandibular skeleton.

Moreover, this operation totally suppresses *Fgf8* expression in BA ectoderm. As mentioned above, the phenotype resulting from surgical extirpation of the cephalic NC is the absence of facial skeleton together with severe defects of forebrain development (Fig. 6A,B). If, in this experimental system the endogenous protein is substituted for by exogenous FGF8, the NCCs from r3 (which normally provides only a few cells to BA1) entirely colonize BA1, induce *Fgf8* expression in BA1 ectoderm and generate a normal jaw skeleton (Fig. 8C-G). In addition, the NCCs, even if they belong to the *Hox*-positive posterior domain of the neural axis, induce *Fgf8* expression in the prosencephalic superficial and neural ectoderm. Restoration of *Fgf8* expression in the prosencephalon is followed by normal forebrain development.[99] Therefore, (i) the cephalic NCCs are necessary for *Fgf8* expression by the epithelial ectodermal cells (ii) in turn FGF8 exerts a strong proliferative effect on cephalic NCCs.

These experiments pointed out to the strong regeneration capacities of r3-derived NCCs and to the role of FGF8 in regulating NCC proliferation and migration. In addition, it has been proposed that, in mice, *Fgf8* expression by the superficial ectoderm specifies the mesectoderm to form either the mandible or the tooth germs and refines the position of the developing jaw joint.[114,115]

The ectoderm of the fronto-nasal process was also shown to play an important role in positioning and refining the shape of the upper beak. This effect is mediated by the two morphogens Sonic hedgehog (SHH) and FGF8, the expression domains of which abut in the frontonasal ectodermal zone, which plays a role in the growth of the underlying mesenchyme of NC origin.[116,117]

Signals emanating from the NCCs are also required for shaping the face. This has been demonstrated by recent experiments in which NCCs were orthotopically exchanged between embryos of quail and duck. These two species were chosen because they show different bill morphologies and develop according to different timings of incubation (17 days for the quail and 28 days for the duck). These experiments revealed that NCCs convey the timing for *Shh* and *Pax6* gene expression to the host ectoderm. NCCs thus impose a donor- rather than host-type molecular pattern for bill morphology.[118]

In similar experiments, Tucker and Lumsden[119] have further analysed the morphology of the quail cartilages that develop within the duck environment (and vice-versa). They found that the shape of the facial cartilages (e.g., the entoglossum and the retroarticular process which strikingly differ between the duck and quail) is always of NC donor-type.

Therefore, once induced by the endoderm to develop into a particular cartilage, NCCs follow a species-specific genetic programme involving a particular growth and morphogenetic pattern. Another important conclusion drawn from these studies is that the dermal ossification of mandibular bones follows a species-specific timing of differentiation.

Thus, during facial morphogenesis, a temporally regulated and multi-step cross-talk occurs between the epithelia (endoderm and ectoderm) and the NCCs.

Heterogeneity of Progenitors and Stem Cells in the Early NC

The final phenotype adopted by NCCs depends mainly on local cues encountered by the cells during and at the end of their journey (see ref. 120 for a review). Single cell studies, essentially carried out in vitro, have revealed that migratory NCCs include highly pluripotent as well as oligopotent progenitors and fate-restricted precursors.[121-127] Some of the pluripotent and bipotent progenitors in the early NC have been shown to be stem cells endowed with self-renewal capacity.[125,127]

In the trunk NC, the pluripotent cells give rise in vitro to pigment cells, glial cells and PNS neurones as well as to myofibroblasts/smooth muscle cells expressing α-smooth muscle actin (αSMA), the latter cell type arising from the NC only at the cephalic level in normal development.[125,127,128]

Quail mesencephalic-rhombencephalic NCCs in culture generate mesectodermal derivatives such as chondroblasts and myofibroblasts.[121-123,127] Subsets of these NCCs exhibit the potential to develop into these mesenchymal as well as glial, neuronal and/or melanocytic cells, with diverse combinations of phenotypes. These cells however constitute a relatively small

proportion (7%) of the clonogenic cephalic NCCs in our culture conditions.[38] Such progenitors, which give rise both to neural-melanocytic (trunk-like) and to mesenchymal (cephalic-like) NC derivatives, could be identified late in NC ontogeny, including the migratory stages, thus arguing against the contention that mesectoderm might be segregated from other NC lineages already in the dorsal neural primordium.[129] The existence of common progenitors for neurones, pigment cells, myofibroblasts and chondrocytes has been also shown in the posterior rhombencephalic NC of the quail[130] and the mouse.[131]

Altogether, these results support the emergence of diversified cell types from highly pluripotent NC stem cells, through the generation of various intermediate progenitors endowed with stem cell properties (Fig. 9).

