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Connecting Teratogen-Induced Congenital Heart Defects to Neural Crest Cells and Their Effect on Cardiac Function

Ganga H. Karunamuni¹, Pei Ma², Shi Gu², Andrew M. Rollins², Michael W. Jenkins^{1,2}, and Michiko Watanabe^{1,*}

¹Department of Pediatrics, Case Western Reserve University School of Medicine, Case Medical Center Division of Pediatric Cardiology, Rainbow Babies and Children's Hospital, Cleveland OH 44106

²Department of Biomedical Engineering, Case Western Reserve University School of Engineering, Cleveland OH 44106

Abstract

Neural crest cells play many key roles in embryonic development, as demonstrated by the abnormalities that result from their specific absence or dysfunction. Unfortunately, these key cells are particularly sensitive to abnormalities in various intrinsic and extrinsic factors, such as genetic deletions or ethanol-exposure that lead to morbidity and mortality for organisms. This review discusses the role identified for a segment of neural crest is in regulating the morphogenesis of the heart and associated great vessels. The paradox is that their derivatives constitute a small proportion of cells to the cardiovascular system. Findings supporting that these cells impact early cardiac function raises the interesting possibility that they indirectly control cardiovascular development at least partially through regulating function. Making connections between insults to the neural crest, cardiac function, and morphogenesis is more approachable with technological advances. Expanding our understanding of early functional consequences could be useful in improving diagnosis and testing therapies.

I. Introduction

Neural crest cells and their derivatives have been the focus of much study for good reason. Their normal development is crucial for many organ systems, including the nervous, cardiovascular, and gastrointestinal systems [reviewed in (Hall, 2008)]. It is very feasible that neural crest cell dysfunction may be responsible for, or at least influence, the severity of many congenital defects. In this review we focus on the role of neural crest cells in cardiac (NC) development and examine the evidence for the role of neural crest derivatives in regulating cardiac function. Abnormal cardiac function may be a common node in the trajectory to CHDs no matter what the original cause. We acknowledge that abnormalities in neural crest are unlikely to be the only cause of CHDs (van den Hoff and Moorman, 2000),

*Corresponding Author: Dr. Michiko Watanabe, Department of Pediatrics, Case Western Reserve University, School of Medicine, 11100 Euclid Avenue, Cleveland OH 44106, 216-844-7361, mxw13@case.edu.

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but an understanding of their influences via abnormal function could elucidate the etiology of many craniofacial and cardiac defects.

I.A. Vulnerability of neural crest cells

Neural crest ablated chicken embryos or mouse embryos in which genes were specifically deleted or mutated in neural crest cells have phenotypes that strikingly resemble those observed in individuals with 22q11 deletion or DiGeorge and related syndromes [reviewed in (Goldmuntz and Emanuel, 1997; Lammer and Opitz, 1986; Walker and Trainor, 2006; Wurdak et al., 2006)]. These phenotypes include craniofacial, glandular, and cardiac defects. Individuals with 22q11 deletion presumably have the same segment of DNA missing from one chromosome (allele) in every cell of the body throughout embryogenesis and yet, it seems that those features requiring normal neural crest cells were particularly drastically affected. These results support that neural crest cells may be more vulnerable than other cells within the developing embryo. If they are more vulnerable, why are they? Speculations are that they are more sensitive to genetic or environmental insult because they proliferate more, travel greater distances, and are multipotent. All of these activities of NCCs require an intact ability to sensitively sense and respond to multiple environmental cues. Furthermore, all of these activities require high energy expenditures that could be impacted by changes in metabolism.

I.B. Neural crest cells and cardiac function

Another set of intriguing findings is that disturbance of neural crest cells has an impact on cardiac function well before NCCs are known to enter the heart to perform their well-known role in outflow tract septation (Conway et al., 1997a; Waldo et al., 1999). Thus, the potential exists for the abnormal cardiac function by itself to be an important early influence in contributing to the development of cardiovascular defects (CHDs). In addition, because so many embryonic and extraembryonic tissues rely heavily on the cardiovascular system for nutrition, oxygenation, and removal of waste, this early abnormal cardiovascular function sets the stage for neural crest abnormalities to indirectly contribute to a global perturbation of development.

I.C. Overview

This review will cover the evidence for the influential role of neural crest cells in development, including their role in controlling cardiovascular structure and function. During the many years of studying the etiology of CHDs, evidence for the following connections have accumulated: (1) NCC disturbances lead to CHDs, (2) teratogen exposure including ethanol exposure induces CHDs, (3) ethanol disturbs NCCs, (4) both ethanol and NCC disturbances affect cardiovascular function, and (5) abnormal cardiovascular function can lead to CHDs. The purpose of this review is to discuss these connections. In reviewing this topic, we expect to reveal where further investigations are required to support these connections and link the connections to each other. This review will also touch on currently available technologies to probe structure and function of the embryonic heart that could be deployed to facilitate these studies.

II. Neural crest disturbances cause congenital defects, including heart defects

The study of neural crest cells, including the subset termed cardiac neural crest cells, has been greatly advanced by the use of animal models, particularly avian and murine embryos. Avian models have been highly useful because they are easily manipulated surgically and specific regions of neural crest can be marked, deleted, and/or dissected for culture. Spatiotemporal specific manipulations are possible. This model is limited to genetic modification using virus infection or electroporation methods. Genetic modification of avian models is underway, but still remains limited. The mouse model has the advantage of being amenable to genetic manipulation, especially with the advent of mouse lines facilitating conditional deletion and overexpression that allow reporter expression and other changes in gene expression, specifically in neural crest cells. Segment or temporally specific manipulations are more difficult at the moment. The zebrafish model has been more recently used for the study of neural crest cell development and provides a powerful model for rapidly advancing the field.

This section will start with background regarding the lineage and role of NCCs that was obtained using mainly avian and mouse models, with a more detailed discussion of NCCs in abnormal heart development.

II. Neural crest cell disturbances cause congenital defects

II.A. Insights from avian models

Neural crest cells were discovered to be important for cardiac development many years ago by neural crest ablation studies and neural crest cell lineage studies conducted primarily using avian embryos [reviewed in (Brown and Baldwin, 2006; Keyte and Hutson, 2012; Kirby, 2007)]. In a series of neural crest ablation studies, the loss of a specific subset of NCCs was discovered to lead to CHDs (Besson et al., 1986; Hutson and Kirby, 2003; Kirby et al., 1983; Kirby et al., 1985). This subset is located in a transitional region of the cranial and trunk neural crest, starting from the middle of the otic placode and ending at the caudal border of somite 3 (Rhombomeres 6,7,8; caudal to the myelencephalon) of a Hamburger and Hamilton (Hamburger and Hamilton, 1992) stage 9–10 chicken embryo. This subset, termed cardiac neural crest (CNC) cells, is responsible for aorticopulmonary septation, the muscular layer of certain regions of the great vessels, and the parasympathetic cardiac ganglia (Kirby, 1987; Kirby et al., 1983; Kirby and Stewart, 1983; Le Lievre and Le Douarin, 1975). At a stage often used for CNC ablations (stage 9–10) the heart is just beginning to beat, that is the cardiomyocytes activate and regularly contract. In these CNC studies the animal models of choice were avian species (chicken and quail), because their mature hearts have four chambers and they are accessible to microsurgies and other experimental interventions. It is possible to specifically ablate segments of the neural crest *in ovo* or in *ex ovo* shell-less culture by using dissection needles, electrothermal cauterization needles, or laser ablation.

The role and fate of the avian cardiac neural crest cells (CNCCs) has been thoroughly investigated through the use of quail-chick chimeras (Bronner, 2012; Dupin et al., 2001; Miyagawa-Tomita et al., 1991) and extensive ablation studies (Bockman and Kirby, 1984;

Kirby et al., 1983; Kirby and Waldo, 1990). Chimeras were created by dissection of a neural crest segment from one species usually quail and grafted onto a chicken embryo (Le Douarin et al., 2008; Teillet et al., 2008). Quail cells have markers that distinguish them from chick cells. When quail tissues were stained for DNA with the Feulgen stain or others, it was noted that interphase nuclei had nucleolar associated heterochromatin that appeared as dark spots within the nuclei in quail cells but not in chick cells. Another useful marker is the antibody QCPN that binds to quail cells but not to chick [reviewed in (Griswold and Lwigale, 2012)]. The most well known of the CHDs associated with CNC ablation are the aortic arch artery anomalies, incomplete outflow tract septation, and great vessel alignment defects accompanied by ventricular septal defects (VSDs).

NCCs (Fig. 1) are known to leave the neural crest as it fuses to form a neural tube (stage 8–9 in chicken embryos), migrate as a “collective” of cells to the circumpharyngeal crest, and those from the CNC eventually fill pharyngeal arches 3, 4 and 6 as proliferating mesenchymal cells (Theveneau and Mayor, 2012a; (Theveneau and Mayor, 2012b). Some envelop the aortic arch arteries and differentiate into smooth muscle and fibroblast components of the vessel walls (Le Lievre and Le Douarin, 1975). Their presence is necessary for the normal morphogenesis of the bilaterally symmetric aortic arch arteries into their final asymmetric form (Bockman et al., 1989; Bockman et al., 1987; Kirby et al., 1997). A subset of NCC derivatives are known to separate from other pharyngeal mesenchymal cells and migrate into the cranial portion of the outflow tract within the endocardial connective tissue and join other mesenchymal cells. These CNCCs appear to concentrate at the sites of fusion of the spiraling endocardial cushions within the outflow tract and are absent when the CNCCs are ablated (Kirby et al., 1983; Waldo et al., 1998). Their absence is presumed to be the cause of the failure of the embryonic outflow tract to separate into the aortic and pulmonary trunks leading to, in the extreme case, persistent truncus arteriosus (PTA)[reviewed in (Kirby, 1987)]. Exactly how these cells have an impact on the fusion of the outflow tract cushions is not known. Is it just a matter of the CNCCs contributing to the volume of the cushions and/or are they instrumental in allowing the dissolution of the endocardium and the fusion of the endocardial cushions? The role of NCCs in the “myocardialization” of the outflow tract has been proposed (van den Hoff et al., 1999; Waldo et al., 1998; Waller et al., 2000; Ya et al., 1998). Myocardialization occurs as a late step in outflow tract septation when the myocardium grows into the connective tissue between the aortic and pulmonary trunks.

Neural crest cells also contribute to the cardiac ganglia and other neurons that innervate the heart (Kirby and Stewart, 1983; Verberne et al., 1998) and play a role in the development of the thymus, thyroid, and parathyroid (Bockman and Kirby, 1984). These roles could also have an indirect impact on heart function but probably during later stages. The cardiac ganglia do not differentiate until 5–10 days of incubation (stages 26–36) and mature between 11 days of incubation (stage 37) to hatching (Baptista and Kirby, 1997). The glands develop later [reviewed in (Romanoff, 1960)] with the thyroid reaching its two-lobed structure at day 5 of incubation (stages 26–27) and showing colloid-filled follicles, histological evidence of secretory function, by the 10th or 11th day (stages 36–37).

While the cardiac and great vessel defects are most easily detected at post-septation stages, CNC ablation has been shown to have consequences to cardiac morphology much earlier. These changes were already visible by stage 14 in chicken embryos and included a shorter abnormally shaped conotruncus and incomplete looping (Leatherbury et al., 1990b; Yelbuz et al., 2002). Other reported early defects were the uneven endocardial jelly distribution and disorganized myofibrils (stage 14–18) [(Waldo et al., 1999; Yelbuz et al., 2002)]. At later stages decreased myocardial volume and compact layer were detected after neural crest ablation (Yelbuz et al., 2003). The early functional disturbances caused by CNC ablation will be discussed in Section IV.A. These changes are attributed to the effect of CNC ablation on the anterior and secondary heart fields.

Another important set of observations regarding CNC ablation is that flexion/torsion of the entire embryo, as well as ventral thoracic wall closure, are altered (Manner et al., 1996). However, these defects did not correlate with cardiac or aortic arch defects in this study. Nonetheless, further studies on the association of embryo flexion/torsion to cardiac defects may be warranted, because of the association of spinal anomalies with cardiac defects (Basu et al., 2002; Liu et al., 2011; Shen et al., 2013).

II.B. Insights from murine models

In mouse embryos, assessing the effects of segment-specific deletion of neural crest is not yet possible. However, it is possible to study spontaneous mutations or to use engineered mouse lines to genetically delete genes or alter expression of genes in neural crest cells and thus to specifically kill or impair NCCs at various times during their development.

II.B.1. Fate mapping the mouse cardiac neural crest cells—In comparison to the avian system, the investigation of the mammalian cardiac neural crest cell (CNCC) population has proven difficult. One limitation was the inability to specifically label mammalian CNCC, either by transplantation or injection, that was compatible with long-term survival of the embryo. Injection of lineage tracers into embryos either *ex utero* or cultured *in vitro* (Fukiishi and Morriss-Kay, 1992; Serbedzija et al., 1992) showed that CNCCs travel through the pharyngeal arches much like their avian counterparts, but failed to demonstrate a morphogenic role due to lack of cell viability or sustained embryo survival. Molecular markers have been used to label the initial migratory population of migrating CNCCs (Conway et al., 1997b; Conway et al., 1997c; Lo et al., 1997; Means and Gudas, 1997), but these studies could not track the complete migratory and functional route of the mammalian CNCCs.

The use of transgenic mouse lines however has yielded somewhat promising results in the area of CNCC lineage tracing, with the caveat that the tracing marks NCCs in general and cannot selectively trace CNCC derivatives. Thus for mouse it is assumed that the NCCs from the level of the otic placode and down several somites are CNCCs. One transgenic line expressed β -galactosidase (β -gal) from the connexin 43 promoter (Lo et al., 1997) in order to map mammalian NCC fate, but unfortunately expression of the transgene did not extend beyond mid to late gestation. This model also suffered from the possibility that molecular

markers may vary in expression after genetic or teratogenic manipulation, which would complicate the mapping of NCCs in an experimental setting.

