The Role of Neural Crest Cells in Vertebrate Cardiac Outflow Development

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Throughout vertebrate evolution, the cardiac outflow vasculature has changed from a branchial arch system to a systemic and pulmonary circulatory system. However, all vertebrate hearts and outflow tracts still develop from a single heart tube. In the chick and mouse, cardiac neural crest cells divide the single outflow tract into the aorta and pulmonary arteries. Additionally, cardiac neural crest cells in mice and chicks provide the smooth muscle of the aortic arch arteries, help to remodel the aortic arch arteries into asymmetrical structures, and contribute to cardiac ganglia. I review the major contributions of cardiac neural crest cells to the outflow vasculature of the chick and mouse and apply this information to study cardiac neural crest cell contributions to vertebrates that lack a divided circulatory system. I re-evaluate the role of cardiac neural crest cells in zebrafish and hypothesize that neural crest cells contribute to the smooth muscle of the gill arch arteries, the ventral aorta and cardiac ganglia, but not to myocardium as previously described. I also study the outflow tract development of the turtle *Trachemys scripta* to understand the process of outflow septation in a vertebrate that has a divided outflow tract but an incomplete division of the ventricle. I compare the chick outflow tract to the turtle. The formation of the proximal versus distal cushions and the appearance of smooth muscle cells within the distal cushions of the turtle are very similar to the cushion position and cell types within the cushions of the chick. In

the chick, smooth muscle markers are expressed in cells within the distal cushions. The cells expressing the smooth muscle markers are derived from cardiac neural crest cells. I hypothesize that cardiac neural crest cells are also responsible for the outflow tract septation of reptiles. These results demonstrate that the pattern of cardiac neural crest cell contribution to vertebrate vasculature remains predictable and consistent, enabling future studies to focus on changes in vascular patterning caused by cardiac neural crest cells among different vertebrate lineages.

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1. Introduction

Throughout the course of evolution, vertebrate cardiac anatomy has most notably changed from a branchial arch to a systemic and pulmonary circulatory system as the site of respiratory exchange changed from the gills to the lungs. In aquatic animals with a branchial arch circulatory system, the heart pumps oxygen-depleted blood to the bilateral gills where it is oxygenated and sent back through the body. In vertebrates that spend time in water and on land, such as lungfish, we see a transitional arrangement of the architecture of the branchial arch arteries. Lungfish gills correspond to arches 2, 5 and 6. These gills exchange CO2 for O2 using diffusion. During air respiration, the valves in these arches close, creating shunts in arches 3 and 4 that pass blood from the ventral to the dorsal aorta. In addition, the 6th arch shunts O2 poor blood to the lungs. (Bemis et al., 1987). Partial divisions in the atria and ventricle as well as valves in the conus also help partition these two systems in the lungfish (Icardo et al., 2005). As vertebrates lose their gills and become exclusively air breathing, the skeletal and muscular elements of the pharyngeal arches are re-purposed as structures in the head 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 aortic arch arteries that

are internalized and remain bilaterally symmetrical. The heart moves caudally, closer to the lungs and has a singular outflow tract with two atria and one ventricle.

In reptiles, there are more partitions within the heart. Reptiles have two atria and two ventricles. Unlike birds and mammals, the ventricles of reptiles are not completely divided, with the exception of the crocodile. Oxygenated and de-oxygenated blood is more separate than in the amphibian condition. The aortic arch arteries remain bilateral but the outflow is now divided into two aortae and one pulmonary artery.

Birds and mammals have a completely divided systemic and pulmonary circulation with two atria, two ventricles and a divided outflow tract. In addition each has lost one of the aortae, so that a single vessel provides oxygenated blood to the systemic circulation (in birds it is the right aorta and in mammals the left). As embryos, these vertebrates also have bilaterally symmetrical arch arteries that are remodeled into the asymmetric great arteries of the heart and a singular outflow that becomes the aorta and the pulmonary artery. The septation of the outflow tract is created by the aorticopulmonary septum, which consists of Cardiac Neural Crest (CNC) cells. (Kirby et al., 1983). CNC cells also aid in the remodeling of the great arteries, creating the variation in aortic arch artery branching observed in birds and mammals. CNC cells play a major role in the morphogenesis of the aortic arch arteries and the outflow tract.

The role of CNC cells in aquatic vertebrates, amphibians and reptiles is less well understood. Because CNC cells are highly involved in processes of aortic arch

remodeling and outflow septation, a reasonable hypothesis would be to suspect that the role of neural crest cells in the heart is limited in fish. Yet previous studies in zebrafish have suggested that neural crest cells contribute to myocardium, a cell fate that is not observed in birds, mammals or any other vertebrate, (Li et al., 2003; Sato and Yost, 2003). In frogs, CNC cells contribute to the aortic arches, like in birds and mammals. Unlike birds and mammals, CNC cells are not found in the spiral valve of the outflow tract (Lee and Saint-Jeannet, 2011). There are no studies on the contribution of CNC cells in reptiles.

In order to begin looking at the role that CNC cells have played throughout the evolution of cardiac development, it is important to understand the interactions of CNC cells within the pharyngeal environment. I address these interactions in the second chapter of my thesis, which is a heavily revised version of a chapter coauthored with M. Kirby and KK Smith and published in the book "Epigenetics: linking genotype and phenotype in development and evolution"(Alonzo et al., 2012). In this chapter the focus is on chick and mouse CNC studies because of the large amounts of data available using these model systems, and because of the variation observed within these vertebrate classes. The body of the chapter focuses on three epigenetic interactions of the CNC cells during development: CNC cells in aortic arch artery remodeling; CNC cells modulating FGF8 signaling in the pharynx; and CNC cells influencing outflow tract septation.

In the third chapter of my thesis, I re-evaluate the role of CNC cells in fish cardiac development. In this chapter I review cardiac and neural crest development in zebrafish to underscore the importance of accurately detecting neural crest cells from cardiac progenitor cells. I report the results of several new experiments. I use a neural crest transgenic zebrafish, to map the fate of neural crest cells with respect to the cardiac and vascular system in zebrafish. My results suggest that neural crest cells do not contribute to myocardium in zebrafish, contradicting previous studies. However, they do contribute to the pericardial wall, cardiac ganglia and cells surrounding the gill vasculature, consistent with neural crest cell fates found in other vertebrates. This chapter is in preparation for submission with co-authors Caroline Burns, Margaret Kirby, Kathleen Smith and Mary Hutson.

Finally, in my fourth chapter I describe the development of the outflow tract in the turtle *Trachemys scripyta*. Although many studies have reviewed general cardiac development in reptiles, none have focused on the septation of the outflow tract. Unlike amphibians, reptiles have a divided outflow tract, which could indicate a role for CNC cells. Using staining techniques that were also applied in chick, I observed the outflow division at different stages of turtle development. The pattern of septation closely follows that of chick outflow septation, specifically the formation of the condensed mesenchyme in the outflow cushions. Although I was not able to label neural crest cells in the turtle, the condensed mesenchyme in the chick outflow cushion is invaded by

CNC cells, which form the aorticopulmonary septum, dividing the outflow tract into the aorta and the pulmonary artery. This descriptive work sets the stage for potential future work on the role of cardiac neural crest in reptile outflow septation.

Finally in Chapter 5 I briefly summarize the results and point towards future directions.

Throughout my thesis, I hope to paint a picture of how neural crest cells have contributed to the cardiac vasculature over the course of cardiac evolution. A review of CNC cell signaling highlights the important pathways that could exert change on the circulatory system during evolution. The data from zebrafish establish the role of the neural crest cells in a branchial circulatory system with no outflow septum. I can then compare neural crest cell contributions to vasculatures that retain a single outflow in adults, like the outflow of the frog. Furthermore, CNC cell actions could be compared between groups of animals that retain a singular outflow as adults and those that have a divided outflow. Data on outflow formation in the turtle will help fill gaps in the knowledge of the role of CNC cells in reptiles. Researchers can then begin to appreciate how CNC cells might mediate vascular changes in the vertebrate transition from water to land.

2. Epigenetic Interactions of Cardiac Neural Crest Cells

2.1 Introduction

Neural crest cells are a migratory cell population that is formed at the border of the neural plate and ectoderm. These cells, found in all vertebrates, migrate away from the neural plate border to ventral structures throughout the body. These cells can be divided into two subsets: cranial and trunk neural crest cells. Cranial neural crest cells migrate from the midbrain and hindbrain in three distinct streams and gives rise to a variety of neural and mesenchymal tissues (Fig. 1)(Le Douarin, 1999). The first cranial neural crest stream arises from the mesencephalon and first and second rhombomeres and populates the fronto-nasal region and first pharyngeal arch. The second cranial neural crest stream arises from the 4th rhombomere and populates the second pharyngeal arch (Le Douarin, 1999; Le Douarin et al., 2007). The third cranial neural crest stream arises from rhombomeres 5-7 and populates pharyngeal arches three, four and six. The cell derivatives from these three streams include cartilage, bone, dentin, mesenchyme, smooth muscle, neurons, glia and melanocytes (Le Dourain 1999). Trunk neural crest arises from the level of the first somite to the last somite and contribute to neurons and glial cells of the peripheral nervous system as well as melanocytes (Erickson, 1985; Le Douarin, 1999). A population of neural crest cells that share both cranial and trunk neural crest properties are called vagal neural crest.

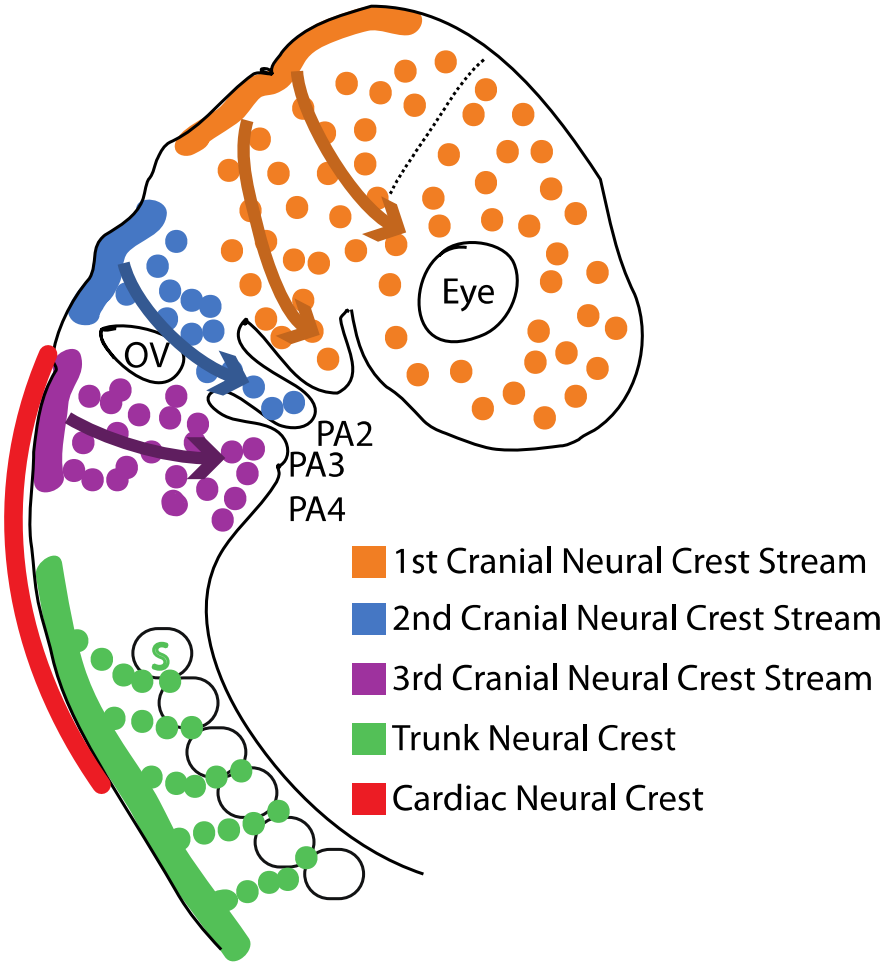

Figure 1 General map of vertebrate neural crest cells. Cardiac neural crest cells contain neural crest from the third cranial neural crest stream and trunk neural crest cells.

Vagal neural crest cells arise in the region between the 1st and 7th somite. In addition to trunk crest cell derivatives, vagal crest cells contribute to the neurons and glial cells of the enteric nervous system. Vagal crest cells also give rise to mesenchyme (Kirby et al., 1983; Kuo and Erickson, 2011; Le Douarin and Jotereau, 1975). Mesenchymal cells originate from the first through fourth somite region of vagal neural crest. These cells contribute mesenchyme to the thymus, thyroid and parathyroid as well as smooth muscle (Bockman and Kirby, 1984; Foster et al., 2008; Le Douarin and Jotereau, 1975).

Cardiac neural crest (CNC) cells are located in a transitional region of the neural crest that encompasses both cranial and vagal neural crest cells. CNC cells arise between the mid-otic placode and the fourth somite boundary of the dorsal neural tube (Jiang et al., 2000; Kirby et al., 1983). From the dorsal neural tube, the cells migrate ventrally to pharyngeal arches three, four and six. CNC cells infiltrate the pharyngeal arches, surround the aortic arch arteries, and differentiate into the smooth muscle within the tunica media layer of the arch artery vasculature. A subset of CNC cells migrates from the pharynx into the cardiac outflow tract. These cells then create the aorticopulmonary septum in avians and mammals and differentiate into cardiac ganglia. Over the years, chick and mouse experiments have demonstrated the importance of CNC cells (Aybar et al., 2002; Bockman and Kirby, 1984; Bockman et al., 1987; Brown et al., 2001; Fukiishi and Morriss-Kay, 1992; Jiang et al., 2002; Jiang et al., 2000; Kirby et al., 1983; Kirby et al., 1997;

Le Douarin, 1975; Lee and Saint-Jeannet, 2011; Li et al., 2003; Lo et al., 1997; Lo et al., 1999; Sato and Yost, 2003; Waldo et al., 1996b; Waldo et al., 1994; Waldo et al., 1998c). In mouse and chick, CNC cells help shape the landscape of the developing aortic arch arteries and the heart.

In this chapter I will discuss the discovery of CNC cells in chick and mouse as well as subsequent studies in other vertebrates. Of particular interest are the interactions that occur between CNC cells and the landscape in which they migrate. I will focus on three CNC cell interactions: aortic arch artery development, secondary heart field addition and outflow septation.

2.2 Cardiac Neural Crest Cell Development in Chick

Kirby and colleagues first described CNC cells in chick e(Nishibatake, 1987 #729)mbryos (Kirby et al., 1983). They ablated pre-migratory neural crest cells to eliminate cardiac innervation. Somewhat surprisingly they found that when neural crest was ablated between the mid-otic placode and somite three, several heart and vascular defects appeared. These defects included incomplete division of the outflow tract, mal-alignment of the aorta and pulmonary artery and mis-patterning of the great arteries of the thorax.

Further work with quail-chick neural crest chimeras clarified the role of this specific neural crest population in cardiovascular development (Kirby et al., 1983; Kuratani and Kirby, 1991; Nishibatake et al., 1987). Similar staged quail cells from the

CNC region were transplanted into chick embryos at stage 9-10 before neural crest migration. A nucleus specific antibody was used to differentiate quail cells from migrating chick cells (Le Douarin and Jotereau, 1975). Quail cells behaved like wild-type chick cells and rescued the CNC ablation defects. Transplanted cells migrated ventrally and paused at the circumpharyngeal ridge, proliferated while in the circumpharyngeal ridge, and re-initiated migration into the pharyngeal arches (Fig. 2) (Kuratani and Kirby, 1991; Waldo et al., 1996a). The quail cells formed a dense sheath of cells around the aortic arch arteries. After quail CNC cells populated the caudal arches, neural crest cells migrated from the pharynx into the arterial pole of the heart (Kuratani and Kirby, 1991). At stage 22 (43-48 somites), CNC cells in the arterial pole populated the cushions of the distal outflow tract (Dodou et al., 2004; Waldo et al., 1999; Waldo et al., 1998c). These cells formed the mesenchymal aorticopulmonary septation complex.

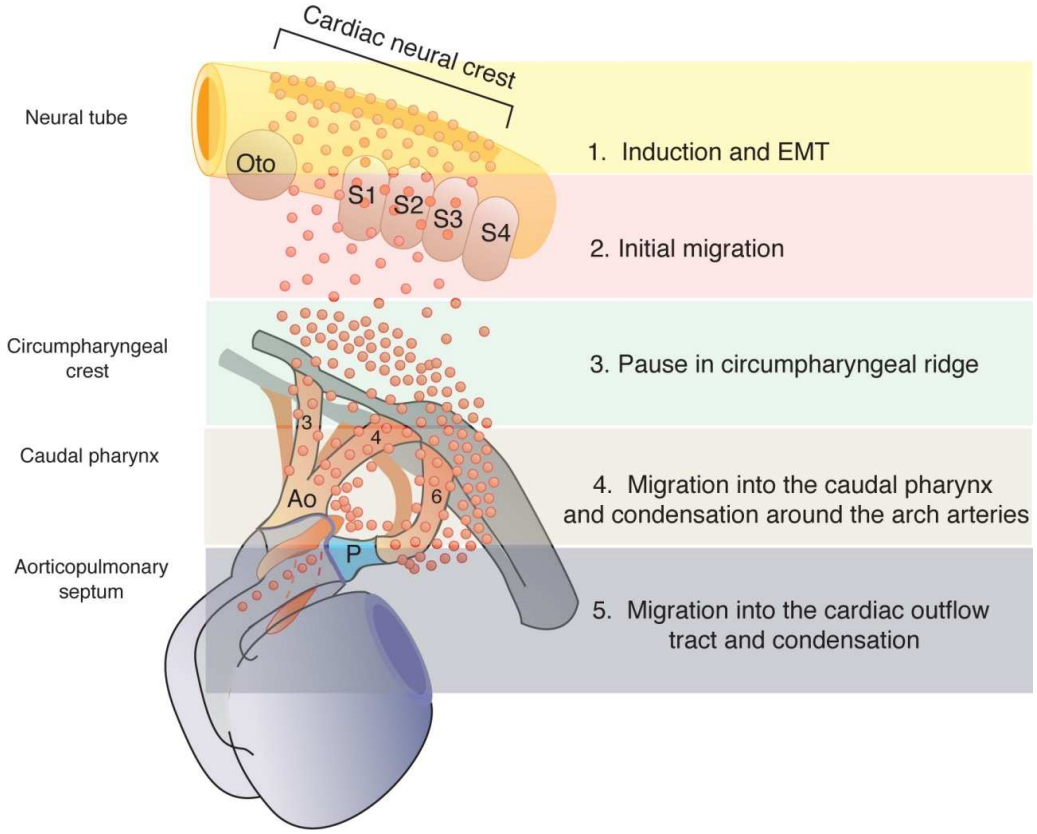

Figure 2 Cardiac Neural Crest Migration. Migration of CNC cells begin between the mid-otic placode and somite three in chick, somite 4 in mouse. As the CNC cells migrate ventrally, they travel through a series of structures: the circumpharyngeal ridge, the caudal pharynx and the cardiac outflow. (Hutson and Kirby, 2007)

The aorticopulmonary septation complex divides the single outflow tract from the ventricles into the aorta and pulmonary artery. During early development the septation resembles a horseshoe, with two prongs extending into the distal cushions of the outflow tract from a mesenchymal shelf (Waldo et al., 1998b). Once in place, the shelf of CNC cells elongates distally into the outflow tract along the length of the prongs.

Because the prongs form by following the spiraling distal outflow cushions, the shelf also spirals as it elongates (Figure 3). Thus the distal outflow septum contains cells derived from CNC cells, as shown by transplanted quail cells. The distal outflow also includes some pharyngeal mesenchyme. This spiraling formation is important for the proper alignment of the aorta and pulmonary vessels with the left and right ventricles, respectively.

CNC cells that migrated through the pharynx also contributed to the parasympathetic cardiac ganglia. The ganglia become the dominant autonomic innervation of the heart. These ganglia are tonically active to slow the intrinsic beat rate in most species (Kirby et al., 1989). In CNC chimeras, quail derived ganglia were most prominent at the base of the outflow tract between the divided outflow tract and the ventricles.

