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Evolutionary and Developmental Origins of the Cardiac Neural Crest: Building a Divided Outflow Tract

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Abstract

The cardiac neural crest cells (CNCCs) have played an important role in the evolution and development of the vertebrate cardiovascular system: from reinforcement of the developing aortic arch arteries early in vertebrate evolution, to later orchestration of aortic arch artery remodeling into the great arteries of the heart, and finally outflow tract septation in amniotes. A critical element necessary for the evolutionary advent of outflow tract septation was the co-evolution of the cardiac neural crest cells with the second heart field. This review highlights the major transitions in vertebrate circulatory evolution, explores the evolutionary developmental origins of the CNCCs from the third stream cranial neural crest, and explores candidate signaling pathways in CNCC and outflow tract evolution drawn from our knowledge of DiGeorge Syndrome.

Keywords

cardiac neural crest; evolution; outflow tract septation; pharyngeal arch artery remodeling; second heart field

Introduction

In the course of tetrapod evolution the site of respiratory exchange has shifted from the gills to the lungs, and this process has necessarily been closely coupled with the evolution of a functionally divided cardiovascular system. Cardiovascular anatomy has evolved from a single-loop, branchial arch system to a double-loop, systemic and pulmonary circulatory system (Fig. 1). In primitive fishes without lungs, such as elasmobranchs, oxygen-depleted blood is pumped through the branchial arch arteries in the bilateral gills where it is oxygenated and then continues through the body. In lungfish, a pulmonary circulation appears with the introduction of lungs, as well as specific adaptations to separate oxygen-rich and -poor bloodstreams. Amphibians and reptiles demonstrate further division of the two bloodstreams, and a structural separation is complete in birds and mammals. Birds and mammals have a completely divided systemic and pulmonary circulation with two atria, two ventricles, and a divided outflow tract. A divided outflow tract arose through the co-option

of the neural crest cells into the distal outflow tract (truncus arteriosus) to form the aorticopulmonary septum. The subpopulation of neural crest cells responsible for aorticopulmonary septation has been termed the cardiac neural crest. In addition to septation of the outflow tract and divisions within the cardiac chambers, the transition from an aquatic to a terrestrial lifestyle brought significant alterations in the branchial arches, particularly in the caudal segments that no longer had to support gills but still contained important vascular structures, the pharyngeal (or aortic) arch arteries. Vertebrates exhibit significant diversity in great vessel branching patterns, and cardiac neural crest cells (CNCCs) were likely instrumental in the rise of this morphological diversity.

This review will explore the various roles of the CNCCs in cardiovascular evolution and development, including critical interactions with nearby cell populations, such as the second heart field. We will highlight the direct and indirect contributions of the CNCCs to cardiovascular development, as well as the signaling pathways that were likely involved in the evolution of the CNCC lineage as the cells were recruited further into the circulatory system and heart. We will also discuss the clinical implications of the disruption of these pathways for human congenital heart disease. The same pathways that are prime candidates for the evolution of a divided circulation clinically relevant are, as disruption of divided circulation is commonly seen in patients with congenital heart defects. CNCCs have been most extensively studied in chick and mouse embryos, so this review begins with an overview of their role in cardiovascular development in these two model systems. CNCCs are required for the normal development of the thymus, thyroid, parathyroids, cardiac conduction system, semilunar valves, parasympathetic innervation of the heart, and outflow septum, as well as proper remodeling of the pharyngeal arch arteries and alignment of the outflow with the ventricles. This review will specifically focus on outflow septation, alignment, and pharyngeal arch artery remodeling.

Developmental Biology of the CNCCs in Birds and Mammals

CNCCs AND PHARYNGEAL ARCH ARTERY REMODELING

The neural crest cells arise from the dorsal neural tube at the ectoderm-neural ectoderm border. This highly migratory, pluri-potent cell population will ultimately differentiate to form a variety of cell types, including melanocytes, cartilage, bone, connective tissue, smooth muscle, and nerves. CNCCs are a subpopulation of cranial neural crest cells that originate from the dorsal neural tube between the mid-otic placode to the posterior border of somite three. CNCCs migrate ventrally, accumulate in the circumpharyngeal ridge, and then continue into pharyngeal arches three, four, and six as each arch develops successively (Fig. 2). The cells migrate between the pharyngeal ectoderm and endoderm, proliferate and fill the arches, and surround the endothelial strands of the forming pharyngeal or aortic arch arteries. The pharyngeal arch arteries develop as a bilaterally symmetrical series of vessels connecting the aortic sac to the paired dorsal aortas. Pharyngeal arch arteries 3–6 are re-patterned later in development into the asymmetric great arteries: the common carotid (right and left third pharyngeal arch arteries), the definitive aortic arch (right or left fourth pharyngeal arch artery), and the ductus arteriosus (right and/or left sixth aortic arch arteries) (Fig. 3). The fifth pharyngeal arch artery regresses.* The CNCCs will form the smooth

muscle tunics of the great arteries (Bergwerff et al., 1998). The CNCCs are not necessary for initial formation of vessels from the endothelial precursors, but are required for subsequent artery remodeling (Bockman et al., 1987).

OUTFLOW SEPTATION

Following the migration into the pharynx, some CNCCs remain in the pharyngeal arches while a subpopulation continues on into the cardiac outflow cushions, cardiac jelly-filled ridges that spiral within the outflow tract. Convergence of the outflow tract cushions separates blood flow from the embryonic left and right ventricles. CNCCs condense within the cushions to form the aorticopulmonary septation complex (Waldo et al., 1998, 1999), which divides the common arterial outflow into the aorta and pulmonary trunks. The cushions are eventually populated by three distinct groups of mesenchymal cells, depending on the proximal-distal location within the outflow tract. In the proximal or conal outflow tract, the cushions are populated by endocardial cells that undergo an epithelial to mesenchymal transformation (EMT) induced by the conal myocardium. These mesenchymal cells will form the bulk of the semilunar valve leaflets. Distally, a small number of pharyngeal mesenchymal cells enter the cushions prior to the arrival of the neural crest cells (Ward et al., 2005). It is unclear what role these non-neural crest cells play in septation. The CNCCs migrate into the cushions and form two centrally placed columns or “prongs”, of condensed cells in the distal or truncal outflow. The prongs join more distally through a shelf of mesenchymal cells protruding into the dorsal wall of the aortic sac forming an upside down “U” or horseshoe-shaped septation complex with the prongs making up the legs of the horseshoe (See Keyte and Hutson, 2012, for a review and images). The shelf is positioned between the origins of the fourth and sixth pairs of arch arteries, which will be remodeled into the arch of the aorta and the ductus arteriosus, respectively. Thus, the shelf is perfectly positioned to divide the systemic and pulmonary outflow. As septation proceeds the shelf grows through the distal outflow at the expense of the prongs dividing the aortic sac and truncus, the most distal segments of the outflow tract. Next, conal or proximal outflow septation occurs in a distal to proximal progression, closing toward the ventricles. This is accomplished by myocardialization in which invading myocardial cells cause the cushions to bulge into the lumen (van den Hoff et al., 1999). The endocardial layer covering the cushions breaks down, allowing mixing of mesenchyme and myocardium from opposing cushions to form the septum. Some CNCCs are also found in the proximal outflow in the subendocardial space and appear as a seam in the septum. It is unclear if they play an active role in the fusion of the cushions (Waldo et al., 1998, 1999).

