Mechanisms driving neural crest induction and migration in the zebrafish and *Xenopus laevis*

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The neural crest is an evolutionary adaptation, with roots in the formation of mesoderm. Modification of neural crest behavior has been critical for the evolutionary diversification of the vertebrates and defects in neural crest underlie a range of human birth defects. There has been a tremendous increase in our knowledge of the molecular, cellular and inductive interactions that converge on defining the neural crest and determining its behavior. While there is a temptation to look for simple models to explain neural crest behavior, the reality is that the system is complex in its circuitry. In this review, our goal is to identify the broad features of neural crest origins (developmentally) and migration (cellularly) using data from the zebrafish (teleost) and *Xenopus laevis* (tetrapod amphibian) in order to illuminate where general mechanisms appear to be in play and, equally importantly, where disparities in experimental results suggest areas of profitable study.

**Introduction**

“Ectoderm and endoderm are primary germ layers; they were the first to appear in animal evolution and are the earliest to form embryologically, being present in the unfertilized egg. Mesoderm is a secondary germ layer; it is not preformed in the vertebrate egg but arises following inductive interactions between ectoderm and endoderm. Like mesoderm, the neural crest arises very early in development and gives rise to very divergent cell and tissue types.”—B.K. Hall

To understand how a tissue such as the neural crest forms and functions, it helps to understand where it comes from, in both evolutionary and embryological terms. Mesoderm has been termed a “secondary” germ layer, since it arises early in embryogenesis through inductive interactions between maternal ectodermal and endodermal determinants.2,3 In that light, neural crest might well be considered a “tertiary” germ layer, since its induction depends upon mesoderm, which acts on ectoderm to induce the differentiation of the neuroectoderm (the nascent nervous system) and neural crest from the embryonic epidermis. The interaction with mesoderm not only induces the neuroectoderm, but also serves to pattern it, with signals from the axial mesoderm/notochord acting to specify the ventral-dorsal axis of the neural tube, while signals from dorsolateral mesoderm are involved in the induction and patterning of the neural crest. Later in development, the neural crest region abuts regions of lateral dorsal mesoderm, which secretes signaling agonists and antagonists as well as extracellular matrix materials through which the neural crest cells migrate toward their final destinations within the later stage embryo.

The nascent epidermis and neuroectoderm-neural tube are characterized by differences in bone morphogenic protein (BMP) (throughout, genes names are indicated by the use of lower case italics, proteins are capitalized and non-italic) signaling. The BMPs are members of the transforming growth factor-β (TGFβ) family of secreted signaling molecules, and act through surface receptors and SMAD-type transcriptional regulator proteins.4-6 BMP signaling, which also corresponds to the most dorsolateral aspect of the neuroectoderm, is high in the epidermis and low in the neuroectoderm; the neural crest forms in the region of intermediate BMP signaling, which also corresponds to the most dorsolateral aspect of the neuroectoderm. In addition to BMP secretion, BMP signaling is controlled by secreted antagonists7 as well as intracellular regulators.8 The simple intermediate-gradient model of neural crest induction implies that neural crest induction occurs after neuroectodermal induction, but there is clear evidence that neural crest induction begins during gastrulation, concomitantly with neural induction.9

**Evolutionary Considerations**

This review focuses on two model organisms, the teleost fish *Dario rerio* (zebrafish) and the amphibian tetrapod *Xenopus laevis*. Much of the pioneering work on neural crest was carried out in other experimental systems, particularly the chick and chick/quail chimeras, pioneered by Nicole Le Douarin,10 and a number of more recent studies have attempted to place the origins of the neural crest in an evolutionary context through studies of organisms such as the lamprey11-13 and the cephalochordate Amphioxus.14,15 Given the length of evolutionary time (in the hundreds of millions of years) since these various organisms shared...
a common ancestor (Fig. 1), it is not surprising that there have been substantial changes in the molecular and cellular underpinnings of what appears, superficially, to be the same process—that is, neural crest formation, migration and differentiation. Such drift can occur in even closely related species and can be mechanistically significant. 16,17 An obvious example, with respect to the neural crest, is the “swapping” of expression patterns between the paralogous zinc-finger transcription factors slug/snail2 and snail1 in different species. 18 This means that the organismic context of a particular study is critical, since results from one organism may or may not be applicable to others; in this light, it is surprising that in many studies the species examined is not identified explicitly in the abstract! This caveat is worth taking seriously, given that mammals are derived from synapsids, rather than archosaurs, like birds (Fig. 1),19 and there can be apparently dramatic differences in the function of orthologous gene products. For example, while slug/snail2 appears to be required for neural crest formation in Xenopus and chick,20-22 this does not appear to be the case in the mouse. 23

