Int. J. Dev. Biol. 47: 541-553 (2003)

Origins and plasticity of neural crest cells and their roles in jaw and craniofacial evolution

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ABSTRACT The vertebrate head is a complex assemblage of cranial specializations, including the central and peripheral nervous systems, viscero- and neurocranium, musculature and connective tissue. The primary differences that exist between vertebrates and other chordates relate to their craniofacial organization. Therefore, evolution of the head is considered fundamental to the origins of vertebrates (Gans and Northcutt, 1983). The transition from invertebrate to vertebrate chordates was a multistep process, involving the formation and patterning of many new cell types and tissues. The evolution of early vertebrates, such as jawless fish, was accompanied by the emergence of a specialized set of cells, called neural crest cells which have long held a fascination for developmental and evolutionary biologists due to their considerable influence on the complex development of the vertebrate head. Although it has been classically thought that protochordates lacked neural crest counterparts, the recent identification and characterization of *amphioxus* and ascidian genes homologous to those involved in vertebrate neural crest development challenges this idea. Instead it suggests that the neural crest may not be a novel vertebrate cell population, but could have in fact originated from the protochordate dorsal midline epidermis. Consequently, the evolution of the neural crest cells could be reconsidered in terms of the acquisition of new cell properties such as delamination-migration and also multipotency which were key innovations that contributed to craniofacial development. In this review we discuss recent findings concerning the inductive origins of neural crest cells, as well as new insights into the mechanisms patterning this cell population and the subsequent influence this has had on craniofacial evolution.

KEY WORDS: *craniofacial, evolution, neural crest cells, amphioxus, ascidians, lampreys, vertebrates*

Historical perspective on the discovery of neural crest cells

In 1868 the Swiss embryologist, Wilhelm His, identified a band of cells sandwiched between the epidermal ectoderm and the neural tube in neurula stage chick embryos. This band of cells which he called Zwischenstrang (the intermediate cord) were the source of cranial and spinal ganglia. Today, we now know the intermediate cord as the neural crest and this term appears to have been used first by Arthur Milnes Marshall (Hall, 1999). Although it was initially associated with the origins of neurons and ganglia, Julia Platt demonstrated in the 1890s that the visceral cartilages of the head and dentine forming cells of the teeth in the mud puppy *Necturus*, also arise from the neural crest (Platt, 1897). Platt's results were extremely controversial as they ran counter to the prevailing germ layer theory of the day and her hypothesis of the cranial skeletogenic origins in the neural crest only truly gained acceptance 50 years later primarily through the seminal work of Sven Horstadius (Horstadius, 1950). The number of cell types that arise from the neural crest is truly astonishing as is the number of tissues and organs to which the neural crest contributes. In addition to forming melanocytes and the neurons and glia of the peripheral nervous system, cranial neural crest cells also contribute most of the cartilage, bone and connective tissue of the face (Fig. 1). These multipotent migrating neural crest cells are capable of some self renewing decisions and based upon this criteria are often considered stem cells or stem cell like (Labonne and Bronner-Fraser, 1998).

Induction of neural crest cells

Neural crest cells arise uniformly at the dorso-lateral edge of the closing neural folds, along almost the entire length of the vertebrate embryo neuraxis (Fig. 2). This region corresponds to the interface

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0214-6282/2003/$25.00

Printed in Spain
www.ijdb.ehu.es

between the non-neural ectoderm (presumptive epidermis or surface ectoderm) and the neural plate (neuroepithelium), a region commonly referred to as the neural plate border. *Slug* and *Snail* are members of the *Snail* zinc-finger transcription factor family, and are two of the earliest known indicators of neural crest cell formation (Fig. 2B) (Nieto, *et al.,* 1994; Sefton, *et al.,* 1998). Neural crest cell induction requires contact mediated interactions between the surface ectoderm and neuroepithelium and importantly, each of these tissues contributes to the neural crest cell lineage (Selleck and Bronner-Fraser, 1995). Lineage tracing studies have demonstrated that a single dorsal neural tube cell can give rise to both neural tube and neural crest derivatives. Thus it is logical to expect that bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and members of the Wnt signalling family which are critical for specifying neural plate induction and determining the boundary of neural and epidermal fate, may also play important roles in inducing and specifying the differentiation of neural crest cells.

BMP signalling plays a critical role in positioning the border of the neural plate during vertebrate gastrulation, and it also functions in the induction and migration of neural crest cells. In open neural plate stage avian embryos, *Bmp4* and *Bmp7* are expressed in the surface ectoderm. During neural tube closure, *Bmp* expression becomes downregulated in the surface ectoderm however it continues to be expressed in the dorsal neural tube. This led to a model whereby BMP proteins, secreted by the surface ectoderm, were hypothesised to function in the neural plate to induce the formation

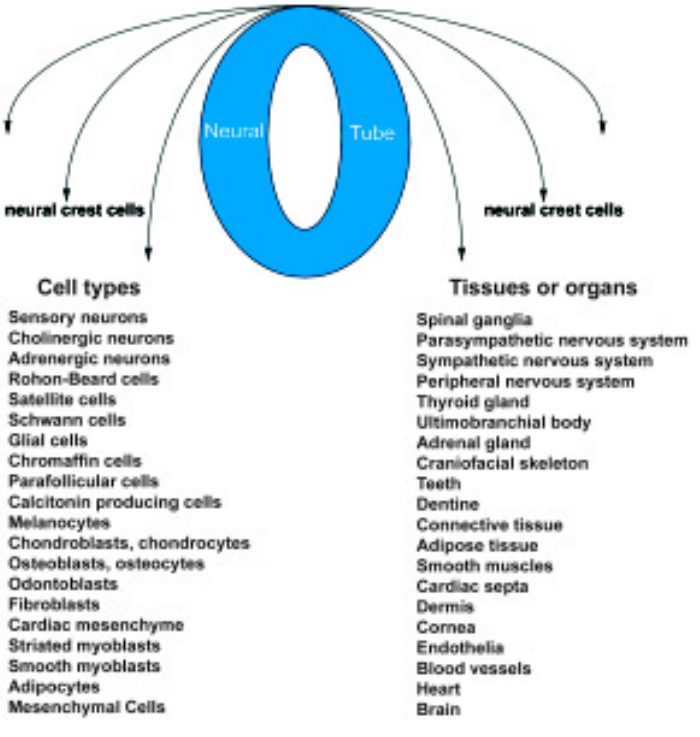

Fig. 1. Neural crest cell derivatives. *Derived from the neural tube, neural crest cells are a pluripotent, mesenchymal population that migrates extensively and gives rise to a vast array of cell types, tissues and organs. Given the wide variety of differentiative fates and a limited capacity for self renewal, neural crest cells are often considered to be a stem cell like population.*

and migration of neural crest cells. Supporting evidence for this model came from the demonstration that BMP4 and BMP7 could substitute for non-neural ectoderm in neural crest cells induction assays, and also that BMP4 induces *Slug* expression and neural crest segregation from avian neural plate explants (Liem, *et al.,* 1995). BMP4 activity is also required for the maintenance of a variety of dorsal neural tube genes including *Slug*, *cadherin6b*, *RhoB*, *Pax3*, *Msx1* and *Msx2*, suggesting that these molecules may mediate a BMP-dependent signalling cascade. Implanting noggin-expressing cells during neural tube closure inhibits neural crest cell formation and migration. Therefore, the dynamic spatiotemporal pattern of expression is consistent with BMP4 playing a role in neural crest cell induction and migration, but the evidence implies that BMP4 signalling in the neural tube may be more important for neural crest induction than earlier BMP4 signalling in the ectoderm.

