

# Trunk neural crest cells: formation, migration and beyond

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**ABSTRACT** Neural crest cells (NCCs) are a multipotent, migratory cell population that generates an astonishingly diverse array of cell types during vertebrate development. The trunk neural crest has long been considered of particular significance. First, it has been held that the trunk neural crest has a morphogenetic role, acting to coordinate the development of the peripheral nervous system, secretory cells of the endocrine system and pigment cells of the skin. Second, the trunk neural crest additionally has skeletal potential. However, it has been demonstrated that a key role of the trunk neural crest streams is to organize the innervation of the intestine. Although trunk NCCs have a limited capacity for self-renewal, sometimes they become neural-crest-derived tumor cells and reveal the fact that that NCCs and tumor cells share the same molecular machinery. In this review we describe the routes taken by trunk NCCs and consider the signals and cues that pattern these trajectories. We also discuss recent advances in the characterization of the properties of trunk NCCs for various model organisms in order to highlight common themes. Finally, looking to the future, we discuss the need to translate the wealth of data from animal studies to the clinical area in order to develop treatments for neural crest-related human diseases.

**KEY WORDS:** *trunk neural crest, migration, neurogenesis, enteric nervous system, neurocristopathies*

## Introduction

One characteristic that separates vertebrates from other metazoans is the cell population called the neural crest (NC). This embryonic tissue is composed of cells arising at the border between the non-neural ectoderm and neural ectoderm. This population is also characterized by having multipotent and stem-cell-like properties. After being induced, neural crest cells (NCCs) undergo a process known as epithelial-to-mesenchymal transition (EMT) and migrate to distinct locations within the embryo to generate a wide diversity of cell derivatives (Duband *et al.*, 1995, Kerosuo and Bronner-Fraser, 2012).

With the exception of the most anterior part of the embryo, NCCs are generated along the entire length of the anterior-posterior (AP) axis. Derivative tissues arising from the NC originate from four different segments of the AP axis: cranial, cardiac, vagal and trunk NC. The cranial NC participates in the formation of the cartilage and bone of the head, nerve ganglia, smooth muscle, connective tissue and pigment cells. The cardiac NC participates in heart development, while the vagal NC contributes to the enteric ganglia of the gut. Finally, the trunk NC will give rise to neurons and glia of

the peripheral nervous system (PNS), secretory cells of the endocrine system and pigment cells of the skin (Etchevers *et al.*, 2001).

The understanding of the development of the NC has important medical implications since a variety of birth defects arise from abnormal processes in the induction, specification and migration of NCCs and affect NC derivatives postnatally. These abnormalities, called neurocristopathies, are one of the most common birth defects in live-born infants, including defects in the cardiac outflow tract, craniofacial malformations, familial dysautonomia, and a range of syndromes including Tracher-Collins, Waardenburg, DiGeorge and Charge syndromes (Etchevers *et al.*, 2006). It is thus of crucial importance to develop experimental approaches that will allow the suitable dissection of the cellular and genetic features of human NCCs.

*Abbreviations used in this paper:* AP, antero-posterior; BMP, bone morphogenetic protein; DRG, dorsal root ganglia; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ENS, enteric nervous system; Eph, ephrin receptor; FGF, fibroblast growth factor; NC, neural crest; NCCs, neural crest cells; NCSCs, neural crest stem cells; NT, neural tube; RA, retinoic acid; tNC, trunk neural crest; tNCCs, trunk neural crest cells; Sem, semaphorin; Shh, Sonic hedgehog

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The stem and pluripotent properties of NCCs, combined with an exceptional diversity of cell type and cell differentiation, have made NCCs an interesting target in regenerative medicine. This is sustained by the fact that NC stem cells (NCSCs) are retained postnatally in the skin, peripheral nerves, gut, heart and bone marrow (Dyachuk *et al.*, 2014).

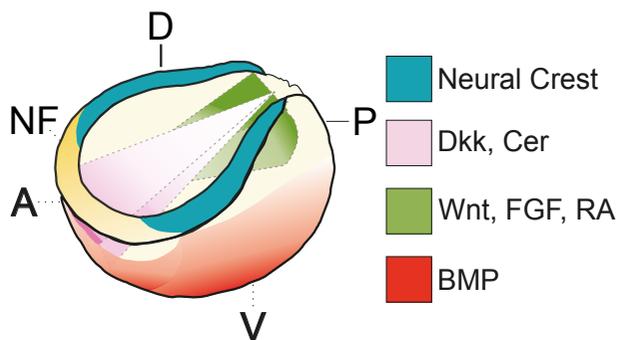
One of the most remarkable properties of the NC is its striking similitude to malignant cancer cells. Malignant tumor cells mimic many behavioral and morphological aspects of NC development. Loss of apicobasal polarity, changes in adhesion, degradation of the ECM, cell proliferation, migration and pluripotency are mechanisms shared by both cell types as they go to their final destinations and differentiate (Kerosuo and Bronner-Fraser, 2012, Maguire *et al.*, 2015).

During the past two decades numerous investigations have documented the induction, specification, delamination and migration of trunk NCCs (tNCCs). Despite this fact, more work is needed to provide straightforward evidence concerning the application of tNCCs in the regeneration of specific cell types, in the repair of damaged tissue and in the potential therapies against various types of cancer.

In this review we will focus on the formation of the trunk NC and its molecular events, and the delamination and migration pathways. We will also address the difference in molecular signature and behavior of the tNCCs between different species, and the differentiation into diverse derivatives. Finally, we will outline several applications of tNCCs in regenerative medicine and cancer therapy.