**General Models of Neural Crest Induction, Specification and Migration**

When we think about the mechanisms (or perhaps better, the circuitry) of developmental processes, it is worth remembering that they are a cascade, with beginnings either in earlier asymmetries or asymmetries that arise through stochastic processes. 24,25 This makes it critical that we distinguish between cellular and molecular events that lead to the initiation of inductive effects from those involved in maintaining and/or “propagating” their effects. Rather than a “clean” linear pathway, it is common (and perhaps universal) to find that the pathways leading to a differentiated phenotype involve a number of interactions that are either co-dependent or required to maintain a (perhaps only slightly) earlier step in the regulatory cascade; feedback and feedforward systems are rampant in embryological processes. As noted previously, 26 “Every neural crest specifier examined thus far also appears necessary and/or sufficient for the expression of the other specifiers in Xenopus.” Because of this “backward” connectedness, it can be difficult (and sometimes meaningless) to identify unambiguously who comes first during a process, such as neural crest induction. This is complicated by the fact that many studies of gene expression during development rely primarily on in situ hybridization visualization of mRNA to determine the order in which genes are expressed. Unfortunately, in situ hybridization does not reveal the on-set of gene expression (it requires the accumulation of sufficient RNA for detection) or when the level of gene product reaches a concentration sufficient to produce physiological effects. So the question becomes, what gene is required to be expressed first? This may be difficult to discern since maintenance and co-dependent effects may be so closely linked to the “initial” step as to be functionally indistinguishable. Moreover, as most investigators who have examined the global effects of manipulating gene expression can attest, generally the expression levels of many hundreds of genes change in response to experimental/genetic perturbations. While this has the advantage of producing (at least for awhile) a steady stream of “new” and “earliest” regulators, it tends to obscure the interconnectedness of the process under study.

Keeping such issues in mind, Sauka-Spengler and Bronner-Fraser 27 have reviewed the hierarchy of genes involved in what they call the neural crest gene regulatory network (reviewed in ref. 26). Their “unified” network includes inducers (BMPs, Wnts and fibroblast growth factors (FGFs)), neural plate border specifiers (zic1, mxe1, mxe2, dlx3, dlx5, pax3 and pax7), neural crest specifiers (snail1, slug/snail2, sox8, sox9, sox10, foxd3, ap-2, twist, c-myc and id family members) and effector genes specifically expressed in each of the various cell types into which neural crest cells differentiate (see below); these include the neurons and glia of the peripheral nervous system, cartilage, bone, connective tissue, pigment cells and sympatho-adrenal cells. 10,28 Additionally, in species that have unpaired fins, such as amphibian tadpoles and fish (e.g., the zebrafish), neural crest cells contribute to the fin mesenchyme. 29-31

Neural crest derivatives occupy various positions along an organism’s rostral-caudal axis and are categorized as cranial, vagal, trunk and sacral. 32 The cranial neural crest forms first and appears to be specified during gastrulation (at least in Xenopus and likely in zebrafish). More posterior neural crest form later and perhaps are subject to different inductive and migratory signals, in analogy to the mechanisms involved in the origin of trunk and tailbud dermomyotome. 33,34 Following their initial specification,
neural crest cells migrate into various regions of the embryo; their fate appears to be determined in part based on their axial location.\textsuperscript{35} While it is obvious (see below) that neural crest cells are migratory, it is interesting that, at least in the chick trunk, inert latex beads and non-migratory cells inserted into the neural crest region “migrate” in a manner similar to neural crest cells, suggesting that at least some of the mechanisms driving neural crest cell migration are passive.\textsuperscript{36,37} Apparently, inert objects (and cells) can be “carried along” by the neural crest stream. While there are general mechanisms common to both Xenopus and zebrafish neural crest development, we have separated the sections to focus on them separately since it is these differences that will help shed light the process. Where they are similar, we will make a note as such.

**Neural Crest Induction and Migration in Xenopus**

Perhaps the most dramatic dichotomy reported in neural crest induction involves the role of mesoderm. Ragland and Raible\textsuperscript{38} reported that signals from mesoderm “are dispensable for zebrafish neural crest induction,” although not for their normal migration (see below). This was a surprising finding, given the report by Bonstein et al.\textsuperscript{39} that paraxial mesoderm, which corresponds to the dorsal lateral marginal zone of the late blastula/early gastrula stage embryo, is required for neural crest induction in Xenopus. This discrepancy may represent a species difference; however, we side with the notion that the mesoderm is generally important for neural crest specification. There are several reasons for our interpretation. First, the mesoderm and neural crest are in very close proximity during development, and it is likely that interactions between them continue throughout neural crest development, including induction and migration. In addition, several factors that are expressed in neural crest cells have been co-opted from the mesoderm, which suggests they may similarly respond to inducer genes. We look forward to analysis of the role of genes such as $slug/snail2$, $snail1$, and $twists$ for their effects on mesoderm formation in zebrafish.

