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Cranial Neural Crest Migration: New Rules for an Old Road

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Abstract

The neural crest serve as an excellent model to better understand mechanisms of embryonic cell migration. Cell tracing studies have shown that cranial neural crest cells (CNCCs) emerge from the dorsal neural tube in a rostrocaudal manner and are spatially distributed along stereotypical, long distance migratory routes to precise targets in the head and branchial arches. Although the CNCC migratory pattern is a beautifully choreographed and programmed invasion, the underlying orchestration of molecular events is not well known. For example, it is still unclear how single CNCCs react to signals that direct their choice of direction and how groups of CNCCs coordinate their interactions to arrive at a target in an ordered manner. In this review, we discuss recent cellular and molecular discoveries of the CNCC migratory pattern. We focus on events from the time when CNCCs encounter the tissue adjacent to the neural tube and their travel through different microenvironments and into the branchial arches. We describe the patterning of discrete cell migratory streams that emerge from the hindbrain, rhombomere (r) segments r1–r7, and the signals that coordinate directed migration. We propose a model that attempts to unify many complex events that establish the CNCC migratory pattern, and based on this model we integrate information between cranial and trunk neural crest development.

Introduction

The vertebrate embryo regulates the programmed invasion of the neural crest, a cell population that makes important contributions to structures that include the head, heart, and peripheral nervous system. In the head, cranial neural crest cells (CNCCs) emerge from the hindbrain (rhombomere (r) segments r1–r7) and are spatially distributed along discrete migratory pathways (Fig 1). During their dorsolateral migration, CNCCs may interact with and receive signals from multiple sources. CNCCs may touch the ectoderm and crawl through microenvironments rich in cranial mesenchyme and extracellular matrix (ECM). Signals arising from within the hindbrain, from other CNCCs, or from the local microenvironments traversed by migratory CNCCs together establish neural crest cell-free zones (Fig. 1). Failure of CNCC migration leads to significant morphological abnormalities of the face, neck and cardiovascular system (Hutson and Kirby, 2007;Tobin et al., 2008), making this an important model system to better understand birth defects.

The long history of NCC tracing and cell behavior analyses by static imaging and time-lapse cinematography (Davis and Trinkaus, 1981; Newgreen et al., 1982), respectively, have provided invaluable data on the CNCC migratory pattern (summarized in (Le Douarin and Kalcheim, 1999)). From early in vitro studies, neural crest biologists realized the complexity of cell migratory behaviors and struggled with determining whether the CNCC migratory

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streams were composed of individual cell movements or collective migration in sheets, and to what extent cells responded to growth of the embryo (Erickson, 1985; Erickson et al., 1980; Le Douarin, 1982; Noden, 1975; Thiery et al., 1982; Tosney, 1982). Detailed investigations of the local ECM in the CNCC microenvironment transitioned studies from mapping cell pathways to providing a basis for how cell microenvironmental interactions influenced neural crest cell direction (Bronner-Fraser, 1993; Newgreen, 1989). From these data and influence from mentors in the cell migration field, such as J.P. Trinkaus and Michael Abercrombie, who also elegantly described cell movements in Fundulus (Trinkaus, 1973) and fibroblasts (Abercrombie and Heaysman, 1954), neural crest biologists derived several models to explain directed cell migration. However, concern that the inability of any single model to explain the CNCC migratory problem suggested the mechanisms in effect were more complex.

In this review, we report recent insights into the molecular signals that direct CNCC behaviors and more detailed cell dynamics analyses that produce the CNCC migratory pattern. First, we will define features of the migratory CNC and cell-to-cell contact dynamics. We will describe participating structures of the CNCC-rich microenvironment and the heterogeneity of cell morphology and proliferative activity that depend on cell position within a migratory stream. Next, we will characterize the selection and plasticity of the CNCC migratory routes and acquisition of orientation and direction after cells leave the hindbrain. Then, we will detail the signaling pathways that have emerged to regulate the CNCC migratory pattern. We will contrast results obtained at multiple spatial scales, from single cell to populations, and propose a unified model for cranial neural crest development. Finally, we will compare cranial and trunk neural crest development in order to highlight common mechanisms.

Cranial Neural Crest Migratory Route Selection

Three phases of cranial neural crest migration

The segmented nature of the hindbrain, into rhombomeres (r), r1–r7, provides a structural and anatomical framework to describe the emergence and early sculpting of CNCCs. The relationship between patterns of gene expression in the hindbrain and branchial arches have been discussed separately (Santagati and Rijli, 2003; Trainor and Krumlauf, 2001). We focus here on defining the phases of CNCC migration after cells exit the neural tube. The initiation of CNC migration begins with inductive cues from non-neural ectoderm and mesoderm that converge at the lateral plate border (Basch and Bronner-Fraser, 2006). These inductive signals initiate signal cascades that result in a remodeling of cellular architecture and adhesive properties characterized by an epithelial-to-mesenchymal transition (EMT) and exit from the neural tube. Alterations to cellular adhesion, mediated principally by changes in the expression of multiple cadherin family members, facilitate delamination from the neural tube (Taneyhill, 2008).

The CNCC invasion program consists of at least three distinct phases of migration that form the basis for our discussion of recent cellular and molecular developments of the CNCC migratory pattern. Acquisition of directed migration along the dorsolateral pathway defines the initial phase of CNCC migration (Fig. 2A,B). After CNCCs leave the hindbrain, they come into intimate contact with the surface ectoderm and cranial mesenchyme adjacent to the hindbrain. The second phase of CNCC migration, homing to the branchial arches, consists of the maintenance of cells in loosely connected streams along the dorsolateral pathway (Fig. 2C,D). The last phase of CNCC migration is entry into and invasion of the branchial arches (Fig. 2E,F).

