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Signals controlling neural crest contributions to the heart

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Abstract

Cardiac neural crest cells represent a unique subpopulation of cranial neural crest cells that are specified, delaminate and migrate from the developing neural tube to the caudal pharynx where they support aortic arch artery development. From the caudal pharynx, a subset of these cells migrates into the cardiac outflow tract where they are needed for outflow septation. Many signaling factors are known to be involved in specifying and triggering the migration of neural crest cells. These factors have not been specifically studied in cardiac crest but are assumed to be the same as for the other regions of crest. Signaling factors like Ephs and Semaphorins guide the cells into the caudal pharynx. Support of the cells in the pharynx is from endothelin, PDGF and the TGF β /BMP signaling pathways. Mutants in the TGF β /BMP pathway show abnormal migration or survival in the pharynx, whereas the migration of the neural crest cells into the outflow tract is orchestrated by Semaphorin/Plexin signaling. Although TGF β family members have been well studied and show defective neural crest function in outflow septation, their mechanism of action remains unclear.

Keywords

cardiovascular development; cardiac neural crest; signaling

Introduction

The cardiac neural crest generates a unique population of cells needed for the normal patterning of the great arteries and for normal cardiac outflow septation [1]. Cardiac neural crest cells originate from the dorsal neural tube between the mid-otic placode and the caudal boundary of the third somite [1]. After they delaminate from the dorsal neural tube, cardiac neural crest cells migrate into the caudal three pharyngeal arches (arches 3, 4, and 6), and from there some of these cells will continue migration into the cardiac outflow tract where they condense and form the outflow septum (Figure 1) [1–4]. While the cardiac neural crest cells are a subdivision of the cranial neural crest, these cells have their own unique properties. Like cranial neural crest, the cardiac neural crest can generate ectomesenchyme and like trunk neural crest, the cardiac neural crest cannot regenerate [5]. Interestingly, these cells are prepatterned in a specific and subdivided *Hox*-dependent manner [6,7].

Since the neural crest cell population is mobile, signaling has to be considered in the context of the cells' location and function at specific time points. While most of the details at each step in the developmental history of cardiac crest cells are not yet available, some aspects of how the cardiac crest respond to various signaling factors are now known. The cardiac crest migrates

Further Reading

Kirby ML. 2007 Cardiac Development. Oxford University Press: New York.

Cross-References

Signaling pathways that underlie DiGeorge syndrome
 Signaling pathways underlying congenital cardiomyopathy
 Cytokine Signaling

stereotypically in a tight timeline in which most events are similar for chick and mouse, the species that have been best studied (Figure 2).

The signaling factors involved in neural crest specification and induction are known from studies of cranial and trunk crest, and these events appear to depend on the same signaling moieties [8]. Migration of the cardiac crest is targeted to pharyngeal arches 3, 4 and 6, and some signaling events are implicated in this targeting. Many signaling pathways are involved in the migration of neural crest cells into the cardiac outflow tract and the condensation of the cardiac crest cells as they form the outflow septum. Interpreting these signaling pathways is complicated because in many cases it is unclear where in the spatiotemporal aspect of the developing embryo the abnormal signaling has affected the cells. We will attempt to show where signaling likely occurs in the context of the developmental history of cardiac neural crest cells (Figure 1).

Specification and induction

The specification and induction of neural crest involves a number of secreted signals [8], including Wnt, Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF) and Retinoic Acid (RA) that are critical for the induction of the neural plate border [9]. Importantly, the canonical Wnt beta-catenin pathway is specifically required for neural crest induction [10]. The combined action of these secreted signals triggers the expression of neural crest specifier genes. Many of the specifier genes are transcription factors, such as Snail2, Sox9 and FoxD3 [11]. Work by several groups has revealed the importance of many signaling factors and their effector targets, including the changes in cadherin expression that are necessary for neural crest cell delamination and migration [12–14]. It is clear that delamination and migration are highly regulated events that are dependent upon multiple signaling cues, but initial BMP4 signaling from the adjacent ectoderm appears to play a major role. Avian neural crest cells synchronously undergo epithelial-mesenchymal transition and emigrate from the neural tube in the S phase of the cell cycle. Inhibition of the transition from G1 to S blocks delamination [15]. These events are likely coordinated by BMP4 signaling via Smad1 which upregulates Slug/Snail2 expression and concomitantly downregulates N-cadherin protein by stimulating its cleavage via a metalloproteinase (ADAM10) into cytoplasmic and soluble fragments. The cytoplasmic fragment of N-cadherin translocates to the nucleus stimulating cyclin D1 transcription [16]. Multiple changes in Cadherin expression coordinate the delamination and emigration of neural crest. For example, Cadherin6b is directly repressed by Snail2, and this regulatory step is critical for the timing of neural crest emigration [14].

