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Neural Crest and Cancer: Divergent Travelers on Similar Paths

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Abstract

Neural crest cells are multipotent progenitors that dynamically interpret diverse microenvironments to migrate significant distances as a loosely associated collective and contribute to many tissues in the developing vertebrate embryo. Uncovering details of neural crest migration has helped to inform a general understanding of collective cell migration, including that which occurs during cancer metastasis. Here, we discuss several commonalities and differences of neural crest and cancer cell migration and behavior. First, we focus on some of the molecular pathways required for the initial specification and potency of neural crest cells and the roles of many of these pathways in cancer progression. We also describe epithelial-to-mesenchymal transition, which plays a critical role in initiating both neural crest migration and cancer metastasis. Finally, we evaluate studies that demonstrate myriad forms of cell-cell and cell-environment communication during neural crest and cancer collective migration to highlight the remarkable similarities in their molecular and cell biological regulation.

Keywords

neural crest stem cells; EMT; collective migration; neuroblastoma; cancer; metastasis

1. Introduction

The neural crest (NC) is a highly migratory, multipotent population of cells found in developing vertebrate embryos that contributes to diverse cell tissues and types such as cartilage, bone, smooth muscle, melanocytes, and neurons and glial cells of the peripheral nervous system (Bronner and LeDouarin, 2012; Le Douarin and Teillet, 1973; Le Douarin et al., 2004). The NC can be divided into four main subtypes, the two largest of which are: the cranial NC, which migrates from the dorsal aspect of the midbrain and hindbrain, and the trunk NC, which migrates from the dorsal aspect of the developing spinal cord. In both regions, migrating streams appear segmentally organized, but in the cranial region, this segmentation is considered intrinsic to the neural tube, while in the trunk the segmental organization is thought to be imparted extrinsically by mesoderm-derived somites (Bronner-Fraser and Stern, 1991; Lumsden et al., 1991). While the NC population as a whole is highly

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plastic, work in multiple species suggests that the trunk NC has greater uniformity among its migratory streams and demonstrates fate restriction earlier than the cranial NC does (Kulesa et al., 2010; Kuo and Erickson, 2010).

NC development first involves signaling via a host of molecules including Wnts, BMPs, FGFs, and retinoic acid in a highly coordinated manner (Bronner and LeDouarin, 2012; Simoes-Costa and Bronner, 2015; Villanueva et al., 2002). After the NC forms along the border of the neural plate, neural crest cells (NCCs) delaminate and undergo an epithelial-to-mesenchymal transition (EMT), whereby polarized epithelial cells lose adhesion and adopt mesenchymal morphologies in preparation to migrate (reviewed in detail in Chen et al., 2017; Kalluri and Weinberg, 2009). Many of the processes underlying the formation and migration of the NC can also play critical roles in cancer progression.

Once NCCs initiate directed migration, they must traverse multiple heterogeneous microenvironments to reach their varied destinations, a process requiring communication among migratory NCCs as well as with the surrounding microenvironment (Kulesa and Fraser, 1998; Szabó and Mayor, 2016; Theveneau and Mayor, 2012a). Several models have been put forward to explain the underlying mechanisms for NC migratory guidance, including leader-follower models (Wynn et al., 2013, 2012), chase-and-run (Theveneau et al., 2013), and contact inhibition of locomotion (CIL) (Carmona-Fontaine et al., 2008). While it is established that NCCs use guidance cues and the local extracellular matrix (ECM) to collectively migrate (Banerjee et al., 2013; McLennan et al., 2010), we lack a thorough mechanistic understanding of NCC collective migration and how it varies between subpopulations of NCCs destined for different locations and fates. Meanwhile, a great deal of work on metastatic cancer cells has revealed striking parallels to NCC EMT and migration. How cancer cells influence the microenvironments and conditions that make EMT and migration possible is an active area of inquiry and can lend insight into both the mechanisms of tumorigenesis and the plasticity and potency of NCCs.

Here, we will discuss how NCCs are specified, how they undergo delamination/EMT, and how they migrate, with a focus on the extraordinary parallels between these developmental processes and those involved in driving cancer metastasis.

2. Potency, Induction, and Specification of Neural Crest and Cancer Stem Cells

NCCs must first undergo induction, delamination, and EMT prior to migration. In this section, we describe factors involved in NCC potency, induction, and specification and outline their known or hypothesized roles in cancer formation and progression.

2.1 Potency

NCCs possess a remarkable and unusual ability to migrate long distances and contribute to a variety of different cell types. How and when NC stem cell potency is initially determined and subsequently refined remains a subject of great debate. This uncertainty is in part due to the shifting cell-cell interactions and new microenvironments encountered over extensive migration paths. Additionally, the presumed ectodermal origin of NCCs is at odds with an

ability to differentiate into tissues classically associated with multiple germ layers (Milet and Monsoro-Burq, 2012). Two distinct and exclusive models may explain the apparent increase in potency of NCCs: a classical model proposes a 'regaining' of potency during or prior to EMT, while a more recent model suggests a retention of potency from earlier embryonic stages (Buitrago-Delgado et al., 2015). EMT has been well studied in cancer cells, and it has been demonstrated that cancer cells may actually gain potency during EMT (Mani et al., 2008). However, as cancer cells often have dramatically altered genomes and aberrant protein expression, these changes could be more responsible for any gain in potency than EMT itself. A primary example of a cancer potency factor is *anaplastic lymphoma kinase (ALK)*, which has been linked to inducing EMT (Voena et al., 2016). *ALK* is a proto-oncogene expressed in the developing peripheral nervous system (Azarova et al., 2011). When human ALK is overexpressed in NC progenitor cells, it results in highly undifferentiated tumors that express stem cell markers but lack normal neuronal or adrenergic differentiation markers (Montavon et al., 2014).