A more successful method of labeling NCCs employed a two-component genetic system based on Cre-Lox recombination. Commonly used promoters to drive Cre recombinase include Wnt1-cre, Pax3-cre, P0-cre, and PlexinA2-cre (Brown et al., 2001; Jiang et al., 2000; Lee et al., 1997). For the most widely used Wnt1-cre model for example, one component was the transgene expressing Cre recombinase under the control of the Wnt1 promoter (Danielian et al., 1998). Wnt1 has been shown to be expressed in the neural plate, the dorsal neural tube, and the initial wave of migrating NCCs. As the NCCs migrate away from the neural tube, they stop expressing Wnt1 and will not express it at later stages (Echelard et al., 1994). The second component was a conditional reporter gene R26R, which expressed β -gal from the Rosa26 locus with Cre-mediated recombination (Soriano, 1999). The Rosa 26 locus is expressed ubiquitously throughout development and is not as susceptible to experimental manipulation (Zambrowicz et al., 1997). With Cre-mediated recombination, the transcript from the Rosa 26 promoter will encode the β -gal protein. Thus, descendants of the cells that go through recombination will be positive for β -gal, even after Wnt1 expression has been extinguished. In this manner, NCCs in the mammalian model were mapped throughout migration and outflow tract morphogenesis, where the cells were found to follow a fate similar to what was demonstrated in the avian model. In summary, mouse NCCs enveloped the pharyngeal arch arteries before populating the aorticoplmonary septum and outflow cushions and surrounding other organs such as the thymus, thyroid, and parathyroid (Jiang et al., 2000).

Some differences in CNCC migration between avians and mice have been noted. For example in quail-chick chimeras, CNCCs travel both subendocardially and submyocardially within the OFT cushion before vessel septation, while mouse CNCCs only migrate subendocardially into the OFT cushions (Waldo et al., 1999). CNCCs in the mouse also extend to the distal conus whereas avian CNCCs only just reach the conus (Waldo et al., 1999). These variations are most likely due to species-specific differences in morphogenesis, as well as timing of developmental events.

II.B.2. Ablation of the mouse neural crest—The first detailed study of mice with genetically ablated neural crest cells (Porras and Brown, 2008) was performed relatively recently compared to similar experiments in the avian model (Kirby et al., 1983), due to problems in sustaining embryo health after the procedure. This limitation was circumvented by using a two component genetic system for the temporal-spatial ablation of the mouse neural crest cells (NCCs). Porras and Brown used the Pu TK selector mouse line (Chen et al., 2004), which expressed a truncated version of the herpes simplex virus-1 thymidine kinase (TK) after Cre recombination. Ganciclovir (GCV) inhibited DNA synthesis in TK-expressing cells by preventing the incorporation of guanosine into elongating DNA, thus leading to cell death (Chen et al., 2004). A Wnt1-Cre mouse line (Danielian et al., 1998) was then used to drive Cre recombinase expression in NCCs. Thus, Porras and Brown were able to ablate mouse NCCs at critical time-points in cardiac development to different extents by varying the timing of GCV administration and the number of GCV doses. Their studies ultimately demonstrated that neural crest ablation in mouse resulted in a spectrum of

cardiovascular and aortic arch patterning defects, whose severity depended on the extent of the ablation. At maximum ablation efficiency, mouse embryos developed PTA, abnormal heart tube looping leading to DORV, VSDs, misaligned great vessels, and anomalies in the patterning of aortic arch vessels, similar to what was shown in the chick ablation model (Farrell et al., 1999; Kirby et al., 1983; Waldo et al., 1999). However, the authors did not observe inflow tract malformations in their study, whereas inflow defects such as tricuspid atresia, straddling tricuspid valve, and double inlet left ventricle were reported in the chick neural crest ablation model (Besson et al., 1986; Nishibatake et al., 1987). It remains to be determined whether the absence of this particular set of defects may be explained due to species-specific differences or individual features of the two different ablation models.

Other mouse models in which various genes were ablated or mutated specifically in neural crest cells have exhibited CHDs similar to those observed in ablation models discussed in the last paragraph. These include NCC-specific deletion of ERK pathway (Newbern et al., 2008), TGF β signaling (Wurdak et al., 2005), and cytokine signaling components (Escot et al., 2013), and Rac1 deletion (Thomas et al., 2010) and deletion of both Foxd2 and Pax3 (Nelms et al., 2011). Deletions that alter tissues in the neural crest environment, rather than the NCCs themselves, have also resulted in similar CHD phenotypes [reviewed in (Papangeli and Scambler, 2013)].

II.B.3. Mouse models of neural crest related human syndromes—CNCC ablation phenocopies several cardiac and craniofacial defects associated with DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS) (Kirby and Bockman, 1984; Van Mierop and Kutsche, 1986). Both syndromes are often caused by a chromosomal 22q11.2 deletion, and defects include interrupted aortic arch, persistent truncus arteriosus (PTA), ventricular septal defects (VSDs), Tetralogy of Fallot (TOF), double outlet right ventricle (DORV), and abnormal thyroid and thymus development (Shprintzen, 2008). A mouse model with the deletion of proximal mouse chromosome 16 is regarded as the first model of 22q11 deletion syndrome (Lindsay et al., 1999). Tbx-1, a T-box transcription factor, was one of the genes located within the deleted region, and further studies indicated that Tbx-1 insufficiency reproduced cardiovascular defects associated with DGS (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Tbx-1 is expressed in the pharyngeal ectoderm, endoderm and secondary heart field (SHF), which comprise the tissues surrounding neural crest cells, but is not actually expressed in the NCCs themselves (Garg et al., 2001; Vitelli et al., 2002). Still, deleting Tbx-1 altered signaling within the NCC environment and altered NCC migration (Calmont et al., 2009; Kochilas et al., 2002; Vitelli et al., 2002). Conditional deletion of Tbx-1 and several of its downstream signaling components resulted in aortico-pulmonary defects and interrupted aortic arch (Calmont et al., 2009; Xu et al., 2004). These findings underscore the importance of non-cell autonomous signaling on neural crest cell development.

Mice that expressed lower levels of FGF-8, a target of Tbx-1, also exhibited defects associated with DGS and VCFS (Brown et al., 2004; Frank et al., 2002; Hu et al., 2004; Moon et al., 2006; Vitelli et al., 2002; Zhang, 2005). While CNCCs migrate into the pharynx, FGF-8 is normally expressed in the pharyngeal ectoderm, endoderm, and splanchnic mesoderm. Diminished FGF-8 expression is thought to alter CNCC migration

and apoptosis (Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003). Recently, FGF-8 was found to act as a chemokine for CNCCs (Sato et al., 2011) and thus may influence both the survival and migratory timing of this cell population. In light of this, FGF-8 signaling has, not surprisingly, been shown to play a crucial role in aortic arch and OFT development, alignment, and septation (Park, 2006; Watanabe et al., 2010).

It appears therefore that Tbx-1 is an important candidate in regulating the progression of DGS and VCFS, along with other genes that are misregulated due to loss of Tbx-1, including Hes1 (van Bueren et al., 2010), retinoic acid signaling (Roberts et al., 2006), other Tbx members, Tbx-2 and Tbx-3 (Mesbah et al., 2012), and a host of other possibilities (Aggarwal and Morrow, 2008). There are however additional genes, apart from Tbx-1, within the 22q11 region that phenocopy many of the defects associated with DGS and VCFS when deleted or mutated. Other potential modifiers include DiGeorge Critical Regions (DGCR) 6 and 8 (Hierck et al., 2004; Shiohama et al., 2003), Crkl (Guris et al., 2001), and Erk2/MAPK1 (Corson, 2003; Newbern et al., 2008). To add to the complexity, there are also genes outside the 22q11 region that when deleted or mutated result in DiGeorge-like phenotypes (Busse et al., 2011; Newbern et al., 2008).

Many of the defects associated with CHARGE syndrome overlap with anomalies seen in DGS and VCFS patients (Siebert et al., 1985). Thyroid and parathyroid development is often affected in CHARGE syndrome patients who can also exhibit aortic arch and OFT defects related to abnormal NCC development. Mutations in CHD7, a member of the chromodomain helicase DNA binding family, have been found in over 90% of the CHARGE patient population (Bergman et al., 2011). CHD7 mouse mutants were found to model CHARGE syndrome as well (Layman et al., 2010). CHD7 has several critical functions, one of which is to regulate transcriptional genes such as Sox9, Twist, and Slug in NCCs (Bajpai et al., 2010). It is also involved in chromatin remodeling, thus indicating that it might have a role as an epigenetic regulator of NCCs during embryo development (Liu and Xiao, 2011).

Cardiac defects associated with Alagille syndrome, which involves mutations in the Notch signaling pathway, include Tetralogy of Fallot and VSDs (Eldadah et al., 2001; Krantz et al., 1999; McElhinney et al., 2002). Notch receptors and ligands are typically expressed in the developing OFT and aortic arch arteries (High et al., 2007; Loomes et al., 2002). In mouse, the targeted inhibition of Notch signaling in NCCs led to defects in aortic arch patterning, pulmonary artery stenosis, and VSDs (High et al., 2007; Loomes et al., 2002). These studies suggested that Notch was required for the differentiation of CNCCs into the smooth muscle of the aortic arch vessels but not for NCC proliferation and their subsequent migration into the OFT.

A number of genes that play a role in the progression of Waardenburg syndrome have been implicated in NCC development. An important player is the transcription factor Pax3, for which mutations have been found in Waardenburg syndrome patients (Tassabehji et al., 1994). Similarly, the Splotch mutant mouse, where Pax3 has been deleted, phenocopies many of the defects related to Waardenburg syndrome (Conway et al., 1997b; Conway et al., 1997c). Loss of Pax3 negatively impacts CNCC migration, leading to fewer cells

contributing to the development of the aortic arches and aortico-pulmonary septation, and thus resulting in PTA and other outflow tract defects (Bradshaw et al., 2009; Conway et al., 2000). Pax3 has ultimately been demonstrated to be critical to the formation of NCC progenitor cells (Olaopa et al., 2011), thus setting the stage early on for the generation of congenital defects in the case of deletion or mutation.

Noonan syndrome is characterized by CHDs, such as pulmonary stenosis and septal anomalies (Burch et al., 1993; Marino et al., 1999), and is often caused by heterozygous mutations in PTPN11, a gene encoding the protein-tyrosine phosphatase SHP2 (Tartaglia et al., 2002; Zenker et al., 2004). Noonan-associated mutations in PTPN11 increase SHP2 activity and signaling through the RAS-MAPK (ERK) pathway (Fragale et al., 2004; Keilhack et al., 2005; Martinelli et al., 2008; Tartaglia et al., 2007). This increase in signaling is the opposite for what is detected for 22q11 deletion and related syndromes. In animal models, deletion of PTPN11 in NCCs does not affect cell migration or proliferation of NCCs, but leads to an absence of these cells within the OFT, resulting in PTA as well as septal and great vessel defects (Nakamura et al., 2009).

In summary, there are several mouse models of aberrant NCC development that phenocopy human diseases involving significant heart defects. There are additional signaling pathways that can regulate CNCC signaling, migration, and development, e.g., semaphorin 3C signaling (Feiner et al., 2001), Foxc1 and Foxc2 (Kume et al., 2001; Seo and Kume, 2006), the TGF/BMP family (Dudas and Kaartinen, 2005; Dudas et al., 2006; Dudas et al., 2004; Kaartinen et al., 2004), and the retinoic acid pathway (Jiang et al., 2002; Kubalak et al., 2002), amongst many others. It is likely that these pathways all contribute to normal CNCC events, with cross-talk occurring among several molecular mechanisms that are cell-autonomous as well as extrinsic. Any upset in the exquisite balance of these pathways could have a deleterious impact on CNCC development and thus trigger the progression of related CHDs. On the other hand, with such a complex network, there is the opportunity for compensatory mechanisms that would modify the outcome. Further investigation of the intricate regulation of these pathways is required in order to prevent and/or rescue associated CHDs, which often require surgical correction and can result in lowered quality of life for the patient, recurrent cardiac events, and even death in some cases.

II.C. Insights from the zebrafish model

Zebrafish CNCCs have very similar fates to those of mouse and avians, except for their apparent ability to become cardiomyocytes (Li et al., 2003; Sato and Yost, 2003). This animal model with the advantages of accessibility to imaging and molecular intervention throughout development could provide new insights into the mechanisms of NCCs in the etiology of CHDs. Morpholino technology allows temporal control of gene knock out in zebrafish and was used to show that *Tbx1* knockdown causes cardiac defects and depressed cardiac function [e.g., (Zhang et al., 2010)]. However, tissue specific control of gene expression is less straightforward with this method. The use of the heat shock protein promoter constructs and the activation of gene expression with lasers allows exquisite spatiotemporal control of gene expression (Halloran et al., 2000; Shoji and Sato-Maeda,

2008). This technique would be valuable in studying neural crest cell developmental biology in the complex environment of the living embryo.

III. Teratogens disturb neural crest cells

Many teratogens have significant effects on the early development of the cardiovascular system. These include environmental toxins and medications, many which affect neural crest cells [(Grimes et al., 2008) and reviewed in (Rosenquist, 2013) (van Gelder et al., 2010)]. Two important classes of teratogenic mechanisms discussed by van Gelder et al.(2010), “folate antagonism” and “neural crest disruption” may be at work in inducing CHDs. Because of their sensitivity and the multiple roles of neural crest cells in normal development, these cells have been used in culture to screen for teratogens (Greenberg, 1982).

Too low or too high levels of retinoic acid (RA) signaling are known to cause cardiac and other effects very similar to those observed after neural crest ablation (Pan and Baker, 2007; Sinning, 1998; Zile, 2004). Lithium, elevated homocysteine, and ethanol may work through a common pathway, the one carbon cycle, that is rescued by folate and other compounds [(Han et al., 2009; Linask and Laties, 1973; Linask and Huhta, 2010) and reviewed in (Rosenquist, 2013; van Gelder et al., 2010)]. This link to a common pathway suggests that an understanding of how one teratogen causes defects may elucidate a general mechanism that might be activated by other teratogens.

In this section, we will focus our attention on the consequences of ethanol exposure during development. Teratogenic effects of ethanol continue to be of significant clinical concern and have been extensively investigated both in the human population and a variety of animal models.

III. A. Prenatal ethanol exposure leads to congenital defects

Exposure of embryos and fetuses to ethanol has long been known to cause growth retardation, and craniofacial and neurobehavioral defects [reviewed in (Clarren and Smith, 1978; Jones and Smith, 1973)]. These consequences have been classified as fetal alcohol syndrome (FAS) and the broader umbrella term fetal alcohol spectrum disorder (FASD). Individuals are identified with FAS if they have these criteria: (1) growth deficiency, (2) specific facial features (narrower eye opening, reduced philtrum, thin upper lip), (3) structural and/or functional central nervous system impairments, and (4) history of maternal drinking during pregnancy (Hoyme et al., 2005). Those with a subset of these criteria are diagnosed as having FASD.