### Trunk neural crest - overview

As mentioned above, the NC gives rise to multiple organs and tissues of the adult organism. The two major population of the NC are the cranial NC and the trunk NC (tNC). Cranial NCCs migrate



**Fig. 1. Model of neural crest (NC) initial induction.** Dorsolateral view of a schematic model embryo. A ventro-medial gradient of BMPs (red) is established in the ectoderm, specifying the neural plate border as neural fold (NF, brown) at a threshold concentration. Posteriorizing signals (green) which correspond to the activities of Wnts, FGFs and RA transform the lateral and posterior part of the neural plate border into prospective NC cells (blue). These signals are also generated in a gradient-like manner, with higher levels in the posterior part of the ectoderm and lower levels in the anterior region. These lower anterior levels are also kept low by antiposteriorizing molecules such as Dickkopf and Cerberus (Wnts antagonists, Dkk and Cer), in pink, produced in the anterior region of the embryo. A, anterior; P, posterior; D, dorsal; V, ventral. Modified figure from Niehrs (2004), redrawn and upgraded.

into three streams: adjacent to the neural tube from the diencephalon to rhombomere 2(r2), adjacent to r4, and adjacent to the postotic region from r6 to r8. NCCs from r3 and r5 migrate rostrally and caudally to join adjacent streams (Kulesa *et al.*, 2010, Kuriyama and Mayor, 2008, Lumsden and Guthrie, 1991). The mechanism and processes involved in cranial NC induction, delamination and migration have been extensively reviewed elsewhere (Gong, 2014, Minoux and Rijli, 2010), and other references).

Trunk NCCs arise from the caudal region of the embryo and migrate along three different pathways: a dorsolateral pathway between the ectoderm and the somites, a ventro-lateral pathway in which cells migrate between and through the somites, and a ventro-medial pathway between the neural tube (NT) and the posterior sclerotome (Gammill and Roffers-Agarwal, 2010, Krispin *et al.*, 2010, McLennan *et al.*, 2015, Richardson *et al.*, 2016). Therefore, the pathways taken by the tNCCs and their final destinations are critically influenced by the surrounding structures, particularly somites and signaling molecules present in this tissue and those from surrounding ones.

The physical structure of the somite provides different kinds of attractive and inhibitory cues that modulate NCCs migration and differentiation. For example, somites are crucial as physical impediments, as a substrate for migration, and as a source of signals. Another case of influence on NC migration comes from the notochord. It repels migratory NCCs, causing them to gather at the dorsal aorta where they differentiate into sympathetic glia (Gammill and Roffers-Agarwal, 2010).

### Induction and multipotency of tNCCs: signals that guide their development

NC progenitors are induced at the neural plate border as a consequence of the integration of complex signaling events involving the BMP, Wnt, RA and FGF pathways (Fig. 1) (Aybar and Mayor, 2002). The signaling molecules and transcription factors that are required for NC specification, maintenance of the specification, migration and differentiation can be explained as a gene regulatory network that defines their individual and combinatorial roles in transcriptional regulation (Betancur *et al.*, 2010a). The transcription factors involved in such processes can be grouped according to their biological role into neural plate border specifiers, NC specifiers, NC/EMT migration factors, and factors that direct the differentiation of NCCs into specific derivative cell types (Taylor and LaBonne, 2007). More specifically, it is known that in the tNC the transcription factors *sox9*, *foxd3* and *slug* are sufficient to specify NCCs and induce EMT (Cheung *et al.*, 2005, Liu *et al.*, 2013, Nitzan *et al.*, 2013). It is important to note that perhaps due to current experimental limitations or to the intrinsic complexities of each model organism analyzed, it has not been possible at present to find inductive mechanisms unique to each particular region of the NC. However, it has been reported that some differences do exist in the development of cranial versus tNCC (i.e. at the onset of gene expression), although similar mechanisms are implicated in NC formation (Osorio *et al.*, 2009).

With respect to the NC potency and migration suggested, it has been proposed that there could be three possible scenarios relating to the properties of the pool of premigratory NCCs. In one case the NC may be assumed to be a homogeneous population of totipotent cells, each with a remarkable developmental poten-

tial. Following migration, the cells could differentiate according to instructive cues from their surroundings, either during migration or at their final destination. In the second case the potentiality of each cell could be characteristic of its NC population. In this scenario, the NC would be composed of a heterogeneous mixture of predetermined cells, each destined to become a particular cell type. A third possibility is that the NC represents a combination of multipotent and predetermined cells (Fig. 2, green and yellow cells) (McKinney *et al.*, 2013). Over the past 25 years since the establishment of these three putative models, numerous investigations have been performed to assess this basic question about the potentiality of NCCs. One group of investigations concluded that NCCs were multipotent *in vivo* (McKinney *et al.*, 2013) and *in vitro* (Dupin and Sommer, 2012). However, other publications reported that the NC was formed by a heterogeneous population of restricted progenitor cells (Krispin *et al.*, 2010). Recently, it has been shown that the vast majority of NCCs, at least in the case of the murine embryo, are multipotent prior to and during migration (Baggiolini *et al.*, 2015, Bronner, 2015).

### EMT and delamination of trunk NCCs

After NCCs have been induced and have become pluripotent to differentiate into various types of derivatives they undergo EMT (epithelial-to-mesenchymal transition). In this transition they delaminate from the NT or from the neuroepithelium and convert from epithelium-like cells to mesenchyme-like cells, which are capable of migrating along the entire embryo (Kerosuo and Bronner-Fraser,

