Review
The Neural Crest: A Versatile Organ System

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The neural crest is the name given to the strip of cells at the junction between neural and epidermal ectoderm in neurula-stage vertebrate embryos, which is later brought to the dorsal neural tube as the neural folds elevate. The neural crest is a heterogeneous and multipotent progenitor cell population whose cells undergo EMT then extensively and accurately migrate throughout the embryo. Neural crest cells contribute to nearly every organ system in the body, with derivatives of neuronal, glial, neuroendocrine, pigment, and also mesodermal lineages. This breadth of developmental capacity has led to the neural crest being termed the fourth germ layer. The neural crest has occupied a prominent place in developmental biology, due to its exaggerated migratory morphogenesis and its remarkably wide developmental potential. As such, neural crest cells have become an attractive model for developmental biologists for studying these processes. Problems in neural crest development cause a number of human syndromes and birth defects known collectively as neurocristopathies; these include Treacher Collins syndrome, Hirschsprung disease, and 22q11.2 deletion syndromes. Tumors in the neural crest lineage are also of clinical importance, including the aggressive melanoma and neuroblastoma types. These clinical aspects have drawn attention to the selection or creation of neural crest progenitor cells, particularly of human origin, for studying pathologies of the neural crest at the cellular level, and also for possible cell therapeutics. The versatility of the neural crest lends itself to interlinked research, spanning basic developmental biology, birth defect research, oncology, and stem/progenitor cell biology and therapy.

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Key words: neural crest; cell migration; neurocristopathy; progenitor cell

Introduction

The neural crest (NC) is a population of early embryonic progenitor cells which has attracted unusual interest, attested to by many book-length monographs (Hörstadius, 1950; Le Douarin, 1982; Hall, 1988, 1999; Gershon, 1998; Le Douarin and Chaya Kalcheim, 1999; Saint-Jeannet, 2006; Nelms and Labosky, 2010; Trainor, 2014). In 1868, Wilhelm His described a structure in the ectoderm whose cells formed, at a distance, sensory ganglia. He uninspiringly named this the *Zwisschenstrang*, literally the "between-strand" (His, 1868), and this brought the novel realization that cells in tissues are not necessarily stationary. This is now termed the NC, and migration forms part of the definition of NC cells. The list of NC derivatives has extended to peripheral neurons (autonomic as well as sensory) and glia (Schwann cells, ganglionic glia), certain endocrine cells and also melanocytes; all neural-related cell types. Twenty years after His' paper came a stunning suggestion: the NC contributes mesoderm cell types (Katschenko, 1888). These included the craniofacial skeleton, myofibroblasts, and connective tissues in glands of the neck and in the walls of the outflow vessels of the heart. This contravened the germ layer "law" of the mutually exclusive fates of the three primary germ layers, the endoderm, mesoderm, and ectoderm. This controversy blazed for over thirty years, and from its ashes arose the term ectomesenchyme, but controversy still persists over details of the fate of cells for ectomesenchyme *vis a vis* the "neural" NC (Dupin et al., 2010; Lee et al., 2013). The origins and fates of NC cells are diagrammed in Figure 1 using the chick embryo as the model.

The ectomesenchymal role led to the novel classification of the NC by Hall as a "fourth germ layer" (Hall, 2000) possessed only by vertebrates. This NC/vertebrate correlation implied that the NC was fundamentally important for the evolution of vertebrates. Its evolutionary appearance permitted the "new head" that boosted the evolutionary prospects of an otherwise unambitious chordate sub-lineage (Northcutt and Gans, 1983). The NC in the strict sense is a vertebrate oddity, but elements of the genetic sub-structure for the NC, and its genes and regulatory network, are present even in nonvertebrate chordates (Yu et al., 2008). Evo-devo NC studies are an important and active dimension for research into the evolution of the chordates. The accelerated "evolution" of a suite of traits in domestic mammals by human selection has also recently been proposed to involve modification to the NC developmental program (Wilkins et al., 2014).

The development of the NC poses basic questions common to the early stages of metazoans—questions of

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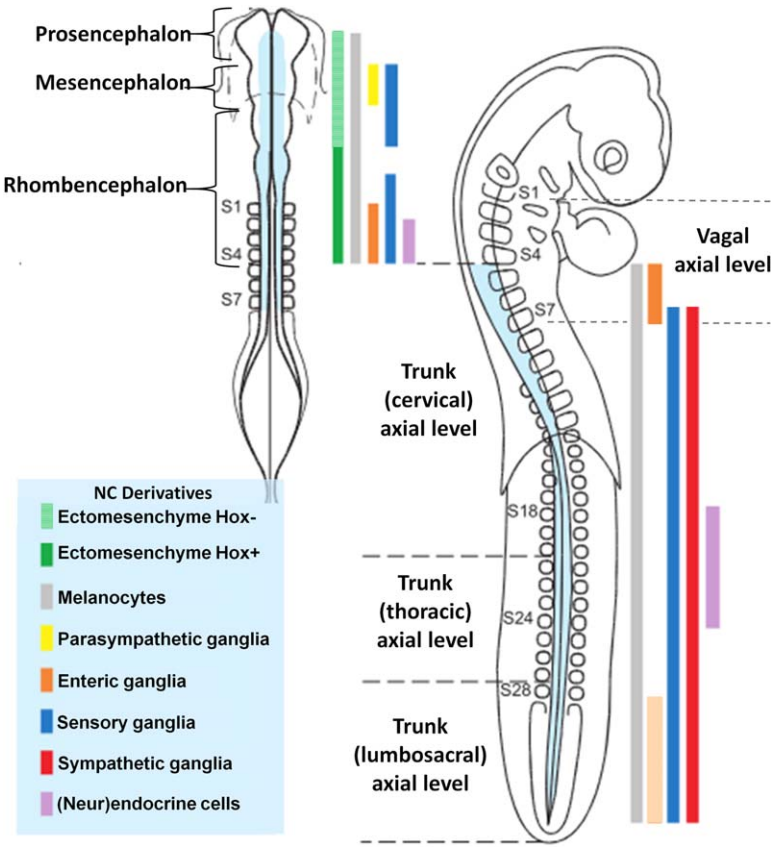

FIGURE 1. *Fate map of the NC superimposed on an E1.5 (left; for cranial levels) and E2.5 (right; for trunk levels) chick embryo. Some NC derivatives have very broad origins (melanocytes, sensory ganglion cells) while others have restricted levels of origin (enteric ganglia, parasympathetic ganglia). Note that the ectomesenchymal derivatives of the NC form two zones defined by presence or absence of Hox gene expression. Adapted from Le Douarin et al. (2004).*

pattern formation, cell identity, morphogenesis, and multilineage differentiation. However, compared to many other early developmental systems, as an object of study the NC is well defined, accurately timetabled, experimentally accessible and manipulable (see Fig. 2A). The development of the NC can be taken as an approachable model for many other developmental events, and provides particularly exaggerated examples of some developmental events, such as EMT and cell migration/invasion. Indeed, these properties also recommend the NC as a model for metastatic cancer (Powell et al., 2013).

Humans and animals show a number of developmental disturbances of the NC, collectively termed neurocristopathies (Bolande, 1997). These include congenital craniofacial and cardiac outflow defects, peripheral neuropathies, and endocrine and pigmentation disorders, and they involve errors of every stage of NC development—specification, migration, and differentiation, and include tumors of NC lineages, such as neuroblastoma and melanoma. Indeed in humans the NC seems to be unusually prone to birth defects, which adds a clinical dimension to the importance of NC studies.

Developmental Biology of the NC

HOW DOES THE NC ORIGINATE?

The NC originates as a result of a chain of interactions beginning at the time of gastrulation, which have been defined by work mostly in *Xenopus* and chick. The interaction signals were identified as growth factors (e.g., FGFs, BMPs, Wnts, Notch, retinoids) and their receptors and modulators, and the responses were also refined to expression of transcription factors. The diversity and accuracy of responses from a modest range of signal molecules is due to the duration, sequence, and strength of the signals in combination, and the responses are also defined by combinatorial expression of sets of transcription factors (Betancur et al., 2010a; Powell et al., 2013) (Fig. 2B). As yet the molecular pathways, or rather networks, between molecular signal and transcription factor gene expression are poorly understood, and the functional genes controlled by these transcription factors are incompletely known.

