Factors controlling cardiac neural crest cell migration

Margaret L. Kirby* and Mary R. Hutson

Departments of Pediatrics and Cell Biology; Duke University; Durham, NC USA

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Cardiac neural crest cells originate as part of the postotic caudal rhombencephalic neural crest stream. Ectomesenchymal cells in this stream migrate to the circumpharyngeal ridge and then into the caudal pharyngeal arches where they condense to form first a sheath and then the smooth muscle tunics of the persisting pharyngeal arch arteries. A subset of the cells continues migrating into the cardiac outflow tract where they will condense to form the aorticopulmonary septum. Cell signaling, extracellular matrix and cell-cell contacts are all critical for the initial migration, pauses, continued migration and condensation of these cells. This Review elucidates what is currently known about these factors.

Introduction

The cardiac neural crest is the most caudal of the cranial neural crest located postotically from the otocyst to somite 3 corresponding to rhombomeses 6, 7 and 8 in the neural tube. Cells from this region undergo an epithelial-to-mesenchymal transition (EMT) beginning at about HH11 in the chick to emigrate from the neural tube along distinct pathways. They migrate first to the circumpharyngeal ridge, an arc-shaped ridge located dorsal to the forming caudal pharyngeal arches. The cells stop because they are destined for the caudal pharyngeal arches 3, 4 and 6, which have not yet developed since the space is occupied by the pericardial cavity. As the pericardial cavity regresses caudally, pharyngeal pouches indent the body wall to delineate the pharyngeal arches. The cells stop because they are destined for the caudal pharyngeal arches 3, 4 and 6, which have not yet developed since the space is occupied by the pericardial cavity. As the pericardial cavity regresses caudally, pharyngeal pouches indent the body wall to delineate the pharyngeal arches. The process proceeds from cranial to caudal, generating arches 3, 4 and 6 in that order. As the arches form they are populated by the cardiac neural crest cells migrating from the circumpharyngeal ridge. All of the cranial neural crest generates neurons, supporting or Schwann cells and ectomesenchyme. Ectomesenchyme, a unique derivative of the cranial neural crest, is important for structural development of the face and cardiovascular system. The ectomesenchymal cells of the postotic cardiac crest are unlike those of the preotic cranial crest that populate arches 1 and 2. The ectomesenchyme of the preotic crest plays a significant role in skeletal development while that derived from the postotic cardiac crest in pharyngeal arches 3, 4 and 6 plays a major role in supporting cardiovascular development. The cells are critical for normal repatterning of the bilaterally symmetrical pharyngeal arch arteries to form the asymmetric great arteries of the thorax. The cells also support development of the thymus and parathyroid glands. A subpopulation of the cardiac neural crest cells in the caudal pharyngeal arches migrates into the cardiac outflow tract, finally stopping when it straddles arches 4 and 6. Cardiac crest in the outflow tract forms: (1) cardiac ganglia and (2) condensed mesenchyme at the junction of the presumptive subaortic and subpulmonary myocardiun of the outflow tract. This condensed mesenchyme is known as the aorticopulmonary seoration complex, which will divide: (1) the most distal outflow into the base of the aorta and pulmonary trunk and (2) the middle outflow into the aortic and pulmonary semilunar valve regions. Crest cells also migrate further into most proximal outflow tract where they may be involved in final closure of the ventricular outflow septum. Finally, cardiac crest cells surround and insulate the bundle of His. This review focuses on the factors that orchestrate cardiac neural crest migration from the neural tube into the pharyngeal arches and heart and factors involved in condensation at various points along their migration pathway (Fig. 1 and Table 1). Few studies have been directed specifically at cardiac crest migration, so our review relies heavily on data from some knockdown studies in chick and many mouse mutants that have cardiac or caudal pharyngeal arch defects.

Epiblast Origin of the Cardiac Crest, Convergent-Extension and Induction

Fate maps of neural crest in chick, particularly with respect to cranial neural crest, indicate that as early as HH4, the neural progenitors are located in the epiblast in a roughly shaped crescent around Henson’s node. The neural crest progenitors are located at the periphery of this crescent. The presumptive caudal hindbrain, which will give rise to the cardiac crest, is located on about the level of the node and extends midway to the area opaca. The cardiac crest, while it has not been specifically mapped, is located at the periphery of the caudal hindbrain progenitors. Convergence and extension during gastrulation bring this region into the neural folds and adjacent ectoderm caudal to the progenitors for the otic placode. The region that gives rise to neural crest is somewhat controversial as the claim has been made that

*Correspondence to: Margaret L. Kirby; Email: mlkirby@duke.edu
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ectomesenchymal cells generated in the cranial region may originate from the adjacent non-neural ectoderm rather than from the neural folds as is more commonly accepted.14