Inductive interactions between the ectoderm and neural plate underlie neural crest formation. In studies carried out in the axolotl, the juxtaposition of neural plate and epidermis gives rise to pigment cells (a neural crest derivative) and both neural plate and epidermal cells can produce neural crest cells.\textsuperscript{40} More recent experiments revealed the upregulation of molecular markers of neural crest, such as $slug/snail2$,\textsuperscript{41} and the appearance of Rohon-Beard sensory neurons, a cell type that forms at the neural plate border and may be related to neural crest cells and the induction of $XBlimp-1$ (PRDM1) in such explants in Xenopus.\textsuperscript{42}

This raises another issue, namely the rather “mixed up” nature of the early Xenopus fate map; a point that was made explicitly by Wardle and Smith,\textsuperscript{43} who noted the presence of “rogue” cells in late blastula/early gastrula stage embryos that expressed gene markers outside of their expected expression domain. This type of behavior is illustrated in Figure 2: an early gastrula stage embryo has been stained in situ for $endodermin$ mRNA, a marker of endoderm; not only are rogue cells found outside of the main endodermal domain, but within the endodermal domain there are cells that fail to express $endodermin$ (we might term them recalcitrant cells); they are presumably expressing markers for other developmental fates. As development proceeds into the late gastrula/early neurula stage, the frequency of these rogue cells decreases.\textsuperscript{43} It would be extremely useful if the pattern of gene expression could be followed in real time by combining real-time imaging with molecular methods like the brainbow technique.\textsuperscript{44,45}

What is clear is that there is substantial mixing of cell fates at early stages. In two recent elegant papers,\textsuperscript{47,48} the investigators injected their experimental reagents into the dorsal-animal blastomere of an 8-cell embryo to target neural crest, and to avoid influencing mesodermal tissues. As can be seen from the fate map (Fig. 3 illustrates the 8/16-, 32-cell and late blastula/early gastrula fate maps, reviewed in refs. 9, 49–52), this is practically difficult if not impossible, and such experiments need to be carefully controlled by looking explicitly for effects on mesodermally-derived tissues; the myotome is a good choice since it is easy to visualize and the expression of muscle markers begins early.\textsuperscript{53} In the absence of explicit evidence, we cannot exclude developmentally significant cross talk between tissues, particularly given the increasing evidence for such cross-talk, mediated by secreted and extracellular matrix factors (see below).

**Developmental Cascades: Mesoderm and Neural Crest Formation**

In Xenopus, and a range of other organisms, there is a maternally-supplied animal-vegetal axis that underlies ectoderm and endodermal germ layer specification (Fig. 4). In Xenopus, signals from endoderm, activated by the maternally supplied and vegetally localized T-box transcription factor VegT act, together with F-type Sox transcription factors to turn on and maintain
the expression of Nodal-type TGFβ family secreted proteins, while animally localized B1-Sox proteins, as well as other factors, such as Eomesoderm,54 Coco55,56 and Xema,57 all targets of Sox3 regulation, act to restrict the extent of Nodal signaling.3 This VegT/Nodal cascade leads to the zygotic expression of a number of genes, among which are those that encode the T-box transcription factors Eomesoderm,58 Brachyury (there are in fact three distinct brachyury genes in Xenopus),59-61 the zygotic splice variant of VegT, known as Antipodean,62,63 and the T-box transcription factors Tbx6 and Tbx6-related.64-67 Recent studies indicate that downregulation of zygotic VegT/Antipodean is critical for paraxial mesoderm patterning;68 in our ongoing studies, we find that inhibiting the expression of antipodean has little effect on neural crest, whereas inhibiting the early embryonic expression of antipodean and xbra together inhibits neural crest and myotome formation.69 In a similar vein, morpholino-based inhibition of slug/snail2, snail1 or twist expression leads to the inhibition of antipodean and xbra expression, myotome and neural crest formation.22,69,70
Defining Mesoderm-Derived Signals Involved in Neural Crest Induction

Given the clear role of mesodermally derived signals in neural crest induction in Xenopus, a number of studies have investigated the nature of these signals. These studies must be considered in light of the fact that the situation in the late blastula/early gastrula embryo is dynamic and self-regulating.\(^{71,72}\) More to the point, during neural crest specification (early during gastrulation) a plethora of secreted factors are expressed. In the dorsal region these include the BMP antagonists Chordin,\(^{73}\) Noggin,\(^{74}\) Follistatin,\(^{75}\) the Wnt antagonists Frzb-1,\(^{76}\) Dickkopf,\(^{77}\) and Crescent;\(^{78}\) the anti-dorsalizing morphogenic protein (ADMP),\(^{79}\) BMP2,\(^{80}\) and IGFBP5,\(^{81}\) and the multifunctional antagonists Cerberus (anti-Wnt, BMP, Nodal)\(^{82}\) and Shisa-1 and Shisa-2 (anti-FGF, anti-Wnt).\(^{83,84}\) Similarly, the ventral organizing center has been found to secrete the anti-chordin Xolloid,\(^{85-87}\) the anti-Wnt and anti-Xolloid Sizzled,\(^{88}\) BMPs,\(^{89-92}\) the BMP signaling modulator Crossveinless-2 (CV2),\(^{93}\) and the BMP antagonist Twisted Gastrulation (Tsg).\(^{94,95}\) In zebrafish, BMP and Wnt requirements for neural crest induction is thought to be conserved (see below), although the details of the directness of these interactions remains to be tested. Later in development, other secreted inhibitors have been found to be involved in neural crest differentiation, e.g., the Wnt antagonist WIF-1,\(^{96}\) and the BMP antagonist Gremlin.\(^{97}\)