The ectoderm gives rise to four major structures: the central nervous system, epidermal ectoderm, cranial

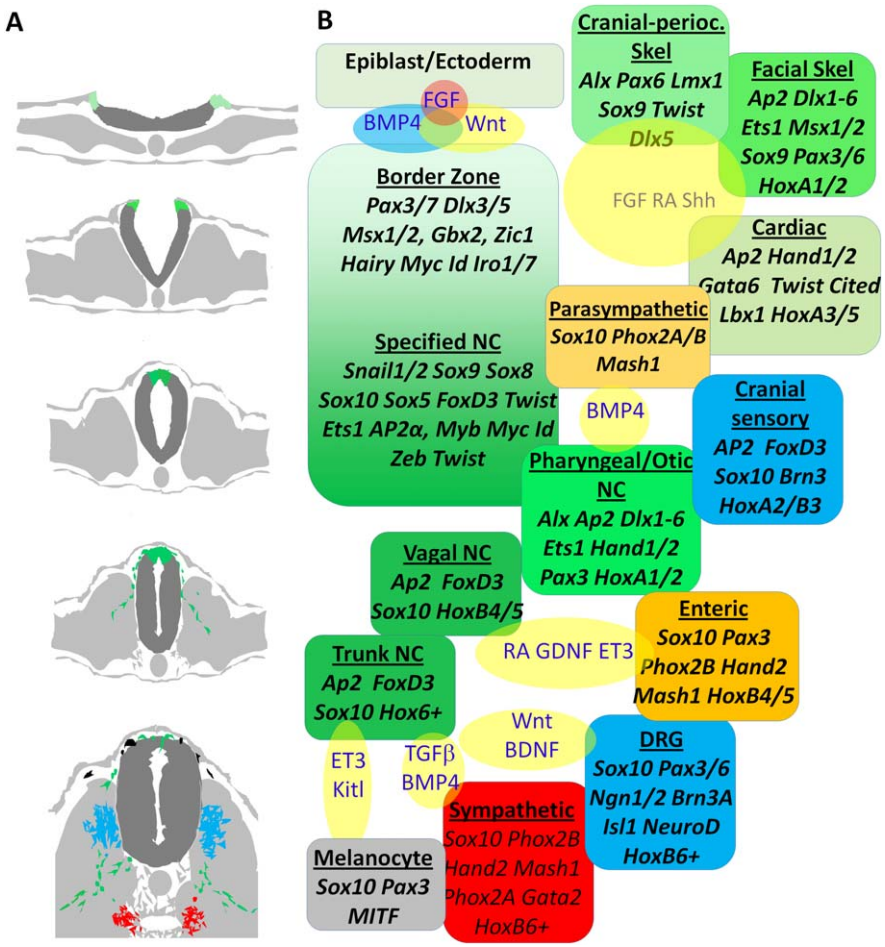

FIGURE 2. A: *Transverse sections at avian trunk of age E1 (top) to age E3.5 (bottom) diagramming NC at specification stage (light green) at the neural plate (dark gray) border, NC cell migration stage (green), and aggregation into dorsal root ganglia (blue) and sympathetic ganglia (red).* **B:** *Diagram showing transcription factors expressed in the specification, migration and differentiation of the NC (light to dark green). Complex cross-regulatory networks connect these factors; no attempt has been made to show these (see Prasad et al., 2012). Ectomesenchymal NC derivatives are colored in shades of green, sensory neural derivatives in blue, autonomic neural derivatives in orange and red, and melanocytes in gray. Signaling molecules important in transitions of state of NC-derived cells are in blue on a yellow field.*

placodes, and NC. Their origin is sometimes depicted as a series of Manichean binary specification choices: ectoderm to either neural or epidermal moieties, neural ectoderm to either central (neural tube), or NC. Early epidermal specification is driven (in part) by BMP signals in a peripheral zone in the chick epiblast opposed in a medial zone by FGF coinciding with neural plate specification (Wilson et al., 2000), both being regulated by Wnt signals (Wilson et al., 2001). The usual model poses signaling between these zones establishing a new zone, the epidermal/neural plate border zone (the *Zwissenstrang* of His) at intermediate/low BMP signaling. This is defined by and requires expression of genes including *Pax7, Dlx3/5, Msx1/2, Gbx2*, and *Zic1* (Betancur et al., 2010a). In *Xenopus* and chick, the roles of *Pax3* and *Pax7* seem to have been at least partially switched. However the appearance of NC cells in epiblast microfragments cultured in "noninducing" conditions suggested that avian NC, at least midbrain/rostral hindbrain NC, is specified very early and independently of neural tissues (Basch et al., 2006), and cell mixing makes strictly regional either/or specifications hard to envisage. This provisional specification of a broad neural plate border region is refined to specification of NC, evidenced by expression of distinctive NC transcription factor genes, such as *Snail1/2, Sox8, Sox9, Sox10, FoxD3, Twist, Ets1, AP2α, cMyc*, and *Id* (see Fig. 2). As with *Pax3/7*, the expression and roles of *Snail1* and *Snail2* seem to be reversed in birds and reptiles, compared with other vertebrates (Locascio et al., 2002). Known or presumed interactions in this regulatory network are complex (Prasad

et al., 2012). For example the enhancers of *Sox10* and *FoxD3* have sites for cranial and vagal/trunk NC, which are responsive to other NC specifier transcription factors, such as Sox9, Ets1, and cMyb (Betancur et al., 2010b). For *FoxD3*, Pax7/Msx/Ets1 bind to the cranial regulatory sequence and Pax7/Msx-Zic1 to the trunk (Simoes-Costa et al., 2012).

Layers of epigenetic regulation of the NC are now being discovered. The DNA methyltransferase DNMT3A is expressed by cells in the border zone; it binds directly to CpG islands of promoters regions of neural plate genes *Sox2* and *Sox3*, and knock down results in expansion of the medial neural plate identity into the NC-fated border zone (Hu et al., 2012). The gene for the histone demethylase, *JumonjiD2A* is also expressed in the border region and its loss downregulates NC specifier genes (Strobl-Mazzulla et al., 2010). The miRNA 200c is expressed in the epidermis lateral to the NC zone (Darnell et al., 2006). In cancer cells this miR down-regulates the pro-EMT genes *Zeb1 (δEF1; Zfhx1a*) and *Zeb2* (*SIP1; Zfhx1b)* (Bracken et al., 2008), the latter being expressed in the NC region (Rogers et al., 2013). This miR may therefore limit the lateral margin of the border zone and hence limit the field of cells that later undergo EMT.

ARE ALL NC CELLS THE SAME?

The NC exists along the entire axial length of the neural *anlage* except at the most rostral forebrain, and gives rise to many types of cells. Some NC derivatives (e.g., Schwann cells, melanocytes) have widespread axial origins, while others, such as ectomesenchyme and enteric nervous system (ENS), are more restricted (Fig. 1). Is this axial division of labor innate to each level of NC, or happenstance, dictated by identical NC cells migrating into different environments? This was tested by a heroic campaign of heterotopic transplantations and tissue combinations chiefly by the Le Douarin group (Le Douarin et al., 2004). Generally transplantation of NC to a more rostral level revealed a deficit in formation of appropriate derivatives, even though the ectopic cells may migrate to the correct location. Notably, cranial NC of rhombencephalic level transplanted rostrally in place of mesencephalic NC resulted in craniofacial skeletal deficits, despite the former's ectomesenchyme capacity (Couly et al., et al., et al., 2002; Lwigale et al., 2004). Likewise trunk NC failed to adequately replace vagal NC as a source of ENS (Le Douarin and Teillet, 1974; Zhang et al., 2010). This suggests that the NC as a population displays a gradual rostral to caudal loss in differentiation potential, and this involves both ectomesenchymal and neural lines of differentiation.

Similar transplants of NC to a more caudal position generally had more normal differentiation. Cranial and vagal-level NC formed sympathetic lineage cells in a trunk microenvironment. However, more detailed analysis revealed level-of-origin differences. Cranial to trunk NC transplants produced ectopic ectomesenchyme (Le Douarin and Teillet, 1974), while some of the sympathetic ganglion cells lacked the characteristic transcription factor HAND2 and metabolic marker tyrosine hydroxylase (Lee et al., 2005). Therefore, although the cranial NC population included cells capable of trunk-appropriate differentiation, it also included cells of different abilities, and this involved both neural and ectomesenchymal lineages.

The disjunction between cranial NC ectomesenchyme at the rhombomere R3 border is *Hox* gene-related: NC rostral to R3 do not express *Hox* genes, unlike more caudal NC (Fig. 1). Moreover, forced expression of *Hox* genes in the rostral NC caused failure of craniofacial skeletal formation (Creuzet et al., 2002). Using quite different assays *in vitro*, cranial and trunk NC differences in survival and differentiation in response to growth factors were the result in part, of differential expression of *Hox* genes (Abzhanov et al., 2003). Therefore there are pre-existing axial level differences in the NC before migration, which partly involves the spatial *Hox* code. Whether this is populational (different levels have different proportions of pre-specified cells) or at the level of individual cell (each cranial NC cell has more options than each trunk cell) is unclear, as is the plasticity or permanence of these differences. The differences currently known are crudely regional, and it is also unclear how fine-grained any differences might be along the NC length.

At a single axial level, the NC also produces multiple different types of cells. Are these differences innate prior to migration, or happenstance? At cranial levels, cloning points to NC cells being similar and multipotent (Dupin et al., 2010), while others argue for a distinct neural/ectomesenchyme dichotomy (Lee et al., 2013). At trunk levels it has long been recognized that the temporal order of NC emigration is reflected by the type of derivative, with more distal derivatives provided by earlier *émigrés* (Weston and Butler, 1966). It has been argued from perturbation experiments that fate-restricted NC cells are stacked in dorso-ventral order in the neural tube prior to emigration (Krispin et al., 2010). In contrast, using live cell labeling, NC cells from different dorso-ventral levels were not found to contribute differentially to NC derivatives (McKinney et al., 2013). Single cell labeling with single cell gene expression will provide further data on this issue.

EMT, MIGRATION, AND LOCALIZATION

The NC transcription factor genes must drive the genes whose functions control the morphogenetic behavior of NC cells (Fig. 2A). *Snail 1/2* is a well-known master gene in cancer EMT and metastasis, as well as in the NC EMT. Part of its role is the repression of genes for cell–cell adhesion molecules (CAMs), especially cadherins. A decrease of cadherin-dependent cell–cell adhesion has long been related to the EMT of the NC (Newgreen and Gooday,

1985). The initial view that cadherin down-regulation gave loss of adhesion, thereby promoting EMT, proved to be grossly simplistic. The initial CAM of interest was the class I cadherin, N-cadherin, but there is dynamic expression of many classic type I and II cadherins and protocadherins, and this varies between NC axial levels in the one species and varies between species (Taneyhill and Schiffmacher, 2013). It is clear that cadherins have roles in cell–cell contacts and cell signaling that go far beyond just adhesion, such as regulating NC cell social dynamics during and after migration (Becker et al., 2013). In addition, CAMs of other families are also regulated, such as the tight junction molecule Claudin-1, whose regulation broadly parallels that of N-cadherin, as well as immunoglobulin family CAMs such as NCAM and L1CAM (McKeown et al., 2013). The way in which NC cells lose epithelial adhesions at EMT varies even at the gross level. Live cell imaging *in situ* has showed that an individual NC cell may detach by cleanly dismantling the adhesion complex, but an otherwise identical neighbor may simply break off its entire adhesion site and leave it behind (Ahlstrom and Erickson, 2009). The suggestive expression studies of diverse CAMs in the NC have not all been matched by functional studies.