BMP4 is homogeneously expressed in the dorsal neural tube. By contrast, the BMP inhibitor noggin is expressed in a cranial-caudal gradient with high expression in the presumptive cardiac crest at the time of induction [17]. Reduction in the level of BMP activity to a precise concentration is required to induce neural crest. This is an intermediate level of BMP activity which leads to increased Msx gene expression in the neural plate border. Msx leads to expression of early neural crest markers like Snail1/2 and FoxD3 at the time of neural crest specification. This suggests a model where a gradient of Bmp activity determined by noggin specifies the expression of Msx genes in the neural folds which in turn upregulates early neural crest specification genes [18].

Migration

Cardiac neural crest cells have definitive migration patterns and stereotypic destinations. The cardiac neural crest cells migrate ventrally through pharyngeal arches 3–6, where they first invest and later form the smooth muscle tunics of the great arteries [19]. From there, cells migrate into the outflow pole of the heart where they form the outflow septum [2,20,21]. The cardiac crest also gives rise to all of the parasympathetic innervation to the heart [1] and

connective tissue insulation of the His-Purkinje conduction system [22], although the signaling involved in determining these cell types has not been investigated.

Cranial neural crest cells originating from the hindbrain express specific sets of Hox genes depending on their craniocaudal origin. The Hox code is determined by FGF8 signaling from the midbrain-hindbrain boundary (isthmus) [23,24]. FGF8 signaling from the isthmus alters HoxA2 expression and consequently pharyngeal arch patterning [24].

In addition to this pre patterning of the neural crest by FGF8 via HoxA2, the signaling factors that govern neural crest migration are segregated. In *Xenopus* embryos, EphA4 and EphB1 are expressed in migrating neural crest cells and in the mesoderm of arch 3 [25,26] while the ephrinB2 ligand is expressed in arch 2 neural crest and mesoderm [25,27]. EphA4/EphB1 receptors and ephrinB2 ligand are involved in restricting the intermingling of third and second arch neural crest and also in targeting third arch neural crest cells [25]. In chick, cranial neural crest cells express a variety of Eph receptors and the ephrinB2 ligand. These cells migrate along pathways bordered by non-neural crest cells expressing ephrin B1 ligand and EphB2 receptor. The neural crest cells strongly prefer to migrate along pathways that contain fibronectin without ephrinB1 or EphB2 [28], suggesting that chick cardiac neural crest cells respond to the same family of repulsive cues as those in *Xenopus*, although the specific roles of each ephrin ligand and Eph receptor have not been rigorously conserved between amphibians and avians.

Throughout their journey, cardiac neural crest cells interact with a multitude of signaling factors, many of which may help guide them and reciprocally, the cardiac crest cells may pattern and contribute to many of the structures in proximity to these secreted factors.

The role for FGF signaling in migrating cardiac neural crest cell development remains somewhat elusive even though we know that cardiac crest cells modulate FGF signaling in the caudal pharynx [29]. Hypomorphic FGF8 mice show apoptosis of the cardiac neural crest cells as they emigrate from the neural tube and in the pharyngeal arches [30]. Even so, Moon and colleagues have reported that the FGF receptor 1/2 double conditional knockout under the Wnt1-cre promoter has a normal phenotype (personal communication). This could be due to redundancy with the other FGFRs. A study of the four FGF receptors and their downstream targets Mkp3 and dpERK showed that they are all present in the dorsal neural tube at the onset of migration [31]. This and the data from FGF8 hypomorph studies suggest a role for FGF8 in cardiac crest development, but further studies are needed to elucidate the specific roles for the FGF family of signaling factors in cardiac neural crest development. Specifically, knockout of all of the FGF receptors should be done. FGF-2 and FGF-8 have chemotactic activity for mouse mesenchymal neural crest cells. FGF-2 expression is promoted by FGF-8 and is a prerequisite for the differential localization of FGF-2 which is essential for chemotaxis of mesencephalic neural crest cell migration [32].