FASD is a worldwide epidemic with an incidence of at least 1% of live births (CDC, 2011; May and Gossage, 2001). Estimations of the prevalence of FASD in school age children in the USA and Europe are 2–5% (May et al., 2009). These estimations do not count miscarriages, stillbirths, and infant death, thus likely underestimating the effects of prenatal ethanol exposure (Bailey and Sokol, 2011).

For FAS, the most severe of the FASDs, 2–7 cases are diagnosed per 1,000 live births in the USA. Among these individuals, the incidence of CHDs is >20%. These CHDs include ASDs, VSDs, pulmonary stenosis, valvular defects, and conotruncal defects (Burd et al., 2007). The reported incidences of CHDs are likely to be underestimations, due to sampling bias, underdiagnosis, misdiagnosis, and selection bias. The bias may result because those embryos and fetuses with heart defects are more likely to die before birth than those without and would not be counted.

Even though the effects of prenatal ethanol exposure are generally known to be bad and women are usually reminded before pregnancy as well as during pregnancy of these negative consequences, the incidence of alcohol consumption during pregnancy has not declined (CDC, 2011). This is partly due to the fact that many women have unintended pregnancies [half in the USA (Finer and Henshaw, 2006)] and by the time they determine that they are pregnant, critical developmental events such as early heart development (10 weeks of gestation) have already occurred. The financial costs of ethanol use during pregnancy are enormous and for FAS alone estimated in the billions per year [reviewed in (Popova et al., 2011)]. The devastating long term cost to the affected individual and their families and society cannot be measured in financial costs alone. To find a way to alleviate if not completely reverse the consequences would be of great value. To this end, the etiology of ethanol-induced congenital defects and disorders and potential therapies are studied in animal models.

The similarity in FAS and DiGeorge (22q11 deletion) craniofacial phenotypes with the neural crest ablation models was the basis for the idea that NCCs may be negatively affected in both FAS/FASD and DiGeorge and related syndromes (Kirby and Bockman, 1984; Sulik et al., 1986; Van Mierop and Kutsche, 1986). Others have raised the connection between FAS and Down syndrome, in that there is an overlap in the defects and affected molecular pathways (Solzak et al., 2013). Individuals with the syndromes overlap in phenotype with microcephaly and other cranial alterations, short stature, and cardiac septal defects. The study comparing mouse models of these syndromes documented similar cranial structural defects using micro CT scans, molecular changes in expression of molecules involved in signaling pathways (Dyrk1a and Rcan1), and elevated levels of the active form of an apoptosis enzyme cleaved Caspase 3 (Solzak et al., 2013). Thus the study of FAS might also elucidate common mechanisms found in many congenital syndromes that include CHDs and that have apparently disparate initiating causes.

III.B. Ethanol exposure leads to congenital heart defects

Congenital heart defects (CHDs) due to ethanol exposure have not been studied as intensively as craniofacial and neurodevelopmental defects, despite a reported prevalence rate of 28.6% for comorbid CHDs and FASD (Burd et al., 2007). CHDs associated with alcohol consumption during pregnancy [e.g. vavuloseptal and conotruncal defects (Grewal et al., 2008)] are among the most life-threatening and require surgical correction.

Animal models have provided strong evidence that CHDs can occur with ethanol exposure very early. The resulting CHDs observed in avian embryos at the earliest stages were wider tubular hearts and cardia bifida (failure of the heart fields to fuse) and delayed and abnormal

cardiac looping (Ross and Persaud, 1986; Serrano et al., 2010). In the same study, pre-cardiac expression of several molecules, *Hex* and *Islet1* mRNA, important in cardiac induction were affected as well, indicating that there is a potential for early steps in cardiac differentiation to be abnormal that could very well impact heart function as early as the tubular heart stages. In another study, the mouse embryo heart at E9 (12 hours after ethanol exposure) was smaller and abnormal in shape, the endocardial cushions of the outflow tract and atrioventricular (AV) canal were smaller, and the alignment of the AV canal was abnormal (Daft et al., 1986). A delay in tubular heart formation was noted in a study of rat embryos exposed to ethanol *in utero* (Ross and Persaud, 1986). Rat embryos incubated *in vitro* in ethanol or its metabolized form acetaldehyde from E9.5 were growth retarded with small branchial arches, and neural tube defects and abnormal hearts (Giavini et al., 1992).

Later stage heart defects (at normal stages post-septation) that were noted in ethanol exposed embryos from animal models were a variety of ventricular septal defects, atrial septal defects, and defects involving the great vessels [(Beauchemin et al., 1984; Bruyere and Stith, 1993; Daft et al., 1986; Fang et al., 1987; Webster et al., 1984) and reviewed in (Ruckman, 1990)]. Valve defects were noted at later stages. Mouse embryos exposed to ethanol *in utero* at gastrulation also exhibited thinner ventricular walls and reduced trabeculation when assayed at post-septation stages (Serrano et al., 2010). The range and severity of CHDs differ with time and dose of ethanol administration, species, and lines/strains within species.

Our studies have revealed that quail endocardial cushion size at stage 19 and atrioventricular valve leaflet volume at stage 34/35 decreases with a single ethanol exposure at gastrulation (Karunamuni et al., 2014). An interesting but complicating finding was that the torsion and flexion of the whole embryo at stage 19 was also abnormal in a proportion of the ethanol treated embryos that makes assessment of structure and function more difficult.

Zebrafish embryos exposed to ethanol during gastrulation and early heart differentiation caused abnormal heart looping, smaller chamber size, and abnormal endocardial cushions at the atrioventricular junction (Sarmah and Marrs, 2013).

Another important aspect of heart development, cardiovascular function, has also been shown to be affected by ethanol-exposure (Table 3). Most of the studies were conducted to assess function at post-septation stages. The few that included analysis of younger stages were all conducted in avian embryos at late looping stages (stage 18/19) and showed that a variety of functional parameters were already affected, including contractility, output, and calcium transients. This area of inquiry into function warrants further investigation to support these promising findings. As mentioned later (Section V), abnormal function has already been shown to lead to CHDs.

The mouse, avian, and zebrafish models provide evidence that cardiac defects result from ethanol exposure at gastrulation stages and that the effect of ethanol could impact the heart very early in its development.

III.C. Ethanol exposure affects neural crest cells

III.C.1. Ethanol and NCCs—Prenatal ethanol exposure at a variety of developmental stages has been shown to disturb many aspects of neural crest development. Even low doses of ethanol exposure (equivalent to one drink) significantly altered the cellular activity of cranial and trunk neural crest cells from *Xenopus* embryos assayed *in vitro* (Czarnobaj et al., 2014). Two of the many mechanisms that may alter NCC number and migratory behavior are discussed next.

III.C.2. Ethanol and NCC cell death—Neural crest cells normally undergo cell death at stage 10–13 in the chicken embryo, with the highest levels between rhombomere 3–5 that are the region of the otic placode and above (Graham et al., 1993; Graham et al., 1996). These cells would normally migrate into pharyngeal arches 2–3. This region overlaps with the cranial region of the CNC (otic placode to somite 3) that migrate into branchial arches 3, 4, and 6 (Fig. 1).

Alcohol causes elevated levels of apoptosis in cranial NCCs [(Cartwright and Smith, 1995; Cartwright et al., 1998; Dunty et al., 2001; Kotch and Sulik, 1992a; b; Rovasio and Battiatto, 2002; Sulik et al., 1981) and reviewed in (Graham et al., 1996) and Smith et al., in this issue] that may explain the craniofacial and other defects resembling those described for FAS/FASD. Several key pathways have been identified to be associated with NC cell death. Sonic hedgehog (Shh) expression and signaling was selectively disrupted with ethanol exposure levels that caused NCC cell death and craniofacial defects in chicken embryos (Ahlgren et al., 2002). In the same study, experimental administration of exogenous Shh rescued the ethanol-induced NCC cell death and craniofacial growth defects. What is upstream of Shh effects is not yet known. Another mechanism for increased apoptosis was traced to an almost immediate (within seconds) induction of an intercellular calcium transient at ethanol exposures as low as 9mM (Debelak-Kragtorp et al., 2003; Garic-Stankovic et al., 2006). This transient was the result of activation of the pertussis toxin-sensitive heterotrimeric G protein, G protein-coupled receptor, and subsequent activation of a phosphoinositide 3-kinase (PI3K) (Garic-Stankovic et al., 2005). More recent work from this group points to the calcium-activated calmodulin kinase II (CaMKII) as the culprit that sustains the calcium transient effect selectively within neural crest cells and leads to their death (Garic et al., 2011). They identified a rapid (<60 seconds) and selective enrichment of activated phosphoCaMKII (Thr286) in the NCCs after ethanol exposure. When this activation was inhibited by CaMKII inhibitors, the excess apoptosis was prevented. The expression of dnCaMKII inhibited apoptosis, while induced expression of caCaMKII enhanced apoptosis. The calcium chelator BAPTA also inhibited apoptosis. This same mechanism was found in zebrafish (Flentke et al., 2014a; Flentke et al., 2014b).

It is clear that ethanol induces higher than normal levels of apoptosis in NCCs that are important for craniofacial development. Thus molecular mechanisms similar to those outlined by others for inducing apoptosis of the more cranial neural crest may be at work in the immediately adjacent CNCCs.

III.C.3. Ethanol and L1—Another proposed mechanism for ethanol to interfere with NCC development is that it intervenes in the function of the cell adhesion molecule L1 by directly binding to L1 that is present on the surface of NCCs and their derivatives. L1 (L1CAM) is a membrane bound neural cell adhesion molecule of the immunoglobulin superfamily that is critical for cell-cell and cell-substrate adhesions in neurons [reviewed in (Schafer and Frotscher, 2012)]. It is highly expressed on neurons in developing neural systems including neural crest cells. L1 plays an essential role in the chain migration and differentiation of neural crest cells (Anderson et al., 2006; Turner et al., 2009).

Ethanol inhibits L1 function by binding directly to L1 through a small hydrophobic pocket on its extracellular domain (Arevalo et al., 2008; Dou et al., 2011; Ramanathan et al., 1996), redistributing L1 within the membrane on lipid rafts (Tang et al., 2011), and interfering with both L1 mediated signaling (Bearer, 2001; Tang et al., 2006; Yeane et al., 2009), and L1 mediated cell-cell adhesions (Ramanathan et al., 1996). This mode of interference by ethanol would be predicted to rapidly impede migration of neural crest cell derivatives over the substrate and other cells and alter the signaling required for neural crest cells to regulate their proliferation and find their way to their final destinations. NCCs have been shown *in vivo* and *in vitro* to alter their morphology and show reduced migratory behavior after ethanol exposure [e.g., (Rovasio and Battisto, 2002)].

IV. Neural crest cell disturbances lead to abnormal function

IV.A. NCCs regulate early as well as late cardiac function

Studies using CNC ablated chicken embryos have revealed changes in cardiac function in early as well as late stages (Table 2). An intriguing hypothesis has developed from the studies supporting that disruption of the NC leads to early abnormalities in cardiac function.

Cardiac function abnormalities have been detected in late stage (incubation days 11 and 15, equivalent to stages 37 and 41) CNCC-ablated chicken embryos (Creazzo, 1990; Creazzo et al., 1997; Godt et al., 1998; Hatcher et al., 1999; Nosek et al., 1997). These included reduced myocardial calcium transients, L-type calcium currents, and caffeine-stimulated calcium transients. The latter indicated abnormalities of the sarcoplasmic reticulum. Late functional changes after the heart normally achieves its 4-chambered morphology (stage 30–32) were expected in order to accommodate the abnormal structural changes in neural crest cell ablated embryo hearts. Studies of cardiac function at earlier stages suggested that the functional deficits preceded the CNCC-ablation induced structural defects of the conotruncus and great vessels.

A surprising finding was that removal of CNCCs had an impact on early cardiac function (stage 14–16) 24–28 hours before the CNCCs derivatives begin to enter the outflow tract (stage 25 in chicken embryos) (Farrell et al., 2001; Leatherbury et al., 1991; Leatherbury et al., 1993; Leatherbury et al., 1990b; Tomita et al., 1991). Cardiac contractility was depressed with reduced shortening fraction and ejection fraction, at stage 18 (68–72 hours or 3 days of incubation). No changes in heart rate, stroke volume, or cardiac output were noted. Calcium transients were depressed in NCC ablated chicken embryos at stage 18 (Waldo et al., 1999). Altered EC coupling and the proliferation rate in the myocardium was increased

and abnormalities in the arrangement of myofibrils were also noted as early as stage 14 (Waldo et al., 1999). Authors suggested that the changes were compensated by ventricular dilation.

In vitro studies provided evidence that the depressed calcium transients could be mimicked by culturing the stage 12 heart with the foregut/pharynx in close proximity and rescued with the neural crest cell replacement and by adding anti-FGF (Farrell et al., 1999; Farrell et al., 2001). The only FGF component that appeared to be present at the right time and place for signaling from the foregut to the heart forming fields and the heart was FGF8.

A proposed hypothesis (Waldo et al., 1999) is that FGF8 induced signaling is normally modified by the presence of neural crest mesenchymal derivatives intercepting the signal to the secondary and anterior heart field. When neural crest cells are depleted, there are not enough of them to regulate FGF signaling and the signaling becomes inappropriately high in the secondary heart field, leading to effects on cardiac differentiation and eventually to early cardiac function. The location of the FGF expressing foregut endoderm and the neural crest cells derivatives compared to the secondary and anterior heart fields in the pharyngeal arches is consistent with this hypothesis (Fig. 2). This theory for the genesis of the early changes in heart function may serve as a significant explanation for the many detrimental effects that result in CHDs.

IV.B. NCCs are involved in cardiac conduction system maturation

Another unexpected finding was that CNCCs are required for cardiac conduction system maturation. Lineage-tracing of CNCCs in chicken embryos revealed that a subset leave the crest late, enter through the inflow, and surround the proximal ventricular conduction system (Gurjarpadhye et al., 2007). Lineage labeled NCCs were also mapped to the proximal cardiac conduction system in mouse embryos (Nakamura et al., 2006). CNCC-ablated chicken embryo hearts had a broader proximal conduction system (common bundle and bundle branches) and were slower to transition from an immature pattern of conduction (base to apex) to a mature pattern (apex to base) (Gurjarpadhye et al., 2007). The hypothesis is that CNCCs enwrap the ventricular conduction system and secrete connective tissue to insulate the conduction system cardiomyocytes from neighboring working cardiomyocytes. The absence of CNCCs resulted in a thicker common bundle that was not clearly delineated from surrounding cardiomyocytes. These results led to the authors' proposal that this structural change in the conduction system may in part explain the delay in differentiation and compromised ability to conduct the impulse to the apex.