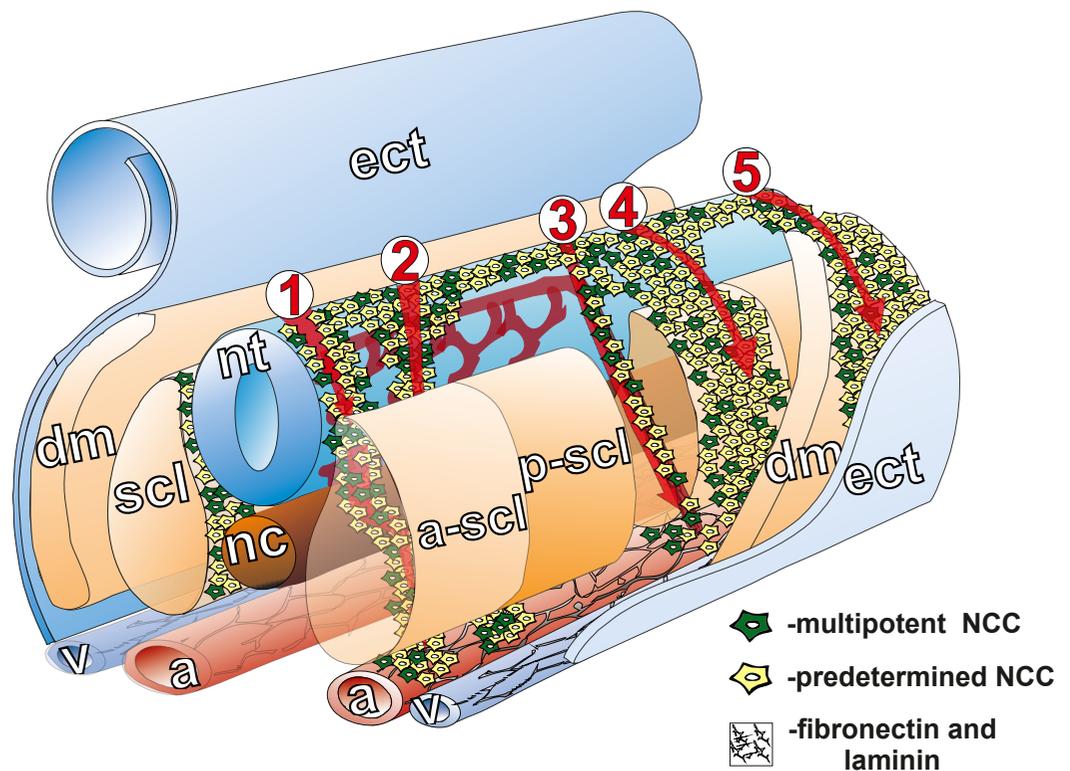
2012, Nieto, 2009).

The EMT is thought to be promoted by the activation of BMP and a simultaneous upregulation of the Wnt signaling pathway (Ahlstrom and Erickson, 2009). Before and after delaminating from the NT and during their migration there is an extensive and complex network of up- and down-regulation of cell adhesion molecules such as cadherins, connexins, occludins and integrins (Taneyhill, 2008). In chick embryos, neural fold cells express cadherin-6B (Nakagawa and Takeichi, 1995). An important factor in the start of migration of NC cells is the downregulation of N-cadherin. This requires the coordinated action of BMP signaling and the proteolytic action of the transmembrane protein ADAM10, which cleaves the extracellular domain of N-cadherin (Osorio *et al.*, 2009, Shoval *et al.*, 2007). In the case of the *Xenopus* embryo, cadherin-11 has an important function, and is expressed in the anterior neural folds and in migrating NCCs (Vallin *et al.*, 1998). In the zebrafish embryo, the Sonic Hedgehog (Shh) receptor and cell adhesion regulator, Cdon, has been shown to play a key role in the directed migration of tNCCs, by regulating N-cadherin localization (Powell *et al.*, 2015). Thus the process of EMT in tNCC is more complex than previously thought, involving several signal molecules and secreted factors, with the addition of cell adhesion molecules, all of which are involved in a complex regulatory network.

### Regional differences between cranial and tNCCs

The molecular mechanisms governing the induction and specification of NCCs vary in accordance to the axial level. An early

**Fig. 2. Trunk neural crest cell migration.** In the trunk of the embryo, neural crest cells (NCCs) migrate in the following ways: **(1)** a minority of NCCs move ventrally through the dorsal anterior sclerotome (a permissive but not preferred substrate) by traveling laterally on myotomal basal lamina to form: a) dorsal root ganglia, b) sympathetic ganglia and c) adrenal medulla; **(2)** between the neural tube and somites to give rise to the sympathetic and sensory ganglia; **(3)** ventrally between the somites along intersomitic vessels. **(4)** After ventral migration is complete, NCCs leave the staging area and enter the dorso-lateral path between the ectoderm and the anterior sclerotome. **(5)** They also migrate dorso-laterally under the ectoderm and over the dermomyotome to give rise to the pigment cells of the skin (melanoblasts or feathers). Differences in



"species-specific" cell surface molecules dictate the various cell migration behaviors. A feature-rich model of the trunk NC migratory streams of this figure is shown in an interactive 3D-pdf in Supplementary Fig. 1. Adapted from Dr. Brian E. Staveley, Memorial University of Newfoundland, redrawn and upgraded. a, dorsal aorta; a-scl, anterior sclerotome; dm, dermomyotome; ect, ectoderm; nc, notochord; nt, neural tube; p-scl, posterior sclerotome; v, vein.

difference between cranial and trunk NCCs is the expression of a distinctive gene marker. In the case of cranial NCCs, the marker that differentiates them from the trunk NC is the *ets-1* gene (Theveneau *et al.*, 2007), whereas a bona fide tNC marker has not yet been found. The kinesin *eg5*, another early gene with expression in the pre-delamination trunk stage, and *ednrb2-c* during migration, could be good candidates as well (Fernandez *et al.*, 2014, Kawasaki-Nishihara *et al.*, 2011, Square *et al.*, 2016).

Another regional difference is the distinct regulation of NC specifier genes. For example, the NC specifier gene, namely *foxd3*, has an enhancer region that receives different inputs with respect to the axial level. The cranial NC enhancer requires an *Ets-1* input, whereas the trunk enhancer requires *Zic1*. Thus there are differential inputs mediating *foxd3* expression in cranial NC versus trunk NC (Simoes-Costa *et al.*, 2012). Another case of differential regulation of protein expression is that of *sox10*. In cranial NCCs the *sox10* gene expression is driven by the *sox10E2* enhancer, while in trunk NC the enhancer is *sox10E1* (Betancur *et al.*, 2010b).

Going into the delamination phase of NC development, there are also differences in the regulation of this process between cranial and trunk NCCs. In mouse and chicken cranial NCCs, the transcription factor *Sip1* is essential for the delamination of the cells (Rogers *et al.*, 2013). On the other hand, in tNCCs, besides the participation of the *Sip1* protein, the transcription factor  $\delta$ EF1 is also required for NCC delamination (Yasumi *et al.*, 2016).