Such secreted factors initially attract interest because manipulating their expression levels produces interesting effects on embryogenesis, and generally a specific signaling pathway is identified as responsible, but this often turns out to be over-simplistic. Take for example the Frizzled-like Wnt inhibitors; these proteins have been found to display a range of effects in various systems, from extending the diffusive reach of Wnt signaling\(^{98}\) to antagonizing each other’s activities.\(^{99}\) In an example particularly relevant to Xenopus, the Frizzled-related protein Sizzled was originally identified as a ventrally expressed Wnt inhibitor but later found to act in a Wnt-independent manner,\(^{100}\) through its effects on the secreted metalloproteinase Tolloid-related,\(^{101}\) which itself is an an inhibitor of Chordin, a dorsally secreted BMP inhibitor. This activity appears to be evolutionarily conserved, at least in the zebrafish.\(^{102}\) Interactions with proteinases may be a common feature of this class of protein, since Frizzled-related protein 2 enhances the activity of the procollagen C proteinase;\(^{103}\) Sizzled and related proteins may regulate the activity of as yet unidentified extracellular proteins.

**Inductive Interactions**

Early studies indicated that Wnts (Wnt1, Wnt3a, Wnt7a) induced increased expression of neural crest markers in Xenopus embryos and ectodermal (animal cap) explants and that Wnt signaling was required for neural crest formation.\(^{104-106}\) The frizzled-7 and -3 Wnt receptors,\(^{107,108}\) the Kremen and Kermit receptor associated proteins,\(^{109,110}\) and the splicing factor/transcription co-regulatory/splicing factor Skip\(^{111}\) have all been implicated in neural crest specification. In their studies of mesoderm-neural crest inductive interactions Monsoro-Burq and colleagues\(^{112,113}\) found a dependence upon FGF-mediated process; they proposed that FGF8 and Wnt signaling acted in parallel, and mediate expression of **msx1** and **pax3**, which in turn activate **slug/snail2** expression. They proposed a role for FGF in neural crest induction.\(^{114}\) FGF signaling can modulate Wnt signaling by effects on the LEP/TCF-binding co-repressor Groucho-related 4.\(^{115}\) Two essential targets of Wnt-induced neural crest specification are **ap2**,\(^{116}\) and **gbx2**, which encodes a homeobox transcription factor.\(^{117}\)

Subsequently, Hong et al.\(^{118}\) reported that FGF acts by inducing Wnt8 in paraxial mesoderm, which in turn acts to induce neural crest, while Wu et al.\(^{119}\) reported that Wnt signaling could induce neural crest even when FGF signaling was blocked. A recent analysis of mesoderm-neural crest interactions\(^{120}\) confirmed and extended Bonstein’s\(^{120}\) original finding that dorsal lateral mesoderm was necessary and sufficient to induce neural crest from animal cap (ectoderm) explants. An analysis of their observation reveals a number of unanswered questions, associated with the dynamic and co-dependent nature of the embryonic system. They found that active canonical (β-catenin-dependent) Wnt signaling was required for the animal cap to respond to mesodermal signals and express neural crest markers (e.g., **slug2/snail2**). That said, inhibition of canonical Wnt signaling appears to be required for neural induction.\(^{120}\) In the embryo, both the requirement for Wnt signaling (for neural crest induction) and its inhibition (in neuroectoderm) are likely to be influenced by the presence of the B1-type Sox proteins, Sox2 and Sox3 (Fig. 5). Sox3 is expressed maternally, and Sox2 and Sox3 are expressed zygotically in the nascent neuroectoderm and both can inhibit canonical Wnt signaling, in part through interactions with β-catenin;\(^{121,122}\) they could play a key role in establishing a Wnt responsive threshold within the epidermal-crest-neuroectoderm domain. At the same time, mesodermal secretion of the Wnt inhibitor Dickkopf1 has been found to inhibit the formation of neural crest at the anterior margin of the neural plate.\(^{124}\)

Mayor et al.\(^{125}\) found that the zinc-finger transcription factor **slug/snail2** is expressed in dorsal mesendoderm, where it acts to repress BMP-4 activity. Our own studies indicate that **slug**/**snail2** and **snail1** are required for both mesoderm and neural crest formation.\(^{20,32,69}\) Since the Snail proteins appear to function exclusively as transcriptional repressors,\(^{126,127}\) the ability of Slug/Snail2 to regulate its own expression\(^ {125}\) is presumably indirect. We have found that the secreted signaling antagonists cerberus and sizzled are immediate-early targets of repression by Slug/Snail2.\(^{69,70}\) Cerberus is expressed in anterior endomesoderm while sizzled is expressed in the ventral region of the embryo; both are involved the interaction network that regulates embryonic patterning.\(^ {128}\)

BMP4 can induce neural crest cells in naïve chick neuroepithelium\(^{129}\) and in Xenopus.\(^ {130,131}\) To examine the role of BMP signaling in neural crest formation, Steventon et al.\(^ {9}\) reduced the level of the BMP antagonist Chordin by anti-chordin morpholino injection in ectoderm/dorsomarginal zone explant studies; this led to the loss of **snail2** in the neural crest; it is not clear whether the expression of earlier crest markers, such as **pax3** and **zic1**,\(^ {32}\) were affected. This is worth knowing since...
Once We Are Neural Crest, We Have to Migrate

The neural crest is the classic example of an epithelial-mesenchymal transition (EMT). This process involves a coordinated set of changes in gene expression and cellular behavior. In the trunk crest, it appears that BMP signaling acts to induce wnt1 expression in somites, which in turn acts to initiate crest migration. Migration begins with the disassembly of the cell-cell adherence junction complex, and the acquisition of a mobile phenotype; cells are maintained in an undifferentiated state in part through the action of the transcription factor Hairy2.