Much of what is known about the roles of cardiac neural crest has been identified from cardiac neural crest ablation studies. Several of the defects seen after neural crest ablation result from abnormal signaling in the caudal pharynx: studies from our lab have shown that the cardiac crest cells are important for modulating signaling in the pharynx, especially FGF8 which is expressed by the pharyngeal endoderm and ectoderm [29]. When the cardiac crest cells do not arrive in the pharynx, FGF8 signaling is much higher than normal, suggesting that the cardiac neural crest cells either reduce the source of FGF8 or in some way sequester/catabolize the FGF8 that is present in the caudal pharynx. The levels of signaling factors in the caudal pharynx are important because they impact the splanchnic mesodermal progenitors of the cardiac outflow tract that are being added to the outflow tract during the time that the cardiac crest cells migrate into the caudal pharynx [33].

The final migration of cardiac crest cells into the outflow tract is guided by Semaphorin 3C. The myocardium underlying the pulmonary side of the undivided outflow tract expresses the semaphorin while the cardiac crest cells express its receptor Plexin A2. The Plexin A2 expressing neural crest cells are attracted by the Semaphorin 3C ligand [34]. Semaphorins are secreted, transmembrane and GPI-linked proteins that were initially found to be important in axonal guidance in the developing nervous system [35,36]. Semaphorin ligands bind neuropilin and plexin receptors which lead to alterations in the cytoskeleton and microtubule network [37]. This signaling is required for the correct migratory patterning of cardiac neural crest cells in the outflow tract [38,39]. Semaphorin is expressed in the outflow myocardium that underlies the nascent pulmonary side of the common trunk [39,40]. The subpulmonary myocardium is the last myocardium added to the outflow tract from the secondary heart field. Seventy-five percent of newborn *Sema3C* mutant mice have incomplete septation of the outflow tract and these animals have a single semilunar valve with four cusps and a ventricular septal defect just below the valve [39,41]. Interestingly, the PlexinA2 coreceptor is expressed by migrating and postmigratory cardiac neural crest cells [41]. This suggests that migration of cardiac crest into the distal outflow tract cushions may be orchestrated by Semaphorin3C-PlexinA2 signaling. The other major cardiovascular defect seen in Semaphorin 3C null embryos is interrupted arch (type B), but the pathogenetic mechanism of this defect is unclear.

Target Sites: Caudal Pharynx and Outflow Tract

The response of cardiac neural crest cells to signaling in the caudal arches and outflow tract is not well understood. Endothelin, Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor Beta (TGF β) family signaling may allow neural crest to function in supporting the arch artery development and repatterning into the great arteries. Failure of the crest cells to function correctly appears to be associated with cell death. Whether the outflow tract is directly affected by these signaling factors or secondary to the failure of cells to populate the outflow tract is unclear. Some of the TGF β family defects are due to aberrations in the extracellular matrix components which bind to TGF β family ligands. For example, mutant mice lacking the long form of latent TGF β binding protein 1 (Ltbp1L), a protein that covalently binds latent TGF β ligand, die at birth from improper septation and remodeling of the arches [42]. While there are other extracellular factors that influence neural crest migration, including matrix metalloproteases, are known to remodel the extracellular matrix as they migrate through it; however, the Ltbp1L is the only extracellular factor known to specifically interact with cardiac neural crest.

Endothelin-1 (ET1) ligand is another signaling factor which undergoes proteolytic cleavage. ET1 is cleaved from an inactive precursor by endothelin-converting enzyme-1 (ECE1) and acts on the endothelin-A (ETA) receptor [43]. ETA is expressed by cardiac neural crest cells in the pharyngeal arches and outflow tract [44,45] while ET1 and ECE1 are expressed by pharyngeal arch epithelia. Treatment of chick embryos with an antagonist to ETA or knockout of ETA or ECE results in abnormal regression of arch arteries 4 and 6 and enlargement of arch artery 3 [43–45]. Initial patterning develops normally but repatterning into the great arteries is abnormal, suggesting endothelin signaling to the cardiac crest helps maintain its ability to pattern the great arteries [43]. Interestingly, ETA null cells are excluded from the walls of the developing arch arteries in chimeric mice, suggesting that ETA signaling is cell autonomous in developing cardiac crest [46].