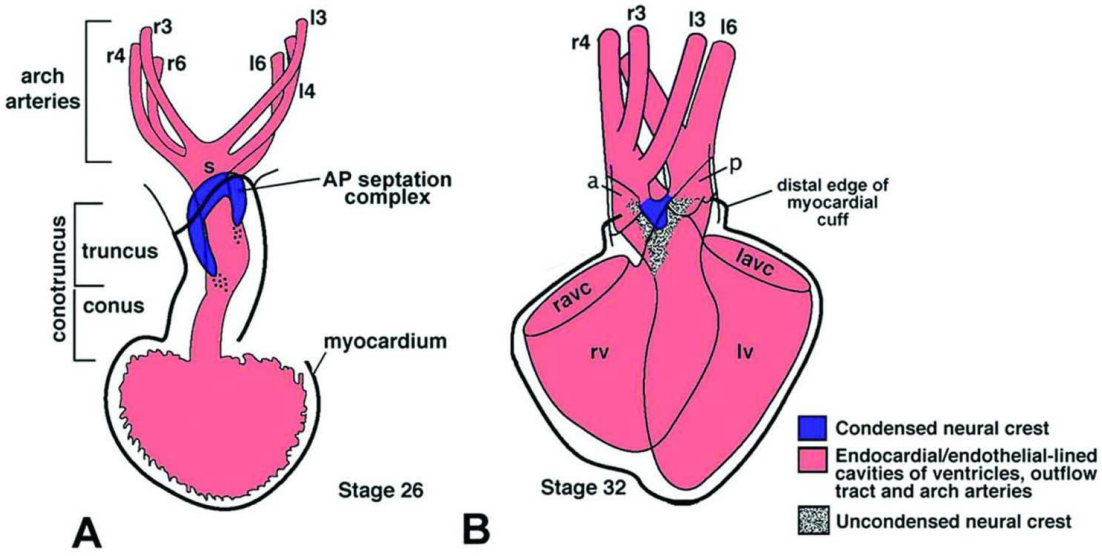

Figure 3 Condensed neural crest cells form the aorticopulmonary septum. (A) The aorticopulmonary septum (blue) takes the shape of a horseshoe, where the shelf is closest to the arch arteries and the prongs have spiraled into the truncus. (B) The shelf elongates into the outflow tract at the expense of the prongs. This divides the outflow into the aorta (a) and pulmonary (p) artery. Illustration reproduced with permission from University of California Press (Alonzo et al., 2012).

2.3 Cardiac Neural Crest Cell Development in Mice

In mice CNC cells share similar derivatives to those found in chick CNC cells. However, in mice CNC cells differ in their origin at the dorsal neural tube and in the timing of migration. As in chicks, CNC cells in mice originate from the dorsal neural tube between the post-otic hindbrain and the fourth somite (Chan et al., 2004b). However, the neural tube adjacent to the second somite was the largest source of CNC cells in mice, unlike quail-chick chimeras that showed the majority of cells migrating from the region of postotic crest and somite one. CNC cells in mice migrated into the pharyngeal arches and outflow septum at E9.5 (20-25 somites) and E10.25-10.5 (32 somite) respectively (Chan et al., 2004a; Jiang et al., 2000). Chick CNC cells migrate later, at 30-36 somites and into the pharynx and outflow tract between 43-48 somites (Kuratani and Kirby, 1992b). Despite these differences in origin, the ultimate trajectory of migration of CNC cells in mouse and chick remains the same. Recently a new mouse study has found neural crest cells surrounding the coronary arteries and contributing smooth muscle. This new neural crest population appears to arise from the second cranial neural crest stream (Arima et al., 2012). This may widen the range of neural crest cells contributing to parts of the heart in mice, but it has yet to be confirmed in chicks and other vertebrates.

2.4 Cardiac Neural Crest Cells in other Vertebrates

CNC cells have not been as extensively studied in other vertebrates. Yet, it appears that much of the initial neural crest migration paths are conserved across species. In all species studied it appears that cranial neural crest cells migrate to facial and neck structures while trunk neural crest migrates to areas below the neck. The three cranial neural crest streams are conserved across all vertebrates that have been studied to date. The third cranial neural crest stream, which includes CNC cells in chick and mice, is also observed in animals as diverse as lamprey (McCauley and Bronner-Fraser, 2003), Australian lungfish (Ericsson et al., 2008), Mexican axolotl (Ericsson et al., 2004), zebrafish (Eisen and Weston, 1993b), Xenopus (Lee and Saint-Jeannet, 2011) and opossum (Vaglia and Smith, 2003). Yet, the study of CNC cell population in these vertebrates has been limited. In *Xenopus*, CNC cells populate the aortic sac and the branchial arches, but they never migrate into the outflow tract (Lee and Saint-Jeannet, 2011). In zebrafish, previous studies suggest that CNC cells give rise to myocardium. The current research available on CNC cells in vertebrates other than chick and mouse does not suggest there is conserved role for neural crest cells among vertebrates. Later in this dissertation, I revisit and challenge the results previously reported in zebrafish (Alonzo-Johnsen Chapter 3) and provide a hypothesis for the conserved role of CNC cells in vertebrates (Alonzo-Johnsen Chapter 5).

2.5 Epigenetic Interactions of Cardiac Neural Crest

In addition to its role in providing cellular material to structures of the heart and pharyngeal arches, recent work demonstrates that CNC cells play an important epigenetic role. In Waddington's definition of epigenetics, there is a causal relationship between genes and their products that brings about a phenotype (Waddington, 1952). A metaphor for this phenomenon would be a person traveling along a path to reach a destination. The many encounters a person has along the road change their fate. Much like a person, the cell encounters gene products along a migration path, changing the cell's fate. With Waddington's definition in mind, three epigenetic interactions of CNC cells can be delineated. First, CNC cells interact with aortic arch arteries. The cells are necessary for normal re-patterning of these bilaterally symmetrical vessels to the asymmetrical great arteries found in mouse and chick. Second, CNC cells modulate signaling factors in the caudal pharynx in chick, such as fibroblast growth factor 8 (FGF8). This role is significant because of the extended period of time over which FGF8-sensitive myocardial progenitors located in the pharynx are added to the outflow tract of the heart. Without the addition of these myocardial cells, significant defects occur in outflow development. Finally, as discussed above, CNC cells are essential to orchestrate septation of the outflow tract as well as to provide the raw material for this structure. Below I will review these epigenetic roles of cardiac neural crest in more detail.

2.5.1 Cardiac Neural Crest cells as an Epigenetic Factor in Aortic Arch Artery Remodeling

Aortic arch arteries in vertebrates develop within the pharyngeal arches and are formed as bilaterally symmetrical vascular conduits that connect the outflow (arterial pole) of the heart to the dorsal aorta. In tetrapods such in chick and mouse, some arch arteries regress and others remodel to form asymmetrical great arteries, in particular the aortic arch and pulmonary artery (Figure 4 B). In fish, the gill arch arteries remain bilaterally symmetrical and undergo remodeling to afferent and efferent arteries (Goodrich, 1930b) (Figure 4 A). In both chick and mouse the CNC cells in arches 3, 4, and 6 form a dense sheath of cells around the endothelium of the developing symmetric aortic arch arteries (Jiang et al., 2000; Kuratani and Kirby, 1991, 1992a). Aortic arch arteries in chicks and mice are capable of developing without CNC cells. However, the aortic arch arteries need CNC cells to instruct their remodeling (Waldo et al., 1996a).

Remodeling aortic arches likely depends on the Hox code expressed by the surrounding neural crest cells (Kirby et al, 1997). Disruption of Hox expression in neural crest causes abnormal patterning of the great arteries even though the neural crest cells populate the arches in a normal fashion. Despite abnormal patterning in the arches due to a Hox code disruption, CNC cells can migrate into the cardiac outflow tract and form a normal outflow septum, suggesting that aortic arch remodeling and outflow septation are two different processes (Kirby et al., 1997).

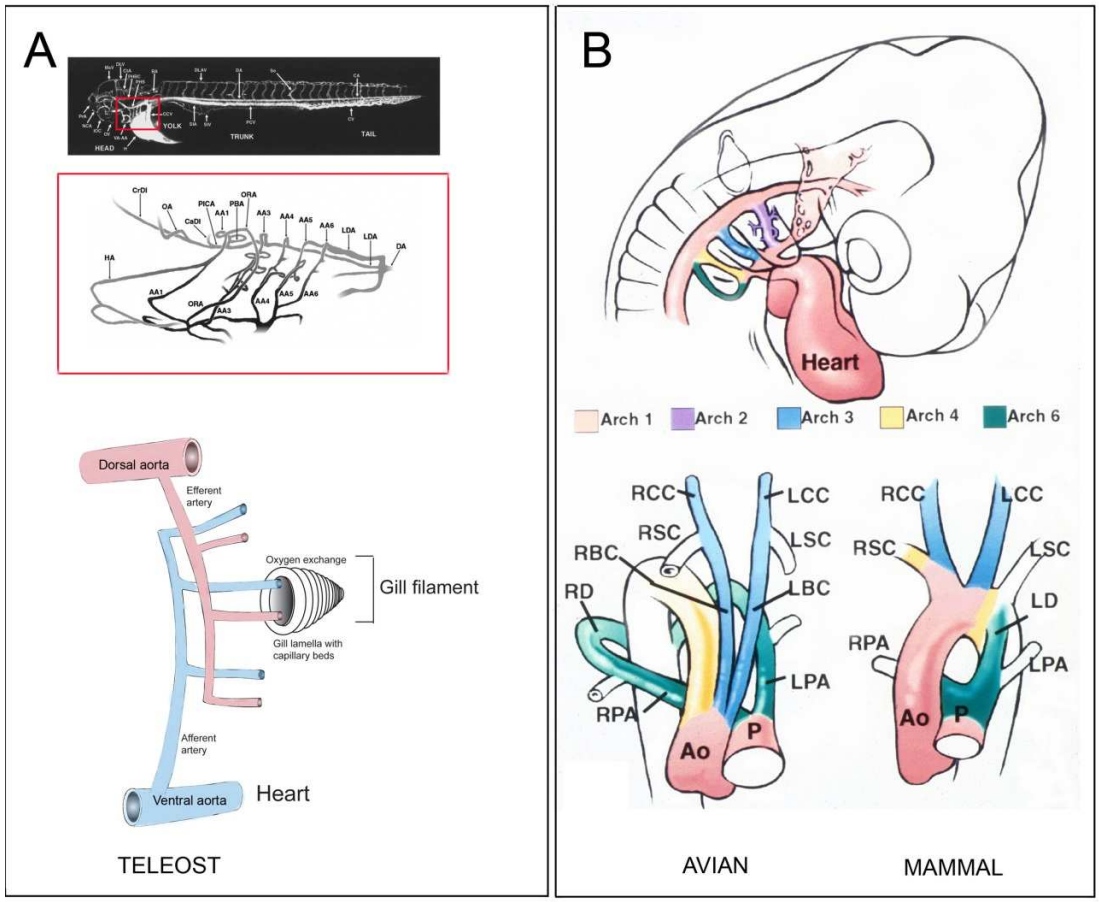

Figure 4 Aortic arch artery remodeling in fish, birds and mammals. (A) Zebrafish gill arch arteries remain bilaterally symmetrical through adult stages. The gill vasculature remodels into the afferent and efferent arteries. (B) Chick and mouse both start with bilaterally symmetrical arches but remodel into asymmetrical aortic arch arteries. Illustration reproduced with permission from University of California Press (Alonzo et al., 2012).

In addition to carrying positioning information from Hox genes, CNC cell also receive signaling guidance from the pharynx. This signaling is via the endothelin pathway, which in addition to its many other roles[1], serves as a guidance cue for CNC cells. Migrating neural crest cells express the endothelin receptor, *endothelin-A (ET_A)* (Betancur et al.). A ligand for this receptor, Endothelin-1 (*ET1*) is expressed in the endothelial cells that form the aortic arch arteries (Kurihara et al., 1999; Yanagisawa et al., 1998a). Mice lacking ET_A have aberrant great artery patterns (Clouthier et al., 1998b; Yanagisawa et al., 1998b). In chimeric embryos, ET_A null neural crest cells are excluded from the pharyngeal arches while WT neural crest migrate to the aortic arch arteries (Clouthier et al., 2003b). This suggests that endothelin signaling is needed for neural crest cells to properly migrate towards the aortic arch arteries. Also observed in these chimeras were ventricular septal defects and right-sided aortic arches; defects also observed in embryos mutant for ET_A (Clouthier et al., 1998a). This could suggest that either certain quantities of neural crest cells are needed for arch artery and heart development or higher levels of endothelin signaling are required to progress to the next stage of arch artery and heart development. According to Waddingtons definition, endothelin signaling provides epigenetic information to CNC cells that is important for migration into the arches and for arch re-patterning.

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[1] Endothelin pathway is involved in tissue differentiation, repair and growth as well as cell proliferation and hormone production.

Downstream of the endothelin pathway is *hand2,* a transcription factor necessary for normal patterning of the great arteries (Thomas et al. 1998). *Hand 2* is a helix-loop-helix transcription factor expressed by pharyngeal neural crest cell derivatives but not migrating neural crest (Srivastava et al., 1995). Expression of *hand2* is observed in the neural crest derived mesenchyme, beneath the most exterior portion of the arches where the tunica media will form (Thomas et al., 1998). Studies have demonstrated that the great arteries fail to pattern correctly in mouse knock-outs of *hand2* (*dHand)* (Srivastava et al., 1997). *ET1* positive endothelial cells are adjacent to the *hand* expressing mesenchyme. In *ET1* null mice, *hand*2 expression is significantly reduced (Thomas et al., 1998). Unlike the ET_A null mice, the neural crest cells in *hand2* null mice continue to migrate to the pharyngeal arches as evidenced by the expression of *msx1* and 2, and homeobox genes expressed in cranial neural crest-derived ectomesenchymal cells (Davidson, 1995). *hand2* may therefore be important for the survival of neural crest cell population once it arrives to the aortic arch arteries. The endothelin and hand2-signaling cascade appears to be a critical in deciding the ultimate patterning of the aortic arch arteries.

The TGF-β superfamily has also been implicated in the remodeling of the aortic arch arteries. CNC cells express Alk5, a TGF-β type I receptor required for the normal re-patterning of the pharyngeal arch arteries to the great arteries (Wang et al., 2006). CNC cells mutant for *alk5* maintain their migration pattern and populate the aortic arch

arteries and aortic sac. However, the aortic arch arteries fail to re-pattern correctly as the great arteries, suggesting that Alk5 could be involved in the downstream remodeling the aortic arch arteries.

Further information on the critical role of CNC cells in patterning the aortic arch arteries arises from experiments that show if CNC cells are ablated, neuronal cells from the nodose placode migrate into all of the unoccupied CNC cell pathways and target sites (Kirby, 1988). Although these neural derivatives of the nodose placode can form a functional cardiac plexus, these cells cannot orchestrate outflow septation, support normal patterning of the great arteries or differentiate into smooth muscle (Kirby et al., 1989). This suggests that CNC cells have specific properties that cannot be replaced by any ectomesenchyme; they are specialized for aortic arch re-patterning and outflow septation. How CNC cells prevent nodose placode cells from migrating remains unknown. A potential mechanism could involve substrate-dependent integrin recycling, which is essential for rapid cranial neural crest migration (Strachan and Condic, 2004). Integrin signaling by neural crest cells could induce downstream pathways that inhibit migration of neighboring nodose-placode cells. Chemotactic signaling used in neuronal path finding such as ephrins and semaphorins could also inhibit neighboring cells from migrating along the path of CNC cells (Palmer and Klein, 2003).

2.5.2 Cardiac Neural Crest Cells Modulate FGF8 Signaling in the Pharynx to Regulate Arterial Pole Development

Previously, it was thought that all of the cells that comprise the myocardium and endocardium of the heart were contained in the initial heart tube (Rosenquist and DeHaan, 1966; Stalsberg and DeHaan, 1969). However, we now know that myocardium and endocardium from the initial heart tube is supplemented over an extended period of time by a cardiac progenitor pool and further cell migration. Myocardial and endocardial cells add to the poles of the heart tube (Dyer and Kirby, 2009). This population of additional cells has been given a variety of names but is currently designated as the second heart field (SHF) (Abu-Issa and Kirby, 2008; Hami et al., 2011; Lazic and Scott, 2011). A substantial number of cells are added to both the arterial (outflow) pole and to the venous (inflow) pole of the heart tube. In this section, I will focus on the influence exerted by CNC cells on the second heart field that adds to the arterial pole.

The SHF is part of the initial heart field progenitors in the pharynx, but is held from differentiation. SHF cells add to the arterial pole after the formation of the initial heart tube and comprises cells of the right ventricle, cells of the proximal and distal outflow myocardium as well as smooth muscle cells at the base of the arterial trunks (Kelly et al., 2001). Cells from the SHF are added sequentially as the anterior portion of the heart tube moves caudally. The first cells that add to the heart are those contributing to the right ventricle and proximal part of the outflow myocardium. These cells are

added from mesoderm located in the position of future pharyngeal arches 1 and 2 prior to the formation of these arches (Kelly et al., 2001). The second set of cells adds to the distal outflow myocardium and proximal smooth muscle of the arterial pole. They are added from the ventral splanchnic mesoderm of the pharynx, underlying pharyngeal arches 3, 4 and 6 (Figure 5.4) (Waldo et al., 2005b; Waldo et al., 2005c; Waldo et al., 2001). This region of splanchnic mesoderm has also been termed the secondary heart field and represents the final subset of SHF cells added to the arterial pole.

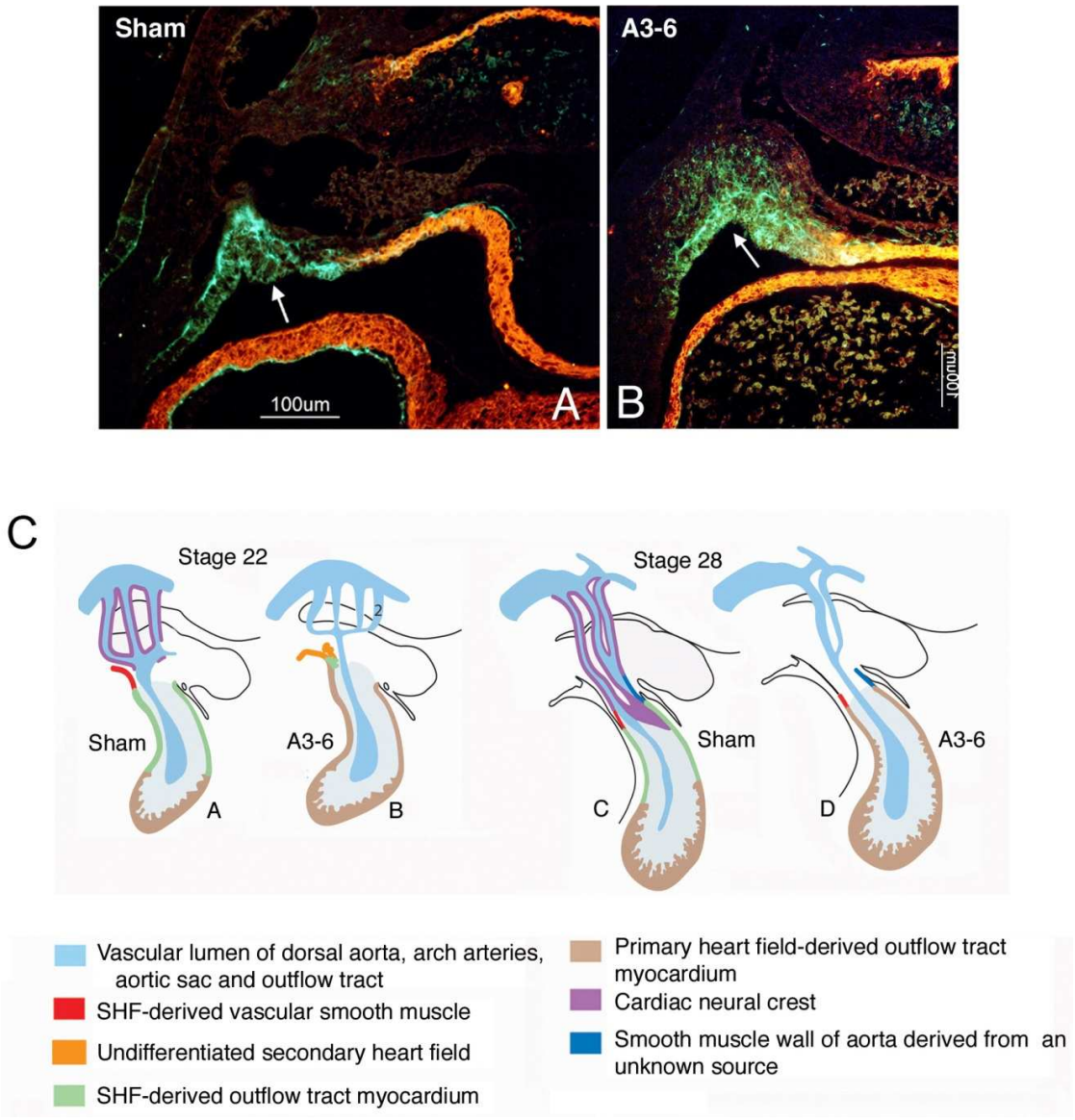

Figure 5 Addition of secondary heart field in Sham and neural crest ablated embryos. (A-B) Secondary heart field myocardium in sham and ablated chick hearts respectively. (B) Myocardium in the ablated embryo is disrupted or reduced. (C) Overview of sham and CNC ablated embryos at stages where secondary heart field is adding to the outflow of the heart. Image reproduced with the permission of University of California Press (Alonzo et al., 2012).