The complex molecular cascade of events required to establish the migratory and multipotent nature of neural crest cells is not fully understood. Wnt, FGF and an intermediate level of BMP signaling are all required for neural crest induction.15 The crest progenitors are located in a region of the epiblast overlapping BMP expression. It has been proposed that the neural crest is induced by BMP earlier than the neural fold stage. However, this is questionable as the neural crest is induced by intermediate levels of BMP signaling provided by negative regulation by BMP antagonists. During neurogenesis, BMP4 and its receptor, BMPR1A, are expressed along the rostrocaudal axis of the neural tube while the extracellular BMP inhibitor noggin is expressed in an opposing low rostral to high caudal gradient.16 The precise level of BMP signaling is important because too much repression caused by overexpression of noggin in the mouse hindbrain leads to failure of crest cells to migrate.17 One of the downstream effectors of BMP signaling is Smad-interacting protein 1 (Sip1), an E-box binding zinc finger transcriptional repressor known to specifically repress E-cadherin in epithelial cells and thus the adhesiveness of the cells.18 Sip1 is expressed by premigratory and migrating cranial neural crest cells,19 which do not migrate in its absence.19 Downregulation of cadherins is important for EMT and migration from the neural tube (see below). Because the postotic crest provides innervation to the gut, Sip1 mutant embryos also have Hirschsprung disease characterized by absence of innervation to the colon. Heterozygous mutations or deletions in the human ZFHX1B gene, which codes for SIP1, have been identified in patients with Mowat-Wilson syndrome.20 This recently described mental retardation-multiple congenital anomaly syndrome is characterized by atypical facial features, severe mental retardation and epilepsy, as well as variable congenital malformations, including Hirschsprung disease and congenital heart defects. Thus, one of the major roles of BMP in the cardiac crest is to activate Sip1, which suppresses expression of E-cadherin, allowing the crest cells to detach from their neighbors in the EMT process. Induction is heralded by the expression of Pax3 and Zic1 transcription factors in the dorsal neural tube followed by activation of neural crest specification genes such as Snail, Slug, AP-2, Sox9 and FoxD in the neural crest cells.15

**Figure 1.** Diagram summarizing the origin and stages of cardiac neural crest migration and condensation in its targeted regions that will be discussed in the review. (Reprinted in modified form from Kirby, 2007116 with permission.)

**EMT, Cell Cycle and Initiation of Cardiac Crest Migration**

The induction process is intimately tied to the next step, EMT, in which the cells lose their cell-cell contacts, reorganize their cytoskeleton and acquire a motile phenotype to leave the dorsal neural tube. Release from cell contacts with adjacent cells allows the cells to interact in three dimensions with extracellular matrix components.21 Migratory neural crest cells are mesenchymal in
that they express the intermediate filament protein, vimentin and are flattened cells with filopodia and lamellipodia that facilitate movement. Release from the neural tube requires downregulation of epithelial cell-cell junctional proteins including cadherin6B, expressed only in the dorsal neural tube. Knockdown of cadherin6B leads to premature neural crest cell emigration, whereas its overexpression prevents migration.22 Vertebrate neural crest cells rapidly alter cadherin expression and localization at the cell surface during migration. Expression of cadherin6B is directly controlled by the Slug/Snail zinc finger family of transcription factors.22-24 Inhibition of Slug25 causes failure of the cells to undergo EMT and thus failure to migrate.26,27 The loss of cell adhesion combined with the membrane blebbing that precedes filopodial extension mark the onset of migration.28 Disruption of myosin II or Rho-kinase (ROCK) activity inhibits neural crest cell blebbing and causes reduced EMT.28

Neural crest cells express a complex collection of integrins, which are receptors that mediate attachment between cells and/or the extracellular matrix. They are important for cell signaling and can influence cell shape, mobility and regulate the cell cycle. The expression of α4β1 integrin by avian neural crest cells shortly after they leave the neural tube seems to be particularly important for both their migration and survival29 though the promiscuous affinity of this receptor for numerous extracellular ligands makes it difficult to narrow its role further. One extracellular matrix glycoprotein that does seem to be critical for neural crest cell motility is tenascinC. Avian neural crest cells make this glycoprotein, which promotes their migration in vitro, shortly after they leave the neural tube. When expression of tenascinC is blocked, the neural crest cells fail to emigrate.30

EMT and migration are linked to the cell cycle. Avian neural crest cells synchronously emigrate from the neural tube in the S phase of the cell cycle and so inhibition of the transition from G1 to S blocks EMT, while arrest at the S or G2 phases of the cell cycle have no effect.31 Genetic studies suggest that Wnt/TCF/Sema3d are in a pathway controlling cell cycle progression and thus initiation of neural crest migration. Canonical Wnt signaling, which activates TCF-dependent transcription, is crucial for the G1/S transition in neural crest cells.32 Repression of TCF causes reduced expression of sema3D, a secreted protein that acts as an inhibitory guidance molecule. Morpholino-mediated knockdown of Sema3d in the rhombencephalon causes G1 to S cell cycle arrest by decreasing cycclinD. This results in reducing the number of neural crest cells able to emigrate from the rhombencephalon.33

The rate of proliferation in the dorsal neural tube also impacts neural crest emigration. Reduction of folate receptor in chick cardiac crest by siRNA reduces proliferation in the neural tube, which impacts neural crest migration to the point that both pharyngeal arch artery and outflow tract are abnormal and resemble the changes seen after cardiac neural crest ablation.34

### Early Migration

In higher vertebrates, the cells in the cranial neural crest migrate in clusters or “streams” and later form cranial nerve ganglia at

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**Table 1. Function of factors involved with specific events in cardiac neural crest migration**

| Induction               | Classification                           |
|-------------------------|------------------------------------------|
| Wnt Signaling Family    | growth factor/growth factor receptors    |
| FGF Signaling Family    | growth factor/growth factor receptors    |
| BMP Signaling Family    | growth factor/growth factor receptors    |
| Noggin                  | BMP signaling inhibitor                   |
| Sip1                    | transcriptional repressor                 |
| Pax3                    | transcription factor                      |
| Zic1                    | transcription factor                      |
| E-cadherin              | cell adhesion                            |

**Specification**

| Snail/Slug Family       | transcription factor                      |
| AP2                    | transcription factor                      |
| Sox9                   | transcription factor                      |
| FoxD                   | transcription factor                      |

**EMT/Initial Migration**

| Snail/Slug              | transcription factor                      |
| cadherin6B             | cell adhesion                            |
| vimentin               | intermediate filament                     |
| myosin II              | cytoskeletal protein                      |
| Rho-kinase (ROCK)      | cell motility regulator                   |

| α4β1 integrin           | receptor mediating cell/cell and ECM interaction |
| tenascinC              | ECM protein                               |
| WNT1                   | growth factor                             |
| TCF                    | transcription factor                      |