The upstream factors that control *Bmp4* expression and restrict the generation of neural crest cells to the neural and non-neural ectoderm boundary have recently been described (Fig. 2B). *Delta1* mediated activation of *Notch* signalling in the epidermis can regulate *Slug* expression through the BMP4 signalling pathway (Endo, *et al.,* 2002). Dominant negative inactivation of *Delta* signalling represses *Slug* expression. This downregulation can be rescued by BMP4, which implies that *Delta1* activates *Notch* to promote *Bmp4* expression in the epidermal ectoderm. Interestingly, however, overexpression of *Notch* decreases *Bmp4* and *Slug* expression, resulting in decreased neural crest cell delamination and migration. This suggests that modest activation of *Notch* by *Delta1* is needed to promote *Bmp4* expression. Furthermore it seems likely that *Delta1* mediated *Notch* activation promotes *Bmp4* expression in the epidermal ectoderm, which subsequently induces neural crest induction at the neural/epidermal junction. While *Notch* promotes or maintains BMP signalling in the epidermis, it also inhibits *Slug* in this region, defining the signals for neural crest specification only in the neural folds. Thus it could be argued that *Notch* expression defines a tightly regulated region for the induction of neural crest, restricting induction by BMP4 to the neural plate border (Endo, *et al.,* 2002).

Collectively the evidence described above implies BMP signalling plays a significant role in neural crest induction. However a recent study has raised doubts concerning some of the *in vitro* analyses ascribing roles for BMP4 and BMP7 signalling in neural crest cell induction (Garcia-Castro, *et al.,* 2002). The problem relates to the use of serum containing, nutrient rich media in explant culture assays. In chemically defined DMEM without supplementation, BMP signalling alone cannot induce neural crest cells in avian neuroepithelial explants. Surprisingly, the same study revealed that Wnt signalling alone is both necessary and sufficient for inducing neural crest cells in avian neuroepithelium explants as evidenced by the activation of *Slug* expression. Furthermore, inhibiting Wnt signalling both in cultured neuroepithelial explants and in whole avian embryos blocks neural crest cell induction. These new findings are consistent with Wnt overexpression experiments performed in *Xenopus* embryos, which result in an increase in several neural crest markers (Saint-Jeannet, *et al.,* 1997). It is also in agreement with the observation that *Wnt1/Wnt3a* double mutant mice exhibit skeletal abnormalities and a marked reduction in melanocytes, cranial and spinal sensory neurons, all of which are derived from neural crest cells

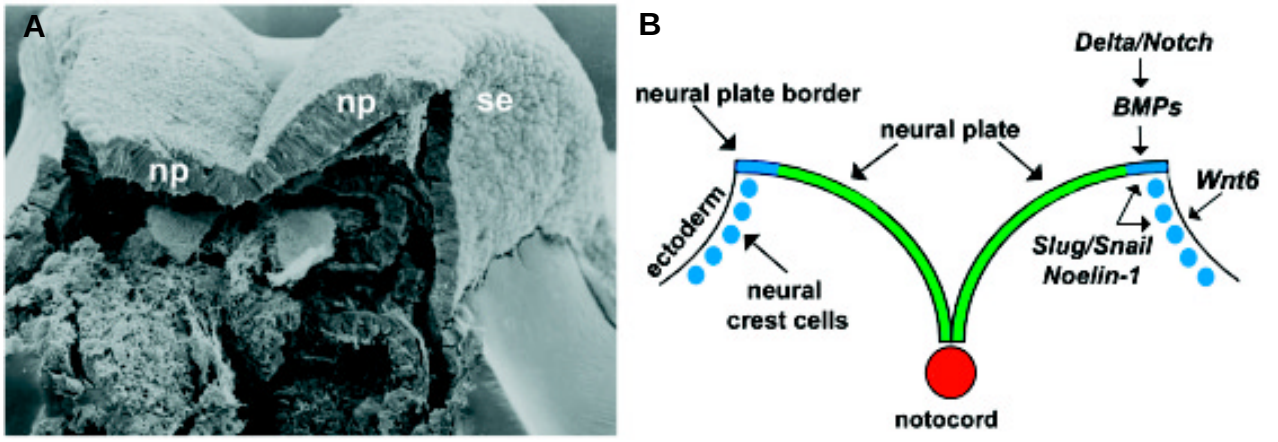

Fig. 2. Genetic regulation of neural crest cell induction. (A) *Neural crest cells are born at the neural plate border, which is the junction between the surface ectoderm (se) and the dorsal region of the neural plate (np). Both the surface ectoderm and neural plate can give rise to neural crest cells.* **(B)** *BMP signalling is essential for establishing the boundary of the neural plate border and for the induction of neural crest cells in the dorsal region of the neural tube. BMP and its effects on neural crest induction can be modified by the Delta/Notch signalling pathway.* Snail, Slug, Dlx, AP-2, and Noelin-1 *genes are all expressed in either pre-migratory and/or migratory neural crest cells and are regulated by BMP signalling.* Wnt6 *is expressed in the surface ectoderm and is also a prime candidate for inducing neural crest cell formation. It remains to be determined whether Wnt and BMP signalling act synergistically during neural crest cell induction. Homologues of vertebrate genes expressed in pre-migratory and migratory neural crest cells have now been identified in protochordates which suggests that the necessary genetic machinery for neural crest formation and migration was already present in protochordates.*

(Ikeya, *et al.,* 1997). These results imply that Wnt signalling is the primary instigator of neural crest cell formation and furthermore that *Wnt6* is the prime candidate for being the inducing signal as it is spatiotemporally expressed in the surface ectoderm at the time of neural crest induction (Fig. 2B). Despite these new findings, we are still left with many unresolved issues. For instance, it is not known whether dominant negative Wnts can block the action of BMPs in serum rich culture media or whether *Wnt6* expression is capable of inducing neural crest cells in the presence of BMP inhibitors. Answers to these questions would surely help to resolve whether Wnts regulate BMP signalling or whether synergy between these two pathways is required for neural crest formation. It could also clarify some of the discrepancies that have been observed between different species. The *in vivo* contact between the neuroepithelium and the surface ectoderm that is integral to the generation of neural crest cells may indicate that a BMP/Wnt signalling interface is necessary for neural crest cell induction.

Recently it has been shown in *Xenopus* that FGF signalling from the mesoderm is also an important player in neural crest induction (Monsoro-Burq, *et al.,* 2003). More specifically FGF signalling exerts its function even in the absence of BMP and Wnt signalling and it will be intriguing in the future to determine if FGF signalling is really at the start of the neural crest induction cascade in other vertebrates as well. Collectively however, these results demonstrate that BMP, Wnt and FGF signalling pathways all play critical roles in inducing neural crest cell formation.

Delamination of neural crest cells

Concomitant with their induction along the dorsolateral edge of the neural plate, neural crest cells undergo an epithelial to mesenchymal transition whereby they delaminate from the neural tube and begin to migrate (Fig. 3). Epithelial to mesenchymal transitions are marked by changes in cell adhesion and cytoarchitecture. During delamination neural crest cells downregulate cell adhesion molecules such as *Ncam*, *N-cadherin* and *cadherin6B*, and upregulate *cadherin7* and *cadherin11* (Akitaya and Bronner-Fraser, 1992; Kimura, *et al.,* 1995; Nakagawa and Takeichi, 1998). This *cadherin* switch demonstrates that a regulated balance of *cadherin* expression is needed for emigration, which is supported by the fact that overexpression of neuroepithelial cadherins prevents neural crest emigration (Nakagawa and Takeichi, 1998). As described above, two of the earliest known indicators of neural crest induction are *Slug* and *Snail* which normally act as transcriptional repressors. *Snail* binds to the promoter of the cell adhesion molecule *E-cadherin*, repressing its expression (Cano, *et al.,* 2000). Thus, ectopic expression of *Snail* in epithelial cell lines results in the downregulation of *E-cadherin*, which represses epithelial to mesenchymal cell transformations and consequently inhibits cell migration. This implies that *Snail* may promote the epithelial to mesenchymal cell transitions associated with neural crest cell delamination and migration from the neural tube through effecting changes in cell adhesion. In agreement with this hypothesis, *Slug* antisense mRNA oligonucleotide treatment of avian (Nieto, *et al.,* 1994) or *Xenopus* (Barembaum, *et al.,* 2000) embryos results in the inhibition of cranial neural crest cell migration. BMP signalling has been shown to induce the expression of both *Slug* and *cadherin6* demonstrating that the same signal can fulfil multiple roles during development. BMP signalling, which is critical for neural crest induction, also plays a role in neural crest delamination (Fig. 2B). Furthermore, *Delta-N*otch signalling promotes *Bmp4* expression while at the same time inhibiting *Slug* expression and this could provide a mechanism for effectively controlling the formation and delamination of neural crest at the neural-epidermal junction (Endo, *et al.,* 2002). Interestingly in *Xenopus*, *Slug* expression increases neural crest production only in territories of endogenous expression implying that other factors besides *Slug* are necessary to regulate neural crest delamination (Labonne and Bronner-Fraser, 2000).