Within the embryo, neural crest cells migrate through spaces between the basal laminae that define the basal surface of the epidermis and the developing dermomyotome. These spaces are occupied by extracellular matrix rich in laminin and fibronectin. Neural crest cells fail to enter regions rich in chondroitin sulphate. Cranial neural crest migrates out in "streams" and the ephrin ligand receptor tyrosine kinase system plays an important role in keeping these streams distinct. In the chick, neural crest cells migrate "principally in loose clusters, with a few single cells in the lead. The cells in these groups display leading-to-trailing edge adhesions and form tongues or streams of cells directed away from the neural tube." Migrating neural crest cells are "elongated and aligned parallel to the direction of migration. Nearly all protrusive activity occurs at their ventral, leading edges."

Neural crest cells, like other normal, that is, non-transformed, substrate-adherent eukaryotic cells, display contact inhibition. The molecular mechanisms of contact inhibition involve non-canonical Wnt signaling, Syndecan-4/Rac1, RhoA, and the PCP pathway. The contact inhibition between neural crest cells serves to "channel" motility forward along the cell's migratory path. The end result is that normal cells do not pile up, but rather tend to form sheets and, in the neural crest, streams. Cadherin-cadherin interactions are generally involved in these cell-cell interactions. Cadherins interact with cytoplasmic catenins, including β-catenin, plakoglobin (γ-catenin) and p120cas, which in turn can interact with and regulate transcription factors. Various signaling pathways, most notably the canonical Wnt signaling, act in part by modulating the stability of these catenins.

While inert latex beads migrate when implanted into the region of the neural crest, it is clear that neural crest cell migration depends upon cellular properties, including interactions with integrins and extracellular matrix proteins. The onset of migration is associated with many changes in cytoskeletal organization and composition; as an example, keratin-type intermediate filaments are replaced by vimentin-type filaments. In Xenopus, vimentin-positive neural crest derived cells are most prominent in the neural crest-derived and dorsal fin mesenchymal cells.

Figure 5. Displays the pattern of expression of B1-type Sox-type transcription factors in the early Xenopus embryo. Top: RT-PCR analysis at various embryonic stages; Center: in situ analysis at blastula stages; Bottom: in situ hybridization at early neural stages (stage 13 and 15) for Sox1 (A and B), Sox2 (C and D) and Sox3 (E and F). Red arrow points to region of reduced Sox expression within the nascent neural plate (Grammer T, Zhang C, Klymkowsky MW, previously unpublished observations).

The accessory protein Irig3 is required for the expression of the neural crest markers slug/snail2, sox9 and foxd3, but not pax3 and zic1. Blocking chordin expression (but not Wnt signaling) eliminated the expression of the neural plate marker sox2, although whether the level of sox2 (or sox3) expression was altered in Wnt-antagonist expressing explants was not reported (see above). Both chordin and wnt8 are expressed in the dorsal marginal zone of the gastrula stage embryo, although whether they are expressed in the same cells is not clear. Their expression in the dorsal marginal zone is lower than that in their maximum expression regions, the dorsal lip and the ventral marginal zones, respectively. More important is the answer to the question, what is the effect of blocking chordin expression on the embryo’s self-regulating BMP gradient, the expression of other agonists (such as wnt8 and bmp4) and antagonists (such as noggin, dickkopf, cerberus, etc.) within the dorsolateral marginal zone?
which have been found to suppress expression of E-cadherin. Analysis of the slug/snail2 promoter indicates that it is regulated by canonical Wnt signals. In Xenopus, disruption of slug/snail2, snail1 or twist expression leads to a dramatic increase in apoptosis. In slug/snail2 morpholino-injected embryos, the increase in apoptosis can be inhibited by injection of RNA encoding anti-apoptotic factors (Bcl2 or Bclx); this also rescues neural crest formation, through NFkB-mediated regulation of slug/snail2, snail2 and twist RNA levels. In mammalian cells, Slug/Snail2 acts to negatively regulate the pro-apoptotic protein Puma; it is an immediate early regulator of the pro-apoptotic caspase, Caspase-9 in Xenopus.