During the addition of secondary heart field cells to the arterial pole of the heart at HH stages 14-18, neural crest cells are migrating from the circumpharyngeal region into the caudal pharyngeal arches. These stages coincide with time cardiac defects are first observed in CNC cell ablated embryos (Leatherbury et al., 1990; Waldo et al., 1996a). When the CNC population is ablated, the secondary heart field fails to add myocardium to the outflow tract (Figure 5) (Waldo et al., 2005c; Yelbuz et al., 2002). Although neural crest cells may not directly interact with the myocardium of the secondary heart field, they are necessary for its normal development.

Because CNC cells are not in direct contact with secondary heart field mesoderm when arterial pole development is abnormal, it is believed that the interaction of the two populations is through an intermediary, currently believed to be *FGF8*. *FGF8* is produced by the pharyngeal endoderm and ectoderm. A variety of studies suggest that there is an elevation of *FGF8* signaling after neural crest ablation (Creazzo et al., 1998; Farrell et al., 2001). For example, CNC ablated chick embryos display depressed myocardial calcium transients, which is rescued by the addition of FGF8b-neutralizing antibody (Hutson et al., 2006). Additional work has shown, that both *FGF8* signaling and downstream target genes are elevated in the caudal pharynx after neural crest ablation (Hutson et al., 2006) (Keyte, unpublished). The increase in downstream targets of *FGF8* coincided temporally with the addition of myocardium by the secondary heart field.

The addition of this cell population was rescued by blocking *FGF8* signaling in neural crest-ablated embryos. Therefore, blocking *FGF8* signaling in neural crest-ablated embryos restored the myocardial calcium transient to normal, as well as restoring proper alignment of the arterial pole (Hutson et al., 2006). These experiments show that CNC cells in the pharynx regulate *FGF8* signaling, directly affecting the formation of the heart. Recent work has shown that neural crest cells may act as a sink to regulate the *FGF8* signal in the pharynx through receptor endocytosis (Yu et al., 2009)(Keyte unpublished).

2.5.3 Cardiac Neural Crest Cells as an Epigenetic Factor in Outflow Tract Septation

A sub-population of CNC cells located in the caudal pharynx enters the heart through the cardiac outflow cushions in chick and mice (Jiang et al., 2000; Phillips et al., 1987). The paired cushions can be divided into two distinct regions. There is a proximal pair that is closer to the ventricle and a distal pair that is closer to the pharynx. The proximal cushions are populated largely by mesenchyme derived from the endocardium via epithelial-mesenchymal transformation (Park et al., 2008). The distal outflow cushions are populated by cardiac neural crest cells migrating from the pharynx (Dodou et al., 2004; Waldo et al., 1999; Waldo et al., 1998c). The CNC cells form a condensed mesenchymal structure called the aorticopulmonary septation complex that consists of a bridge or shelf of tissue that joins two prongs extending into the distal cushions (Waldo et al., 1998b). The bridge of neural crest cells crosses the outflow tract between the

fourth and sixth arch arteries. Once the septation complex is in place, the shelf of neural crest cells elongates distally into the outflow tract along the length of the prongs. Because the prongs follow the spiraling distal outflow cushions, the shelf also spirals as it elongates. This spiraling formation is important for the proper alignment of the aorta and pulmonary vessels with the left and right ventricles, respectively (Figure 3).

The proximal cushions divide the proximal outflow tract in a different manner that does not involve condensed mesenchyme from CNC cells (Waldo et al., 1998b). The proximal cushions undergo an epithelial to mesenchymal transition (EMT) by myocardial cells, which cause the cushions to bulge toward each other. As the cushions bulge, the endocardium underlying each cushion touches and subsequently begins to break down, allowing the mixing of myocardial and mesenchymal cells. Although neural crest cells do not play a physical role in the closure of the proximal cushions, they are present at the seam where the fusion of the cushions occurred. CNC cells migrate past the levels of the semilunar valves and underneath the endocardium. The CNC cells remnants appear at the seams when the endocardium breaks down. The proximal cushions connect the aorta with the left ventricle and the pulmonary with the right ventricle underneath the level of the semilunar valves.

The septation of the outflow tract cannot be accomplished without CNC cells. Meltrin β, a member of the ADAM (A Disintregin And Metalloprotease) family of proteins is expressed in CNC cells, has been shown to be important for complete

septation of the outflow tract (Kurohara et al., 2004). Mice mutant for Meltrinβ appeared to have normal cardiac neural crest migration and normal EMT in endocardial cushions (Komatsu et al., 2007). Although these processes appeared normal, mice had incomplete outflow septation with ventricular-septal defects (VSD) and defective pulmonary and tricuspid valves (Kurohara et al., 2004; Zhou et al., 2004). When meltrinβ was expressed in neural crest cells in a meltrin β mutant background, VSD and to some extent the pulmonary and tricuspid valves were rescued (Komatsu et al., 2007). When meltrinβ was expressed in the endocardial epithelial cells of the meltrinβ mutant background mice, its expression in the endothelial cells did not rescue septation. This suggests that in addition to septation of the distal outflow cushion, neural crest cells can signal to the proximal outflow cushion.

2.6 Discussion

Cardiac Neural Crest cells interact with multiple structures throughout development. In the arch arteries, CNC cells instruct arch artery formation and smooth muscle differentiation. While in the pharynx they also modulate signaling factors for the secondary heart field. Finally, CNC cells migrate into the outflow tract to orchestrate a crucial step in air breathing vertebrates, dividing the systemic and pulmonary circulation. Division of the septum is the hallmark of CNC cells in chick and mice, but along their migratory path they do much more.

CNC cell can carry information to their respective aortic arch arteries by way of hox gene expression. Hox gene information carried by CNC cells can provide a blueprint for arch artery remodeling in chick and mice (Makki and Capecchi, 2012; Waldo et al., 1996b). When hox genes are mis-expressed solely in CNC cells, aberrant aortic arch artery patterning is observed in chicks and mice. This occurs without changing the identity of the pharyngeal arches, which also express a specific set of hox genes. A change in hox gene expression within neural crest cells could lead to changes in vascular identity, and ultimately, from bilateral to asymmetrical aortic arch arteries. However, few studies have separated the role of hox gene expression in CNC cells from hox gene expression in pharyngeal arches that affect vasculature. By changing the hox information carried by CNC cells, the cells in turn may change how they perceive the environment within the pharyngeal arches.

Another signaling pathway important for CNC cell interaction within the pharyngeal arches is the endothelin pathway. Endothelin is conserved within the vertebrates and has two components: the signal expressed by the endothelium in the pharynx and the receptor expressed by neural crest cells. In mice, CNC cells without an endothelin receptor can migrate away from the dorsal neural tube but cannot respond to the ligand within the pharyngeal environment. Differential gene expression within the arches could cause neural crest cells to migrate towards certain arches as compared to others. Upon entering the pharyngeal arches, neural crest cells in the arches of chicks and mice may receive signaling that determines their fate in that specific arch. Although this pathway is common to most vertebrates, slight variations between animals could give rise to a completely new pattern of aortic arches.

A recently discovered role for CNC cells is directing the addition of the secondary heart field into the outflow tract. This outflow tract addition is crucial for lengthening the outflow tract and setting up the spiraling cushions. Without proper cushion alignment, CNC cells appear to enter askew and cannot septate the outflow tract. This is due in part because the outflow tract has not properly elongated and there is not enough cellular material for two cushions to develop. CNC cells may control the addition of secondary heart field cells through FGF signaling. The Hutson lab has suggested that CNC cells control the level of FGF through receptor-mediated endocytosis. CNC cells could create a sink to modulate FGF signal within the pharynx

(Yu et al., 2009)(Keyte 2013). In this way, CNC cells change the environment in which another population of cells travels through. I should note that the secondary heart field is not limited to chicks or mice but also is found in zebrafish and presumably other vertebrates (Hami et al., 2011; Zhou et al., 2011). Although zebrafish do not have a septum, is possible that neural crest disruption could affect the addition of the secondary heart field.

Chick and mouse CNC cells are absolutely required for dividing the aorta and pulmonary artery. But, what of cues that attract neural crest cells to the outflow tract? *MeltrinB* expression in neural crest cells is required for cell migration into the outflow tract. Meltrin has been associated with regulating endocytosis of the Epherin receptors (Yumoto et al., 2008). Controlling epherin signaling in the outflow tract could be a possible epigenetic mechanism for maintaining cushion formation in the outflow tract. Future research could focus on the role of ephrin signaling (and other axonal finding signaling pathways such as netrins, semaphorins and slits) as a possible pathway that drove CNC cells into the outflow tract for septation.

There are many ways that CNC cells change the environment in which they travel. CNC cells can manipulate the structures they invade, such as the pharyngeal arch arteries and outflow tract. In the pharyngeal aortic arch arteries, arches persist or regress according to the signals given or received by CNC cells. In the outflow tract, they orchestrate the rotation of the cushions via the secondary heart field as well as the

septation of the aorta and pulmonary artery. Without these cells, the arterial pole develops incorrectly. These mesenchymal cells have a powerful epigenetic role in pharyngeal and cardiac development. When they move through the pharyngeal landscape, the landscape is forever transformed.

3. Cardiac Neural Crest in Zebrafish

As discussed in the previous chapter, Cardiac Neural Crest (CNC) cells form the septation complex that divides the aorta and pulmonary arteries in chick and mouse. Although CNC cells are a subset of neural crest cells, which are found all vertebrates, studies of CNC cells have been limited to chick, mouse and *Xenopus*. In vertebrates without a separate pulmonary and systemic circulation such as zebrafish, the role of CNC cells is unresolved. Previous studies of CNC in zebrafish have yielded unexpected results. These studies claimed that neural crest cells contributed to myocardium, a cell fate not found in chick, mouse or *Xenopus*. In this study I use a variety of methods to trace the fate of CNC in zebrafish and re-evaluate previous studies of CNC in zebrafish. First I will briefly review neural crest and heart development in zebrafish.

3.1 Neural crest cells in Zebrafish

In zebrafish, as in all vertebrates, cranial and trunk neural crest cells contribute to a wide range of structures. Cranial neural crest cells contribute to cartilage, bone, connective tissue, cranial ganglia, glial and pigment cells in the head (Eisen and Weston, 1993a). Cranial neural crest cells types originate in the midbrain and hindbrain and migrate in three distinct streams (Figure 1). The first steam migrates from the midbrain and 1st and 2nd rhombomeres to the face and jaws. The second stream migrates from the 4th rhombomere level to the second arch or hyoid region. The third stream migrates from the 6th rhombomere level to pharyngeal arches 3-7 (Schilling, 1997). Neural crest

cells caudal to the arches are designated trunk neural crest cells (Schilling, 1997). These cells contribute to neurons of the peripheral nervous system, Schwan cells and pigment cells (Raible et al., 1992a).

In zebrafish neural crest cells begin migration at 13 hours post fertilization (hpf) and progress in a cranial to caudal fashion. Cranial neural crest cells reach their targets by 24hpf, while trunk neural crest cells continue to migrate until 48hpf (Lamers et al., 1981; Raible et al., 1992a). Neural crest cells move away from the neural keel which later hollows out to form the neural tube. Neural crest cells in zebrafish are larger and fewer in number than has been reported in chick and mouse. In chick, there are three to five times more neural crest cells than there are in zebrafish (Raible et al., 1992a). Zebrafish neural crest migration occurs slightly earlier in development at 8 somite stage (ss); in the chick migration begins at HH10 or 9-11 somite stage (ss) (Khudyakov and Bronner-Fraser, 2009).

3.2 Cardiac Progenitors in Zebrafish

The heart in zebrafish, as in other vertebrates, develops from lateral plate mesoderm. These cells are called cardiac progenitor (CP) cells and in zebrafish, they can be identified at the shield stage just prior to gastrulation (Keegan et al., 2004). As the embryo undergoes gastrulation, CP cells are detected in the caudal portion of the anterior lateral plate mesoderm (ALPM) on either side of the midline. The bilateral CP cell population lies below the hindbrain. At 12 hpf, equivalent to 6ss, it is found beneath

rhombomere 2 and extends back to the tip of the notochord (Schoenebeck et al., 2007). At 14 ss, CP cells begin to differentiate, and express cardiac myosin light-chain 2 (Huang et al., 2003; Yelon et al., 1999). At 16 ss, CP cells start to move medially under the hindbrain. At 18ss the posterior CP cells join together forming "v" shape and by 20 ss, the anterior cells join together so that the CP cells as a whole form a cone. Within the cone, the inner most cells will give rise to the ventricle and the outer most cells will give rise to the atria (Figure 5). After the 20 ss, the cone begins to involute on the posterior right side (IV quadrant). The cells that involute become the ventral floor of the heart tube as they move to meet the atrial cells on the anterior left side (III quadrant) (Rohr et al., 2008). At 30 ss, the cone has telescoped out to the left and formed a tube (24 hpf) (Rohr et al., 2008; Smith et al., 2008). From 36-72 hpf, the heart tube starts to bend at the atrio-ventricular junction and move towards the midline with the ventricle on the right and the atria on the left (Yelon, 2001; Yelon and Stainier, 1999). Cells continue to add to the atria and ventricle during this time period via the secondary heart field, which is a continuation of the ALPM (Hami et al., 2011; Lazic and Scott, 2011).

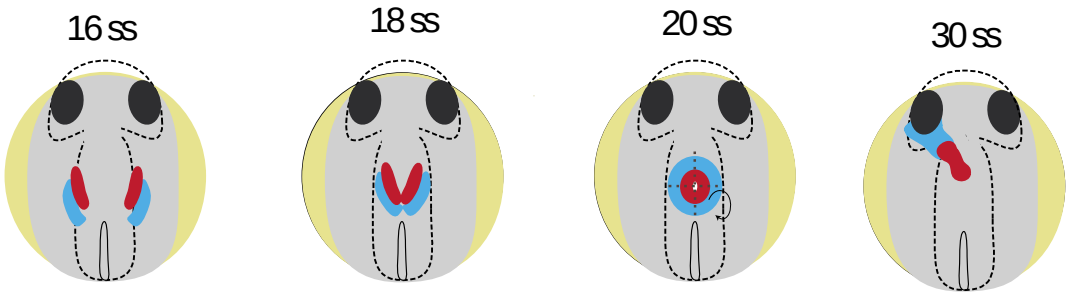

Figure 6 Cardiac progenitor migration in zebrafish between 16-30ss. CP cells start out as two separate sections of mesoderm located in the ALPM. The medial most cells in red contribute to the ventricle and arterial pole. The lateral most cells in blue contribute to the atria and venous pole. CP cells move towards the midline and form a cone at 20 ss. Cells from quadrant IV involute and migrate under the cells in quadrant II. The CP cells form the heart tube that telescopes to left side of the zebrafish embryo.

3.3 Spatial and temporal relationship between cardiac progenitors and neural crest cells in zebrafish

In zebrafish, neural crest cells and mesodermal cardiac progenitors develop and migrate at similar times and are in close proximity to each other. When neural crest cells start migration at the 8ss, CP cells are already located in a bilateral formation at the axial level of the hindbrain. During hindbrain formation at 8ss-20ss, the bilateral CP cells are moving closer to the midline immediately below the hindbrain. When the heart begins to telescope to the left after 20ss, it moves anteriorly underneath the head and remains close to the cranial neural crest streams. After 24hpf, the heart moves more ventrally as the neural crest form the structures that will develop into the branchial cartilages. At this

time all three germ layers are thinly spread over the embryonic yolk (Kimmel et al., 1995). The only cells that migrate into the zebrafish heart after 24hpf are the cells of the secondary heart field(Hami et al., 2011; Nevis et al., 2013).

The close spatial and temporal migration of the two populations in zebrafish is in contrast to the condition in chick and mouse (Abu-Issa and Kirby, 2007; Khudyakov and Bronner-Fraser, 2009; Kirby and Waldo, 1995). In chick, cardiac progenitors are located in the ALPM at stage HH7 (Hamburger and Hamilton, 1992). Between chick stages HH9-HH10, CP cells have met at the midline and formed the linear heart tube. It is only after the chick has formed a heart tube at HH10 that neural crest cells initiate migration (Khudyakov and Bronner-Fraser, 2009). Neural crest cells in chick are found in the pharyngeal arches[2] between HH13-HH16 and in the outflow tract at HH23. At HH23 in chick the heart is not completely divided, but much of the atria and ventricles are defined (Kirby and Waldo, 1995). In chick, the folding of the head pushes the heart tube ventrally away from the neural crest cells before they migrate. The CNC cells in chick travel through many structures before they can migrate into the outflow tract (Kuratani and Kirby, 1992b). Thus, unlike chick, CP cells and neural crest develop at the same time in zebrafish.

[2] The term pharyngeal arch refers to arches that bear aortic arch arteries. The term branchial arch refers to arches that bear gills.

Previous studies of zebrafish concluded that CNC cells contribute to the myocardium of the heart (Li et al., 2003; Sato and Yost, 2003). These results were surprising because despite extensive studies, CNC cells have never been shown to differentiate into myocardium in chick, mouse or any other vertebrate. However, the studies of Sato and Li did not include methods that identified only neural crest cells before labeling. Because these two cell populations develop in such close proximity, it is critical to independently identify neural crest cells from the surrounding mesenchyme. In this chapter, I report on studies that use new techniques that allow me to accurately identify neural crest cells and ensure that only neural crest cells are labeled. By correctly identifying neural crest cells before labeling, I created a detailed and accurate map of CNC cells in zebrafish. After tracing neural crest cells through 72hpf, I argue that CNC cells in zebrafish do not contribute to myocardium.

3.4 Methods

3.4.1 Zebrafish Lines

We used several zebrafish transgenic lines to identify neural crest cells and cardiac progenitors. The *Tg (sox10:egfp)* zebrafish line marked pre-migratory and migrating neural crest cells. Green fluorescent protein (EGFP) was under the control of the Sry-like 10 (*sox10*) promoter, which is expressed in pre-migratory and migrating neural crest cells during early stages of zebrafish development (Carney et al., 2006). To identify cardiac progenitor cells, I used the *Tg (cmlc2:egfp)* zebrafish line that expresses EGFP under the cardiac myosin light chain-2 promoter. Finally, I used the *Tg* (*sox10:cre)* transgenic line to permanently label cells that at some time in development have expressed *sox10.* It was crossed to *Tg* (*EF1alpha:loxp-egfp-pA-loxp-dsred-pA*) fish, which initially express EGFP under the Elongation Factor 1 promoter, and converts it to dsRed once sox10 is expressed (Kelsh , 2013).