Mouse and chick are the most common animal models used to study outflow septation. The process appears to occur in a similar fashion in both models, but with some notable differences in the timing of events and distribution of the CNCCs. For example, CNCCs

*It has been mistakenly believed that amniotes, and thus humans, do not have a fifth pharyngeal arch artery during embryonic development. However, numerous sources have reported the transitory appearance of the fifth pharyngeal arch artery in amniotes including chick, mouse, pig, and human embryos (Reinke, 1910; Bockman et al., 1987; Hiruma and Hirakow, 1995; Lorandau et al., 2011; Geyer and Weninger, 2012; Bamforth et al., 2013). Congenital cardiovascular malformations in humans, including double aortic arch and double-lumen aortic arch, have been attributed to abnormal persistence of the fifth pharyngeal arch artery, and some defects can only be explained on the basis of its presence (Van Praagh and Van Praagh, 1969; Gerlis et al., 1989; Zhao et al 2007; Yu 2010; Bamforth et al., 2012).

arrive in the distal outflow in the mouse at E9.5, approximately one day after emigration from the neural tube, while in the chick the neural crest-derived cells enter the outflow tract 3.5 days after leaving the neural tube. Another notable difference is the route by which CNCCs take to enter the outflow tract cushions (Waldo et al., 1999). Quail-chick chimeras show that the cardiac neural crest-derived cells enter by two distinct paths, subendocardially and submyocardially. This is in contrast to a single subendocardial route taken by the CNCCs, as shown in the Cx43-lacZ-marked CNCCs in the mouse. In addition CNCCs in the mouse migrate as far as the distal conus of the outflow tract, while they stop short of the conus in the chick (Waldo et al., 1999). These variances are likely due to differences in the timing of developmental events in cardiovascular development, as well as morphological differences between birds and mammals. The important similarity is that in both the mouse and the chick the CNCCs are required for truncal septation, which appears to be a key evolutionary step in physically dividing the systemic and pulmonary circulations.

THE SECOND HEART FIELD

Another important requisite to a divided pulmonary and systemic circulation is concordance or alignment of the divided outflow tract vessels with the ventricles. Alignment and septation involve distinct cell populations and separate events during development. As discussed above, outflow septation is dependent on ingression of CNCCs that form the aorticopulmonary septum. In contrast, arterial pole alignment is reliant on lengthening of the of the heart tube, which allows the inflow and outflow poles to converge with adequate length in the loop for the outflow tract to rotate and wedge correctly. This allows the atria and outflow vessels to be in concordance with the ventricles. Heart tube elongation is driven by the addition of cardiac progenitor cells to the arterial and venous poles of the heart tube. These progenitors, termed the second heart field (SHF), are located in the splanchnic mesoderm of the pharynx and generate the atria, atrial septum, and right ventricle, as well as the conal and truncal segments of the outflow tract. Perturbation of SHF development results in a shortened heart tube, leading to arterial pole malalignment defects, including double outlet right ventricle (DORV) and overriding aorta.

While alignment and septation involve distinct cell populations as discussed above, the SHF and CNCCs must act together to form a divided outflow tract. For example, inhibition of Notch signaling within the second heart field results in abnormal migration of CNCCs into the outflow tract, suggesting that a Notch-dependent signal from the SHF can modulate CNCC behavior (High et al., 2009). Further, following neural crest ablation the undivided outflow tract is dextroposed, implicating abnormal addition of the SHF. Cell proliferation within the SHF is also increased, demonstrating that the presence of the neural crest cells in the pharynx is required for normal development of the SHF (Waldo et al., 2005). Finally, once the CNCCs arrive in the outflow, coordinated morphogenesis of the two cell populations is essential to form the septation complex.

DEFINING THE CNCCS

Nearly 30 years ago Kirby et al. (1985) defined the “cardiac neural crest”, not because the cells migrated exclusively to the heart but because they were found to be critical for normal heart development. The region of neural crest critical for normal cardiovascular

development was defined in chick as arising from the dorsal neural tube between the mid-otic placode to the posterior border of somite three. Ablation of this population of cells results in defective development of the cardiac outflow tract; abnormal myocardial function; and defective development of the derivatives of the caudal pharynx, including arch arteries, pharyngeal glands, and the secondary heart field (Hutson and Kirby, 2003). A population of “preotic” neural crest cells has recently been found to contribute to the smooth muscle of the coronary arteries in mouse and chick (Arima et al., 2012), and is therefore arguably cardiac neural crest, as well. As will be discussed below, a broader definition of the CNCCs makes sense given that these cells likely have a common evolutionary and developmental origin. Genetic profiling and further developmental studies of these cell populations may allow us to define a broader group of CNCCs, based on common molecular markers.

The CNCCs are clearly critical in birds and mammals for the normal development of the great arteries of the heart, alignment of the outflow vessels with the ventricles, and the aorticopulmonary septation complex. In addition, the SHF is an essential partner to the CNCCs in orchestrating outflow septation. But what developmental and genetic changes occurred during vertebrate evolution that allowed the neural crest cells to reinforce the pharyngeal arch arteries and regulate artery remodeling? What developmental and genetic changes were required for neural crest cell recruitment into the aorticopulmonary septum? At what point during vertebrate evolution do CNCCs become required for pharyngeal arch artery remodeling, proper alignment, and outflow septation? In the next section, we develop a framework for addressing these questions by first describing major transitions in the evolution of the vertebrate cardiovascular system.

Evolution of the Vertebrate Heart and Circulatory System

Over the course of vertebrate evolution the structure of the heart and patterns of circulation have been significantly modified (Figs. 1, 3 and 4). Major changes in the heart and arch arteries occurred as the primary site for gas exchange shifted from gills to lungs with the move from water to land, and later with the evolutionary advent of endothermy.