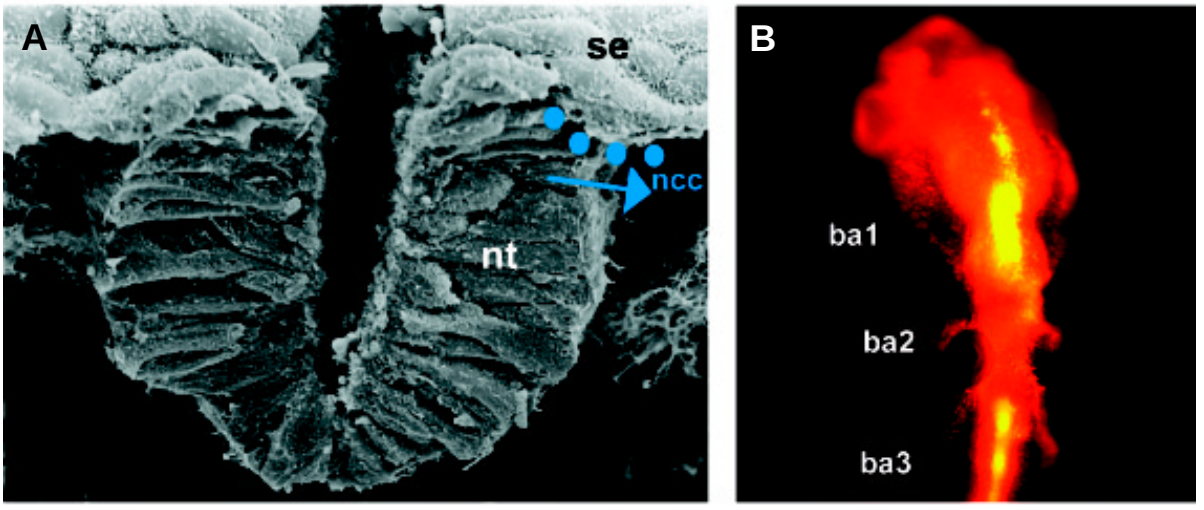

Fig. 3. Cranial neural crest cell migration. (A) *During neurulation, the neural plate curls up to form a hollow neural tube (nt) and during this time neural crest cells are induced to form at the neural plate border. Neural crest cells undergo an epithelial to mesenchymal transition, delaminate from the neural tube and commence their migration to the periphery of the head.* **(B)** *In the vertebrate head, the majority of neural crest cells are derived from the hindbrain and the neural crest cells migrate in three discrete segregated streams into the branchial arches (ba). The neural crest cell derivatives are distinct for each branchial arch.*

One of these additional signalling factors is the GTP-binding protein *RhoB*, which is a member of the ras gene superfamily. *RhoB* has been implicated in playing a role in the delamination of neural crest cells as it is selectively expressed by cells in the dorsal neural tube and transiently expressed by the neural crest. *RhoB* expression is also induced by BMPs, and the inhibition of *RhoB* prevents neural crest delamination from dorsal epithelium (Liu and Jessell, 1998). The delamination and migration of neural crest cells from the neural tube following the epithelial to mesenchymal transition of neuroepithelial precursors is a transient event. The maintenance of the competence of the neural epithelium to generate neural crest cells appears to be regulated by the glycoprotein Noelin-1 (Barembaum, *et al.,* 2000). *Noelin-1* is expressed initially along the lateral edges of the neural plate but is also expressed later in migrating neural crest cells. The overexpression of *Noelin-1* in the neural tube by retroviral infection results in a prolonged period of neural crest production and migration (Barembaum, *et al.,* 2000). Similarly overexpression of *Noelin-1* prolongs the period during which neural crest cells can regenerate after dorsal neural tube ablation. These findings suggest that *Noelin-1* may maintain the period during which the neural epithelium is competent to generate neural crest cells.

Specification of the pathways of neural crest cell migration

Following delamination from the neural tube, neural crest cells migrate along specific pathways to their final destinations (Fig. 3). In the vertebrate head, the majority of cranial neural crest cells are derived from the hindbrain, which migrate ventrolaterally from the neural tube in three distinct sub-ectodermal streams adjacent to the even numbered rhombomeres (r2, 4 and r6; Fig. 3B). The three streams of neural crest cells populate the first, second and third branchial arches respectively in keeping with their craniocaudal axial origins (Lumsden, *et al.,* 1991) and give rise to a wide variety of cell lineages (detailed in Fig. 1), which are distinct for each branchial arch (Noden, 1983; Kontges and Lumsden, 1996). The mechanism by which the neural crest exclusion zones adjacent to the odd numbered rhombomeres (r3 and r5) are generated and their function in segregating neural crest cells into distinct streams remains to be resolved.

Analyses in avian embryos reported elevated levels of cell death in the pre-migratory neural crest populations resident in r3 and r5 and inter-rhombomeric signalling was believed to be a key player in modulating this process (Graham, *et al.,* 1993; Graham, *et al.,* 1994). It was reported that signals originating from the even numbered rhombomeres and mediated by *Bmp4* induced *Msx2* expression in r3 and r5 led directly to the apoptotic elimination of premigratory neural crest cells in r3 and r5 and hence segregated the neural crest cells into discrete streams. *Bmp4* and *Msx2* are normally expressed in r3 and r5 during the period of neural crest formation in avian embryos. Recently it was shown that overexpression of a Wnt antagonist *sFRP2* could inhibit BMP signalling thereby preventing programmed cell death (Ellies, *et al.,* 2000). This lead to a more recent hypothesis in which the apoptotic elimination of neural crest precursors was modulated by a WNT-BMP signalling loop and that this apoptotic cell death was instrumental in the evolutionary elimination of unnecessary muscle attachment sites (Ellies, *et al.,* 2002). *In vitro* models proposing *Bmp4/Msx2* mediated cell death in odd numbered rhombomeres as a mechanism for segregating streams of neural crest cells remain slightly controversial because similar to the controversy surrounding assays of neural crest induction described earlier, the early examinations of neural crest cell death were performed in serum rich containing media. Repeat analyses using chemically defined media instead of serum containing media failed to demonstrate any causal link between BMP signalling and the specific apoptotic elimination of neural crest cells from odd numbered rhombomeres (Farlie, *et al.,* 1999). Furthermore, it is important to note that blocking neural crest cell death in the odd numbered rhombomeres does not disrupt the segregation of neural crest streams (Ellies, *et al.,* 2000), which together with more recent investigations emphasizes the importance of the microenvironment adjacent to the neural tube in regulating the pathways of neural crest

cell migration (Kulesa and Fraser, 1998; Golding, *et al.,* 2000; Trainor, *et al.,* 2002).