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Acquisition of Directed Migration Along the Dorsolateral Pathway

Filopodial dynamics and cell contact behaviors: evidence for cell communication

There is an impressive body of literature detailing the spatio-temporal emergence and pathway selection of an aggressive neural crest cell, and more recently its cellular features and cell-to-cell contact dynamics (Birgbauer et al., 1995; Epperlein et al., 2000; Kulesa and Fraser, 1998; Lumsden et al., 1991; Schilling and Kimmel, 1994; Sechrist et al., 1993; Serbedzija et al., 1992) (Fig. 2). For example, use of fluorescent reporters targeted to the cell membrane and nucleus, combined with in vivo chick time-lapse confocal microscopy have revealed the unexpected finding that an individual CNCC extends long filopodia well beyond its center of mass to reach distant cells and ectoderm overlying the migratory route (Teddy and Kulesa, 2004) (Fig 2). CNCC morphologies vary depending on cell position within a migratory stream. Chick CNCCs at the migratory front of a stream display protrusive activity in multiple directions, and trailing cells have a bipolar shape with equal leading and trailing edge protrusive activity aligned along the migratory route (Kasemeier-Kulesa et al., 2008) (Fig. 2). Furthermore, CNCC filopodial extensions appear early, as cells exit the neural tube, as visualized in vivo in the zebrafish embryo (Berndt et al., 2008). Membrane blebbing activity, characteristic of CNCCs in the neural tube, transitions to lamellipodial and filopodial extensions when cells exit the neural tube (Berndt et al., 2008). Extensive filopodial dynamics have also been described during NC-derived cell migration in the embryonic mouse gut (Druckenbrod and Epstein, 2005; Young et al., 2004). In the gut, enteric NCCs travel rostrocaudally in strands and display filopodial extensions as cells crawl over each other and reach into the unpopulated tissue to extend the strand. Enteric NCCs have a subpopulation of cells that are disconnected from the migratory front, called advance cells (Druckenbrod and Epstein, 2007). Advance cells have multiple filopodial extensions, some of which extend backward and appear to guide bipolar shaped cells within the strands (Druckenbrod and Epstein, 2007).

Cell contact between migratory CNCCs has provided evidence for cell communication. Contact between two CNCCs may be local (within a cell diameter or two) or non-local, up to 70um away (Teddy and Kulesa, 2004) (Fig. 2). Detailed analysis of filopodial dynamics in the chick CNCC migratory streams has shown that cell–to-cell contact occurs when a thin process extends to contact the trailing edge of a lead cell. Then, either the process remains near that trailing edge and the cell body moves forward, or the process retracts back to the cell body and the cell body moves forward to the position of contact (Teddy and Kulesa, 2004) (Fig. 2B). CNC cell-to-cell contact may also take the form of more membrane surface area contact and result in cell movement away from the contact, termed contact inhibition of movement (Abercrombie and Heaysman, 1954), recently visualized in Xenopus CNCCs (Carmona-Fontaine et al., 2008). In vivo evidence to the contrary in the zebrafish trunk has shown that NC cell-to-cell contact does not result in filopodial retraction, but a continued contact and movement in the direction of the contact (Jesuthasan, 1996). Whether this is due to behavioral differences in the cranial versus trunk NC or species differences is unclear.

Communicating positional information within the cranial microenvironment

The CNC are a heterogeneous population of stem and progenitor cells. CNCCs contribute to a variety of cell types that are neural and non-neural. Derivatives that include bone, cartilage and other mesenchymal structures, neurons, glia, and melanocytes, arise from the same regions of the CNC. Extensive experimental data suggest the CNCC-rich microenvironment contains powerful signals that may reprogram cells enroute to head and neck targets (reviewed in (Crane and Trainor, 2006; Le Douarin et al., 2008; Le Douarin et al., 2004)). The neural crest microenvironment can also reprogram small numbers of neural crest cells transplanted from a different axial level (Noden and Trainor, 2005; Sandell and Trainor,

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2006; Trainor et al., 2002a). Together with single cell tracing data of chick trunk NCCs (Bronner-Fraser and Fraser, 1988), this suggests the subpopulation of CNCCs within a migratory stream consists of a mix of multipotent cells and more restricted progenitors whose developmental potential may be instructed by local microenvironmental signals. Indeed, the ability of highly aggressive CNCCs to express multiple cellular phenotypes and engage in functional plasticity, such as changes in trajectory and proliferative activity, defines their multipotency.

Contributions of cranial neural crest cells from rhombomeres 3 and 5

It is well known that CNCC migratory streams are composed of cells from multiple rhombomeres. Each CNCC migratory stream is sculpted by a combination of extrinsic and intrinsic cues. The contribution of r3- and r5-CNCCs to neighboring migratory streams has been previously described in detail (Graham et al., 2004; Kulesa et al., 2004). Briefly, discrete CNCC migratory streams are separated by NCC-free zones adjacent to both r3 and r5. Prevailing explanations for the lack of CNCC migration into the regions flanking r3 and r5 include diminished CNCC production or increased apoptosis associated with these odd-numbered rhombomeres, or the restricted movement of CNCCs generated by r3 and r5.

Each of these processes may play an important role in the early stages of neural crest segmentation, but may vary widely among species. For example, analyses in avian embryos demonstrated increased apoptosis in neural crest populations residing in r3 and r5, resulting in part from BMP-4-induced Msx2 expression in these respective rhombomeres at the time of neural crest induction and migration (Graham et al., 2004; Lumsden et al., 1991). Similarly, studies in Xenopus have demonstrated an important role for *msx1* in regulating cell death in specific areas of the neural folds as a means of generating precise neural crest territorial boundaries (Tribulo et al., 2004). However, this report contradicts previous studies in Xenopus that demonstrated an absence of rhombomere-specific apoptosis (Hensey and Gautier, 1998). Furthermore, blocking neural crest cell death in r3 and r5 is not sufficient to disrupt neural crest migratory segregation (Ellies et al., 2002). Considering that studies in mouse and zebrafish have revealed an absence of rhombomere-specific apoptotic patterning (reviewed in Kulesa et al., 2004), these findings suggest that the potential role of apoptosis in segmenting neural crest migratory streams is widely variable across species and remains poorly understood. Alternatively, there may be diminished CNCC production in r3 or r5; this mechanism appears to be species specific and is discussed in more detail in Kulesa et al., 2004. Lastly, CNCCs that emerge from r3 and r5 may have restrictive lateral movement. Static and time-lapse analysis of chick r3- and r5-NCCs shows that CNCCs may enter these regions lateral to the hindbrain, but CNCCs either change direction to move towards a neighboring migratory stream or collapse filopodia and stop (Sechrist et al., 1993; Birgbauer et al., 1995; Kulesa and Fraser, 1998).