The predominant cadherin in the early Xenopus embryo is C-cadherin; beginning in the late blastula an E-cadherin-like molecule is expressed in the developing epidermis and is absent from the developing neural plate. Beginning at gastrulation, cadherin-11 is expressed in a Wnt-dependent manner; later it is expressed in migrating neural crest cells, together with the protocadherin PCNS167 and N-cadherin. Functional studies of cadherin-11 indicate that its overexpression, or the overexpression of a mutant polypeptide missing its cytoplasmic β-catenin binding domain, blocks cranial neural crest migration and leads to their neural differentiation. In contrast, a mutant form of cadherin-11 missing its extracellular domain induced the premature migration of neural crest cells, and led to a decrease in the levels of twist expression. Such a version of cadherin-11 (ΔN-cadherin-11) would be expected to act as an inhibitor of β-catenin-mediated Wnt signaling, and so it is not completely surprising that the overexpression of β-catenin (which mimics the effects of a canonical Wnt signal) can rescue twist expression in mutant cadherin-11 expressing embryos. In any case, this provides further evidence for the role of Wnt signaling in migrating neural crest.

During migration, the extracellular domain of cadherin-11 is cleaved by the ADAM metalloproteinase, to produce an extracellular polypeptide and a cellular polypeptide, similar to ΔN-cadherin-11; the extracellular fragment promotes neural crest cell migration. It is possible that it acts by inhibiting cadherin-11-mediated cell-cell interactions. The membrane-associated, cytoplasmic region of cadherin-11 interacts with the guanine nucleotide exchange factor Trio and stimulates filopodia. Such filopodia interactions are important for contact inhibition of migration between neural crest cells, a behavior mediated by syndecan-4/trc1 and non-canonical Wnt signaling. Loss of function studies have implicated Wnt11r in crest migration. A component of the non-canonical Wnt signaling pathway PTK7 is required for normal neural crest migration, while pescadillo, which appears to be regulated by non-canonical Wnt4 signaling, is also involved in crest migration.

Neural Crest Induction and Migration in Zebrafish: Extrinsic Signals Involved in Neural Crest Induction in Zebrafish

When the ectoderm segregates into neural and non-neural ectoderm, neural tissue lies dorsally while epidermal ectoderm is located laterally and ventrally; the neural plate border (from which the neural crest arises) lies at the junction between these two tissues. Neural crest cells appear after the neural and non-neural ectoderm have segregated from one another but before the neural tube has closed. Rohon-Beard sensory neurons, mechanosensory primary neurons and cranial placodes, which form neurons and glia of the cranial ganglia, arise from the anterior neural plate border. During the stages when the neural plate border is beginning to form, a gradient of BMP activity is established in the ectoderm (see above). Similar to Xenopus, neural crest cells (as well as Rohon-Beard sensory neurons) are not specified or are shifted ventrally in BMP pathway zebrafish mutants. Wnt signaling is required for neural crest induction in zebrafish. Activation of a heat shock version of a mutant tcfβ, which does not bind beta-catenin and so acts as a dominant antagonist of canonical Wnt signaling, leads to the absence of foxd3 expressing neural crest cells. However, wnt8a and βb are required for early neural crest induction, but later markers of neural crest cells either do not require Wnt signaling or there is some recovery of the later neural crest markers, such as sox10 and mitfa.

Notch/Delta is another juxtacrine signaling system by which cells within an equivalence domain diversify their cell types. Early during neurulation in the zebrafish, single cells or small clusters of cells express high levels of deltaA at the neural plate border. A dominant negative mutation in deltaA also leads to the formation of supernumerary Rohon-Beard sensory neurons with a concomitant decrease in neural crest-derived pigment cells and cells of the dorsal root ganglia. Similarly a mutation of mindbomb (mib), which encodes a RING ubiquitin ligase involved in Notch/Delta signaling, leads to supernumerary Rohon-Beard sensory neurons and a decrease in neural crest-derived pigment cells. These data suggest that Notch/Delta signaling functions to specify a Rohon-Beard sensory neuron at the expense of other neural crest derived cell types, presumably from a common progenitor.

Intrinsic Signals Involved in Specification

Early experiments in chick and the mouse revealed that injecting a single cell with a lineage tracer dye prior to the onset of migration resulted in several types of labeled neural crest derivatives at later developmental stages. In zebrafish, single cell labeling of early neural crest cells results in labeling of more than one, and often all combinations of neural crest derivatives, similar to what was previously seen in chick. At the same time labeling of trunk neural crest cells just after they segregated from the neural tube rarely gives rise to multiple neural crest derivatives suggesting that neural crest cells may adopt their fates earlier in zebrafish than in other species, a conclusion supported by the observation that labeling cells at gastrulation generally results in...
that of other neural crest specifiers and homozygous mutant xblimp1 phic (or null) allele. The Xenopus homolog of "knock-down" embryos suggests that the mutant is not an amorphic (or null) allele. The Xenopus homolog of prdm1a, known as xblimp1, is expressed at the neural plate border and in the anterior mesenchyme, loss of ceratobranchial cartilage and fewer neurons per ganglion in the dorsal root ganglia. During neurulation, the neural plate morphs into a cylindrical tube. In most species, this occurs when the neural plate border regions elevate to form neural folds, which then converge toward the midline and eventually fuse together to form the dorsal aspect of the neural tube. In teleost fish, however, there are no neural folds. Rather, the neural keel
(analogous to the neural tube) forms through the ventral thickening of the ectoderm, a process known as secondary neurulation.\textsuperscript{213,214} Neural crest cells migrate from their position in or immediately adjacent to the dorsal region of the neural tube to various positions within the embryo. The eventual location of each neural crest cell depends on the type of neural crest derivative that it will become. For example, pigment cells are located throughout the epidermis.\textsuperscript{215} Cells in the dorsal root ganglia are lateral to the neural tube.\textsuperscript{216} and caudal fin mesenchyme is located within the mesenchymal region of the tail. Depending on the species of teleost, neural crest cell migration begins either during or after neural tube closure.\textsuperscript{217} Trunk neural crest cells migrate along two routes: ventromedially through the somites and dorsolaterally between the epidermis and the somites. Those crest cells that migrate along the medial pathway give rise to more ventral derivatives, such as dorsal root ganglia, sympathetic ganglia, enteric neurons and glia; those that migrate more dorsally give rise to pigment cells (melanocytes). Inductive interactions with the migratory environment, particularly the somites, is thought to determine/influence to cell fate. The regulatory ability of neural crest cells was demonstrated by ablation studies; cells can regulate to fill in the gap of missing neural crest derivatives, even if they would not normally do so.\textsuperscript{218}

