CHAPTER 8

Neural Crest Contribution to the Cardiovascular System

Christopher B. Brown* and H. Scott Baldwin

Abstract

Normal cardiovascular development requires complex remodeling of the outflow tract and pharyngeal arch arteries to create the separate pulmonic and systemic circulations. During remodeling, the outflow tract is septated to form the ascending aorta and the pulmonary trunk. The initially symmetrical pharyngeal arch arteries are remodeled to form the aortic arch, subclavian and carotid arteries. Remodeling is mediated by a population of neural crest cells arising between the mid-otic placode and somite four called the cardiac neural crest. Cardiac neural crest cells form smooth muscle and pericytes in the great arteries, and the neurons of cardiac innervation. In addition to the physical contribution of smooth muscle to the cardiovascular system, cardiac neural crest cells also provide signals required for the maintenance and differentiation of the other cell layers in the pharyngeal apparatus. Reciprocal signaling between the cardiac neural crest cells and cardiogenic mesoderm of the secondary heart field is required for elaboration of the conotruncus and disruption in this signaling results in primary myocardial dysfunction. Cardiovascular defects attributed to the cardiac neural crest cells may reflect either cell autonomous defects in the neural crest or defects in signaling between the neural crest and adjacent cell layers.

Introduction

The neural crest are a pluripotent population of cells responsible for the formation or remodeling of a large number of tissues and organ systems. Neural crest cells can form a wide variety of cell types including neurons, glia, Schwann cells, cartilage, bone, and smooth muscle.[1] The neural crest is divided into two major regions termed cranial (mid-diencephalon to somite 5) and trunk (cells arising caudal to somite 5) based upon the rostral-caudal position at which they arise and their subsequent developmental potential.[2] The cranial neural crest form ectoderm derived mesenchyme (ecto-mesenchyme or mesectoderm) that is characterized by the ability to differentiate into numerous cell types normally associated with mesoderm including muscle and bone.[3] Trunk neural crest however, are restricted in developmental potential to melanocytes, neurons and their support cells.[2] A sub-population of cranial neural crest provide material contribution of pericytes and smooth muscle to the cardiovascular system as well as the neurons and ganglia of symapthetic and parasympathetic cardiac innervation.[3,4] This population of neural crest cells has been termed the "cardiac neural crest" (Fig. 1A).[5]

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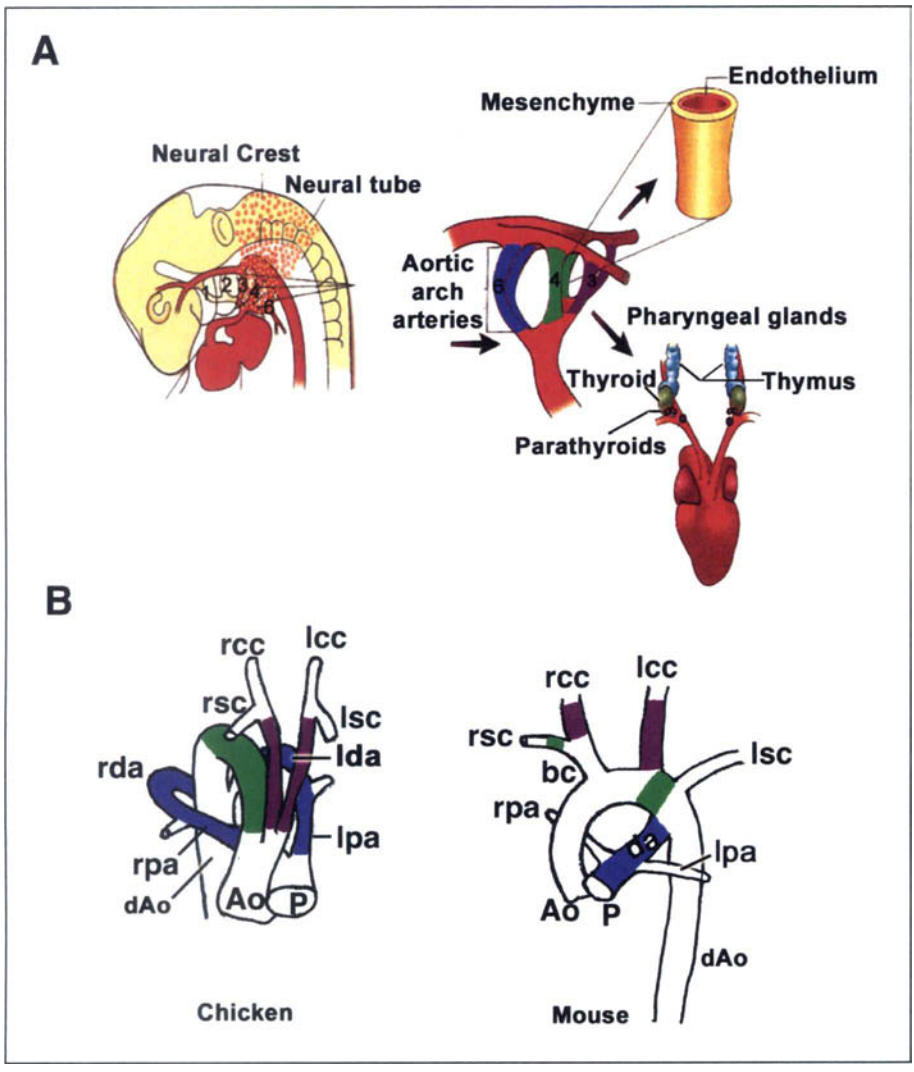

Figure 1. Diagram illustrating the contribution of cardiac neural crest cells to the pharyngeal arch arteries and pharyngeal glands. A) Neural crest form the smooth muscle of the great vessels and connective tissues of the thyroid, parathyroids, and thymus. B) Remodeling of the pharyngeal arches results in a right-sided aortic arch in chickens and a left-sided aortic arch in mouse and human. The third arches in chickens (purple) form the right and left brachiocephalic arteries and form portions of the common carotids in mice. In chickens, left and right ductus arteriosus are formed from the sixth arches (blue). A single ductus arteriosus forms in the mouse (blue). The right fourth arch (green) forms the aortic arch in the chicken, while the left fourth arch forms the transverse segment of the aortic arch in mice. Ao, aorta; P, pulmonary trunk; rpa, right pulmonary artery; lpa, left pulmonary artery; rda, right ductus arteriosus; lda, left ductus arteriosus; da, ductus arteriosus; rsc, right subclavian artery; rcc, right common carotid artery; lcc, left common carotid artery; lsc, left subclavian artery; bc, brachiocephalic artery; dAo, descending aorta. Adapted from: Kirby ML. Contribution of neural crest to heart and vessel morphology. In: Rosenthal RPHN, ed. Heart Development. New York: Academic Press, 1999:179-193;[156] ©1999 with permission from Elsevier.

Identification and Characterization of the Cardiac Neural Crest

Neural crest contribution to the cardiovascular system was first demonstrated in avian models of quail-chicken chimeras.[3,4] Le Douarin and colleagues transplanted the entire rhombencephalon primordium from quail into chicken and observed quail cell contribution to the walls of the brachiocephalic arteries, the carotid arteries, the pulmonary trunk and proximal aorta.[3] Margaret Kirby and colleagues utilized the quail nuclear marker QCPN coupled with small neural tube transplants in quail-chicken chimeras for more detailed analysis of neural crest contribution to the cardiovascular system.[4] These chimera experiments confirmed the results of Le Douarin and localized the cardiac neural crest to the region of the neural tube between the mid-otic placode and somite three (rhombomeres 6-8). Subsequent fate mapping analysis in chicken and mouse have suggested that the caudal boundary of the cardiac neural crest may extend to somite four.[6,7] These cells populate pharyngeal arches 3, 4 and 6 and the outflow tract (Fig. 1A).[8,9] Neural crest entering pharyngeal arches 3 and 4 interact with the endoderm to produce the pharyngeal glands (thymus, and parathyroid glands) (Fig. 1A).[10] This population of neural crest cells also forms the enteric ganglia of the midgut and hindgut (Fig. 2C).[11,12] Thus the term "cardiac neural crest" refers to the unique role of this cell population in cardiovascular patterning and does not imply that these cells are restricted to cardiovascular lineages.