The influence of the microenvironment is clearly evident from lineage tracing and time-lapse imaging of neural crest cell migration in avian embryos which have demonstrated that r3 and r5 do in fact generate small numbers of neural crest cells. Rather than migrating laterally like neural crest cells derived from even numbered rhombomeres, odd rhombomere derived neural crest cells migrate anteriorly and posteriorly and join the even numbered rhombomere neural crest streams. On occasions when an r3 neural crest cell does delaminate and migrate laterally, its filopodia can be observed collapsing as it contacts the environment adjacent to r3 (Kulesa and Fraser, 1998). Interestingly, frog and zebrafish neural crest cells migrate laterally adjacent to rhombomere 5 (Smith, *et al.,* 1997). Together these results demonstrate that the presence of neural crest free zones and the segregation of neural crest cells into discrete streams is not an intrinsic property of the vertebrate hindbrain and odd numbered rhombomeres in particular. These results also underscore the importance of the environment adjacent to the neural tube in regulating the pathways of neural crest cell migration and raise the possibility of distinct mechanisms being used by individual species.

Recent investigations of neural crest patterning in mouse embryos revealed that the dynamic patterns of cell death in the neural tube do not correlate with the generation or migration of neural crest cells (Trainor, *et al.,* 2002). Furthermore, reciprocal cell transplantations between odd and even rhombomeres demonstrate that odd and even rhombomeres have a similar intrinsic capability to generate large numbers of neural crest cells and that specific tissue environments are inhibitory to neural crest cell migration (Trainor, *et al.,* 2002). Importantly, this mechanism may be conserved between mouse and chick. This implies that inter-rhombomeric signalling is less important than the combinatorial interactions between the hindbrain and adjacent paraxial tissue environment in the process of restricting the generation and migration of neural crest cells. This mechanism appears to be supported by analyses of neural crest cell migration in *ErbB4* null mutant mice in which r4 derived neural crest cells acquire the ability to migrate through the dorsal mesenchyme adjacent to r3 (Golding, *et al.,* 2000). The aberrant migration arises as a consequence of changes in the paraxial mesenchyme environment and is not autonomous to the neural crest cells. Since *ErbB4* is normally expressed in r3 and r5 this phenotype reflects defects in signalling between the hindbrain and the adjacent environment. It also highlights the presence of additional mechanisms for restricting the mixing of neural crest cells since ventrally, the neural crest streams remain segregated as they migrate into the branchial arches in mutant embryos.

To date few molecules that influence the path finding of cranial neural crest cells have been identified. However the presence of a neurite outgrowth inhibitor in the mesenchyme adjacent to r3 may be part of the mechanism that restricts the lateral migration of neural crest cells from r3 (Golding, *et al.,* 1999). Evidence obtained primarily from analyses in frog embryos suggests that bi-directional *Eph/ephrin* cell signalling plays an important role in keeping the neural crest cell streams segregated ventrally (Smith, *et al.,* 1997). Future analyses of other gene families involved in axon guidance such as the *semaphorins*, *slit/robo* and *neuropilins,* may also prove to be important regulators of cranial neural crest cell migration.

The different mechanisms observed in various species may be related to the specific tissue interactions the neural crest cells experience during their migration. In frogs and zebrafish, neural crest cells primarily interact with the endoderm whereas in mice and chicks, interactions with mesoderm (Trainor and Krumlauf, 2000; Trainor, *et al.,* 2002), ectoderm (Couly and Le Douarin, 1990; Tucker and Sharpe, 1999; Golding, *et al.,* 2002) and endoderm (Veitch, *et al.,* 1999; Couly, *et al.,* 2002) have all been reported. Irrespective of the mechanism that is used to pattern the migration of cranial neural crest cells, the segregation of neural crest cells into discrete streams in the vertebrate head is essential for keeping *Hox* expressing and non-*Hox* expressing neural crest cells separated from each other. This is borne out by the fact that overexpression of *Hoxa2* in first branchial arch neural crest cells, transforms the identity of the first arch into that of a second arch, which subsequently suppresses jaw formation (Grammatopoulos, *et al.,* 2000; Pasqualetti, *et al.,* 2000). Conversely in *Hoxa2* null mutant mice, the second arch is transformed into a first arch identity resulting in duplicated first arch skeletal structures. This highlights the incompatibility of *Hoxa2* expression and jaw formation (Couly, *et al.,* 1998; Creuzet, *et al.,* 2002). The segregation of neural crest streams also prevents cranial ganglia fusions as is evident in a number of mouse mutations where neural crest streams were able to mix. Therefore the segregation of neural crest cells into discrete streams is a pre-requisite for proper jaw formation and subsequently normal head development.

The embryological and genetic origins of neural crest cells

The appearance of neural crest cells coincided with the emergence of vertebrates and since the diversification of neural crest cell derivatives is associated with vertebrate radiations, considerable attention has been given to identifying the origins of neural crest cells. Vertebrates belong to the phylum Chordata, which includes two protochordate groups: the cephalochordates represented by *amphioxus* and the urochordates, represented by ascidians (Fig. 4). Protochordates (cephalochordates plus urochordates) share a similar body plan to vertebrates along with several other characteristic features such as a dorsal tubular nervous system, notochord and pharyngeal gill slits. Despite the simplicity of the protochordate neural tube, its basic structural and genetic organization highlights the high degree of conservation between protochordates and vertebrates (Holland and Chen, 2001; Wada and Satoh, 2001). Dorso-ventral differentiation of the neuraxis in protochordates and vertebrates certainly appears to be controlled by similar genetic mechanisms (Shimeld, 1999). *Shh* expression is confined to the ventral region of the neural tube in *amphioxus* (Shimeld, 1999) and in *ascidians BMPs* are expressed in the ectoderm adjacent to the neural plate (Miya, *et al.,* 1997). Despite the conservation of the basic dorso-ventral patterning systems, it has been classically assumed that neural crest cells are a unique vertebrate cell type. Neither ascidians nor *amphioxus* possess cells that fit the traditional definition of neural crest cells, that is, migratory cells derived from the boundary between the neural epithelium and epidermis. Such migratory populations that give rise to typical neural crest derivatives such as neurons, glia, melanocytes, cartilage and bone are thought to be lacking in both non-vertebrate chordates (urochordates and cephalochordates) and also in non-chordate deuterostomes (echinoderms and hemi-

chordates) (Turbeville, *et al.,* 1994; Wada and Satoh, 1994). If neural crest evolution was truly a vertebrate specific event, then it may have been a consequence of genome duplication (Baker and Bronner-Fraser, 1997). Gene duplications are permissive for the evolution of new gene functions since one of the duplicated genes can diverge and become co-opted for new functions. Although gene duplication might enable the evolution of new cell types, its occurrence cannot on its own tell us how new cell types evolved, because even the most ancient living (jawless) vertebrates, hagfish and lampreys, already have most of the spectrum of neural crest derivatives (Baker and Bronner-Fraser, 1997).

What if neural crest cells were not a vertebrate specific characteristic? As described earlier, contact mediated interactions between the neural epithelium and epidermis are essential for the induction and specification of neural crest cells in all vertebrates (Fig. 2) (Selleck and Bronner-Fraser, 1995). Therefore it is logical to assume that neural crest cells probably evolved in the equivalent region at the junction between the neuroepithelium and epidermis in the vertebrate ancestors (Holland and Holland, 2001; Wada and Satoh, 2001). The cephalochordate *amphioxus* is generally acknowledged to be the closest extant relative of the vertebrates and recent evidence suggests that two cellular features of the dorsal-midline epidermis in *amphioxus* are very neural crest like. Firstly, distinct ectodermal cells lie at the lateral border of the neural plate and secondly these epidermal cells migrate over the neural tube towards the midline (Holland, *et al.,* 1996). Similarly ascidians (urochordates) have a population of cells in the boundary between the neural tube and epidermis that emerge from the remnant of the embryonic neural tube and from which both neuronal cells such as peripheral neuroendocrine cells and epidermal cells differentiate (Mackie, 1995; Powell, *et al.,* 1996). This raises the tantalizing prospect that the precursors for the vertebrate neural crest already existed in protochordates and may have originated from the dorsal midline epidermis (Wada and Satoh, 2001). One needs to be cautious however in drawing this conclusion since the criteria that is used for defining "neural crest properties" in this case is restricted narrowly to the ability of the cell population to migrate and its very limited differentiation capacity.