Patterning of NC cells into distinct streams and their precise targeting to specific tissues are controlled by a plethora of negative and positive guidance cues than can work by two different models, follow-the-leader or cell-cell-contact. In the follow-the-leader model, leader and follower identities are acquired before the start of migration and remain fixed after that (Kelsh *et al.*, 2009, Kuo and Erickson, 2010, McLennan *et al.*, 2012, Wynn *et al.*, 2013). In the cell-cell contact, the cell needs a co-attraction and contact inhibition of locomotion, and identities could be acquired after the onset of migration (Broders-Bondon *et al.*, 2016, Szabo and Mayor, 2016). In chick and zebrafish it has been shown that whereas cranial NCCs do not require leader cells for their migration and all the cells present similar migratory capacities, tNCCs require leader cells to direct movement and cell-cell contact. It has also been shown that the leader and follower identities are acquired before the initiation of the migration and remain fixed after that (Richardson *et al.*, 2016). This could also be an intrinsic difference between cranial and tNC that lies in the migration mechanism.

## The tNCC migration routes

Right after delamination, the NCCs of the trunk region of the embryo start to migrate along three different routes (Fig. 2). The first NCCs that delaminate and migrate start doing so in a ventral way around the epithelial somites, mostly along blood vessels in the intersomitic space. They also migrate between the somites and the NT. After the somites have matured and dissociated into dermomyotome and sclerotome, a second set of NCCs begins to invade the sclerotome and migrate ventrolaterally through different parts of the somite, depending upon the particular species of organism (Serbedzija *et al.*, 1990). A third wave of NCCs migrate along a dorsolateral pathway between the epidermal ectoderm and the dermomyotome (Erickson *et al.*, 1992). The possible mechanisms guiding or controlling the migration of each cellular group are

discussed below. An increasing amount of evidence suggests that the transition from ventral to dorsal pathway preference is NCCs autonomous and not due to changes in the environment (Kuo and Erickson, 2011, Simkin *et al.*, 2013).

### Species-specific differences in tNC migration pathways

Developmental biology has taught us that the development of very different species is strikingly similar. Nonetheless, regarding the timing and trajectories of migration of trunk NCCs, there are a few differences between the four major model organisms, i.e. *Xenopus laevis*, *Mus musculus*, *Gallus gallus* and *Danio rerio*.

In mouse and chick embryos, tNCCs start migrating ventrally in a non-segmented way between the NT and the somites, favoring the intersomitic space. After the maturation of the somite, NC cells pass through the anterior half of the sclerotome and along the basement membrane of the dermomyotome (Hall, 2008). As mentioned above, the third pathway undertaken by tNCCs is the dorsolateral one. In mouse embryos both routes are invaded simultaneously, while in chick this third wave invades the dorsolateral pathway with a 48-hour delay (Kuo and Erickson, 2010).

In zebrafish, trunk NCCs start migrating along a medial pathway in-between the somites and the NT. These NCCs align to and are affected by slow muscle cells in the middle part of the somite (Honjo and Eisen, 2005). After 4hs of the start of migration through the medial pathway, NCCs commence migrating on the dorsolateral pathway between the epidermis and the somite (Raible *et al.*, 1992). In contrast to other species, zebrafish melanocytes precursors are composed of NCCs arriving from the dorsal pathway as well as from the medial pathway (Kelsh *et al.*, 2009). In *Xenopus*, as opposed to chick and mouse, trunk NCCs pass through the caudal region of the somite during their ventromedial migration. In addition, few NCCs migrate along the lateral pathway under the ectoderm (Collazo *et al.*, 1993).

The migration of the NC has been studied not only in these major vertebrate organisms, but also in more basal vertebrates such as the lamprey and hagfish. In lamprey we can distinguish three major NC populations: the trigeminal crest (TC), the hyoid crest (HC) and the branchial crest (BC). Although the head-trunk differentiation in the lamprey is not yet completed and there is no clear differentiation in trunk and cranial NC in this species, BC cells can be considered similar to tNC since the branchial region is expanded caudally. In regard to their migration, BC cells are found superficially to the arch mesoderm, indicating that these cells migrate along the dorsolateral pathway (Mukendi *et al.*, 2016). All the results mentioned above stress the fact that migration of NCCs varies between different species and among various phyla.

### Light traffic control during tNCCs migration

tNCCs have the potential to differentiate into a multitude of cell derivatives including neurons and glia of the sensory and autonomic nervous system. Such cell derivatives also include the neurons and glia of the dorsal root (DRG) and sympathetic ganglia, Schwann cells that line the ventral roots, adrenal chromaffin cells, pigment cells (melanocytes), cells from the adrenal medulla thyroid glands and the neurons of the enteric nervous system (ENS) (Le Douarin and Kalcheim, 1999). Besides the genetically encoded potentiality of NCCs to become a specific derivative, the final differentiation of these cells depends to a large extent on the environment through which they migrate and the cues that they receive. To reach their