Homing to the Branchial Arches

Each cranial neural crest cell migratory stream has its own characteristics

What is striking and worth describing in more detail is that each CNCC migratory stream is distinct in terms of the shape and population density of the migratory front and individual cell migratory behaviors (Fig. 1). For example, CNCCs that emerge from the midbrain to mid-r3 and mid-r5 through r7 emerge in a wide front (the width of 2–3 rhombomeres) of cells that move in a directed manner to the periphery (Fig. 1B,C,E). After an initial wavefront of individual migratory cells, trailing cells from r7 (fewer in number) form chain-like arrays (Fig. 1E) that follow-the-leader to the trailing edge of the wavefront (Rupp and Kulesa, 2007). In contrast, the CNCC migratory stream that emerges lateral to r4 (comprised of CNCCs from mid-r3 to mid-r5) consists of a migratory front (the width of one

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rhombomere) that narrows in rostrocaudal width, back towards the neural tube (Fig. 1B,D). Analysis of chick CNCC trajectories within the migratory front reveals that cells that emerge from rostral- or caudal-r4 tend to migrate near the borders of the stream and NCC-free zones (Kulesa et al., 2008). In contrast, cells that emerge first from mid-r4 spread out into all regions of the migratory front (Kulesa et al., 2008). This suggests that a permissive corridor lateral to r4 emerges. CNCCs may travel to the branchial arches without contact with neural crest cell-free zones. Indeed, when premigratory r4 CNCCs are reduced by ablation and replaced by a smaller number of r4 NCCs, fewer numbers of cells emerge and travel directly down the middle of the region lateral to r4, then spread out into the ba2 (McLennan and Kulesa, 2007).

Cranial neural crest cell proliferative activity along the migratory route

CNCCs proliferate along their migratory route and the activity is key to the complete invasion of the branchial arches and formation of head and neck structures. The proliferation of cranial NCCs appears to occur in a regulated manner that involves the FGF/TGF-beta signaling pathways (see Table 1 for a list of known signals and references). Specifically, a subpopulation of CNCCs within the front portion of a typical migratory stream proliferate at a higher rate than the trailing cells (Kulesa et al, 2008). Higher cell proliferation within the migratory front may be triggered by space availability in the local microenvironment and less physical limitations, in the form of cell crowding, on the lead CNCCs. Lead CNCCs may in turn respond to molecular signals that stimulate proliferative activity. Alternatively, lead CNCCs may possess an intrinsic mechanism that regulates cell proliferative activity, independent of microenvironmental signals or cell crowding. Further investigation of differences in CNC proliferative activity depending on cell position within a stream and cell orientation during division will help to shed light on mechanisms that regulate proliferation of CNCCs along the migratory route.

Differences in NCC proliferative activity depending on cell position within a migratory stream have been revealed during enteric NC migration. In the mouse gut, NCCs within the front of the cell strands actively proliferate more than trailing cells (Simpson et al., 2007). This spatial bias of cell proliferative activity was not due to intrinsic mechanisms within NCCs at the front, but rather a function of proximity to uninvaded tissue (Simpson et al., 2007). Therefore, cell proliferation is an important component of directed NCC target invasion.

Cranial neural crest cells are not restricted to a particular migratory stream

CNCCs that enter a particular discrete migratory stream are not restricted to remaining in this stream and to populating the segmental branchial arch target directly lateral to the rhombomere level at which the cell exited. Cell contact between CNCC migratory streams tends to occur near the branchial arches. CNCCs that reach the branchial arches, only to find the arch full of other NCCs may change direction to contact a neighboring migratory stream (Kulesa and Fraser, 2000). The cell contact between streams is not a wholesale rearrangement, but rather a small number of cells form a cellular bridge between the streams by entering typically neural crest cell free zones at the entrances to the branchial arches. This cell-to-cell interaction between CNCC migratory streams can be exacerbated when either the cell's ability to read local inhibitory signals of the microenvironment are disrupted or the surface ectoderm adjacent to the presumptive neural crest cell-free zones is ablated (we discuss this in a later section).

Plasticity of cranial neural crest cell trajectories

CNCC trajectories are plastic and cells may respond to changes in their local microenvironment. CNCCs may alter their trajectories when physical barriers are introduced

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in advance of the migratory front. Interestingly, chick CNCCs can overcome a physical barrier or ablation of neighboring cells and re-target to branchial arch destinations. When a barrier is placed in front of a CNCC migratory stream, lead cells encounter the barrier and some lead and trailing cells are able to re-orient around the barrier and move towards the branchial arch target (Kulesa et al., 2005). CNCCs also respond to ablation of premigratory neighboring CNCCs and alter their trajectories to fill into less populated targets (Saldivar et al., 1997). When r5–r6 premigratory chick NCCs are ablated, neighboring r7 NCCs reroute their trajectories and fill in the second branchial arch, a target they do not normally invade (Kulesa et al., 2000). CNCCs also fill in for missing ablated neighbors in response to the addition of factor-soaked beads placed in the microenvironment (Creuzet et al., 2004). When mesencephalic and r1–r2 premigratory NCCs are ablated in chick, r3 NCCs are stimulated by exogenous Fgf8 to proliferate and migrate in a rostrolateral direction to fill in for missing neighboring cells in the first branchial arch (Creuzet et al., 2004). Thus, CNCC cell trajectories are not hardwired.

Cranial and trunk neural crest cells share migratory behavior characteristics

Features of CNCC migratory behaviors are mimicked in the trunk. In the trunk, spatio-temporal signals in the somites along the length of the neural tube guide NCCs within multiple migratory pathways that travel adjacent to, through, and dorsal to the somites to organize structures such as the peripheral nervous system (see review by Gammill and Roffers-Agarwal in this volume). Trunk NCCs may migrate in chain-like arrays (Kasemeier-Kulesa et al., 2005; Krull et al., 1997) or reverse direction back towards the neural tube (Kasemeier-Kulesa et al., 2005). Interestingly, trunk NCC trajectories are also unpredictable. Whether trunk NCCs also spread out along the dorsal neural tube before selecting a ventral migratory pathway or are restricted to a particular axial level is not clear. Cell labeling studies of premigratory trunk NCCs and endpoint analysis at the sympathetic ganglia show discrete ganglia are composed of NCCs that emerged from multiple axial levels (Yip, 1986). One explanation for this has been provided by data that show trunk NCCs that travel from the dorsal neural tube within a discrete migratory stream reach the dorsal aorta and spread out in the anteroposterior direction before ending up in a particular sympathetic ganglia (Kasemeier-Kulesa et al., 2005). Additionally, exchange of trunk NCC cells between neighboring discrete migratory streams may also take place within the somites along the migratory route (Kasemeier-Kulesa et al., 2005).