Neural crest cell determination is tightly linked to the specification of dorsal neural identity. In support of the midline origins of neural crest cells, protochordate genes homologous to those involved in pre-migratory and migratory vertebrate neural crest specification are expressed at the edges of the neural plate and non-neural ectoderm in *amphioxus* and ascidians (Holland, *et al.,* 1996; Holland and Holland, 1998; Shimeld and Holland, 2000; Wada and Satoh, 2001). This is certainly evident in the function of BMP signalling in distinguishing neural from non-neural ectoderm. In *Drosophila*, as well as in *amphioxus*, *Bmp2/4* homologues are expressed in non-neural ectoderm only (Wilson and Hemmati-Brivanlou, 1995; Panopoulou, *et al.,* 1998). Similarly *Bmpa*, an ascidian homologue of *Bmp7,* is expressed in ectodermal cells during gastrulation and becomes upregulated in the dorsal midline epidermal cells during neurulation (Holland and Holland, 2001). In vertebrates, both BMP4 and BMP7 are able to substitute for the activity of the non-neural ectoderm in inducing neural crest cells from the neural plate (Liem, *et al.,* 1995).

Several other genes such as members of the *Pax*, *Dlx*, *Snail* and *AP-2* families are also expressed in this boundary region of the neural plate and are implicated in neural crest cell specification (Fig. 2B). *Pax3* and *Pax7* are initially expressed throughout the entire neural plate of vertebrates before being refined to the dorsal neural tube and neural crest. Vertebrate mutations in both *Pax3* and *Pax7* result in neural crest differentiation defects. *Pax3* and *Pax7* have a single gene homologue in ascidians, *HrPax-3/7*, which exhibits a pattern of expression similar to its vertebrate counterparts (Wada, *et al.,* 1997). *HrPax-3/7* is expressed in cells destined to form the dorsal neural tube and also in those cells destined to form dorsal midline epidermis. The *amphioxus* homologue of *Pax3/7* is expressed in the lateral part of the neural plate, which subsequently occupies the dorsal part of the neural tube (Holland, *et al.,* 1999).

Vertebrate *Distal-less* genes are also expressed in migrating and differentiating neural crest cells (Fig. 2) (Akimenko, *et al.,* 1994; Qiu, *et al.,* 1995). The ascidian homologue of *Dlx* is expressed in the dorsal midline epidermis (Wada, *et al.,* 1999).

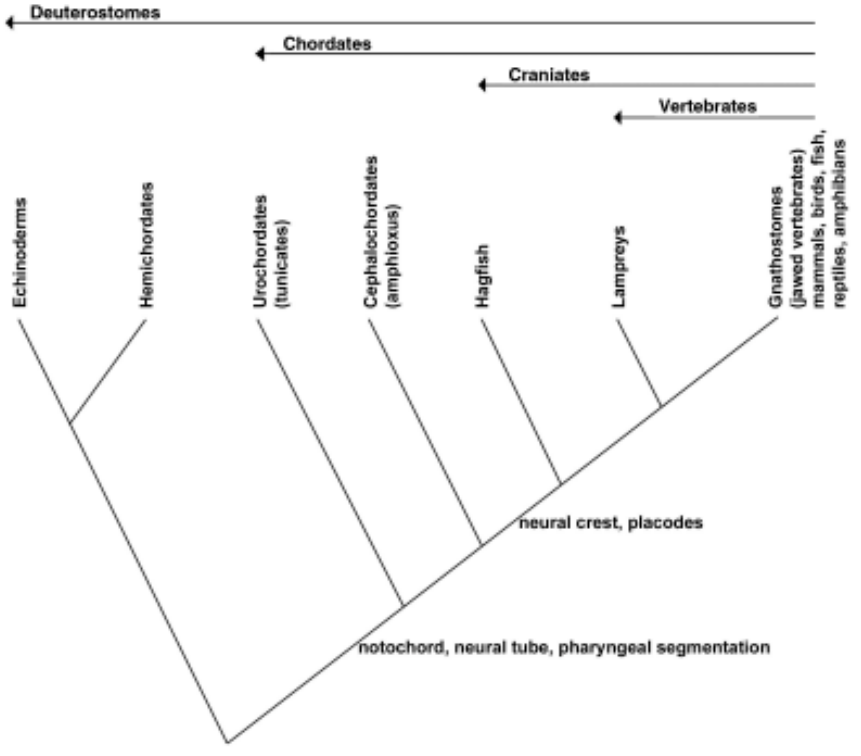

Fig.4. Evolution of vertebrate characteristics. *Cladogram showing that the transition from invertebrate to vertebrate chordates was a multistep process involving the formation and patterning of many new cell types and tissues. The emergence of the first vertebrates, the jawless fish (hagfish and lampreys), was accompanied by the formation of new specialized sets of cells, such as neural crest cells and placode cells. Based on homologous gene comparisons, it has been argued that neural crest precursors could have already been present in* amphioxus *and that during evolution, they acquired the ability to migrate along with a multipotent differentiative capacity, which are characteristics of vertebrate neural crest cells.*

AmphiDll, the *amphioxus* homologue of *Dlx* is expressed in the dorsal neural tube and in the epidermis adjacent to the neural plate (Holland, *et al.,* 1996) which is intriguing given that both the epidermis and the neural plate contribute to neural crest cells in vertebrates.

Similar to the *Dlx genes, Snail* and *Slug* are two closely related zinc finger genes that are also used extensively as markers of neural crest cells. *Snail/Slug* genes are expressed in both pre-migratory and migratory populations of neural crest cells in all vertebrate species examined. Their role in neural crest development appears to be to induce the process of epithelial-to-mesenchymal transition that allows cells from the neural tube to detach and migrate as individual cells (Cano, *et al.,* 2000). *Snail* and *Slug* have a single homologue in protochordates (Corbo, *et al.,* 1997; Langeland, *et al.,* 1998). The *amphioxus* homologue of *Snail/Slug* is expressed in the dorsal region of the neural tube (Langeland, *et al.,* 1998). *Ci-Sna*, the ascidian homologue is expressed in cells at the lateral neural plate border and similar to *amphioxus* is also not expressed in the epidermis (Corbo, *et al.,* 1997; Erives, *et al.,* 1998), which is consistent with playing a role in the migration of lateral cells over the neural tube during ascidian neurulation.

The transcriptional activator *AP-2* is also a robust marker of pre- and post-migratory vertebrate cranial neural crest cells and is essential for their proper development (Zhang, *et al.,* 1996). Although there are four vertebrate *AP-2* genes, only a single *amphioxus* homologue has been identified (Meulemans and Bronner-Fraser, 2002). The expression of *AP-2* in non-neural ectoderm is a feature conserved between *amphioxus* and vertebrates, which suggests an ancient functional role for *AP-2* in this tissue and in the evolution of neural crest cells.

It is important to note that there are some differences in *Bmp*, *Pax*, *Dlx*, *Snail* and *AP-2* gene expression between *amphioxus*, ascidians and vertebrates. However, overall the genetic machinery for generating neural crest cells appears to have been in place in the common ancestor of *amphioxus* and the vertebrates (Holland and Holland, 2001). One can speculate therefore, that co-option of genes such as *Slug*, *Snail*, *Dlx* and *AP-2* by the neural crest were crucial events in vertebrate evolution. However, given the recent identification of *Wnt6* expression in the ectoderm and its role as a prime candidate for vertebrate neural crest induction, one important unanswered question that remains is whether an *amphioxus* or ascidian homologue of the vertebrate *Wnt6* gene exists with equivalent spatiotemporal expression and function.