3.4.2 Single Cell Labeling

In order to label single zebrafish neural crest cells, I injected *Tg (sox10:egfp)* transgenic embryos at the single cell stage with a cocktail of caged rhodamine-dextran (MW 10,000) and caged fluorescein-dextran (MW 10,000)(Molecular Probes) dissolved in 0.2M KCl. Injecting at the one cell stage ensured that the cocktail was distributed evenly among all the cells in the embryo. After embryos had been injected with the rhodamine-

fluorescein cocktail, they were incubated at 28.5 °C until they reached the 8 ss (10.9 hours). Embryos were screened for faint red and green fluorescence to see if the dextran dyes were incorporated throughout the embryo. Embryos were then mounted on 10-well glass depression slides and oriented in profile, with the right side of the embryo facing up. A solution of 2% low-melt agarose, made with embryo medium, held the embryos in place. Embryo medium was added on top of the embryos in agarose to keep them from drying out and provided a liquid interface for the 40X microscope water immersion objective lens. An Olympus microscope was used to visualize and uncage the GFP-positive neural crest cells. Single GFP positive cells were targeted with a 368nm nitrogen laser under the 40X water-immersion objective. After single cells were uncaged, the embryos were removed from the agarose and placed in fresh embryo medium. The embryos were kept in the dark using aluminum foil and placed at 28.6 °C. At 22 hpf, 0.003% 1-Phenyl-2-Thiourea (PTU) was added to the embryo medium to prevent pigment formation. Uncaged embryos were fixed at 36, 48 and 72hpf.

3.4.3 Immunohistochemistry

Once the uncaged embryos were fixed, the uncaged dyes were identified by immunohistochemistry. Fixed embryos were dehydrated in a graded series of methanol diluted with Phosphate Buffered Saline and 0.1% Tween (PBST) for 5 minutes each. They were left in 100% methanol overnight at -20 °C. Embryos were rehydrated in a graded series of PBST for 5 minutes and washed in PBST twice for 5 minutes. They were

digested with 10 ug/ml proteinase-K in Phosphate Buffered Saline (PBS) for 15, 25 and 35 minutes for 36, 48 and 72 hpf. Embryos were rinsed twice with PBST and post-fixed with 4% paraformaldehyde for 20 minutes. After three 5- minute washes with PBST, embryos were heat-inactivated in a 65°C oven for 10 minutes. Embryos were placed in a blocking solution of 5% sheep serum, 2 mg/ml Bovine Serum Albumin (BSA) in PBST for 2 hours. They were incubated in blocking solution and 1:5000 AP-conjugated fluorescein antibody (Roche) at 4°C overnight. After washing six times for 5 minutes in PBST, embryos were incubated in alkaline phosphatase buffer (0.1 ml 5M NaCl. 0.5 ml 1M Tris, pH9.5, 0.05 ml 10% Tween-20 and 4.35 ml of distilled water) and NBT/BCIP (2%) to detect the uncaged cells. Embryos were post-fixed for 20 minutes in 4% paraformaldehyde and stored in PBS.

3.4.4 In-situ Hybridization and Immunohistochemistry

In order to determine whether neural crest cells were found in the heart at 24hpf, a sensitive antibody assay was used to detect EGFP in the myosin positive heart. Embyros were fixed in 4% PFA for 1 hour at room temperature. Then they were washed with PBST 5 times for 5 minutes, de-hydrated in a methanol-PBST series, washed in 100% methanol twice for 5 minutes and stored at -20 °C for 24 hours. Next, embryos were washed in methanol and 3% hydrogen peroxide for 15 minutes, then re-hydrated in the methanol/PBST series and washed in PBST 3 times for 5 minutes. Embryos at 24 and 36 hpf were then treated with proteinase-K (10 ug/ml) in PBS for 10 and 20 minutes,

respectively. They were then washed in PBST quickly three times and post-fixed with 4% paraformaldehyde for 30 minutes. After washing again in PBST, the embryos were placed in blocking solution containing BSA (2 mg/ml), New Goat Serum (10%), Triton-X (0.1%) and 1X PBS. Afterwards, embryos were blocked for an hour and then placed in the blocking solution containing primary antibodies for anti-GFP (ab-cam 6662) at a concentration of 1:1000 and MF20 (Developmental Studies Hybridoma Bank) at a concentration of 1:10. Embryos were left in primary antibody solution overnight at 4°C. Embryos were washed six times for 10 minutes with PBST and placed in blocking solution for an hour. They were incubated in blocking solution with secondary goat anti-rabbit-HRP (Roche) for GFP and goat anti-mouse alexa 488 (Invitrogen) at a concentration of 1:200 for two hours at room temperature. Embryos were washed with PBST six times for 10 minutes before being placed in a 1:1000 solution of tetramethylrhodamine-tyramide and amplification diluent solution from Perkin-Elmer (NEL742001KT). The embryos were incubated in solution for 15 minutes and then washed with PBST five times for 1 minute. Reaction was stopped with 6% hydrogen peroxide in distilled water then placed in PBS before processing. The half-life of GFP is ~24 hours (Corish and Tyler-Smith, 1999).

To order to visualize the neural crest and cardiac cells earlier in development, I performed an *in situ* hybridization and antibody detection assay within the same *Tg(sox10:egfp)* embryo. The *in situ* hybridization was performed with cmlc2 probe at 14,

16, 18 and 20ss according the protocol used by Hami et. al. (2011). After the *in situ* hybridization, the embryos were prepared for the GFP antibody as previously described.

Finally, in order to detect dsRed in *Tg*(*sox10:cre)*embryos crossed to *Tg*(*EF1alpha:loxp-egfp-pA-loxp-dsred-pA*), I anesthetized embryos in 0.016% tricaine containing 0.1 M potassium chloride to relax the heart chambers in diastole. Embryos were fixed in 2% paraformaldehyde containing 5% sucrose for 1 hour at room temperature. They were dehydrated in a methanol series and stored at -20°C for 24 hours. Embryos were re-hydrated and treated with proteinase-K (10 ug/ml) for 35, 45, and 55 minutes respectively. After washing twice with PBST, embryos were washed in PBST and 0.1% DMSO for 15 minutes. Embryos were placed in blocking solution consisting of PBS with sheep serum (10%), BSA (2 mf/ml), saponin (0.2%) and DMSO (0.1%) for 1 hour and incubated with MF20 (1:10) and 1:1000 Living Colors anti-dsRed polyclonal antibody (Clonetech Laboratories) overnight at 4°C. After rinsing four times for 15 minutes in PBST 0.1% DMSO, embryos were incubated for 2 hours in the dark with 1:200 Alexafluor 668 for dsRed (Invitrogen).

3.4.5 Paraffin Sections

To determine the exact location of labeled neural crest cells within the embryos, they had to be sectioned. Labeled embryos were washed in a series of increasing ethanol/PBS culminating in two 5-minute 100% ethanol washes. The embryos were placed in Histo-clear solution (National Diagnostic) for a 5- and 10-minute wash.

Embryos were transferred to molten paraffin wax, incubated at 56°C in a vacuum oven for two changes of 15 and 25 minutes respectively, and embedded in paraffin. Paraffin blocks were sectioned at 10 microns. Sections were then mounted on the slides and allowed to dry before they were placed on a warming tray overnight. Slides were then placed in a 56°C oven for an hour to melt the paraffin. They were washed in 100% xylene twice for 3 minutes and taken to water by dipping in an ethanol series (100%, 90%, 70%). After being washed in distilled water for 10 minutes, they were cover-slipped with Fluorsave Reagent (Calbiochem 345789).

3.4.6 Acetylcholinesterase Stain

Cardiac ganglia are derived from cardiac neural crest cells. Cardiac ganglia also produce acetylcholinesterase, and can be detected through acetylcholinesterase stain. In order to detect acetylcholinesterase in chick and zebrafish hearts, embryonic chick and zebrafish adult hearts were perfused with 1X PBS and cold 10% formalin. Hearts were removed and fixed in 10% formalin O/N at 4°C. Hearts were washed in 1X PBS 5 times, for 10 minutes, followed by the Karnovsky-Roots protocol for acetylcholinesterase stain. In this protocol acetylcholinesterase reaction medium was made by dissolving 5 mg acetylthiocholine iodide into 6.5 ml of 0.1M sodium acetate/sodium phosphate at pH 6.0. Then the following chemicals were added to the solution: 0.5 ml of 0.1M sodium citrate, 1 ml of 30mM $CuSO_4$, 1 ml of dH_2O and 1 ml of 5mM potassium ferricyanide. Hearts were transferred from the PBS solution and immersed in solution and placed on a rocker

for 5 minutes and checked continuously until stain developed. Finally, the hearts were washed and stored in PBS.

3.5 Results

3.5.1 Neural crest cells in relation to cardiac progenitors

Tg(sox10:egfp) transgenic zebrafish were used to study pre-migratory and migrating neural crest cells directly. Although *sox10* is expressed in other cells at later times (most notably early cartilage), pre-migratory and migrating neural crest are the only cells that express this gene early on. Therefore, this transgenic line allowed me to examine the migration of neural crest cells and from the 8ss to 24 hpf. After 24hpf, *sox10* expression is down regulated in neural crest cell derivatives except cartilage, dorsal root ganglia and Schwann cells (Carney et al., 2006). At these time points, I could not detect any neural crest cells in the heart in whole mount preparations.

In order to determine the location of neural crest cells relative to CP cells earlier in development, I staged matched *Tg (sox10:egfp* embryos and *Tg(cmlc2:efgp)* embryos in which cardiac progenitor cells are labeled (Figure 7). This study revealed that CP cells migrated towards the midline underneath and in close proximity to the second and third cranial neural crest streams (Fig. 7 D,H). CP cells begin expressing cardiac genes at the 14-16ss, but the GFP signal from the neural crest cells obscured the view of the CP in whole-mount. Transverse sections of doubly labeled embryos for neural crest and CP cells allowed me to closely trace the spatial proximity of these two populations. Labeled CP cells were 3-5 cells away from migrating neural crest in *Tg(sox10:egfp)* embryos (Figure 7 I'). Again, no neural crest cells could be seen within the CP population

between the 14-16ss. Between the 18 and 20ss, CP cells approach the midline and form a cone, while cranial neural crest cells became more distinct. However, at this time, no neural crest cells were seen in the CP population.

Taking advantage of the long half-life of GFP, I examined the hearts in *Tg(sox10:egfp)* transgenic fish after heart tube formation to see if there were any Sox10-expressing cells in the heart. Using a sensitive immunofluorescence amplification assay that detects low levels of GFP, I looked through serial sections of doubly labeled embryos at 24 and 36 hpf (Figure 8). Sox10 positive GFP cells did not co-localized with myosin, a myocardial marker. The embryos had no *sox10-egfp* positive cells in the heart (Figure 7). These studies demonstrate that neural crest cells remained a distinct population from cardiac cells through 36 hpf.

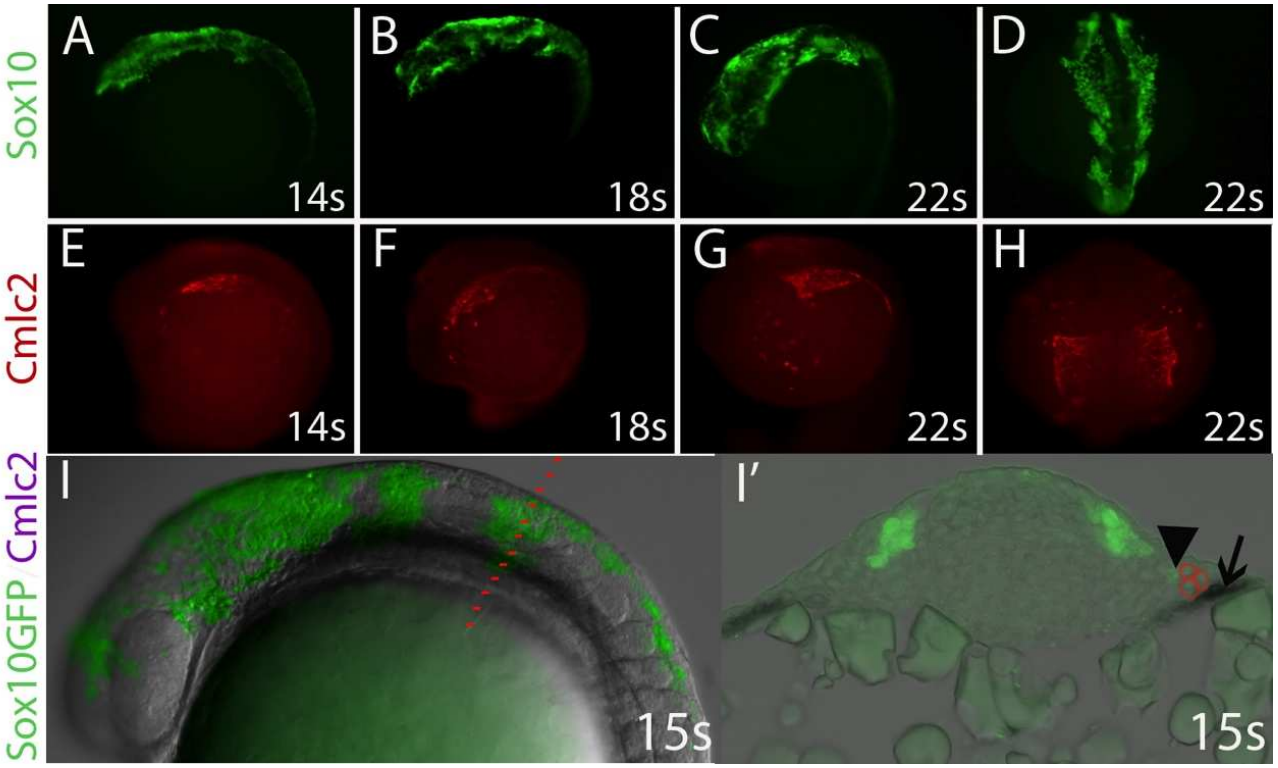

Figure 7 Underneath the migrating neural crest the CP cells are moving towards the midline. (I) At 15ss, a section was taken through the embryo at the level of the third cranial neural crest stream just posterior to the otic placode. (I') Neural crest cells expressing Sox10 (arrow head) are in close proximity to cardiac cells expressing cmlc2 (arrow). (I') Three cells are circled in red and represent the distance between the cardiac progenitors and neural crest cells.

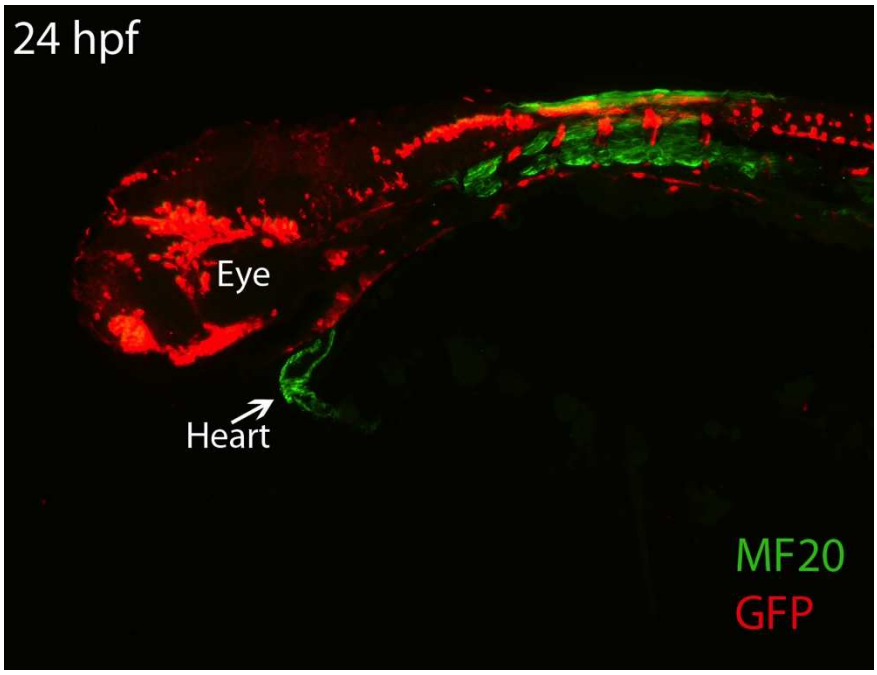

Figure 8 A Sox10-egfp embryo was immunostained for GFP in red. The heart was immunostained with MF20 (myosin) in green. Neural crest cells were not found in the heart tube at 24hpf.

3.5.2 Mapping neural crest cells by labeling single cells

In order to follow neural crest cells beyond 36hpf, I employed an uncaging technique to track cells as they migrated away from the dorsal neural tube. This technique allowed me to follow neural crest cells after the down-regulation of *sox10* expression in migrating neural crest cells. It also allowed me to track the origination of individual neural crest cells. Using the *sox10:egfp* transgenic line, I injected uncaged rhodamine/fluorescein into single cell embryos. These embryos were divided into four groups and in each group I examined a different population of cells. The groups were:

cells from cranial neural crest stream one, two or three and neural crest cells at somite level 1-3. Once the embryos in each group reached the 8ss, I uncaged a single cell at the leading edge of the migrating stream (Figure 8). I collected embryos in each group at 36, 48 and 72hpf.

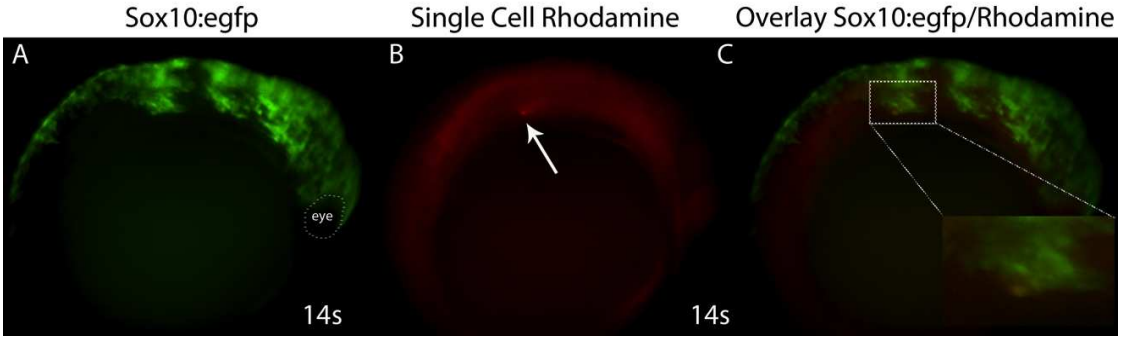

Figure 9 Single cells in *Tg(Sox10:egfp)* embryos (A) were uncaged with a laser. The uncaged cell is brighter than the light red haze produced by the rhodamine dye (B, arrow). (C) An overlay shows that a single Sox10 positive cell was uncaged in the caudal portion of the third cranial neural crest stream.

In previous zebrafish CNC cell studies, cells from the first cranial neural crest stream were found to contribute to cardiac cells (Sato and Yost, 2003). When I labeled single cells in the first cranial neural crest stream, I observed cells in the lower jaw and frontal processes at 24hpf. At 36 and 72hpf, I saw contributions to the same region frontal region of the zebrafish embryo. I did not find any neural crest cells from the first cranial neural crest stream contributing to the heart (Table 1).

Previous studies also reported that neural crest cells from the second cranial neural crest streams contributed to cardiac cells (Li et al., 2003; Sato and Yost, 2003). Therefore I also labeled single cells in the second cranial neural crest streams and

observed their derivatives at 36, 48 and 72hpf. Labeled cells from this stream were found caudal to the eye, in the region where the hyoid develops. This is consistent with previous findings (Raible et al., 1992b). There were also labeled neural crest cells that remained at the level of the hindbrain (Figure 10 A, D, G). At 48hpf, some embryos appeared to have labeled cells very close to the pericardial sac, but none were found in the heart as confirmed by sectioning (0/76). In some of the 72hpf whole- mount embryos I noticed cells in the heart and pericardial region (Figure 10 G). To determine whether they were in the heart or part of the pericardial sac, these embryos were sectioned (12/70). Upon sectioning, cells were identified in the pericardial wall but not in the heart (Table 1) (Figure 11).