One of the first direct comparative analyses of mouse and chicken neural crest contribution to cardiovascular patterning used a connexin 43 (Cx43) enhancer-*Lac Z* transgene.[13] These experiments demonstrated that mouse cardiac neural crest cells are targeted to the pharyngeal arches, outflow tract and cardiac ganglia consistent with results in the chicken (Fig. 1). Definitive fate mapping of cardiac neural crest was subsequently preformed using the CreLoxP system in transgenic mice. At least four transgenic mouse lines that drive expression of Cre in neural crest cell populations have been reported.[14-17] Three of these lines, *P0*-Cre, *Pax3*-Cre and *Wnt1*-Cre, are active in the dorsal neural tube and allow for lineage tracing of neural crest from the time of emergence into the final mature structures when mated to a *Lac Z* reporter line (R26R).[14-16,18] Human tissue plasminogen activator (*ht-PA*) Cre is not active in the neural tube and labels later migratory and post-migratory neural crest.[17] All four lines demonstrate cardiac neural crest contribution of smooth muscle to the aortic arch, the pulmonary trunk, brachiocephalic artery, the right subclavian artery, the right and left common carotid arteries and the remnant of the ductus arteriosus (ligamentum arteriosum)(Fig. 2A,C-E). The left subclavian artery, pulmonary arteries and descending aorta are not stained in neural crest lineage analysis, reflecting their mesodermal origin (Fig. 2A,E). Fate mapping using a Tbx1-Cre mouse line that labels pharyngeal mesoderm confirms the mesodermal origin of these vessels and demonstrates that the conal portions of the aorta and pulmonary trunk are mesodermally derived (Fig. 2B).[19] The cardiac ganglia and parasympathetic nerve fibers are also labeled as neural crest derivatives (Fig. 2C,D).[15-17] Cardiac neural crest are seen in the leaflets of the aortic and pulmonic valves and the interventricular septum although their role in these tissues is unknown.[15,17] A few fate-mapped cells of unknown function have also been observed in unexpected locations including in the inflow tract, the epicardium, the coronary arteries, the myocardium and in close apposition to the conduction system.[20-23] Although labeling of cells is highly consistent between the Cre lines, the *Wnt1*-Cre mouse line has become the predominant line used for fate mapping and conditional gene inactivation. Some controversy remains over the exact number of cells labeled with each mouse line, and over which mouse line most faithfully labels the true cardiac neural crest niche.

Cardiac Neural Crest Function in Cardiac Development and Remodeling

The first functional heart is a linear tube composed of an endocardial layer and a myocardial layer separated by specialized extracellular matrix. At the heart tube stage, blood enters through a common atria and ventricle, and exits through a common outflow tract (conotruncus). Blood flows through paired pharyngeal arch arteries and into paired dorsal

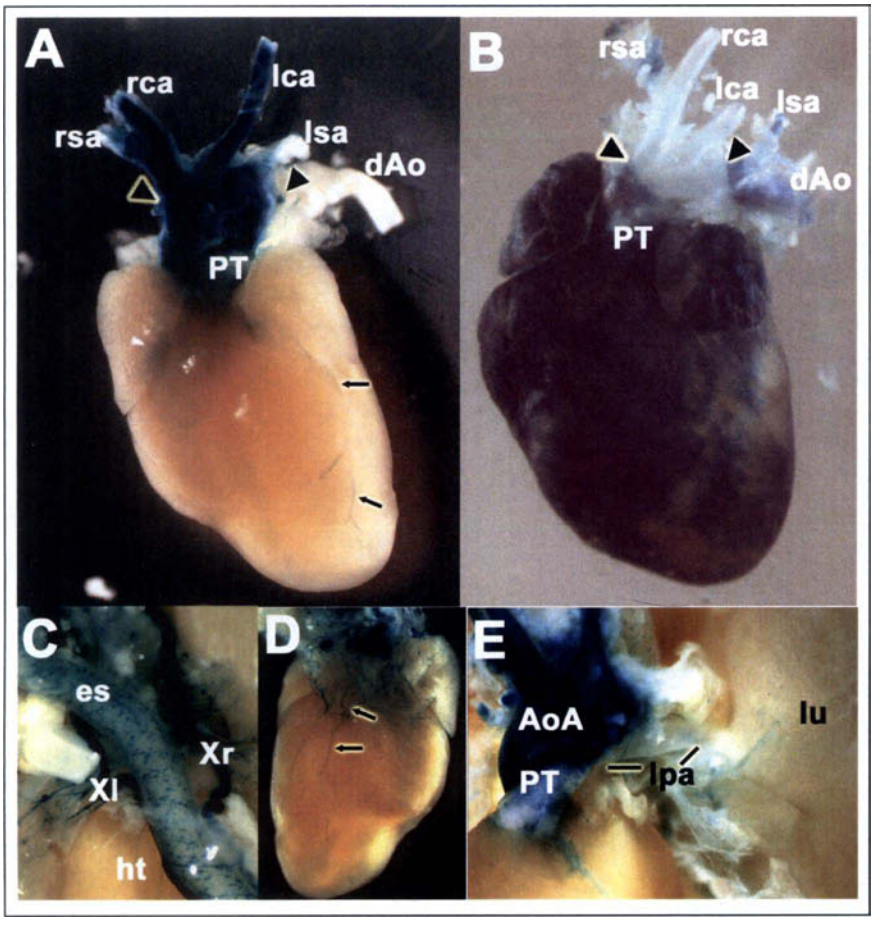

Figure 2. Fate mapping of cardiac neural crest cells with Wnt1-Cre and R26R. A) Ventral view of an X-gal stained neonatal Wnt1-Cre::R26R heart revealing cardiac neural crest derivatives. Note staining in the aortic arch to the level of the left subclavian artery (between black arrowheads). Small arrows denote neuronal staining associated with the coronary arteries. B) Ventral view of an X-gal stained Tbx1-Cre::R26R neonatal heart demonstrating mesodermal contribution to the aortic arch arteries. The proximal pulmonary trunk (PT) and aorta are labeled. The aortic arch is not labeled showing a reciprocal pattern to the neural crest staining (compare region between arrowheads in A, B). The right subclavian artery, left subclavian artery and dorsal aorta (dAo) are labeled using Tbx1-Cre and are not neural crest derived. C) Dorsal view of a Wnt1-Cre::R26R neonatal heart with esophagus and nervous tissue in place. Labeled neural crest contribute to the enteric ganglia in the esophagus, and the left (Xl) and right (Xr) vagal branches. D) Dorsal view of the heart in C with noncardiac tissue removed. Extensive labeling of the neural crest derived cardiac ganglia and nerve tracts is observed (arrows). E) Left lateral view of the Wnt1-Cre::R26R heart from C,D. Neural crest contributes extensively to the aortic arch (AoA). The pulmonary trunk shows lower levels of neural crest contribution with no expression in the conus. The left pulmonary artery (lpa) and lung (lu) show no neural crest contribution.

aorta (Fig. 1A) (for reviews see refs. 24,25). As development proceeds, the conotruncus is split into the pulmonary trunk and aorta while the symmetrical arch arteries are remodeled into the asymmetrical mature forms (for review see refs. 11,26). Ablation of the cardiac neural crest in chickens demonstrated that these cells are required for both septation of the

conotruncus, and for the remodeling of the pharyngeal arch arteries.[5,27] Final separation of the four heart chambers is accomplished by elaboration of atrial and ventricular septa and by the formation of the mitral and tricuspid valves. Chamber formation is not believed to be dependent on the activity of neural crest, although defects may be observed which reflect compensatory changes secondary to defective remodeling of the outflow tract and pharyngeal arch arteries.