Thus, advances in cell labeling and improved in vivo imaging techniques have yielded two important facts: first, many factors contribute to the segmentation and the shaping of the caudal hindbrain streams; and second, significant species-specific differences exist in how the neural crest streams are shaped. The culmination of these features builds the picture of loosely connected, individually migrating CNCCs that display local and non-local cell contacts and complex cell contact behaviors that influence a cell's choice of direction.

Models of directed cell migration applied to the neural crest

There are several model mechanisms that have been proposed to explain the CNCC migratory pattern (reviewed in (Weston, 1982). We focus on the set of model mechanisms that seek to explain how CNCCs are directed from the hindbrain to the branchial arches along discrete migratory pathways. These model mechanisms include cell chemotaxis, cell nudging, population pressure and contact inhibition of movement, and polarized cell movement. In this section, we briefly describe details of the models and fill in emerging molecular data that support aspects of each model. Although it is conceivable that one unique model mechanism could account for the complex dynamics of the CNCC migratory pattern, we consider a scenario in which several model mechanisms could function in a

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coordinated manner to propagate the typical CNCC stream and produce the global migratory pattern.

First, contact inhibition of movement suggests that cells will move away from densely populated regions into less populated regions by direct cell-to-cell contact. Contact inhibition of movement was first described in fibroblasts in culture in the 1950s (Abercrombie and Heaysman, 1953; Abercrombie and Heaysman, 1954) and has recently been discovered as an in vitro and in vivo behavior in Xenopus CNCCs (Carmona-Fontaine et al., 2008). Time-lapse imaging has shown that Xenopus CNCCs move away from each other upon contact. By combining a fluorescent reporter for RhoA activity, the authors were able to show an increase in RhoA during CNCC neighbor contact (Carmona-Fontaine et al., 2008). The authors show that CNCC contact leads to contact inhibition of movement that in turn activates the planar cell polarity signaling pathway (Carmona-Fontaine et al., 2008). The authors suggest that propagation of a CNCC migratory stream is the result of contact inhibition of movement.

It is evident that short-term interactions between NCCs may result in contact inhibition of movement, but whether this is the driving mechanism is unclear. In order for contact inhibition of movement to propagate a CNCC stream along a particular migratory pathway, there must be an additional mechanism(s), otherwise emerging CNCCs would spread out concentrically from a high-to-low density. Local inhibitory signals, that exist adjacent to r3 and r5 (Farlie et al., 1999; Trainor et al., 2002) could restrict CNCC movements to permissive corridors adjacent to r1-r2, r4, and r6–r7. When population pressure from newly emerging cells is combined with this restriction of AP movement, contact inhibition of movement would act to propagate the CNCCs towards the branchial arches. Although this model mechanism is conceivable, there is evidence that chick, zebrafish, and mouse NCCs use cell contact to promote movement, shown both in vivo (Jesuthasan, 1996; Schilling and Kimmel, 1994; Teddy and Kulesa, 2004) and in vitro (Davis and Trinkaus, 1981; Druckenbrod and Epstein, 2005; Young et al., 2004), and convey guidance information by touch (Fig. 2B,C). Additionally, cell-to-cell contact is necessary to propagate the stream forward in a contact inhibition of movement model. However, imaging of migratory NCCs in the mouse gut (Druckenbrod and Epstein, 2005; Young et al., 2004) and in vivo in axolotl (Keller and Spieth, 1984) have shown that directed migration can occur in absence of contact between NCCs. So, alternatively, CNCC contact inhibition of movement behaviors in the Xenopus embryo as compared to other animal model systems could be due to species differences.

CNCC contact with neighboring cells can lead to forward movement by a process called cell nudging, first described in Fundulus (Tickle and Trinkaus, 1976). In this model mechanism, CNCCs would exert a mechanical influence on each other (tension) that would cause membrane blebbing on the opposite side of the cell contact. Membrane blebbing would lead to lamellipodia protrusive activity and directed migration would follow. It is plausible that cell-to-cell contact would cause either intracellular signaling or even exchange of information through membrane channels to move a CNCC forward in the manner suggested by Tickle and Trinkaus (1976). Interestingly, Jesuthasan (Jesuthasan, 1996) found that NCCs can propel beads in culture and hypothesized the same force could be exerted on other NCCs by the observation they can adhere to one another and their protrusions thicken after contact. Further investigation with fluorescent activity reporters will be necessary to decipher whether this model mechanism plays a role in CNCC migration.

The third model suggests that NCCs have an intrinsic cell polarity that drives their directed migration towards the branchial arches. A polarized cell type has been linked to directed cell migration since the 1960s (Trelstad et al., 1967). In vitro culture assays of the eye (Bard and

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Hay, 1975) showed that NCCs move in a directed manner with a polarized morphology. This model mechanism is dependent on the ability of CNCCs to emerge from the neural tube and adopt a polarized morphology that is sustained over long distances. Time-lapse imaging of zebrafish CNCCs has recently shown that cells emerge and acquire direction shortly after delaminating from the neural tube (Berndt et al., 2008). However, other time-lapse imaging results have shown that CNCCs can reverse direction, adopt a diagonal trajectory from the neural tube to join a neighboring stream, or change direction after encountering a local inhibitory region (Kasemeier-Kulesa et al., 2005; Kulesa and Fraser, 1998; Kulesa and Fraser, 2000), that would be contradictory to sustained polarized cell movement. Also, it is not clear whether cell polarity results in directed migration or whether polarity is a consequence of directed migration. For example. polarized morphology may be a feature adopted by cells within a particular region of the migratory stream and influenced by local microenvironmental or cell-to-cell communication. This has been observed as a feature in trailing cells within chick CNCC streams that display a bipolar cell morphology whereas lead cells have multiple filopodia extending in many directions (Teddy and Kulesa, 2004).

The last model mechanism proposed for CNCC migration involves receptor-ligand mediated guidance cues and the chemotactic response of cells to microenvironmental signals. Recent exciting data have discovered evidence for NCC chemoattractants and inhibitory signals in the head, gut, and trunk. CNCCs may respond to non-permissive cues present in the NCC-free zones (Farlie et al., 1999; Kulesa and Fraser, 1998). When tissue overlying the CNCC migratory pathway is removed, cells may pass through these NCC-free zones and form cellular bridges between neighboring migratory streams (Golding et al., 2000). Alternatively, attractive or permissive cues could directly guide the NCCs to their final destinations, the branchial arches (McLennan et al., 2010). These ideas were supported by studies in which regions of the neural tube was rotated (Sechrist et al., 1994). When a portion of the neural tube was rotated 180°, so that rhombomeres 3 and 4 were transposed, cranial neural crest cells followed their normal migratory streams (Sechrist et al., 1994). Thus, the CNCC migratory streams may be sculpted by a combination of attractive and inhibitory cues.