In primitive fishes without lungs, the six branchial aortic arch arteries are interrupted by capillary networks in the bilateral gills (Fig. 3). Oxygen-depleted blood is pumped through the branchial arteries in the gills, where it is oxygenated and then continues through the body (Fig. 1A). The African lungfish (*Protopterus*) has retained some of the branchial circulation of primitive fishes, but may alternatively take up oxygen from the air via a pulmonary circulation that carries blood to the lungs and back to the heart. They exhibit a transitional arrangement in the architecture of the branchial arch arteries and heart. Lungfish gills, corresponding to branchial arches two, five, and six, exchange CO₂ for O₂ by diffusion. The ductus arteriosus, an evolutionary innovation of the sixth arch, allows blood flow to bypass the lungs when the animal is in aquatic breathing mode. During air respiration valves in arches two, five, and six close, creating shunts through arches three and four that pass blood from the ventral to the dorsal aorta. By constricting the ductus arteriosus, the sixth arch shunts oxygen-poor blood away from the gills in that arch and to the lungs via the pulmonary artery (Bemis et al., 1987). Partial divisions in the atria and

ventricle, as well as the spiral valve in the conus, also help partition oxygenated and deoxygenated blood in the two circulations (Icardo et al., 2005).

As terrestrial vertebrates become exclusively air breathing, the skeletal and muscular elements of the gills or branchial arches are re-purposed to pharyngeal structures in the neck and the vasculature is remodeled to support a systemic and pulmonary circulation. This transition can be observed in amphibians that begin as larvae with gills, but metamorphose into adults with internalized vasculature (DeLong, 1962). During metamorphosis, the gill structures are remodeled, resulting in pharyngeal arch arteries that are internalized and remain bilaterally symmetrical (Fig. 3). The first and second embryonic arch arteries regress and the third becomes a portion of the internal carotid artery. Adult salamanders usually retain the fourth and fifth arch arteries; the fifth arch is lost in frogs. The heart moves caudally into the thoracic cavity, closer to the lungs and consists of two atria, one ventricle, and an incompletely divided singular outflow tract (Fig. 4). The flow of oxygenated and deoxygenated blood is directed to the systemic and pulmocutaneous arteries by an incomplete spiral valve in the outflow and divisions within the bifurcated ventral aorta (DeGraaf, 1957; Farmer, 1999; Kolker et al., 2000; Mohun et al., 2000; Lee and Saint-Jeannet, 2011).

In reptiles[†], the extent of separation of the pulmonary and systemic circulations is more complete, due to additional partitions within the heart and outflow (Fig. 4). As in most amphibians, the two atria in reptiles are completely divided. The ventricles are completely septated in crocodiles and partially divided in other reptiles. In most reptiles, two aortae and one pulmonary artery exit a fully divided outflow. A unique arrangement occurs in crocodilians, in which the blood vessels draining the left and right ventricles possess an interconnecting aperture, called the Foramen of Panizza, that allows for some mixing of blood beyond the ventricles and a bypass of the pulmonary circulation. As in other semi-aquatic vertebrates, bypassing the pulmonary circulation is advantageous to this diving reptile. In all reptiles, the pharyngeal arch arteries remain bilateral (Fig. 3). The third arch arteries contribute to the internal carotids, the fourth arch arteries form the systemic aortic arches, the fifth arch arteries are lost, and the sixth arch arteries contribute to the ductus arteriosus and base of the pulmonary arteries.

Birds and mammals have completely divided systemic and pulmonary circulations with two atria, two ventricles, and a divided outflow tract (Fig. 4). As mentioned above, in both groups of animals, septation of the truncus arteriosus of the outflow tract requires the CNCCs. In addition each group has lost one of the aortae, so that a single vessel provides oxygenated blood to the systemic circulation (Fig. 3). In birds, the right fourth pharyngeal arch artery becomes the arch of the aorta. In mammals, by contrast, the left fourth pharyngeal arch artery becomes the arch of the aorta and the right fourth the base of the right subclavian. The loss of one of the fourth arch arteries may reflect the recruitment of the

[†]Note that the group “reptiles” is not monophyletic, and therefore does not include all of the descendants of the ancestor of all reptiles, most significantly birds. Sauropsida is the group of amniotes that includes all living reptiles “and birds” as well as their fossil ancestors. Therefore when discussing the evolutionary origin of certain characters in reptiles, keep in mind that these changes may have occurred in an ancestor to extant reptiles and birds.

neural crest to the outflow septation complex. Indeed, many outflow tract malformations in humans and animal models are accompanied by defects in fourth arch derivatives.

Origins of the Cardiac Neural Crest: The Third Cranial Neural Crest Stream in Non-Avian and Non-Mammalian Vertebrates

The CNCCs in chick and mouse originate from the third of three cranial neural crest streams (Fig. 2). Depending on the organism, the third cranial stream is also termed the “branchial” or “postotic” stream. The presence of three cranial streams is a conserved character of vertebrates; even the most basal vertebrates, jawless fishes such as the lamprey, have three cranial neural crest streams (Horigome et al., 1999; McCauley and Bronner-Fraser, 2003). The multipotency and migratory range of third stream neural crest cells in basal vertebrates provides clues to the evolutionary developmental origin of the CNCCs. Unfortunately, studies examining the contribution of neural crest cells to vertebrate cardiovascular development outside birds and mammals have been limited for a number of reasons. In many species direct labeling of neural crest cells for lineage studies is extremely difficult. Access to live embryos may not be possible, or when possible, the embryos may not survive long enough under experimental conditions to determine whether the neural crest cells contribute to the aortic arch arteries and outflow tract. Accurate labeling is also often technically challenging. When using many traditional lineage-tracing techniques, such as DiI labeling, great care must be taken to avoid spurious, non-neural crest cell labeling. Additionally, there is no universal, cross-species marker that is exclusive to neural crest cells, making it difficult to definitively identify neural crest cells without lineage tracing (Hall and Gillis, 2013). Because of these obstacles, studies of CNCCs have been largely restricted to a small number of developmental model systems.

Lampreys are the most primitive extant vertebrates readily available for embryonic manipulation (Jandzik et al., 2014). Although the lamprey has three cranial neural crest streams as in other vertebrates, it differs in that the posterior border of the branchial neural crest stream is not well defined (McCauley and Bronner-Fraser, 2003). The cells from the branchial stream enter the gill arches and contribute to the branchial arch skeletal structures that support the gills (Languille and Hall, 1988; Horigome et al., 1999; McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2004). In addition to the gill cartilage support structures, cells that originate from the branchial stream also have extensive rostral and caudal migration and are found in structures of the hyoid arch, as well as structures in the trunk (McCauley and Bronner-Fraser, 2003). It is unknown if neural crest cells in lampreys contribute to the smooth muscle of the gill arch arteries.

The three cranial neural crest streams of elasmobranchs (sharks, skates, and rays) have been studied by SEM in the cat shark, *Scyliorhinus torazame*, and the branchial stream has been shown to enter the gill arches (Kuratani and Horigome, 2000). In the skate *Leucoraja erinacea*, DiI labeling of neural crest-derived pharyngeal arch mesenchyme of the first gill arch showed that these cells gave rise to chondrocytes of the gill arch endoskeleton (Gillis et al., 2013). Other derivatives were not the focus of this study, so it is unknown if neural crest cells gave rise to smooth muscle of the gill arch arteries. Another recent study reported labeling trunk neural crest in the bamboo shark, *Chiloscyllium punctatum*, and observing

DiI-positive cells in the branchial arches, as well as two sets of migrating cells in the outflow tract of the heart (Juarez et al., 2013). It is unclear what derivatives were labeled in the arches and outflow tract as no co-localization with cell-type specific markers or sections were presented, confounding the conclusions that may be drawn from this study.