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. Conditional double knockout of PDGFR α/β in the cardiac neural crest is more severe than either of the single knockouts [47]. These double knockouts all display persistent truncus arteriosus and retroesophageal origin of the right subclavian artery associated with

abnormal regression of the right fourth aortic arch artery [47]. It appears that crest cells begin migrating normally and populate the pharyngeal arches and outflow tract, but over the next few days fewer cells are seen in these locations. This suggests that PDGF signaling is important in either maintaining viability or triggering proliferation of the cardiac crest cells.

The TGF β superfamily (Figure 3), including BMP and TGF β ligands, is one of the most widely studied signaling families that affect cardiac neural crest development. Knockout of TGF β 2 ligand [48], or simultaneous inactivation of BMP6 and BMP7 leads to cardiovascular defects but tell us little about cardiac neural crest signaling [49]. Conditional knockout of intracellular components of the BMP and TGF β signaling pathways, i.e. specific receptors and Smads using Wnt1cre have been more informative (see below).

The TGF β subfamily ligands signal via a receptor complex composed of two type II receptors and two type I receptors (Figure 3) [50]. Upon ligand binding, type II receptors, which are constitutively active kinases, phosphorylate and activate type I receptors (ALKs) [51–53]. Type I receptors phosphorylate and activate a specific set of downstream signaling molecules called Smads. In general terms, TGF β s bind to the TGF- β type II receptor (TGF β RII) and TGF β type I receptor (ALK5) activating Smads 2 and 3, while BMPs bind to the BMP type II receptor and type I receptors ALK2, -3, or 6, activating Smads 1, 5 and 8. However, it is likely that these signaling interactions are more complex *in vivo*, possibly allowing formation of heterotetrameric complexes composed of different type II and type I receptors [54]. Also novel ligands, like growth and differentiation factors (GDFs) 8 and 9 have been found to bind combinations of receptors in the TGF β family and activate Smads. Because of these multiple pathways for crosstalk, phenotypes associated with null mutation of one receptor might not correspond to those seen in a null mutation of its usual binding partner. 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 cytoskeleton [55]. This would suggest 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.

Alk5 (TGF β R1) conditional mutants have a significant percentage of retroesophageal right subclavian arteries and right arches in addition to persistent truncus arteriosus [56]. Cardiac neural crest cell migration and proliferation are not affected but there is a dramatic increase in the number of dying cells in tissues surrounding the aortic sac including the site where the outflow septum begins to form. The phenotype of TGF β R2 conditional null mutant is persistent truncus arteriosus and interrupted aortic arch (IAA) in mutant embryos. The pathogenesis of persistent truncus arteriosus is unclear because the cells migrate into the outflow tract, form the septation complex but fail to form an outflow septum. Proliferation and cell death are not altered in these embryos. The pathogenesis of the IAA is clearer in that at E12.5: the 4th arch artery connection between the ascending aorta and the dorsal aorta is surrounded by dying smooth muscle actin-positive cells [57]. Thus, it seems that ALK5 mediates a somewhat wider spectrum of signaling events than TGF β RII in cardiac neural crest cells during outflow development and arch artery patterning.

It has been shown that individual BMPs elicit distinct cellular responses and bind to different type I receptors with different binding affinities. ALK2, ALK3 and ALK6 are able to bind and transduce signaling from many different BMPs although there are preferences [58].

Alk2 and *Alk6* have been conditionally deleted in cardiac neural crest. In *Alk6* conditional mutants, neural crest cells migrate normally into the pharyngeal arches and the aortic arch

arteries and their derivatives are correctly patterned. Some crest cells migrate into the outflow tract but these are not able to form the outflow septum leading to persistent truncus arteriosus [59]. By contrast, conditional *Alk2* mutants show regression of the 6th pair of aortic arch arteries. While an initial septation complex forms it fails to extend prongs into the truncal cushions. This suggests that *Alk2* is needed either for normal migration and/or maintenance of cardiac crest cells in the pharyngeal arches and outflow tract. It is unclear if a minimum population of cells is required in the pharyngeal arches to allow further migration of cells into the outflow tract. Thus the dependence of crest on *ALK2* to migrate into the outflow tract may be secondary to the requirement for *ALK2* in the pharynx [60].