NC cells must escape and migrate directionally following stereotyped routes. The escape phase has been most related to the expression of extracellular proteases, MMPs and ADAMS. The simplistic idea was initially that these proteases enabled NC cells to breach the basal lamina, though in some species at some axial levels a NC basal lamina never exists (Martins-Green and Erickson, 1987). Nevertheless these proteases are important for NC migration, by cleavage of CAMs (Monsonego-Ornan et al., 2012) and modification of ECM (Cousin et al., 2012), as organizing molecules between NC cells (Anderson, 2010) and signal transduction within cells (Cousin et al., 2011). As with CAMS, the expression data are not matched by functional studies.

The routes of NC cell migration were first related to the distribution of adhesive ECM, the archetype being fibronectin (Newgreen and Thiery, 1980), but many other ECM molecules have been related to NC migration (Perris, 1997). Parallel studies on ECM receptors have revealed NC cells possess integrins, particularly $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (Testaz and Duband, 2001). In some cases the ECM/integrin interaction seems to influence cell movement, but integrins may also convey survival signals (Goh et al., 1997). Diverse perturbations of specific NC cell/ECM interactions *in vivo* affect NC cell morphogenesis, but often less dramatically than expected from *in vitro* studies (Boucaut et al., 1984; Breau et al., 2009); this has been presumed to relate to redundancy in ECM *in vivo*. NC migration routes are equally defined by exclusion zones (Farlie et al., 1999) created by molecules that disfavor NC cell adhesion or locomotion. The first inhibitory/repulsive molecules were large chondroitin sulphate proteoglycans of the ECM (Newgreen, 1982), but others are known, including Slit with receptor Robo (De Bellard et al., 2003) and tenascin-C with integrin receptors (Breau et al., 2009).

NC migration directionality has long been of interest. NC repulsion based on diffusible molecules can limit and direct NC migration. Molecules with this function include Semaphorins (6A, 6B 3A) with receptors Plexin-A2 and Neuropilin-1 (Anderson et al., 2007; Toyofuku et al., 2008), and Sonic Hedgehog (Testaz et al., 2001). Diffusible molecules are also known to favor NC migration by chemotaxis or chemokinesis; these could provide target-derived directionality signals. The molecules involved include GDNF (receptor: Ret) and Netrin-1 (receptor: DCC) for enteric NC cells (Young et al., 2001; Jiang et al., 2003), VEGF (receptor: neuropilin-1) for cranial NC (McLennan et al., 2010), Semaphorin3C (receptor: plexin-D1/neuropilin-1) for cardiac NC (Toyofuku et al., 2008) and SDF-1 (receptor: CXC4R) in several locations (Kasemeier-Kulesa et al., 2010; Theveneau et al., 2010; Saito et al., 2012).

Experimental placement of NC at the distal "target" has revealed that the cells can migrate along the normal path but in reverse direction (Hörstadius and Sellman, 1946; Burns et al., 2002). This cannot be controlled by target-derived factors, and answers have been sought within the NC cell population. Contact inhibition of locomotion (CIL), a concept introduced by Abercrombie in the 1960s (Abercrombie, 1970) was described in NC cells *in vitro* (Newgreen et al., 1979). As well as inhibiting motility at the point of contact, CIL promotes locomotion in a new direction (Newgreen, 1990), so this could explain the "reverse migration" results. It is now clear that CIL occurs *in vivo* in the amphibian cranial NC (Carmona-Fontaine et al., 2008) and in avian corneal NC-derived cells (Bard and Hay, 1975), and in mouse enteric NC cells in organ culture (Young et al., 2014). Molecules transducing these directional signals are also now apparent, and in cranial NC include Wnt/PCP (including Par3) signaling which activates RhoA to inhibit protrusions at Cadherin-11 contacts (Becker et al., 2013; Moore et al., 2013). However, Eph/ephrin and Nectin/integrin $\alpha v\beta 3$/PDGFR can mediate CIL in other cell types (Takai et al., 2008; Batson et al., 2013) and are expressed by NC cells; these are therefore also candidates. Above this level of direct control of individual NC cell motile behavior are several population-based explanations. NC cells showing random walk motility coupled to proliferation up to a carrying capacity set by the environment would show "frontal expansion" into uncolonized regions (see next section). This has been recognized in the colonization of the intestine and face by NC cells (Simpson et al., 2007; Kulesa et al., 2008). The environmental factors are presumed to be mitogens, such as GDNF (Hearn et al., 1998) or VEGF (McLennan et al., 2010), and it is assumed that these are consumed by NC cells. This would create a gradient of factor across the NC front, and both these factors are chemotactic (Young et al.,

2001; McLennan et al., 2010), so this would provide additional directional signals for migrating NC cells.

Cranial NC cells localize and assemble into the precise and complex forms of the facial skeleton *anlagen*. This clearly involves tissue interactions in the context of the particular axial NC type (Couly et al., 2002), but is formidably complex. The assembly of neural ganglia is a more approachable problem (see Fig. 2A). For the sympathetic ganglia, the ventral dispersion of NC cells requires chemotaxis of CXCR+ NC cells to an SDF target region (Kasemeier-Kulesa et al., 2010). Assembly into ganglia is then driven by the CAM N-cadherin, while the cells simultaneously avoid interganglionic regions due to Eph/ephrin repulsion (Kasemeier-Kulesa et al., 2006). The development of evenly spaced and sized ganglia of the ENS from chains of migrating NC cells has also been examined, and a model of CAM function, proliferation, and cell motility has duplicated this morphogenesis (see next section).

DIFFERENTIATION

After specification of the NC, an initial task is to prevent differentiation in early migration. SoxE genes (Haldin and LaBonne, 2010) such as *Sox10* inhibit the differentiation of NC cells and maintain progenitor properties (Kim et al., 2003). However, the role of Sox10 is made more complex as it is also involved later in the differentiation of peripheral glia and melanocytes) (Mollaaghababa and Pavan, 2003). Sox10 may bind as a monomer or cooperatively with other transcription factors (Peirano and Wegner, 2000). High Sox10 level may inhibit differentiation whereas Sox10 plus Pax3 proteins bind separately to activate *MITF* for melanocyte differentiation. In the ENS, the same two transcription factors interact to regulate the neuroglial gene *Ret* via the Pax3 site (Bondurand et al., 2000; Lang and Epstein, 2003). Another NC specifier gene, *FoxD3*, has been implicated in maintaining multipotent progenitor status while suppressing ectomesenchymal options (Mundell and Labosky, 2011).

In the head, the early NC genes are downregulated as the migrating cells reach the pharyngeal arches. Here ectomesenchymal genes are upregulated via FGF signals (Blentic et al., 2008) which are in turn influenced by Shh from the pharyngeal endoderm (Haworth et al., 2007). Prominent in the ectomesenchymal suite of genes is re-expression of *Sox9*. This NC specifier is uneccessary for migration, but essential for later craniofacial cartilage differentiation (Mori-Akiyama et al., 2003), via an extensive and complex set of regulatory elements (Bagheri-Fam et al., 2006).

A neural tube-derived TGF-β member is necessary for differentiation of sympathetic neurons, as well as BMP4 which is produced by the dorsal aorta, directly adjacent to the sympathetic localization site. BMP4 induces expression of DNA-binding proteins required for both neuronogenesis and cell type-specific expression of noradrenergic marker genes (Schneider et al., 1999). These include the homeodomain proteins Phox2b and Phox2a, the basic helix-loop-helix DNA binding proteins achaete-scute homolog1 (Mash1 mouse) and Hand2, and the zinc finger protein Gata3 or Gata 2 (mouse and chick) (Moriguchi et al., 2006). Phox2b is a master regulator for the autonomic nervous system (ANS), as deletion results in loss of sympathetic, parasympathetic, and enteric neurons (Howard, 2005). In contrast, deletion of Hand2 results in loss of sympathetic neurons (Schmidt et al., 2009) but this has no clear effect on parasympathetic neurons. Loss of Hand2 in Nestin-expressing ENS cells reduces proliferation and differentiation, disturbs the ENS ganglionic organization and axonal architecture, and depletes neurons, especially NOS and VIP neurons (Lei and Howard, 2011). The different effects of loss of Hand2 in the generation of sympathetic, compared to other ANS neurons, suggest the existence of additional instructive cues for specification of neurotransmitter phenotypic characteristics. The roles of various molecules in sympathetic, parasympathetic, enteric, and sensory neuron differentiation are presented in Figure 2B. As development proceeds, the peripheral NC neurons express receptors of the Trk family and become dependent on target-derived factors including NGF, BDNF, and ET3 (Ernsberger, 2009).