Neural crest cells from the third cranial stream populate the caudal pharyngeal arches. In chick and mice, CNC cells are a subpopulation of cells from the third cranial stream. Therefore I also labeled cells in this cranial stream, hypothesizing that it would contain CNC cells. When I labeled single cells in the third cranial stream, the cells migrated caudally around the otocyst and into caudal branchial arches as predicted. Cells were identified around the heart in a subset of whole-mount embryos at 72hpf. To determine if these cells were in the heart these embryos were sectioned (n=10) (Figure 11). Upon sectioning, I found no cells in the myocardial layer of the heart through I did see cells in the pericardial wall (Table 1). At later stages pigment cells develop in the

pericardial wall, which are a neural crest derivative, confirming that in our tracing studies we labeled neural crest cells, which developed into pigment cells.

The fourth group of neural crest cells labeled came from the first through the third somites. At the 8ss, cells at the level of the somites have not completely begun migration but were still visible. I labeled neural crest cells within somite region 1-3 and fixed embryos at 36, 48 and 72hpf. Labeled cells did not migrate to the heart at any of these stages. The labeled cells remained in the trunk, going between the somites (Figure 10).

Table 1 Labeled neural crest cells that contributed to the pericardium. Embryos at 72hpf appeared to have cells in the heart in wholemout. Upon sectioning no labeled cells were present in the heart (0/5 and 0/10) in 2nd and 3rd Cranial neural crest streams respectively.

	1st Cranial stream	2nd Cranial stream	3rd Cranial stream	Somite 1-3
36 hpf	0/25	0/25	0/30	0/30
48 hpf	0/33	0/40	0/102	0/25
72 hpf	0/45	5/76	10/90	0/50

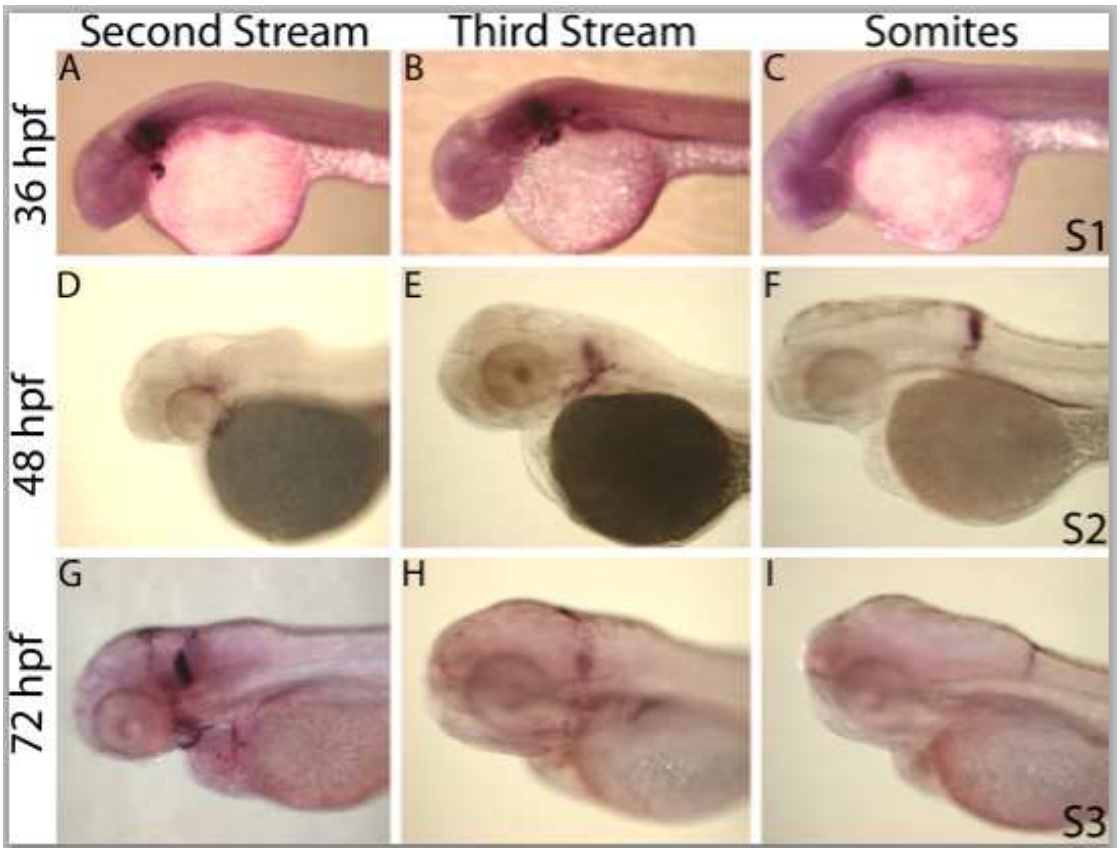

Figure 10 Un-caged cells in the second and third cranial stream at 36, 48 and 72hpf. None of the neural crest cells uncaged in this experiment were found in the heart at 36, 48 or 72hpf. Cells uncaged at the level of the somites did not migrate cranially at 36, 48 or 72hpf to contribute to the heart.

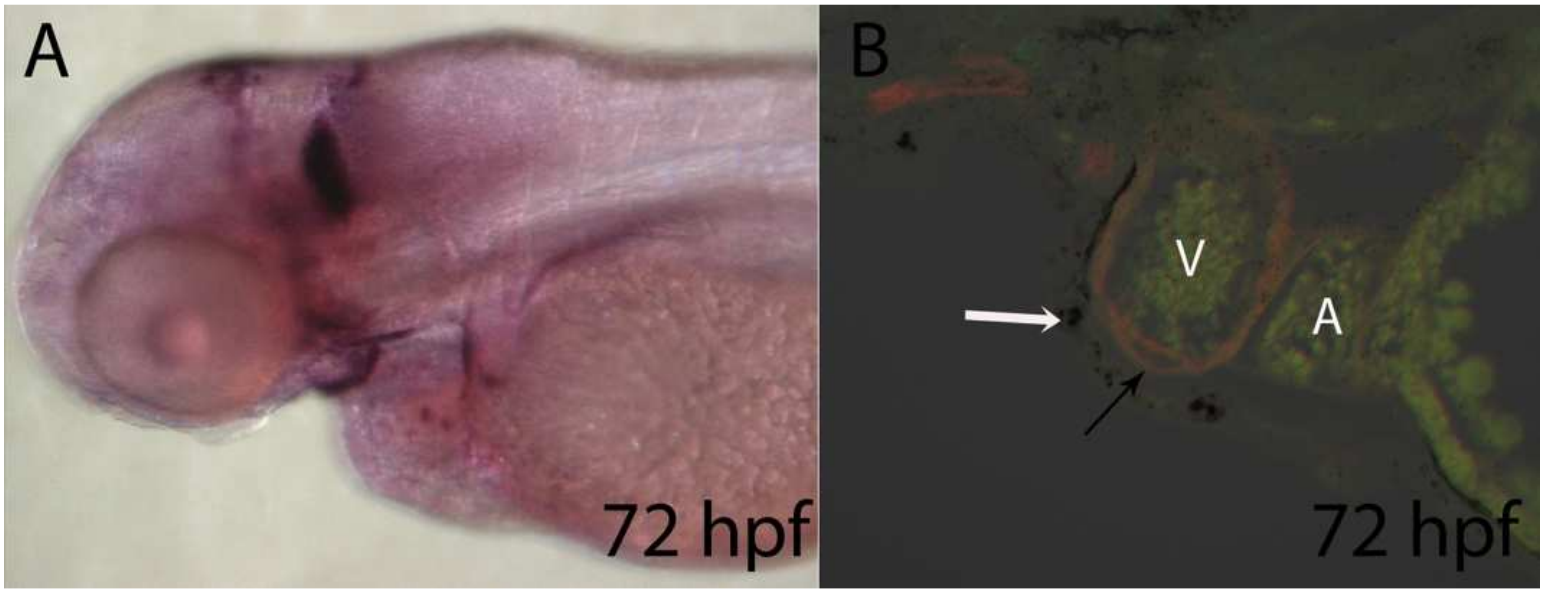

Figure 11 (A) Second cranial neural crest stream single cells at 72hpf. (B) Sections of the embryo reveal that the labeled cells are in the pericardium (white arrow) not in the myocardial layer (black arrow). A: atria, V: ventricle

3.5.3 Multiple Cell Uncaging

I uncaged a larger group of cells in order to ensure that my single cell labeling study did not miss cells migrating into the heart. This larger population included sox10 gfp-positive cells and the cells adjacent, which were non-gfp positive cells. The cells that were not gfp positive were not neural crest cells. I focused on the second and third cranial neural crest streams because cells from these streams appeared close to the heart at 72hpf in the single cell uncaging experiments. In these experiments at 72hpf, labeled cells from the second stream were found in the arches as well as the heart. Sectioned embryos revealed that some uncaged cells were located within the myocardium. At 72hpf, labeled cells from the third cranial stream were also found in the heart. The cells that migrated into the myocardium appeared at the arterial pole, similar to the previous secondary heart field studies done in the lab (Hami et al., 2011). I repeated this study specifically focusing on gfp-positive cells. When I uncaged 2-3 individual gfp cells in the injected embryos, I did not find cells in the heart and my results were similar to those in my single cell uncaging study. This study shows how important it is to identify neural crest cells, for neural crest cells are in close proximity of other cell populations. Without an independent neural crest marker, cells from the mesodermal layer could be labeled.

3.5.4 Genetic lineage tracing: Sox10-Cre zebrafish

The uncaging strategy I employed could not be carried out past 72hpf so I used a transgenic zebrafish that allowed for longer genetic lineage tracing. The *Tg:(sox10-cre)*

transgenic permanently labels all cells that have produced sox10 protein with dsRed. It thus labels cells derived from the neural crest, as well as otic epithelium. *Tg:(sox10-cre)* embryos at 72 hpf did not show any labeled dsRed cells in the heart, confirming our tracing studies. At later time points of 96-120 hpf, labeled dsRed cells were detected in the heart region in whole mount preparations. At 96 hpf, the dsRed positive cells were sparse and appeared in the ventricle and outflow portion of the heart. At 120hpf, dsRed cells could also be detected in the ventral aorta but could be more clearly seen in the adult structures (Fig. 14).

Because neural crest is known to contribute to the cardiac ganglia in the heart I examined the distribution of dsRed by the sox10:cre in adult zebrafish. I also mapped the distribution of acetylcholinesterase, as acetylcholinesterase identifies cardiac ganglia. In comparing the morphology of the glia in chick hearts to those found in zebrafish hearts, I found that they were both stained for acetylcholinesterase and were both found at the outflow tract (Figure 12). I then compared the distribution of acetelcholinesterase positive cells to dsRed positive cells in the adult zebrafish heart (Figure 13). The acetylcholine positive ganglia that run across the adult zebrafish ventricle match closely to the dsRed positive cells in that ventricle. Some of the cells that appear sox10 positive could be cardiac ganglia.

Figure 12 Chick (A) and zebrafish (B, C) hearts. The white arrows in chick and zebrafish (A, B) show the location of the ganglia stained with acetylcholinesterase. In the dsRed positive zebrafish (C) the red fluorescence appears around the bulbus structure similar to staining in (B).

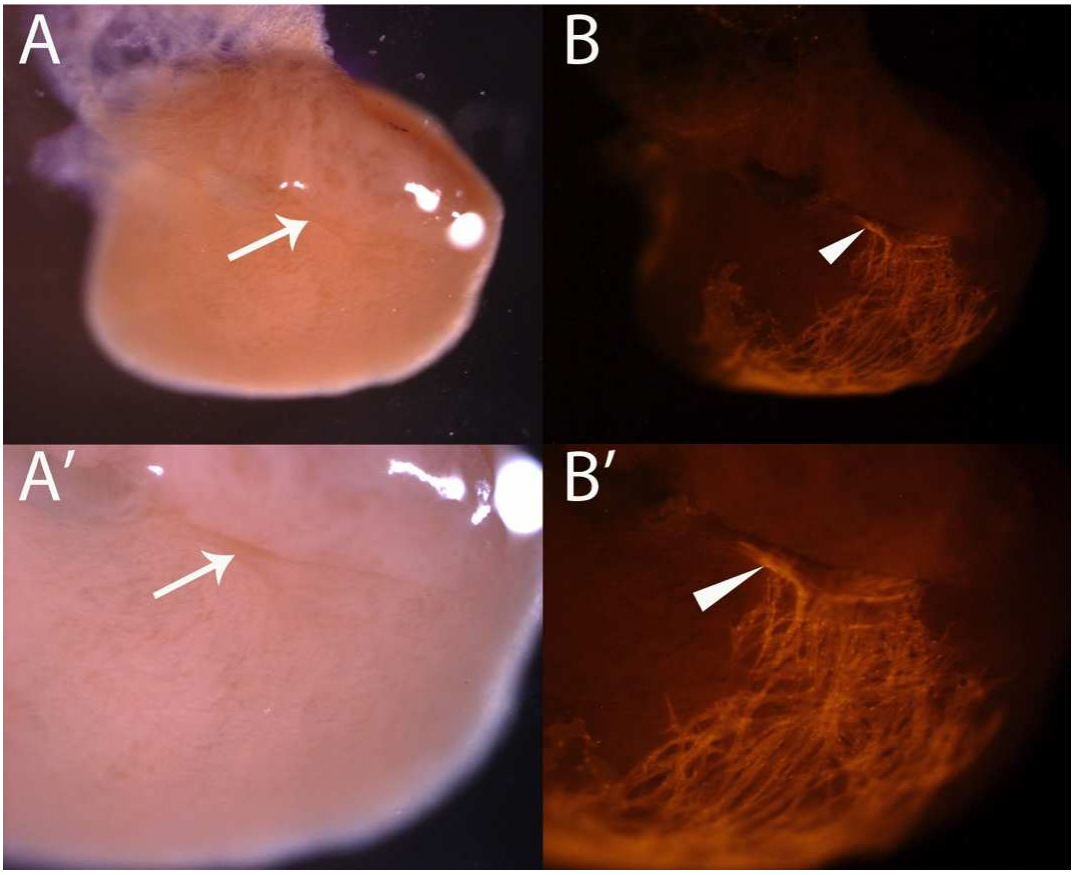

Figure 13 Acetylcholinesterase and dsRed in adult zebrafish hearts. The line of ganglia on the zebrafish ventricle (A, arrow) matches closely to the most dorsal dsRed positive lines (B, arrowhead).

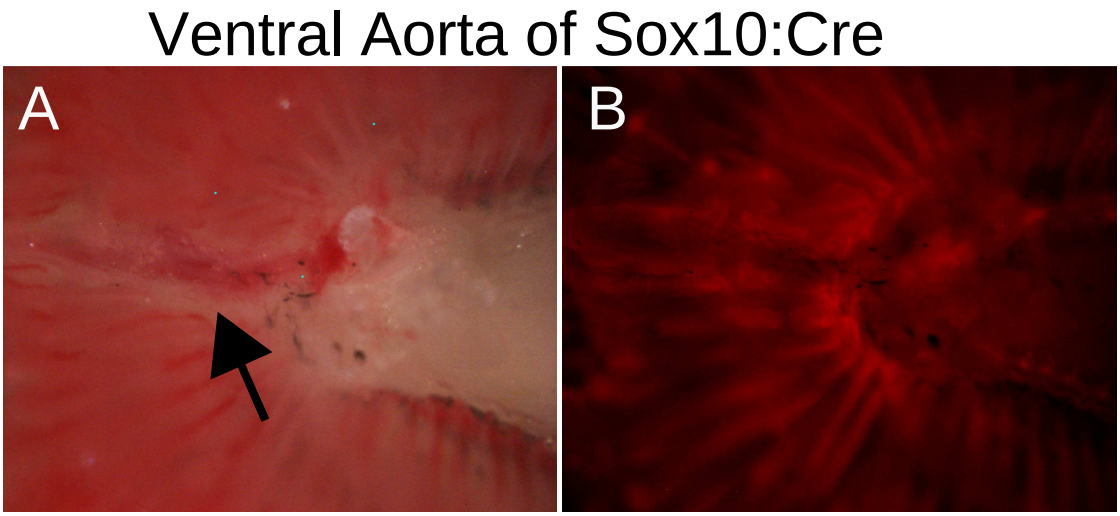

Figure 14 The ventral aorta of an adult Sox10:Cre. The zebrafish adult has a large ventral aorta (A, black arrow) where blood exits from the bulbus arteriosus and is distributed to the gills. In the Sox10:Cre adult, the red sox10 positive neural crest cells surround the ventral aorta (B).

3.6 Discussion

In this study I use three different techniques to trace the migration of neural crest cells to the cardiac region from the onset of migration through 120hpf: 1. *Tg (Sox10:egfp)* labeling of migrating neural crest; 2. uncaging of labeled dye in individual or groups of cells of known neural crest origin; 3. use of transgenics that labeled all cells that expressed Sox 10 at any time, thus permanently labeling cells of neural crest origin. These techniques allowed me to trace neural crest cells for an extended period and to definitively distinguish cells of neural crest and mesodermal origin. These studies indicate that cells of neural crest origin do not contribute to the heart myocardium at least before 120hpf. However, cells of neural crest origin are found in the pericardium at 72hpf, the ventral aorta at 96 hpf, and in the region of the cardiac neural ganglia in adult hearts. I also demonstrate that neural crest cells and mesodermally derived cardiac progenitor cells originate and migrate in close spatial and temporal proximity in zebrafish. This spatial and temporal proximity differs from other model organisms such as mice and chicks. I show that if care is not taken to distinguish neural crest and mesodermal cells, then it is likely that mesodermal cells adjacent to the neural crest cells will be labeled; these labeled cells will subsequently be detected in the heart myocardium. These results show that neural crest cells in zebrafish do not contribute to the myocardium in early stages of heart development, yet neural crest cells do contribute to cardiac innervation.

This study contradicts previous findings that CNC cells in zebrafish give rise to myocardial cells in the heart (Li et al., 2003; Sato and Yost, 2003). Li et al. traced neural crest cells by injecting caged fluorophores in the whole embryo and uncaging cells in the dorsal neural tube at the 2-3 somite stage (ss). However, the authors had no way of distinguishing neural crest and mesodermal cardiac progenitor cells during the labeling process and thus lacked specificity. When Li et al. followed the uncaged, labeled cells from 2-3ss to 48 hpf, the labeled cells co-localized with MF20, a striated muscle marker. Li et al. also ablated cells they believed to be neural crest cells. In these ablations, zebrafish heart rate decreased and the heart failed to loop. Due to the large ablation area, it is likely that mesodermal cells that contribute to the initial heart tube were ablated as well. Like Li et al., Sato and Yost (2003) also traced neural crest cells. Unlike Li et al., they started their experiments at the 8ss and ended at 72hpf. Sato used three labeling techniques. The first technique injected caged fluorophores into zebrafish at the one cell stage. The fluorophores were uncaged in cells dorsal to the neural tube at 8ss. The locations of the uncaged cells were examined at 72hpf. Second, they activated GFP in the dorsal neural tube of *hsp70-gfp* transgenic fish at 8ss to follow neural crest cells through 72hpf. Finally, they used cell transplantation to follow neural crest cells from 8ss to 72 hpf. The progeny of the uncaged and heat-shocked cells were observed in the heart myocardium as early as 24hpf. Again, Sato and Yost (2003) did not use an independent method of identifying neural crest cells. They presumed that the cells labeled in their

three labeling techniques were neural crest based solely on the location of the cells. In their transplant experiments for example, the cells that they identified in the dorsal neural tube were not identified through transverse sections. During gastrulation, both heart progenitors and neural crest cells move towards ventral positions and could appear to take similar migration pathways. Sato and Yost could not ensure that only neural crest cells, and not cardiac progenitors, were labeled.