**Early Migration: Guidance**

| FoxD3                  | transcription factor                      |
| Sox10                  | transcription factor                      |
| FGF8                   | growth factor                             |
| Semaphorin Family      | guidance molecules that can attract or repel |
| neuropilin1/2          | co-receptor for Semaphorins               |
| plexin A1/2            | co-receptor for Semaphorins               |
| Ephrin/Eph family      | guidance molecules/receptors              |
| HoxA1/B1               | transcription factors                     |

**Early Migration: Cell-Cell interaction**

| cadherin11             | cell adhesion                            |
| Cx43                   | cell-cell communication                   |
| N-Cadherin             | cell adhesion                            |
| p120 catenin           | cell motility regulator                   |
| Wnt family             | growth factor/growth factor receptors     |
| RhoA                   | cell motility regulator                   |

**Early Migration: ECM interaction**

| α5β1 integrin           | receptor mediating cell/cell and ECM interaction |
| arginyltransferase     | modifying enzyme                          |
| ADAM13                 | zinc metalloprotease                      |
| TIMP2                  | metalloprotease inhibitor                 |
| Pax3                   | transcription factor                      |
The migrating cells can have many shapes and while the number and directionality of cell processes are dynamic, within the three cranial neural crest streams, cells at similar locations have similar shapes. In addition, cells in the three streams migrate in patterns characteristic for each stream. The caudal stream comprises most of the cardiac crest. The majority if the crest emanate from the even numbered rhombomeres. Crest in rhombomeres 3 and 5 die and this may contribute to the separation of the streams at these rhombomeres (Fig. 2). In the third stream (postotic region) where the cardiac crest originates, dorsal somites and ventral pharyngeal arches coexist at the same axial level. Proximally the cardiac crest cells follow the dorsolateral pathway only where somites are absent. They divert around the occipital somites rostrally, making an arc that represents the caudal limit of the dorsolateral pathway of cranial crest cells. This represents the head/trunk interface dorsally. Cardiac crest cells localize in postotic pharyngeal arches 3, 4 and 6, as well as in an arc-shaped ridge, called the circumpharyngeal ridge. The circumpharyngeal ridge and arch 6 represent the caudal limit of the pharynx and thus the head/trunk interface ventrally. The third stream crest cells also distribute in the innervation region of cranial nerves IX and X.

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expression of a hox code depending on the distance from the 
FGF source. To a certain extent this plays a role in the final 
fate of the neural crest cells, making it important that the cells 
maintain their streams during migration. Since the cardiac crest 
is the most caudal stream, it is thus the farthest from the FGF8 
source. The low FGF signal may influence the cardiac crest cell 
fate because transplantation of more cranial crest into the caudal 
(cardiac) stream results in cartilage nodules, a cell fate of the 
preotic streams, in the outflow tract.

As mentioned above, crest cell death in rhombomeres 3 and 
5 may contribute to the separation of the streams. However, it 
is not the only mechanism for establishing the crest-free zones. 
Semaphorins, a family of guidance molecules, which can attract 
or repel cells, are important in defining the breaks in the three 
cranial streams of crest cells. In general, chick neural crest cells 
avoid substrates with semaphorin 3A, 3F and 6, though they 
are attracted by semaphorin 3C. Cells from rhombomeres 3 
and 5 move rostrally and caudally to join the migratory streams 
emerging from rhombomeres 2, 4 and 6–8. Semaphorins 3A 
and 3F provide repulsive guidance cues and are expressed in 
rhombomeres 3 and 5. Emerging cranial crest cells express the 
semaphorin receptors neuropilin1 and 2 and the coreceptor for 
neuropilins, plexin A1. These repulsive cues are also likely 
to play a role in placement of the ganglia of cranial nerves V, 
VII and IX. X. The semaphorin code is slightly different in 
zebrafish where Semaphorins 3F and 3G and neuropilin 2 function 
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In zebrafish, the cranial crest cells migrate medially over the 
neural keel before beginning their migration ventrally toward 
the branchial arches. Foxd3 and Sox10, transcription factors 
are expressed early in migration but turned off before the cells 
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Foxd3 and Sox10 expression in migrating neural crest cells. 
Knockdown of DISC1 led to the cells remaining in their medial 
position before beginning the longrange migration ventrally. 
Decreasing Sox10 expression in the DISC1 morphants restored 
normal migration.

Three equally important factors are central for guidance of 
migrating neural crest cells: secreted signaling molecules acting 
through their protein kinase receptors and interactions of the 
cells with each other and with their surrounding extracellular 
matrix. We will discuss these in the next sections.

**Signals Guiding Early Migration**

Neural crest cells emigrating from the hindbrain receive an FGF8 
signal from the midbrain-hindbrain boundary that promotes 
ablation of rhombomere 5–6 crest allows neighboring rhombo-
mere 7 crest cells (caudal stream) to reroute their trajectory to 
fill in arch 2 (middle stream) but rhombomere 5 crest cells 
repopulate neither the caudal stream emigrating from rhombo-
meres 6, 7 and 8 nor arches 3, 4 and 6 when the caudal stream 
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semaphorin 3F and 3G expression, the neural crest streams are fused. Ephrins are a group of signaling molecules that were initially described in axon and endothelial cell guidance. Eph receptors and their ephrin ligands have been suggested to play a role in maintaining the different migratory streams, but this has been difficult to test because different vertebrates use different combinations of Eph receptors and ephrins. The Eph receptors are subdivided based on sequence similarity and ligand-binding characteristics. The EphA receptors bind EphrinAs, membrane-bound ligands that attach to the cell membrane via a glycosyl phosphatidylinositol linkage. EphrinBs are transmembrane-bound ligands that bind EphB receptors. The EphB receptors can activate reverse signaling through EphrinBs. EphrinB-EphB interactions regulate the formation of boundaries and prevent cell mixing. Ephrin signaling is likely more important for directing migration into the pharyngeal arches rather than guidance away from the neural tube as seen with the semaphorins. Interestingly, EphA2 gene expression in rhombomere 4 and arch 3 crest is directly regulated by Hoxa1 and Hoxb1. This suggests that the Hox genes through ephrin signaling maintain the “regional identity” of the caudal stream by providing an identity code for cells within the rhombomere as well as the neural crest.