The idea of 'cellular neoteny,' in which cells retain potency during development, was discussed almost 30 years ago with respect to NCCs that give rise to the adrenal medulla (Anderson, 1989). Recent work manipulating factors identified as critical to NCC development in *Xenopus* indicates that NCC potency may have substantial similarities to that of blastula-stage animal pole cells (Buitrago-Delgado et al., 2015). This study demonstrates that some of the underlying factors responsible for the potency of NCCs, such as Sox5 and Snail1 (a member of the Snail superfamily; Nieto, 2002), are also required to maintain the potency of blastula stage cells (Buitrago-Delgado et al., 2015), suggesting the possibility of retained potency instead of a *de novo* gain.

There is also evidence from chick embryos to suggest that NC identity may be determined even before neural plate formation. During gastrulation in chick, patches of cells that eventually contribute to the NC express Pax7. When Pax7 translation is blocked, proper NCC formation fails and typical NCC markers are not expressed (Basch et al., 2006). Although Pax7 does not specifically mark NCCs, Basch et al. suggested that the process of NC formation may begin before or during gastrulation, leaving open the possibility that a selection of highly potent cells is set aside as the future NC. Furthermore, new evidence indicates that cells along the border of the neural plate that go on to form the NC express markers traditionally associated with both the NC and neuronal fate. Altering the balance of Pax7 and Sox2 tips the cells towards either a neuronal or NC fate, respectively (Roellig et al., 2017). Another factor expressed in a similar spatiotemporal pattern as Pax7 in chick is cMYC (Kerosuo and Bronner, 2016). The MYC family transcription factors cMYC, N-MYC, and L-MYC are important in promoting cell growth and proliferation (Stine et al., 2015). When cMYC is knocked down in chick embryos using morpholinos, the number of NCCs decreases significantly and there is a significant increase in apoptosis. Additionally, siRNA and *in vitro* colony forming assays show that NC proliferation is not affected, but there is a decrease in self-renewal (Kerosuo and Bronner, 2016). It may be that cMYC plays a critical role in maintaining the stem cell-like characteristics of NCCs.

Interestingly, the MYC family also appears to play a role in the potency of cancer stem cells (CSCs) (Galardi et al., 2016), a multipotent population of cancer cells that have the capacity

to self-renew and proliferate (Clarke et al., 2006; Garner and Beierle, 2015). When MYC activity was inhibited in glioblastoma stem-like cells, there was a decrease in their ability to self-renew (Galardi et al., 2016). Sox2 and Nanog are other factors necessary to maintain self-renewal of stem cells, and Pandian et al (2015) demonstrated a significant increase in expression levels of both proteins in CSCs. Quantitative transcriptional profiling showed increases in the expression of 29 stem cell-related molecules including BMPs, Notch2, Slug and Twist1. Pandian et al also described high levels of cellular plasticity and increased pluripotency of aggressive metastatic neuroblastoma cells in comparison to their parental cell lines. Together, these lines of evidence suggest that many of the proteins known to play roles in embryonic stem cell potency, including in NCCs, may have similar roles to play in controlling the potency of CSCs.

Neuroblastoma is the most common type of cancer in the first year of human life and arises from the NC-derived sympathetic lineage (Matthay et al., 2016). During embryonic development, NC-derived sympathoadrenal progenitor cells migrate to the dorsal aorta, then undergo a secondary migration to form the sympathetic nervous system and chromaffin cells (Huber, 2006). One of the genes known to be involved in neuroblastoma progression is *MYCN*, which can initiate tumorigenesis when it is misexpressed (Weiss et al., 1997; Westermark et al., 2011). Using a zebrafish model, Zhu et al. (2012) revealed that when N-MYC is overexpressed, differentiation into chromaffin cells is lost and neuroblasts become hyperplastic. A small proportion of these neuroblasts eventually form a heterogeneous tumor (Zhu et al., 2012). Further investigation of N-MYC, making use of a murine NC progenitor cell line, revealed that stable overexpression of either N-MYC or ALK-F1174L in conjunction with an absence of cMyc activity results in a neuroblastoma-like tumor upon transplantation into mice (Schulte et al., 2013). These results suggest that a NC progenitor cell can become cancerous with only a few genetic modifications. Meanwhile, neuroblastoma treated with the drug berberine, which has recently been implicated in inhibiting cell proliferation (Ortiz et al., 2014), demonstrates increased expression of terminally differentiated neuronal markers and decreased expression of stem cell markers (Naveen et al., 2016). These results could suggest that some pediatric cancers such as neuroblastoma may retain potency from earlier stages in development to become tumorigenic.

2.2 Induction

Major molecular changes are necessary to initiate formation of the NC. A classical model dictates that NC development is a multi-step process that begins during gastrulation and continues throughout neurulation. Multipotent progenitors capable of giving rise to the NC – in addition to neural tissue, placodal derivatives, and the epidermis – are initially induced at the neural plate border, an area between the neural plate and the non-neural ectoderm (Basch et al., 2006; Bronner-Fraser, 1995; Groves and LaBonne, 2014; Selleck and Bronner-Fraser, 1995). Within this region, a combination of cell-autonomous gene regulatory events and non-cell-autonomous interactions with the neural plate, non-neural ectoderm, and the underlying mesoderm progressively define NC identity (Milet and Monsoro-Burq, 2012; Simoes-Costa and Bronner, 2015). Here, we focus on several factors of particular interest

due to their roles not just in forming the presumptive NC in its proper spatial arrangement but also the induction of cancer proliferation, survival, and/or metastasis.

In *Xenopus* and zebrafish, BMP signaling exhibits graded activity at the time of NC induction, with high levels in the epidermis and low levels in the neural plate (Marchant et al., 1998; Nguyen et al., 1998). The intermediate BMP signaling levels present at the neural plate border have been shown to induce NCCs (Marchant et al., 1998; Nguyen et al., 1998; Selleck and Bronner-Fraser, 1995). However, in chick, BMP signaling is initially extinguished in the epiblast at the onset of neural streak formation (Faure et al., 2002). During neurulation, BMP is inactivated in the prospective neural plate and strongly activated at the neural plate border and in the epidermal ectoderm (Faure et al., 2002; Liem et al., 1995). Furthermore, BMP4 and BMP7 are capable of promoting the differentiation of NCCs from ventral neural plate explants in culture (Liem et al., 1995). Together, these wide-ranging results suggest that graded levels of BMP signaling may induce NC identity in a concentration-dependent manner. Consistent with this model, reducing BMP signaling by overexpressing the BMP antagonist chordin in *Xenopus* embryos and explants induces the expression of early NC markers. However, these crest markers are expressed at far lower levels than in the endogenous situation, suggesting that modulation of BMP signaling alone does not explain NC formation (LaBonne and Bronner-Fraser, 1998).