In zebrafish, a teleost, the postotic neural crest stream has been shown to contribute to the neurons of the proximal and jugular ganglia, as well as components of the gill cartilages: basibranchial, hypobranchial, and ceratobranchial cartilages (Schilling and Kimmel, 1994; Mongera et al., 2013). It is widely assumed in zebrafish that the neural crest cells provide the smooth muscle of the main gill arch artery, but this has not been formally demonstrated. Recently, neural crest cells have been identified in the branchial region associated with primary and secondary lamellae of the gill filaments (Mongera et al., 2013). The primary lamellae branch off of the main gill arch artery, and the secondary lamellae branch off of the primary lamellae. In the primary lamellae the neural crest cells do form the smooth muscle cells of the blood vessels (Mongera et al., 2013). In the secondary lamellae, the neural crest cells are associated with pillar cells, which support the respiratory epithelium to form lumina, which provide an area where gas exchange occurs (Bettex-Galland and Hughes, 1973). The study by Mongera et al. (2013) did not directly trace the neural crest cells from their origin along the rostro-caudal axis. However because these cells are in close proximity to the supporting gill cartilages that arise from the branchial stream, it is highly likely that these gill lamellae neural crest cells are also from the third or branchial stream.

The zebrafish embryo has been the subject of a number of CNCC studies (Li et al., 2003; Sato and Yost, 2003; Sato et al., 2006; Xia et al., 2013). Zebrafish do not have a divided outflow, and thus it was not expected that neural crest cells would be found beyond the aortic arch arteries. However, two studies in the early 2000s unexpectedly identified neural crest cells capable of generating myocardium (Li et al., 2003; Sato and Yost, 2003). These results are controversial, given that CNCCs have not been found to contribute myocardium in any other species studied thus far. (While the studies in the shark reported neural crest cells in the heart, the cells were not verified with myocardial markers). The myocardial lineage studies should be reconsidered for several reasons. Zebrafish is now commonly used to model human cardiac malformations and, although the initial studies were done with the best tools at the time, there were several technical issues with the studies. For example, the neural crest labeling and ablations were done without an independent, real-time marker of the neural crest. These studies were also done before the more elegant cre-lox system and other genetic color switching systems were regularly used in the zebrafish model (Mongera et al., 2013). *Sox10* has been reported to be specifically expressed in all neural crest during early stages and is maintained in only a subset of neural crest derived structures. A recent study used a *Sox10*-inducible cre line and reported some colocalization of *Sox10*-positive cells with cardiomyocytes (Mongera et al., 2013). However, the authors also noted that they observed rare spurious muscle clones that may be of non-NC origin (Mongera et al., 2013). Because of the lack of evidence of a myocardial cell fate in other species, the controversial myocardial cell fate of CNCCs should be revisited using these more targeted approaches.

The Australian lungfish *Neoceratodus* also has three neural crest streams, of which the postotic stream contributes to the developing ceratobranchial cartilages (Ericsson et al.,

2008). However, no studies have examined other postotic neural crest cell derivatives in any lungfish species. In contrast to *Neoceratodus*, the African lungfish *Protopterus* is one of the few fish species that possess true lungs in combination with reduced gills. With a partially septated ventricle and outflow tract, the separation of oxygen-rich and poor blood is more complete than in amphibians (Icardo et al., 2005). As such, the study of CNCCs in *Protopterus* could be especially revealing.

In axolotl, the neural crest cells of the postotic stream contribute to the ceratobranchial cartilages that support the gills (Epperlein et al., 2000; Ericsson et al., 2004). In addition to the postotic cartilages in axolotl, the neural crest cells contribute to the connective tissue of the external gills (Epperlein et al., 2007; Ericsson et al., 2004). Neural crest cells of the postotic stream were observed in the prospective heart region of axolotl, but further studies are needed to establish the exact location and determine the identity of the specific neural crest derivatives (Epperlein et al., 2000).

The first study to perform a long-term labeling of neural crest cells in anurans came from *Bombina orientalis*, the oriental fire bellied-toad (Olsson et al., 2001). This study revealed that the ceratobranchial cartilages in *B. orientalis* are derived from the branchial neural crest stream. Neural crest cells surround the developing thymus in *Xenopus*, but they originate from the second, hyoid stream, not the third, branchial stream, as seen in chicks and mice (Lee et al., 2013).

The specific contribution of neural crest cells to the cardiovascular system of amphibians has been studied in *Xenopus* (Lee and Saint-Jeannet, 2011; Martinsen et al., 2004; Sadaghiani and Thiébaud, 1987). In orthotopic transplantation experiments using *Xenopus borealis*-*Xenopus laevis* chimeras to lineage trace neural crest cells, a small number of neural crest cells were observed in the wall of the truncus arteriosus (Sadaghiani and Thiébaud, 1987). The fate of these cells was not determined. More recent studies that have found, as in chick and mouse, that the myocardium of the amphibian outflow tract is derived entirely from the second heart field (Gessert and Kuehl, 2009; Lee and Saint-Jeannet, 2011; Martinsen et al., 2004). The outflow tract cushion mesenchyme is entirely generated by epithelial-to-mesenchymal transformation of the endocardial cells lining the cushions. Using two distinct labeling techniques, Lee et al. (Lee and Saint-Jeannet, 2011) demonstrate that CNCCs never enter the outflow tract cushions in *Xenopus*, but rather remain in the aortic sac and arch arteries. They conclude that CNCC migration into the cushions is a derived characteristic of higher vertebrates (birds and mammals). They propose that the recruitment of the CNCCs into the outflow cushions allowed for an increase in the mass of the outflow tract septum to complete separation of the systemic and pulmonary circulations at the arterial pole. Therefore, the participation of CNCCs in outflow tract septation is an acquired characteristic of higher vertebrates (at least of birds and mammals) that is associated with the evolutionary need for a more fully divided circulation (Lee and Saint-Jeannet, 2011). Studies in other amphibians are needed to determine if the absence of CNCCs in the outflow is common to the group as a whole. *Xenopus* is aquatic, and if pulmonary respiration has been the selective pressure to recruit neural crest cells to the arterial pole for septation, CNCCs may migrate into the outflow of terrestrial amphibians (Chin et al., 2012).