**Cell-Cell Interactions in Early Migration**

In addition to maintaining contact with their microenvironment via extracellular matrix, migrating cranial neural crest cells maintain constant contact with each other. Local contact is maintained via lamellipodia or short, thin filopodia while long distance contact up to 100 um is also maintained by filopodia. The cell-to-cell contacts appear to influence a cell to change direction in favor of a neighboring cell’s trajectory. A complete picture of what the cells communicate is still not available.

As discussed above, reducing the cell-cell adhesion is an important characteristic of the neural crest in order to leave the neural tube. A certain amount of cell adhesion is required for the neural crest to maintain their migratory streams. Cadherin-11 is expressed by cranial neural crest cells during migration. Overexpression of cadherin-11 in Xenopus prevents migration suggesting that the amount of cadherin-11 expressed is important.

The cell-cell adhesion facilitates cell-cell communication. Cx43, a gap junction protein that forms channels between cells is important in the speed and directionality with which the crest cells migrate. Transgenic mice with altered Cx43 function in cardiac neural crest cells have outflow tract obstruction and altered patterning of the coronary artery stems. Elevated Cx43 function enhances the rate of neural crest cell migration and hence the abundance of neural crest cells in the outflow tract. Reduction or inhibition of Cx43 function reduces the rate of neural crest cell migration and thus decreases the number of crest cells arriving in the outflow tract. Neural crest cells with reduced Cx43 expression have increased cell proliferative activity, which is accompanied by the loss of polarized cell movement. The inhibitor of integrin activity and chemorepellent, Semaphorin 3A, normally inhibits neural crest motility. However, in crest cells deficient for or overexpressing Cx43, the cell processes fail to retract in the presence of Semaphorin 3A.

Cx43 is also involved in mediating neural crest cell adhesivity and thus, motility. In chick embryos, N-cadherin is expressed in migrating neural crest cells and blocking its function perturbs the onset of neural crest migration. N-cadherin, a protein involved in modulating cell motility and localized to adherens junctions, binds p120 catenin, another protein involved in modulating cell motility. Both are expressed in neural crest cells and colocalize with Cx43. Significantly, the distribution of p120 catenin is altered in Cx43- and N-cadherin-deficient neural crest cells suggesting that interactions of N-cadherin, p120 catenin and Cx43 are important in regulating the migration of cardiac crest.

While cell-cell contact is important for cell communication, neural crest cells in culture move away from each other and this seems to be affected in vivo by contact inhibition. The Wnt planar cell polarity pathway is involved in contact inhibition. Cells expressing a dominant negative form of disheveled (Dsh), a member of the Wnt protein family, which specifically inhibits the planar cell polarity pathway, were not able to collapse their lamellipodia when contacting another cell and were unable to avoid other cells. When two cells collide, Dsh is normally relocalized to the point of cell-cell contact with subsequent change in direction. Wnt-frizzled activation is needed for this signaling; however, localization of Dsh at the cell membrane can also be achieved by ephrinB1, which is also expressed by cranial neural crest (see above). RhoA, a small GTPase protein that regulates the actin cytoskeleton is a known downstream effector of planar cell polarity via Dsh during neural crest migration and inhibition of Rho-associated kinase (ROCK), a downstream target of RhoA, leads to a complete loss of contact inhibition of locomotion.

**Extracellular Matrix in Early Migration**

The extracellular matrix is thought to provide a permissive environment on which the crest cells can migrate. Cranial neural crest cells express α5β1 integrin receptor, which allows them to migrate on fibronectin, but not laminin, vitronectin or type I collagen in vitro. The central cell-binding domain of fibronectin contains the RGD (Arginine-Glycine-Aspartic acid) sequence and an adjacent site that acts in synergy with RGD as well as the second heparin-binding domain that allows α5β1 integrin-dependent cell spreading and cell migration. Blocking α5β1 integrin prevents neural crest cell migration on the central cell-binding domain of fibronectin.

The post-translational addition of arginyl groups to newly synthesized proteins is critical for embryonic development. Arginylation of proteins is a poorly understood post-translational modification mediated by arginyltransferase. This modification exerts a number of intracellular effects by altering proteins.
involved in cell motility and the actin cytoskeleton. Conditional deletion of arginyltransferase in neural crest using Wnt1cre results in delayed neural crest migration. The mice survive to birth and ultimately 13% live to adulthood, but they are small and many have craniofacial defects. In these mice the pattern of migration of the cardiac (caudal) stream of crest cells into arches 3 and 4 is altered.69 Focal adhesions appeared smaller and fewer in the knockout cells, which would be expected to affect migration. However, if migration of the cardiac stream were dramatically affected, the cardiac defect expected to result from absence of cardiac crest in the outflow tract is persistent truncus arteriosus a complete non-division of the outflow into a pulmonary and aortic outlet.70 This defect would be lethal at or prior to birth, suggesting that the embryos did not have persistent truncus arteriosus, further suggesting that while neural crest migration is affected by arginylation, it is not required.