While the role of BMP may explain why the NC is induced at the neural plate border, it does not account for its anterior-posterior localization. Indeed, the anterior neural plate does not adopt a NC identity and instead contributes to the forebrain (Milet and Monsoro-Burq, 2012). During early neural patterning, Wnt, FGF, and retinoic acid inhibit anterior identities and promote posterior identities (Kudoh et al., 2002). Several studies have demonstrated that in addition to BMP, NC induction requires FGF and Wnt activity (Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Luo et al., 2003; Mayor et al., 1997; Monsoro-Burq et al., 2003). Further, anterior neural plate tissue subject to intermediate levels of BMP activity can be ectopically transformed into NC by FGF, Wnt, and retinoic acid signals (Villanueva et al., 2002). Together, these studies have led to a “two-step model” of NC induction in which intermediate levels of BMP in addition to Wnt, FGF, and retinoic acid signals are required for proper NC formation (Aybar and Mayor, 2002).

Of the major signaling molecules described above, Wnts have been one of the most extensively studied in cancer, in part because of interactions with the MYC family. When the canonical Wnt pathway is activated in cancerous cells, target genes such as members of the MYC family are transcribed (He et al., 1998; Rennoll and Yochum, 2015). Overactivation of canonical Wnt signaling supports self-renewal of cancer cells, while broadly inhibiting Wnt signaling impairs self-renewal (Tammela et al., 2017). It has been suggested that Wnt signaling-mediated effects may occur in part through interactions with N-MYC (Duffy et al., 2016). In addition, the NC-inducing factor retinoic acid, mentioned above, induces cultured neuroblastoma to differentiate (Thiele et al., 1985) and improves the prognosis of children with neuroblastoma (Matthay et al., 1999). Interestingly, *MYCN*-amplified neuroblastoma cell lines treated with retinoic acid alone or alongside other chemotherapy agents exhibit a decrease in N-MYC expression (Aktas et al., 2010). Given these multiple lines of evidence,

it is intriguing to consider whether multiple pathways that play a role in driving NC induction also have significant roles to play in driving neuroblastoma self-renewal.

2.3 Specification

Many of the signaling pathways introduced above lead to the expression of a specific set of transcription factors at the neural plate border. These, in turn, drive the expression of a regulatory module that specifies NC identity. Once specified, NCCs undergo gene regulatory changes that ultimately regulate differentiation into specific cell fates (Meulemans and Bronner-Fraser, 2004). As described above, BMP, FGF, Wnt, and retinoic acid signals are critical for the induction of NC identity. Together, these pathways drive the expression of a set of genes known as neural plate border specifiers that are initially transcribed during gastrulation (Meulemans and Bronner-Fraser, 2004; Simoes-Costa and Bronner, 2015). Notably, border specifier genes *Gbx2, Pax3* and *Msx1* appear to be regulated by Wnt signaling (Bang et al., 1999; Li et al., 2009). The activation of *Pax3* may be indirect, as Wnt signals also activate expression of Tfap2, which in turn binds directly to *Pax3* regulatory elements (de Croze et al., 2011). Other neural plate border specifier genes, namely *Zic1/3, Dlx5*, and *Msx1* are activated by the attenuation of BMP activity (Aruga et al., 2002; Luo et al., 2001; Tribulo et al., 2003). Finally, FGF8 has been shown to regulate the expression of *Zic5, Msx1*, and *Pax3* (Monsoro-Burq et al., 2005, 2003). In summary, these neural plate border transcription factors act downstream of Wnt, BMP, and FGF signals and may serve as pathways to define the presumptive NC.

Subsequently, neural plate border specifier genes cooperate with upstream signaling pathways to activate a suite of genes termed the NC specifiers. Pax3 and Zic1, for example, are direct upstream regulators of *Xenopus Snail1/2, Foxd3, Twist1, Sox8/9*, and *Tfap2b* (Bae et al., 2014; Plouhinec et al., 2014). Cooperation with signaling inputs also plays an important role. In chick, Wnt signaling is necessary for the expression of *Snai2 and FoxD3* even after neural plate border specification (Garcia-Castro et al., 2002). Similarly, Wnt signaling cooperates with *Pax3* and *Zie1* to initiate NC differentiation in *Xenopus* (Sato et al., 2005). Together, these findings suggest that NC specifier genes act downstream of both neural plate border specifiers as well as Wnt.

ALK and MYC, discussed above, can be co-opted to result in a heterogeneous neuroblastoma tumor composed of inappropriately-differentiated cells from multiple NC lineages (Louis and Shohet, 2015). Overamplification of *MYCN*, present in approximately 25% of neuroblastoma cases, leads to a highly aggressive phenotype which exhibits rapid progression and a poor prognosis (Ishola and Chung, 2007). ALK cooperates with N-MYC, resulting in a significantly higher penetrance of neuroblastoma and accelerated tumor initiation (Zhu et al., 2012). Prosurvival signals provided by the activation of ALK result in early-onset neuroblastoma that expresses high levels of N-MYC and ALK and progresses rapidly (Montavon et al., 2014). Other pro-survival signals have been found to play a role as well. While p53 mutations are rare in neuroblastoma, there is often an overexpression of Twist-N, which in turn causes downregulation of p53 and inhibits cell death (Valsesia-Wittmann et al., 2004). When Slug/Snail expression is decreased in cultured neuroblastoma, there is an increase in apoptosis and a decrease in cell survival signals (Vitali et al., 2008).

Given these results, along with previously discussed studies revealing that NCCs can become neuroblastoma cells with only a few genetic changes (Schulte et al., 2013) and that neuroblastoma can be induced to differentiate via retinoic acid treatment (Matthay et al., 1999; Thiele et al., 1985), it is intriguing to compare neuroblastoma cells alongside NCCs that may maintain potency from earlier stages.