The expression patterns of *Bmp*, *Pax*, *Dlx*, *Snail* and *AP-2* genes are reasonably consistent with the idea that the origins of the neural crest are to be found in the dorsal midline epidermis. However, in vertebrates the dorsal midline epidermis is not the sole source of neural crest cells. Cell lineage analyses have shown that a single cell can give rise to neural tube, neural crest and epidermal cells (Bronner-Fraser and Fraser, 1988). Holland and colleagues (Holland, *et al.,* 1996) have argued therefore that the neural crest origins may be found in both the dorsal midline epidermis and the dorsal region of the neural tube. Even if one accepts the idea that neural crest cells originated in the protochordate dorsal midline epidermis and the dorsal neural tube, there are remarkable differences in pluripotency and cell migratory behaviour in vertebrate neural crest cells compared to their protochordate equivalents.

Recently Wada (Wada and Satoh, 2001) provocatively proposed that the evolution of the vertebrate neural crest may not have required the birth of a new cell type. Rather the protochordate precursors of the neural crest acquired specific cellular properties such as pluripotency that are now considered characteristic of neural crest cells. Vertebrate neural crest cells differentiate into numerous cell types including pigment cells, glia, peripheral sensory and sympathetic neurons, cartilage and bone just to name a few (Fig. 1). Similar to vertebrates, protochordates possess peripheral nerves and pigment cells. In contrast *amphioxus* possesses many multipolar peripheral sensory neurons (Bone, 1958), dorsal medullary cells, columns of sensory spinal neurons but no peripheral ganglia (Fritzsch and Northcutt, 1993). Ascidians on the other hand possess peripheral gonadotrophin expressing neuroendocrine cells (Mackie, 1995; Powell, *et al.,* 1996) calcitonin positive endocrine cells (Thorndyke and Probert, 1979), a single ganglion (Bollner, *et al.,* 1995) and two melanin containing pigment cells types. The first step in neural crest evolution therefore may coincide with the development of a specific dorsal neural population that contributed to sensory processing (Shimeld and Holland, 2000).

Although dorsal midline epidermal cells in protochordates such as *amphioxus* can migrate, these cells do not delaminate. They migrate as a sheet of cells, similar to convergence-extension movements during vertebrate gastrulation. *Amphioxus* and ascidian *Snail* genes are expressed at the lateral border of the neural plate, as well as in mesoderm, in an equivalent location where vertebrate neural crest forms. However it should be remembered that cell migration is not purely a neural crest specific trait. If the conserved role of *Snail* from *Drosophila* to vertebrates is to allow cells to detach from an epithelia (Nieto, 2002), why does this not occur in protochordates? An intriguing possibility is that the environment through which these cells would migrate does not allow their movement, therefore precluding the existence of crest-like migration to occur. Vertebrate neural crest cells migrate along very specific pathways and we can speculate that if the notion described above is true, that neural crest precursors were already present in the protochordates, then perhaps the environment adjacent to the midline epidermis/dorsal neural tube evolved in such a way that it became permissive for cell migration. As a consequence, the migratory characteristic of neural crest cells could have been born and the evolution of neural crest derivatives would have directly followed as a consequence of the neural crest encountering novel environments along their new migration pathways. The influence of the paraxial tissue environment on cranial neural crest migration was discussed earlier, however below we consider the roles played by the same environment on patterning the identity of neural crest cells and the effects this could have on craniofacial evolution.

Neural crest cell patterning and models for craniofacial development

The classical view of craniofacial development and neural crest patterning was that regional diversity in the vertebrate head was a consequence of patterning information provided by the neural crest (Noden, 1983; Hunt, *et al.,* 1991). This was based largely on the fact that when first arch (mandibular) neural crest cells were grafted posteriorly in place of second (hyoid) or third (visceral) arch neural crest, the transplanted neural crest cells formed duplicate first arch skeletal elements (Noden, 1983). Not only were the duplicated crest-derived structures inappropriate for their new

location but the muscle cell types and attachments associated with the ectopic structures were also characteristic of a first arch pattern. This suggested firstly that neural crest cell fate may be pre-programmed prior to emigration from the neural tube and secondly, that myogenic populations and other cell types receive spatial cues from the invading neural crest-derived connective tissue. The majority of cranial neural crest cells are derived from the hindbrain and the same restricted domains of *Hox* gene expression found in the hindbrain are emulated in the migrating neural crest cells and then later in the ganglia and branchial arches, reflecting the origins of the neural crest cells contributing to these tissues (Hunt, *et al.,* 1991). Taken together, these observations led to the proposal of the neural crest pre-programming model, whereby it was thought that positional information encoded by the *Hox* genes was carried passively from the hindbrain to peripheral tissues and branchial arches by the neural crest (Hunt, *et al.,* 1991).

Recently there have been significant advances in our understanding of craniofacial patterning through neural crest cell transpositions within the hindbrains of mouse (Golding, *et al.,* 2000; Trainor and Krumlauf, 2000) and zebrafish embryos (Schilling, 2001). In contrast to the classic analyses performed in avian embryos, these new studies uncovered a consistently high degree of cranial neural crest cell plasticity. In heterotopic transplantations of neural crest cells within mouse and zebrafish hindbrains, graft derived neural crest cells migrate into the nearest arch without any evidence of path finding and concomitantly downregulate inappropriate *Hox* gene expression in these cells (Trainor and Krumlauf, 2000). In zebrafish embryos, the transplanted cells were followed for longer periods such that the cells activated new gene expression programmes and they differentiated and contributed to the pharyngeal cartilages appropriate to their new axial location (Schilling, 2001). These results demonstrate that the axial character of cranial neural crest cells are neither fixed nor passively transferred from the hindbrain to the branchial arches and periphery of the head. The plasticity observed in neural crest cell patterning correlates with molecular analyses that have identified distinct cis-regulatory elements controlling *Hox* gene expression in different tissue such as the neural tube and neural crest (Maconochie, *et al.,* 1999). Consequently, these results support a new model called the "neural crest plasticity and independent gene regulation model" as a mechanism for describing neural crest and craniofacial development. This argues that neural crest cells can respond and adapt to the environment in which they migrate and furthermore that the cranial mesoderm plays an important role in patterning the identity of the migrating neural crest cells (Golding, *et al.,* 2000; Trainor and Krumlauf, 2000).

Importantly, these new findings for neural crest plasticity and independent gene regulation can be reconciled with the classic studies promoting neural crest pre-programming. Firstly, the differences in the degree of plasticity observed in mouse and zebrafish embryos compared with avians can be attributed partially to cell community (i.e. size of the tissue transplanted) and timing effects (Trainor and Krumlauf, 2000). When whole hindbrains and pairs of rhombomeres are transplanted, inter-rhombomeric signalling and neighbouring cell signalling can help to reinforce and maintain the identity of the transplanted cells. In the case of small sub-rhombomeric cell populations (mouse and zebrafish) and single cells (zebrafish), the transplanted cells lack this neighbouring reinforcement. The graft derived neural crest cells migrate as a dispersed population and therefore are more likely to respond to the new environment in which they migrate. Secondly, the potential inclusion of the isthmus (midbrain-hindbrain junction) in the original avian neural crest transplantations can account for the skeletal duplications that were observed (Trainor, *et al.,* 2002). The isthmus is a clear neural tube landmark, which was probably used for delineating the anterior and posterior limits of the frotonasal and first arch crest tissues respectively to be transplanted. This helps to clarify why transplantations of frontonasal neural crest (which also probably included the isthmus) and first arch neural crest produce similar duplicated skeletal elements, whereas during normal development these cell populations would give rise to distinct parts of the craniofacial skeleton. The landmark neural crest transplantations were performed in the 1980's and it wasn't until the mid 1990's that the isthmus and its patterning or organizing capabilities were realized (Martinez, *et al.,* 1995; Crossley, *et al.,* 1996). It is now known that the isthmus has the capacity to repress *Hoxa2* in second branchial arch neural crest cells and in the absence of *Hoxa2* the second branchial arch is transformed into a first arch identity (Trainor, *et al.,* 2002), which results in first arch skeletal duplications in chick embryos mimicking the duplications observed in *Hoxa2* null mutant mice (Gendron-Maguire, *et al.,* 1993; Rijli, *et al.,* 1993).