The real challenge is to better understand how individual CNCCs respond to guidance cues and each other, and to develop strategies that include the interrogation of cell behaviors across multiple scales. The presence of both multipotent and more restricted progenitors within a migratory stream and differences in cell morphologies and filopodial dynamics make it clear we should assess whether every cell responds in a similar manner to the same signal. The first step is to identify the input and output of an individual cell and how spatial position is communicated between neighbors. Toward this goal, new technologies such as photoactivation cell labeling, fluorescent activity reporters, and targeted electroporation have enabled neural crest biologists to specifically visualize, and perturb CNCCs within different microenvironments along their migratory routes. We are now in a position to address key questions of the CNCC migratory pattern. First, what signals are provided by the CNC microenvironment to drive directed migration? Second, how are local signals communicated between CNCCs to produce a coordinated migratory stream?

Mechanisms of Cranial Neural Crest Cell Migration

Signals That Sculpt the Early CNCC Migratory Streams

Many guidance molecules have been shown to play a role in cranial NCC migration (see Table 1 for a list of known cues). To review the main molecular players in cranial NCC migration, we will evaluate the separate phases of migration. Several guidance cues found within the local microenvironment have been reported to sculpt and maintain the early aspects of the CNCC streams. These include ErbB4 (Golding et al., 2004;Golding et al.,

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2000), Eph/ephrin interactions (Adams et al., 2001;Davy et al., 2004;Mellott and Burke, 2008;Smith et al., 1997), chemokines (Olesnicky Killian et al., 2009) and neuropilin/ semaphorin interactions (Gammill et al., 2007;Schwarz et al., 2008).

ErbB4, a receptor for neuregulin that is typically expressed within r3 and r5, is involved in maintaining the NCC-free zone adjacent to rhombomere 3 in both mouse and chick (Golding et al., 2004; Golding et al., 2000). Mouse embryos lacking erbB4, as well as chick embryos electroporated with a dominant negative form of erbB4, display misrouted neural crest cells into dorsolateral r3 mesenchyme (Golding et al., 2004; Golding et al., 2000).

Ephs and ephrins have been shown to be involved in the maintenance of the cranial NCC streams in the chick as well as Xenopus and mouse (Adams et al., 2001; Davy et al., 2004; Mellott and Burke, 2008; Smith et al., 1997), however their specific function in different species is poorly conserved. In Xenopus, cranial NCCs originating from different rhombomeres express different Ephs and/or ephrins and it is this expression that plays a role in preventing intermingling between the cranial NCC streams (Smith et al., 1997). In the chick, neural crest cells express a variety of Eph receptors and membrane-bound ephrin ligands, which interact in a repulsive manner with cognate Eph/ephrins expressed in the mesenchyme to demarcate stream boundaries (Mellott and Burke, 2008). In the mouse, mutations in ephrin-B1 and ephrin-B2 disrupt neural crest guidance, resulting in NCCs breaching the borders that separate the streams (Adams et al., 2001; Davy et al., 2004). Such inconsistencies related to specific protein function combined with significant species-specific differences in function bring into question the extent to which the role of these guidance molecules is conserved in cranial NCC migration.

Signals that influence neural crest cell homing to the branchial arches

The most recently discovered key guidance receptors for directed CNCC migration are chemokines and neuropilins and their ligands. Chemokines, a family of small secreted cytokines, are found to play many roles during embryonic development to shepherd cells over long distances (Raz and Mahabaleshwar, 2009). Recently, Artinger and colleagues have shown an important role for CXCR4/SDF-1 signaling in the condensation and patterning of CNCCs in the pharyngeal arches (Olesnicky Killian et al., 2009). Neuropilin-1 and neuropilin-2 are expressed by cranial NCCs in both mouse and chick (Chilton and Guthrie, 2003; Eickholt et al., 1999; Gammill and Bronner-Fraser, 2002; Gammill et al., 2007; McLennan and Kulesa, 2007; Osborne et al., 2005; Schwarz et al., 2008). Cranial NCCs also express Plexin-A1 transcripts, a co-receptor for neuropilin, while semaphorin 3A and semaphorin 3F transcripts are expressed in odd-numbered rhombomeres (Eickholt et al., 1999; Osborne et al., 2005). Finally, neuropilin-1, neuropilin-2, semaphorin3A or semaphorin3F mutant mice display NC-cellular bridges directly adjacent to rhombomere 3, linking the r1/r2 stream to the r4 stream (Gammill et al., 2007; Schwarz et al., 2008). Chick CNCCs avoid substrates containing semaphorin 3A in vitro (Eickholt et al., 1999), suggesting that semaphorin-neuropilin interactions play a role in the initial sculpting CNCC streams.

Signals that influence neural crest cell entry into and invasion of the branchial arches

After the CNCCs undergo their initial migration in the segmental streams, they must invade their target destinations and then properly assemble into differentiated structures. For example, the NCCs in the rhombomere 4 stream must invade branchial arch 2 before forming facial bone and cartilage as well as cranial ganglia. Recently it has been shown that this is not a passive event but rather a highly regulated one that involves multiple guidance cues (see Table 1 for a list of known cues).

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Neuropilins not only play a role in the initial sculpting of the CNCC streams, but also in the invasion of target sites. When neuropilin-1 expression was knocked down in chick r4 NCCs (using Np-1 siRNA (Bron et al., 2004)), their initial migration was normal, but then they failed to properly invade branchial arch 2 (McLennan and Kulesa, 2007). This phenotype was specific to branchial arch 2 invasion as when premigratory Np-1 siRNA transfected CNCCs were transplanted directly into the branchial arch 2 microenvironment, they failed to migrate from the transplant site (McLennan and Kulesa, 2007). Furthermore, Np-1 siRNA CNCCs that had migrated to the entrance of branchial arch 2 and were transplanted back into r4, regained their migratory abilities (McLennan and Kulesa, in preparation). Recently, it has been shown that the ectoderm of branchial arch 2 expresses the neuropilin-1 ligand, vascular endothelial growth factor (VEGF) and VEGF has been shown to be a strong attractive cue for CNCCs both in vitro and in vivo (McLennan et al., 2010). This is an exciting example of chemoattraction-mediated NCC target invasion.