3. Delamination and Epithelial-Mesenchymal Transition (EMT)

Following induction, NCCs must delaminate from the neural tube and undergo EMT (Kalluri and Weinberg, 2009) to begin migrating to their future targets. Although NC delamination and EMT are often used interchangeably, these processes do not always occur simultaneously or in the same order in all organisms (Theveneau and Mayor, 2012b). Additionally, it has been recently noted that EMT may not just be a binary choice but that in certain circumstances, cells may take on particular traits while not fully adopting all stereotypical epithelial or mesenchymal characteristics (Campbell and Casanova, 2016; Nieto et al., 2016). For the purposes of this review, we define delamination and EMT separately, with delamination indicating the separation of NCCs from the neural tube and EMT describing the complex process by which a cell with stereotypical epithelial characteristics transitions to a cell with stereotypical mesenchymal characteristics.

The processes of delamination and EMT differ in fundamental ways between the trunk and cranial regions. In the trunk, NCCs invade the somites in a segmental fashion (Teillet et al., 1987). Cranial NCCs, on the other hand, are not confronted with somitic mesoderm, with the exception of NCCs exiting from the posterior-most rhombomeres of the hindbrain which coincide with the first few somites (Lumsden et al., 1991). In light of the different barriers and microenvironments NCCs confront at various anterior-posterior levels, it is not surprising that different molecular microenvironments regulate EMT in a region-specific and sometimes species-specific fashion. In chick trunk NCCs, BMP4 and its inhibitor Noggin coordinate interactions between NCCs and the lateral plate/intermediate mesoderm to promote NC delamination (Sela-Donenfeld and Kalcheim, 2002, 1999). BMP signals also promote delamination via canonical Wnt signaling, in particular by stimulating the transcription of Wnt1 (Burstyn-Cohen et al., 2004). The cascade of BMP and canonical Wnt signaling activates Slug/Snail, Foxd3, and members of the SoxE family such as Sox9 and Sox10 (Burstyn-Cohen et al., 2004; Cheung et al., 2005; Cheung and Briscoe, 2003; Sela-Donenfeld and Kalcheim, 2002). Thus, multiple families of transcription factors, many of which are NC specifier genes, are primed to induce EMT in premigratory NCCs. Notably, Sox9 in combination with Slug/Snail is sufficient to induce EMT (Cheung et al., 2005). Together with Foxd3, Slug/Snail and members of the SoxE family regulate the expression of cell-cell adhesion molecules that are key to EMT (Chalpe et al., 2010; Cheung and Briscoe, 2003).

Cadherins are transmembrane proteins that mediate cell-cell contact, build tissues, and provide stability (Maître and Heisenberg, 2013). Many cadherins have been implicated in NC EMT; please see Taneyhill and Schiffmacher (2017) for a thorough review of this topic. Here, we will focus specifically on E-cadherin and N-cadherin's relevance to both the NC and cancer. In many cell types including the NC, EMT has long been associated with a

cadherin switch that involves decreased E-cadherin and increased N-cadherin (Araki et al., 2011; Gravdal et al., 2007; Lade-Keller et al., 2013; Scarpa et al., 2015; Wheelock et al., 2008), although the importance of this particular cadherin switch remains a point of significant debate due to the perdurance of E-cadherin post-EMT in some cases (Dady et al., 2012; Dady and Duband, 2017; Huang et al., 2016). During EMT in cancer, there is usually a similar transition entailing a downregulation of E-cadherin at cell-cell junctions and an upregulation of N-cadherin (Kuphal and Bosserhoff, 2006; Thiery, 2002), but as with the NC, there may be exceptions to this behavior (Yan et al., 2016). N-cadherin has also been found to be required for NCC migration (Theveneau et al., 2010), and the switch from E-cadherin to N-cadherin allows cell protrusions to become polarized and NCCs to migrate (Scarpa et al., 2015). This transition is important not just for EMT but also for CIL (Scarpa et al., 2015), a behavior seen in migrating NCCs whereby a cell contacts a neighboring cell, collapses its protrusions, and subsequently repolarizes in a different direction (Carmona-Fontaine et al., 2008). Cultured NCCs, after expressing N-cadherin and initiating migration, display stereotypical CIL behavior after cell-cell collision at a much higher frequency than do premigratory, E-cadherin-expressing NCCs (Scarpa et al., 2015). Sip1 and Slug are two factors that may mediate the switch from E-cadherin to N-cadherin and cause a downregulation of E-cadherin in a synergistic manner in human gastric carcinoma cells (Castro Alves et al., 2007). Sip1 was also investigated in chick embryos via morpholino knockdown in NCCs and found to be required for the E-cadherin to N-cadherin switch (Rogers et al., 2013). Meanwhile, Sip1 can inhibit intercellular BMP signaling (van Grunsven et al., 2007), while canonical Wnt signaling is important in regulating Slug expression (Monsoro-Burq et al., 2005). Given the similar and synergistic roles that Sip1 and Slug might play in cancer, it is tempting to speculate that they may exist as a point of intersection in controlling EMT in NCCs through downregulation of E-cadherin and/or upregulation of N-cadherin. Substantial questions remain regarding the precise spatiotemporal roles of cadherins, the degree to which cadherin switching can be taken to indicate binary choices between states of NCCs, and species-specific differences in the use of particular cadherins.

In order to metastasize, cancer cells must decrease their adhesiveness and dissociate from the primary tumor mass. The loss of adhesiveness is manifested through either up- or downregulation of proteins that regulate tight junctions (Martin and Jiang, 2009). This alteration of tight junctions modifies the existing tissue structure and creates an environment conducive for EMT (Ikenouchi et al., 2003). EMT in cancer cells is influenced by the ECM, growth factors, and genetic mutations (e.g., in *CDH1* (*E-cadherin*)) (Kalluri and Weinberg, 2009; Leonel et al., 2017). The signaling pathways directing EMT can be triggered by transcription factors that are active in both NC development and cancer progression. For example, Wnts and BMPs activate transcription factors Snail 1/2, which are responsible for the repression of cell-cell adhesion molecules such as E-cadherin, in turn decreasing adhesiveness and allowing EMT to occur (Zhang et al., 2014). As with the NC, EMT is likely not just a binary process in cancer. It has been observed using both *in vivo* and *in vitro* approaches that some cancer cells downregulate cadherins and lose apical-basal polarity but lack stereotypical mesenchymal morphology (Bronsert et al., 2014). Furthermore, there are some breast cancer populations that appear to exist in both epithelial and mesenchymal

states due to incompletion of either EMT or mesenchymal-to-epithelial transition (Grosse-Wilde et al., 2015).