My study differs from these previous studies in two key ways. First, the studies by Li et al. and Sato and Yost found labeled cells in the myocardium of the heart, which they presumed were of neural crest origin. However due to the lack of an independent method to distinguish neural crest from the underlying mesodermal cells, it is likely that the authors labeled both premigratory neural crest and premigratory mesoderm. When I purposefully labeled a non-neural crest cell adjacent to a neural crest cell, I found labeled derivatives in the myocardium. Labeling a larger area of neural and non-neural crest cells increased the contribution of labeled cells to the heart. From this I conclude that because the previous studies did not use a marker that clearly identified neural crest cells, they mistakenly labeled cardiac progenitor cells. Second, I found that neural crest cells contribute to the pericardium, ventral aorta and adult cardiac ganglia. I believe that these neural crest cell derivatives were overlooked in the studies of Sato and Yost and Li et al. in favor of the labeled cells found in the myocardium. I extended my study through 120hpf and additionally examined adults, whereas the previous studies ended

at 72hpf. At 72hpf labeled neural crest cells appear in the pericardium, but neural crest cells in the ventral aorta and cardiac ganglia appear after 72hpf and were missed in the Sato and Yost study (Fig. 13, 14). My study increased the window of time in which I could evaluate the role of neural crest cells in the context of zebrafish heart development.

Using the data I collected in this study, I can begin to compare the role of neural crest cells in zebrafish heart development to neural crest cells in other vertebrate hearts such as the frog. *Xenopus* embryos, like fish, have bilateral gill arch arteries and a common aorta as tadpoles. As tadpoles undergo metamorphosis, the gill arches remodel into six aortic arch artery branches: two carotids, two aortas and two pulmocutaneus arteries. These aortic arch arteries originate from an undivided outflow tract. CNC cells in *Xenopus* surround the gill arches and are detected in the aortic arch arteries but not in the outflow tract (Lee and Saint-Jeannet, 2011). The outflow tract in frogs develops from secondary heart field cells, much like the bulbus arteriosus in zebrafish (Hami et al., 2011; Zhou et al., 2011). In zebrafish and in *Xenopus,* the proximal outflow tract is derived from cells of the secondary heart field and the distal portion, the arch arteries, have contributions from neural crest cells.

The position of neural crest cells in the arch arteries relative to the cardiac outflow derived from secondary heart field is maintained throughout evolutionary changes in outflow tract anatomy. The outflow tract of *Xenopus* develops a spiral septum

to aid in the shunting of blood to the systemic or pulmonary system. In mice and chick, the systemic and pulmonary systems are fully divided because of the separate aorta and pulmonary artery. Yet, neural crest cells continue to surround the aortic arch arteries up to the point where they meet with secondary heart field derived smooth muscle.

The evolutionary changes in cardiac outflow tract anatomy require additional roles of neural crest cells. Neural crest cells in avians and mice change the signaling environment of the pharynx. The secondary heart field, which moves under the pharynx, is sensitive to signaling changes. The changes detected by the secondary heart field could affect the elongating heart tube. This indirect role of neural crest cells in secondary heart field addition could be tested on fish and frogs. Receptors in neural crest can be knocked down though morpholinos in fish and frogs. Receptors of intrest are FGF and Endothelin, because neural crest cells continue to migrate but they cannot react to the liginds. In knocking down the receptors, I could look at the addition of secondary heart field structures in the fish and frog. Using these two model organisms one could distinguish the role neural crest cells may have on secondary heart field addition from the role neural crest cells have in septation. Septation of the outflow tract is another important task for neural crest cells in mice and chicks. CNC cells are crucial in creating the aorticopulmonary septum that divides the aorta and pulmonary artery. In addition to birds and mammals, reptiles also have division of the aorta and pulmonary system, albeit incomplete. The outflow tract of reptiles consists of a

pulmonary and two aortas. These arteries arise from a singular ventricle or a ventricle with incomplete divisions. A study on reptilian outflow tract development could help to elucidate the steps required to achieve a divided circulation and determine if the CNC population potentially plays a role.

Cardiac neural crest was first defined with the discovery of the contribution of neural crest to the aorticopulmonary septum, in chicks, and later mammals. Subsequently it has been shown that CNC cells aid in remodeling the aortic arch arteries in animals that possess a divided septum. Zebrafish do not possess a septum, and they undergo minimal arch artery remodeling to afferents and efferents, however, neural crest cells do contribute to the cells that surround the ventral aorta and gill arch arteries. We see a similar situation in *Xenopus*, chicks and mice where the neural crest cells surround the aortic arch arteries. This leads us to question the definition of CNC. As originally defined, CNC was considered to be the cells that contribute to outflow septation. As we examine a variety of vertebrates we see that neural crest that migrates to the aortic arch area and heart performs a variety of roles. Given these changing roles we might ask do zebrafish possess CNC or do we need to change the conventional definition of CNC?

A wide variety of studies of the head have shown the role of neural crest in contributing to patterning changes during evolution. Further study of the role of crest in aortic arch remodeling in transitional species, including amphibians and reptiles will

allow us to document the potential role of neural crest in mediating the changes we see in the circulatory system in terrestrial vertebrates.

4. Outflow tract development in the turtle *Trachemys scripta*

Fully aquatic animals like zebrafish have an undivided heart, an un-septated outflow and bilaterally symmetrical gill arch arteries (Isogai et al., 2001; Stainier and Fishman, 1992). Amphibians like *Xenopus*, have bilaterally symmetrical gill arch arteries as tadpoles that undergo remodeling during metamorphosis. In *Xenopus* and zebrafish neural crest cells contribute to the vasculature of the arch arteries, but not to structures within the outflow tract. In this chapter, I will review what is known about outflow tract formation in amphibians and reptiles and describe the development of outflow septation in the turtle, *Trachemys scripta.* Using this information I take a closer look at the role neural crest cells could potentially take in the development of the outflow tract in reptiles.

4.1 Review of outflow tract structure in Amphibians and Reptiles

Amphibians have a single, undivided ventricle, and a single outflow tract (Figure 13). This tract is divided by a spiral valve that runs along its length (Goodrich, 1930a). This spiral valve is created by cushion-like septum that helps to partition oxygenated and deoxygenated blood to the systemic, subcutaneous and pulmonary arteries respectively. In the salamander *Amphiuma tridactylum,* the single outflow contains two different regions: the bulbus proximally and the truncus distally (Johansen and Hanson, 1968). The bulbus is at the base of the outflow and contains three semilunar cusps that

prevent blood from returning to the ventricle. The spiral valves, similar to those in the frog, are found in the truncus region of the outflow (Johansen and Hanson, 1968). Studies in *Xenopus laevis* have shown that CNC cells do not contribute to the spiral valves of the outflow tract but that it is made up of cells from the secondary heart field (Lee and Saint-Jeannet, 2011). Unfortunately outflow tract development in amphibians has only been studied in *Xenopus*, therefore we do not know if these results pertain to all amphibians.

Compared to the amphibian heart, the reptilian heart has more partitions. Reptiles have one ventricle, but the septa in the ventricles partially divide the left systemic and right pulmonary portions (Goodrich, 1930b). Along with the increased division of the heart, the outflow tracts in reptiles also show a division beyond that seen in amphibians. In Tuataras (*Sphenodon punctatus*) the base of the outflow tract is partially divided and has been described as being similar to amphibian cushion-like spiral valves (Simons 1965). A cartilaginous rod divides the distal portion of the outflow tract. Cartilage in the head is derived from neural crest cells, suggesting that the cartilaginous rod in Tuataras could be neural crest cell derived. The distal outflow tract branches into the pulmonary and systemic arches. The division of the arteries in snakes and lizards occurs closer to the ventricle, with a muscular partition dividing the pulmonary and the two systemic arteries (Hart 1968, O'Donoghue 1918). The right systemic artery is more closely associated to the pulmonary than the left systemic artery. In the crocodile, there

is a rotation of the cardiac outflow tract septum so that the left systemic artery is more closely associated with the pulmonary artery and carries more oxygenated blood. Crocodiles, unlike snakes and lizards, have a completely divided ventricle, although the Foramen of Panizza serves as a pathway between the right and left systemic arteries (O'Donoghue 1918, Bremer 1928, Hart 1968, van Mierop 1985, Koshiba-Takeuchi 2009). In the reptiles discussed so far, all the branches of the outflow tract, two systemic aortas and one pulmonary artery, have a base in the ventricle.[1] Thus the outflow is completely divided.

Like other reptiles the heart in the adult turtle has two divided atria, a partially divided ventricle and three separate arch arteries that arise from that ventricle (Hicks and Malvin, 1992; Kutsche and Van Mierop, 1988) (Figure 13). The right and left aorta as well as the pulmonary artery arise from the base of the ventricle. The ventricle is sub-divided by partial septa into three compartments: the cavum venosum, the cavum arteriosum and the cavum pulmonle. These compartments help maintain the separation of oxygenated and de-oxygenated and ensure blood goes to corresponding arch artery.

[1] Living reptiles unlike birds and mammals have two systemic aortae – a right and a left. Birds and mammals have each separately lost one of these systemic arteries and have single systemic aortae.

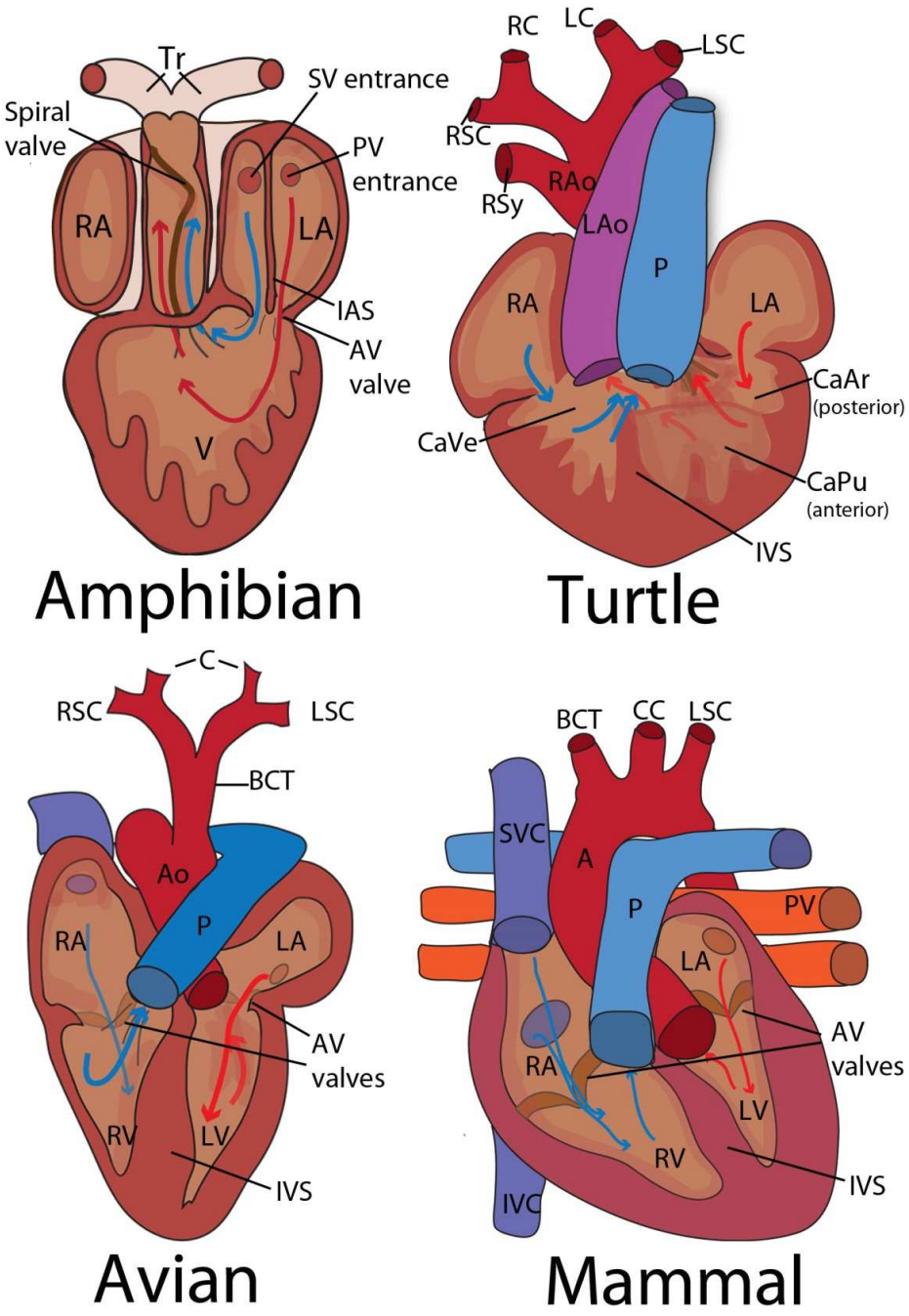
Tr
SV entrance
Spiral valve
PV entrance
RA
LA
IAS
AV valve
V
Amphibian
RC
LC
LSC
RSC
RAo
RSy
LAo
P
RA
LA
CaAr
(posterior)
CaVe
CaPu
(anterior)
IVS
Turtle
C
RSC
LSC
BCT
Ao
P
RA
LA
AV valves
RV
LV
IVS
Avian
BCT
CC
LSC
SVC
A
P
PV
LA
AV valves
RA
LV
RV
IVS
IVC
Mammal

Figure 15 Amphibian, Turtle, Avian and Mammal Adult Hearts. The outflow of the adult amphibian heart remains undivided, while the turtle, bird and mammal outflow tracts are divided. In the turtle, the ventricle is partially divided into three parts: Cavum Pulmonale (CaPu) which is located on the left anterior side, Cavum Arteriosum (CaAr) located on the left posterior side and the Cavum Venosum located on the right side. The avian and mammal hearts are shown for comparison. The blue arrows depict deoxygenated blood while the red arrows depict oxygenated blood.

RA=Right Atrium, LA=Left Atrium, V= Ventricle, RV=Right Ventricle, LV= Left Ventricle, IAS= Interarterial Septum, PV= Pulmonary Vein, SV=Sinus Venosus, Tr= Truncus, IVS= Intraventricular Septum, LSC= Left Subclavian, RSC= Right Subclavian, RSy= Right Systemic, BCT= Brachiocephalic Trunk, SVC= Superior Vena Cava, IVC=Inferior Vena Cava

4.2 Cardiac blood flow in Turtles

In turtles (and other reptiles without completely separated circulations) de-oxygenated blood enters the sinus venosus into the right atria (Figure 13). Following the right atria, the blood enters the right ventro-lateral space of the ventricle, which is divided into the cavum venosum and the cavum pulmonale compartments. De-oxygenated blood primarily from the cavum pulmonale enters the pulmonary artery. Oxygenated blood returns from the lungs into the left atria. This blood is sent to the left dorso-lateral portion of the ventricle, the cavum arteriosum. During atrial systole, oxygenated blood is pumped into the cavum arteriosum and then the cavum venosum (Holmes, 1976). During ventricle systole, the oxygenated blood is pumped from the cavum venosum to the left and right aorta (White, 1968; Johansen and Burgren, 1980;

Hicks and Wang, 1996). In periods of apnea or when the turtle dives, there is an increase in pulmonary vascular resistance and blood circulates through the cavum arteriosum to the cavum venosum to sustain circulation (White 1968, Hicks and Wang, 1996).

4.3 Development of the heart in reptiles

Turtles, like other vertebrates, have a heart that begins as a linear tube. The heart tube in turtles first loops right as noted in vertebrates like zebrafish, chick and mice (Stainier and Fishman, 1992). The fold that is created by the linear tube looping right separates the atria from the ventricles (Bertens et al., 2010). In the pond turtle, heart looping is important for septa formation between the atria and ventricle, as well as the partial septa that divide the ventricle (Bertens et al., 2010). As the heart loops and forms the atria and ventricle, the outflow is elongated and has two bends, one proximal to the ventricle and one distal, which creates a twist in the outflow tract. Inside the outflow tract, the endocardial tissues bulge to touch each other, creating the divisions between the systemic and pulmonary arteries (March 1968, Bertens 2010).

Although two aortas persist in the turtle, the position of the pulmonary artery relative to the aortas can be compared to the position of the pulmonary and aorta in chick. As the outflow tract develops in the chick it elongates by the addition of cells. The elongation of the outflow is important for positioning of the outflow cushions and subsequently, neural crest cell invasion. In chicks, like in turtles, the outflow elongates and rotates with the addition of the secondary heart field (Dyer et al., 2010). The proper

elongation of the outflow tract is important for alignment of the outflow vessels to the correct cardiac chambers. Chick CNC cells invade the distal outflow cushions and are needed for full division of the aorta and pulmonary artery. However the potential role of CNC in the formation of a divided outflow tract has never been studied in reptiles.

In this chapter I detail the development of the septation of the outflow tract in *Trachemys scripta.* I present images of whole mount specimens to help describe the orientation of vessels in outflow tract and then describe sectioned material. I describe cushion development and formation of the septa that divides the aortas and the pulmonary artery using histological methods to visualize the sections. Finally, using immunohistochemistry I look at the types of cells found within the developing cushions and septa. My goal is to present a CNC cell model of outflow septation in reptiles to further our understanding of the CNC cell population across vertebrates.

4.4 Methods

4.4.1 Turtle Heart Fixation

I studied the development of the turtle heart in collaboration with students Lindsey Mork and Michael Czerwinski in Blanche Capel's Lab in the department of Cell Biology at Duke University. I collected the hearts at the stages in which the students in the Capel lab collected turtle gonads. *Trachemys scripta* hearts were collected between stages 15 and 26 (Greenbaum 2002). Hearts and outflow tracts were removed from the embryos and fixed with Methcarn (30 ml Methanol, 15 ml Chloroform, 5 ml Acetic Acid) or 4% Paraformaldehyde (PFA). The outflow tracts were severed closest to the pharyngeal wall, and included as much of the aortic arch artery branches as were available. The hearts were stored overnight in fixative at 4 degrees Celsius. Methcarn fixed hearts were transferred to 70% ethanol for long-term storage. Hearts fix with 4% PFA were transitioned into Methanol and placed in -20 degree Celsius for long-term storage. Whole heart photos were taken in 70% ethanol for Methcarn fixed hearts and in PBS for 4% PFA fixed hearts.

Table 2: Stages Collected of Trachemys scripta embryonic hearts

	Stage 15	Stage 17-18	Stage 23	Stage 26	Stage 28
Total	11	14	25	23	15

4.4.2 Heart histology and immunohistochemistry

In order to examine the type of cells present in the turtle outflow cushions, I stained the hearts with specific cells markers. H&E allowed me to visualize condensed mesenchyme within the outflow cushions from muscle cells in the rest of the heart. In order to identify the types of cells in the hearts, I used antibodies for MF20 (striated muscle) and SM22-alpha (smooth muscle) and HNK-1 (migrating cells). Fixed specimens were completely dehydrated and placed in Xylene before infusing with paraffin. All hearts were embedded in paraffin for sectioning. Sections for immunohistochemistry and immunofluorescence were sectioned at 8um. Sectioned hearts were stained for H&E following the protocol in Hutson et. al. 2008. Sections destined for immunofluorescence were de-paraffinized in Xylene and re-hydrated stepwise from 100% ethanol to 70% ethanol. After a 10-minute water bath, sections were incubated with 0.1 M Tris-Tween, 0.1 M Tris and 0.1 M Tris-2% Fetal Bovine Serum each for 5 minutes. Hearts fixed with Methcarn were doubly stained with MF20 and SM22-alpha. All hearts were incubated with Hoechst, which when bound to dsDNA, emits blue fluorescence (Buys and van der Veen, 1982). All section hearts were visualized on a Leica DMRA2 compound microscope with either a Q Imaging Micropublisher Digital color camera or a Retiga EX Black and white digital camera with fluorescent cubes. Pictures were captured using OpenLab Software from PerkinElmer.