In most species, neural crest cells travel through the anterior half of each somite, their migration along the caudal region of the somite is inhibited by the expression of specific inhibitors, such as Ephrin-B1 and T-cadherin.\textsuperscript{217,219} In the zebrafish, migration occurs through the middle of the medial surface of each somite,\textsuperscript{220,221} whether the same "molecular migration signposts" are involved remains to be established. Because crest cells migrate along the middle of the somites in zebrafish, and given that a large portion of the somite forms after zebrafish neural crest cells have started migrating, it has been suggested that the sclerotome is not required for their migration. Instead, it appears that myotome, specifically the slow twitch muscle fibers, plays the role of sclerotome. Mutations that reduce sonic hedgehog signaling (\textit{syn, smu}), reduce the amount of slow twitch muscle and disrupt neural crest cell migration. Replacing the precursors of slow twitch muscle rescues these defects, suggesting that slow twitch muscle is required for zebrafish neural crest migration. Mutations that affect the formation of mesoderm (\textit{spf, ntl}) and the segmentation of somites (\textit{bea, fsi}), disrupt neural crest migration, but not its induction.\textsuperscript{221}

The lateral surface of the somite induces contact inhibition of migrating neural crest cells, blocking their invasion into the somite and channeling the movement\textsuperscript{48} (see above). Neural crest cells change direction when confronted with another neural crest cell along its migratory route leading to directed (forward) cell migration in the absence of an attractant. Syndecan-4 morphants have normal neural crest induction but no neural crest cell migration. A series of elegant experiments suggest that Syn4 inhibits Rac while Wnt/Dsh promotes RhoA activity which coordinates the directionality of migration.\textsuperscript{247} Members of these pathways localize to the site of cell-cell contact and thus control the directed cell protrusions required for migration in a directed fashion. Cadherin 11 is localized to the leading edges of the migrating cells where it presumably mediates interactions with epithelial cells via the regulation of Rac, RhoA and cdc42.\textsuperscript{165}

**General and Molecular Mechanisms of Cranial Neural Crest Migration in Zebrafish**

Cranial neural crest cells migrate in an anterior stream toward the eye and three streams, with the midbrain and anteriormost hindbrain rhombomere (R) 1/2 cells emigrate first, followed by the emigration of the more caudal R4 and R6/7 cells. The segmental fate of the cranial neural crest is dictated by the premigratory position of neural crest cells along the antero-posterior axis of the embryo. Neural crest cells in the pharyngeal arches give rise to the ventral pharyngeal skeletal elements. Specifically, pharyngeal arch one gives rise to the Meckel’s and palatoquadrate cartilages, arch two forms the basihyal, ceratohyal and hyosymplectic cartilages and arches three through seven give rise to the ceratobranchial cartilages.\textsuperscript{222,223} The dorsal craniofacial cartilages make up the neurocranium or “braincase.” The anterior neurocranium is composed of two rod-like structures, termed trabeculae, which fuse anteriorly and connect to the ethmoid plate. The anterior neurocranium is derived from the anteriormost cranial neural crest, which migrate out from the dorsal neural tube and reach the dorsal anterior aspect of the head following a path along the optic stalks, medial to the eyes.\textsuperscript{224,225} Migration in the zebrafish cranial region begins at 12 h post fertilization (hpf) with the anterior most cells delaminating and migrating and by 15–16 hpf the cells move into the pharyngeal arches, as shown by Schilling.\textsuperscript{227} Interestingly fate-mapping data indicates that neural crest cells are segmentally restricted, meaning there is very little mixing between pharyngeal arch compartments.\textsuperscript{228} This is in contrast to the chick embryo, where there is substantial mixing during migration between hindbrain compartments.\textsuperscript{229}