In order to progress normally through the extracellular matrix, neural crest cells in vitro secrete proteases that break down cell-cell and cell-matrix adhesions and other extracellular matrix components. Xenopus cranial neural crest cells express a disintegrin and metalloprotease (ADAM)13, a protease that cleaves matrix glycoproteins. Overexpression of ADAM13 in Xenopus neural crest cells leads to a massive invasion of these cells into the surrounding tissues, whereas the expression of a dominant negative construct leads to an inhibition of neural crest cell motility in situ.71 Matrix metalloproteases are secreted into the extracellular matrix as inactive proforms that have to be proteolytically activated. Tissue inhibitors of metalloproteases (TIMPs), regulate matrix metalloprotease enzymatic activity. TIMP2 is uniquely expressed in early migrating cardiac neural crest cells. This expression domain expands to include other mesenchymal cells once cardiac neural crest cells enter the pharyngeal arches.72 Overexpression of TIMP2 in chick results in increased neural crest cell motility while inhibition by antisense oligonucleotides results in decreased neural crest migration.

One other factor that may play a role in neural crest cell migration because of its effect on extracellular matrix is the paired box homeodomain transcription factor Pax3. Homozygote Pax3-null embryos have multiple caudal pharyngeal arch abnormalities. Pax3-positive neural crest cells emigrate from the neural tube relatively normally but are much reduced entering the caudal pharynx without affecting cell proliferation or apoptosis.73 Early studies suggested that Pax 3-null cells had disrupted or delayed neural crest migration resulting in cardiac outflow tract defects.73-75 In Splotch embryos with mutant Pax3, the expression of chondroitin sulfate and heparan sulfate proteoglycans is enhanced76 and there is a marked overexpression of versican in the neural crest cell migration pathways.77 These mutants also show altered sialylation of NCAM, an important cell adhesion molecule expressed by neural crest cells, that could contribute to decreased migration of cells. However, though this suggests that Pax3 controls factors that might regulate migration, this issue has become complicated by newer findings. Further expression analysis revealed that Wnt1, but not Wnt3a, is expressed at decreased levels in Pax3 mutants and that the cardiac neural crest cells fail to undergo normal proliferative expansion prior to migration while still in the neural folds. Also, when placed into a wild-type matrix or a tissue culture environment, the Pax3 mutant cardiac crest cells migrated normally.74 However, in another study using mouse-chick chimeras mouse neural crest cells with mutant Pax3 did not migrate normally.79

### Pause in the Circumpharyngeal Ridge

The cells migrate to the circumpharyngeal ridge and in the chick pause while the caudal pharyngeal arches form (Fig. 3). All the factors involved in homing to the circumpharyngeal ridge and in their pause have mostly not been elucidated. In chick EphB3 and B4 are expressed in mesoderm near the dorsal neural tube80 and this could potentially be the signal for the crest cells to pause here.

So far we have discussed many of the factors required by the neural crest to achieve the extraordinary feat of coordinated cell migration in which cells detach from the neural tube migrate toward the pharynx and heart before finally differentiating and participating in the formation of important cardiovascular structures. Now we will focus on targeting the neural crest to their final destinations in the caudal pharyngeal arches and the heart as well as the factors involved in their condensation and differentiation.

### Migration into the Caudal Pharynx and Condensation Around the Arch Arteries

As the cells leave the circumpharyngeal ridge and populate arches 3, 4 and 6, the cells that form the leading edge of the migration front have long filopodia extended in the direction of migration and toward the ectoderm.78 Cells in the middle of the migrating group are bipolar with equal protrusive activity on the leading and trailing edges aligned with the migratory route. Cell contact between two cells can be up to 70 um away.79 The variety of protrusive activity of the crest cells also provides abundant opportunities for interaction with the extracellular environment as well as cell-cell communication during migration that the cells use to continue to maintain directionality along the entire migratory path.

The course of the three streams of neural crest must be further refined to reach specific pharyngeal arches because each arch has a unique structure and function supported by the neural crest cells that populate them. The crest cells respond to a variety of growth factors that target them to a particular arch. For example, vascular endothelial growth factor (VEGF) is a strong attractant for second stream neural crest cells entering arch 2. VEGF in arch 2 signals the cells via neuropilin receptors.81 However, VEGF does not seem to play a role in attracting cardiac crest from the circumpharyngeal ridge. On the other hand FGFR8 expressed by the pharyngeal endoderm and ectoderm has been shown in unpublished studies to be chemokinetic and chemotactic for cardiac crest cells (Kirby, unpublished). In addition, FGF8 signaling by the pharyngeal ectoderm is needed for viability of neural crest cells in arch 4.82
Receptors leads to scattering of arch 3 cells into adjacent regions. This suggests that the mixing of neural crest destined for arches 2 and 3 is blocked by the complementary expression of EphA4/EphB1 receptors and ephrin-B2 so that the cells are correctly targeted to arch 3. A similar mechanism is utilized in the mouse. In embryos with conditional knockout of EphrinB1, cells in the caudal stream invade territories that are normally devoid of crest cells. Consequently, the branching and fasciculation patterns of cranial nerves IX and X are not normal. However, other cells in the cranial crest are also abnormal so EphrinB1 is not specifically necessary for cardiac crest. EphA4 receptor, detected by a lacZ knockin allele, is expressed by cells migrating to the second and third arches in homozygous ephrinB2-null mutants and the second arch is either absent or reduced in size. Because Eph/ephrins have only been seen in the more cranial arches, this pathway does not seem to be critical for cardiac crest migration except possibly for arch 3 cardiac crest.