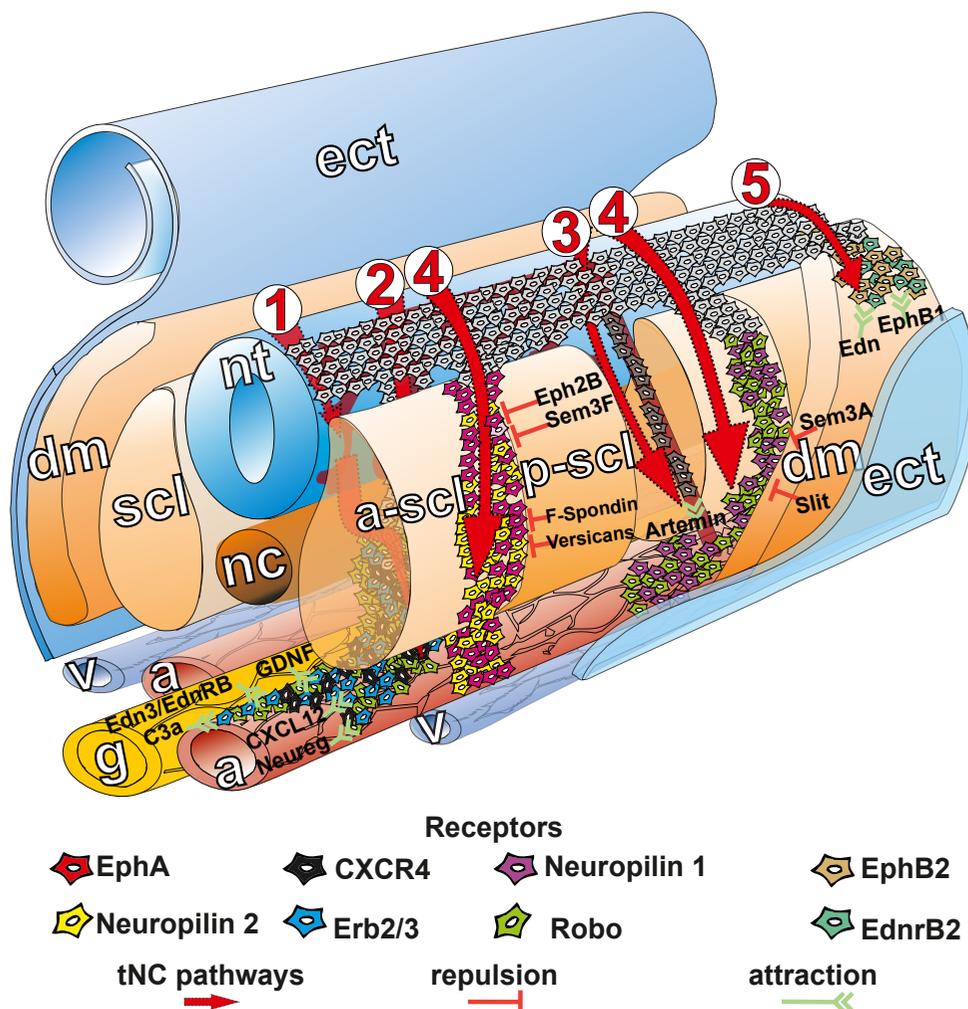
final destination NCCs emit and receive signals that are crucial to determine the path they go through. In the paragraphs below we explain in detail the signals and factors that modulate tNCCs migration.

In the three migratory pathways described before, the proteins laminin and fibronectin are permissive substrates for NC migration. In chick, fish and frog, one of the first cues that direct recently delaminated NCCs to go ventrally to the sclerotome is the chemokine Cxcl12, which attracts Cxcr4 expressing NCCs to the dorsal aorta (Fig. 3) (Gordon *et al.*, 2011).

In mouse and chick, migrating tNCCs are restricted to the anterior sclerotome because they express the receptors Ephrin-A/B (Eph-A/B) while the posterior sclerotome expresses Ephrin-B ligands (Baker and Antin, 2003). Another restriction to the migration of NCCs in the somite is the expression pattern of semaphorins

(Sem). Dermomyotome cells express Sem3A, which prevents Neuropilin-1 positive NCCs from entering it (Fig. 3), while the caudal sclerotome expresses Sem3F, which restricts Neuropilin-2 expressing cells to the anterior part of the somite (Fig. 3) (Schwarz *et al.*, 2009). The semaphorin guidance cue is potentiated by proteoglycans (F-spondin and Versicans) that are expressed in the caudal sclerotome (Casini *et al.*, 2008). The role of Eph/Ephrin and semaphorin pathways in *Xenopus* and zebrafish tNC migration has not been assessed yet, thus providing an interesting field for future research.

The signaling Slit/Robo is essential in preventing tNCCs from entering the dorsolateral pathway and restricting them to the ventral one. The dermomyotome secretes the Slit protein, which prevents Robo-expressing NC from entering the dorsolateral pathway (Fig. 3) (Jia *et al.*, 2005). Another recently found role of the Slit/Robo



**Fig. 3. Environmental cues guiding trunk neural crest migration.** (1) After delaminating from the neural trunk, trunk neural crest cells (tNCCs) migrate along various streams. (2) Ventromedial pathway: cells expressing Cxcr4 are attracted by the ligand Cxcl12. Neuregulin attracts Enrb2/3 expressing cells. C3a and GDNF from the gut mesenchyme attracts cells expressing receptors C3ar and GFR $\alpha$ 1, respectively. Also, endothelin3 is an attractant of cells expressing EdnrB. Finally, the Slit factor excludes Robo expressing cells from entering the gut. (3) Ventrally between the somites along intersomitic vessels, by Artemin attraction. (4a) Ventrolateral pathway across the somites: Eph2B and Sem3F signals repel EphA and Neuropilin1 expressing cells, respectively. (4b) Slit and Sem3A generate a repulsive signal from the dermomyotome to cells expressing Robo and Neuropilin1, respectively. This prevents cell migration from the sclerotome to the dermomyotome. (5) Dorsolateral pathway: NCCs along this pathway are attracted to migrate between the dermomyotome and the ectoderm by EphB1 and Endothelin proteins which attracts cells expressing EphB2 and EdnrB2 receptors, respectively. a, dorsal aorta; a-scl, anterior sclerotome; dm, dermomyotome; ect, ectoderm; g, gut; nc, notochord; nt, neural tube; p-scl, posterior sclerotome; v, vein.

signaling is the restriction to the entrance of tNCCs into the developing gut. Slit expressing cells that are near the entrance to the gut generate a negative cue to migrating tNCCs that express the receptor Robo (Fig. 3) (Zuhdi *et al.*, 2015).