Modeling NC Development

Mathematical models of biological phenomena have suffered from the charge of not being closely linked to the biology (Mogilner et al., 2006), but recently a series of papers has appeared which integrate experimental developmental biology of the NC with modeling. Mathematical modeling at the most trivial level may provide a *post hoc* mathematical description to match a biological observation, or may merely validate in mathematical terms a prior intuitive model. However, in the face of simultaneous interacting variables, intuition is untrustworthy and in this circumstance modeling may demonstrate how a known outcome could arise from the interaction of known variables. An even more useful result of formal modeling is to demonstrate that, even for a favored intuitive model, the known outcome can never emerge under any reasonable values of the input variables. Perhaps the most satisfying outcome of modeling, however, is where the model not only realizes its original intent, but additional "hidden parameters"(Aylaj et al., 2011) emerge which, when one is alerted to them, are later uncovered in the biological example (Table 1).

MODELS OF CRANIAL NC MIGRATION

A major focus of modeling studies of NC morphogenesis is cranial NC migration. This has the advantage that the dense cranial NC streams in the avian head are favorable for *in vivo* time lapse imaging and analysis (Teddy and Kulesa, 2004), which anchors models to reality. In addition

TABLE 1. *Contributions of Modelling to NC Migration, Ganglionation, Cell Lineage and Disease*

		Previous			
NC system property	**Process**	**Observ[n]**	**Predict[n]**	**Emergent property**	**Confirmat[n]**
CrNC & NC/ENS collective migration	Migration and Proliferat[n]	Y	Y	Y	
CrNC & NC/ENS self-generated chemotactic gradient		N	Y	Y	Not yet
CrNC leader/trailer differences		N	Y	Y	Y
CrNC & NC/ENS invasion wave		Y	Y	Y	
CrNC & NC/ENS frontal expansion		N	N	Y	Y
NC/ENS rear proliferation (back-filling)		N	N	Y	
CrNC and NC/ENS provisional leader/trailer cells		N	Y	Y	Y
NC/ENS "transmesenteric" dominance of distal colon		Y	N	Y	Y
NC/ENS reverse migration		Y	N	Y	
CrNC & NC/ENS chain stability/single cell instability		Y	N	Y	
NC/ENS chains, increasing spatial complexity		Y	N	Y	
CrNC & NC/ENS effect of domain growth on colonisation[n]		Y	N	Y	Y
NC/melanoblast dermal/epidermal prolif difference		Descript[n]	Quantific[n]		
NC/melanoblast dermal/epidermal flow		Descript[n]	Quantific[n]		
NC/ENS aggregation/ganglionation	Ganglion formation	Y	Y	Y	
NC/ENS multiple small stable ganglia		Y	N	Y	
NC/ENS ganglion internal organisation		N	Y	Y	Y
NC/ENS clones: quantitative heterogeneity (superstars)	Lineage	N	N	Y	Y
NC/ENS clones: qualitative heterogeneity		N	N	Y	Not yet
NC/ENS clones: spatial heterogeneity		N	N	Y	Y
NC/melanoblast progenitor number (trunk)		N	N	Y	Not yet
ENS/HSCR as proliferation defect	Disease	N	N	Y	Y
ENS/HSCR discordance/MZ twins		Y	N	Y	Not yet

References: Aylaj et al. (2011), Cheeseman et al. (2014), Hackett-Jones et al. (2011), Lanman et al. (2011), Luciani et al. (2011), McLennan et al. (2012), Simpson et al. (2007), Teddy and Kule (2004), Wynn et al. (2012), Young et a1. (2014) and Zhang et al. (2010)

these migrations are constrained to corridors by flanking exclusion domains; and this justifies mathematical simplifications (Wynn et al., 2012). These models incorporated short and long-range cell interactions between NC agents, chain and "follow the leader" behavior, proliferation, and attractive effects of diffusible factor(s), in this case VEGF. In addition the domain in which migration occurs is also growing. The modeling posed that chemotaxis was established by the NC cells, which consumed a factor to produce a gradient. However in the model this was insufficient to explain the observations without invoking differences between the leading and trailing cells. These were indeed discovered by gene expression profiling. The leading cells also showed different morphology ("hairy cells") and heightened cell proliferation, elsewhere termed frontal expansion. Nevertheless, these differences were provisional, since transposition of lead and trailing cells predicted changes in cell behavior appropriate to the new position (McLennan et al., 2012). These studies significantly extended understanding of cranial NC migration at a system level.

MODELS OF ENS FORMATION

Another NC example of mathematical modeling has centered on the colonization of the intestine by NC cells, the later aggregation (ganglionation) process to form the ENS, and the numerical and spatial evolution of clonal enteric NC derivatives. The NC population in this case colonizes as a wave of agents with a net unidirectional movement, and at the cell level this has been time lapse-imaged in gut in organ culture (Young et al., 2014). The biological advantage of ENS formation is that it is lengthy, well described, and accurately timetabled. Moreover it occurs in a quasi-two dimensional environment that can be idealized as the

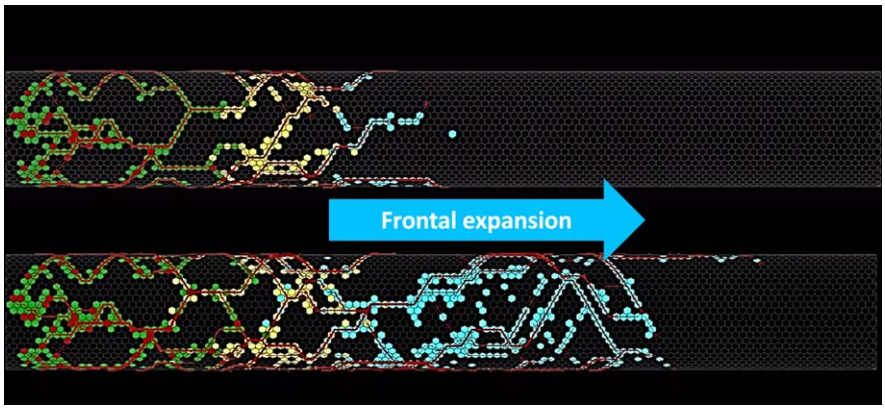

FIGURE 3. *In an agent-based model, the wall of the gut tube is colonized by NC-agents (arbitrarly colored green, yellow, and blue) in a rostro-caudal wave (left to right in this diagram). These agents can move and proliferate under encoded conditions, and also differentiate into neuron-agents and extend axons (red lines). Daughter-agents maintain the same color coding as the original agent. The model correctly predicted that colonization of the gut tube is mainly accomplished by movement and proliferation of the NC-agents at the front ("frontal expansion") but there is considerable mixing of NC-agents behind the front. Reproduced from Landman et al. (2011).*

surface of a cylinder. Two cell behavioral properties were initially modeled in attempts to reproduce the population-level cell migratory wave (Simpson et al., 2007); these were (i) unpredictable NC agent movement coupled with (ii) logistic NC agent proliferation (i.e., dependent on the intestinal domain). In this process the domain of migration, the intestine itself, is also elongating: the intersection of directional net NC migration and directional domain growth offer interesting possibilities, both mathematical and biological (Landman et al., 2003). In detail enteric NC cells when isolated show random movement directions but when in contact, they form interweaving chains, and this dynamic individual cell behavior has been the subject of modeling (Cox, 2011; Landman et al., 2011). Additional features of adhesive and repulsive cell–cell and cell–axon interactions, and chemotaxis by self-consumption of a factor (such as GDNF) have also been incorporated into models. Many of the properties of ENS colonization, such as frontal expansion (Fig. 3), emerged from these models, and were later confirmed biologically.

The aggregation phase that forms the enteric ganglia has been modeled with motile proliferative NC agents which differentiate into neurons, the two having different affinity strengths, which is consistent with the observed differences in labeling for CAMs. These properties were sufficient to dictate aggregation of ENS agents into groups (ganglia), segregation of NC and neuronal agents within the groups, and the size, spacing, and stability of the groups (Hackett-Jones et al., 2011). Clonal analysis performed mathematically using the previous cell proliferation and movement parameters showed that the expansion of the ENS as a whole was carried out by a few progenitors ("superstars"), leaving a multitude of progeny while most underwent very few cycles of division. This frequency distribution was completely unexpected, but clonal single-cell labeling using a replication proficient label showed that it indeed occurred in the embryonic ENS (Cheeseman et al., 2014). In the model there was no difference encoded between the agents that became "superstars" and those that did not, the differential outcome was chance. There was no obvious difference between any of the enteric NC cells at the beginning of the biological assay, so it may be that the real outcome is also chance. This widely variable, chance-driven clonal development pattern has implications for how the ENS develops into such a consistent set of different cell types (around 15 types of neurons) of tightly regulated numbers and proportions.

MODELS OF MELANOCYTE POPULATION DYNAMICS

Linked biological and mathematical treatments have centered on the proliferative and dispersive behavior of mouse melanoblasts between the dermis and epidermis after their specification from NC cells but before they colonize the hair follicles (Larue et al., 2013). This system in the mouse skin has the advantage for modeling of easily visualized distribution of melanoblasts, and the separation of these cells facilitates single cell tracking and enumeration of their massive proliferation and dispersal (Aylaj et al., 2011). Moreover, changes in cell number involve only proliferation, without having to account for loss through cell death or loss of expression of cell markers. Melanoblasts cross from the dermis into the epidermis, but never in the reverse direction. In addition these early events are reflected later in the easily observed coat color pattern, of which there are numerous mutants. The modeling used measurements of the number of melanoblast and of their proliferation in the epidermis and in the dermis

(Luciani et al., 2011). The analyses indicated numerical parameters for proliferation in these two regions that accounted for the observations in wild type and in β-catenin loss and gain of function mutants, and were consistent with the dermal population maintaining itself while also providing cells for the epidermal zone. In addition the models could be extrapolated back in time to suggest the number of originating melanoblast progenitors in the trunk of the mouse.