Another important signaling pathway affecting neural crest in the pharyngeal arches is via endothelin/endothelin receptor. The endothelin ligands (Endothelin 1, 2 and 3) are a family of 21 amino acid peptides. These active ligands are produced by proteolysis of larger precursors that are known as big endothelins by an enzyme called endothelin-converting enzymes. Endothelins signal through endothelin receptors that are members of the family of G protein-coupled receptors with seven transmembrane domains (Pla and Larue 2003). Three subtypes of endothelin receptors are recognized, on the basis of their affinity for the various endothelin ligands. The endothelin receptor A (ETA) has a high affinity for endothelin 1 and 2 and a low affinity for endothelin 3. Endothelin receptor B (ETB) has a high affinity for endothelin 3. There is some variation in expression patterns.

Transcription factors also contribute indirectly to the homing of migrating neural crest to the correct pharyngeal arch. Tbx1, a t-box transcription factor regulates, the expression of the homeobox transcription factor Gbx2. Gbx2 regulates Slit expression in the pharyngeal endoderm and ectoderm. The Slit ligand, a secreted glycoprotein that has been shown to act as a guidance molecule, recognizes its transmembrane receptor Roundabout (Robo), which is expressed by cardiac crest cells. In the absence or in a decreased environment of Slit, cardiac crest cells destined for pharyngeal arch 4 migrate instead to arches 3 and 6. Lacking their cardiac crest sheath, the endothelial cells in arch 4 specifically fail to maintain patency of the 4th arch artery, resulting in interrupted aortic arch. Thus Slit/Robo signaling in arch 4 seems to be an attractant for arch 4 crest. In trunk crest Slit/Robo signaling is an important repellant maintaining migrating trunk neural crest cells in the ventral pathway.

As mentioned previously the Ephrin signaling family is likely to be involved in attracting the crest to the pharyngeal arches. EphrinA signaling occurs in all of the pharyngeal arches. In Xenopus, treatment with dominant negative EphA2 caused crest cells destined for arch 3 to migrate into the domain of the 4th arch crest. However, mouse EphA2 mutants do not show a defective neural crest phenotype suggesting that other Ephs can substitute in its absence.

In Xenopus EphA4 and EphB1 are both expressed in crest cells migrating to arch 3 while EphB1 only is expressed in arch 4 crest. The ephrin-B2 ligand is expressed in the adjacent arch 2 neural crest and mesoderm. Truncation of the EphA4 and EphB1 receptors leads to abnormal migration of arch 3 crest cells in that they are misdirected into arches 2 and 4. Ectopic activation of the receptors by overexpression of ephrin B2 or truncation of Eph receptors leads to scattering of arch 3 cells into adjacent regions. This suggests that the mixing of neural crest destined for arches 2 and 3 is blocked by the complementary expression of EphA4/EphB1 receptors and ephrin-B2 so that the cells are correctly targeted to arch 3. A similar mechanism is utilized in the mouse. In embryos with conditional knockout of EphrinB1, cells in the caudal stream invade territories that are normally devoid of crest cells. Consequently, the branching and fasciculation patterns of cranial nerves IX and X are not normal. However, other cells in the cranial crest are also abnormal so EphrinB1 is not specifically necessary for cardiac crest. EphA4 receptor, detected by a lacZ knockin allele, is expressed by cells migrating to the second and third arches in homozygous ephrinB2-null mutants and the second arch is either absent or reduced in size. Because Eph/ephrins have only been seen in the more cranial arches, this pathway does not seem to be critical for cardiac crest migration except possibly for arch 3 cardiac crest.
between vertebrates. For example, ETA is expressed by mouse cranial neural crest during EMT and early migration, but not expressed in chicken cranial crest during early migration.91-93

Endothelin 1 ligand is produced by the endothelium of the arch arteries91 and ETA is expressed in both chick and mouse by ectomesenchymal cardiac crest populating the pharyngeal arches. This reciprocal expression pattern allows the endothelium to signal to the neural crest. This signaling is important for cardiovascular development because ETA-null or endothelin 1-null mice embryos have VSD and arch artery patterning defects.91 In the chick, ETA-specific antagonists caused reduction and dysmorphogenesis of the jaw, heart and aortic arch artery repatterning. Interestingly, the dorsal derivatives of pharyngeal arches 1 and 2 (temporomandibular joint, incus, stapes) show a normal mixture of neural crest and mesenchymal cells while the ventral elements are affected.92 These defects suggest a patterning role for endothelin signaling to crest cells but the mechanism is still unclear. One way this may be achieved is by regulating proliferation of the neural crest cells. ETA-null mouse embryos show significantly less proliferation at E10.5 and more apoptosis after E11.5 after the cells have populated the pharyngeal arches resulting in small jaw cartilages. Another possibility is that the ETA expressing neural crest are attracted by the endothelin1 expressing endothelial cells. In chimeras, ETA-null crest cells are excluded from the caudal pharyngeal arches when mixed with normal cells in wild-type hosts, which results in a failure to form a normal aortic arch artery.92 94 It is not clear if the crest cells fail to migrate to the arches because endothelin is needed for signaling or if the cells are unable to function properly once in the arches and then die. Ablation of ETB in the cardiac crest population results in failure of the cells to migrate to the distal gut resulting in Hirschsprung's disease. Thus, there is precedence for endothelin signaling to be important in migration but the finding of increased cell death in the arches suggests that it is cell viability rather than migration that is important in endothelin signaling in the cardiac crest in the caudal arches.