The critical importance of CNCCs in outflow tract septation was first recognized in neural crest-ablation studies in the chick (Kirby et al., 1983). CNCC ablation resulted in persistent truncus arteriosus (unseptated outflow tract), pharyngeal arch artery remodeling defects, as well as looping defects related to abnormal development of the second heart field. Neural crest ablation studies in *Xenopus* have differing results, depending on the extent of the premigratory neural crest domain ablated (Lee and Saint-Jeannet, 2011; Martinsen et al., 2004). Martinsen et al. (2004) ablated neural crest cells from the entire cranial region to the mid-trunk level of the *Xenopus* embryo. They observed abnormal cardiac development including an elongated, unlooped heart tube, pericardial edema, as well as a lack of normal heart tube formation, and concluded that the presence of the neural crest cells is required for normal heart development (Martinsen et al., 2004). In contrast, Lee and Saint-Jeannet (2011) ablated a series of more restricted domains of premigratory neural crest, and did not observe any major cardiac anomalies, except for the loss of the aortic sac and arch arteries. Spiral septum formation was normal. These results are in agreement with labeling experiments showing that the CNCCs never enter the amphibian outflow (Lee and Saint-Jeannet, 2011). It is possible that the larger neural crest cell domain ablated by Martinsen et al. represents a SHF defect as observed in the chick and mouse (Hutson et al., 2006; Waldo et al., 2005; Yelbuz et al., 2002). The abnormal artery patterning and heart looping defects are similar to the physical and genetic CNCC ablation studies in chick and mouse.

While it has been established that the CNCCs contribute to septation of the avian and mammalian aorticopulmonary septum, and that CNCCs do not participate in formation of the spiral valve in a model amphibian, it is unknown what role the CNCCs play in outflow septation in reptiles. The aorticopulmonary septum is complete in reptiles. Thus, if the CNCCs do indeed move into the outflow in order to increase the mass of the septum and complete the septation complex, CNCCs may well septate the outflow tract of reptiles. Septation of the outflow tract by the CNCCs could therefore be a derived character of all amniotes. Lineage-tracing studies of neural crest cells in many reptiles are difficult, given that the eggshell is soft and embryos do not survive the opening of their eggs (Cebra-Thomas et al., 2007). In order to determine whether neural crest cells are present in the reptile outflow, this technical hurdle will need to be overcome or alternative methods to identify CNCCs in reptiles will need to be devised.

The Evolution of the Cardiac Neural Crest

The first role of the CNCCs was most likely to stabilize the branchial arch arteries through formation of a smooth muscle tunic. It is well established that, in amphibians and amniotes, the CNCCs contribute smooth muscle to the arch arteries (Lee and Saint-Jeannet, 2011); however, the origin of this character within the vertebrate evolutionary tree is unknown. It is widely assumed that the branchial arteries of teleosts contain a CNCC-derived smooth muscle layer, but this has yet to be formally demonstrated. As mentioned above, the neural crest cells form the smooth muscle cells of the blood vessels in the primary lamellae branching off the main gill arch arteries of zebrafish (Mongera et al., 2013). It is possible that a CNCC-derived smooth muscle tunic surrounding the arch arteries is a shared vertebrate character (synapomorphy). However, there are currently too many gaps in our knowledge of vertebrate CNCC derivatives, particularly among more basal vertebrates, to

know when the neural crest cells evolved the ability to reinforce the arch arteries by differentiating into a smooth muscle layer. Lineage tracing from jawless (lamprey) and cartilaginous fishes (sharks, skates, rays) would be especially helpful in this regard.

The CNCCs may participate in remodeling of the aortic arch arteries in non-mammalian and non-avian vertebrates, but again, our knowledge is limited. The role of the CNCCs in the formation of the unique cardiovascular configurations in lungfish warrants study, as well as the Foramen of Panizza in crocodilians, a structure of the arterial pole whose embryonic origin may involve the CNCCs. It is also unknown if the CNCCs serve a function in pharyngeal arch artery remodeling in amphibians and reptiles, for example in the regression of the fifth arch artery.

We hypothesize that at some point after the CNCCs were recruited for smooth muscle reinforcement and remodeling of the pharyngeal arch arteries, they were then recruited further into the heart to aid in septation of the outflow tract. Key to this recruitment is targeting the CNCCs to the outflow myocardium. Pigment cells and cardiac ganglia, presumably of neural crest origin, are present in the hearts of amphibians, which do not have a fully septated outflow. Therefore, prior to the evolution of outflow septation, neural crest cells were able to follow migratory cues to the heart. A highly plausible mechanism for the evolution of outflow septation by the CNCCs is recruitment of the cells from the caudal arches. The ventral aspect of these arches is in close proximity to the outflow tract, and within developing chick and mouse embryos, CNCCs traveling to the outflow first pass through the caudal pharyngeal arches. The disruption of specific signaling pathways in mouse or chick, such as the semaphorins discussed below, results in the CNCCs halting their migration just prior to entering the outflow (Feiner et al., 2001). These pathways may have been instrumental in the evolution of outflow septation by the CNCCs, and are excellent candidates for study in transitional organisms, such as amphibians and reptiles. As reviewed above, reptiles are the most basal vertebrates to possess fully septated outflows, but further study of neural crest cell lineages is needed in these animals to determine if septation involves the CNCCs.

THE ROLE OF THE SECOND HEART FIELD IN CNCC EVOLUTION

A discussion of the evolution of the CNCCs and outflow septation is, in reality, a discussion of CNCC and SHF co-evolution. One of the derivatives of the SHF is the outflow myocardium, which in higher vertebrates can be divided into truncal and conal segments. The conus is able to produce a valve via epithelial to mesenchymal transition (EMT) whereas the truncal myocardium lacks the ability to induce EMT. The CNCCs interact primarily with the truncal myocardium, septating this portion of the outflow rather than the conus. As will be discussed in further detail below, signals from the truncus are critical for CNCC migration into the outflow. Thus, the evolution of the truncal portion of the outflow tract was likely a prerequisite for CNCC involvement in outflow tract septation.

While lengthening of the heart tube by the SHF is required to divide the amniote heart into systemic and pulmonary chambers, the SHF also exists in vertebrate species with partial or no septation. Indeed, a SHF has recently been identified in amphibian, fish, and agnathan embryos (Brade et al., 2007; de Pater et al., 2009; Gessert and Kuehl, 2009; Hami et al.,

2011; Kokubo et al., 2010; Lee and Saint-Jeannet, 2011; Zhou et al., 2011), and a SHF-like group of cells has been found in the early chordate *Ciona* (Stolfi et al., 2010). Grimes et al. (2010) have conducted an extensive, cross-species comparison of the myocardial and smooth muscle components of the outflow tracts of a number of different vertebrate species (Grimes et al., 2010). They suggest that in all vertebrates, the outflow tract consists of three distinct components: a proximal, myocardial component; a distal, smooth muscle component; and a middle component forming an overlapping seam between the proximal, myocardial, and distal, smooth muscle outflow tract components. This common construction of the outflow suggests that all vertebrates have a SHF.