Neural Crest Ablation

The functional requirement for cardiac neural crest in normal cardiovascular development was first demonstrated in the chicken embryo by mechanical or laser ablation (Fig. 3).[5,28] Following ablation, no new cells arise from the ablated regions, and these gaps are not repopulated by adjacent neural crest populations. The outflow tract fails to elongate resulting in altered cardiac looping.[29] Cardiovascular defects following ablation include double outlet right ventricle (DORV), Teratology of Fallot, persistent truncus arteriosus (PTA), and anomalous development of the pharyngeal arteries (Fig. 3).[28,30] The severity of

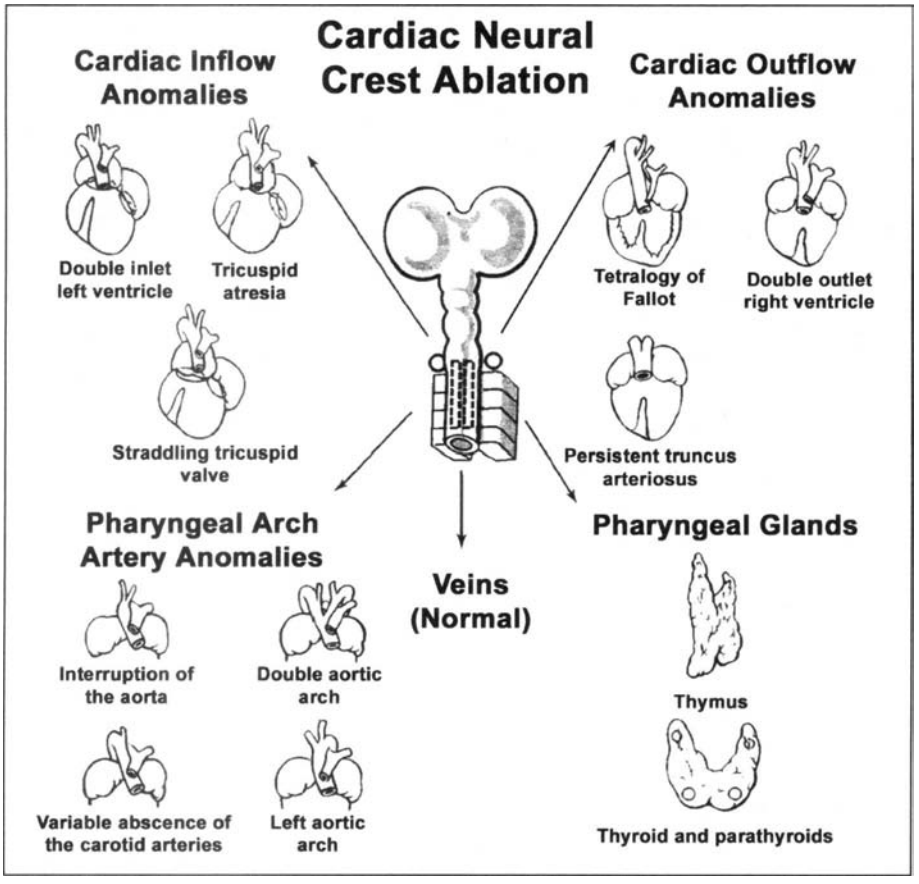

Figure 3. Phenotypic consequences of cardiac neural crest ablation in the chicken. Ablation of the cardiac neural crest causes defective inflow morphology, defective pharyngeal arch patterning, defective outflow septation and alignment defects. Hypoplasia or aplasia of the pharyngeal glands is also noted. Modified from: Kirby ML. Trends Cardiovasc Med 1993; 3(1):18-23;[157] ©1993 with permission from Elsevier.

the defects observed depends on the number of cells ablated.[30] In the absence of neural crest persistence of pharyngeal arch arteries is variable with unpredictable loss or maintenance of individual vessels. These experiments demonstrated that the cardiac neural crest cells are required for persistence rather than formation of the arch arteries.[31] Hypoplasia or aplasia of pharyngeal glands is also seen following ablation (Fig. 3).[10] Deletion of smaller numbers of neural crest cells causes misalignment of the pulmonary trunk and aorta without a septation defect. This suggests an indirect role for neural crest in alignment of the outflow vessels. In addition to defects in patterning, neural crest ablation also causes heart failure.[32-34]

Cardiac Neural Crest Function in Pharyngeal Arch Patterning

The pharyngeal arches are transient "bulges" of mesoderm that arise on the ventral surface of the head flanking the primitive foregut.[35] Each of the pharyngeal arches houses a single artery.[31,36] In chicken, mouse and human five pairs of pharyngeal arch arteries arise in symmetric pairs and connect the common outflow tract to the paired dorsal aortae (Fig. 1A). The arch arteries arise as endothelial tubes. The endothelial cells of the arteries are mesodermally derived and are one of the few cell types not formed by neural crest. Cardiac neural crest form pericytes and smooth muscle in the arteries as they mature.[37,38] During remodeling of the pharyngeal arch system, asymmetric contributions of neural crest cells is thought to be a major determinant of whether a particular vascular component persists or is reabsorbed.[36]

During remodeling, pharyngeal arch arteries 1 and 2 are remodeled into capillary beds.[39,40] Arch arteries three, four and six persist and are remodeled to become components of the mature vasculature (Figs. 1, 4). There are several important differences between birds and mammals in the remodeling of the pharyngeal arches (Fig. 1B).[11,39] In birds remodeling is predominantly right-sided while in the mouse the left side is dominant. In chicken, remodeling occurs between the initiation of circulation at Hamburger and Hamilton stage 12 (45-49 hours) and stage 34 (approximately day 8) (Fig. 5).[39] In birds, the third arch arteries are remodeled into two branchiocephalic arteries (left and right) (Figs. 1B, 4A,D). The left and right common carotid and subclavian arteries originate from the branchiocephalic arteries. The arch of the aorta forms from the right fourth arch while the left fourth arch regresses. Each sixth arch forms a ductus arteriosus (an embryonic vascular shunt that directs circulation away from the developing lungs).[39] It is important to note that while the pulmonary arteries connect to the sixth arch arteries during patterning, they are not "derived" by remodeling from the sixth arch vessels. Thus, pulmonary arteries with ectopic origin are formed in embryos lacking the sixth arch. In the mouse, remodeling occurs between embryonic day 10.5 and 13 (Fig. 5).[40] The right third and fourth arch arteries along with the proximal right dorsal aorta are remodeled to form a single brachiocephalic artery from which originates the right subclavian and right common carotid arteries (Figs. 1B, 4E). The left fourth arch forms the "bridge", or transverse segment, connecting the ascending aorta to the descending aorta while the right fourth arch regresses (Fig. 1B). The left common carotid and left subclavian arteries originate from the aortic arch (Fig. 4B,E). The left sixth arch forms a single ductus arteriosus while the right sixth arch regresses.[40] In both mammals and birds, the ductus arteriosus closes in the early neonatal period in response to a sudden increase in oxygen concentration.