IV. C. Mouse neural crest and cardiac function

As predicted from the similarities between the findings from avian and mouse neural crest cell studies, abnormalities in heart function have been detected in mouse embryos with disturbed neural crest cells. One study assessed early cardiac function using echocardiography (UBM-Doppler system) and found that embryos with neural crest cell specific disruption of a BMP receptor had reversed flow in the dorsal aorta and reduced cardiac output at E11.5 and E12.5 that may explain the embryonic lethality at E12.5 (Nomura-Kitabayashi et al., 2009). Most studies have focused on later function (at the time

of ventricular septation), probably due to technical limitations. The Splotch mutant mouse (described in section II.B.) that has a Pax3 mutation and is deficient in neural crest cells has been shown to have reduced cardiac function by video microscopy imaging of 13.5 dpc heart *in situ* and reduced calcium transients, as assessed in ventricular muscle strips from as embryos early as 12.5 dpc (Conway et al., 1997a). The latter assay revealed that excitation-contraction coupling was impaired likely by the 3.2 fold reduction in Ca^{2+} current. These findings could account for the depressed cardiac function and the embryonic lethality.

At later stages, neural crest may impact innervation of the heart. The SHP-2 deletion in mouse embryo neural crest cells resulted in abnormal cardiac sympathetic innervations that would have consequences for regulating cardiac function later in development (Lajiness et al., 2014).

Labeled NCC fate studies show that NCC derivatives map to valves of the aortic and pulmonary arteries and the septal leaflets of the AV valves in later fetal stages and in the adult (Nakamura et al., 2006). Studies of various NCC deficiency models show abnormal semilunar valves of the aortic and pulmonary arteries and abnormal flow, as detected by echocardiography at post-septation stages (Jain et al., 2011). Thus NCC disruption, in addition to altering valve development by the indirect mechanism of altering shear force on valve primordia, has the potential to directly alter later valve leaflet differentiation by depriving developing leaflets of the NCC component.

Another late impact of neural crest ablation would be by disturbing thyroid development and consequently thyroid function. Neural crest cells are known to be critical for normal thyroid development, in that neural crest ablation or disturbance results in the absence, abnormal location, or small size of the thyroid and other glands in the neck region [e.g. (Newbern et al., 2008) and reviewed in (Adams and Bronner-Fraser, 2009)]. The molecular mechanisms for how neural crest cells contribute to gland development are not well understood [reviewed in (Adams and Bronner-Fraser, 2009; Fagman and Nilsson, 2010)]. Once the thyroid differentiates, it normally secretes critical thyroid hormone at highly controlled levels postnatally. Thus abnormal thyroid development could lead to abnormal thyroid hormone levels that are known to affect adult cardiac function (Danzi and Klein, 2014; Sharma et al., 2013) and could very well affect the heart of the embryo at least in later stages. It is not clear how early impaired thyroid function of the embryo would have an influence on cardiac development in the embryo.

IV. D. Zebrafish neural crest ablation and abnormal cardiac function

NC ablation in zebrafish results in abnormal cardiac function that included heart rate. Even for those ablated embryos with normal heart rates there was decreased stroke volume, ejection fraction, and cardiac output, as assessed by videomicroscopy images (Li et al., 2003).

IV. E. Summary of NCC and function

Avian, mouse, and zebrafish all manifest abnormal cardiac function during development when NCCs are disturbed. Given that abnormal function by itself has been shown to alter

heart and great vessel morphogenesis (see section V), these findings bring up the possibility that CNCC ablation contributes to structural defects by altering cardiac function.

Many questions still remain. A thorough analysis of cardiac function after neural crest cell ablation in animal models is still warranted because we do not know specifically in what way early cardiac function is compromised. Exactly how does the CNCC impact cardiac function through regulating FGF signaling of the secondary/anterior heart field cells? How much of the late stage functional defects are due to altering other NCC derivatives, the glands (thyroid), and parasympathetic innervations. Do individuals with 22q11 syndrome and other NCC-associated defects or mouse models of NCC defects have compromised early cardiac function? What is the capacity of the embryo to compensate for abnormal function? How does abnormal cardiac function affect the embryo overall and the extraembryonic vasculature within the yolk or placenta?

V. Abnormal cardiac function leads to congenital heart defects

A number of studies have demonstrated that abnormal cardiac function and blood flow is likely to lead to CHDs. In children, abnormal calcium handling which is critical for cardiac contraction has been linked to CHDs (Vittorini et al., 2007). Gross disturbance of cardiovascular function in animal models by constricting a major cardiac vessel, atrial chamber, or vitelline vessel results in obvious CHDs [e.g., (Hogers et al., 1997; 1999; Hove et al., 2003; Lucitti et al., 2006; Sedmera et al., 2002; Tobita and Keller, 2000)]. Disruption of the heart beat also results in abnormal heart development and early death of the embryo (Bartman et al., 2004; Bi et al., 1999; Fritz-Six et al., 2003; Koushik et al., 2001). Increasing or decreasing the viscosity of the blood that would impact blood flow and shear force (Lucitti et al., 2007; Vermot et al., 2009) also results in cardiac defects. Disturbing the electrical activation of the heart can also alter cardiac development (Chi et al., 2010; Linask and Linask, 2010).

The zebrafish has been a useful model for the study of the relationship between blood flow, molecular, and cellular changes, and CHDs, particularly atrioventricular valve abnormalities (Auman et al., 2007; Bartman et al., 2004; Berdougou et al., 2003; Hove et al., 2003; Vermot et al., 2009). Zebrafish embryos (48–96 hpf), in which myocardial function was compromised genetically or pharmacologically, failed to form valve structures at the atrioventricular junction. The retrograde flow fraction (RFF), the fraction of the cardiac cycle during which there is retrograde flow, was quantified using high speed imaging in transparent zebrafish embryos between 22–28 hours post-fertilization (hpf) prior to valve formation. The largest RFF was detected in the AV canal prior to valve formation coincident with the restricted RNA expression of three shear force responsive genes, *notch1b*, *klf2a*, and *bmp4*. When the RFF was reduced by reducing blood viscosity by inhibiting blood cell formation or by inducing the heart to beat at higher than normal pace with drug applications, the expression of some of the shear forces responsive genes were reduced in expression and valve leaflets remained thick and immature, likely due to the reduced endocardial cell number and abnormal endocardial cell shape.

VI. Putting it all together for future directions

Based on links provided by the literature, we propose the following scenario (Fig. 3). Ethanol kills or compromises the CNCCs that undergo excess apoptosis or fail to migrate and proliferate, resulting in a paucity of NCC mesenchyme in the branchial arches 3, 4, and 6. This results in abnormal signaling received by the heart forming fields and abnormal cardiomyocyte differentiation. The abnormal cardiomyocytes fail to pump blood adequately to provide enough proper biomechanical stimulation to allow epithelial-mesenchymal transition (EMT) and other aspects of endocardial cushion development. This result leads to abnormal endocardial cushion volumes and content, and eventually abnormal valve leaflet differentiation.

In the avian embryo, ethanol exposure that mimics binge drinking at gastrulation can cause molecular misexpression in the precardiac mesoderm, as early as gastrulation stages that are crucial for heart development (Serrano et al., 2010). At this stage, specification of cardiac mesoderm is ongoing, including the primary, anterior, and secondary heart fields (Kelly and Buckingham, 2002), and the newly identified region that gives rise to pulse-generating cardiomyocytes (Bressan et al., 2013). There is therefore the potential that cardiac function could be compromised by ethanol exposure as early as at the first heart beat, by directly affecting cardiomyocyte differentiation (Fig. 3, question mark).

Ethanol clearly has effects on neural crest cells that appear to be particularly sensitive to any type of experimental intervention. More NCCs die after ethanol exposure at cranio-caudal levels that likely include the CNCCs. Thus, it would be important to determine whether the NCCs moving to and populating the pharyngeal arches 3, 4, and 6 are either fewer in numbers and/or not functioning properly to regulate the differentiation of cardiomyocytes. Engineered mice might help in this analysis. If abnormal cardiomyocytes have difficulty in contracting at a level that would keep up with the demands of the embryo and the extraembryonic tissues, these tissues could be compromised in their development. Should the cardiomyocytes fail to function properly in pumping blood, the endocardial cushions may not form properly due to abnormal shear forces, cardiomyocytes and supporting cells may not differentiate properly because of abnormal stress and strain, tissues throughout the embryo might suffer by not receiving proper level of circulation, and there would be growth delay.

There is the possibility that ethanol has direct effects on abnormal heart function by altering cardiomyocytes and abnormal endocardial cushion formation (boxes with question marks in Fig. 3). Direct effects of ethanol have been documented in altering propagation and maturation of embryonic cardiomyocytes in culture (Adickes et al., 1993), as well as adult cardiomyocyte function (Hajnoczky et al., 2005; Iacovoni et al., 2010; Walker et al., 2013). Ethanol has also been shown to promote expression of molecules for EMT in cancer cells (Elamin et al., 2014; Forsyth et al., 2010).

VI. A. Analysis of cardiac function in the early embryo using newly developed technology

Few studies have delved into the early functional abnormalities in embryos (Tables 2 and 3). The investigation of functional parameters in sculpting normal and abnormal cardiac

structures has been missing from many studies, because of the difficulty in accurately measuring these parameters in small beating hearts that are undergoing rapid morphogenesis. Recent advances in technology allow us to simultaneously and longitudinally follow structure and function of the embryonic heart [reviewed in (Gu et al., 2011; Jenkins et al., 2012)]. This ability allows us to reexamine the genesis of CHDs in detail and discover novel ways to intervene.

Many of the key biophysical parameters can now be assessed in the developing avian heart. Our capabilities are to capture (1) activation sequence and impulse conduction patterns using sensitive high resolution optical mapping using voltage sensitive dyes (Wang et al., 2014) with correction for the 3-D structure (Ma et al., 2014) and (2) contraction and flow patterns *in vivo* using optical coherence tomography (OCT) (Gu et al., 2012; Jenkins et al., 2010; Peterson et al., 2012). Pulsed Infrared light can also be used to pace the embryonic heart to probe electrophysiological parameters (Wang et al., 2014). OCT can also be used for structural analysis, i.e., (3) quantification of endocardial cushion size and shape using 3-D reconstruction of OCT and optical coherence microscopy (OCM) images *in vivo* and in fixed tissues (Garita et al., 2011; Karunamuni et al., 2014), and (4) the identification of mesenchymal cells and other structures within the endocardium using OCM (Garita et al., 2011; Jenkins et al., 2012). These and other advanced imaging technologies could be deployed in the study of the role of function in creating CHDs.

VI.B. Predicting and preventing CHDs

One of the practical goals of studying CHD etiology is to be able to find ways to predict the severity of defects to prepare both the family and the medical team. Early detection is useful for decision making by both parties. Even with timely detection of the defect, current strategies used to alleviate CHDs are not always successful and multiple surgeries are often needed. If we better understood the genesis of each CHD, we could improve the ability to predict which individuals would react positively or negatively to a particular treatment strategy.

Several limitations prevent us from going earlier than necessary to detect cardiac abnormalities in humans. One is that we have a limited ability to detect cardiac function at the time of most active heart morphogenesis (up to 10 weeks after conception in humans). With the use of the latest echocardiography technology and the transvaginal approach, the heart rate of the embryo/fetus can be detected as early as 5 weeks of gestation and echocardiography can be performed as early as 10–13 weeks (American Institute of Ultrasound in Medicine, 2011; Johnson and Simpson, 2007; Mirza et al., 2012). The functional parameters that indicate heart disease are abnormal ductus venosus waveforms and tricuspid regurgitation, but these indicators may not be enough to definitively predict CHDs or to indicate healthy heart development.

A second important goal is to find a method to alleviate or prevent CHDs. The prevention of CHDs in the human population has been elusive. Many compounds appear to prevent the effects of ethanol on cardiovascular development if administered at the proper time. These compounds include retinoic acid, curcumin, vitamin C, folic acid by itself, or folic acid with myoinositol (Memon and Pratten, 2009; Serrano et al., 2010; Twal and Zile, 1997; Wang et

al., 2012). A potentially promising avenue is the alleviation or prevention of CHDs by the use of folate that is already being used in supplements and as additives to staple foods to prevent neural tube defects [reviewed in (Czeizel et al., 2011; Czeizel et al., 2013; Osterhues et al., 2013; Peake et al., 2013)]. The preclinical data show promise for folate/folic acid to reduce CHDs resulting from many causes, including ethanol exposure.

Folate/folic acid is recommended widely to women during the periconceptional stage for its ability to prevent neural tube defects (NTDs). The level currently recommended for women of child bearing age and especially for those planning pregnancy is 400 µg/day [reviewed in (Wilson et al., 2003)]. The recommendation for women who have already given birth to a child with NTD is to take a higher dose of folic acid starting even earlier, at least a month ahead of conception. In many countries including the USA folic acid is added to staple foods like bread and flour. The incidence of NTDs has dramatically dropped since folic acid use has become common and represents a rare success story in the prevention of congenital birth defects (Committee on Genetics, 1999). The explanation for why NTDs occur and how folate works to prevent NTDs is under investigation.

Less well known and less definitive is the effect of folic acid on reducing CHDs (Bailey and Berry, 2005). Several studies have shown that periconceptual intake of multivitamins containing folic acid (800 µg) reduces that incidence of CHDs [reviewed in (Czeizel et al., 2013)]. Animal models of FAS/FASD have been useful in probing the mechanisms for ethanol-induced congenital defects and in investigating potential preventative therapeutics, such as folic acid, choline, and myoinositol (Ballard et al., 2012; Cole et al., 2012; Sant'Anna and Tosello, 2006; Serrano et al., 2010; Smith, 1997; Wilson and Cudd, 2011). There are promising findings that folic acid either alone or in combination with other compounds reduces the incidence of ethanol-induced NTDs and CHDs in animal models (Sarmah and Marrs, 2013; Serrano et al., 2010).

The question remains whether assessing early cardiac function could be useful to predict the severity of defects and the efficacy of prevention strategies.