The TGFβ superfamily includes BMP and TGFβ ligands and is one of the most widely studied signaling families that affect cardiac neural crest development; however, evidence of the family's role in migration is scanty. Knockout of various ligands in the family leads to cardiovascular defects but tell little about their role in cardiac neural crest migration.95,96 A recent study demonstrated direct interaction of the cytoplasmic domain of BMPRII with the cytoskeletal regulator LIM kinase 1 as well as BMP-induced regulation of the kinase activity of LIM kinase 1. These data provide evidence for direct signaling by a type II receptor, through LIM kinase 1, to the cytoskeleton97 suggesting that BMP signaling might be important in cardiac crest migration. Unfortunately, the conditional knockout experiments that have been done with the BMP receptor family to date have not resulted in a simple association of this signaling family with migration. Specific deletion of intracellular components of the BMP and TGFβ signaling pathways in neural crest cells including specific receptors and Smads using Wnt1cre have been more informative. Knockdown of the BMP receptor 1 in premigratory cardiac crest in chick embryos does not alter early migration (Fig. 4).

TGFβ family defects can be due to aberrations in the extracellular matrix components, which bind to TGFβ family ligands. Mutant mice lacking the long form of latent TGFβ binding protein 1 (Ltp1L), a protein that covalently binds latent TGFβ ligand, die at birth from improper septation and remodeling of the arches.98

Platelet-derived growth factor (PDGF) has been thought to be important in cardiac crest development from the Patch mutant mouse phenotype. Both of the PDGF receptor (PDGFR) subtypes α and β are expressed in cardiac crest and conditional double knockout of PDGFRα/β in the neural crest leads to atresia of the right 4th aortic arch artery (retroesophageal right subclavian) and failure of outflow septation.99,100 However, neural crest migration to the pharynx is normal in these embryos and the cells slowly disappear, suggesting that PDGF is needed for some aspect of survival rather than migration.

Once the neural crest cells have been properly targeted to the correct pharyngeal arch, a subpopulation of cells condenses around the pharyngeal arch arteries to form first a sheath, which subsequently develops into the tunica media. Condensation of the neural crest cells around the aortic arch arteries 91 and ETA is expressed in both chick and mouse by EMT and early migration, but is not expressed in chicken cranial crest during early migration.91-93

Condensation of the cardiac neural crest cells around the aortic arch arteries and patterned depends on chemokine signaling via stromal cell-derived factor (Sdf1) and its receptor Cxcr4.101 Cxcr4 and Sdf1 are expressed in the migrating neural crest and in the migratory pathway in the mouse and has been shown to be important for positioning of the dorsal root ganglia.102 Ablation of the Sdf1 receptor Cxcr4a in zebrafish allows the cells to migrate through the arches and onto the yolk sac.103 In addition, the cells in the caudal arches fail to condense normally. Thus the pause or stop in migration may also be linked to the initial stages of condensation.

Another molecule associated with condensation of the neural crest in the pharynx is Rac1. Rac1 is generally associated with formation of lamellipodia suggesting that it might play a role in neural crest migration. However, it has been found to be critical for cell adhesion and condensation of the cells in the pharyngeal arches and outflow tract. Wnt1cre conditional knockout of Rac1 is associated with mispatterned aortic arch arteries, failed smooth muscle differentiation of the neural crest derived tunics and failure of outflow tract septation.103

Migration into the Cardiac Outflow Tract and Condensation

The final steps in cardiac crest migration are entry into the distal outflow tract cushions where the cells condense. From there a small population migrates into the proximal outflow tract. These cells do not form condensed mesenchyme in contrast to the distal outflow crest. Finally, cells migrate into the interventricular septum where they form a sheath around the AV bundle. Little is known about what factors attract neural crest cells into the outflow tract cushions. In Xenopus, Sdf is expressed in the heart and has been proposed but not tested to regulate entry of cardiac crest (expressing the Sdf receptors Cxcr4 and Cxcr7) into the heart.104

Once the cardiac neural crest cells reach their target, i.e., the outflow cushions, the cardiac neural crest cells condense to form the aorticopulmonary septum. The TGFbeta/BMP signaling
family has been implicated in this process. The BMP receptors, Alk2 and Alk6 (BMP receptor IA) have been conditionally deleted in neural crest. When Alk6 is deleted in neural crest cells they migrate normally into the pharynx and outflow tract but do not condense to form the outflow septum.105 Conditional deletion of Alk2 leads to regression of the 6th pair of aortic arch arteries and while an initial septation complex forms it fails to extend prongs into the truncal cushions.106 Because of the combination of arch and outflow defects, the dependence of crest on Alk2 to migrate into the outflow tract may be secondary to the requirement for Alk2 in the pharynx.106 Both TGF and BMP signaling require Smad4 as a coactivator. Conditional deletion of Smad4 in cardiac neural crest cells leads to failure of outflow tract septation. While the cardiac crest cells migrate normally to the caudal pharyngeal arches, large clumps of dying cells can be seen in the arches and the cardiac neural crest cell contribution to the outflow tract is dramatically reduced.107 Because of the reduction in the crest population in the pharynx with altered TGF family signaling, it seems unlikely that the TGF family plays a direct role in crest migration.