Cardiac Neural Crest in Outflow Tract Septation and Alignment

In addition to mediating pharyngeal arch remodeling, cardiac neural crest cells are also required to separate the outflow tract into the pulmonary trunk and aorta (Figs. 5, 6).[4,5,13,27,41] While it is universally accepted that cardiac neural crest cells play a fundamental role in this process and provide material contribution of smooth muscle to the aorta and pulmonary trunk, the specifics of septation are a matter of controversy. In embryological terms, the conotruncus

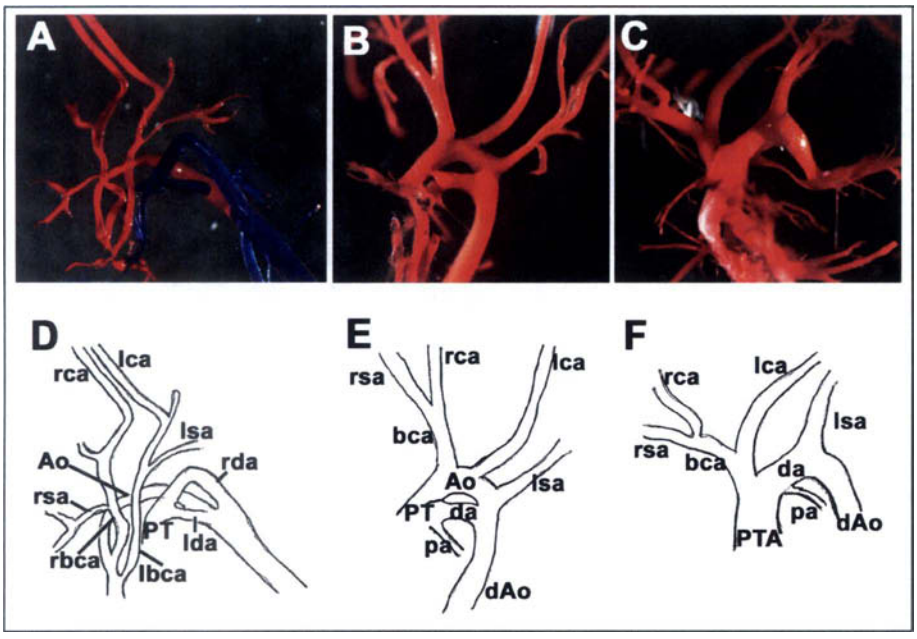

Figure 4. Acrylic resin cast analysis of chicken and mouse pharyngeal arch arteries. A,D) Acrylic resin cast of the pharyngeal arch arteries from a 10 day in ovo chicken embryo. Red acrylic resin was injected into the left ventricle and blue resin in the right ventricle. The pulmonary trunk (PT) has become dissociated from the ascending aorta during processing and is shifted up artificially in this image. D) Labeled sketch of the arteries shown in A. Note that the first branch from the ascending aorta(Ao) is the left brachiocephalic artery (lbca) followed by the right brachiocephalic artery (rbca). The carotid and subclavian arteries branch from the brachiocephalic arteries. There is both a left (lda) and right (rda) ductus arteriosus in chickens. B,E) Single color resin injection demonstrating normal pharyngeal arch structure in a neonatal mouse. E.) The pulmonary trunk (PT) arises anterior to the aorta (Ao) and here is backfilled through the single left sided ductus arteriosus (da). The first branch from the aortic arch is the single brachiocephalic artery (bca). The right subclavian (rsa) and right common carotid (rca) branch from the brachiocephalic. The left common carotid (lca) and left subclavian (lsa) arise directly from the aortic arch as independent branches. The pulmonary arteries (pa) originate from the pulmonary trunk. C,F) Abnormal arch patterning in a Semaphorin3C null embryo. F) This embryo has persistent truncus arteriosus (PTA) and an interrupted aortic arch type B between the left common carotid (lca) and left subclavian (lsa) arteries. The ductus arteriosus is greatly distended and serves as the vascular channel for systemic circulation in this embryo. Pulmonary arteries (pa) arise from the ductus arteriosus.

is generally synonymous with "outflow tract". The conus refers to the proximal portion below the level of the valves while the truncus refers to the region above the valves and below the aortic sac (Fig. 6A). The aortic sac is the nonmuscularized connection between the conotruncus and the arch arteries.[41] Septation involves a twisting or "spiraling" of the outflow tract, fusion of the endocardial cushions and significant investiture of the cushions with cardiac neural crest.[27,41-43] One popular model argues that the neural crest form an aorticopulmonary septum in the roof of the aortic sac that connects to prongs of neural crest that invade the truncal cushions (Fig. 6).[13,27] In this model the aorticopulmonary septum processes down the truncus separating the aorta and pulmonary artery. Other investigators argue that there is no aorticopulmonary septation complex and that separation of the vessels is achieved through cushion fusion and neural crest mediated formation of the facing walls of the aorta and pulmonary artery.[41] The conus region below the level of the valves is also extensively remodeled, and

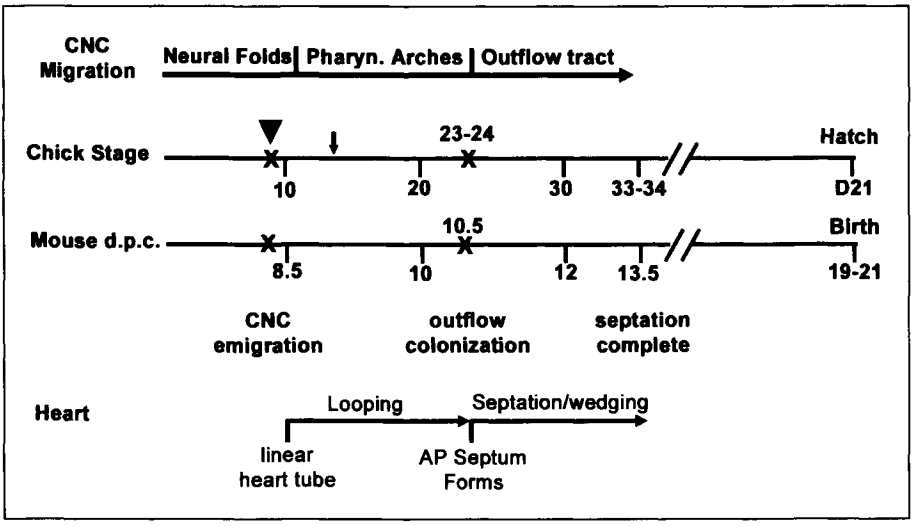

Figure 5. Timeline of cardiac neural crest migration in the chicken and mouse. Timelines for cardiac neural crest migration are presented indicating neural crest position and cardiovascular patterning events relative to developmental age. The black arrowhead on the chick timeline indicates the timing of neural crest ablation at Hamburger and Hamilton stage 9-10. The black arrow indicates the onset of detectable myocardial dysfunction at H&H stage 13-14. Cardiac neural crest do not contact primary myocardium until H&H stage 23-24. Cardiac neural crest cells colonize the mouse outflow tract around E10.5 and septation is completed by E13.5.

is likely to be involved in an additional contribution of myocardial cells (myocardialization) from the inner curvature of the heart.[44,45] Remodeling of the conus causes a change in position of the aorta and pulmonary trunk resulting in a shift from side by side alignment to the correct anterior-posterior positioning. The process of rearrangement and valve placement is called aortic wedging (Fig. 6B).[26,27] The aortic valve comes into fibrous continuity with the mitral and tricuspid valves, whereas the pulmonary valve is elevated by a band of muscle. Defective remodeling of the conus can produce malalignment in the presence of a fully septated truncus indicating that alignment and septation are independent processes.