4. Migratory Mechanisms and Communication

Migration of NCCs to their target tissues involves a unique ability to navigate as a collective stream responding to a complex and dynamic microenvironment, which we define here as non-NC cells and the ECM that NCCs migrate through. NCCs migrate as loosely associated cells that are guided by inhibitory cues that surround the stream and chemoattractants in front of the stream (Belmadani, 2005; Koestner et al., 2008; McLennan and Kulesa, 2010; Robinson et al., 1997; Theveneau et al., 2013). NCCs may use a variety of mechanisms to collectively migrate through their microenvironments including CIL, chase-and-run, and leader-follower (Carmona-Fontaine et al., 2008; Richardson et al., 2016; Roycroft and Mayor, 2016; Stramer and Mayor, 2016; Szabó and Mayor, 2015; Theveneau et al., 2013). Here, we will focus on some of the mechanisms that both migratory NCCs and cancer cells use to traverse through and interact with their microenvironments.

4.1 Cell-Environment Communication

NCCs migrate through a variety of regions within the developing vertebrate embryo to reach their final destinations. As cranial NCCs migrate along the rhombomeres, subpopulations migrate into and populate some of the branchial arches (Kuo and Erickson, 2010). The NCCs of the hyoid stream traverse over rhombomere 4 in a wide, densely packed stream, while those of the branchial stream migrate over rhombomere 7 in narrow, linear chain-like arrays (Wynn et al., 2013). Although these cells have different migratory characteristics, NCCs swapped between rhombomeres 4 and 7 in chick are able to adopt the local migratory style (Wynn et al., 2013). It is intriguing to consider what mechanisms are at play to enable the transplanted NCCs to adapt to their new microenvironments and take on local migratory patterns. Similarly, xenotransplantation has shown that metastatic melanoma cells can behave like NCCs by migrating along NC trajectories and not entering NCC-free zones. A subset of these transplanted melanoma cells go on to express melanocyte or neuronal markers (Kulesa et al., 2006). In this section, we will discuss factors present in the microenvironment that have been shown to influence how NCCs migrate and how these factors might impact the metastatic capabilities of cancers.

The complex interplay between NCCs and their microenvironment relies upon multiple signaling cues and has intriguing parallels to the methods of communication between metastatic cancer cells and their microenvironment. For example, semaphorin ligands and neuropilin receptors have been repeatedly implicated in NC migratory behavior. Neuropilin-1 (Nrp1) is involved in directing cranial NC migration into branchial arches in chick (McLennan and Kulesa, 2010, 2007). Semaphorin 3A (Sema3A), expressed in the lens placode of the chick, prevents Nrp1-expressing NCCs from migrating into the periocular region until a subset of NCCs downregulates Nrp1 (Lwigale and Bronner-Fraser, 2009). In mouse, Sema3A and 3F along with Nrp1 and 2 guide trunk NC migration into specific regions of the somite (Schwarz et al., 2009). Additionally, subpopulations of cranial and trunk NCCs express either Nrp1 or Nrp2 in non-overlapping patterns that appear to indicate

what neuronal types the NCCs will differentiate into (Lumb et al., 2014). Overall, semaphorins primarily act as inhibitory guidance cues to prevent NCCs from invading incorrect targets or entering tissues prematurely. Intriguingly, semaphorins may play a role in cancer migration as well. An increase in the expression of Sema3B (a known tumor suppressor gene) appears to correlate with histopathology indicating a more favorable outcome for neuroblastoma patients (Nair et al., 2007). If neuroblastoma cells are induced to differentiate (lose their stem-like characteristics and lessen their potential to metastasize) with retinoic acid, a correlated increase in Sema3B expression is also seen (Nair et al., 2007). VEGF, another ligand of Nrp1, is present in the second branchial arch where cranial NCCs invade (McLennan et al., 2010). Loss of endogenous VEGF leads to cranial NCCs being unable to properly migrate into branchial arch two, while ectopic sources of VEGF cause cranial NCCs to migrate away from their native migratory path (McLennan et al., 2010). These observations suggest that VEGF acts as a chemoattractant for this population of cranial NCCs and promotes their migration into VEGF-expressing tissues. In breast cancer, Nrp1 and VEGF similarly impact metastatic abilities (Luo et al., 2016). Silencing Nrp1 in breast cancer cells significantly decreases the number of metastatic tumors formed in mice. Additionally, increasing Nrp1 and VEGF enhances the invasive abilities of breast cancer cells *in vitro* (Luo et al., 2016). These data suggest that Nrp1 and VEGF may function similarly in NCCs and cancers by increasing migratory capabilities.

Eph receptors and their ligands, ephrins also help guide migratory NCCs. Both the ligand and receptor are cell surface proteins. In *Xenopus*, ephrin-B2 is expressed in the second branchial arch NC population while its receptors EphA4 and EphB1 are expressed in the adjacent third branchial arch NC population (Smith et al., 1997). Normally, the NCCs of the second and third arch migrate as separate streams. Inhibition of EphA4 and EphB1 leads to aberrant migration of third arch NCCs into the second arch (Smith et al., 1997). Similarly, in chick, EphB3 and ephrin- B1 are important in guiding trunk NCCs into the rostral half of the somite (Krull et al., 1997). A diversity of Eph receptors are expressed in chick cranial NC in unique patterns which may speak to their roles in guiding NCC migration (Mellott and Burke, 2008). Interestingly, melanoma cells (formed from a NC derivative) express high levels of various Eph receptors, which may contribute to their ability to traverse along native NC migration pathways when transplanted into chick (Bailey and Kulesa, 2014). Ectopic EphB6 expression causes transplanted melanoma cells to migrate into areas devoid of NCCs without impairing cell-cell interactions (Bailey and Kulesa, 2014). The authors suggest that this deviation in migration may be due to the ability of EphB6 to interact with other Eph receptors and ephrins, which in turn could impair the ability of melanoma cells to respond to the embryonic environment. These data speak to a possible conservation in the roles of Eph receptors in both NCCs and cancer cells to migrate through their respective microenvironments.