The diffusible protein Draxin, which is better known for its role as an axon guidance molecule, has an inhibiting role as a signaling cue during chick and mouse NC migration. In the first wave of migration, Draxin expressed in the dorsal lip of the dermomyotome restricts early migrating NC cells from entering the dorsolateral pathway. This inhibitory function lies in the ability of Draxin to alter cell polarization by cytoskeleton remodeling (Zhang *et al.*, 2016)

As expressed above, cadherins are important in the process of EMT and during the migration of trunk NCCs. In regard to the migration of NCCs through the anterior part of the sclerotome, T-cadherins are essential to reduce the adhesion of NCCs to the caudal sclerotome (Ranscht and Bronner-Fraser, 1991).

The cells that migrate along the ventrolateral pathway reaching the dorsal aorta are guided by four distinct signaling cues. Neuregulin from the mesenchyme around the dorsal aorta attracts Erb2/3 expressing NCCs to the ventral side to pass the sclerotome (Fig. 3) (Fantauzzo and Soriano, 2015). The second signal is the above mentioned Cxcl12 chemokine which attracts NCCs expressing Cxcr4 to the dorsal aorta. In addition to these signals, the neurotrophic factor Artemin, secreted by the peripheral blood vessel cells, attracts NC sympathetic precursors and allows their proliferation (Fig. 3, stream 3) (Honma *et al.*, 2002). The fourth signal has a repulsive mechanism; Sem3A expressed in limbs, dermomyotome and notochord restricts Neuropilin-1 expressing NCCs near the dorsal aorta (Kawasaki *et al.*, 2002).

Zebrafish tNCCs require Notch/Delta signaling. The trunk region contains an equivalence domain of cells that can become Rohon-Beard cells but, when exposed to Notch/Delta signaling, become tNC instead (McGraw *et al.*, 2012). During their migration along the ventrolateral pathway, NC cells not only receive signals from the environment but also induce certain changes in neighboring tissues. For instance, tNCCs migrating underneath the dermomyotome trigger myogenesis by transiently activating Notch/Delta signaling in muscle precursors (Rios *et al.*, 2011).

Apart from the vast evidence and information about the ventromedial pathway in mouse and chick, little is known about the particular molecular cues that guide *Xenopus* tNCCs to this ventromedial pathway. Further work is needed to understand the underlying mechanisms that govern the migration of *Xenopus* tNCCs.

Migration of tNCCs along the dorsolateral pathway is controlled by several signaling molecules. Cells that are determined to the melanocytic lineage upregulate Eph-B2 and are attracted to the dorsal ectoderm by Ephrin-B1 (Fig. 3) (Santiago and Erickson, 2002). The dorsolateral route contains Endothelins, which prevents EdnrB-expressing neuronal and glial precursors from entering the dorsolateral pathway and at the same time stimulate EdnrB2-expressing melanocytic precursors to the migration onto this pathway (Harris *et al.*, 2008). In the case of the mouse, the kit ligand expressed in the dermomyotome attracts melanoblasts that express the kit receptor (Wehrle-Haller *et al.*, 2001). Also in mouse, the chemokine Sdf1 guides the melanocytes from the skin to the hair follicle (Belmadani *et al.*, 2009). Not only particular guidance molecules direct the migration of tNCCs but entire tissues do so as well. It has been shown that the emerging dermis supplies a yet uncharacterized diffusible attractant that stimulates melanoblasts

to enter the dorsal pathway (Tosney, 2004)

In contrast to the vast quantity of cells that enter the dorsolateral pathway in mouse and chick, in *Xenopus* only a small portion of cells migrate through this pathway; they use the medial pathway instead and then move laterally, below the somites, to reach the epidermis (Collazo *et al.*, 1993). In the case of zebrafish, NCCs use both the ventromedial and the dorsolateral pathway to form melanocyte derivatives (Kelsh *et al.*, 2009).

The directionality of the migration of tNCCs cannot emerge only from a balance of positive and negative cues that promotes the invasion of specific areas of the embryo. The interplay between inhibiting signals and attracting cues cannot fully explain the overall directionality in the migration of tNCCs. Therefore, it is more likely that this directed migration is controlled on a local scale in between a certain migratory stream.

For this reason extensive research has been done to understand the molecular mechanisms that delineate the movements of the NCCs during their migration. In *Xenopus laevis* it has been shown that the collective migration of NCCs involve a combination of mechanisms, including contact inhibition of locomotion (CIL), repolarization, chemoattraction and coattraction, at least in the case of cranial NCCs. More specifically, it is known that NCCs are attracted to each other via ligand receptor interaction mediated by Complement3a (C3a) and the C3a receptor (Broders-Bondon *et al.*, 2016). These cells are also attracted by a secreted factor called SDF, which plays a key role in patterning NC cells into forward moving streams. This factor is expressed in the ectoderm facing the early migrating NCCs (Theveneau *et al.*, 2010).

Recently, molecular level-studies and computational simulations were performed to analyze the intrinsic molecular signature within an NCC migratory stream. The results showed that there are regional differences in gene expression within a particular NC stream and that there is a consistent and stable molecular signature unique to the cells in the distal portion of the migratory stream (McLennan *et al.*, 2015).

### Signaling pathways involved in the specification and differentiation of tNCCs

After migrating along the three pathways mentioned before, the NCCs settle in discrete sites and differentiate into diverse cell types. This process of cell differentiation requires the deployment of differentiation gene batteries, which are sets of genes that confer its terminal identity to each cell (Erwin and Davidson, 2009). Gene batteries are controlled by different drivers, which are regulators that control the transition between the progenitor and differentiated states (Peter and Davidson, 2015). The SoxE transcription factors are important in activating differentiation gene batteries during NC differentiation. More particularly, Sox10 is involved in the differentiation of NCCs into neurons, glia and melanocytes (Kelsh, 2006), while Sox9 participates in the stimulation towards a chondrocyte fate (Mori-Akiyama *et al.*, 2003). The misexpression of *SOX10* during NC development in human causes Waardenburg-Hirschsprung disease (Pingault *et al.*, 1998).