Myocardial Dysfunction, Elongation, Alignment and the Secondary Heart Field

Myocardial dysfunction is observed in cardiac neural crest ablated chicken embryos. Following neural crest ablation, the heart has reduced ejection fraction and ventricular dilation accompanied by reduced calcium current and dysregulated excitation-contraction coupling. Similar myocardial dysfunction has been observed in the *Splotch mouse*.[46,47] Myocardial dysfunction is too early to be explained by direct neural crest interaction with myocardium in the heart tube (Fig. 5). Another unexplained outcome of neural crest ablation is the failure of outflow tract elongation that results in altered looping. Classic embryological experiments in the chicken embryo demonstrated that outflow tract elongation occurs through addition of cells from outside of the heart tube.[48] The issue of outflow tract elongation has recently been revisited using a variety of molecular markers. These experiments demonstrated that elongation is due to cell addition from a population of anterior pharyngeal splanchnic mesoderm (Fig. 7).[49-53] In the chicken this mesoderm population gives rise to the conotruncus and is called the secondary heart field.[51] In the mouse a similar region of pharyngeal mesoderm gives rise to the conotruncus and right ventricle and has been called the anterior heart-forming

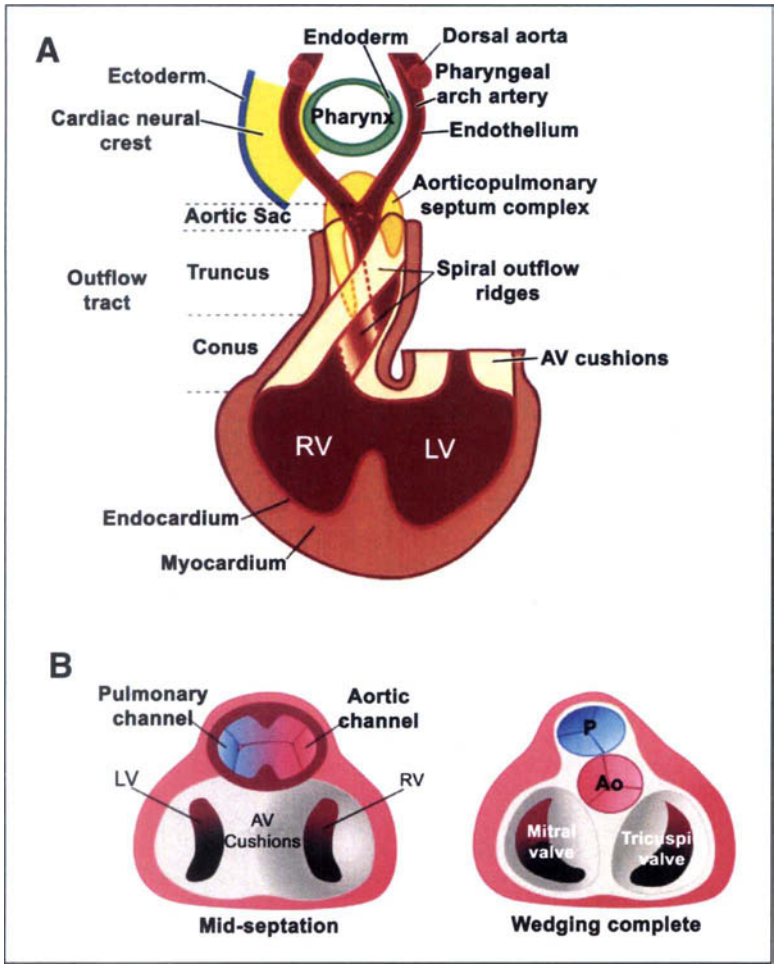

Figure 6. Diagram of neural crest mediated septation of the outflow tract and aortic valve wedging. A) Illustration of the popular model of outflow tract septation involving an aorticopulmonary septation complex and spiraling ridges of conotruncal cushions. B) Diagram of aortic valve wedging. The aortic valve is wedged between the mitral and tricuspid valves and the valves are in fibrous continuity with no intervening myocardium (pink). Panel A is from: Waldo KL et al. Dev Biol 1999; 208(2):307-323;[13] 1999 with permission from Elsevier. Panel B is from: Hutson MR, Kirby ML. Birth Defects Res C Embryo Today 2003; 69(1):2-13;[26] ©2003 with permission from Wiley-Liss, Inc., a subsidiar of John Wiley & Sons, Inc.

field[49,50] (reviewed in ref. 52). Loss of early reciprocal signaling between the cardiac neural crest and secondary heart field mesoderm in the pharynx presumably explains the myocardial dysfunction phenotype in neural crest deficient embryos, and this signaling in chickens appears to involve fibroblast growth factors.[33] Outflow tract elongation is deficient in ablated embryos due to lack of cell addition from the secondary heart field. It has also been proposed that continuous addition of cells to the truncus provides a mechanical force important for wedging.[29,54] Neural crest thus affects both elongation and septation of the outflow tract as well as alignment of the great vessels by influencing the elaboration or differentiation of cells from the secondary/anterior heart field.

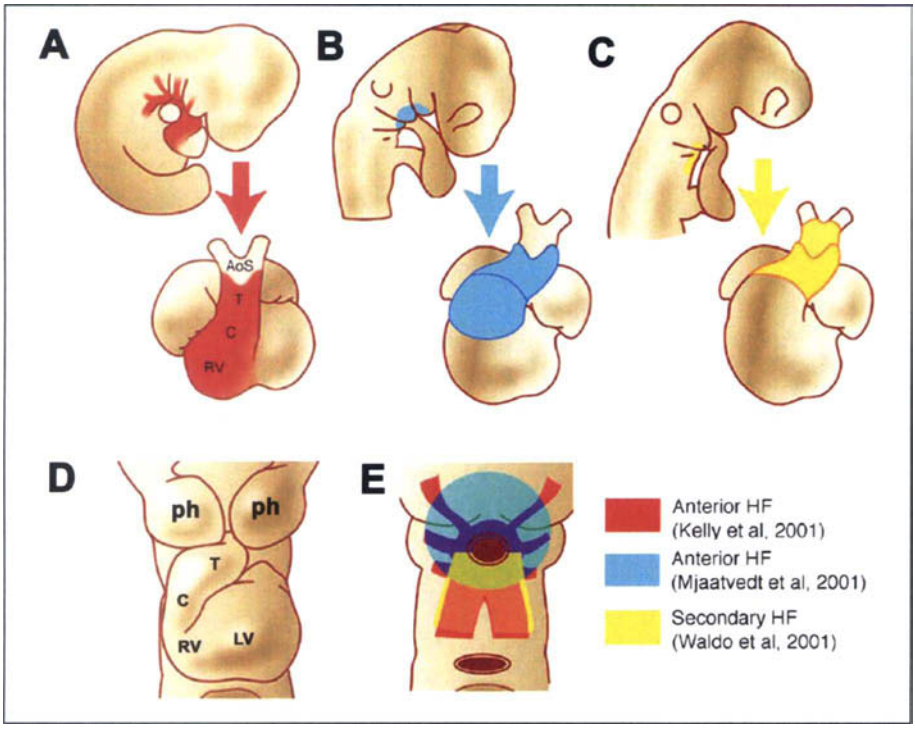

Figure 7. Comparison of the extent, location and contribution of cells from the anterior or secondary heart field to the outflow tract and right ventricle. A) Lateral view of an E9.5 embryo and ventral view of an E11.5 mouse heart demonstrating the origin and contribution of the "anterior heart field" (red) to the conotruncus and right ventricle as described by Kelly et al using an Fgf10-lacZ mouse line.[50] B) Lateral view of a chick stage 16 embryo and ventral view of a stage 22 heart demonstrating the origin and contribution of the "anterior heart field"(blue) mesoderm to the distal conus and truncus as defined by Mjaatvedt et al.[53] C) Lateral view of the "secondary heart field" (yellow) as described by Waldo et al. at stage 14 in the chick and limited contribution of secondary heart field mesoderm to the distal truncus myocardium of the heart at stage 22.[51] D) Ventral view of anE9.5 mouse (comparable to stage 12 chicken) demonstrating relationship of the heart and pharyngeal arches (ph). E) Ventral view of the embryo in D with the heart removed demonstrating the overlap in described secondary or anterior heart field pharyngeal mesoderm. Red ovals are sites of heart connection to the vasculature at the arterial (upper oval) and venous (lower flat oval) poles. Abbreviations: Aos,aortic sac; T,truncus; c, conus; RV,right ventricle; LV, left ventricle. Modified from: Abu-Issa R et al. Dev Biol 2004; 272(2):281-285;[52] ©2004 with permission from Elsevier.