More recently, the ECM protein versican was shown to act as a guiding factor in NC migration (Szabó et al., 2016). Versican is inhibitory to NCC migration *in vitro*, whereas loss of versican in *Xenopus* embryos results in NCCs being unable to migrate dorsolaterally along the embryo (Szabó et al., 2016). These results suggest the possibility that versican's inhibitory effect could be required for funneling NCCs into migratory streams. In human renal cancer, however, elevated versican expression is correlated with an increased risk of

metastasis, and when versican is knocked-down *in vitro*, renal cancer cells have decreased invasive capacity (Mitsui et al., 2017). It may be that versican has different roles to play in the guidance of NCCs and cancer cells. Whether through conserved use of similar receptors and ligands or, as is the case with versican, diverse responses to the same molecule, the studies discussed here point to multiple parallels between NC migration and cancer metastasis as these cells interact with their microenvironments.

4.2 Cell-Cell Communication

Communication between migrating NCCs is a critical aspect of establishing and maintaining migratory streams. For example, coattraction signals such as C3a and its receptor C3aR may maintain NCC cohesiveness during migration in *Xenopus* (Carmona-Fontaine et al., 2011). Additionally, *in silico* modeling has proposed that a balance of coattraction and CIL is important for the directional migration of NCCs (Woods et al., 2014). Other modeling suggests that NCCs may have two general roles: leader cells located toward the front of the migratory stream and follower cells trailing behind the leaders (McLennan et al., 2012). Based on this computational model, leader cells would respond more to long-range signals within the microenvironment, while follower cells would respond to short-range signals coming from NCCs ahead of them (McLennan et al., 2012). Together, NCCs may be using various forms of communication ranging from short-range paracrine signaling to direct cell-cell contact in order to maintain directed collective migration. Highly reminiscent of NC migration is the multicellular streaming of loosely- or non-cohesive cancer cells, one of the multiple modes that cancer cells use to invade the ECM – the others being highly cohesive clumps or single-cell migration (Clark and Vignjevic, 2015). While migratory streams of cancer cells may appear similar to migratory NCCs, it is not clear how communication between cancer cells directs invasion. As previously mentioned, it has been shown that xenotransplanted melanoma cells can incorporate themselves throughout chick cranial NC streams (Bailey et al., 2012; Kasemeier-Kulesa et al., 2008; Kulesa et al., 2006). One potential explanation for this observation is that melanoma cells are interacting with and/or responding to cues from nearby NCCs, opening up the possibility that metastatic cancer cells communicate with each other as well.

Proposed leader-follower models may explain some general behaviors of migratory NCCs. In this paradigm, ‘leader’ cells of NC migratory streams interpret signals from the microenvironment and chart the course for ‘follower’ cells (Wynn et al., 2013, 2012). However, identifying these leader cells genetically has been challenging, perhaps in part due to a dynamic identity and/or shifting population. In one of the first experiments to demonstrate leader-follower dynamics, cranial NCCs were shown to migrate around physical barriers introduced into chick embryos. Upon encountering a barrier, the front-most NCCs paused while more distally located cells in the stream eventually migrated around and become the new leader cells (Kulesa et al., 2005). Within cranial migratory streams, there appear to be distinct molecular profiles for leading edge cells versus for those trailing behind (McLennan et al., 2012). When NCCs are transplanted from one part of the migratory stream to another, the transplanted cells appear to change their genetic profile to more closely match that of the cells in their transplanted area (McLennan et al., 2012). More recently, a unique molecular signature was found to be consistently expressed throughout

migration in a subset of NCCs at the leading edge of the migrating stream. When this molecular signature is perturbed in cranial NCCs, migration distances and patterns are disrupted (McLennan et al., 2015). However, subsequent work has shown that physical ablation of NCCs at the leading edge does not appear to impact cranial NC migration (Richardson et al., 2016). Interestingly, cranial NCCs appear to alter positions relative to each other while migrating; cells starting off in a lead position appear to fall back and are overcome by cells that began as followers (Richardson et al., 2016). These results suggest that leader and follower roles in migratory cranial NCCs may be dynamic, in contrast with observed behavior in trunk NCCs, which appear to require leader cells to properly migrate (Richardson et al., 2016). If cranial NCCs have naturally dynamic leader- follower roles during migration, it may help to explain the observations from Kulesa et al. (2005) and McLennan et al. (2012) in which follower cells are seen taking on leader positions and vice versa. It is also possible that, as migrating NCCs are changing their locations within the stream, they are also changing their molecular signatures to match their new roles. Taken together, these intriguing data suggest that migrating cranial NCCs may be a highly dynamic population of cells with some flexibility in taking on leader or follower profiles. It remains unclear why cranial NCCs have such dynamic and plastic mechanisms while migrating and how these mechanisms are used to direct collective migration over time.