4.5 Development of the heart in Trachemys scripta

The heart of *Trachemys scripta* begins as a single undivided heart tube. At stage 15 (Figure 16), the heart is linear but has started to loop to the right. The heart also begins to bend to the right at the junction of the developing ventricle and truncus, creating an outer and inner curvature. At stages 15-16, the outflow tract lengthens as the ventricle and atria expand. The aortic arch arteries remain bilaterally symmetrical branching from the aortic sac. At stage 18, the aorta and pulmonary arteries could be detected distal to the heart, but the outflow remains undivided proximal to the ventricle (Fig. 16). At stage 23, the vessels can be clearly traced with the pulmonary located on the left side of the left aorta, entering the cavum pulmonale in the ventricle (Fig 16). The left aorta is located between the pulmonary and right aorta. The pulmonary is the most ventral artery, followed by the left aorta. The right aorta is located further behind the left aorta and originates from the cavum venosum. At the base of the left aorta and pulmonary artery appears to be a thickened band, presumably where the semilunar valves will develop. At stage 26, both the pulmonary and left aorta are distinguishable as two separate vessels in whole mount specimens (Figure 17).

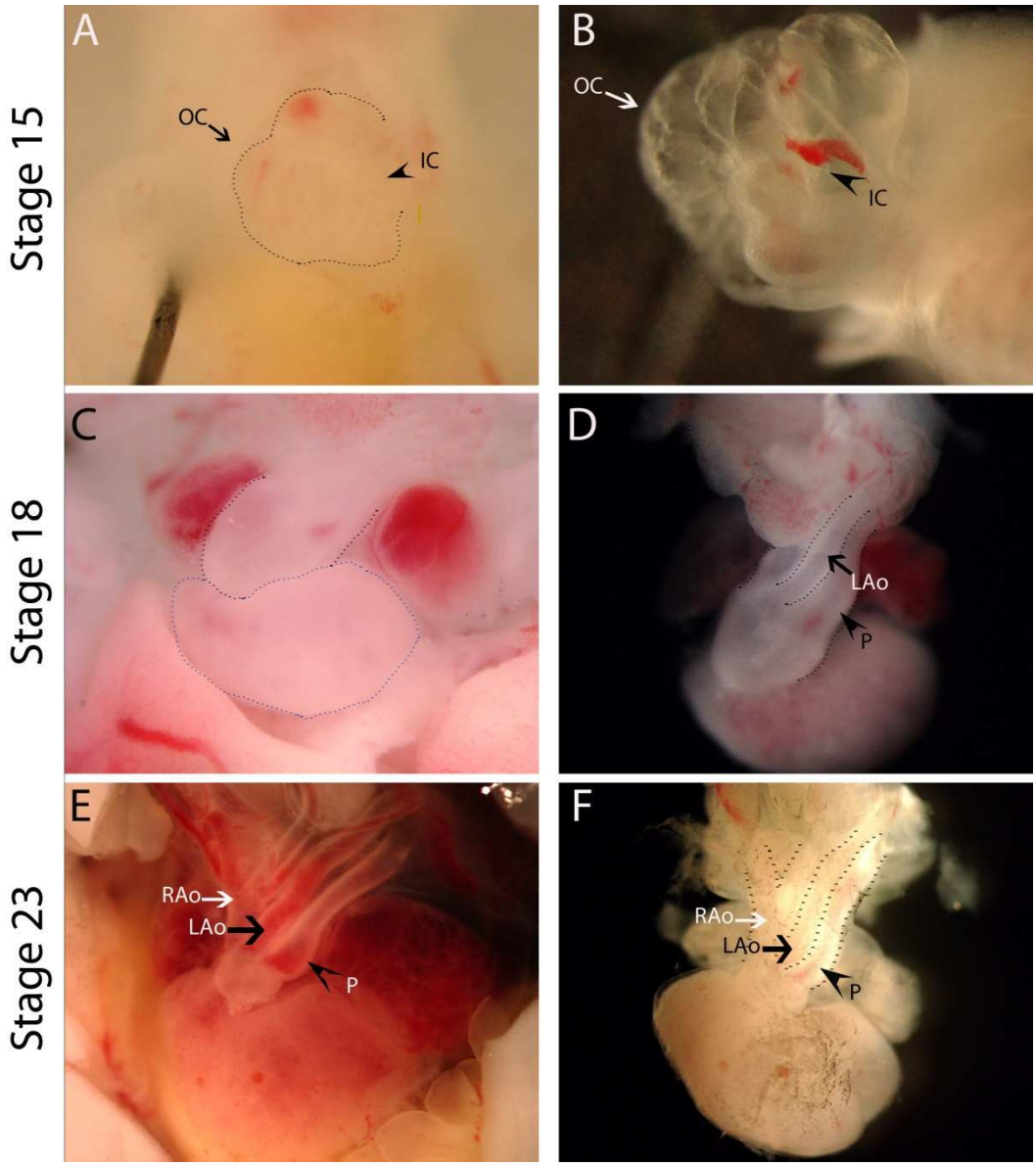

Figure 16 ***Trachemys Scripta*** **hearts in situ in the chest cavity (A, C, E) and dissected out. The red represent blood still remaining in the heart and its vasculature (B, D. F). (A-B) At this stage the tubular heart is bending to the right. The outer curvature (OC) of the heart is looped to the right while the inner curvature (IC) remains close to the body wall at stage 15. (C-D) At stage 17-18, the heart has formed the ventricle and two atria and the outflow has elongated. The left aorta and the pulmonary (P) artery start to separate distally. The proximal outflow has not yet divided. (E-F) At stage 23, the outflow has a clear division**

of the right aorta (Hiraoka et al.), left aorta (Snider et al.) and pulmonary (P) artery.

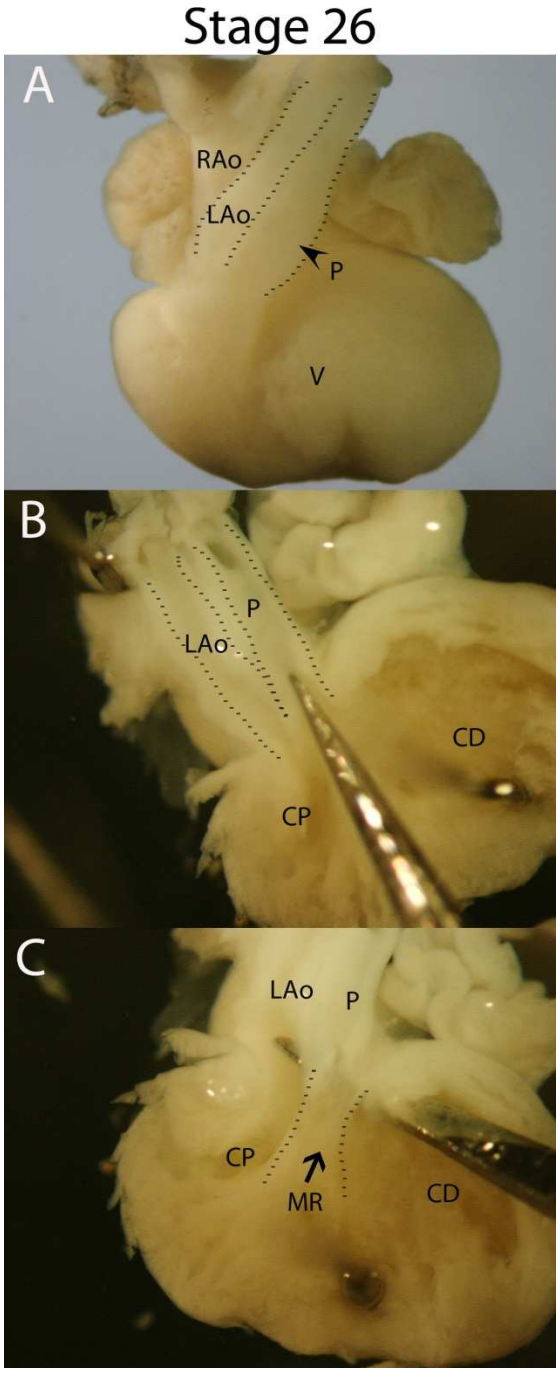

Figure 17 The heart in *Trachemys scripta* at stage 26. (A) The ventricle has grown and the vessels are fully rotated. (B,C) Heart is cut

longitudinally to display the pulmomary and left aorta as well as the ventricle and pulmonary artery. The cavernum pulmonale (CP) and the cavernum dorsale (CD) both lead to their respective aortic arch arteries. MR=myocardial ridge, the incomplete septum.

4.6 Septation of the outflow tract in Trachemys Scripta

At stage 23 the turtle heart has distinguishable aortas and a pulmonary artery. I stained the sections of the outflow tract histochemically with Eosin and Haematoxylin to to reveal the internal cellular structures. At the base of the outflow tract the right aorta is distinguishable, but it has not fully separated from the rest of the outflow. At this level, cushions separate the blood flow between the right aorta and the rest of the outflow (Figure 18 B). The right aorta is separated from the main outflow tract through a mass of cells that extend ventrally through the cardiac jelly, to the opposite end of the outflow wall (Fig. 18 B arrowhead). Distal to these sections, the right aorta is fully separated and changes from a ventral to a dorso-lateral position, becoming flush with the opening of the left aorta and pulmonary (Fig. 18 A, D). Eventually the right aorta will lie behind the left and pulmonary artery.

At the base of the outflow tract, the left aorta and pulmonary artery have not completely separated (Fig.18 B). The division of these two vessels occurs more distal in the outflow tract. Mesynchymal cells appear to extend from the left side of the wall of the right aorta and bisect the outflow tract from right to left (Fig 18 C). A smaller group of condensed meseynchymal cells forms underneath the cardiac jelly from the left side of the outflow tract. The two cushions meet in the middle and create a frontal division of

the outflow tract (Fig. 18 D). The pulmonary is located ventrally and the left aorta is in the doral position, behind the pulmonary artery.

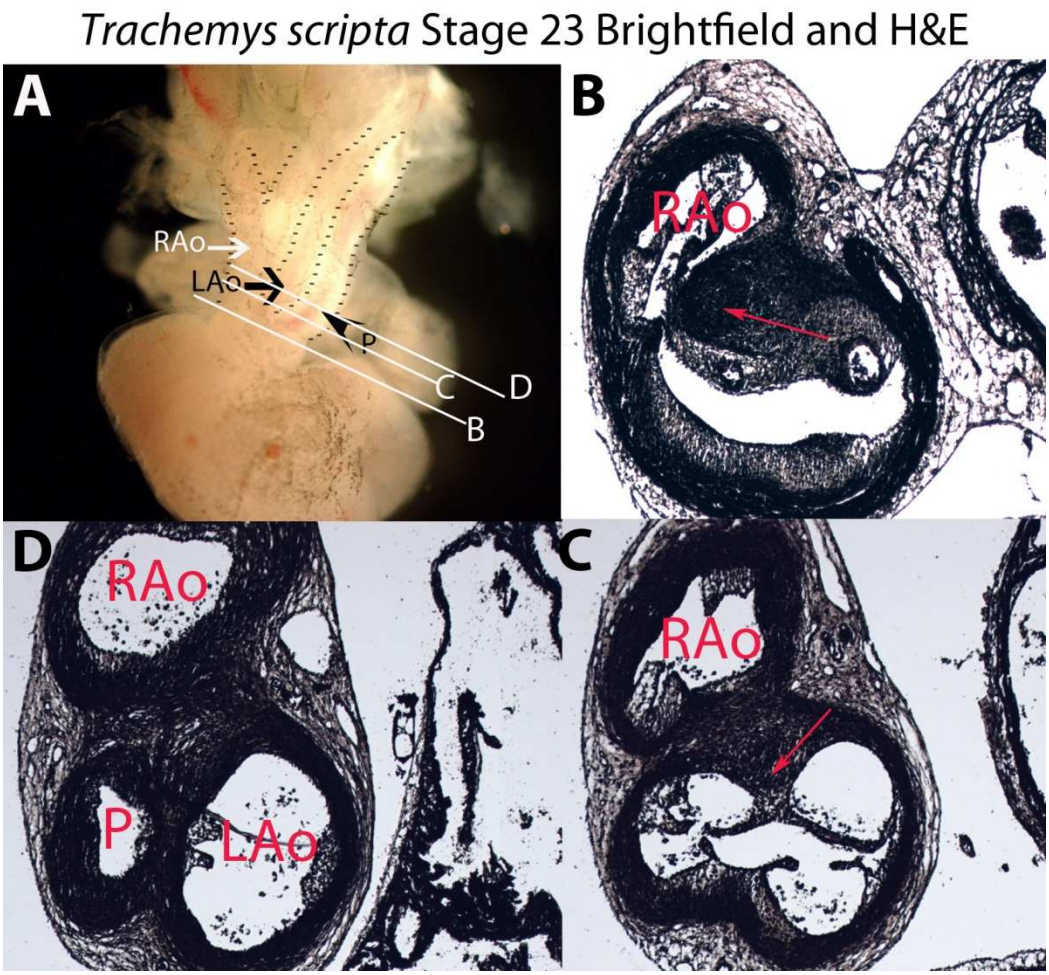

Figure 18 Division of the *Trachemys scripta* outflow tract by condensed mesenchyme. The bright field image of the stage 23 heart is a reference for sections (A). The first section is the most proximal to the ventricle, subsequent sections move away from the ventricle. The first distinguishable artery, proximal to the ventricle is the right aorta (B). A mass of condensed mesenchyme (red arrow) bisects the outflow tract sagittally, isolating the right aorta from the rest of the outflow tract (B). The pulmonary and left aorta division occurs more distal to the ventricle (192 um distance from B to D). The condensed mesenchyme (red arrow) from the newly formed right aorta moves to the left. When the mass of condensed mesenchyme meets, it bisects the outflow in the frontal plane, creating a ventral pulmonary and a dorsal left aorta (C, D). Sections stained with Eosin and Haematoxylin. RAo= right aorta, LAo= left aorta, P= pulmonary

4.7 Myocardial and Smooth Muscle cell types of the developing outflow tract

Immunohistochemistry was used to determine the cells types within the outflow tract of *Trachemys scripta*. I used MF20 for cells destined to be striated muscle and SM22 for smooth muscle. The striated muscle is indicative of the cells in the ventricle and the base of the outflow tract, while smooth muscle is indicative of the outflow tract vessel walls. In chick, the striated cardiac muscle is derived from mesoderm while the smooth muscle is derived from a combination of mesoderm and neural crest cells (Waldo et al., 2005a). I observed embryonic hearts at stage 15-16 and at stage 18 to determine the type of cells that surrounded the outflow tract and the cells found within the outflow cushions.

At stage 15-16, the base of the outflow tract is undivided and surrounded by MF20 positive cells (Figure 19 B). Proximally, the outflow has no distinguishable arteries but the cushions are visible and they divide the blood flow into two streams (Fig. 19 C). In subsequent distal sections, the MF20 positive cells are no longer detected around the outflow. In these areas, smooth muscle positive cells can be detected and sometimes overlap with MF20 cells, which indicate the future area of outflow valve development. (Fig. 19 E). Smooth muscle positive cells are detected on either side of the outflow at the base of the outflow cushions. MF20 positive cells are detected around the dorsal half of the outflow, the area adjacent to the ventricle. The walls of the ventral half of the outflow are void of MF20 positive cells, and the opening will become the right aorta. In a

subsequent distal section, the cushions of the outflow tract have merged and contain smooth muscle positive cells that correspond to condensed mesenchyme (Fig. 19 F). Smooth muscle cells are also observed in the following sections within the cushions dividing the dorsal and ventral half of the outflow tract (Fig. 19 G).

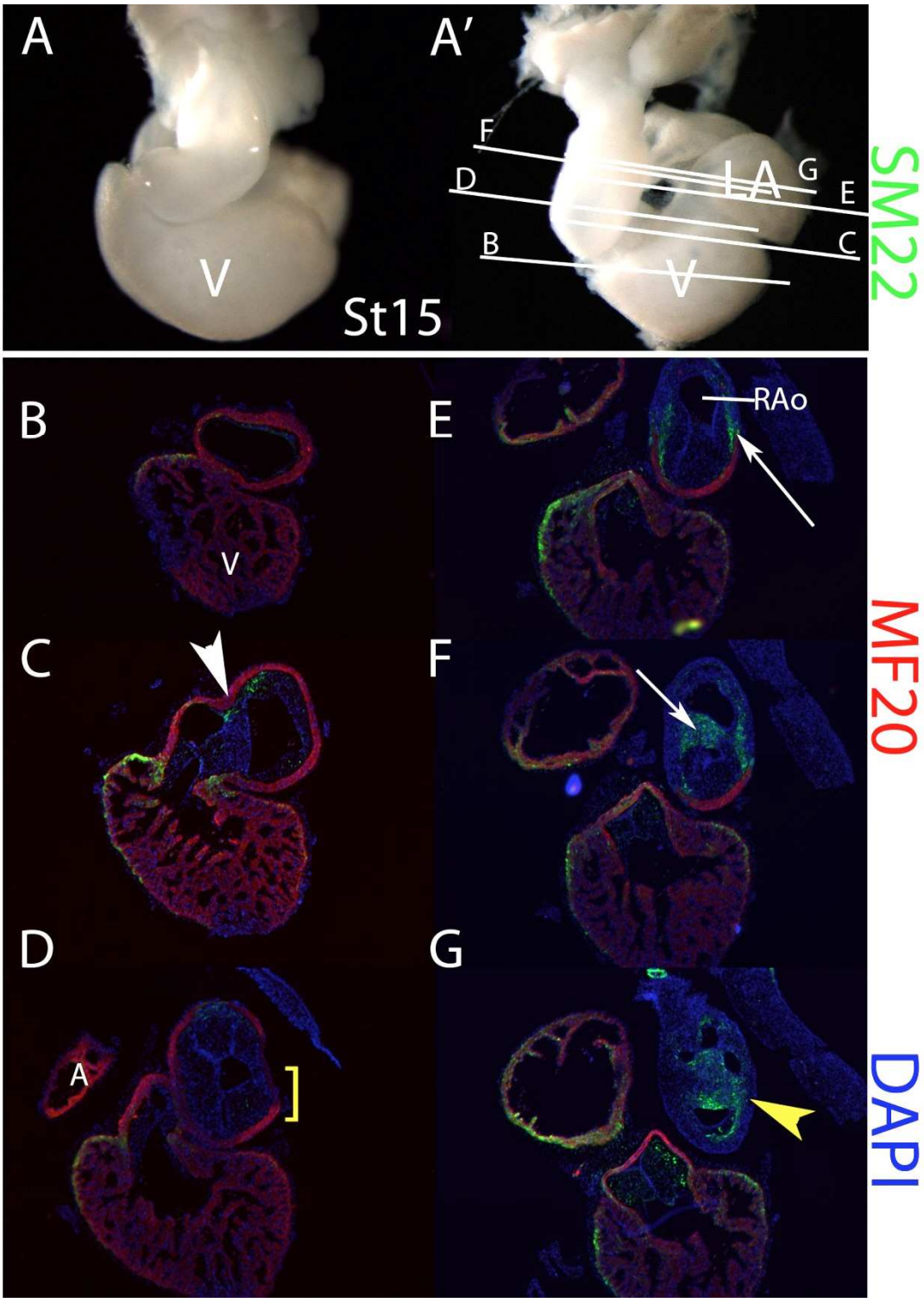
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Figure 19 Sections of stage 15-16 *Trachemys scripta* outflow tract. Two whole hearts are for reference (A, A'). The sections are labeled with smooth (SM22 green) and striated (MF20 red) muscle markers. Sections proximal to the ventricle are surrounded by myocardium only (B). In section C, outflow tract cushions are visible, dividing the blood flow in two (white arrowhead). The subsequent section shows the cushions with some smooth muscle overlapping with the outer striate muscle. Some of the striated muscle is being replaced (D, red bracket). In E, the right aorta is distinguishable, divided by two forming cushions. Smooth muscle cells are present at the base of the cushion (white arrow). Section F shows that the cushions have fused and have smooth muscle positive cells in the middle (white arrow). In G, smooth muscle cells lie within the septum (yellow arrow head) as well as surrounding the branches of the right aorta. The distance between section B and G is 432 um. RAo= right aorta, LAo= left aorta, P= pulmonary, RSy=right systemic, BCT= brachiocephalic trunk, LBC= left brachiocephalic, RBC= right brachiocephalic

At stage 18, the three arteries can be detected in the most distal portion of the outflow tract (Figure 20 A). Proximally, the outflow tract is undivided with the outer portion surrounded by MF20 positive cells and inner portion with smooth muscle positive cells. There are more smooth muscle cells overlapping with MF20 muscle markers than in previous stages. As the outflow cushions become more robust distally, two channels for blood flow become more apparent (Fig. 20 C). The outflow cushions divide of blood between the developing right aorta and left aorta/pulmonary opening. Eventually, the leading edge of the cushions are no longer visible, creating a mass shelf of cells that partition the right aorta from the single outflow tract. In the most distal section of the outflow tract, smooth muscle positive cells surround the outer and inner walls of the outflow arteries (Fig. 20 G, H). No MF20 muscle markers are observed at this level.