Hence rather than providing evidence for pre-patterning, the early neural crest transplantation experiments (Noden, 1983) highlight the effects of local signalling centres on anterior-posterior patterning and regulation of *Hox* gene expression. Neural crest cell development and patterning therefore is based upon a balance between the neural plate and rhombomeric signals they receive during their formation and their response to the environmental signals and interactions with the tissues through which they migrate. The developmental plasticity of cranial neural crest cells has important implications for head and ultimately vertebrate evolution.

Evolutionary significance of neural crest plasticity in craniofacial development: evolutionary diversification in action

Craniofacial evolution is considered fundamental to the origin of vertebrates (Gans and Northcutt, 1983). The observations described above that neural crest cells are plastic and that gene expression is independently regulated in different tissues of the head provides a mechanism for how neural crest cells can be modified or evolve in response to the environment through which they migrate independently of the neural tube from which they are derived. Although the hindbrain exerts a profound influence in establishing the foundations of vertebrate head development, a rigid neural crest pre-patterning model in which the program for head morphogenesis is set in the neural tube would offer very restricted opportunities for diversifying head structures. In contrast the neural crest plasticity and independent gene regulation model could provide the flexibility and adaptability that facilitates diversity and we can speculate that it might be one reason for the successful radiation of vertebrates into new environments. This is because neural crest plasticity and independent gene regulation offers the potential for generating substantially distinct cranial phenotypes by subtle minor changes of the primordial pattern.

Successful adaptation and developmental flexibility is particularly evident in the bird beak, which has undergone extensive

diversification and modification during evolution. Bird beaks are used for numerous diverse functions including eating, defence, scratching, courting and preening. Even though beak morphology is remarkably variable between species and even within species, each beak is derived from comparable tissues during development. The lower beak is bipartite and is formed by the fusion of the paired mandibular cartilages. In contrast the tripartite upper beak is derived from the fusion of the frontonasal mass to the paired lateral nasal and maxillary cartilages. All of the beak cartilage and connective tissue is derived from neural crest cells (Schneider and Helms, 2003). The outer cornified layers of the beak come from the ectoderm while the mastication/articulation muscles come from the mesoderm (Noden, 1983). Evolutionary modifications in beak morphology could be driven by any if not all of these tissue components.

Recently, an elegant series of interspecies neural crest cell transplantations have revealed that neural crest cells direct beak morphology in a species dependent autonomous manner (Schneider and Helms, 2003). When quail neural crest cells, which normally give rise to narrow, short beaks were transplanted into duck hosts, the resulting chimeras exhibited beak morphologies characteristic of the quail. Conversely, when duck neural crest cells, which normally produce longer flatter bills were transplanted into quail hosts the resulting chimera developed beaks typical of ducks. Interestingly, the donor neural crest cells also transformed host derived structures. The morphology of the beak epidermis and egg tooth, and the positioning of the external nasal openings in the chimeras were all characteristic of the species from which the neural crest cells were derived. This is despite the fact that the egg tooth and epidermis are host tissue derived structures. These results resemble earlier classic interspecies transplantation experiments between frogs and salamanders (Andres, 1949; Wagner, 1959) and between quails and chicks (Noden, 1983). Most significant, however, was the observation in the quail/duck chimeras that the spatiotemporal patterns of gene expression in the facial mesenchyme (donor derived) and the ectoderm (host derived) were always characteristic of the donor. Hence when donor quail neural crest cells were transplanted into ducks, the chimeras exhibited accelerated gene expression programmes characteristic of the quail even in the tissues derived from the duck hosts. This demonstrates that neural crest cells pattern beak development in a species specific manner by maintaining their own molecular programmes and by regulating the patterns of gene expression and differentiation in adjacent tissues. Neural crest cells therefore can regulate the spatial patterns of cellular differentiation around them during craniofacial development.

Another example of morphological changes that can be attributed to neural crest occurs during early craniofacial development in marsupials. Methatherian mammals are born after a relatively short gestation period and complete their embryonic development outside of the body of the mother (Smith, 2001). This means that at the moment of birth, they require some basic morphological requirements, not present in similar stage embryos of other vertebrates (around 10.5-12.5 days post coitum in mouse embryos). Among these, a functional suckling mouth is of paramount importance and the jaw structures are derived mainly from neural crest and cranial mesoderm from the first branchial arch. Analyses of early marsupial embryos reveal an accelerated growth of the mandibular and maxillary processes of the first arch compared to similarly staged embryos of placental mammals. This is related to the fact that neural crest migration apparently commences much earlier in placental mammals than typical mammalian models such as mice (Vaglia and Smith, 2003). This de-coupling of the timing of first arch neural crest induction and migration in placental mammals (a classic heterochrony), allows the neonate to be able to nurture itself through later phases of development (Smith, 2001).

All of these results highlight clear species specific differences in the spatio-temporal differentiation of neural crest cells, but what does it tell us about craniofacial evolution? Morphometric measurements of the beaks obtained in quail/duck chimeras revealed only minor size and shape changes. The beaks that developed in the chimeras were more characteristic of the species origin of the donor neural crest cells rather than the host into which they were transplanted. The implication of this observation is that during evolution the ectodermal tissues surrounding the neural crest derived beak may influence neural crest cell differentiation only to a minor extent. This may not be that surprising given that neural crest derived cartilage and bone, to which soft tissues such as the muscles and connective tissue must adhere, provides the scaffolding for the vertebrate head.

Collectively these results argue that a far more likely evolutionary scenario is one in which beak and by extrapolation craniofacial evolution occurs primarily through a combination of changes to the neural crest cells themselves (neural crest cell plasticity) and by their subsequent influence on the differentiation of tissues surrounding them. Bird beaks continue to undergo extensive evolutionary modification and selection in response to rapid environmental changes which affect foraging and feeding habits. A prime example are the changes that have been afflicting the Galapagos Island finches during the past decade (Grant and Grant, 1993; Grant and Grant, 2002) and we can speculate that cranial neural crest cells are the primary conduit for effecting these changes. The demonstration that craniofacial morphology can change rapidly under selective conditions offers the tantalizing prospect of being able to correlate evolutionary diversification with observable cellular and molecular changes in rapidly evolving populations.

Mechanisms for the evolution of neural crest cells and their influence on craniofacial morphology

Understanding the genetic programs and tissue interactions that direct neural crest cell development are critical not only for elucidating the establishment of the primordial origins of neural crest formation but also its evolution, such as in the case of Galapagos Island finches and marsupials. The generation of morphological diversity is believed to be based upon changes in the processes that control development. Comparative biology indicates that these changes are regulatory, affecting the spatiotemporal expression of developmentally important genes (Averof and Patel, 1997; Sucena and Stern, 2000).