As cancer often invades into the surrounding ECM as collective streams, there may exist populations of cells similar to NC leaders and followers within a tumor. A potential example of leader-follower dynamics is seen when squamous cell carcinoma is co-cultured with fibroblasts. In these co-cultures, fibroblasts are frequently seen at the leading edge of invasive streams (Gaggioli et al., 2007). Interestingly, it appears that processing of the ECM by fibroblasts is sufficient to induce invasion of carcinoma cells, whereas factors secreted from fibroblasts are insufficient. While these results suggest that remodeling of the ECM by fibroblasts may be sufficient for cancer metastasis, other studies have found that cancer cells themselves may behave as leaders. *In vitro* work has identified molecularly distinct cells at the leading edge of invasive cancer (Cheung et al., 2013; Westcott et al., 2015). In Cheung et al. (2013), a subtype of cancer cells expressing the gene K14 are frequently found to be at the front of breast cancer invasive streams. When K14 is knocked down, there is a lower level of invasion both *in vitro* and *in vivo* (Cheung et al., 2013), suggesting that these cells may be required for tumor invasion. Similarly, in Westcott et al. (2015), a molecularly distinct subpopulation of cells was frequently found at the edge of the invasive streams coming off of triple-negative breast cancer tumors. These leader cells are required for another subpopulation of cells, termed opportunists, to invade. When these two subpopulations of cells are cultured nearby but not in contact, the opportunists are poorly invasive, suggesting that diffusible factors alone are not sufficient to induce opportunist invasion (Westcott et al., 2015). Together, these studies suggest that, in at least a subset of cancers, leader cells modify the ECM to induce tumor invasion. NCCs also likely need to modify the ECM to properly migrate. Matrix metalloproteases appear to be upregulated in the leading edge of the NC migratory stream (McLennan et al., 2012), supporting the possibility that NCCs modify the ECM during collective migration. Alfandari et al. (2001) found that ADAM13, a matrix metalloprotease, is located within NC migratory streams in *Xenopus* and that migratory streams are greatly impaired when ADAM13 activity is

knocked down. Additionally, matrix metalloproteinase 9/gelatinase B may also be required for proper migration. When this protein is knocked down in chick, premigratory NCCs fail to properly delaminate and begin migration, and when matrix metalloproteinase 9/gelatinase B activity is inhibited during migration, NCCs cease migrating (Monsonego-Ornan et al., 2012). Collectively, these results demonstrate that NCCs need to modify and degrade the ECM to properly migrate. Current evidence does not appear to indicate that cancer leader-follower dynamics rely directly on cell-cell communication, but it is intriguing to speculate that some of the cell-cell communication mechanisms used in NC migration could be co-opted in cancer.

4.3 Wnt Signaling in Migration and Metastasis

As NCCs migrate to target tissues, cells remain behind in some cases to populate the regions through which they are migrating, as seen with melanocyte development, while the rest invade more distant target tissues and intermix such as in the development of many cranial sensory organs (Chan and Tam, 1988; D'Amico-Martel and Noden, 1983; Erickson et al., 1992; Kuo and Erickson, 2010; Saxena et al., 2013; Steventon et al., 2014; Waldo and Kirby, 1993; Wang et al., 2011). As previously discussed above, canonical Wnt signaling has been well-established in both the induction and specification of the NC. Additionally, Wnt signaling has been implicated in the subsequent differentiation of NCCs (Dorsky et al., 1998), but it is unclear if the precise role of canonical Wnt signaling remains consistent or changes throughout these processes. For example, increased canonical Wnt signaling in premigratory or migratory NCCs in mice decreases or increases melanocyte production, respectively (Hari et al., 2012; Lee et al., 2004). A role for canonical Wnt signaling in migratory NCCs across species is supported by a more recent study in zebrafish. Increasing canonical Wnt signaling after NCCs are already migratory leads to an increase in melanocytes (Vibert et al., 2017). In *Xenopus*, however, canonical Wnt signaling may need to be strictly regulated for NCCs to become migratory, as both increasing or inhibiting canonical Wnt activity in premigratory NCCs prevents them from becoming migratory (Maj et al., 2016). Canonical Wnt signaling may also work cooperatively with sonic hedgehog (Shh) signaling – a morphogen known to be important in brain patterning and limb development (De Luca et al., 2016; Lopez-Rios, 2016) – to promote NC-placode intermixing in the trigeminal placode in mouse (Kurosaka et al., 2015). Additionally, the balance of Shh and canonical Wnt appears to be critical in promoting the survival of cranial NCCs (Kurosaka et al., 2015). Meanwhile, canonical Wnt signaling has been thoroughly studied in cancer. Overexpression of canonical Wnt signaling may support the self-renewal and migration of CSCs (Jang et al., 2015). In neuroblastoma, inhibition of canonical Wnt activity decreases its ability to proliferate and in some cases appears to induce a more differentiated state (Duffy et al., 2016). When exposed to Wnt3a or Wnt5a, neuroblastoma cells become more proliferative and migratory and when exposed to small interfering RNA targeting Fzd2 (a Wnt receptor), neuroblastoma cells become less proliferative and migratory (Zins et al., 2016). In sum, balanced canonical Wnt signaling is important in multiple stages of NC development, including migration, and increased activation of Wnt signaling appears to promote cancer proliferation and/or migration.

In addition to canonical Wnt signaling playing an important role in migratory NCCs, the non-canonical Wnt/Planar Cell Polarity (PCP) pathway has also been implicated in *Xenopus* NC. The Wnt receptor Fzd7 may be required as a receptor for Wnt11 in NCCs (De Calisto et al., 2005). Both Fzd7 and Disheveled have been described as essential for the CIL mode of migration (Carmona-Fontaine et al., 2008). In zebrafish, another PCP molecule, Prickle1b, may be implicated specifically in the migration of cranial NC, perhaps in concert with other PCP molecules (personal communication, KA and Victoria Prince). With respect to cancer, another Frizzled receptor, Fzd6, activates the non-canonical Wnt pathway in Fzd6-positive, highly metastatic neuroblastoma cells. When Fzd6 is removed, these aggressive neuroblastoma cells lose some of their tumorigenic abilities in mouse transplants (Cantilena et al., 2011). Altogether, it appears that non-canonical Wnt signaling may also play a critical role in NC migration and cancer metastasis.

5. Concluding Remarks

NC migration is a complex process that requires numerous events to proceed correctly. Prior to migration, the potency of NCCs is either gained or maintained through precise regulatory cascades. After induction, via both extrinsic and intrinsic factors discussed in this review, NCCs delaminate from the neural tube and undergo EMT. They subsequently respond to their microenvironment and communicate with each other to collectively migrate. To better understand the cellular and genetic mechanisms used by migratory NCCs and how they might differ between NC subpopulations, it may be helpful to employ new conditional approaches in future studies that allow for greater spatiotemporal specificity such that the dynamically changing roles of developmental regulators can be clearly separated out. Furthermore, the migratory mechanisms used by NCCs often parallel the mechanisms used by invading and metastasizing cancer cells, and investigating how migratory cancer cells interpret unique *in vivo* microenvironments, including in the developing vertebrate embryo, may help identify new targets for inhibiting cancer metastasis in humans.