Taken together, these modeling papers have produced confirmatory and new results that have significantly widened our understanding of how the NC operates as a system. Some of the contributions of modeling to understanding NC development are outlined in Table 1.

Neurocristopathies

Neurocristopathies, a concept introduced by Bolande in 1974, describes diseases of NC development (Bolande, 1997). This concept can help clinical understanding and build a bridge with basic science. We will briefly discuss Treacher Collins syndrome (TCS), Hirschsprung disease (HSCR), and 22q11.2 deletion syndromes, three of the most extensively studied neurocristopathies, in a clinical context to illustrate the complexity of NC cell biology and their fate from their formation to the constitution of the target organ.

TREACHER COLLINS SYNDROME

Description, genetic, and developmental causes. TCS, also known as mandibulofacial dysostosis or zygoauromandibular dysplasia, affects 1/50,000 live births (Trainor et al., 2009) and is a facial dysmorphism that involves mainly structures arising from the first and second pharyngeal arches (Rogers, 1964), to which the cranial NC makes a major ectomesenchymal contribution (see Fig. 1). Mutations in *TCOF1*, *POLR1C*, or *POLR1D* are currently known to be the cause of TCS. In humans, up to 93% of individuals with TCS are heterozygous for a mutation in *TCOF1* (Splendore et al., 2000). In mice, *Tcof1* is strongly expressed in the neuroepithelium at E8.5 and in the frontonasal and pharyngeal arch ectomesenchyme at E9.5 (Dixon et al., 1997), and haploinsufficiency of *Tcof1* mimics severe TCS in humans (Dixon et al., 2006). In fact, NC cells exhibit elevated apoptosis in the neural ectoderm, together with decreased proliferation in *Tcof1*+/- mice (Dixon et al., 2006). By inhibition of p53 in these mice, Jones et al. (2008) were able to restore the NC cell population, and thus rescue the pathogenesis. It has been demonstrated that Treacle, the nucleolar protein encoded by *TCOF1*, is involved in ribosome biogenesis (Gonzales et al., 2005). In addition, mutations in *POLR1C* and *POLR1D* which have been detected in affected individuals without a *TCOF1* mutation (8% of individuals with TCS) (Dauwerse et al., 2011) lead to diminished functional RNA polymerases I and/or III. Collectively, this suggests that mutations in these genes result in insufficient numbers or function of mature ribosomes in the neural ectoderm and the NC cells at a critical time, resulting in apoptosis of cranial NC progenitors of the first and second pharyngeal arches and consequent cranioskeletal insufficiency. Neural derivatives of the cranial NC are spared in TCS.

Diagnosis. The major features of TCS are hypoplasia of the facial bones (80% of cases), particularly the zygomatic complex and the mandible, external ear abnormalities (80% of cases), lower eyelid abnormalities (70% of cases), and family history compatible with autosomal dominant inheritance (40% of cases) (Katsanis and Jabs, 1993). The minor clinical features include ophthalmologic defects, such as refractive errors (58% of cases) or strabismus (37% of cases) (Hertle et al., 1993), hypoplasia of the external auditory canals and the ossicles of the middle ear, cleft palate (28% of cases), and preauricular hair displacement (Katsanis and Jabs, 1993; Cobb et al., 2014). The distribution of dysgenetic skeletal and soft tissue structures in TCS is generally bilateral but not necessarily symmetrical (Marsh et al., 1986). The patient with a complete form of TCS can be recognized at birth with midface hypoplasia; however, phenotypic variations are wide (Teber et al., 2004) and mildly affected patients can remain undetected. Clinical examination and radiological findings guide genetic testing to reach a diagnosis.

Management. As there is no phenotype-genotype correlation (Teber et al., 2004) and prenatal diagnosis is not routinely practiced, early planning is currently impossible. Evaluation and treatment are done according to age and are tailored to individual needs by a multidisciplinary team (pediatrician, plastic surgeon, maxillo-facial surgeon, otorhinolaryngologist, ophthalmologist, geneticist, dentist/orthodontist, speech pathologist, audiologist, and psychologist). Four periods of management can be distinguished (Posnick et al., 2004; Cobb et al., 2014): neonatal, infancy, childhood, and maturity. At birth, airway management takes precedence: immediate problems are managed by a nasopharyngeal airway or a tracheostomy. Mandibular distraction has been proposed in selected cases (Tahiri et al., 2014). During infancy, interventions are aimed at functional improvement: hearing (surgical correction of hearing loss, bone-anchored hearing bands); speech; surgical correction of the cleft palate if necessary; and eye protection. During childhood and adolescence, patients will need multiple craniofacial reconstruction procedures which should coincide with facial growth pattern (Posnick et al., 2004). Zygomatic and orbital reconstructions are undertaken around the age of six or when the orbitozygomatic bony growth is complete, as well as external ear reconstruction. Maxillo-mandibular reconstruction should be performed according to the severity of the deformity and the skeletal maturity. Nasal reconstruction, as well as surgical procedures to improve the soft tissue deficiencies,

should follow orthognathic reconstruction. Long-term assessments show that functional deficiencies in hearing, speech, or closure of the eyelids are as important as facial appearance (Plomp et al., 2013).

HIRSCHSPRUNG DISEASE (HSCR)

Description, genetic, and developmental causes. HSCR is the most common congenital gut motility disorder with an incidence of 1/5,000 live births (Blesa et al., 2009), which is characterized by an absence of ENS neurons in a variable length of the distal bowel. ENS precursors, vagal NC (see Fig. 1), colonize the developing distal intestine as a proximo-distal wave (Le Douarin and Teillet, 1973) and a distal shortfall in this process results in HSCR. Neurocristopathy seems an adequate framework to understand the complex interactions between genetics, cellular, and developmental mechanisms, and severity (length of the aneuronal bowel, severity of obstructive symptoms and post-operative evolution).

Mutations in many genes (*RET, GDNF, NTN, EDNRB, EDN3, KBP, β-1 Integrin, L1Cam, PHOX2B, HASH1, HAND1, SIP1/ZFHX1B, SOX10)* have been described in HSCR patients or in murine models (Heanue and Pachnis, 2007). Generally, HSCR is believed to be a multigenic disorder and the genotype-phenotype correlation is poor. HSCR occurs as an isolated trait in 70% of cases but is also part of numerous syndromes (Amiel et al., 2008), and other neurocristopathies are frequently associated with HSCR (MEN type 2A, neuroblastoma, congenital central hypoventilation syndrome, Waardenburg syndromes).

Diagnosis. HSCR can be classified according to the extent of the aneuronal gut: absence of neurons in the rectum and the sigmoid is most frequent (75–80%), and extension proximal to the sigmoid colon accounts for 20% of cases (Suita et al., 2005). Rare cases (3.8% of HSCR patients, Suita 2005) can involve the whole colon and portion of the small bowel. Three further variants of HSCR have been reported: total intestinal HSCR (Ikeda and Goto, 1986), ultra-short segment HSCR (Neilson and Yazbeck, 1990) and skip lesions, or zonal aganglionosis (Kapur et al., 1995). The gut between the aneuronal and the neuronal bowel, the transition zone, shows reduced enteric neuron density and its length is difficult to predict (Doi et al., 2005). Male patients are affected three to four times more often than female in recto-sigmoid HSCR, while the sex ratio is more even in more extensive forms (Blesa et al., 2009).

Lack of neurons in the distal bowel results in tonic contraction with delayed passage of meconium, vomiting, or abdominal distension. Subsequently, clinical presentation is mainly bowel obstruction or enterocolitis, which is a life-threatening complication. Nowadays, 80–90% of HSCR patients are diagnosed as neonates (Singh et al., 2003) by rectal suction biopsies and/or anorectal manometry (de Lorijn et al., 2006). Patients diagnosed later in childhood present with chronic constipation (Orr and Scobie, 1983). Enterocolitis (abdominal distension, fever and septicemia), can be present in the preoperative period as well as after the surgical treatment, with incidence rates of 18–50% and 5–35%, respectively (Vieten and Spicer, 2004).

Management. HSCR is treated by surgical resection of the aneuronal bowel. Four main surgical techniques have been described: Swenson's abdomino-perineal pull-through, Duhamel's retro-rectal pull-through, Soave's transanal endorectal pull-through, and Rehbein's technique. The most recent trend is one-stage transanal endorectal pull-through often assisted by laparoscopy (De la Torre-Mondragon and Ortega-Salgado, 1998). The reports on long-term outcomes of bowel function after surgical repair of HSCR are conflicting. Fecal incontinence persisting in up to 48% of the adult patients is the principal problem (Jarvi et al., 2010). The Swenson and the Duhamel techniques were reported to provide a majority of patients with normal bowel control (Sherman et al., 1989; Bourdelat et al., 1997). However, more recent studies including age-matched healthy controls, with a range of follow-up between 8.4 and 31 years, showed significantly decreased bowel control regardless of the surgical technique (Rintala and Pakarinen, 2006). Increased age at diagnosis is a predictor of poor outcome (Jarvi et al., 2010) and total colonic extension of the disease gives poorer outcomes with higher rates of post-operative enterocolitis and fecal incontinence (Menezes et al., 2008). Residual aneuronal bowel or transition zone bowel after pull-through (Friedmacher and Puri, 2011), dysmotility due to intestinal neuronal dysplasia (Kobayashi et al., 1995) are possible causes of poor outcomes.