Canonical TGF β or BMP signaling results in phosphorylation of different Smads but these phosphorylated Smads all bind the universally used co-Smad4. The Smad4 complex is then translocated to the nucleus where it alters transcription. Conditional deletion of Smad4 in cardiac neural crest cells using *Wnt1cre* 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. The cardiac neural crest cell contribution to the outflow tract is dramatically reduced [61].

Conclusion

Much of our understanding of signaling to cardiac crest is extrapolated from studies that alter gene expression in all neural crest. While specification, induction and early migration are likely the same as in crest originating at other axial levels other aspects of signaling in cardiac crest are likely to be specific because of its unique migratory pathway, functions and final targets. More studies that specifically target cardiac crest await a cardiac neural crest-specific promoter driven cre for excision of gene function. Much can be learned specifically about cardiac crest by returning to avian models and particularly quail-chick chimeras. Electroporation or viral delivery of constructs to premigratory crest can target the cells for overexpression or knockdown of specific genes along with fluorescent tracers that allow visualization of the cells as they migrate into the pharyngeal arches and outflow tract. Several lines of transgenic avian embryos are also now available [62,63]. New techniques for visualization allow monitoring behavior of marked cells i.e. migration, proliferation and homing to target sites in real time. A popular method for creating transgenic quail has employed lentiviral vectors. The infected cells follow normal migratory routes and undergo normal differentiation [64,65] and express sufficient levels of fluorescent protein to allow *in vivo* time-lapse video-microscopy, laser scanning confocal microscopy, and two-photon microscopy. The viruses can be targeted to various subcellular locations and express various GFP color variants [66]. The ability to localize different GFP variants to specific cellular organelles allows these structures to be specifically imaged, cell divisions to be followed, and morphological changes to be dynamically observed [67]. These new techniques will allow finer resolution of the role of signaling in cardiac neural crest.

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Figure 1.

Composite representation of a transverse section of a chick embryo. Neural crest, represented in yellow, emigrate from the dorsal neural tube. (1) Signals involved in specification and delamination of neural crest (blue triangles) are represented at stages 10–12 in the developing chick embryo although specification likely occurs much earlier, and it is clear that Wnt, Bmp, Fgf and RA are involved in these developmental processes. (2) Cardiac neural crest migrate into pharyngeal arches 3, 4, and 6 from stages 13–18 (blue squares). Eph/Ephrins are important signaling cues, and it is likely that Fgf is also involved. (3) Endothelin and PDGF signaling supports the survival and maintenance of the neural crest in the pharyngeal region from stages 18–22. (4) While it remains unclear exactly how neural crest forms the outflow septation complex from stages 26–34 (blue pentagons), Semaphorin signaling is involved in this process. TGF/BMP signaling is also involved in this process, but its specific role is unresolved.

Timeline of Cardiac Neural crest Migration in the Chick and Mouse

Figure 2.

The critical developmental steps in cardiac neural crest migration occur in the same order and are conserved between chick and mouse. Delamination from the neural tube, the population of the pharyngeal arches, and septation of the cardiac outflow tract occur similarly across species with divided systemic and pulmonary circulations.

Ligand	Type I	Type II	Smad
TGF β	ALK5(TGFBRI) ALK1 ALK2	TGFBRII	Smad 2/3 Smad1/5
BMP	ALK1 (ActRI) ALK3 (BMPRIA) ALK6 (BMPRIB)	BMPRII	Smad1/5/8

Figure 3.

Components of TGF β superfamily signaling many of which have been evaluated by conditional deletion in the neural crest. TGF β ligands typically activate Smads 2/3 or 1/5, whereas BMP ligands activate Smads 1/5/8. Smads 1,2,3,4 and 8 are also known as receptor-regulated or R-Smads. Smad 4 is a co-Smad and is activated via both types of ligands. Upon activation, the Smad4 complex is translocated to the nucleus where it drives transcription of target genes. The Smads can interact with multiple transcriptional regulators, one of which is Lef/TCF.