Molecular Pathways

Numerous signaling pathways and individual genes have been implicated in cardiac neural crest induction, migration and differentiation following the observation of outflow tract or pharyngeal arch artery malformations in homozygous null mouse embryos. Analysis of the regulation of gene function and signaling pathways in the cardiac neural crest is complicated by the complex cellular interactions associated with normal cardiac morphogenesis. Defective development, or disruption of signaling pathways in any of these cell populations can result in cardiovascular abnormalities consistent with disrupted cardiac neural crest. The use of tissue specific-Cre mouse lines for conditional gene inactivation in the mouse is beginning to unravel the cell-specific gene requirements during neural crest mediated cardiovascular remodeling. Here we focus on a subset of genes that have known impact on cardiac neural crest mediated cardiovascular remodeling.

Mouse Model of Cardiac Neural Crest Deficiency

The *Splotch* mouse is the most extensively studied mouse model of cardiac neural crest dysfunction. The first *Splotch* allele was identified in 1954 and is named for the white belly spot apparent in heterozygous mice.[55,56] Homozygous *Splotch* mice die in utero by day 14 and resemble the chick neural crest ablation phenotype of persistent truncus arteriosus, pharyngeal arch patterning defects and hypoplasia or aplasia of the thymus and parathyroid glands.[56-58] The exact defect in cardiac neural crest in *Splotch* embryos is controversial. *Splotch* alleles represent mutations in the *Pax3* gene, a member of the paired box family of transcription factors.[59-61] *Pax3* is known to regulate cell migration through transcriptional regulation of the scatter factor receptor *c-met*.[62] Transgenic expression of *Pax3* in neural crest cells in *Splotch* null embryos rescues the cardiovascular defects arguing for a cell autonomous function.[63] Cardiac neural crest cells are formed and colonize the pharyngeal arches and conotruncal cushions in *Splotch* null embryos, albeit in reduced number and lacking proper positional identity arguing against a primary migration defect.[58] However, several investigators have suggested a delay in cardiac neural crest emigration from the neural tube that may be consistent with a role of *Pax3* in regulating factors required for migration.[6,57] It has also been proposed that cardiac neural crest cell numbers in *Splotch* may be decreased due to a failure of expansion of the neural crest stem cell population. Inappropriate or precocious differentiation would result in a decrease in the total number of neural crest cells.[64] This role in stem cell regulation would be consistent with the recent observation that *Pax3* in melanocyte stem cells plays a dual role in initiating lineage restriction and maintenance of the lineage restricted stem cell population.[65] A similar role is played by the related paired box gene *Pax7* in skeletal muscle stem cells.[66,67] The relative importance of *Pax3* in mediating lineage restriction and maintenance of the cardiac neural crest stem cell niche to the observed *Splotch* phenotypes remains to be determined.

Neurotrophins

The neurotrophins (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT4/5) are a family of growth factors that regulate neural survival and differentiation.[68] Neurotrophins signal through the Trk class of tyrosine kinase receptors.[68,69] Neurotrophin-3 (*NT-3*) and its receptor *trkC* are expressed in a subset of neural crest, in the neural crest-derived subendothelial mesenchyme of the aorta and pulmonary trunk, and in the neurons of the cardiac ganglia.[70-72] *NT-3* is also expressed by some endothelial cells.[73] Knockout of the *NT-3*[72] or it's receptor *trkC*[74] results in cardiovascular defects including atrial and ventricular septal defects, abnormal valves and conotruncal defects including persistent truncus arteriosus and Tetralogy of Fallot at low penetrance. Neurotrophins have been proposed to function in maintenance and lineage restriction of the neural crest stem cell niche. Youn and colleagues analyzed *trkC* null cardiac neural crest cells in explant culture.[73] Three types of neural crest stem cells were identified in explant culture. Cardiac neural crest stem cells (CNC-SC) were able to self renew and could assume any terminal fate including neurons, Schwann cells, pigment cells, chondrocytes and smooth muscle. Restricted cardiac neural crest cells (CNC-RC) form mostly smooth muscle cells, and do not form pigment cells or neurons. Smooth muscle stem cells (CNC-SmC) were committed to the smooth muscle lineage with little proliferative capacity.[73] Explants from *trkC* null embryos contained more lineage-restricted stem cells (CNC-RC) and reduced levels of uncommitted neural stem cells (CNC-SC). The decrease in uncommitted stem cell numbers in *trkC* null embryos suggests that neurotrophins function to retain pluripotency in the cardiac neural crest stem cell niche.

Forkhead Transcription Factors FoxC1/FoxC2

Foxc1/Mf1 and *Foxc2/Mfh1* are closely related forkhead/winged helix transcription factors.[75,76] Both genes are expressed in head mesoderm, pharyngeal arch mesenchyme and endothelial

cells and *Foxc1* is expressed in cardiac neural crest. Both *Foxc1* and *Foxc2* nulls exhibit cardiovascular defects including coarctation and interruption of the aortic arch and ventricular septal defects.[76,77] *Foxc1/Foxc2* compound heterozygote and homozygote embryos display more severe cardiovascular defects indicating that these genes compensate for one another and that function is dose dependant.[78] Migration of cardiac neural crest cells and expression of neural crest markers appears normal in *Foxc1* and *Foxc2* nulls suggesting that the cardiovascular defects observed are not due to a cell-autonomous neural crest defect.[76] *Foxc1* and *Foxc2* can regulate expression of the T-box transcription factor *Tbx1* in tissues where they are coexpressed.[79] *Tbx1* is a transcription factor implicated in the etiology of DiGeorge syndrome, the most common congenital heart syndrome in humans.[80,81] *Tbx1* has an important non cell-autonomous role in regulating cardiac neural crest maintenance and differentiation through a signaling cascade involving Fibroblast growth factor(Fgf) ligands.

Tbx1, Fibroblast Growth Factors and DiGeorge Syndrome

DiGeorge syndrome, velocardiofacial syndrome, and conotruncal anomaly face syndrome (DiGeorge spectrum disorders) are the most common human congenital cardiovascular disorders affecting as many as 1 in 4000 births. The majority of patients with these syndromes have chromosome 22q11 deletions.[82-86] These patients exhibit an incompletely penetrant phenotype including hypoplasia of the thymus and parathyroid, craniofacial and skeletal abnormalities, cardiac abnormalities, and speech and learning disabilities. Common heart defects include interrupted aortic arch type B, persistent truncus arteriosus, tetralogy of Fallot, tetralogy of Fallot with pulmonary atresia, and posterior malalignment ventricular septal defect.[87] These defects are consistent with neural crest dysfunction, and the DiGeorge spectrum disorders have long been considered "neurocristopathies". Mouse models of DiGeorge syndrome have been developed by deletions on mouse chromosome 16 in regions syntenic to human chromosome 22 (the DiGeorge critical region)[88,89] and these models helped to identify the transcription factor TBOX1 (TBX1) as a candidate gene.