22Q11.2 DELETION SYNDROMES (22Q11.2DS)

Description, genetic, and developmental causes. 22q11.2DS presents as a complex, variable, major dysgenesis of the face, the pharyngeal arch derivatives, including the vessels of the cardiac outflow tract, the thymus, the parathyroid, and the thyroid glands, all of which involve cranial NC (see Fig. 1). 22q11.2DS is the most common microdeletion syndrome with a calculated prevalence rate of 1/4,000 live births (Bassett et al., 2011). *TBX1*, a candidate gene for 22q11.2 DS, is located in the 3 million-base pair deletion on chromosomal region 22q11.2. *Tbx1*$^{-/-}$ mice present with a phenotype characteristic of the 22q11.2 deletion syndromes, including cardiac outflow tract abnormalities, facial deformities, hypoplastic thymus, and parathyroid glands (Jerome and Papaioannou, 2001). *Tbx1* is expressed in pharyngeal ectoderm and endoderm cells for outflow tract development but not by NC cells. In *Tbx1* mutation, NC migration (Vitelli et al., 2002) or proliferation (Garg et al., 2001), rather than differentiation (Xu et al., 2004), are affected. Therefore, NC cells are involved in syndromic features of 22q11.2DS, but defects in the NC cells are not necessarily the causative agent.

TABLE 2. *Common Genes in NC Cells, Melanoma and Neuroblastoma*

Gene	Function in NC	Marker/function in NC derived tumor (over-expression, activation or amplification)
ALK(BMP receptor)	Cranial NC and sympathetic neuron survival/differentiation.	Neuroblastoma: Activation/amplification. Poor prognosis.
Endothelin-3/EDNRB	Survival/proliferation/inhibition of differentiation° (melanocyte and ENS).	Melanoma: Proliferation/survival/inhibition of differentiation. Poor prognosis.
KCTD11	Regulator of neuron differentiation.	Melanoma: "extravascular migratory metastasis"
MITF	Melanocyte survival/differentiation.	Melanoma: Survival/cell viability. More favourable prognosis.
MMP-1,2,9	NC EMT/migration.	Melanoma: Invasion/tumour growth.
N-cadherin	Lower expression: migration. Higher expression: aggregation	Melanoma: Epidermal detachment/dermo-vascular invasion. Neuroblastoma: lower expression: higher apoptosis and invasion.
MYCN	Prospective NC/EMT/migration and neuronal differentiation.	Neuroblastoma: Proliferation/reduced apoptosis/reduced differentiation/ tumour-vascular interactions. Poor prognosis.
NT-3	Survival/proliferation/neuron differentiation.	Neuroblastoma: Up-regulation: growth/metastasis/ inhibition TrkC-induced apoptosis.
p75	Multipotent NC progenitor cell marker.	Melanoma: Up-reg.: metastasis. Poor prognosis. Enriched in Human Melanoma Initiating Cells. Neuroblastoma: NGF-induced apoptosis.
S100	Expressed in NC derived tissues	Melanoma: Serum marker for malignancy/tumour progression/recurrence.
Snail family	Prospective NC/EMT.	Melanoma: EMT/invasion.
Sox9/10	Prospective NC/EMT/migration.	Melanoma: Migration/metastasis marker.
TCOF1	NC generation/proliferation.	Melanoma: Over-expressed in angiotropic (highly metastatic) melanoma.
Trk A, B, C	Neuron differentiation/ maintenance.	Neuroblastoma: High TrkA and C: apoptosis/ differentiation/low MYCN amplification. Favorable prognosis. High TrkB/BDNF: MYCN amplification/aggressive tumors. Poor prognosis.
Twistl	NC ectomesenchyme migration/differentiation.	Melanoma: EMT/progression/metastasis.

References contained in: Bailey et al. (2012), Cheung and Dyer (2013), Jiang et al. (2011), Miller and Mihm (2006) and Powell et al. (2013).

Tbx1 controls development of the pharyngeal arch arteries by controlling *Gbx2* expression: ablation of *Gbx2* in *Tbx1*-expressing domain leads to pharyngeal arch artery defects by misrouting NC migration into the fourth pharyngeal arch (Calmont et al., 2009). Interaction of Tbx1 with several other pathways, including FGF8, Shh, Pitx2, retinoic acid, and VEGF, have been described. In humans, TBX1 mutations have been linked with 22q11.2 DS phenotypes (Yagi et al., 2003). However, isolated loss of *TBX1* function alone is unlikely to be a common cause (Gong et al., 2001).

Diagnosis. 22q11.2DS is a multi-system syndrome with a marked variability in clinical presentation and age at presentation, as well as severity of expression (Bassett et al., 2005). Diagnosis is made using various molecular genetic testing (FISH, chromosomal microarray or targeted analysis) in patients presenting two or more of the following features (Bassett et al., 2011): dysmorphic features (>90% of cases: long narrow face, malar flatness, hooded eyelids, dysplastic nose, small mouth); learning disabilities or developmental delay (90%); cardiac anomaly (50–75%; predominantly conotruncal defects such as tetralogy of Fallot, interrupted aortic arch or ventriculoseptal defect); palatal abnormalities (75%); immune deficiencies (80%; thymic hypoplasia, T-cells impaired function); hypocalcaemia (60%); renal anomalies (10–31%); failure to thrive and/or gastro-oesophageal reflux; neuropsychiatric disorders (20% of children with autism spectrum disorders, 20% of adults with schizophrenia); skeletal abnormalities; and ophthalmologic abnormalities. Mortality is mainly due

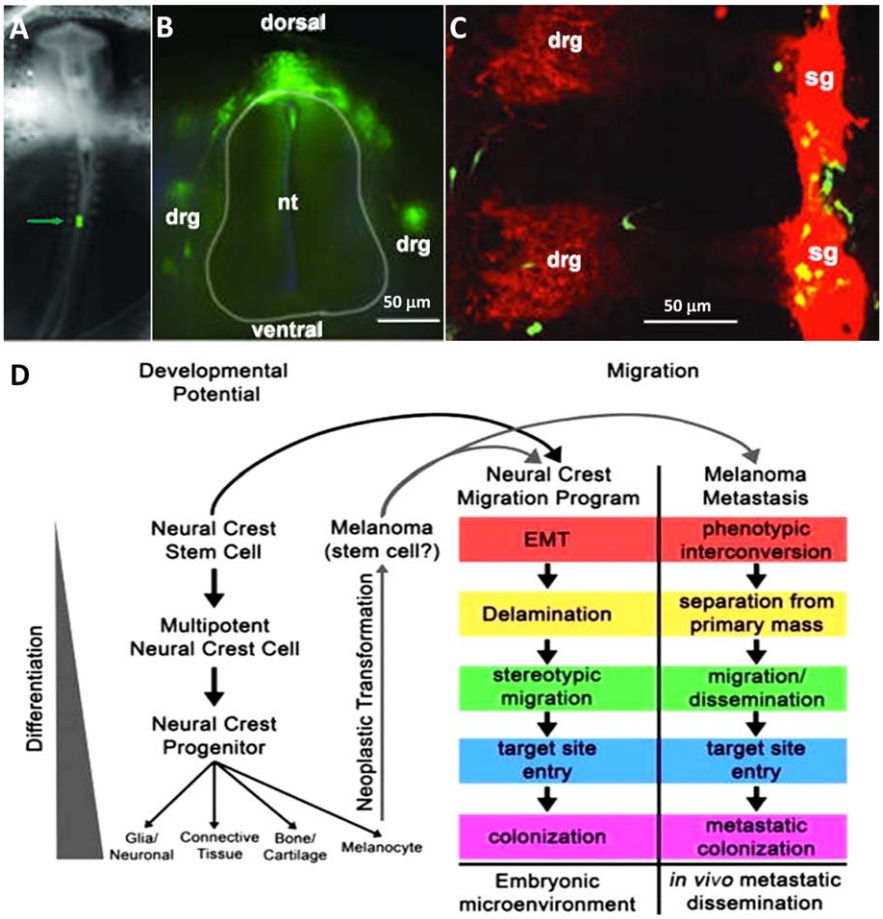

FIGURE 4. *Human melanoma cells in a normal embryonic migratory NC environment undergo stereotyped NC-like migration.* **A:** *GFP-expressing human metastatic melanoma cells transplanted into the E1.5 chick embryo trunk neural tube (nt).* **B:** *Two days later, GFP+ melanoma cells have followed the medioventral migratory path to the dorsal root ganglia (drg).* **C:** *GFP+ melanoma cells distributed with NC cells labeled with HNK-1 (red) in the sympathetic ganglia (sg).* **D:** *Diagrammatic representation of normal embryonic NC development and melanoma generation and metastasis, highlighting the parallels between normal migratory morphogenesis, differentiation/dedifferentiation and pathological invasion. A–C modified from Kulesa et al. (2006); D from Kulesa et al. (2010).*

to cardiac anomalies (Ryan et al., 1997). Management is made by multidisciplinary teams and is tailored to the individual, their age or developmental stage, and the severity of the associated features.

In summary, neurocristopathies can result from a disrupted NC development during formation, migration, or differentiation. They are often characterized by a wide phenotypic variation without genotypic correlation, the most extreme being discordancy of disease in monozygotic twins (Stoll et al., 1984), an illustration of the complexity of NC development. Lack of routine prenatal diagnosis and prognostic factors prevent early treatment being planned in detail in advance. Understanding the etiology and the pathogenesis of individual neurocristopathies could help in diagnosing, developing therapeutic approaches, and eventually preventing these distressing conditions.