Since migratory NCCs are multipotent, environmental cues play a key role in determining the fate of their progeny. These cues are integrated by the signaling pathways that are active in the cells along the migratory route. In this section we describe the different molecules and signaling pathways involved in the differentiation

of tNCCs into their particular derivatives.

Kit signaling is essential in the differentiation of NC into melanocytes (Parichy *et al.*, 1999). Furthermore, *MitfA* and *Sox10* are crucial to promote melanoblast specification. However, subsequently, *MitfA*-dependent activation of the histone deacetylase complex *Hdac1* represses *sox10* expression in melanoblasts, promoting both differentiation and fate commitment (Greenhill *et al.*, 2011).

In addition, BMP has been shown to participate in the differentiation of cells into the autonomic nervous system lineage (Saito *et al.*, 2012), whereas the Wnt pathway has been associated with the formation of sensory neurons and melanocytes (Lee *et al.*, 2004). Another example of a signaling pathway responsible for the differentiation of NCCs is the Endothelin-receptor B signaling, which is required for enteric nerve cell formation and melanocyte development (Dupin and Le Douarin, 2003). It has been suggested that another receptor of the same family, the Endothelin receptor A, plays a key role in the induction of *Xenopus laevis* melanocytes (Bonano *et al.*, 2008). Also in *Xenopus*, a study has shown that the *Edn3-Ednrb2* signal is required for the migration of melanoblasts and that the receptor is expressed in melanoblast while the ligand *Edn3* is expressed at the destination sites of their migration (Kawasaki-Nishihara *et al.*, 2011).

Another signaling molecule important for the differentiation of tNC into a specific cell type is Retinoic Acid, which influences cells to develop as sympathetic neurons (Ito and Morita, 1995). Moreover, *ErbB/Neuregulin* signaling is involved in tNC migration and differentiation into DRG sensory neurons and sympathetic ganglia (Britsch *et al.*, 1998).

Until now we have only discussed the signaling molecules and survival factors involved in the formation of trunk NC derivatives. However, these factors inevitably cooperate to activate a transcriptional cascade that in turn establishes the functional and morphological changes that are coincident with differentiation. For example, the transcription factor *Achaete-scute homolog 1 (Ascl)* is the first indicator of neuronal differentiation in the autonomic lineage (Groves *et al.*, 1995). It is also a master proneural gene for the specification of the sympathetic lineage (Sommer *et al.*, 1995). Another important regulator for the development of the autonomic nervous system is the transcription factor *Phox2b*, which is hierarchically equivalent to *Ascl1* (Pattyn *et al.*, 1999). In regard to another aspect of PNS development, *Hand1* and *Hand2* transcription factors are important in the acquisition of noradrenergic characteristics among the peripheral system neurons (Morikawa *et al.*, 2005). In the development of the sensory nervous system, signaling by the *Trk* receptors is of vital importance during the neurogenesis of the DRG (Fantauzzo and Soriano, 2015).

The enteric neural system (ENS) is formed from enteric NCCs (ENCCs) migrating from the vagal and sacral region, which colonize the entire length of the gut wall forming all the neurons of the enteric system. The ENS is sometimes called the “second brain” because of the diversity of neuronal cell types and complex, integrated circuits that permit the ENS to autonomously regulate many processes in the bowel (Panza *et al.*, 2012). The signaling through the receptor tyrosine kinase gene, *RET*, is essential for ENS development (Panza *et al.*, 2012). The receptors involved in this signaling are the glial cell line-derived neurotrophic factor (*GDNF*) and its glycosylphosphatidylinositol (GPI)-anchored coreceptor, *GFR $\alpha$ 1*. Expression of *Ret* and *Gfra1* is activated in ENCCs upon entry into the foregut, whereas *Gdnf* is detected in

gut mesenchyme prior to ENCCs arrival in the foregut (Natarajan *et al.*, 2002). The Endothelin B signaling pathway through the Endothelin 3 ligand and the *EdnrB* receptor has also a very important role in the development of the ENS, for the mutation in the genes of these proteins delay colonization in the gut (Lee *et al.*, 2003)

Even though knowledge about the differentiation mechanisms in the NC is continuously growing, further work is required to address all the steps necessary to transform a multipotent migratory NC cell into a differentiated progenitor.

### NCCs and tumor cells: two distinct but similar cell populations

Cancer cells use certain molecular and genetic tools similar to developing NCCs. Malignant cells mimic many of the behavioral, genetic, molecular and morphological aspects of NC development (Karunasena *et al.*, 2015, Kerosuo and Bronner-Fraser, 2012, Maguire *et al.*, 2015, Nguyen *et al.*, 2009).

In cancer cells, *TGF $\beta$*  and *Wnt* are upregulated. There is also an increase in the signaling by tyrosine kinase receptors (Thiery and Sleeman, 2006). These upregulated signaling pathways are reminiscent of the BMP, *Wnt* and *FGF* signals implicated in NC induction and the BMP/*Wnt* cascade that triggers the EMT. In addition, the *Hippo/Yap* signaling, which has known roles in the migration of cancer cells, has recently been described as promoting an early NC phenotype (multipotency) and migration which occur during human NC development (Hindley *et al.*, 2016, Uribe and Bronner, 2015). This emphasizes the relationship between NC development and cancer progression.

After delaminating from their original place, NCCs and tumor cells undergo solitary and collective cell migration. Both of these cell types use existing tissues and cellular structures to migrate and colonize new territory; i.e. tumor cells migrate along nerves and use blood vessels to disseminate (Nguyen *et al.*, 2009), while NCCs use the basement membrane of the ectoderm and dermomyotome and also migrate along nerves (Hall, 2008).

Besides the fact that cancer cells are similar to NCCs, there is a group of cancers that are direct descendants of NCCs, some of which are described below. One example is the group of tumors from the sympathetic ganglia lineage known as neuroblastomas and ganglioneuromas, which evidence a high expression of the Hedgehog signaling pathway (Gershon *et al.*, 2009) or *TrkB/BDNF* signaling (Gryz and Meakin, 2003). Paragangliomas and pheochromocytomas are highly vascularized tumors arising from NC-derived tissues in the paraxial autonomic ganglia or in the chromaffin cells of the adrenal medulla, respectively (Dahan *et al.*, 2005). One of the best known cancers derived from the NC are malignant melanomas, which arise from the melanocytic lineage (Davies *et al.*, 2002). Neurofibromatosis consists of tumors that are derived from Schwann cells and are genetically inherited (Evans *et al.*, 2010).