Resident NC Stem Cells Are Present in Differentiated Tissues up to Adulthood

At post-migratory stages, pluripotent NC stem cells were identified in the PNS nerves, DRG and in the gut of fetal and postnatal rat.[132-135] These findings further document the results of previous in vivo back-transplantations of quail NC derivatives, wherein grafted PNS ganglia gave rise to migratory cells able to colonize new targets and to differentiate into alternative phenotypes in the chick host tissues.[6]

The '*boundary cap cells*' of the dorsal root entry zone and motor exit points in the embryonic spinal cord were identified recently as a source of NC stem cells during neurogenesis.[136,137] These cells, which are derived from late-emigrating trunk NCCs, produce Schwann cells but also differentiate into subsets of sensory neurones and satellite glial cells in the mouse DRG, as shown by Cre-recombinase fate mapping using the *Krox20* locus.[136] Moreover, mouse '*boundary cap cells*' in clonal cultures comprise self-renewing progenitors for glia, sensory neurones and myofibroblasts.[137]

In the mammalian skin, progenitors isolated either from the dermal papillae[138,139] or the epidermal bulge area[140,141] of hair follicles, behave in vitro as pluripotent stem cells endowed with both neural and mesenchymal lineage potentials. Genetic tracing using *Wnt1-Cre* transgenic mice revealed that these progenitors are of NC origin. In the head region, the NCCs invade the presumptive skin early in mouse embryogenesis and comprise pluripotent stem cells, which thus persist up to adulthood in the vibrissae whisker follicles and may serve to regenerate multiple cell types during skin repair.

Therefore, a certain degree of differentiation plasticity and regeneration capacities characterize subsets of NC-derived cells long after PNS and skin organogenesis is completed. In mammals, the opportunity to prospectively isolate NC stem cell populations using cell surface markers combined with gene targeting mutations, allows further understanding the mechanisms of self-renewal[142] and maintenance of pluripotency.[143,144]

Plasticity of NC-Derived Phenotypes

How the NC-derived phenotypes are specified and thereafter maintained in NC derivatives is still poorly understood. We have shown recently that pigment cells and Schwann cells can change their phenotype and convert into each other in vitro upon mitogenic stimulation by endothelin-3 (ET3) peptide.

Schwann cells isolated from quail embryonic nerves are capable of changing their lineage programme and generate pigment cells in long-term cultures supplemented with ET3.[145] In addition to melanocytes, these cells give rise to myofibroblasts, which are obtained in culture earlier than melanocytes and independently of the cytokine ET3.[146] Moreover, after transplantation into the BA1 of younger chick embryos, the Schwann cells spread into the host mesenchyme and generate myofibroblasts that are recruited to participate in the formation of the peri-vascular smooth muscle layer.[146]

The melanocyte phenotype also displays a high plasticity in vitro. Single pigment cells purified from the quail epidermis until hatching stage, are able to yield glial and myofibroblastic cells in addition to parental-like melanocytes, following exposure to ET3 (see ref. 147 and Real et al submitted) (Fig. 10). This phenotypic reprogramming involves the dedifferentiation of

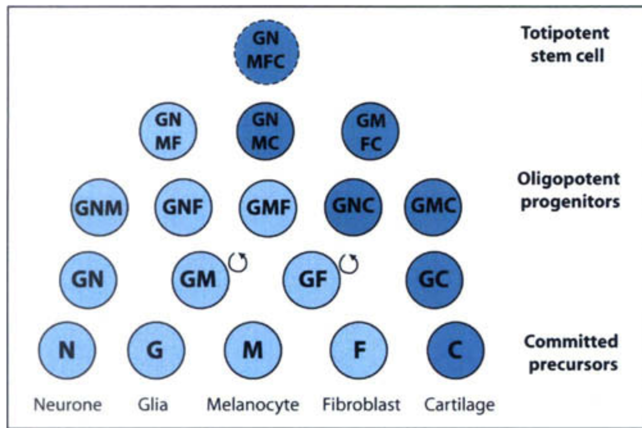

Figure 9. Model for the diversification of NC-derived lineages. The progenitors identified in the quail NC by in vitro clonal cultures are classified according to their number of potentialities. Data[122,127] support a hierarchical model for lineage segregation, according to which progressive restrictions of a putative pluripotent stem cell (broken circle) give rise to oligopotent progenitors and finally to precursors committed to each of the main NC-derived phenotypes, namely, glia (G), neurones (N), melanocytes (M), myofibroblasts (F) and cartilage (C). All the progenitors able to yield cartilage (dashed circles) are present in the cephalic but not in the trunk NC. At least some of the oligopotent progenitors exhibit the ability to self-renew in vitro (curved arrows).