NC TUMORS

Classification of NC-derived tumors. Tumors arising from cells of NC-derived structures (see Fig. 1) can be classified into five groups (Dundr and Ehrmann, 2012): (1) peripheral neuroblastic tumors (olfactory neuroblastoma, neuroblastic tumors of the sympathetic nervous system and the adrenal gland), (2) melanocytic tumors (melanocytes nevi, malignant melanoma, dermal melanocytoses and blue nevi, diffuse leptomeningeal melanocytosis, melanocytoma of the leptomeninges), (3) tumors of peripheral and cranial nerves (Schwannoma, malignant peripheral nerve sheath tumors, benign and malignant granular cell tumors, neurofibroma, perineurioma, neurothecoma, nerve sheath maxima), (4) paraganglioma group (termed pheochromocytomas for those arising from the adrenal medulla), and (5) other tumors of NC origin (medullary thyroid carcinoma).

Identification of cells with NC stem markers in mammalian tissues that are not NC-derived is consistent with the possibility that NC-derived tumors might also arise outside the recognized sites of NC-derived cells. For example, apparent NC stem cells have been isolated in rodents from bone marrow (Nagoshi et al., 2008) and in humans from cord and adult peripheral blood (Wang et al., 2013).

Tumor classification with respect to embryonic origin can be bewildering, and some tumors have been ascribed to the NC mainly because of presumed metabolic similarities. Endocrine cell cancers ("APUDoma") of the gastrointestinal tract were postulated to be of NC origin (Pearse and Polak, 1971), but were later proved to be of endodermal origin (Cox and Pierce, 1982). A NC origin has been suggested for PEComas (Perivascular epithelioid cell tumors), but only indirect evidence supports this (Fernandez-Flores, 2011). Some non-NC tumors can be confused with those of NC origin. Peripherally located primitive neuroectodermal tumors (pPNETs) like Ewing's sarcoma family of tumors may be of diverse origins, but their genetic lesion (e.g., *EWS-FLI1* fusion) confers NC-like properties (Hu-Lieskovan et al., 2005). Conversely, many tumors that are of NC origin are mistakenly classified as pPNETs (Berman, 2009).

NC tumor genetics and markers. Although mostly sporadic, some NC tumors also occur in hereditary syndromes (Dundr and Ehrmann, 2012). These include paraganglioma and pheochromocytoma of the carotid bodies and adrenal medulla, respectively. Neurofibromatosis type I (*NF1* gene, protein neurofibromin 1) and the much rarer type 2 (*NF2* gene, protein Merlin) involve loss of tumor suppressor function, generally in Schwann cells, while some Schwannomatoses involve mutation in *SMARCB2*. Von Hippel-Lindau syndrome almost always involves mutation in the eponymous *VHL* gene. A considerable proportion of Familial Medullary Thyroid Carcinoma and MEN2A and type 2B, Carney triad, and Carney-Stratakis dyad, all involve mutations in the *RET* gene that results in constitutive activation of the RET receptor. This contrasts with the loss of function *RET* mutations in HSCR (see previous section).

Antibodies to the low affinity nerve growth factor (NGF) receptor p75 and the HNK-1 glycotype are widely used in NC developmental studies. These antigens can be detected in neurofibromas and pheochromocytomas (Ross et al., 1984), and in metastatic melanoma (Thies et al., 2004). These are useful markers, but both antigens are also expressed in non-NC tissues (Suzuki et al., 2008). In addition, when a tumor with neuronal differentiation but lacking Schwann or glial cells components, such as neuroectodermal tumor of infancy, is associated with high urinary excretion of vanillylmandelic acid, this suggests NC origin instead of CNS origin (Borello and Gorlin, 1966). Therefore there is no single histological, immunological, or metabolic marker that can identify with surety tumors of NC origin, but a panel of markers can give strong indications.

Invasiveness of NC-derived tumors—embryological throw-back or fantasy?. The migratory behavior of NC cells during development and the invasive phase of cancer metastasis (not just NC-derived cancers) share characteristics like EMT and cell movement. NC EMT, its molecular triggers, and its genetic regulatory network has placed it as an uncannily accurate model of tumor cell EMT and metastasis (Powell et al., 2013). NC cancers are often regarded as being more likely to be aggressive, the somewhat glib explanation being that they may aberrantly *repeat* the expression of genes involved in normal NC EMT, proliferation, migration, and survival (Trainor, 2014). Speculatively, this could be due to retained lineage-specific epigenetic memory, but alternatively it could be that the tumor is derived from previously quiescent NC progenitor cells, that are not so much repeating as *resuming* their earlier behavior. Despite this, many cancers of NC origin are not aggressive, such as Schwannoma and paraganglioma (Heth, 2004; Enoz et al., 2006). We will briefly focus on two of the more aggressive NC-derived cancers (Table 2).

Melanoma. Melanoma is one of the most aggressive types of skin cancer, typically in adults, and late detection and misdiagnosis often leads to inadequate treatment and poor prognosis (Grant-Kels et al., 1999). Melanoma is heterogeneous, composed of not only melanocytes but also cells with neuronal, glial, chondrocytic, and adipocytic features (Shakhova, 2014). Heterogeneity has also been established through gene expression studies (Haqq et al., 2005; Jeffs et al., 2009). The first step in melanoma formation is proliferation of normal melanocytes to form a benign nevus followed by dysplasia in an already existing nevus or in a new location (Clark et al., 1989). Misregulation of genes, such as *BRAF, CDKN2A, INK4A, PTEN, CCND1*, and the MITF and MAPK signaling pathways, are implicated in progression of a benign nevus into malignant melanoma (Miller and Mihm, 2006).

Multipotent differentiation capacity of melanoblasts after their migration into epidermis (Motohashi et al., 2009) suggests that they retain ancestral NC characteristics (Gupta et al., 2005). This is often linked to the frequently aggressive behavior of melanoma cells (Gupta et al., 2005; Uong and Zon, 2010). Transcriptional plasticity has also been identified experimentally in human malignant melanoma cells (Hoek et al., 2008). In a more extreme test of plasticity, human and mouse melanoma cells grafted in embryonic chick NC microenvironment (Fig. 4A–C) assumed stereotyped NC-like migratory and differentiative behaviors (Kulesa et al., 2006; Oppitz et al., 2007). However, when grafted to a non-NC site, the mouse melanoma cells resumed metastatic invasion. When harvested and analyzed through laser capture microscopy and comparative gene expression, the grafted human

melanoma cells had re-expressed sub-sets of genes associated with NC induction, EMT, and migration (Kulesa et al., 2010, 2013) (see Fig. 4D). These studies show that at least some cells in metastatic melanoma can change to a more normal behavioral phenotype in response to specific microenvironments. The changed gene expression patterns detected by Kulesa et al. (2010) could throw a developmental light on the control of metastasis in melanoma.

Neuroblastoma. Neuroblastoma is the most common solid tissue cancer diagnosed during the first year of life. The incidence of neuroblastoma is 10.2 cases per million children under 15 years of age in the United States. NC progenitor cells are widely accepted as the cell of origin of neuroblastoma (Maris, 2010; Jiang et al., 2011), with most arising from the adrenal gland or the lumbar sympathetic ganglia (Cheung and Dyer, 2013). NC cells and neuroblastomas share also common expressed genes: germline mutations in *PHOX2B* (Trochet et al., 2004) and *ALK* (Mosse et al., 2008), which are expressed by sympathoadrenal NC precursors, have been shown in familial neuroblastomas, possibly in conjunction with HSCR or congenital central hypoventilation syndrome; and *ALK* mutations are the most frequent mutations in sporadic neuroblastomas (Pugh et al., 2013). Amplification of *MYCN* is the most common chromosomal aberration, observed in ∼20% of sporadic neuroblastomas (Maris, 2010). In a murine model, immortalized, multipotent NC progenitor cells form neuroblastoma-like tumors upon transformation by *MYCN* and an oncogenic variant of *ALK* (Schulte et al., 2013).

Neuroblastoma is a heterogeneous malignancy with prognosis ranging from poor survival rate, for tumors refractory to treatment, to very high. Interestingly, a subset of initially progressing neuroblastomas can regress spontaneously. Patients are categorized into prognostic subsets, according to risk based on clinical, genetic, and molecular features of the tumor (Maris, 2010). Amplification of *MYCN* and *ALK* mutations are associated with poor prognosis. While ALK inhibitors are a promising therapeutic target (Mossé et al., 2013), there is no clinical trial targeting MYCN.

NC Progenitor Cells

The NC, when first specified, and at subsequent stages of morphogenesis and differentiation, seems to be a population of cells with individual cells showing variable degrees of restriction of potency, although overall the restriction gradually increases with developmental time (Ruhrberg and Schwarz, 2010; Dupin and Sommer, 2012). Following isolation and growth of NC and NC-derived cells from embryonic and fetal stages, multiple cell types within the NC "family" of cells, may differentiate, although with a diversity that varies with the site and that declines with donor age (Sieber-Blum and Cohen, 1980; Morrison et al., 1999; Kruger et al., 2002; Mosher et al., 2007; Dupin et al., 2010; Motohashi et al., 2014). NC cells are multipotent rather than pluripotent, and their self-renewal potential is not established, therefore, the term NC stem cell is problematic; here we use the term NC progenitor cell (NCPC).

USES OF NCPCS

In pluripotent cell biology, the information flow was initially from developmental studies to stem cell practice; now however there is an important reverse flow. NCPCs can be used as an *in vitro* workhorse for probing the fine details of the basic biological mechanisms of control of the generation, proliferation, and differentiation of NC cell types. This is particularly important in the human context where early embryonic NC cells are mostly unavailable. Second, NCPCs could be used in a "disease in a dish" model to study the genetic and physiological causes of cell autonomous neurocristopathies, including using cells derived from patients (Lee et al., 2009; Valensi-Kurtz et al., 2010). This technology can expedite mutation correction trials and drug screening for NC defects (Lee et al., 2012). Third, NCPCs could also be exploited therapeutically either by providing trophic assistance or as cell replacements for deficiency of NC-derived cells, as in craniofacial disorders (Jones and Trainor, 2004) and in the ENS disorder HSCR (Hotta et al., 2009a).