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Abbreviations

ASD	atrial-septal defect
AV	atrioventricular
CHDs	congenital heart defects
CNC	cardiac neural crest
CNCC	cardiac neural crest cells
EMT	epithelial mesenchymal transition
FAS	fetal alcohol syndrome

FASD	fetal alcohol spectrum disorder
DGCR	DiGeorge critical regions
DGS	DiGeorge syndrome
NC	neural crest
NCC	neural crest cells
RA	retinoic acid
VCFS	Velocraniofacial syndrome
VSD	ventricular septal defect

References

- Abu-Issa R, Smyth G, Smoak I, Yamamura K, Meyers EN. Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development*. 2002; 129(19):4613–4625. [PubMed: 12223417]
- Adams MS, Bronner-Fraser M. Review: the role of neural crest cells in the endocrine system. *Endocr Pathol*. 2009; 20(2):92–100. [PubMed: 19377845]
- Adickes ED, Mollner TJ, Makoid MC. Ethanol-induced teratogenic alterations in developing cardiomyocytes in culture. *Alcohol Alcohol Suppl*. 1993; 2:283–288. [PubMed: 7748312]
- Aggarwal VS, Morrow BE. Genetic modifiers of the physical malformations in velo-cardio-facial syndrome/DiGeorge syndrome. *Dev Disabil Res Rev*. 2008; 14(1):19–25. [PubMed: 18636633]
- Ahlgren SC, Thakur V, Bronner-Fraser M. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. *Proc Natl Acad Sci U S A*. 2002; 99(16):10476–10481. [PubMed: 12140368]
- American Institute of Ultrasound in Medicine. AIUM practice guideline for the performance of fetal echocardiography. *J Ultrasound Med*. 2011; 30(1):127–136. [PubMed: 21193716]
- Anderson RB, Turner KN, Nikonenko AG, Hemperly J, Schachner M, Young HM. The cell adhesion molecule 11 is required for chain migration of neural crest cells in the developing mouse gut. *Gastroenterology*. 2006; 130(4):1221–1232. [PubMed: 16618414]
- Arevalo E, Shanmugasundararaj S, Wilkemeyer MF, Dou X, Chen S, Charness ME, Miller KW. An alcohol binding site on the neural cell adhesion molecule L1. *Proc Natl Acad Sci U S A*. 2008; 105(1):371–375. [PubMed: 18165316]
- Auman HJ, Coleman H, Riley HE, Olale F, Tsai HJ, Yelon D. Functional modulation of cardiac form through regionally confined cell shape changes. *PLoS biology*. 2007; 5(3):e53. [PubMed: 17311471]
- Bailey BA, Sokol RJ. Prenatal alcohol exposure and miscarriage, stillbirth, preterm delivery, and sudden infant death syndrome. *Alcohol Res Health*. 2011; 34(1):86–91. [PubMed: 23580045]
- Bailey LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. *Am J Clin Nutr*. 2005; 81(5):1213S–1217S. [PubMed: 15883454]
- Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, Chang CP, Zhao Y, Swigut T, Wysocka J. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature*. 2010; 463(7283):958–962. [PubMed: 20130577]
- Ballard MS, Sun M, Ko J. Vitamin A, folate, and choline as a possible preventive intervention to fetal alcohol syndrome. *Med Hypotheses*. 2012; 78(4):489–493. [PubMed: 22285196]
- Baptista CA, Kirby ML. The cardiac ganglia: cellular and molecular aspects. *Kaohsiung J Med Sci*. 1997; 13(1):42–54. [PubMed: 9130822]

- Bartman T, Walsh EC, Wen KK, McKane M, Ren J, Alexander J, Rubenstein PA, Stainier DY. Early myocardial function affects endocardial cushion development in zebrafish. *PLoS biology*. 2004; 2(5):E129. [PubMed: 15138499]
- Basu PS, Elsebaie H, Noordeen MH. Congenital spinal deformity: a comprehensive assessment at presentation. *Spine (Phila Pa 1976)*. 2002; 27(20):2255–2259. [PubMed: 12394903]
- Bearer CF. Mechanisms of brain injury: L1 cell adhesion molecule as a target for ethanol-induced prenatal brain injury. *Semin Pediatr Neurol*. 2001; 8(2):100–107. [PubMed: 11464956]
- Beauchemin RR Jr, Gartner LP, Provenza DV. Alcohol induced cardiac malformations in the rat. *Anat Anz*. 1984; 155(1–5):17–28. [PubMed: 6721179]
- Berdougo E, Coleman H, Lee DH, Stainier DY, Yelon D. Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development*. 2003; 130(24):6121–6129. [PubMed: 14573521]
- Bergman JE, Janssen N, Hoefsloot LH, Jongmans MC, Hofstra RM, van Ravenswaaij-Arts CM. CHD7 mutations and CHARGE syndrome: the clinical implications of an expanding phenotype. *J Med Genet*. 2011; 48(5):334–342. [PubMed: 21378379]
- Besson WT 3rd, Kirby ML, Van Mierop LH, Teabeaut JR 2nd. Effects of the size of lesions of the cardiac neural crest at various embryonic ages on incidence and type of cardiac defects. *Circulation*. 1986; 73(2):360–364. [PubMed: 3943168]
- Bi W, Drake CJ, Schwarz JJ. The transcription factor MEF2C-null mouse exhibits complex vascular malformations and reduced cardiac expression of angiopoietin 1 and VEGF. *Dev Biol*. 1999; 211(2):255–267. [PubMed: 10395786]
- Bockman DE, Kirby ML. Dependence of thymus development on derivatives of the neural crest. *Science*. 1984; 223(4635):498–500. [PubMed: 6606851]
- Bockman DE, Redmond ME, Kirby ML. Alteration of early vascular development after ablation of cranial neural crest. *Anat Rec*. 1989; 225(3):209–217. [PubMed: 2817439]
- Bockman DE, Redmond ME, Waldo K, Davis H, Kirby ML. Effect of neural crest ablation on development of the heart and arch arteries in the chick. *Am J Anat*. 1987; 180(4):332–341. [PubMed: 3425561]
- Bradshaw L, Chaudhry B, Hildreth V, Webb S, Henderson DJ. Dual role for neural crest cells during outflow tract septation in the neural crest-deficient mutant *Splotch*(2H). *J Anat*. 2009; 214(2):245–257. [PubMed: 19207986]
- Bressan M, Liu G, Mikawa T. Early mesodermal cues assign avian cardiac pacemaker fate potential in a tertiary heart field. *Science*. 2013; 340(6133):744–748. [PubMed: 23519212]
- Bronner ME. Formation and migration of neural crest cells in the vertebrate embryo. *Histochem Cell Biol*. 2012; 138(2):179–186. [PubMed: 22820859]
- Brown CB, Baldwin HS. Neural crest contribution to the cardiovascular system. *Adv Exp Med Biol*. 2006; 589:134–154. [PubMed: 17076279]
- Brown CB, Feiner L, Lu MM, Li J, Ma X, Webber AL, Jia L, Raper JA, Epstein JA. PlexinA2 and semaphorin signaling during cardiac neural crest development. *Development*. 2001; 128(16):3071–3080. [PubMed: 11688557]
- Brown CB, Wenning JM, Lu MM, Epstein DJ, Meyers EN, Epstein JA. Cre-mediated excision of *Fgf8* in the *Tbx1* expression domain reveals a critical role for *Fgf8* in cardiovascular development in the mouse. *Developmental Biology*. 2004; 267(1):190–202. [PubMed: 14975726]
- Bruyere HJ Jr, Stith CE. Strain-dependent effect of ethanol on ventricular septal defect frequency in White Leghorn chick embryos. *Teratology*. 1993; 48(4):299–303. [PubMed: 8278929]
- Bruyere HJ Jr, Stith CE. Ethyl alcohol reduces cardiac output, stroke volume, and end diastolic volume in the embryonic chick. *Teratology*. 1994; 49(2):104–112. [PubMed: 8016742]
- Burch M, Sharland M, Shinebourne E, Smith G, Patton M, McKenna W. Cardiologic abnormalities in Noonan syndrome: phenotypic diagnosis and echocardiographic assessment of 118 patients. *J Am Coll Cardiol*. 1993; 22(4):1189–1192. [PubMed: 8409059]
- Burd L, Deal E, Rios R, Adickes E, Wynne J, Klug MG. Congenital heart defects and fetal alcohol spectrum disorders. *Congenit Heart Dis*. 2007; 2(4):250–255. [PubMed: 18377476]

- Busse T, Graham JM Jr, Feldman G, Perin J, Catherwood A, Knowlton R, Rappaport EF, Emanuel B, Driscoll DA, Saitta SC. High-Resolution genomic arrays identify CNVs that phenocopy the chromosome 22q11.2 deletion syndrome. *Hum Mutat.* 2011; 32(1):91–97. [PubMed: 21120947]
- Calmont A, Ivins S, Van Bueren KL, Papangeli I, Kyriakopoulou V, Andrews WD, Martin JF, Moon AM, Illingworth EA, Basson MA, Scambler PJ. Tbx1 controls cardiac neural crest cell migration during arch artery development by regulating Gbx2 expression in the pharyngeal ectoderm. *Development.* 2009; 136(18):3173–3183. [PubMed: 19700621]
- Cartwright MM, Smith SM. Increased cell death and reduced neural crest cell numbers in ethanol-exposed embryos: partial basis for the fetal alcohol syndrome phenotype. *Alcohol Clin Exp Res.* 1995; 19(2):378–386. [PubMed: 7625573]
- Cartwright MM, Tessmer LL, Smith SM. Ethanol-induced neural crest apoptosis is coincident with their endogenous death, but is mechanistically distinct. *Alcohol Clin Exp Res.* 1998; 22(1):142–149. [PubMed: 9514299]
- CDC. Drinking while pregnant still a problem. CDC; 2011. Alcohol use among pregnant and nonpregnant women of childbearing age--United States, 1991–2005; p. 529–532.
- Chen YT, Levasseur R, Vaishnav S, Karsenty G, Bradley A. Bigenic Cre/loxP, puDeltat conditional genetic ablation. *Nucleic Acids Res.* 2004; 32(20):e161. [PubMed: 15561996]
- Chi NC, Bussen M, Brand-Arzamendi K, Ding C, Olgin JE, Shaw RM, Martin GR, Stainier DY. Cardiac conduction is required to preserve cardiac chamber morphology. *Proc Natl Acad Sci U S A.* 2010; 107(33):14662–14667. [PubMed: 20675583]
- Chuck ET, Freeman DM, Watanabe M, Rosenbaum DS. Changing activation sequence in the embryonic chick heart. Implications for the development of the His-Purkinje system. *Circ Res.* 1997; 81(4):470–476. [PubMed: 9314827]
- Chuck ET, Meyers K, France D, Creazzo TL, Morley GE. Transitions in ventricular activation revealed by two-dimensional optical mapping. *Anat Rec A Discov Mol Cell Evol Biol.* 2004; 280(2):990–1000. [PubMed: 15372433]
- Clarren SK, Smith DW. The fetal alcohol syndrome. *Lamp.* 1978; 35(10):4–7. [PubMed: 251812]
- Cole GJ, Zhang C, Ojiaku P, Bell V, Devkota S, Mukhopadhyay S. Effects of ethanol exposure on nervous system development in zebrafish. *Int Rev Cell Mol Biol.* 2012; 299:255–315. [PubMed: 22959306]
- Committee on Genetics. Folic acid for the prevention of neural tube defects. *American Academy of Pediatrics. Committee on Genetics. Pediatrics.* 1999; 104(2 Pt 1):325–327. [PubMed: 10429019]
- Conway SJ, Bundy J, Chen J, Dickman E, Rogers R, Will BM. Decreased neural crest stem cell expansion is responsible for the conotruncal heart defects within the splotch (Sp(2H))/Pax3 mouse mutant. *Cardiovasc Res.* 2000; 47(2):314–328. [PubMed: 10946068]
- Conway SJ, Godt RE, Hatcher CJ, Leatherbury L, Zolotouchnikov VV, Brotto MA, Copp AJ, Kirby ML, Creazzo TL. Neural crest is involved in development of abnormal myocardial function. *J Mol Cell Cardiol.* 1997a; 29(10):2675–2685. [PubMed: 9344762]
- Conway SJ, Henderson DJ, Copp AJ. Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant. *Development.* 1997b; 124(2):505–514. [PubMed: 9053326]
- Conway SJ, Henderson DJ, Kirby ML, Anderson RH, Copp AJ. Development of a lethal congenital heart defect in the splotch (Pax3) mutant mouse. *Cardiovasc Res.* 1997c; 36(2):163–173. [PubMed: 9463628]
- Corson LB. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development.* 2003; 130(19):4527–4537. [PubMed: 12925581]
- Creazzo TL. Reduced L-type calcium current in the embryonic chick heart with persistent truncus arteriosus. *Circ Res.* 1990; 66(6):1491–1498. [PubMed: 1693103]
- Creazzo TL, Brotto MA, Burch J. Excitation-contraction coupling in the day 15 embryonic chick heart with persistent truncus arteriosus. *Pediatr Res.* 1997; 42(6):731–737. [PubMed: 9396550]
- Czarnobaj J, Bagnall KM, Bamforth JS, Milos NC. The different effects on cranial and trunk neural crest cell behaviour following exposure to a low concentration of alcohol in vitro. *Arch Oral Biol.* 2014; 59(5):500–512. [PubMed: 24631632]