Tbx1 is one of the genes contained within the DiGeorge critical region in humans and mice.[90] Targeted inactivation of *Tbx1* results in cardiac defects (similar to those seen in patients with 22q11 deletions) in mice heterozygous or homozygous for the mutations.[88,89,91] Recently, three independent cases of TBX1 mutations in human patients with conotruncal anomaly face syndrome were reported[92] confirming that TBX1 is a major genetic determinant in the DiGeorge spectrum disorders. Tbx1 is expressed in pharyngeal mesoderm and endoderm but not in neural crest cells indicating that the neural crest defects are non cell-autonomous.[93] Tbx1 is also expressed in precursors of the secondary heart field suggesting a cell autonomous role in conotruncal patterning.[94]

Fibroblast growth factor (*Fgf8* and *Fgf10*) signaling has been reported as a critical mediator of aortic arch development and conotruncal septation downstream of *Tbx1*.[95-97] *Fgf8* and *Fgf10* expression is down regulated in *Tbx1* nulls, and *Fgf8* hypomorphic embryos exhibit cardiovascular defects reminiscent of *Tbx1* nulls. Neural crest cells migrate normally in *Fgf8* hypomorphs, but there are increased levels of apoptosis of neural crest cells within the pharyngeal arches.[95,96] This suggests that *Fgf8* expression in the pharyngeal arches is required for neural crest cell survival. Tissue specific inactivation of *Fgf8* in the ectoderm results in defective pharyngeal arch artery patterning.[98] Deletion of *Fgf8* in the *Tbx1* expression domain results in defects of conotruncal septation and malpositioning of the proximal great vessels.[94] Thus, *Fgf8* is required both for neural crest survival/differentiation and in the secondary heart field. Hu et al recently identified an *Fgf8* enhancer that is dependent on *Tbx1* in vivo for regulating expression specifically in the cardiac outflow tract, but were unable to show direct transcriptional activation by *Tbx1*.[99] Direct transcriptional regulation of *Fgf10* by *Tbx1* has recently been demonstrated.[100] Determination of the relative roles of *Fgf8* and *Fgf10* in cardiovascular patterning is an area of active research.

Retinoic Acid

It has long been known that too much or too little Vitamin A, or the biologically active form retinoic acid, causes neural crest dependent cardiovascular defects.[101-103] Retinoic acid(RA) is synthesized from retinol by the action of retinol and retinal dehydrogenases. In mouse the enzyme retinaldehyde dehydrogenase 2 (RALDH-2) is apparently the primary rate-limiting enzyme for RA synthesis.[104] Deletion of RALDH-2 recapitulates the full spectrum of RA deficiencies.[105] RA signals through heterodimers of retinoic acid receptors (RAR α, β and γ) and retinoid X receptors (RXR α, β and γ).[106] RARs can be activated by RA or 9-cis RA[107] whereas only 9-cis RA efficiently activates RXRs.[108] Further complexity is generated through different receptor splice isoforms. RA also binds to cellular retinol binding proteins I and II and cellular retinoic acid binding proteins I and II and this binding may regulate RA signaling by decreasing free RA in the cell.[109]

Stereotypical retinoic acid deficient phenotypes are only observed in compound RAR (α1β2, αβ2, αγ) nulls indicating functional receptor redundancy.[109] The cardiovascular defects observed following retinoic acid exposure or compound receptor knockout include persistent truncus arteriosus, interrupted aortic arch and double outlet right ventricle. These defects are consistent with defects in the cardiac neural crest.[109] Neural crest fate mapping analysis in *RARα1/RARβ* compound null embryos demonstrated normal migration and differentiation of cardiac neural crest in animals exhibiting persistent truncus arteriosus.[110] Wnt1-Cre mediated tissue specific RAR deletion does not result in cardiovascular defects suggesting that RA effects on the neural crest are non cell-autonomous.[110] Cardiovascular defects may arise due to altered signaling between the cardiac neural crest and a retinoic acid responsive neighboring cell population. RA regulation of Fibroblast growth factor ligand expression may explain the observed cardiovascular defects. *Fgf8* is expressed in the pharyngeal ectoderm and endoderm and is required for maintenance or differentiation of both neural crest and secondary heart field cells. Retinoic acid has been shown to directly activate expression from an *Fgf8* genomic enhancer in vitro[111] and to induce *Fgf10* expression in vivo.[112] Both Fgfs are proposed to have a non cell-autonomous role in regulating neural crest function in the pharyngeal arches and conotruncus.

Cell-Cell Contact (Connexins)

Connexin 43, also known as alpha1 connexin, is a gap junction protein expressed in cardiac neural crest.[113] Gap junctions are membrane channels that allow passage of low molecular weight molecules and ions between cells. Dye coupling experiments have shown that cardiac neural crest cells maintain inter-cell continuity while migrating. Cx43 is involved in cardiac neural crest migration. Both increase and decrease of Cx43 levels in cardiac neural crest result in outflow patterning defects.[114-116] Deletion of Cx43 results in outflow tract obstruction and conotruncal defects.[114] Loss of Cx43 expression or expression of a dominant negative form of Cx43 in cardiac neural crest results in decreased migration and decreased cardiac neural crest in the outflow tract.[114,115] Overexpression of Cx43 causes the opposite effect with increased cell motility and more cells in the outflow tract.[116] These results suggest a primary role for Cx43 in regulating neural crest cell motility. In addition to neural crest, Cx43 is also expressed in the pro-epicardium and is required for normal formation of the coronary arteries. Cx43 deficient proepicardial cells display increased proliferation and decreased migration rates in culture.[117]

Platelet Derived Growth Factor (PDGF)

Platelet-derived growth factors are broadly expressed growth factors that have been implicated in regulation of cell migration, survival and proliferation.[118] Interest in PDGF stems from the observation that the PDGF α receptor (*PDGFRα*) is deleted in the *Patch* mouse *Patch* (Ph).[119] Heterozygous *Patch* mice exhibit defective melanocyte migration causing a white belly spot. *Patch* homozygotes exhibit PTA, interrupted aortic arch, decreased thymus and other defects associated with deficient cardiac neural crest.[120] Homozygous null *PDGFRα* mice phenocopy *Patch*.[121]

While the *Patch* deletion also encompasses some enhancers for the c-kit gene, similarity in phenotype suggests that loss of *PDGFRα* is the primary defect. Tissue specific inactivation of *PDGFRα* in neural crest causes PTA and abnormal patterning of the right subclavian artery.[122] However, the exact role of PDGF signaling in neural crest is unclear. There are no obvious defects in migration, proliferation or survival of cardiac neural crest in conditional null animals and patterning defects are seen in only slightly more than 50% of conditional null embryos.

Endothelin

Endothelins (ET) are a family of small signaling peptides (*ET-1, ET-2, ET-3*).[123] The active forms of Endothelin are generated from large precursor proteins through the activity of endothelin converting enzyme-1 and-2 (*ECE-1* and *ECE-2*).[124] Endothelins signal through two G-protein coupled receptors named *ET-A* and *ET-B. ET-A* binds *ET-1* and *ET-2* but not *ET-3*.[125,126] *ET-A* and its receptor *ET-1* are expressed in complimentary patterns. In the mouse, *ET-1* is expressed in the endothelium of pharyngeal arch vessels and *ET-A* is expressed on migratory neural crest and in neural crest derived mesenchyme of the pharyngeal arches.[127] Mice lacking the ligand *ET-1*, the receptor *ET-A*, or the converting enzyme *ECE-1* have defective pharyngeal arch and conotruncal patterning. The converting enzyme *ECE-1* is expressed in both endothelium and mesenchyme in the arches.[124,127] The most common malformations in *ECE-1* and *ET-A* null embryos are type B aortic arch interruption and absent right subclavian artery. Outflow tract defects include overriding aorta, double outlet right ventricle and rare cases of PTA. Most embryos also have a peri-membraneous ventricular septal defect. Cardiac neural crest appear to migrate normally in *ECE-1* and *ET-A* null embryos and cardiovascular defects appear to result from deficient paracrine signaling between pharyngeal arch endothelial cells and neural crest derived mesenchyme.[127]