Evolutionary change in neural crest cells could conceivably occur in two ways. Within the neural crest itself, there could be changes in the target genes of compartment specific selector proteins such as the products of *Hox* genes, or phenotype-determining factors such as *Snail*. Alternatively, molecular and cellular changes could occur in the periphery (i.e. neural crest cell migration) in relation to the expression of specific organizer molecules such as FGFs, BMPs, Shh, Wnts or retinoic acid which could effect

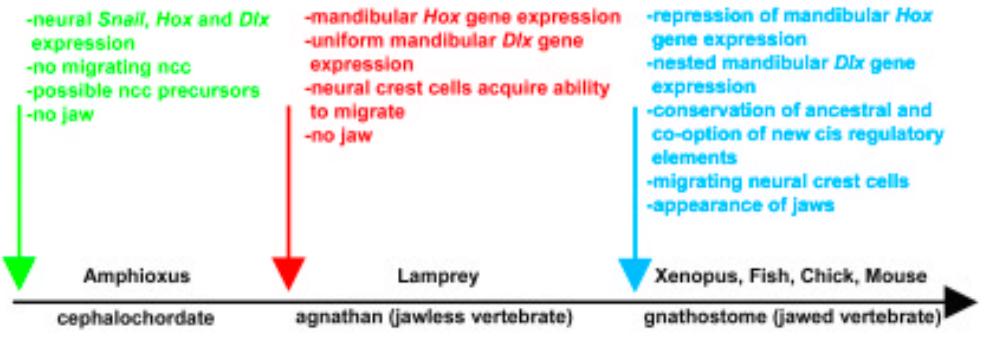

Fig. 5. The origins of neural crest cells and jaws during craniofacial evolution. *Cephalochordates such as* amphioxus *exhibit regional expression of* Snail, Hox *and* Dlx *genes amongst many others in the dorsal epidermal/neural region which is morphologically homologous to the territory in which neural crest cells form in vertebrates. Despite having the genetic machinery,* amphioxus *lacks true vertebrate-like neural crest cells. The transition to agnathans (jawless vertebrates) coincided with the formation of a migrating neural crest cell population, a greater differentiative repertoire along with mandibular* Hox *and* Dlx *expression. Later during evolution, the loss of mandibular* Hox *gene expression combined with the appearance of nested domains of* Dlx *gene expression appears to have precipitated jaw formation and the transition to a predatory lifestyle. Many cis-regulatory elements are conserved between vertebrates and* amphioxus *but during evolution it is also clear that additional elements have been co-opted by emerging cell populations which opened the way for new domains of expression and function.*

or create new sites of neural crest deposition, condensation or differentiation.

The evolution of body form involved changes in expression of developmental genes, largely through alterations in cis-regulatory elements which exhibit rapid evolutionary turnover (Averof and Patel, 1997; Sucena and Stern, 2000). A recent study utilizing cross-species transgenesis has provided some important insights into the relationship between the evolution of gene regulatory regions and the evolution of neural crest cells (Manzanares, *et al.,* 2000). The expression patterns of a series of reporter constructs containing the regulatory regions of the 3' *amphioxus Hox* genes *AmphiHox1*, *AmphiHox2* and *AmphiHox3* were analysed in vertebrates. These *Hox* genes are normally involved in anterior-posterior patterning of the neural tube and certain populations of neural crest cells in vertebrates. The *AmphiHox1A* regulatory region was able to drive reporter *lacZ* expression in the neural tube of chick and mouse embryos and importantly it was responsive to retinoic acid which is known to play an essential role in establishing basal *Hox* gene expression in vertebrates. Remarkably the *AmphiHox1A* element also directed expression to the neural crest and placodes in vertebrates, despite the fact that *amphioxus* lack both of these cell populations. This result indicates that at least some of the regulatory elements necessary for directing *Hox* expression in neural tube, neural crest and placode cells existed prior to the evolution of neural crest cells and vertebrates (Manzanares, *et al.,* 2000). This study also revealed that other cis-regulatory elements must have been recruited or co-opted by these novel cell populations and were crucial for establishing new spatiotemporal domains of gene expression and function. It is only a matter of time before interspecies homologous gene replacement studies are performed such as with the *amphioxus* and mouse *Hox* and *Snail* genes. Determining how the ancestral gene functions in the vertebrate environment would surely start to address the issue of whether vertebrate downstream targets are missing in the ancestor species or whether changes in the environment permitted the onset of neural crest cell migration.

One question that remains is how characteristic craniofacial structures such as the jaw evolved once neural crest cells had acquired the ability to migrate. Analyses undertaken in lampreys have been critical to our understanding since they possess neural crest cells but are jawless vertebrates (agnathans). Jaw development was a critical event in vertebrate evolution because it ushered the transition from a sessile, filter feeder such as *amphioxus* into an active predator, facilitating vertebrate radiation into new environments (Northcutt and Gans, 1983). At the cellular level, comparative anatomy assumes that the upper and lower jaw cartilages evolved through dorso-ventral modification of the most rostral branchial arch of primitive vertebrates. At the genetic level, signalling cascades involving genes such as *Msx*, *Fgfs* and *Bmps*, which are important for mandibular development are generally conserved between lampreys and vertebrates (Shigetani, *et al.,* 2002). Two important differences however are firstly, that lampreys express at least a single *Hox* gene in the mandibular arch whereas jawed vertebrates (gnathostomes) do not (Fig. 5) (Cohn, 2002). *Hox* gene expression as described earlier is incompatible with jaw development (Couly, *et al.,* 1998; Creuzet, *et al.,* 2002). Secondly, *Dlx* cognates are expressed in different parts of the facial mesenchyme in comparisons between lampreys and gnathostomes (Shigetani, *et al.,* 2002). Lampreys exhibit uniform *Dlx* expression in the mandibular arch in contrast to the nested domains of *Dlx* expression found in vertebrates. The importance of *Dlx* genes in vertebrate jaw development have been well documented through numerous mouse null mutations but none more so than two recent analyses characterising the phenotype of *Dlx5/6* double mutants (Beverdam, *et al.,* 2002; Depew, *et al.,* 2002). The loss of both these genes results in homeotic transformations of the lower jaw into an upper jaw suggesting that the advent of nested *Dlx* expression within the branchial arches contributed to the transition from jawless to jawed vertebrates.

This allows us to propose a speculative model in which jaw evolution occurred through the loss of *Hox* gene expression in the mandibular arch of lampreys (*Hox* genes are incompatible with jaw bone development) in combination with spatiotemporal changes resulting in nested *Dlx* expression domains and the promotion of proximo-distal arch differentiation (Fig. 5). It is interesting to note that *Fgf8* has been shown to be able to downregulate *Hox* gene expression in the head (Irving and Mason, 1999) as well as regulate *Dlx* gene expression in the branchial arches (Ferguson, *et al.,* 2000; Thomas, *et al.,* 2000). Therefore lying at the heart of the processes of jaw and craniofacial development is an increased importance or functional role of growth factor/organiser mediated tissue interactions during neural crest cell migration and differentiation. In further support of this idea, alterations to local BMP,

retinoic acid (Lee, *et al.,* 2001) and FGF (Trainor, *et al.,* 2002) signalling have dramatic effects on neural crest cells and the differentiation of the facial skeleton.

Conclusions

Although it has been classically thought that protochordates lacked neural crest counterparts, the recent identification and characterization of homologues of vertebrate neural crest specific genes in protochordates suggests that neural crest cells may not be a novel vertebrate cell population. The evolution of the neural crest cells could be reconsidered in terms of the acquisition of new cell properties such as delamination-migration and also multipotency, which were key innovations that contributed to craniofacial development. It seems clear that alterations to ancestral expression patterns of transcriptional regulation genes combined with the general plasticity of neural crest cells resulted in profound changes in the morphology of craniofacial structures and contributed to the radiation of vertebrates and their success in adapting to new or rapidly changing environments. The dramatic effects of tinkering with gene regulation on body form have revealed many of the mechanisms that regulate morphological evolution and the challenge in the future will be to visualize the genetic and cellular interactions occurring in the neural crest cells of rapidly evolving populations, which should aid our understanding of the specific factors regulating selection driven evolution.

Acknowledgements

The authors would like to thank Robb Krumlauf for generously providing the mouse embryo scanning electron micrographs and to members of the lab for comments on the manuscript. This work was supported by a March of Dimes Basil O'Connor Scholar Research Award (P.A.T.), a Children's Mercy Hospital Clinical Scholarship (K.R.M.) the Stowers Institute for Medical Research (P.A.T. and K.R.M.) and the Spanish Ministry of Science and Technology (M.M.).

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