The importance of the SHF in the evolution of the vertebrate heart is highlighted by its two defining properties: continued proliferation and differentiation delay. This suggests that this progenitor cell population initially evolved not to drive septation but to facilitate morphogenesis of a longer heart tube. As divided circulations evolved in vertebrates, the differentiation delay and proliferative capacity of the SHF allowed for the addition of more and more cardiac segments. As the outflow lengthened during vertebrate evolution, a truncal portion became distinct at the distal end of the outflow. The truncus is the portion of the outflow that attracts the CNCCs and contains the cushions into which the crest cells will migrate. It also is the myocardium that interacts with the CNCC-septation complex to physically divide the outflow tract. Therefore, we hypothesize that only after the addition of the truncus were the CNCCs able to participate in outflow septation.

Unfortunately there is much confusion in the anatomical and developmental literature as to what parts of the outflow are referred to as “truncus” and “conus” in different vertebrates (Grimes et al., 2010). For example, according to some sources, in *Xenopus* the “truncus” is the base of the bifurcated aorta, and the “conus” is the portion that contains the spiral valve (Liem et al., 2001). Others label the portion containing the spiral valve the “truncus”, and the portion below, which is incorporated into the ventricle as development proceeds, is referred to as the “conus” (Kolker et al., 2000). Without a consistent nomenclature across taxa and without knowing the homology of the different components of the outflow tract in different species, reconstructing the history of outflow tract evolution that led to the recruitment of the CNCCs is problematic. For example in *Xenopus*, the outflow portion containing the spiral valve is not capable of recruiting the CNCCs and completing outflow septation (Lee and Saint-Jeannet, 2011). It is unknown if this particular segment is homologous to the truncus or conus of chick and mouse. Therefore, it is unclear if this segment is a truncus that gained the ability to recruit CNCCs in amniotes, or if distal to this segment an amniote-like truncus was added through further lengthening of the heart tube. Such a distinction would help reconstruct the evolutionary history of CNCC involvement in outflow septation.

As discussed above, the CNCCs also play a role in the regulation of signaling for the SHF and are required in chick and mouse for normal addition of the SHF to the heart tube. Neural crest ablation studies in other vertebrates will be necessary to first determine when this role evolved. We speculate that this function of CNCCs could have very early roots in vertebrate evolution, given that CNCCs are required for regulation of signaling prior to their entry into the outflow tract and that the SHF appears to be common to all vertebrates.

Candidate Signaling Pathways in Cardiac Neural Crest Evolution

The co-option of the neural crest for outflow tract septation arose through modification of existing developmental regulatory programs. For example, regulatory programs may be changed by the co-option of new upstream regulators or by the employment of new downstream gene targets, thus placing existing networks in a novel context (Donoghue et al., 2008). Modifications of the timing or levels of gene expression can likewise result in significant alterations in developmental form (Keyte and Smith, 2014). The following sections describe a subset of candidate signaling pathways that, based on their perturbation in animal models or mutation in humans, are likely to have been involved in the co-option of the neural crest cells for their involvement in SHF development and outflow tract septation. We have chosen to focus on *Tbx1* and DiGeorge Syndrome, but a number of signaling pathways are also known to be critical for CNCC and SHF development, and have been linked to human malformations (Notch, TGF β , Endothelin, and Retinoic Acid, for example). Comprehensive reviews of pathways involved in CNCC and SHF development have recently been conducted, and we refer readers to these for more information (Keyte and Hutson, 2012; Lin et al., 2012; Zaffran and Kelly, 2012).

DIGEORGE SYNDROME: A FAILURE OF CNCC AND SHF FUNCTION

A fitting illustration of a failure in the evolutionarily derived functions of CNCCs and the SHF – aberrant pharyngeal arch artery remodeling and a lack of outflow alignment and septation – can be seen in DiGeorge Syndrome (DGS). The congenital defects in DGS have historically been described as neurocristopathies, but upon closer inspection the DGS defects result from perturbation of the formation of the pharyngeal apparatus that includes the ectoderm, endoderm, and mesoderm, as well as the neural crest cells. Thus the cardiovascular defects seen in DGS (interrupted aortic arch type B, persistent truncus arteriosus, tetralogy of Fallot with pulmonary atresia or stenosis, and double outlet right ventricle) are attributable to abnormal development of both the CNCCs and SHF. The syndrome is frequently caused by a chromosomal deletion, 22q11, which encompasses a cohort of genes including *Tbx1*. Deletion and complementation experiments in mice have shown that *Tbx1* deficiency replicates the cardiovascular abnormalities seen in DGS, and mutations of *Tbx1* without the classical 22q11 deletion have been found in human patients (Jerome and Papaioannou, 2001; Lindsay, 2001; Merscher et al., 2001; Yagi et al., 2003). In mice, *Tbx1* is expressed in pharyngeal ectoderm, endoderm, and the SHF, but is not expressed in CNCCs (Garg et al., 2001; Vitelli et al., 2002a). Thus, mutations in *Tbx1* do not directly impact the CNCCs, but instead affect the environment through which the CNCCs migrate. As a consequence, signaling between the CNCCs and surrounding tissues is impacted, and neural crest cell migration is altered (Frank et al., 2002; Kochilas et al., 2002; Vitelli et al., 2002a; Calmont et al., 2009). Conditional *Tbx1* mutants display aorticopulmonary septal defects due to fewer CNCCs migrating through pharyngeal arch four and reaching the outflow. In addition to the indirect regulation of CNCC migration, *Tbx1* positively regulates cell proliferation in the SHF for normal elongation of the primary heart tube and correct outflow tract alignment (Xu et al., 2004). The severity of the DGS-like phenotype in mice can be modulated by dosage of *Tbx1* expression. For example aortic arch artery defects are observed at 50% of normal levels of *Tbx1* while persistent truncus

arteriosus is observed in mice expressing 20% of normal levels (Zhang and Baldini 2007). Consequently, *Tbx1* levels must be tightly regulated to coordinate normal arch artery patterning, outflow tract alignment, and septation. Thus, *Tbx1* and its transcriptional targets (see below) comprise a pertinent developmental regulatory program within the pharynx that would be amenable to evolutionary modification for outflow tract septation.