Recently, other CNCC chemoattraction factors have been identified. One example of CNCC attraction involves platelet-derived growth factor (PDGF). Experiments in zebrafish have demonstrated that PDGF attracts CNCCs to the oral ectoderm, and that this mechanism is modulated by the microRNA Mirn140 (Eberhart et al., 2008). The role of PDGF signaling in mouse CNCCs is less clear, but it is suggested to be involved not in migration but in differentiation or extracellular matrix deposition (Tallquist and Soriano, 2003). Finally, the branchial arch ectoderm expresses fibroblast growth factor receptor 1 (FGFR1) and at a lower level, FGFR2 (Trokovic et al., 2005). In hypomorphic FGFR1 mouse mutants, CNCCs fail to invade branchial arch 2 in a non-cell-autonomous manner (Trokovic et al., 2005).

Future Perspectives

The CNC model for cell migration includes complex cell behaviors that lead to programmed cell invasion in at least three phases of migration that include the acquisition of directed migration along the dorsolateral pathway, homing to the branchial arches, and entry into and invasion of the branchial arches (Figs. 1,2). We have learned that CNCCs sample their microenvironment and each other with short- and long-range filopodial dynamics. Cell contact between CNCCs promotes local follow the leader behavior or contact inhibition of movement. Filopodial dynamics and their consequence in CNCC behaviors offer overwhelming evidence for cell communication during migration that imparts direction information.

We also learned there are differences in CNCC morphologies and cell behaviors depending on position with respect to the migratory front. This appears to be a common feature of NCC migration in other regions of the vertebrate embryo, including gut and trunk. It is possible that lead cells experience microenvironmental signals and transmit information to trailing cells through filopodial contact or alteration of the microenvironment. In this way, cell behaviors may be distinct depending on cell position and acquisition, transmission, or receiving of guidance information. In contrast, phenotypic differences between migratory cells that begin to adopt a particular fate may alter their behaviors. Indeed, immature enteric neurons migrate slower and display a long-leading process that is distinct from the multipolar, multiple short filopodial extensions on neighboring, other enteric NCCs (Hao et al., 2009). Given the heterogeneous composition of the CNCC migratory streams as multipotent and more restricted progenitors cells, it is possible that differences in cell migratory behaviors are manifested along the migratory routes to the branchial arches. Thus, it will be very exciting to correlate differences in cell migratory behaviors with both cell position and cell fate.

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The identification of the neuropilin/semaphorin/VEGF signaling pathway and interplay of distinct co-receptors at different phases of the CNCC migration program suggest a strategy of chemoattraction and local inhibition (Gammill et al., 2007; McLennan and Kulesa, 2007; McLennan et al., 2010). It is conceivable that the CNC respond to chemoattraction and cell contact guidance that in combination with local inhibitory cues provide directional information for cells to maintain discrete streams and reach long distance targets. However, it is important to recognize that expression of VEGF in the surface ectoderm is not a typical gradient form until near the entrance to the 2nd branchial arch (McLennan et al., 2010). Thus, VEGF signal may play a role to stimulate non-directed CNCC movement to the 2nd branchial arch entrance, then play a chemotactic role to direct CNCCs into and within the branchial arch. A similar model mechanism appears in the trunk. Early emerging trunk NCCs are restricted to migrate through the rostral somites by an interplay of neuropilin/semaphorin signaling (Gammill et al., 2006) and are thought to be guided over long distances to the dorsal aorta by chemokine signaling (Kasemeier-Kulesa, in preparation; Y. Takahashi, personal communication). When neuropilin/semaphorin signaling is disrupted in mouse, cells continue to reach and are sculpted into discrete sympathetic ganglia (Gammill et al., 2006); the latter morphogenesis by local molecular mechanisms in the normal embryo (Kasemeier-Kulesa et al., 2006). This evidence demonstrates that local inhibitory cues and chemoattraction may work in unison to maintain a discrete NCC migratory stream to a precise location.

For VEGF to play a CNCC chemoattractant role all along the migratory route, an interesting issue is then how does a VEGF chemotactic gradient response become established from a uniformly expressed VEGF signal in the ectoderm? One explanation is that lead CNCCs may simply bind VEGF ligand to create a VEGF sink, proximal to the migratory front. CNCCs within the migratory front would sense the distal gradient and continue to move forward. Guidance information would have to be communicated from cells within the migratory front to trailing cells. This model assumes the lack of resupply of VEGF ligand to the migratory route microenvironment proximal to the migratory front, within the timeframe of CNCC migration. Alternatively, mesenchymal tissue or trailing CNCCs may sequester VEGF ligand to create a sink, such that lead cells sense a gradient and continue proper directed migration downstream. In support of this model, recent data in zebrafish primordial germ cell migration has shown that ubiquitously expressed SDF-1a ligand is sequestered by somatic cells expressing CXCR7 (Boldajipour et al., 2008), thus revealing a local source-sink mechanism to direct CXCR4b-expressing cells toward their targets. Whether this type of mechanism is mimicked during CNCC migration and what molecular mechanisms control the distribution of VEGF chemoattractant in vivo are for future investigation.

We presented several potential model mechanisms that might regulate the CNCC migratory pattern. What is emerging is the potential for multiple model mechanisms to co-exist, but play a critical role during the different phases of CNCC migration. This is supported by changes in cell morphology, migratory behaviors, and proliferative activity depending on cell position within a CNCC migratory stream. We suggest a unified model in which chemoattractant and repulsive mechanisms integrate with cell contact guidance and contact inhibition of movement to generate the CNCC migratory pattern. One possibility is that contact inhibition of movement may be an active mechanism for CNCCs at the migratory front. This would allow lead CNCCs to survey larger subregions of uninvaded tissue, without hindrance from cell-to-cell contact, and select a direction of migration from external cues. Once lead CNCC direction is chosen, this information may be communicated to trailing CNCCs that rely on cell-to-cell contact guidance information for direction. Future studies to dissect out how information is propagated between CNCCs will be important.