- Czeizel AE, Bartfai Z, Banhidy F. Primary prevention of neural-tube defects and some other congenital abnormalities by folic acid and multivitamins: history, missed opportunity and tasks. *Ther Adv Drug Saf.* 2011; 2(4):173–188. [PubMed: 25083211]
- Czeizel AE, Dudas I, Vereczkey A, Banhidy F. Folate deficiency and folic acid supplementation: the prevention of neural-tube defects and congenital heart defects. *Nutrients.* 2013; 5(11):4760–4775. [PubMed: 24284617]
- Daft PA, Johnston MC, Sulik KK. Abnormal heart and great vessel development following acute ethanol exposure in mice. *Teratology.* 1986; 33(1):93–104. [PubMed: 3738814]
- Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol.* 1998; 8(24):1323–1326. [PubMed: 9843687]
- Danzi S, Klein I. Thyroid Disease and the Cardiovascular System. *Endocrinol Metab Clin North Am.* 2014; 43(2):517–528. [PubMed: 24891175]
- Debelak-Kragtorp KA, Armant DR, Smith SM. Ethanol-induced cephalic apoptosis requires phospholipase C-dependent intracellular calcium signaling. *Alcohol Clin Exp Res.* 2003; 27(3):515–523. [PubMed: 12658119]
- Dlugos CA, Rabin RA. Structural and functional effects of developmental exposure to ethanol on the zebrafish heart. *Alcohol Clin Exp Res.* 2010; 34(6):1013–1021. [PubMed: 20374213]
- Dou X, Menkari CE, Shanmugasundararaj S, Miller KW, Charness ME. Two alcohol binding residues interact across a domain interface of the L1 neural cell adhesion molecule and regulate cell adhesion. *J Biol Chem.* 2011; 286(18):16131–16139. [PubMed: 21367865]
- Dudas M, Kaartinen V. Tgf-beta superfamily and mouse craniofacial development: interplay of morphogenetic proteins and receptor signaling controls normal formation of the face. *Curr Top Dev Biol.* 2005; 66:65–133. [PubMed: 15797452]
- Dudas M, Kim J, Li WY, Nagy A, Larsson J, Karlsson S, Chai Y, Kaartinen V. Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. *Dev Biol.* 2006; 296(2):298–314. [PubMed: 16806156]
- Dudas M, Sridurongrit S, Nagy A, Okazaki K, Kaartinen V. Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. *Mech Dev.* 2004; 121(2):173–182. [PubMed: 15037318]
- Dunty WC Jr, Chen SY, Zucker RM, Dehart DB, Sulik KK. Selective vulnerability of embryonic cell populations to ethanol-induced apoptosis: implications for alcohol-related birth defects and neurodevelopmental disorder. *Alcohol Clin Exp Res.* 2001; 25(10):1523–1535. [PubMed: 11696674]
- Dupin E, Real C, Ledouarin N. The neural crest stem cells: control of neural crest cell fate and plasticity by endothelin-3. *An Acad Bras Cienc.* 2001; 73(4):533–545. [PubMed: 11743601]
- Echelard Y, Vassileva G, McMahon AP. Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS. *Development.* 1994; 120(8):2213–2224. [PubMed: 7925022]
- Elamin E, Masclee A, Troost F, Dekker J, Jonkers D. Activation of the epithelial-to-mesenchymal transition factor snail mediates acetaldehyde-induced intestinal epithelial barrier disruption. *Alcohol Clin Exp Res.* 2014; 38(2):344–353. [PubMed: 24033729]
- Eldadah ZA, Hamosh A, Biery NJ, Montgomery RA, Duke M, Elkins R, Dietz HC. Familial Tetralogy of Fallot caused by mutation in the jagged1 gene. *Hum Mol Genet.* 2001; 10(2):163–169. [PubMed: 11152664]
- Escot S, Blavet C, Hartle S, Duband JL, Fournier-Thibault C. Misregulation of SDF1-CXCR4 signaling impairs early cardiac neural crest cell migration leading to conotruncal defects. *Circ Res.* 2013; 113(5):505–516. [PubMed: 23838132]
- Fagman H, Nilsson M. Morphogenesis of the thyroid gland. *Mol Cell Endocrinol.* 2010; 323(1):35–54. [PubMed: 20026174]
- Fang TT, Bruyere HJ Jr, Kargas SA, Nishikawa T, Takagi Y, Gilbert EF. Ethyl alcohol-induced cardiovascular malformations in the chick embryo. *Teratology.* 1987; 35(1):95–103. [PubMed: 3563941]
- Farrell M, Waldo K, Li YX, Kirby ML. A novel role for cardiac neural crest in heart development. *Trends Cardiovasc Med.* 1999; 9(7):214–220. [PubMed: 10881754]

- Farrell MJ, Burch JL, Wallis K, Rowley L, Kumiski D, Stadt H, Godt RE, Creazzo TL, Kirby ML. FGF-8 in the ventral pharynx alters development of myocardial calcium transients after neural crest ablation. *J Clin Invest*. 2001; 107(12):1509–1517. [PubMed: 11413158]
- Feiner L, Webber AL, Brown CB, Lu MM, Jia L, Feinstein P, Mombaerts P, Epstein JA, Raper JA. Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. *Development*. 2001; 128(16):3061–3070. [PubMed: 11688556]
- Finer LB, Henshaw SK. Disparities in rates of unintended pregnancy in the United States, 1994 and 2001. *Perspect Sex Reprod Health*. 2006; 38(2):90–96. [PubMed: 16772190]
- Flentke GR, Garic A, Hernandez M, Smith SM. CaMKII represses transcriptionally active beta-catenin to mediate acute ethanol neurodegeneration and can phosphorylate beta-catenin. *J Neurochem*. 2014a; 128(4):523–535. [PubMed: 24117889]
- Flentke GR, Klingler RH, Tanguay RL, Carvan MJ 3rd, Smith SM. An evolutionarily conserved mechanism of calcium-dependent neurotoxicity in a zebrafish model of fetal alcohol spectrum disorders. *Alcohol Clin Exp Res*. 2014b; 38(5):1255–1265. [PubMed: 24512079]
- Forsyth CB, Tang Y, Shaikh M, Zhang L, Keshavarzian A. Alcohol stimulates activation of Snail, epidermal growth factor receptor signaling, and biomarkers of epithelial-mesenchymal transition in colon and breast cancer cells. *Alcohol Clin Exp Res*. 2010; 34(1):19–31. [PubMed: 19860811]
- Fragale A, Tartaglia M, Wu J, Gelb BD. Noonan syndrome-associated SHP2/PTPN11 mutants cause EGF-dependent prolonged GAB1 binding and sustained ERK2/MAPK1 activation. *Hum Mutat*. 2004; 23(3):267–277. [PubMed: 14974085]
- Frank DU, Fotheringham LK, Brewer JA, Muglia LJ, Tristani-Firouzi M, Capecchi MR, Moon AM. An *Fgf8* mouse mutant phenocopies human 22q11 deletion syndrome. *Development*. 2002; 129(19):4591–4603. [PubMed: 12223415]
- Fritz-Six KL, Cox PR, Fischer RS, Xu B, Gregorio CC, Zoghbi HY, Fowler VM. Aberrant myofibril assembly in tropomodulin1 null mice leads to aborted heart development and embryonic lethality. *J Cell Biol*. 2003; 163(5):1033–1044. [PubMed: 14657235]
- Fukiishi Y, Morriss-Kay GM. Migration of cranial neural crest cells to the pharyngeal arches and heart in rat embryos. *Cell Tissue Res*. 1992; 268(1):1–8. [PubMed: 1323420]
- Garg V, Yamagishi C, Hu T, Kathiriyi IS, Yamagishi H, Srivastava D. *Tbx1*, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development. *Dev Biol*. 2001; 235(1):62–73. [PubMed: 11412027]
- Garic-Stankovic A, Hernandez M, Flentke GR, Smith SM. Structural constraints for alcohol-stimulated Ca²⁺ release in neural crest, and dual agonist/antagonist properties of n-octanol. *Alcohol Clin Exp Res*. 2006; 30(3):552–559. [PubMed: 16499497]
- Garic-Stankovic A, Hernandez MR, Chiang PJ, Debelak-Kragtorp KA, Flentke GR, Armant DR, Smith SM. Ethanol triggers neural crest apoptosis through the selective activation of a pertussis toxin-sensitive G protein and a phospholipase Cbeta-dependent Ca²⁺ transient. *Alcohol Clin Exp Res*. 2005; 29(7):1237–1246. [PubMed: 16046880]
- Garic A, Flentke GR, Amberger E, Hernandez M, Smith SM. CaMKII activation is a novel effector of alcohol's neurotoxicity in neural crest stem/progenitor cells. *J Neurochem*. 2011; 118(4):646–657. [PubMed: 21496022]
- Garita B, Jenkins MW, Han M, Zhou C, Vanauker M, Rollins AM, Watanabe M, Fujimoto JG, Linask KK. Blood flow dynamics of one cardiac cycle and relationship to mechanotransduction and trabeculation during heart looping. *Am J Physiol Heart Circ Physiol*. 2011; 300(3):H879–891. [PubMed: 21239637]
- Giavini E, Broccia ML, Prati M, Bellomo D, Menegola E. Effects of ethanol and acetaldehyde on rat embryos developing in vitro. *In Vitro Cell Dev Biol*. 1992; 28A(3 Pt 1):205–210. [PubMed: 1582996]
- Godt RE, Fogaca RT, Nosek TM. Alterations of myocardial contraction associated with a structural heart defect in embryonic chicks. *Adv Exp Med Biol*. 1998; 453:453–458. discussion 459. [PubMed: 9889857]
- Goldmuntz E, Emanuel BS. Genetic disorders of cardiac morphogenesis. The DiGeorge and velocardiofacial syndromes. *Circ Res*. 1997; 80(4):437–443. [PubMed: 9118473]

- Graham A, Heyman I, Lumsden A. Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development*. 1993; 119(1):233–245. [PubMed: 8275859]
- Graham A, Koentges G, Lumsden A. Neural Crest Apoptosis and the Establishment of Craniofacial Pattern: An Honorable Death. *Mol Cell Neurosci*. 1996; 8(2/3):76–83.
- Greenberg JH. Detection of teratogens by differentiating embryonic neural crest cells in culture: evaluation as a screening system. *Teratog Carcinog Mutagen*. 1982; 2(3–4):319–323. [PubMed: 6130631]
- Grewal J, Carmichael SL, Ma C, Lammer EJ, Shaw GM. Maternal periconceptional smoking and alcohol consumption and risk for select congenital anomalies. *Birth Defects Res A Clin Mol Teratol*. 2008; 82(7):519–526. [PubMed: 18481814]
- Grimes AC, Erwin KN, Stadt HA, Hunter GL, Gefroh HA, Tsai HJ, Kirby ML. PCB126 exposure disrupts zebrafish ventricular and branchial but not early neural crest development. *Toxicol Sci*. 2008; 106(1):193–205. [PubMed: 18660518]
- Griswold SL, Lwigale PY. Analysis of neural crest migration and differentiation by cross-species transplantation. *J Vis Exp*. 2012; (60)
- Gu S, Jenkins MW, Peterson LM, Doughman YQ, Rollins AM, Watanabe M. Optical coherence tomography captures rapid hemodynamic responses to acute hypoxia in the cardiovascular system of early embryos. *Dev Dyn*. 2012; 241(3):534–544. [PubMed: 22275053]
- Gu S, Jenkins MW, Watanabe M, Rollins AM. High-speed optical coherence tomography imaging of the beating avian embryonic heart. *Cold Spring Harb Protoc*. 2011; 2011(2) pdb top98.
- Guris DL, Fantes J, Tara D, Druker BJ, Imamoto A. Mice lacking the homologue of the human 22q11.2 gene CRKL phenocopy neurocristopathies of DiGeorge syndrome. *Nat Genet*. 2001; 27(3):293–298. [PubMed: 11242111]
- Gurjarpadhye A, Hewett KW, Justus C, Wen X, Stadt H, Kirby ML, Sedmera D, Gourdie RG. Cardiac neural crest ablation inhibits compaction and electrical function of conduction system bundles. *Am J Physiol Heart Circ Physiol*. 2007; 292(3):H1291–1300. [PubMed: 17172273]
- Guzman LV, Mayoral PV, Valencia JP, Pine SS, Gomez CS. Developmental pattern of the right atrioventricular septal valve leaflet and tendinous cords. *Anat Rec (Hoboken)*. 2010; 293(1):55–61. [PubMed: 19899118]
- Hajnoczky G, Buzas CJ, Pacher P, Hoek JB, Rubin E. Alcohol and mitochondria in cardiac apoptosis: mechanisms and visualization. *Alcohol Clin Exp Res*. 2005; 29(5):693–701. [PubMed: 15897712]
- Hall BK. The neural crest and neural crest cells: discovery and significance for theories of embryonic organization. *J Biosci*. 2008; 33(5):781–793. [PubMed: 19179766]
- Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY, Shoji W. Laser-induced gene expression in specific cells of transgenic zebrafish. *Development*. 2000; 127(9):1953–1960. [PubMed: 10751183]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn*. 1992; 195(4):231–272. [PubMed: 1304821]
- Han M, Serrano MC, Lastra-Vicente R, Brinez P, Acharya G, Huhta JC, Chen R, Linask KK. Folate rescues lithium-, homocysteine- and Wnt3A-induced vertebrate cardiac anomalies. *Dis Model Mech*. 2009; 2(9–10):467–478. [PubMed: 19638421]
- Hatcher CJ, Godt RE, Nosek TM. Excessive microtubules are not responsible for depressed force per cross-bridge in cardiac neural-crest-ablated embryonic chick hearts. *Pflugers Arch*. 1999; 438(3):307–313. [PubMed: 10398860]
- Hierck BP, Molin DG, Boot MJ, Poelmann RE, Gittenberger-de Groot AC. A chicken model for DGCR6 as a modifier gene in the DiGeorge critical region. *Pediatr Res*. 2004; 56(3):440–448. [PubMed: 15333760]
- High FA, Zhang M, Proweller A, Tu L, Parmacek MS, Pear WS, Epstein JA. An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. *Journal of Clinical Investigation*. 2007; 117(2):353–363. [PubMed: 17273555]