As mentioned above the evolution of outflow septation requires novel adaptations of the CNCCs, as well as the outflow myocardium derived from the SHF. The SHF-derived aortic and pulmonary myocardium of the outflow tract are developmentally and molecularly distinct, a fact that has been revealed in part from study of *Tbx1* mouse mutants (Bajolle et al., 2008; Bertrand et al., 2011; Rochais et al., 2009; Théveniau-Ruissy et al., 2008). SHF progenitors in the pharyngeal and splanchnic mesoderm are prepatterned in discrete myocardial subdomains that first occupy the superior and inferior walls of the outflow tract, and subsequently the base of the aorta and the pulmonary artery, respectively. In *Tbx1* mutant hearts the subpulmonary myocardium is specifically affected, and *Semaphorin3C* (*Sema3C*) is downregulated (Théveniau-Ruissy et al., 2008), suggesting that the subpulmonary myocardium is missing or mispatterned. The semaphorins, a group of secreted ligands, and their receptors are also important players in the targeted migration of the CNCCs to the arterial pole of the heart. The semaphorin receptors *Plexin-A2*, *Plexin-D1* and *Neuropilin-1* (*Nrp1*) are expressed by the CNCCs. *Sema6A* and *6B* have been shown to repel neural crest cells, while *Sema3C* attracts them. *Sema3C* is expressed in the outflow tract, whereas *Sema6A* and *6B* are expressed in the dorsal neural tube and lateral pharyngeal mesenchyme (Toyofuku et al., 2008). This suggests that CNCCs are driven from the neural tube by *Sema6A* and *6B*, and attracted to their target, the outflow tract, by *Sema3C*. Downregulation of each receptor in neural crest cells by RNAi knockdown results in the failure of the cells to migrate into the cardiac outflow tract (Toyofuku et al., 2008). Moreover, both *Plexin-A2* and *Np1*-null mice exhibit interrupted aortic arch and persistent truncus arteriosus, associated with a decrease of neural crest cells at the septation site (Brown et al., 2001; Kawasaki et al., 1999; Toyofuku et al., 2008). Mice with null mutations for *Sema3C* also display interrupted aortic arch and persistent truncus arteriosus (Feiner et al., 2001). *Sema3C* is specifically required by CNCCs; in these mutants, all other neural crest derivatives are normal. Thus the regulation of *Sema3C* signaling by *Tbx1* seems to be an important evolutionary step toward a divided circulation in that *Sema3C* provides a different molecular signature to the pulmonary myocardium of the outflow tract, as well as important chemoattractant signals to the CNCCs. *Sema3C* is expressed in the heart of *Xenopus* (Koestner et al., 2008), but it is unknown if transcript is present specifically in the outflow or if the expression pattern is asymmetric. We would predict that if *Sema3C* is in fact expressed in the amphibian outflow tract, that message would be evenly distributed rather than asymmetric. Examining the *Xenopus* outflow for the expression of genes known to be asymmetrically expressed in the pulmonary and aortic myocardium of amniotes could be particularly illuminating for understanding the evolution of outflow tract septation.

Retinoic acid (RA) signaling is also highly conserved among vertebrates and is up-regulated in *Tbx1* mutant mice. In chick, knockdown of the RA degrading enzyme, *Cyp26*, phenocopies DGS (Roberts et al., 2006). RA has important functions in neural crest cells,

and either an excess or deficit of RA can result in conotruncal and aortic arch artery defects (Coberly et al., 1996; Lammer et al., 1985). When RA receptors *RAR α* and *RAR β* are specifically deleted in the neural crest cells, the number, migration, and fate of the CNCCs are all normal, but the ability of the cells to septate the outflow tract is compromised (Jiang et al., 2002). The CNCCs do not utilize retinoid receptors or respond to RA during septation. Therefore, the truncus phenotype is non-cell autonomous for the neural crest cells. This indicates that the outflow tract myocardium may respond directly to RA, and then induces or allows the CNCCs to commence septation (Jiang et al., 2002). RA signaling has additionally been shown to be important in SHF development, and specifically in delimiting the posterior border of the SHF. In RA receptor mutant mice, the outflow tract is shortened, malaligned, and mis-specified along its proximal-distal axis, resulting in ectopic expression of *TGF β 2* and ectopic EMT (P. Li et al., 2010). Mouse mutants for *Raldh2*, the RA synthesis enzyme, have an expanded SHF (Ryckebusch et al., 2008; Sirbu et al., 2008). Known targets of RA signaling include the Hox genes, and *Hoxa1* null embryos exhibit outflow tract alignment defects, including tetralogy of Fallot (Makki and Capecchi, 2012). *Hoxb1*, *Hoxa1*, and *Hoxa3* define distinct progenitor subdomains within the second heart field (Bertrand et al., 2011). *Hoxa1* and *Hoxa3* are restricted to the distal outflow tract, suggesting that proximo-distal patterning of the outflow is related to SHF subdomains determined by combinatorial Hox gene expression. Additionally, SHF progenitor cells expressing *Hoxb1*, *Hoxa1*, and *Hoxa3* contribute to subpulmonary myocardium but not aortic myocardium, supporting previous studies indicating that progenitor cells that will give rise to subpulmonary myocardium are prepatterned in distinct subdomains (Bajolle et al., 2008). Manipulation of RA signaling altered Hox expression in the SHF, with reduction of RA signaling leading to a defect of the inferior wall of the outflow tract, which gives rise to the subpulmonary myocardium (Bertrand et al., 2011). We hypothesize that small changes in RA signaling during amniote evolution could have allowed the outflow to lengthen and gain new identity, one that was capable of interacting with CNCCs to form the septation complex.

Another important target of *Tbx1*, which underlies the etiology of DGS, is the secreted growth factor, *Fgf8* (Frank et al., 2002; Hu et al., 2004; Moon et al., 2006; Vitelli et al., 2002b; Zhang et al., 2005). Although not contained within the 22q11-deleted region, *Fgf8* expression is nevertheless reduced in mice mutant for *Tbx1*, and *Fgf8* hypomorphs phenocopy DGS (Vitelli et al., 2002b). As the CNCCs enter the arches, FGF8 is expressed at high levels in the pharyngeal endoderm and ectoderm and at lower levels in splanchnic mesoderm. The *Fgf8* hypomorph phenotype is likely the result of reduced FGF8 signaling from the pharyngeal endoderm and ectoderm to the CNCCs migrating through the arches (Frank et al., 2002). *Fgf8* hypomorphic and conditional mouse mutants exhibit abnormal apoptosis of the CNCCs (Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003). In addition, FGF8 has been demonstrated to be chemokinetic for the CNCCs, and when FGF8 is overexpressed within the pharynx, greater numbers of CNCCs migrate more quickly into the arches. These results suggest that FGF8 affects crest cell survival, as well as the timing and targeting of migration through the pharyngeal arches (A. Sato et al., 2011).

Fgf8 also has tissue-specific functions in SHF development. *Fgf8* produced by the mesoderm is needed for proper outflow tract alignment, while *Fgf8* from the pharyngeal

endoderm regulates outflow septation (Park et al., 2006). Following the experimental reduction of FGF8 expression during the period of SHF addition, arterial pole alignment defects are seen in chick embryos in the absence of persistent truncus arteriosus (Hutson et al., 2006), indicating that overall levels of FGF8 are important for SHF development. In addition to levels, the source of FGF8 is also important. *Fgf8* expression is missing from the pharyngeal endoderm of *Tbx1* null mutants (Vitelli et al., 2002b) but ectodermal expression of *Fgf8* is not reduced. This suggests that endodermally-derived FGF8 is critical for outflow tract development. Thus, the multiple roles of FGF8 in neural crest cell survival and migration, as well as in SHF development and outflow septation, make the FGF8 signaling pathway a compelling target for modification of the developmental program in the evolution of outflow tract septation.