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The discovery and further investigation of cell guidance and fate determination signals within the CNC microenvironment have the potential to provide significant value to cancer biology (Fig. 3). We have discussed how multipotent NCCs demonstrate the ability to spatially distribute along programmed pathways and undergo cell fate specification. Interestingly, two of the most aggressive cancer cell types include NC-derived melanoma and neuroblastoma, however it is unclear whether these cancer cell types recapitulate aspects of their embryonic invasion program during metastatic events (Hendrix et al., 2007; Yang and Weinberg, 2008). During cancer progression, multipotent melanoma cells secrete and receive molecular cues that promote tumor growth and metastasis (Postovit et al., 2006;Uong and Zon, 2010). Whether signals within the embryonic NC microenvironment may reprogram the metastatic phenotype of their ancestrally-related cancer cell types is a fertile area of research with potential for differentiation and anti-metastatic therapies (Abbott et al., 2007). In vivo and in vitro studies are underway to understand the embryonic signals that downregulate and silence the expression of genes associated with the metastatic phenotype(Hendrix et al., 2007;Kasemeier-Kulesa et al., 2008;Kulesa et al., 2006). Furthermore, information is emerging on signals that regulate CNC stem and progenitor cells (Dupin et al., 2010; Le Douarin, 2004; Sieber-Blum and Hu, 2008; Sommer, 2006). Together, it will be exciting to determine the potential for reprogramming the metastatic phenotype by working at the interface between embryonic and tumorigenic signaling pathways associated with the NC and NC-derived cancers.

In summary, we have gained new insights into the potential of the CNC microenvironment to sculpt discrete cell migratory streams and direct cells into specific branchial arches throughout the three phases of CNCC migration. What has clearly emerged is a picture whereby single models proposed over thirty years ago may be brought together in combinations to explain how complex mechanisms of CNCC migration are coordinated in space and time to produce the CNC migratory pattern. Data from in vivo imaging and targeted molecular perturbation have begun to add a molecular basis to the complexity of CNCC migratory behaviors. Now that some of the CNCC guidance signals are known, future migration studies will need to determine how dynamic changes in the guidance signals are regulated in space and time. The discovery of key signaling pathways that underlie CNCC migration may help to devise new therapeutic strategies to migration-derived birth defects and allow us to better understand events of NC-derived cancer cell invasion.

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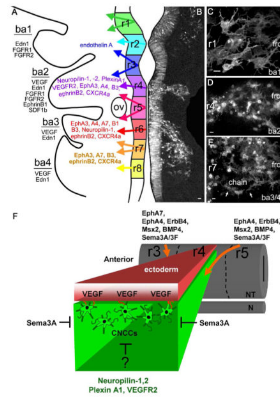

Figure 1. The cranial neural crest cell migratory pattern; cellular features and signaling pathways

(A) A schematic representation showing recently discovered key guidance cues involved in CNCC migration. (B) The cranial NCCs migrate in 3 distinct streams as seen by membrane (Gap43-GFP) and nuclear (H2B-mCherry) labeling (introduced into premigratory NCCs by electroporation delivery) reduced to grayscale for clarity. (C) CNCCs that emerge from mid-r3 and more rostral migrate in a broad wave and display multiple filopodial protrusions. (D) CNCCs that emerge mid-r3 to mid-r5 are sculpted into a tight stream adjacent to r4 that spreads out at the front (E) Post-otic NCCs that emerge from mid-r5 and more caudal migrate as an initial wave, followed by NCCs that form chain-like arrays. The arrows point to cells that travel in a chain-like array. (F) A schematic representation of the molecules guiding the r4 NCC stream. The r4 NCCs express neuropilins, Plexin A1 and VEGFR2. The overlaying ectoderm expresses VEGF, which is a NCC chemoattractant. R3 and r5 secrete semaphorin3A, which is a NCC inhibitor. A guidance cue that prevents the r4 NCCs migrating ventromedially is as yet unknown (?). r, rhombomere; ba, branchial arch; OV, otic vesicle, NT, neural tube, N, notochord. The scale bars are 20um in (B) and 10um in (C–E).

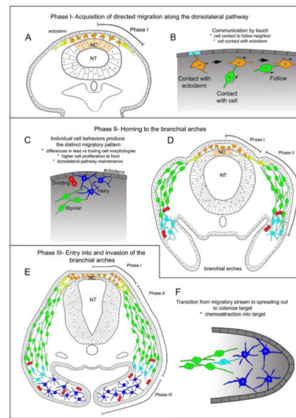

Figure 2. The three phases of cranial neural crest cell migration and characteristic cell behaviors. Phase 1: Acquisition of directed migration along the dorsolateral pathway

(A) Cranial NCC migration starts around Hamburger and Hamilton (HH) Stage 11 in chick. During Phase I, the lead cranial NCCs emerge from the dorsal neural tube (beige). Initially the cranial NCCs do not exhibit directed orientation (orange NCCs), but within a short distance from the dorsal neural tube, they acquire directionality (yellow NCCs). (B) The cranial NCCs communicate with each other and the microenvironment, by touch. First, a NCC touches the ectoderm and receives direction information. Second, there is follow-the-leader behavior, where one NCC touches another, and then with or without filopodia retraction, follows the lead NCC. **Phase II: Homing to the branchial arches.** (C) After acquiring directionality, cranial NCCs migrate in a directed manner and exhibit a bipolar phenotype (green NCCs). Along the migratory route, cranial NCCs stop, retract filopodia and divide (red NCCs). As they invade the target site, the cranial NCCs extend multiple filopodia in all directions (light blue NCCs). The migrating cranial NCCs have intimate contact with the overlaying ectoderm and local microenvironment. (D) Cranial NCCs continue their migration toward their target sites through HH St14 in chick. During Phase II, NCCs migrate in a highly directed manner towards their target site, in this case branchial ach 2. **Phase III: Entry into and invasion of the branchial arches.** (E) Cranial NCCs continue to migrate and by HH St 17, they have invaded and colonized their target sites. During Phase III, NCCs transition from being loosely connected with one another to spreading out to fill the entire target site, branchial arch 2. (F) As the cranial NCCs enter the arch, they spread out from one another and display multiple filopodia in all directions (dark blue NCCs). NT, neural tube; NC, neural crest.