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Abbreviations

CIL	contact inhibition of locomotion
EMT	epithelial-to-mesenchymal transition
ECM	extracellular matrix
NC	neural crest
NCCs	neural crest cells

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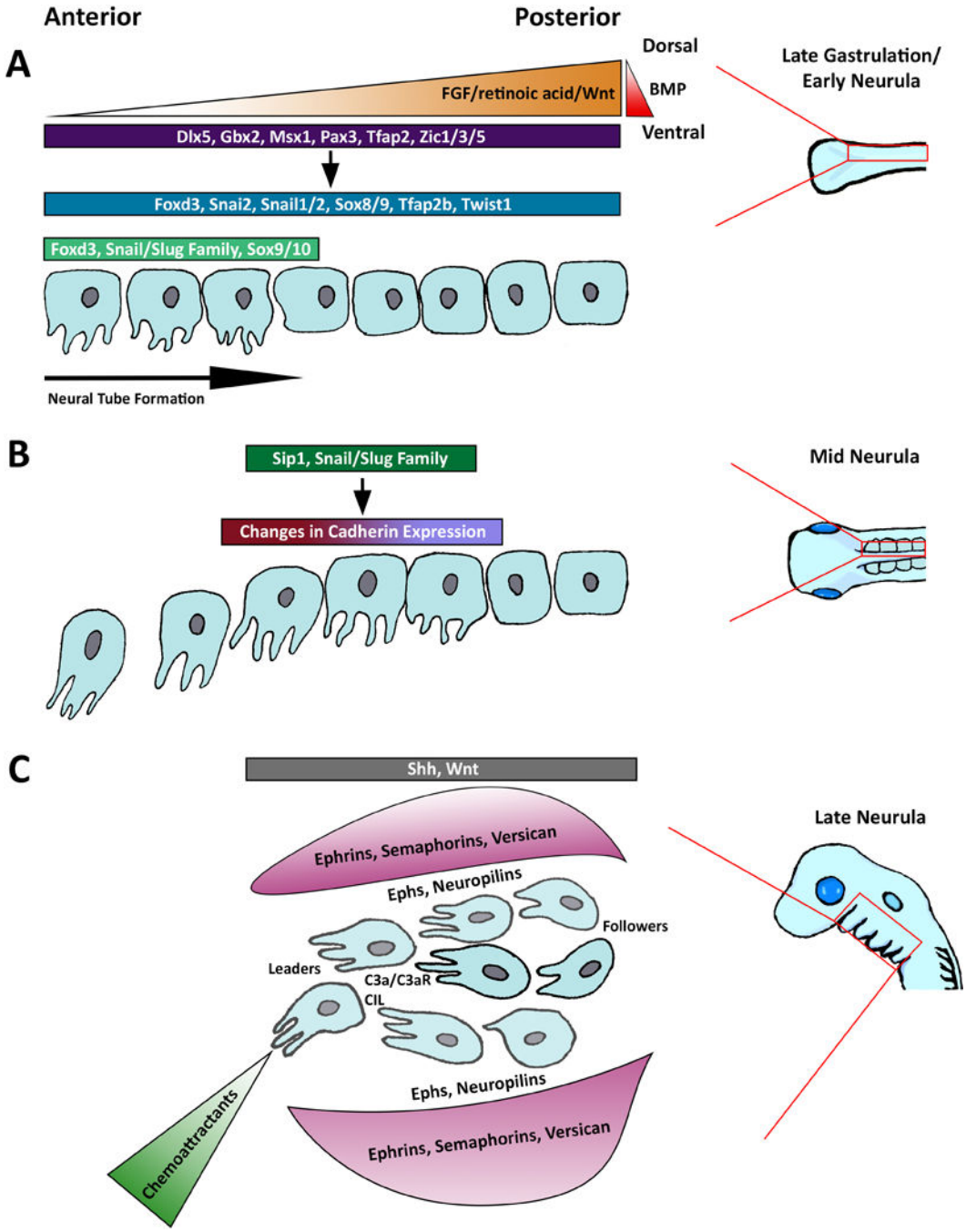

Figure 1. Schematic representing the major processes and controlling factors discussed in this review

Cartoons represent late gastrulation/early neurula **(A)**, mid neurula **(B)**, and late neurula **(C)** stages in vertebrate embryos. Illustrated processes are important for neural crest development and migration in multiple species (chicken, frog, mouse and/or zebrafish). Relevant sections of the review are listed below. **(A)** FGF, retinoic acid, and Wnt anterior-posterior gradients (orange triangle; section 2.2) and an orthogonal BMP gradient (red triangle; section 2.2) are together critical for the first step of neural crest induction. Neural

border specifiers (purple rectangle; section 2.3) are involved in separating the neural ectoderm from the non-neural ectoderm. Subsequently, neural crest specifiers (blue rectangle; section 2.3) thought to be important in the initial formation of the neural crest are activated, followed by proteins involved in neural crest delamination (light green rectangle; section 3). **(B)** Delaminating neural crest cells undergo epithelial-mesenchymal transition (EMT), which is guided in part by the Snail/Slug family and Sip1 (dark green rectangle; section 3) and results in cadherin switches (dark red to purple rectangle; section 3). **(C)** Directional migration of neural crest cells is coordinated by several mechanisms, which may include regulation of cohesion (e.g., C3a/C3aR), contact inhibition of locomotion (CIL), and leader-follower dynamics (section 4.2). Attractive cues for migration include the chemoattractant VEGF, and inhibitory cues include Eph/ephrins, neuropilins/semaphorins, and versican (section 4.1). Both canonical and non-canonical Wnt signaling have been implicated in neural crest migration and/or differentiation, and the former can work in coordination with sonic hedgehog (Shh) (section 4.3).