- Hogers B, DeRuiter MC, Gittenberger-de Groot AC, Poelmann RE. Unilateral vitelline vein ligation alters intracardiac blood flow patterns and morphogenesis in the chick embryo. *Circ Res.* 1997; 80(4):473–481. [PubMed: 9118477]
- Hogers B, DeRuiter MC, Gittenberger-de Groot AC, Poelmann RE. Extraembryonic venous obstructions lead to cardiovascular malformations and can be embryolethal. *Cardiovasc Res.* 1999; 41(1):87–99. [PubMed: 10325956]
- Hove JR, Koster RW, Forouhar AS, Acevedo-Bolton G, Fraser SE, Gharib M. Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. *Nature.* 2003; 421(6919):172–177. [PubMed: 12520305]
- Hoyme HE, May PA, Kalberg WO, Kodituwakku P, Gossage JP, Trujillo PM, Buckley DG, Miller JH, Aragon AS, Khaole N, Viljoen DL, Jones KL, Robinson LK. A practical clinical approach to diagnosis of fetal alcohol spectrum disorders: clarification of the 1996 institute of medicine criteria. *Pediatrics.* 2005; 115(1):39–47. [PubMed: 15629980]
- Hu T, Yamagishi H, Maeda J, McAnally J, Yamagishi C, Srivastava D. Tbx1 regulates fibroblast growth factors in the anterior heart field through a reinforcing autoregulatory loop involving forkhead transcription factors. *Development.* 2004; 131(21):5491–5502. [PubMed: 15469978]
- Hutson MR, Kirby ML. Neural crest and cardiovascular development: A 20-year perspective. *Birth Defects Research Part C: Embryo Today: Reviews.* 2003; 69(1):2–13.
- Iacovoni A, De Maria R, Gavazzi A. Alcoholic cardiomyopathy. *J Cardiovasc Med (Hagerstown).* 2010; 11(12):884–892. [PubMed: 20308914]
- Jain R, Engleka KA, Rentschler SL, Manderfield LJ, Li L, Yuan L, Epstein JA. Cardiac neural crest orchestrates remodeling and functional maturation of mouse semilunar valves. *J Clin Invest.* 2011; 121(1):422–430. [PubMed: 21157040]
- Jenkins MW, Peterson L, Gu S, Garghesha M, Wilson DL, Watanabe M, Rollins AM. Measuring hemodynamics in the developing heart tube with four-dimensional gated Doppler optical coherence tomography. *J Biomed Opt.* 2010; 15(6):066022. [PubMed: 21198196]
- Jenkins MW, Watanabe M, Rollins AM. Longitudinal Imaging of Heart Development with Optical Coherence Tomography. *IEEE Photonics Society (IPS) Journal of Selected Topics in Quantum Electronics.* 2012; (99)
- Jerome LA, Papaioannou VE. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat Genet.* 2001; 27(3):286–291. [PubMed: 11242110]
- Jiang X, Choudhary B, Merki E, Chien KR, Maxson RE, Sucov HM. Normal fate and altered function of the cardiac neural crest cell lineage in retinoic acid receptor mutant embryos. *Mech Dev.* 2002; 117(1–2):115–122. [PubMed: 12204252]
- Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. *Development.* 2000; 127(8):1607–1616. [PubMed: 10725237]
- Johnson B, Simpson LL. Screening for congenital heart disease: a move toward earlier echocardiography. *Am J Perinatol.* 2007; 24(8):449–456. [PubMed: 17992711]
- Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. *Lancet.* 1973; 302(7836):999–1001. [PubMed: 4127281]
- Kaartinen V, Dudas M, Nagy A, Sridurongrit S, Lu MM, Epstein JA. Cardiac outflow tract defects in mice lacking ALK2 in neural crest cells. *Development.* 2004; 131(14):3481–3490. [PubMed: 15226263]
- Karunamuni G, Gu S, Doughman YQ, Peterson LM, Mai K, McHale Q, Jenkins MW, Linask KK, Rollins AM, Watanabe M. Ethanol exposure alters early cardiac function in the looping heart: a mechanism for congenital heart defects? *Am J Physiol Heart Circ Physiol.* 2014; 306(3):H414–421. [PubMed: 24271490]
- Keilhack H, David FS, McGregor M, Cantley LC, Neel BG. Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. *J Biol Chem.* 2005; 280(35):30984–30993. [PubMed: 15987685]
- Kelly RG, Buckingham ME. The anterior heart-forming field: voyage to the arterial pole of the heart. *Trends Genet.* 2002; 18(4):210–216. [PubMed: 11932022]
- Keyte A, Hutson MR. The neural crest in cardiac congenital anomalies. *Differentiation.* 2012; 84(1):25–40. [PubMed: 22595346]

- Kirby ML. Cardiac morphogenesis--recent research advances. *Pediatr Res*. 1987; 21(3):219–224. [PubMed: 3562119]
- Kirby, ML. Cardiac Development. New York: Oxford University Press; 2007. Neural crest, great arteries, and outflow septation; p. 143-160.
- Kirby ML, Bockman DE. Neural crest and normal development: a new perspective. *Anat Rec*. 1984; 209(1):1–6. [PubMed: 6731866]
- Kirby ML, Gale TF, Stewart DE. Neural crest cells contribute to normal aorticopulmonary septation. *Science*. 1983; 220(4601):1059–1061. [PubMed: 6844926]
- Kirby ML, Hunt P, Wallis K, Thorogood P. Abnormal patterning of the aortic arch arteries does not evoke cardiac malformations. *Dev Dyn*. 1997; 208(1):34–47. [PubMed: 8989519]
- Kirby ML, Stewart DE. Neural crest origin of cardiac ganglion cells in the chick embryo: identification and extirpation. *Dev Biol*. 1983; 97(2):433–443. [PubMed: 6852374]
- Kirby ML, Turnage KL 3rd, Hays BM. Characterization of conotruncal malformations following ablation of “cardiac” neural crest. *Anat Rec*. 1985; 213(1):87–93. [PubMed: 4073565]
- Kirby ML, Waldo KL. Role of neural crest in congenital heart disease. *Circulation*. 1990; 82(2):332–340. [PubMed: 2197017]
- Kochilas L, Merscher-Gomez S, Lu MM, Potluri V, Liao J, Kucherlapati R, Morrow B, Epstein JA. The role of neural crest during cardiac development in a mouse model of DiGeorge syndrome. *Dev Biol*. 2002; 251(1):157–166. [PubMed: 12413905]
- Kotch LE, Sulik KK. Experimental fetal alcohol syndrome: proposed pathogenic basis for a variety of associated facial and brain anomalies. *Am J Med Genet*. 1992a; 44(2):168–176. [PubMed: 1456286]
- Kotch LE, Sulik KK. Patterns of ethanol-induced cell death in the developing nervous system of mice; neural fold states through the time of anterior neural tube closure. *Int J Dev Neurosci*. 1992b; 10(4):273–279. [PubMed: 1414440]
- Koushik SV, Wang J, Rogers R, Moskopidhis D, Lambert NA, Creazzo TL, Conway SJ. Targeted inactivation of the sodium-calcium exchanger (Ncx1) results in the lack of a heartbeat and abnormal myofibrillar organization. *FASEB J*. 2001; 15(7):1209–1211. [PubMed: 11344090]
- Krantz ID, Smith R, Colliton RP, Tinkel H, Zackai EH, Piccoli DA, Goldmuntz E, Spinner NB. Jagged1 mutations in patients ascertained with isolated congenital heart defects. *Am J Med Genet*. 1999; 84(1):56–60. [PubMed: 10213047]
- Kubalak SW, Hutson DR, Scott KK, Shannon RA. Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos. *Development*. 2002; 129(3):733–746. [PubMed: 11830573]
- Kume T, Jiang H, Topczewska JM, Hogan BL. The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev*. 2001; 15(18):2470–2482. [PubMed: 11562355]
- Kuratani SC, Kirby ML. Migration and distribution of circumpharyngeal crest cells in the chick embryo. Formation of the circumpharyngeal ridge and E/C8+ crest cells in the vertebrate head region. *Anat Rec*. 1992; 234(2):263–280. [PubMed: 1384396]
- Lajiness JD, Snider P, Wang J, Feng GS, Krenz M, Conway SJ. SHP-2 deletion in postmigratory neural crest cells results in impaired cardiac sympathetic innervation. *Proc Natl Acad Sci U S A*. 2014; 111(14):E1374–1382. [PubMed: 24706815]
- Lammer EJ, Opitz JM. The DiGeorge anomaly as a developmental field defect. *Am J Med Genet Suppl*. 1986; 2:113–127. [PubMed: 3146281]
- Layman WS, Hurd EA, Martin DM. Chromodomain proteins in development: lessons from CHARGE syndrome. *Clin Genet*. 2010; 78(1):11–20. [PubMed: 20507341]
- Le Douarin N, Dieterlen-Lievre F, Creuzet S, Teillet MA. Quail-chick transplantations. *Methods Cell Biol*. 2008; 87:19–58. [PubMed: 18485290]
- Le Lievre CS, Le Douarin NM. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J Embryol Exp Morphol*. 1975; 34(1):125–154. [PubMed: 1185098]

- Leatherbury L, Braden DS, Tomita H, Gauldin HE, Jackson WF. Hemodynamic changes. Wall stresses and pressure gradients in neural crest-ablated chick embryos. *Ann N Y Acad Sci.* 1990a; 588:305–313. [PubMed: 2357019]
- Leatherbury L, Connuck DM, Gauldin HE, Kirby ML. Hemodynamic changes and compensatory mechanisms during early cardiogenesis after neural crest ablation in chick embryos. *Pediatr Res.* 1991; 30(6):509–512. [PubMed: 1805144]
- Leatherbury L, Connuck DM, Kirby ML. Neural crest ablation versus sham surgical effects in a chick embryo model of defective cardiovascular development. *Pediatr Res.* 1993; 33(6):628–631. [PubMed: 8378123]
- Leatherbury L, Gauldin HE, Waldo K, Kirby ML. Microcinéphotography of the developing heart in neural crest-ablated chick embryos. *Circulation.* 1990b; 81(3):1047–1057. [PubMed: 2306815]
- Lee M, Brennan A, Blanchard A, Zoidl G, Dong Z, Tabernero A, Zoidl C, Dent MA, Jessen KR, Mirsky R. P0 is constitutively expressed in the rat neural crest and embryonic nerves and is negatively and positively regulated by axons to generate non-myelin-forming and myelin-forming Schwann cells, respectively. *Mol Cell Neurosci.* 1997; 8(5):336–350. [PubMed: 9073396]
- Li YX, Zdanowicz M, Young L, Kumiski D, Leatherbury L, Kirby ML. Cardiac neural crest in zebrafish embryos contributes to myocardial cell lineage and early heart function. *Dev Dyn.* 2003; 226(3):540–550. [PubMed: 12619138]
- Linask J, Laties GG. Multiphasic absorption of glucose and 3-o-methyl glucose by aged potato slices. *Plant Physiol.* 1973; 51(2):289–294. [PubMed: 16658317]
- Linask KK, Huhta J. Folate protection from congenital heart defects linked with canonical Wnt signaling and epigenetics. *Curr Opin Pediatr.* 2010; 22(5):561–566. [PubMed: 20844350]
- Linask KL, Linask KK. Calcium channel blockade in embryonic cardiac progenitor cells disrupts normal cardiac cell differentiation. *Stem Cells Dev.* 2010; 19(12):1959–1965. [PubMed: 20624035]
- Lindsay EA, Botta A, Jurecic V, Carattini-Rivera S, Cheah YC, Rosenblatt HM, Bradley A, Baldini A. Congenital heart disease in mice deficient for the DiGeorge syndrome region. *Nature.* 1999; 401(6751):379–383. [PubMed: 10517636]
- Lindsay EA, Vitelli F, Su H, Morishima M, Huynh T, Pramparo T, Jurecic V, Ogunrinu G, Sutherland HF, Scambler PJ, Bradley A, Baldini A. Tbx1 haploinsufficiency in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature.* 2001; 410(6824):97–101. [PubMed: 11242049]
- Liu Y, Xiao A. Epigenetic regulation in neural crest development. *Birth Defects Res A Clin Mol Teratol.* 2011; 91(8):788–796. [PubMed: 21618405]
- Liu YT, Guo LL, Tian Z, Zhu WL, Yu B, Zhang SY, Qiu GX. A retrospective study of congenital scoliosis and associated cardiac and intraspinal abnormalities in a Chinese population. *Eur Spine J.* 2011; 20(12):2111–2114. [PubMed: 21533853]
- Lo CW, Cohen MF, Huang GY, Lazatin BO, Patel N, Sullivan R, Pauken C, Park SM. Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. *Dev Genet.* 1997; 20(2):119–132. [PubMed: 9144923]
- Loomes KM, Taichman DB, Glover CL, Williams PT, Markowitz JE, Piccoli DA, Baldwin HS, Oakey RJ. Characterization of Notch receptor expression in the developing mammalian heart and liver. *American Journal of Medical Genetics.* 2002; 112(2):181–189. [PubMed: 12244553]
- Lucitti JL, Jones EA, Huang C, Chen J, Fraser SE, Dickinson ME. Vascular remodeling of the mouse yolk sac requires hemodynamic force. *Development.* 2007; 134(18):3317–3326. [PubMed: 17720695]
- Lucitti JL, Visconti R, Novak J, Keller BB. Increased arterial load alters aortic structural and functional properties during embryogenesis. *Am J Physiol Heart Circ Physiol.* 2006; 291(4):H1919–1926. [PubMed: 16648183]
- Ma P, Wang YT, Gu S, Watanabe M, Rollins AM, Jenkins MW. 3D correction of conduction velocity in the embryonic heart using integrated optical mapping and optical coherence tomography. *Journal of Biomedical Optics.* 2014 In Press.
- Macatee TL, Hammond BP, Arenkiel BR, Francis L, Frank DU, Moon AM. Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during

- cardiovascular and pharyngeal development. *Development*. 2003; 130(25):6361–6374. [PubMed: 14623825]
- Manner J, Seidl W, Steding G. Experimental study on the significance of abnormal cardiac looping for the development of cardiovascular anomalies in neural crest-ablated chick embryos. *Anat Embryol (Berl)*. 1996; 194(3):289–300. [PubMed: 8849676]
- Marino B, Digilio MC, Toscano A, Giannotti A, Dallapiccola B. Congenital heart diseases in children with Noonan syndrome: An expanded cardiac spectrum with high prevalence of atrioventricular canal. *J Pediatr*. 1999; 135(6):703–706. [PubMed: 10586172]
- Martinelli S, Torrieri P, Tinti M, Stella L, Bocchinfuso G, Flex E, Grottesi A, Ceccarini M, Palleschi A, Cesareni G, Castagnoli L, Petrucci TC, Gelb BD, Tartaglia M. Diverse driving forces underlie the invariant occurrence of the T42A, E139D, I282V and T468M SHP2 amino acid substitutions causing Noonan and LEOPARD syndromes. *Hum Mol Genet*. 2008; 17(13):2018–2029. [PubMed: 18372317]
- Martinsen BJ. Reference guide to the stages of chick heart embryology. *Developmental Dynamics*. 2005; 233(4):1217–1237. [PubMed: 15986452]
- May PA, Gossage JP. Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Res Health*. 2001; 25(3):159–167. [PubMed: 11810953]
- May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Dev Disabil Res Rev*. 2009; 15(3):176–192. [PubMed: 19731384]
- McElhinney DB, Krantz ID, Bason L, Piccoli DA, Emerick KM, Spinner NB, Goldmuntz E. Analysis of cardiovascular phenotype and genotype-phenotype correlation in individuals with a JAG1 mutation and/or Alagille syndrome. *Circulation*. 2002; 106(20):2567–2574. [PubMed: 12427653]
- Means AL, Gudas LJ. The CRABP I gene contains two separable, redundant regulatory regions active in neural tissues in transgenic mouse embryos. *Dev Dyn*. 1997; 209(1):59–69. [PubMed: 9142496]
- Memon S, Pratten MK. Developmental toxicity of ethanol in chick heart in ovo and in micromass culture can be prevented by addition of vitamin C and folic acid. *Reprod Toxicol*. 2009; 28(2):262–269. [PubMed: 19473809]
- Merscher S, Funke B, Epstein JA, Heyer J, Puech A, Lu MM, Xavier RJ, Demay MB, Russell RG, Factor S, Tokooya K, Jore BS, Lopez M, Pandita RK, Lia M, Carrion D, Xu H, Schorle H, Kobler JB, Scambler P, Wynshaw-Boris A, Skoultschi AI, Morrow BE, Kucherlapati R. TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome. *Cell*. 2001; 104(4):619–629. [PubMed: 11239417]
- Mesbah K, Rana MS, Francou A, van Duijvenboden K, Papaioannou VE, Moorman AF, Kelly RG, Christoffels VM. Identification of a Tbx1/Tbx2/Tbx3 genetic pathway governing pharyngeal and arterial pole morphogenesis. *Hum Mol Genet*. 2012; 21(6):1217–1229. [PubMed: 22116936]
- Mirza FG, Bauer ST, Williams IA, Simpson LL. Early fetal echocardiography: ready for prime time? *Am J Perinatol*. 2012; 29(4):313–318. [PubMed: 22143968]
- Miyagawa-Tomita S, Waldo K, Tomita H, Kirby ML. Temporospatial study of the migration and distribution of cardiac neural crest in quail-chick chimeras. *Am J Anat*. 1991; 192(1):79–88. [PubMed: 1750383]
- Moon AM, Guris DL, Seo JH, Li L, Hammond J, Talbot A, Imamoto A. Crkl deficiency disrupts Fgf8 signaling in a mouse model of 22q11 deletion syndromes. *Dev Cell*. 2006; 10(1):71–80. [PubMed: 16399079]
- Nakamura T, Colbert MC, Robbins J. Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system. *Circ Res*. 2006; 98(12):1547–1554. [PubMed: 16709902]
- Nakamura T, Gulick J, Colbert MC, Robbins J. Protein tyrosine phosphatase activity in the neural crest is essential for normal heart and skull development. *Proceedings of the National Academy of Sciences*. 2009; 106(27):11270–11275.
- Nelms BL, Pfaltzgraff ER, Labosky PA. Functional interaction between Foxd3 and Pax3 in cardiac neural crest development. *Genesis*. 2011; 49(1):10–23. [PubMed: 21254333]