TGFβ Superfamily Members

Bone morphogenetic proteins (BMP) and transforming growth factor beta (TGFβ) are members of the transforming growth factor beta superfamily of signaling molecules and are important mediators of embryogenesis.[128,129] TGFβ family ligands signal through heteromeric serine-threonine kinase receptor complexes of a Type II receptor (*TBR2*) and a Type I receptor (activin-like kinase 5 (*Alk5*), also known as *TBR1*).[130,131] BMPs similarly utilize a single BMP type 2 receptor (*Bmpr2*) but multiple type I receptors (*Alks*) to transduce signals from different ligands.[132] One type I receptor, *Alk2* appears to be utilized by both TGFβ and BMP in certain cell types.[133,134]

There are three Tgfβ ligands (Tgfβ1, Tgfβ2, and Tgfβ3). Tgfβ1 null embryos die in early gestation from a defect in yolk sac vasculogenesis.[135] Tgfβ3 knockouts die shortly after birth and display cleft palate.[136] Knockout of the ligand TGFβ2 results in cardiovascular defects including DORV, short or absent brachiocephalic artery, and retroesophageal right subclavian artery.[137] Both Type II TGFβ (*TBR2*) receptor and Type I TGFβ (TBR1) receptor null mice die before E10 from defective vascularization of the yolk sac and placenta.[138,139] Wnt-Cre mediated deletion of *TBR2* in neural crest cells results in PTA, ventricular septal defects, pharyngeal artery remodeling defects as well as defects in the thymus, parathyroids and craniofacial structures.[140,141] Neural crest migration and survival appear normal in conditional TBR2 nulls but neural crest fail to form smooth muscle. Traditional knockout of the bi-functional *Alk2* receptor results in embryonic lethality at gastrulation. Wnt1-Cre mediated deletion of *Alk2* in the neural crest results in PTA and abnormal pharyngeal arch maturation with right ventricular hypertrophy.[142] Cardiac neural crest migration is decreased and smooth muscle differentiation of neural crest is impaired in *Alk2* conditional nulls suggesting that *Alk2* may be the functional Type I Tgfβ receptor in cardiac neural crest.[142]

BMP signaling is known to play a role in induction and maintenance of the neural crest. Double knockout of *Bmp6* and *Bmp7* leads to cardiac outflow tract, valve and septal defects, and expression of a hypomorphic *Bmpr2* causes an interrupted aortic arch with an unusual

subvalvular PTA.[143] Neural crest specific deletion of the BMP receptor IA (*Bmpr1a* also called *Alk3*) causes shortened cardiac outflow tract, defective septation and acute heart failure with reduced proliferation of the myocardium.[22] These defects are consistent with the previously described indirect effect of reciprocal signaling from the cardiac neural crest to the secondary heart field and not a primary defect in neural crest differentiation. Nkx2.5Cre deletion of BMP4 (a *Bmpr1a* ligand) in the caudal pharyngeal arches, splanchnic mesoderm and truncus results in defective septation, aortic arch interruptions, abnormal arch artery remodeling with decreased smooth muscle recruitment, decreased myocardial differentiation in the truncus, and hypoplastic conotruncal cushions.[144] These defects imply a multi-tissue role for BMP4 in cardiac neural crest, secondary heart field and cushion tissues. BMP7 expression in the conditional BMP4 mutant embryos may prevent more severe conotruncal defects since BMP7 null embryos with reduced levels of BMP4 have a shortened outflow tract consistent with a BMP requirement in the secondary heart field.[144]

Semaphorins

Semaphorins were originally identified as neural pathfinding molecules providing predominantly repulsive axon guidance cues.[145] It has subsequently been appreciated that semaphorin signaling can be attractive or repulsive depending on the cell type and environmental context.[146] Class 3 semaphorins are secreted ligands known to signal through a heteromeric complex of class A plexins and either neuropilin-1 (*npn-1*) or neuropilin-2 (*npn-2*).[147] Semaphorin 3C (Sema3C) null embryos die at birth of interrupted aortic arch and PTA (Fig. 4C).[148] *Sema3C* is expressed in the conotruncal myocardium and pharyngeal arch mesenchyme at E10.5. The Semaphorin receptor *PlexinA2* is expressed in cardiac neural crest suggesting a role for semaphorin signaling in guidance during migration.[94] Decreased levels of cardiac neural crest are observed in the conotruncal cushions of *Sema3C* nulls, consistent with a defect in neural crest cell migration. However, normal levels of neural crest were observed in the pharyngeal arches in *Sema3C* nulls indicating that the *Sema3C* phenotype was not due to a global defect in neural crest homing or migration. Recent experiments suggest an additional role for class 3 semaphorin signaling through plexin D1 in endothelial cells of the pharyngeal arch arteries.[149,150]

Pitx2 and Laterality

One of the enduring mysteries of pharyngeal arch patterning is how stereotyped left sided (mouse) or right-sided (chicken) asymmetry is achieved.[151] The bicoid-related homeodomain transcription factor Pitx2 plays a critical role in directing asymmetric cardiovascular remodeling (for review see ref. 152). Three isoforms of Pitx2 are produced by alternate splicing and alternate promoter use but only the Pitx2c isoform is expressed asymmetrically in the developing heart.[153] In the cardiac crescent stage Pitx2c is expressed only in the left heart field. At the linear and looped heart stages Pitx2c retains left sided expression in the entire heart tube and extending into the body wall at both the arterial and venous poles. Between E9.5 and E10.5 Pitx2c is asymmetrically expressed in the left pharyngeal arch mesoderm, splanchnic mesoderm and outflow tract myocardium. This left-sided expression suggests a role for the Pitx2c isoform in asymmetric pharyngeal arch patterning.[153]

Pitx2 expression is regulated by a wnt signaling pathway involving disheveled 2 (*Dvl2*) and β-catenin.[154,155] The global knockout of all three Pitx2 isoforms (Pitx2abc) causes right atrial isomerism (RAI), PTA, DORV, and atrial and ventricular septal defects.[153] Knockout of Dvl2 or Wnt1-Cre mediated deletion of β-catenin results in loss of expression of all Pitx2 isoforms.[154] Global loss of Pitx2 expression results in decreased numbers of cardiac neural crest cells due to an arrest in proliferation.[154,155] Thus, Pitx2 has a cell autonomous effect in regulating cardiac neural crest proliferation.

Pitx2c null embryos exhibit most of the cardiovascular phenotype of the Pitx2abc null, but do not exhibit PTA. In addition, Pitx2c nulls display pharyngeal arch patterning anomalies seen only with this knockout.[153] Null Pitx2c mice display patterning defects including right-sided

aorta and double aortic arch, although apparently normal levels of cardiac neural crest are observed in the pharyngeal arches and outflow tract. These mutations are believed to result from a loss of reciprocal signaling between the cardiac neural crest expressing all three Pitx2 isoforms and pharyngeal mesenchyme expressing only Pitx2c.[153] The laterality pathway downstream of Pitx2 is unknown, however the asymmetric expression of Semaphorin 3C in the conotruncus is mediated by Pitx2c.[153]

Summary

Our understanding of neural crest contribution to cardiovascular development has increased greatly since the early observations of quail cells in the great vessels of chimeric chicken embryos. The primary challenges now facing the field involve deciphering the complex reciprocal signaling events between the cardiac neural crest and the myriad cell populations with which they interact, and in deciphering the pathway relationships between the ever expanding list of genes with cardiac neural crest associated phenotypes. There exists a surprisingly large number of knockout mice with "cardiac neural crest defects" in which there is no demonstrable defect in migration, survival or differentiation of the neural crest. The defects in these mice must lie in either poorly understood tissue layer interactions, or in as of yet undiscovered aspects of neural crest biology. The generation of new tissue specific Cre-recombinase mouse lines and conditional alleles will be critical for the careful molecular dissection of tissue specific gene function during cardiovascular patterning. The recent realization of the importance of reciprocal signaling between neural crest and the secondary/anterior heart field demonstrates that many important aspects of cardiac neural crest biology remain to be elucidated.

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