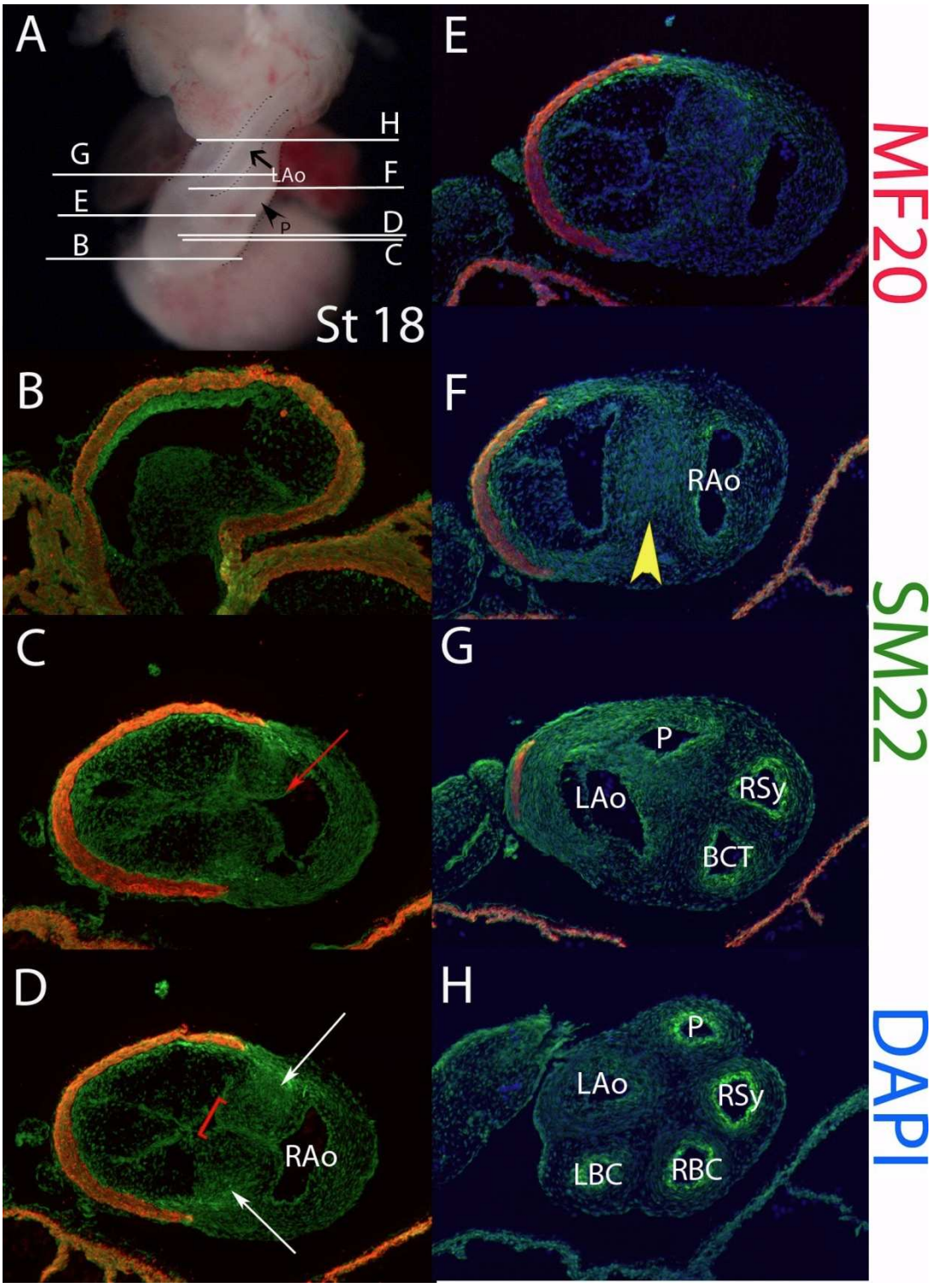
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Figure 20 Sections of a stage 18 *Trachemys scripta* outflow tract. The sections are labeled with smooth (SM22 green) and striated (MF20 red) muscle markers. Sections proximal to the ventricle are surrounded by myocardium with an inner layer of smooth muscle on the left side (B). In section C, the outflow tract cushions isolating the right aorta are visible. The subsequent section shows a high concentration of smooth muscle positive cells within the cushions (D, white arrows). The most distal sections show the development of the left aorta and the pulmonary, from the branches of the right aorta (G, H). In E and F, the walls of the cushions have broken down and the septum between the right aorta can be detected (F, yellow arrowhead). The distance between section B and G is 320 um. RAo= right aorta, LAo= left aorta, P= pulmonary, RSy=right systemic, BCT= brachiocephalic trunk, LBC= left brachiocephalic, RBC= right brachiocephalic

4.8 Discussion

The development of the outflow tract in *Trachemys scripta* progresses from a singular tube to a fully divided outflow, sharing many similarities to chick outflow development. In my turtle study, I looked at two aspects of turtle outflow development and compare them with chick. The first is the division of the outflow tract and how it progresses in a distal to proximal fashion. The second is the cell types within the outflow tract and how they potentially relate to neural crest cell location within the outflow tract. Taking these two facets of turtle septation and comparing them to chick outflow septation, I propose that CNC cells also play a role in the development of the outflow tract in *Trachemys scripta*.

In the turtle, the distal portion of the outflow tract is divided before the proximal as in chick. In chick, the outflow tract is also divided from distal to proximal direction (Kirby et al., 1983). Chick outflow septation starts as the bulging of cardiac jelly filled cushions into two longitudinal ridges divides the lumen of the outflow tract. In the middle of the outflow tract, the ridges create a twist, which offsets the ridges in the distal portion from those in the proximal outflow tract (Waldo et al., 1998a). The distal portion of the outflow is termed the truncus while the proximal portion is called the conus (Waldo et al., 1998a). In the turtle outflow, the lumen is also divided by the bulging of the cardiac jelly. At stage 15, the cushion at the base of the outflow appears to bulge in a dorsal ventral orientation, while distally it is in a left-to-right position,

indicating a spiraling of the forming septum. At stages 18 and 23, the differences in distal versus proximal were not as apparent. The distal cushions in the turtle are clearly defined and filled with condensed mesenchyme. The difference in distal and proximal location within the turtle outflow could indicate that turtles also have a conus and truncus, although the exact area of each could not be determined in this study.

In the chick, neural crest cells surround the aortic arch arteries, the aortic sac and form the aorticopulmonary septum in the truncus. These chick neural crest cells express early smooth muscle markers such as SM22 and α Smooth Muscle Actin and are the only source of smooth muscle cells in the distal outflow. In the chick proximal outflow, smooth muscle cells are derived from the secondary heart field. Because the neural crest cells in chick express smooth muscle cell markers during outflow septation, I also used this cell marker to observe outflow septation in the turtle. In the chick, the formation of condensed mesenchyme consisting of neural crest cells in the truncus is the aorticopulmonary septum. In this U-shaped aorticopulmonary septum, the upper shelf of the U divides the aortic sac between the fourth and sixth pairs of aortic arch arteries (Erickson, 1985; Kuratani and Kirby, 1991; Sumida et al., 1989). The prongs of the U are located within the truncus cushions. The conal cushions consisting of chick mesenchymal cells from the mesdoderm divide the proximal outflow tract. In the proximal cushions neural crest cells are located at the seam of division between the aorta and pulmonary arteries. Neural crest cells in the chick are also detected beneath the

outflow myocardial layer at the areas where the myocardium will cinch the presumptive aorta and pulmonary artery. Smooth muscle positive cells in turtle stage 15 outflow tracts are present in high concentrations within the distal cushions. Subsequent proximal sections show the developing distal cushions with smooth muscle positive cells at the base. At stage 23, the distal cushions that are closed have a high concentration of smooth muscle positive cells. In subsequent proximal sections, the cushions that are bulging toward the middle of the lumen also contain smooth muscle positive cells. At stages 15 and 23, the smooth muscle cells in turtle parallel the prong shape detected in the chick.

In the proximal portion of the turtle outflow smooth muscle cells do not populate the cushions; the smooth muscle positive cells are just inside the striated muscle layer. In the chick, smooth muscle cells overlap with myocardial cells in the in the chick conus, but the smooth muscle cells of the wall at the base of the outflow tract are derived from Secondary Heart Field mesoderm. The neural crest cells in the chick proximal outflow can be found in the outflow valves and in the seam between the walls of the aorta and pulmonary. At turtle stage 15, smooth muscle positive cells are found in the areas underneath the myocardium and in the space between the future division of the right aorta and left aorta/pulmonary artery. Within the proximal outflow tract of turtles, additional cell labels are needed to determine whether the smooth muscle positive cells are of mesodermal or neural crest cell origin.

Taking the data collected above, I suggest two hypotheses: 1. Smooth muscle positive cells in the distal cushions are, as in chick, neural crest cells; 2. Smooth muscle positive cells in the turtle, as in the chick, divide the outflow into the aortae and pulmonary artery. The location of smooth muscle positive cells in the distal outflow tract in the turtle parallel the positions of cardiac neural crest cells in the chick. In the chick, neural crest cells express smooth muscle markers early, but will not necessarily differentiate into smooth muscle. In stages 15 and 18, the sections through the distal outflow tract contain smooth muscle cells that create a U-shaped prong separating the right aorta and the left aorta/pulmonary artery. In the proximal outflow tract, the cushions are oriented differently and the smooth muscle cells are located underneath the myocardial muscle cells. These cells appear to be like the neural crest cells of chick in that they are found in the area between where the right aorta and left aorta/pulmonary artery would cinch. Yet, these cells could also be mesodermal cells forming the base of the outflow tract. The smooth muscle cells in the proximal outflow tract are more diffuse that those of the distal outflow tract.

More studies in turtles and reptiles in general, are needed in order to identify whether the smooth muscle cells in the outflow tract are indeed cardiac neural crest cells. The current methods of lineage tracing cells in chick have not been successful in the turtles due to the high mortality rate of embryos when the shells are opened. New techniques are needed to lineage trace of neural crest cells in turtles. In this study I was

limited in the material available. In order to have a full study of heart development in turtles I would need additional stages in between stage 15 and stage 18 so as to clearly visualize the orientation of cushions that form in the proximal versus distal outflow. By adding more intermediate stages, I could track the offset development of the cushions to: 1. determine the shape of the cushions that create the three outflow arteries; 2. distinguish between the conal and truncal sections of the outflow. Within these cushions, I would use additional smooth muscle markers to observe potential neural crest cells in the distal versus proximal cushions. Additionally, I would collect embryos post stage 23 in order to follow through the final septation of the proximal outflow tract. At later stages, I would focus on the differences between smooth muscle positive cells in the outer walls of the outflow arteries versus the smooth muscle cells found at the seams of the aortae and pulmonary artery.

Once the contribution of neural crest to heart septation in reptiles has been fully studied, a better picture of the role of these cells throughout the evolution of vertebrate heart formation could be developed. Among vertebrates, reptiles are an important intermediate. They have completely septated outflow tracts but (except for crocodiles) have an incomplete division of the ventricle. Reptiles also have two aortae, a condition that is more similar to amphibians like *Xenopus* that have an incomplete division of the outflow tract. An understanding of the role of CNC cells in reptiles would provide us

with critical information on the evolution of septation in the outflow tract in vertebrates and the potential role that neural crest has played in this major evolutionary step.

5. Conclusion

In this thesis I focused on the development of the cardiac vasculature and outflow tract in vertebrates. Specifically, I observed the contributions of neural crest cells in zebrafish and the outflow tract development of the turtle *Trachemys scripta*. The data collected from the zebrafish offers a new perspective on the cardiac neural crest cell population in vertebrates with an unseptated heart. Although the outflow tract is not septated in the fish, neural crest cells still contribute to the walls of the outflow vasculature and innervation of the heart. The data collected from my turtle heart specimens is the first study to look specifically at outflow septation in reptiles. I studied the cell morphology in the outflow tract as well as the early striated and smooth muscle markers being expressed by cells within the outflow tract. By using the chick for comparison, we hypothesized that the cells contributing to outflow septation could be cardiac neural crest cells. With the work completed in this thesis, I offer a new perspective on the contributions of cardiac neural crest cells in vertebrate outflow development.

The model that I propose of cardiac neural crest cells in zebrafish is congruent with the models of cardiac neural crest cells in chick and mouse. With the exception of outflow septation, neural crest cells are found within the ventral aorta, gill arch arteries and cardiac innervation. The previous studies of cardiac neural crest in zebrafish never

addressed the neural crest contribution to the outflow vasculature. As zebrafish are a widely used developmental model, these studies prevented a comparative investigation of neural crest cells in the vasculature of fishes to other vertebrates. Unlike the bilaterally symmetrical orientation of gill arch arteries in adult fishes, chick and mouse aortic arch arteries are sites of considerable change. CNC cells do not only contribute to the smooth muscle of the aortic arch arteries in chick and mouse, but are required for the remodeling of the aortic arch arteries from bilaterally symmetrical structures into the asymmetrical structures seen in adults. In fish, we first observe functional changes in the vascular arches of lungfish. Although the arch arteries are bilaterally symmetrical, the shunts in the lungfish vasculature create a division between gill and lung respiration. The aortic arch arteries appear to be an area of much change in vertebrate outflow vasculature.

In order to study how neural crest cells effect change in the outflow vasculature of vertebrates, more models of CNC cell development are needed. Studies in reptilian heart development have focused on chamber formation and heart looping (Bertens et al., 2010; Bremer, 1928). The data I have collected enhances previous work by focusing on outflow tract development and includes a study of cell types within the outflow tract. When these data are compared to data in chick, I hypothesized that neural crest cells contribute to reptilian outflow tract septation. When sufficient technological methods to trace neural crest cells and increase embryo viability, I will be able to test this

hypothesis. Following neural crest cells in turtles would allow the comparison of migration patterns of neural crest cells between reptiles, amphibians and birds. I emphasize these three vertebrates because there is variability between them in aortic arch artery patterning. *Xenopus* aortic arch arteries are surrounded by cardiac neural crest cells and undergo some remodeling but the arteries still remain bilateral as adults. Turtles and chicks have asymmetrical arch arteries, although their patterns of arch artery persistence vary: Turtles have two aortae and chicks only have one.

Additional information that can be gleaned from more turtle studies is difference between the distal and proximal outflow tract. Neural crest cells migrate into the distal portion of the outflow tract of chick and mouse, into the cushions arising from the mesoderm termed the secondary heart field. Studies in Zebrafish and Xenopus have shown that the distal portion of the outflow tract also arises from secondary heart field cells, mesoderm that adds post heart tube formation (Hami et al., 2011; Lee and Saint-Jeannet, 2011; Nevis et al., 2013). Yet, in fish and amphibians, the outflow portion is not divided into the conus and truncus structures seen in chick (although the outflows are often referred to as the conotruncus). If neural crest cells in turtle migrate into the same areas as they do in chick and mouse, we can see if the distinction between the conus and truncus is conserved. If these two areas of the outflow tract are conserved, it raises questions about the relationship of the secondary heart field and neural crest cells. In chick, the neural crest cells are responsible for proper addition of the secondary heart

field (Hutson et al., 2010). With more comparative models, questions are raised such as: Do neural crest cells create two separate identities for the conus and truncus population? Are the secondary heart field cells responsible for attracting neural crest cells into the cardiac outflow? Comparative models allow us to focus on specific contributions of cardiac neural crest development, such as aortic arch artery formation and give us tools to separate neural crest functions.

Studies of cranial development have shown the labile nature of cranial neural crest cells in creating the vertebrate skull. As the vertebrate skull changed so did the vasculature, yet similar studies of neural crest cells in outflow vasculature have not been conducted. With the data presented in this thesis, I hope to emphasize the importance of including intermediate vertebrate models of outflow vasculature in studies of cardiac neural crest development.

Studies of outflow development could focus on the three aspects of cardiac neural crest influence; signaling within the arch arteries, signaling to the secondary heart field and the act of outflow septation. When focusing on the influence of neural crest cells within the arch arteries, we have a model of bilaterally symmetrical arches in fish and those that are asymmetrical in reptiles, avians and mammals. An important signaling pathway in arch artery remodeling in mouse and chick is the endothelin pathway (Clouthier et al., 2003a; Miller et al., 2003). Without endothelin signaling, the aortic arch arteries do not remodel properly. In zebrafish, endothelin is responsible for

cartilage development in the branchial arches (Kimmel et al., 2003). When endothelin signaling is reduced in zebrafish, it creates hypoplastic gill arch arteries in addition to reduced cartilage. Exploring the endothelin signaling pathway and its downstream effectors could provide new insights into the changes in neural crest cells direction within the pharyngeal arches.

The influence of neural crest on the addition of the secondary heart field and outflow septation could be more difficult to parse out because they appear to be interconnected. In chick, cardiac neural crest cells help to modulate Fgf signaling in the pharynx (Hutson et al., 2006). Without this Fgf modulation, the secondary heart field does not complete addition to the heart. Furthermore, neural crest cells that migrate into the outflow tract of Fgf perturbed specimens migrate unevenly, creating a single prong of neural crest cells instead of two. Zebrafish and Xenopus models could provide answers in determining whether or not there is a neural crest influence in the addition of the secondary heart field, without compounding it with septation. Neural crest signaling could also be examined for involvement in creating a separation of the secondary heart field mesodermal cells. Neural crest cells could be influencing the identity of conal and truncal precursors, and in essence, preparing the cushions to receive the neural crest cells. One could compare the secondary heart field mesoderm in Zebrafish and *Xenopus* versus the secondary heart field in chick and mouse. If there is a difference, a test could be developed to determine whether the mesoderm in turtle undergoes a similar

instructional cue. Having multiple vertebrate models in which to compare neural crest and cardiac development is vital to the study of changes within the cardiac neural crest lineage.

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Biography

Martha Alonzo-Johnsen was born on July 15th, 1982 in the windy city of Chicago, Illinois. She attended the University of Chicago and in 2004 earned a Bachelor of Arts in Biology. As an undergraduate, she worked in the lab of Dr. Martin Feder studying Heat Shock Proteins in *Drosophila melanogaster*. Upon graduation, she received an NIH post-baccalaureate fellowship to work in the lab of Dr. Victoria Prince at the University of Chicago. There she studied pancreas development in Zebrafish.

She arrived at Duke University in 2006 and joined the Kirby lab in 2007 to study the development of cardiac neural crest cells in zebrafish. She has presented posters at the Weinstein heart development conference in Amsterdam and Chicago and given talks to the Duke Biology Department regarding her work on turtles and zebrafish. While at Duke, she was awarded a Duke Biology Grant-in-Aid, Sigma Xi mini grant and a NIH-NIGMS Diversity Supplement.

Martha has published the following book chapter:

Alonzo, M., Kirby, M., Smith, K., 2012. Epigenetic Interactions of the Cardiac Neural Crest in: Hallgrímsson, B.H., Brian K (Ed.), Epigenetics: linking genotype and phenotype in development and evolution, 1 ed. University of California Press, San Francisco 181-200