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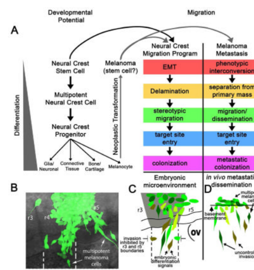

Figure 3. Common features of the multipotent neural crest cell and neural crest-derived cancer cell metastatic program

The neural crest migration program shares many similarities to melanoma metastasis. (A) A cartoon depicting the neural crest migration program and developmental potential. Neural crest stem cells give rise to a multipotent neural crest cell population that emigrates to a specific, defined site of differentiation and gives rise to diverse cell types including pigment cells. Following neoplastic transformation, melanocytes display many stem-cell-like traits, suggesting that melanoma cells reacquire specific neural crest attributes. The neural crest migratory program parallels many aspects of melanoma metastasis, and when aggressive human melanoma cells are transplanted into the chick embryonic neural crest microenvironment, they exhibit behaviors typical of neural crest migration. (B) GFP-labeled c8161 human melanoma cells transplanted into the chick neural tube at the rhombomere 4 (r4) axial level exit the dorsal neural tube and migrate along the r4 neural crest migratory pathway while generally avoiding the NC-free zones. (C) The schematic shows that human melanoma cells respect the host embryonic neural crest cell-free zones adjacent to r3 and r5, and a subset of the invading human melanoma cells may be influenced by the host embryonic neural crest microenvironment to express genes characteristic of a neural crest-like phenotype (data in Kulesa et al., 2006). The neural tube region of r4 and the boundaries between the host r4 NCC migratory stream and neural crest cell-free zones are highlighted. (D) In comparison, a schematic representation of in vivo metastatic dissemination highlights the unprogrammed invasion of NC-derived tumor cells in the human microenvironment.

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Table 1

Phase of migration	Cue	Proposed Role	Reference
Delamination	Slug	Involved in the epithelial to mesenchymal transition	(Nieto et al., 1994)
Delamination	RhoB	Necessary for correct delamination of NCCs	(Liu and Jessell, 1998)
Delamination	Cadherins	Cell-cell adhesion molecules that control the timing of emigration, delamination and migration	(Borchers et al., 2001; Coles et al., 2007; Kashef et al., 2009; McCusker et al., 2009; Taneyhill, 2008)
Migration	Hox genes	Maintain segmental identity of cranial NCCs	Reviewed by (Trainor and Krumlauf, 2000)
Migration	Integrins	Mediate NCC motility on fibronectin in avian, Xenopus and mouse	(Alfandari et al., 2003; Strachan and Condic, 2003; Strachan and Condic, 2008)
Migration	Chemokines	Regulate cell migration and patterning in zebrafish	(Olesnicky Killian et al., 2009)
Migration	EphA4, EphB1 and ephrin-B2	Prevent intermingling of third and second arch Xenopus NCCs	(Smith et al., 1997)
Migration	Multiple Ephs and ephrins	Restricts avian and murine NCCs into streams by Inhibiting migration into NCC- free zones	(Adams et al., 2001; Davy et al., 2004; Mellott and Burke, 2008)
Migration	Neuropilin-1 and Semaphorin- 3A, -3F	Avian and murine cranial NCCs express neuropilin-1 and are repelled by semaphorin-3A	(Eickholt et al., 1999; Gammill et al., 2007; Osborne et al., 2005; Schwarz et al., 2008)
Migration	Neuropilin-1a,- 1b, -2a, -2b and Semaphorin- 3Fa, -3Ga	Restricts zebrafish NCCs into streams by inhibiting migration into NCC-free zones	(Yu and Moens, 2005)
Migration	Wnt11r	Promotes Xenopus cranial NCC migration	(Matthews et al., 2008)
Migration	Myosin-X	Promotes migration and segregation of Xenopus cranial NCCs	(Hwang et al., 2009; Nie et al., 2009)
Induction, Migration and Differentiation	BMPs	Multiple roles	Reviewed by (Nie et al., 2006)
Migration	Retinoic Acid	Mediates the segmental migration of cranial NCCs	(Dupe and Pellerin, 2009; Menegola et al., 2004); (Lee et al., 1995); (Pratt et al., 1987)
Migration	RhoA	Influences migration rate and filopodia dynamics	(Rupp and Kulesa, 2007)
Migration and differentiation	Laminin alpha5	Required for proper migration and timely differentiation of a subset of murine cranial NCCs	(Coles et al., 2006)
Migration and differentiation	Disc1	Represses transcription of foxd3 and sox10	(Drerup et al., 2009)
Migration	ErbB4	Maintains the r3-adjacent NCC-free zone	(Golding et al., 2004; Golding et al., 2000)
Migration	Chokh/rx3	Mutant chokh/rx3 zebrafish lack eyes and have disorganized NCC dorsal anterior migration	(Langenberg et al., 2008)
Target invasion	Neuropilin-1 and VEGF	VEGF attracts neuropilin-1 expressing NCCs into branchial arch 2	(McLennan and Kulesa, 2007; McLennan et al., 2010)

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Phase of migration	Cue	Proposed Role	Reference
Trigeminal ganglion formation	Neuropilin-2 and Semaphorin- 3F	Mice with null mutations in either molecule display improperly formed ganglia	(Gammill et al., 2007)
Trigeminal ganglion formation	Robo2 and Slit1	Disruption of either molecule results in disorganized ganglia	(Shiau et al., 2008)
Palatogenesis	PDGF and MicroRNA Mirn140	PDGF is required for NCCs to contribute to cranial mesenchyme and attracts zebrafish NC-derived palatal precursors	(Eberhart et al., 2008; Tallquist and Soriano, 2003)
Target invasion	FGFR1	Provides a permissive environment for NCC migration into branchial arch 2	(Trokovic et al., 2005)
Target invasion	Endothelin-1 and endothelin A receptor	Required for proper migration into or within the arches	(Abe et al., 2007; Clouthier et al., 2003; Pla and Larue, 2003); (Clouthier et al., 2000)
Survival and proliferation	Msx1 and Msx2	Mouse mutants display impaired cranial NCC patterning, survival and proliferation	(Han et al., 2003; Ishii et al., 2005)
Survival and/or differentiation	B-catenin	Conditional inactivation of B- catenin results in increased apoptosis in mouse cranial NCCs and craniofacial malformations	(Brault et al., 2001)
Survival	Sonic Hedgehog	Reduction in sonic hedgehog signaling leads to increased neural tube and NCC death	(Ahlgren and Bronner-Fraser, 1999; Jeong et al., 2004)
Survival and differentiation	Dlx2	Involved in survival of zebrafish cranial NCCs and differentiation of sensory ganglia	(Sperber et al., 2008)
Survival, proliferation and differentiation	Pinch1	Required for multiple steps for the development of murine cranial NCC-derived structures	(Liang et al., 2007)
Proliferation	TGF-beta	Mediates FGF signaling which is required for cranial NCC proliferation	(Iwata et al., 1999; Oka et al., 2008; Sasaki et al., 2006)
Proliferation and differentiation	FGF2	Depending on the concentration of FGF2, either proliferation is enhanced or cartilage differentiation is induced	(Sarkar et al., 2001)