Zebrafish neural crest cells also migrate in an anterior stream around the eye, which is important for development of the neurocranium.\textsuperscript{225,226} The microRNA Mirn140 modulates the attraction of cranial neural crest cells via platelet derived growth factor (\textit{pdgfaa}) signaling during zebrafish neurocranium development. In embryos lacking Mirn140, cranial neural crest cells accumulate around the optic stalk near the attractive Pdgfa signal and fail to migrate further to the oral ectoderm, resulting in loss of the ethmoid plate and in palatal clefting.\textsuperscript{230} The eye is important for organizing neural crest cell migration in the zebrafish. In wild-type embryos, neural crest cells migrate anteriorly around the eye and are not detected on the eye surface until 23–24 hpf. Soon after, however, cranial neural crest cells will cover the entire surface of the eye. \textit{chokh} mutant embryos fail to develop eyes and cranial neural crest cells fail to migrate anteriorly and are disorganized in a region posterior to the normal eye location. Analysis of craniofacial cartilages in \textit{chokh} mutant larvae shows loss of the ethmoid plate and fusion of the trabeculae. \textit{chokh} mutant larvae do however have normal viscerocranium development.\textsuperscript{226}

Chemokines are small, secreted chemoattractants that have been well studied for their roles in regulating cell migration during embryogenesis, immune response and cancer.\textsuperscript{231} The
chemokine Stromal cell-derived factor (Sdf1), binds to the receptors Cxcr4 and Cxcr7.\(^232,233\) Cxcr4 has also been shown to be expressed in migrating neural crest cells in the mouse embryo\(^34\) while Sdf1 is expressed within the neural crest cells migratory path. In the chick, an Sdf1 expression domain is associated with the branchial arch region.\(^30\) In the mouse Sdf1/Cxcr4 signaling has been found to mediate the positioning of dorsal root ganglia.\(^234\) In the zebrafish, Sdf1a signaling has been implicated in melanophore patterning; melanocytes are attracted to the site of the implantation of an Sdf1a coated bead.\(^236\)

There are two Cxcr4 genes in zebrafish, Cxcr4a and Cxcr4b; only Cxcr4a is expressed within neural crest cells.\(^237\) Loss of Cxcr4a, but not Cxcr7b, results in aberrant cranial neural crest cell migration in the anterior stream, resulting in neurocranial defects and cranial ganglia dismorphogenesis.\(^238\) Gain of function of either Sdf1b or Cxcr4a causes aberrant cranial neural crest cell migration and results in ectopic craniofacial cartilages. This suggests that Sdf1b signaling from the pharyngeal arch endoderm and optic stalk to Cxcr4a-expressing cranial neural crest cells is important for both the proper condensation of these cells into the pharyngeal arches and the subsequent patterning and morphogenesis of neural crest-derived tissues, such as proper positioning of the partially neural crest-derived trigeminal sensory ganglia.\(^239\)

Interestingly, Sdf1 signaling also plays a role in neural crest migration in Xenopus (R. Mayor, personal communication). Although Cxcr4a is expressed by all arch neural crest cells and Sdf1b by the endodermal pouches surrounding the arches in which they are migrating, there is no significant defect of cells derived from the pharyngeal arches, suggesting multiple redundant mechanisms for neural crest migration, especially in the cranial region.

Ephrins and Ephrin receptors have been implicated in regulating the migration of cranial neural crest cells in other vertebrates and are thought to function by altering cell adhesion, likely through interactions with integrins. In the mouse, Ephrin-B2 is important for crest cell migration into branchial arch 2, while Ephrin-B1s required for multiple steps during crest migration; mutant mice exhibit a cleft palate.\(^240,241\) To date, no such phenotype has been observed in either zebrafish or Xenopus. The Neuruplin family has also been shown to be required for neural crest migration. Knockdown of Neuruplin-1 function in the chick results in a reduction in the number of crest cells that reach the branchial arches.\(^242\) Signaling through Neuruplin-2 by Semaphorin-3F has been implicated for proper cranial neural crest cell migration and condensation of the trigeminal sensory ganglia in mouse. Interestingly, while cranial neural crest cell migration is affected in these mutants, no skeletal defects were apparent.\(^243\) Studies in zebrafish implicate sema3F and sema3G in cranial neural crest migration. sema3F and 3G are expressed in neural crest-free regions, while nrg2a and nrg2b are expressed in cranial neural crest cells.\(^244,245\) In zebrafish, mutations in pbx4 is suppressed following knockdown of sema3F/3G and overexpression of sema3Gb permits crest cells to migrate in normally crest-cell-free zones.\(^246\) Other genes, such as Disc1, have more recently been shown to be involved in cranial neural crest cells migration.\(^247\)

### Summary

While it is common to treat neural crest as a conserved feature of vertebrate development, it is clear that different molecules and mechanisms have come to assume distinct functions in different lineages (e.g., the different roles of snail-family proteins in zebrafish, Xenopus and mouse). Similarly, while there are parallels between neural crest and cancer metastasis, the differences are likely to be profound.\(^248\) What is clear is that multiple redundant mechanisms are at play and required for the proper induction and migration of neural crest. We propose that species differences in neural crest specification are of interest as they are likely to provide insights into a range of processes involved in evolutionary, developmental and pathogenic processes.

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