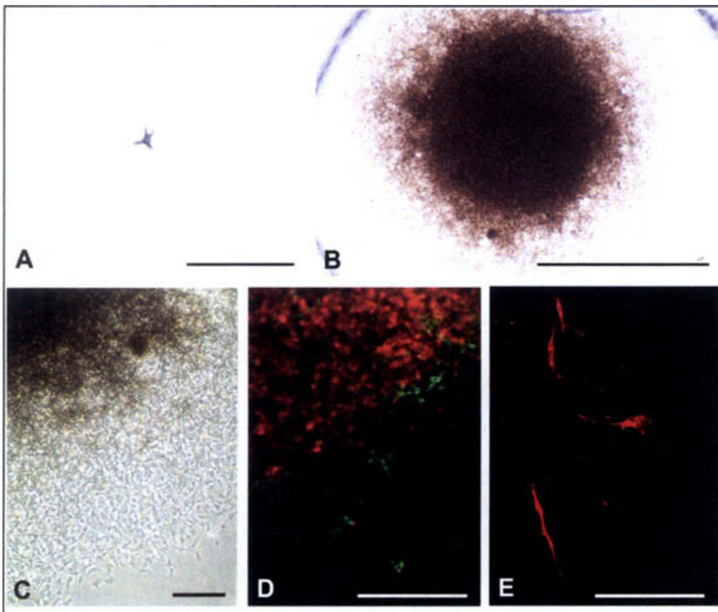

Figure 10. Pigment cells generate multiphenotypic clonal progeny in vitro. A) Culture of a single melanocyte from quail embryonic epidermis 4 hours after clonal seeding. B) After 13 days in the presence of endothelin-3 (ET3), the melanocyte has generated a large colony with a highly pigmented core. C) Detailed view of (B)—the periphery of the colony comprises both unpigmented and pigmented cells. D,E) Phenotypic analysis show that colony contains (D) melanoblasts and glial cells (immunoreative to MelEM and SMP markers)[147] as well as (E) myofibroblasts (expressing α-smooth muscle actin αSMA). Bars: 100 μm in (A) and (E), 200 μm in (C) and (D), 1 mm in (B).

dividing pigment cells into cells that reexpress NC early transcription factors such as *Sox10, FoxD3, Pax3* and *Slug*. Single melanocytes were shown to generate multipotent progenitors able to self-renew along serial subcloning, thus exhibiting characteristic properties of stem cells. Some of these stem cells harbour the HNK1 cell surface marker that is expressed by early NCCs and which had been extinguished in the parental differentiated pigment cells (Real et al submitted).

Therefore, when removed from their *'niche'* and subjected to new environmental in vitro conditions, embryonic pigment cells and Schwann cells can reverse their differentiation programme and recapitulate early properties of their NC ancestors. These findings suggest that differentiated cell types derived from the NC are phenotypically unstable and capable of broad differentiation plasticity. In the skin and nerves, the dedifferentiated NCCs thus might be mobilized for tissue repair, alternatively or complementary to resident undifferentiated NC stem cells.

Concluding Remarks

During the last ten years, the NC has become a popular subject in cell and developmental biology. This is justifiable by the widely diversified interests offered by this system. First, in the group of chordates characterized by a common body plan, the NC is present only in vertebrates. It is considered to have been essential in the transition from cephalochordates to vertebrates since specific vertebrate traits within the chordate phylum such as skeletal tissues, PNS and spectacular head and brain development, are linked to the NC and its derivatives.

The first emphasis on the role of the NC in the evolution of chordates was brought about in a well-acknowledged article by Carl Gans and Glenn Northcutt in 1983.[148] These authors proposed that the NC, which gives rise to most of the skull, the meninges and the vascularization of forebrain and face (as revealed by embryological studies, see ref. 2 for references), was responsible for the formation of a *'New Head'* characterized by the spectacular development of the forebrain and associated sense organs. According to Gans and Northcutt, these attributes were related to a radical change in the life style of the vertebrates as compared to the cephalochordates: vertebrates became able to look for their food and even became predators, whereas the cephalochordates, like their extant form the *Amphioxus* (considered as closely related to the vertebrate ancestors), were filter-feeders. It is noticeable that the first organ of predation that appeared in vertebrates is the jaw, which is entirely NC-derived (see above).

In addition to its interest in vertebrate evolution, the NC is a system on which a large register of developmental problems can be studied in a privileged manner. As mentioned above, the NCCs are endowed with migratory properties and, in this respect they can be a model for studying the molecular mechanisms that regulate cell migrations which commonly occur in normal and pathological conditions.

The NC is a highly pluripotent structure. Several laboratories have demonstrated the presence of NC stem cells both in the embryo and in the adult derivatives of this structure. NCCs of birds and mammals can be cultivated in vitro and their plasticity was shown to be remarkable. A lot remains to be discovered, in terms of cytokines active on the various NC-derived lineages and of their mode of action in these well-defined experimental models.

Finally, by its ubiquitous ramifications all over the organism, the PNS is critical in coordinating the functions of various cellular components of the vertebrate body.

Many key problems remain to be investigated about the NC, among which, the genetic control of its formation in the embryo is particularly interesting owing to its possible evolutionary implications.

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