Tbx1 is not the only gene within the DiGeorge critical region implicated in the pathogenesis of DGS. Among a number of other genes, *Crkl* is also located within the 22q11-deleted region and codes for an adapter protein which functions in intracellular receptor tyrosine kinase signaling cascades. *Crkl* protein is expressed strongly in neural crest cell derivatives, and disruption of *Crkl* function causes cardiovascular defects observed in DGS (Guris et al., 2001). *Crkl* deficiency has been demonstrated to interfere with *Fgf8* signaling and alter MAPK activation (Moon et al., 2006). *Crkl* and *Tbx1* interact in a dose-dependent manner, suggesting that in many patients, DGS is in fact a contiguous gene syndrome (Guris et al., 2006). *Crkl* is a crucial element of an FGF8-induced feed-forward loop, which is permissive for activation of the mitogen-activated protein kinase *Erk1/2*, also required for CNCC development. *Crkl* is essential for *Fgf8*-induced chemotaxis in vitro (Moon et al., 2006), suggesting a function for *Crkl* in CNCC migration.

THE EVOLVING ROLE OF TBX1

The expression of *Tbx1* is highly conserved in the pharyngeal arches of vertebrates, including lamprey (Sauka-Spengler et al., 2002). In *Amphioxus*, a descendant of the last common invertebrate ancestor of the vertebrates, *Tbx1/10*-mediated branchial arch endoderm and mesoderm patterning functions are conserved, and thus predated the origin of neural crest cells (Mahadevan et al., 2004). As demonstrated by the *van gogh* (*vgo*) mutant, *Tbx1* is required for SHF proliferation in zebrafish (Hami et al., 2011; Nevis et al., 2013; Piotrowski et al., 2003). The neural crest-derived cartilages are abnormal and the aortic arch arteries are reduced in size or interrupted altogether, but neural crest induction and migration appear normal (Piotrowski et al., 2003; Piotrowski and Nusslein-Volhard, 2000). Therefore, *Tbx1* first functioned to positively regulate cell proliferation in the SHF and was later co-opted for the regulation of third stream neural crest cell migration. In *Xenopus*, *Tbx1* knockdown results in abnormal heart looping, pericardial edema, and aortic arch abnormalities (Ataliotis et al., 2005; Tazumi et al., 2010; Tran et al., 2011). In addition to abnormal SHF development, neural crest cell migration is altered. Therefore, the ability of *Tbx1* to regulate neural crest cell migration was present prior to the recruitment of CNCCs for outflow tract septation. Regulatory changes upstream of *Tbx1* or in downstream effectors may have been involved in the co-option of CNCCs into the outflow tract of amniotes and warrant further study.

In summary, we propose that there are four novel adaptations of the SHF and the CNCCs required for outflow tract septation: (1) lengthening of the outflow tract through addition of the truncus, (2) molecularly distinct outflow tract myocardium with designated “aortic” and “pulmonary” sides, (3) targeting of the CNCCs to the outflow tract cushions, and (4) interaction between the outflow myocardium and the CNCCs within the cushions to physically divide the outflow tract.

Conclusions

The CNCCs have clearly been important in the evolution of the vertebrate cardiovascular system, but our knowledge of their evolutionary history is still rather sparse. Labeling studies in a number of vertebrates are necessary to understand the contribution of CNCCs to the evolution of the heart and circulatory system. Once this information has been gathered, specific hypotheses concerning the evolutionary-developmental mechanisms by which neural crest cells were recruited for cardiovascular development can be developed. In order to fully understand how outflow septation arose, consideration of second heart field/outflow tract co-evolution with the crest cells will be essential.

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FIGURE 1.

Circulation of oxygenated and de-oxygenated blood. (A) In most fish the circulation of blood creates a single loop. (B, C) In amphibians and reptiles, two loops form with the addition of the pulmonary circulation. However, the separation of oxygenated and de-oxygenated blood is more complete in reptiles than in amphibians. (D) Birds and mammals possess two separate circuits of oxygenated and de-oxygenated blood. A, atrium; V, ventricle; RA, LA, right and left atrium; RV, LV, right and left ventricle.

FIGURE 2.

Cranial neural crest streams in relation to the developing cardiac vasculature of divided (**A**) and un-divided (**B**) outflow tracts. (**A**) In mammals and birds, the cells arising from the third cranial neural crest stream (shown in green) contribute to the aortic arch arteries and eventually the outflow tract (dashed green arrow). (**B**) In teleosts, cells from the third cranial neural crest stream contribute to the branchial arches that will bear gills arch arteries.

FIGURE 3.

The evolution of aortic arches and cardiac chambers in vertebrates. **(A)** Primitive fishes, represented by sharks, have six paired gill arches. **(B)** In teleosts, the gill arch arteries are reduced to form four pairs in the caudal branchial arches. **(C)** Lungfish have both gills and a pulmonary circulation with the gill arches corresponding to arches two, five, and six. During air respiration, the blood is shunted through arches three and four, while the ductus arteriosus in arch six shunts oxygen-poor blood away from the gills and to the lungs. **(D)** In adult amphibians, the gill arches are lost and the aortic arch vasculature remains bilaterally symmetrical. Oxygenated and de-oxygenated blood enter the ventricle through the right and left atrium and leaves the heart through a single outflow tract containing a spiral valve. **(E)** In mammals, the fourth aortic arch arteries become bilaterally asymmetrical and the outflow tract separates into two distinct outflow vessels. **(F)** Birds also have a completely divided outflow tract with asymmetrical aortic arch arteries. **(G)** In reptiles such as the turtle, aortic arch artery four remains bilateral but is divided at the base of the outflow tract. The outflow tract is divided into three arteries: right and left aortic arch arteries and the pulmonary artery. In all figures cranial is to the top and caudal is to the bottom.

FIGURE 4.

Comparative anatomy of vertebrate hearts. (A) Teleosts have a single atrium and ventricle with an outflow consisting of the bulbus arteriosus. Only deoxygenated blood pumps through the teleost heart. (B) Frogs have two atria and one ventricle in which oxygenated and de-oxygenated blood can mix. The blood exits the ventricle through a single outflow vessel containing a spiral septum. (C) Turtles have two atria and a ventricle that is partitioned into three sections. The dorsal left atrial space directs blood to the right aorta while the ventral left ventricle directs blood to the left aorta. The right side of the ventricle

directs blood to the pulmonary artery. (**D, E**) In the avian and mammalian hearts, the ventricle is completely divided with a single aorta and a pulmonary artery.