Cells in the distal outflow tract condense to form the aortico-pulmonary septation complex. Semaphorin 3C and neuropilin 1/plexinA2 signaling appear to be important for not only normal migration, as discussed above, but also for condensation of the cardiac crest cells in the distal outflow tract. Neuropilins are transmembrane receptors but with a short cytoplasmic tail that does not participate in transduction of the semaphorin signal. The cytoplasmic domain of the co-receptor plexins have signaling function and are thought to be the signal transducer of neuropilin-plexin complexes. Sema3C is an attractive guidance signal for cardiac crest and it is also expressed in the cardiac outflow tract.108-110 Mice null for Sema3C or PlexinA2 have disorganized crest in the outflow tract. Sema3C is also expressed by cardiac crest cells themselves. Sem3A and Sem3E are agonists with receptor complexes containing neuropilin 2, but block Sem3D-signaling at receptor complexes containing neuropilin 1.111 This suggests that a role for sema3C in cardiac crest in the outflow tract might be to mute the response of cells to sema3C expressed by the subpulmonary myocardium expressing sema3C.108

Another factor that is important in condensation of crest cells to form the aortico-pulmonary septation complex is N-cadherin.112 With crest-specific knockout of N-cadherin the migration and homing of crest cells to the outflow tract is normal but the cells are unable to form the septation complex resulting in persistent truncus arteriosus in the majority of mutant embryos. The cells showed abnormal morphology in that they were unable to elongate and align properly in the middle of the outflow tract and showed limited contact with their neighbors. Rotation of the outflow tract was also incomplete suggesting that alignment of the channels is dependent on N-cadherin-generated cytoskeletal forces with the outflow myocardium. Condensation of the crest cells in the pharyngeal arches to form the aortic arch artery sheaths was normal so the requirement for N-cadherin in condensation is specific to the distal outflow tract.

**Table 1. Function of factors involved with specific events in cardiac neural crest migration**

| Function                                   | ECM Protein | Growth Factor | Transcription Factor | Signaling Pathway                              | Cell Adhesion |
|--------------------------------------------|-------------|---------------|---------------------|------------------------------------------------|---------------|
| Migration/Condensation: Outflow Tract       | Fibronectin | Chondroitin   | Versican            | Bmp1L/Eta/etb                                    | N-cadherin    |
| Migration/Condensation: Pharyngeal Arches  | Chondroitin | Tbx1/Eph     | Gbx2               | Tgf/Fgf                                         | Smad4         |
| Migration/Condensation: Circump. Ridge     | Chondroitin | Slt/Robo     | Endothelin          | Lgr/FGF                                        | Smad4         |
| Cell Adhesion & Migration                  | Chondroitin | Ephrin/Eph   | N-cadherin          | Smad4 Smad 1                                    | Ets1          |

**Migration into the Proximal Outflow Tract**

The mesenchyme of the proximal cushions is derived largely from EMT of the endocardium to form cushion mesenchyme. Even so a sparse population of crest cells migrates into the proximal cushions. Because most perturbations that interfere with cardiac crest affect distal outflow septation, it has been unclear whether the cells migrating proximally are important for the final steps in septation. Some light has recently been thrown on this issue. Many tyrosine kinase receptors activated by a variety of growth factors rich in the pharynx and cardiac outflow tract, including FGFRs, activate the intracellular MEK-ERK signaling pathway. Ets1 is a transcription factor activated by MEK-ERK signaling. Ets1 knockout mice have a white belly spot suggesting that late migration of neural crest-derived melanocytes is dependent on Ets1 activation. Cardiac crest also fails to migrate into the proximal (conal) cushions suggesting that this final migration...
is controlled by Ets1. Because these mice have membranous ventricular septal defects, this suggests that cardiac crest cells, although a minor cell population in the proximal outflow tract, are important in closure of the conal portion of the ventricular septum. However, a confounding factor in this interpretation is that there are intracardiac cartilage nodules derived from cardiac crest. Interestingly, MEK/ERK signaling upstream of Ets1 is required to block cartilage formation.

Formation of the Cardiac Ganglia

The cardiac ganglia are formed entirely by cardiac crest: both the neuronal cell bodies and supporting cells are derived from cardiac crest. Virtually nothing is known about the factors that control their separation from the cardiac crest forming the aortico-pulmonary septum or their condensation as ganglia. However, cardiac crest cells also participate in formation of the nodose ganglion. This is the distal sensory ganglion of the vagus nerve. The nodose ganglion is formed from neurons derived from the nodose placode located dorsal to pharyngeal arches 4/6. Cells migrate from this placode to coalesce with cardiac crest to form the nodose ganglion. Condensation of this ganglion depends on N-cadherin and signaling by Slit/Robo signaling. In cranial crest Slit1/Robo signaling in conjunction with N-cadherin is important for coalescence of crest cells and placode-derived neurons into ganglia. N-cadherin and Robo2 are expressed by placode neurons and Slit1 is on neural crest cells. If either N-cadherin or Robo2 is knocked down, the ganglia do not coalesce properly.

Conclusion

Remarkable progress continues to be made in the identification of signaling pathways, environmental cues and transcription factors that control the formation, migration and differentiation of cardiac neural crest cells. We have discussed some of the factors that influence the specification of the cardiac neural crest in the neural tube, initiation of migration, organization into the 3rd migratory stream and the unique path they take first to the circumpharyngeal ridge, then the pharyngeal arches and finally to the heart. Many questions remain. The finding that the same signals, such as BMPs and Ephrins, control multiple aspects of neural crest development raises the intriguing question of how a signal controls different responses at different stages. What are the cues that cause the caudal stream to pause in the circumpharyngeal ridge? What are the molecular cues and differences between the cardiac neural crest that stop and condense to participate in arch artery development in the caudal pharyngeal arches and the subpopulation that continues to migrate into the outflow tract? How do these cardiac crest cells further segregate into those that will form the outflow septum and those that will form the cardiac ganglia? These differences may be initiated before the crest cells leave the neural tube and/or by the dynamic contacts between neural crest cells themselves, the neighboring cells and the environment. Finally, the least well understood and studied aspect of cardiac neural crest development is the process of condensation of the crest at their final destinations. This seems to be a common theme in how the neural crest initiates differentiation. While much remains to be learned about the amazing journey cardiac neural crest takes from the neural tube to the heart, we do know that disrupting any phase of the journey can have serious consequences on cardiovascular development.

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