HOW ARE NCPCS OBTAINED?

NCPCs can be obtained from embryos of laboratory animals, but some of the above uses require human NCPCs, and often of autologous origin, and realistically these can only be obtained from postnatal donors. Do NCPC persist postnatally, and can they be obtained? Cells with properties of NCPC have been isolated from peripheral nerves (Morrison et al., 1999), dental pulp (Tatullo et al., 2014), and hair follicles (Sieber-Blum and Hu, 2008). Several lines of evidence support the existence of multipotent progenitor cells in, for example, the postnatal ENS. First, after disruption, the ENS can be reconstituted from intrinsic cells (Katsui et al., 2009), although the degree to which this occurs is contentious (Joseph et al., 2011). Second, ENS cells harvested from postnatal mouse and human gut are able to give rise to neurons and glia when transplanted into a recipient gut (Metzger et al., 2009; Hotta et al., 2013). Third, some cells in the ENS show SOX10 expression combined with the absence of both glial (such as S100β or GFAP) and neuronal markers (PGP9.5 and nestin); this is consistent with an ENS NCPC identity (Metzger et al., 2009; Azan et al., 2011). However, a lack of differentiation markers may not always be a prerequisite for NC-lineage multipotentiality. Recently, cells expressing markers of NC-derived glia have been shown in mouse to differentiate into nonglial cell types, including parasympathetic neurons (Dyachuk et al., 2014) and ectomesenchymal lineage dental pulp cells and odontoblasts (Kaukua et al., 2014). Whether such transdifferentiation

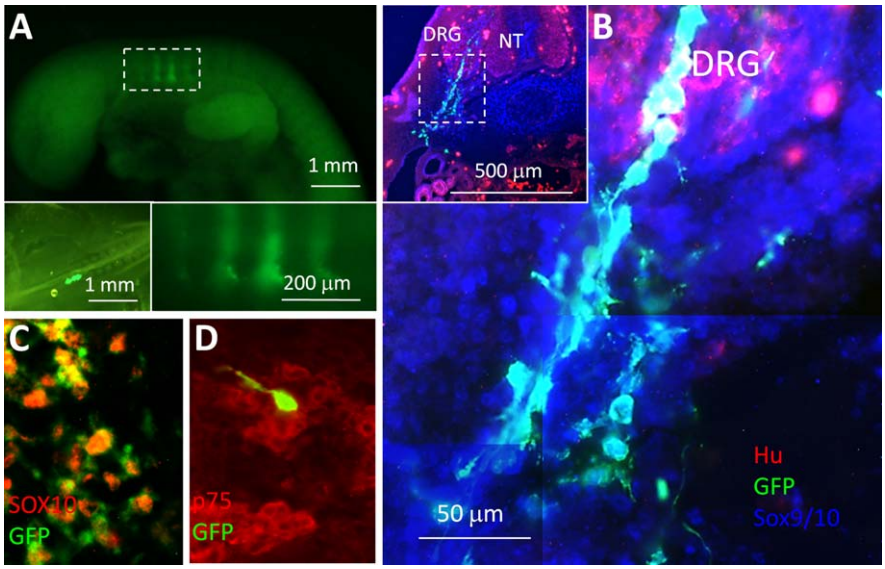

FIGURE 5. *Human NCPCs derived in vitro from ES cells behave like NC cells in vivo when transplanted into chick embryos.* **A:** *GFP-expressing NCPCs implanted in the trunk of an E2.5 embryo spread in the somites along characteristic hemisegmental NC pathways.* **B:** *NCPCs extend in the medioventral NC pathway along with host NC cells of the DRG and ventral root.* **C:** *Many NCPCs express the NC marker SOX10.* **D:** *Some NCPCs develop axons when localized in host sympathetic ganglia. Images provided by M. Denham, M. Dottori, and D. Zhang.*

persists postnatally in human NC-derived glia is not yet known.

Initial methods of harvesting NCPCs from complex tissues depended on the notion that, once dissociated, NCPCs would self-segregate to form neurosphere-like bodies, which could then be serially passaged (Almond et al., 2007; Silva et al., 2008). Greater specificity of selection has been sought by harvesting NC-lineage cells from tissues immunologically by flow cytometry or magnetic bead immunoaffinity, most commonly using antibodies to p75 (Wong et al., 2006; Jiang et al., 2009; Geisbauer et al., 2012), with other positive (α2 integrin) (Joseph et al., 2011) or negative selectors (Pzero) (Morrison et al., 1999). However, it is clear that NCPC persist in low numbers in postnatal tissues and, when compared with NCPCs from younger donors, their mitotic rate and multilineage differentiation capacity is reduced (Morrison et al., 1999). For purposes where a cell population permissive of large scale expansion and full-range differentiation is required, these signs are not encouraging.

Attention has turned to the creation of NC from other sources which offer the possibility of almost limitless expansion: from pluripotent stem cells (PSC). Successful differentiation of embryonic stem (ES) cells into cells of NC lineage was first achieved with mouse and primate ES cells (Mizuseki et al., 2003). This required the differentiation of ES cells into neural ectoderm, following coculture with stromal cells and carefully staged exposure to BMP4. Similarities to generation of the NC in the embryo are obvious. Subsequently, it was demonstrated that SOX10 could be used as reporter to detect and purify NC cells from mouse ES cells, and these cells could differentiate into neurons when placed in fetal gut (Kawaguchi et al., 2010). Similarly, it was demonstrated that NC cells could be generated from human ES cells by exposure to a stromal feeder layer which promoted neural differentiation, specifically the formation of rosettes representing the early developing neuroepithelium. Cells within these rosettes expressed p75 and HNK-1 and other markers to define their NC identity (Lee et al., 2007). Functionally, p75-sorted cells from this source migrated like *bone fide* NC cells and formed cells with neural and glial markers when grafted into chick embryos (Fig. 5), and human ES-derived Schwann cells were able to myelinate rat DRG neurons *in vitro* (Liu et al., 2012). Currently, both human ES cells and human iPS cells have been also been used to generate NC-like cells and subsequently NC-derived differentiated lineages (Lee et al., 2007; Zhou and Snead, 2008; Hotta et al., 2009b; Jiang et al., 2009; Lee et al., 2010; Denham and Dottori, 2011; Menendez et al., 2011; Liu et al., 2012). The precise methods have varied, but, in all cases neural ectoderm differentiation is induced whether through coculture with a stromal feeder layer, small molecule, or growth factor exposure.

OUTLOOK FOR NCPCS

Considerable technical improvements are still being made to overcome the variability and yield problems of NCPCs derived from ES or iPS cells. The use of feeder layers and complex 3D aggregates (neurospheres) to achieve steps of

differentiation show that these steps are attainable, but present "known unknowns". The use of growth factors suffers from lot-variability, half-life, and expense problems. These are being supplanted by controlled cell environments, which should also include O_2 tension, synthetic small molecule mimetics of both of soluble (growth) factors, and bound (ECM) factors, as well as synthetic surroundings with designed biophysical properties. In addition there remains the possibility of achieving NC lineage differentiation via directed transgenesis, without passing through a pluripotent intermediate stage. This has been achieved experimentally by expressing a small number of neuronal lineage-determining transcription factors in non-neuronal cells of both neural (glial) and non-neural (fibrobast) type (Vierbuchen and Wernig, 2012); direct NC lineage conversion cannot be too far away.

The scope for the employment of NCPCs, especially of autologous human origin via iPS cell technology, is expanding rapidly, and some of the uses mentioned previously are well underway. However, one problem that will need to be addressed is the need to produce exact sub-types of NCPC. We have earlier discussed that covert differences exist in NC cells along the axis; these differences will have to be replicated faithfully to obtain optimal models of NC cell biology and NC cell function from NCPCs produced in the laboratory. An example of this is the quest for a NCPC therapy to provide an ENS for HSCR. Not all NC cells are efficient at this, and the ability is positionally encoded prior to migration (Zhang et al., 2010). The basic biology of positional information will have to be enlisted to create this sub-type of NCPC, perhaps involving manipulation of retinoic acid exposure (Marshall et al., 1992). Moreover positional information may require monitoring because it is labile normally (Trainor and Krumlauf, 2000) and in culture (Jesús Santa-Olalla, 2003). However, positionally appropriate NC cells are still not capable of forming ENS without further changes, which seem to occur partly in response to retinoic acid after migration has commenced (Simkin et al., 2013). This also will have to be replicated for NCPCs, with the obvious problem that, if retinoic acid is the agent for achieving two different steps; keeping them separate will require very careful control of exposure times, durations, and levels.

Conclusions

The NC is one of the primary organ systems, and of unparalleled versatility since it generates a range of cell types, taking in not only typical neural ectoderm-like derivatives but also many cell types associated with mesoderm. Because of its later evolutionary appearance it can be viewed as somewhat of an "add-on" system to the three primary germ layers, and this "add-on" quality may account for the frequency of neurocristopathy in live births: defects involving the primary germ layers are typically early embryonic lethal whereas those of the NC often become a crucial problem only after birth. Study of the NC has made important contributions to the understanding of vertebrate developmental biology in general, and it is fitting that the NC should also be at the forefront of attempts to use applied developmental biology, in the form of NCPCs, for furthering the understanding and treatment of defects in human development.

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