The use of embryonic models and particularly NCCs to study tumor cell phenotype determination affords an innovative approach for investigating the boundaries of tumor cell plasticity and the potential for reprogramming deadly cell types by targeting the convergence of embryonic and tumorigenic signaling pathways. A common problem in the testing of clinical drugs is that it is a long and expensive process limited only to cultured cancer cells or artificial tumor models. However, embryos from model organisms

can serve as a unique *in vivo* system for screening the effects of specific factors or for high-throughput screening of small molecule libraries (Maguire *et al.*, 2015).

Cancer treatments at present include surgery, chemotherapy, and radiation therapy, all of which are done at the cost of a lower quality of life. It is of extreme importance to design therapies that reduce collateral damage. Targeting signaling factors and processes have proven very effective; small molecule inhibitors of *Snail* (Azmi *et al.*, 2013) or *c-Myc* (Muller *et al.*, 2014) are being tested for their therapeutic potential. There is also a wide field of research in the targeting of *hedgehog* signaling molecules for cancer treatment (Abidi, 2014). Moreover, recent efforts are aimed at identifying inhibitors of EMT in order to inhibit the invasive capacity of cancer cells (Chua *et al.*, 2012).

With a growing capacity to target particular signaling pathways and molecules we will be able to tackle several pathologies imposed by this disease. Given the above results and evidence, it is important to say that the understanding of NC development can have potentially important implications for the treatment of several types of cancers.

### NCCs as stem cells: characteristics and potential applications

Due to the amazingly diversified array of cell types that they generate, tNCCs represent an attractive model in the stem cell field. The tNCC population has multipotent and bipotent cells endowed with self-renewal, a *bona fide* property of stem cells. These cells have the potency to yield glial cells, autonomic neurons and myofibroblast/smooth muscle cells as well as melanocytes (Bittencourt *et al.*, 2013). They are called NC stem cells (NCSCs) (Achilleos and Trainor, 2012). These cells were first isolated from mammalian multipotent NCCs taking advantage of the expression of the p75 marker (Stemple and Anderson, 1992).

In rat and mouse, BMP and Wnt signaling are involved in the maintenance of the undifferentiated state of NCCs (Kleber *et al.*, 2005). The transcription factor Sox10, which is expressed in premigratory and migratory NCCs, functions in the maintenance of multipotentiality of these cells both *in vitro* and *in vivo* (Kim *et al.*, 2003). Other regulators that control the pluripotent capacity of NCCs are the chromatin remodelers CHD7 and SWI/SNF-B, which act in conjunction with BMP and Wnt signaling (Fujita *et al.*, 2014). NCSCs are found not only in premigratory NCCs, but also in NC derivatives such as the peripheral nerve, the DRG and the gut, demonstrating that the migratory stream of some NC populations are a mixture of specified and multipotent cells (Morrison *et al.*, 1999).

It has recently been shown in *Xenopus laevis* that, through the process of gastrulation, NCCs retain pluripotency factors inherited from stem cells of the blastula stage. These are the Snail1 and Sox5 factors, which are responsible for keeping the developmental capacities characteristic of NCCs (Buitrago-Delgado *et al.*, 2015).

Major advances in this area have allowed scientists to unravel the specific molecules responsible for favoring a specific NC lineage, taking as a source NCSCs. Such is the case for TGF $\beta$ , BMP4, Neuregulin-1, Retinoic Acid, Edn3 and Delta-1, all of which favor the development of specific NC sublineages in an instructive or permissive manner (Le Douarin and Dupin, 2016).

A major breakthrough in NC biology has occurred recently, with

scientists being able to differentiate human pluripotent stem cells (hPSC) into subtype specific NC cells, namely trunk NCCs (in the presence of Retinoic Acid) and cranial NCCs (in the absence of Retinoic Acid) (Huang *et al.*, 2016). This discovery offers a renewable human cell model to study NC biology and to develop therapies against diseases arising from abnormal NC formation.

Therefore, the study of NC derived stem cells is of crucial importance because of their capacity to give rise to fully differentiated and functional cell types, which contributes to the development of regenerative medicine.

### Concluding remarks

Fifteen years ago, many people argued that tNC played could play a pivotal role in the development and differentiation of sensory neurons and glia of the peripheral nervous system as well as in the differentiation of adenomedullary cells and pigment cells of the skin. Nowadays, advances in visualization and embryonic manipulation technologies have allowed us to understand the mechanisms that underlie the formation of the tNC in a much deeper way, thus making possible a more specific assessment of the key role of the tNC in the development of the peripheral and enteric nervous system. However, the ability of tNC to generate neural derivatives is not unique to this axial level, but instead is shared by all crest cells. This ability, as well as with all other crest potentials, is modulated by environmental cues.

Importantly, elucidating how and when different cues are expressed and discovering the interacting proteins during the formation, migration and differentiation of the trunk NC will contribute to our overall knowledge of the molecular basis of tNC formation. Dissecting these and many other key issues will yield important insights into the molecular control of NCCs development and help to define the pathogenesis of various developmental abnormalities (human disorders, diseases and syndromes) that occur upon aberrant tNC development. Also, identification of the signals required for trunk NCCs formation will facilitate ways to generate hESCs, which will be beneficial for the application of NCCs to regenerative medicine.

Thus, today our views of the significance of the tNC have changed. Continued attention to these areas should unveil numerous key players in the regulation of NCCs formation and development over the next few years. Future research will focus on understanding how these diverse signals work together to pattern trunk NCCs. Further investigation into these questions will clarify the mechanisms that dictate patterned tNC migration and segmental peripheral nervous development.

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