- Newbern J, Zhong J, Wickramasinghe RS, Li X, Wu Y, Samuels I, Cherosky N, Karlo JC, O'Loughlin B, Wikenheiser J, Gargsha M, Doughman YQ, Charron J, Ginty DD, Watanabe M, Saitta SC, Snider WD, Landreth GE. Mouse and human phenotypes indicate a critical conserved role for ERK2 signaling in neural crest development. *Proc Natl Acad Sci U S A*. 2008; 105(44):17115–17120. [PubMed: 18952847]
- Nichols CA, Creazzo TL. L-type Ca²⁺ channel function in the avian embryonic heart after cardiac neural crest ablation. *Am J Physiol Heart Circ Physiol*. 2005; 288(3):H1173–1178. [PubMed: 15539421]
- Nishibatake M, Kirby ML, Van Mierop LH. Pathogenesis of persistent truncus arteriosus and dextroposed aorta in the chick embryo after neural crest ablation. *Circulation*. 1987; 75(1):255–264. [PubMed: 3791607]
- Nomura-Kitabayashi A, Phoon CK, Kishigami S, Rosenthal J, Yamauchi Y, Abe K, Yamamura K, Samtani R, Lo CW, Mishina Y. Outflow tract cushions perform a critical valve-like function in the early embryonic heart requiring BMPRIA-mediated signaling in cardiac neural crest. *Am J Physiol Heart Circ Physiol*. 2009; 297(5):H1617–1628. [PubMed: 19717734]
- Nosek TM, Fogaca RT, Hatcher CJ, Brotto MA, Godt RE. Effect of cardiac neural crest ablation on contractile force and calcium uptake and release in chick heart. *Am J Physiol*. 1997; 273(3 Pt 2):H1464–1471. [PubMed: 9321838]
- Olaopa M, Zhou HM, Snider P, Wang J, Schwartz RJ, Moon AM, Conway SJ. Pax3 is essential for normal cardiac neural crest morphogenesis but is not required during migration nor outflow tract septation. *Dev Biol*. 2011; 356(2):308–322. [PubMed: 21600894]
- Osterhues A, Ali NS, Michels KB. The role of folic acid fortification in neural tube defects: a review. *Crit Rev Food Sci Nutr*. 2013; 53(11):1180–1190. [PubMed: 24007422]
- Pan J, Baker KM. Retinoic acid and the heart. *Vitam Horm*. 2007; 75:257–283. [PubMed: 17368319]
- Papangelis I, Scambler P. The 22q11 deletion: DiGeorge and velocardiofacial syndromes and the role of TBX1. *Wiley Interdiscip Rev Dev Biol*. 2013; 2(3):393–403. [PubMed: 23799583]
- Park EJ. Required, tissue-specific roles for Fgf8 in outflow tract formation and remodeling. *Development*. 2006; 133(12):2419–2433. [PubMed: 16720879]
- Peake JN, Copp AJ, Shawe J. Knowledge and preconceptional use of folic acid for the prevention of neural tube defects in ethnic communities in the United Kingdom: systematic review and meta-analysis. *Birth Defects Res A Clin Mol Teratol*. 2013; 97(7):444–451. [PubMed: 23873812]
- Peterson LM, Jenkins MW, Gu S, Barwick L, Watanabe M, Rollins AM. 4D shear stress maps of the developing heart using Doppler optical coherence tomography. *Biomed Opt Express*. 2012; 3(11):3022–3032. [PubMed: 23162737]
- Popova S, Stade B, Bekmuradov D, Lange S, Rehm J. What do we know about the economic impact of fetal alcohol spectrum disorder? A systematic literature review. *Alcohol Alcohol*. 2011; 46(4):490–497. [PubMed: 21515625]
- Porras D, Brown CB. Temporal-spatial ablation of neural crest in the mouse results in cardiovascular defects. *Dev Dyn*. 2008; 237(1):153–162. [PubMed: 18058916]
- Ramanathan R, Wilkemeyer MF, Mittal B, Perides G, Charness ME. Alcohol inhibits cell-cell adhesion mediated by human L1. *J Cell Biol*. 1996; 133(2):381–390. [PubMed: 8609170]
- Roberts C, Ivins S, Cook AC, Baldini A, Scambler PJ. Cyp26 genes a1, b1 and c1 are down-regulated in Tbx1 null mice and inhibition of Cyp26 enzyme function produces a phenocopy of DiGeorge Syndrome in the chick. *Hum Mol Genet*. 2006; 15(23):3394–3410. [PubMed: 17047027]
- Romanoff, AL. *The Avian Embryo: Structural and functional development*. New York: Macmillan; 1960.
- Rosenquist TH. Folate, homocysteine and the cardiac neural crest. *Dev Dyn*. 2013; 242(3):201–218. [PubMed: 23335187]
- Ross CP, Persaud TV. Cardiovascular primordium of the rat embryo following in utero exposure to alcohol and caffeine. *Can J Cardiol*. 1986; 2(3):160–163. [PubMed: 3719451]
- Rovasio RA, Battiatto NL. Ethanol induces morphological and dynamic changes on in vivo and in vitro neural crest cells. *Alcohol Clin Exp Res*. 2002; 26(8):1286–1298. [PubMed: 12198407]
- Ruckman RN. Cardiovascular defects associated with alcohol, retinoic acid, and other agents. *Annals of the New York Academy of Sciences*. 1990; 588:281–288. [PubMed: 2192646]

- Ruckman RN, Messersmith DJ, O'Brien SA, Getson PR, Boeckx RL, Morse DE. Chronic ethanol exposure in the embryonic chick heart: effect on myocardial function and structure. *Teratology*. 1988; 37(4):317–327. [PubMed: 3394107]
- Sant'Anna LB, Tosello DO. Fetal alcohol syndrome and developing craniofacial and dental structures—a review. *Orthod Craniofac Res*. 2006; 9(4):172–185. [PubMed: 17101024]
- Sarmah S, Marrs JA. Complex cardiac defects after ethanol exposure during discrete cardiogenic events in zebrafish: Prevention with folic acid. *Dev Dyn*. 2013; 242(10):1184–1201. [PubMed: 23832875]
- Sato A, Scholl AM, Kuhn EN, Stadt HA, Decker JR, Pegram K, Hutson MR, Kirby ML. FGF8 signaling is chemotactic for cardiac neural crest cells. *Dev Biol*. 2011; 354(1):18–30. [PubMed: 21419761]
- Sato M, Yost HJ. Cardiac neural crest contributes to cardiomyogenesis in zebrafish. *Dev Biol*. 2003; 257(1):127–139. [PubMed: 12710962]
- Schafer MK, Frotscher M. Role of L1CAM for axon sprouting and branching. *Cell Tissue Res*. 2012; 349(1):39–48. [PubMed: 22370595]
- Sedmera D, Hu N, Weiss KM, Keller BB, Denslow S, Thompson RP. Cellular changes in experimental left heart hypoplasia. *Anat Rec*. 2002; 267(2):137–145. [PubMed: 11997882]
- Seo S, Kume T. Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. *Dev Biol*. 2006; 296(2):421–436. [PubMed: 16839542]
- Serbedzija GN, Bronner-Fraser M, Fraser SE. Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development*. 1992; 116(2):297–307. [PubMed: 1283734]
- Serrano M, Han M, Brinez P, Linask KK. Fetal alcohol syndrome: cardiac birth defects in mice and prevention with folate. *Am J Obstet Gynecol*. 2010; 203(1):75 e77–75 e15. [PubMed: 20451895]
- Sharma AK, Arya R, Mehta R, Sharma R. Hypothyroidism and cardiovascular disease: factors, mechanism and future perspectives. *Curr Med Chem*. 2013; 20(35):4411–4418. [PubMed: 24152286]
- Shen J, Wang Z, Liu J, Xue X, Qiu G. Abnormalities associated with congenital scoliosis: a retrospective study of 226 Chinese surgical cases. *Spine (Phila Pa 1976)*. 2013; 38(10):814–818. [PubMed: 23197014]
- Shiohama A, Sasaki T, Noda S, Minoshima S, Shimizu N. Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region. *Biochem Biophys Res Commun*. 2003; 304(1):184–190. [PubMed: 12705904]
- Shoji W, Sato-Maeda M. Application of heat shock promoter in transgenic zebrafish. *Dev Growth Differ*. 2008; 50(6):401–406. [PubMed: 18430027]
- Shprintzen RJ. Velo-cardio-facial syndrome: 30 Years of study. *Dev Disabil Res Rev*. 2008; 14(1):3–10. [PubMed: 18636631]
- Siebert JR, Graham JM Jr, MacDonald C. Pathologic features of the CHARGE association: support for involvement of the neural crest. *Teratology*. 1985; 31(3):331–336. [PubMed: 4012643]
- Sinning AR. Role of vitamin A in the formation of congenital heart defects. *Anat Rec*. 1998; 253(5):147–153. [PubMed: 9811123]
- Smith SM. Alcohol-induced cell death in the embryo. *Alcohol Health Res World*. 1997; 21(4):287–297. [PubMed: 15706739]
- Solzak JP, Liang Y, Zhou FC, Roper RJ. Commonality in Down and fetal alcohol syndromes. *Birth Defects Res A Clin Mol Teratol*. 2013; 97(4):187–197. [PubMed: 23554291]
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21(1):70–71. [PubMed: 9916792]
- Stewart DE, Kirby ML, Sulik KK. Hemodynamic changes in chick embryos precede heart defects after cardiac neural crest ablation. *Circ Res*. 1986; 59(5):545–550. [PubMed: 3802428]
- Sulik KK, Johnston MC, Daft PA, Russell WE, Dehart DB. Fetal alcohol syndrome and DiGeorge anomaly: critical ethanol exposure periods for craniofacial malformations as illustrated in an animal model. *Am J Med Genet Suppl*. 1986; 2:97–112. [PubMed: 3146306]
- Sulik KK, Johnston MC, Webb MA. Fetal alcohol syndrome: embryogenesis in a mouse model. *Science*. 1981; 214(4523):936–938. [PubMed: 6795717]

- Tang N, Farah B, He M, Fox S, Malouf A, Littner Y, Bearer CF. Ethanol causes the redistribution of L1 cell adhesion molecule in lipid rafts. *J Neurochem.* 2011; 119(4):859–867. [PubMed: 21884525]
- Tang N, He M, O’Riordan MA, Farkas C, Buck K, Lemmon V, Bearer CF. Ethanol inhibits L1 cell adhesion molecule activation of mitogen-activated protein kinases. *J Neurochem.* 2006; 96(5): 1480–1490. [PubMed: 16478533]
- Tartaglia M, Kalidas K, Shaw A, Song X, Musat DL, van der Burgt I, Brunner HG, Bertola DR, Crosby A, Ion A, Kucherlapati RS, Jeffery S, Patton MA, Gelb BD. PTPN11 mutations in Noonan syndrome: molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. *Am J Hum Genet.* 2002; 70(6):1555–1563. [PubMed: 11992261]
- Tartaglia M, Pennacchio LA, Zhao C, Yadav KK, Fodale V, Sarkozy A, Pandit B, Oishi K, Martinelli S, Schackwitz W, Ustaszewska A, Martin J, Bristow J, Carta C, Lepri F, Neri C, Vasta I, Gibson K, Curry CJ, Sigüero JP, Digilio MC, Zampino G, Dallapiccola B, Bar-Sagi D, Gelb BD. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. *Nat Genet.* 2007; 39(1):75–79. [PubMed: 17143282]
- Tassabehji M, Newton VE, Leverton K, Turnbull K, Seemanova E, Kunze J, Sperling K, Strachan T, Read AP. PAX3 gene structure and mutations: close analogies between Waardenburg syndrome and the Splotch mouse. *Hum Mol Genet.* 1994; 3(7):1069–1074. [PubMed: 7981674]
- Teillet MA, Ziller C, Le Douarin NM. Quail-chick chimeras. *Methods Mol Biol.* 2008; 461:337–350. [PubMed: 19030809]
- Theveneau E, Mayor R. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. *Dev Biol.* 2012a; 366(1):34–54. [PubMed: 22261150]
- Theveneau E, Mayor R. Neural crest migration: interplay between chemorepellents, chemoattractants, contact inhibition, epithelial-mesenchymal transition, and collective cell migration. *Wiley Interdiscip Rev Dev Biol.* 2012b; 1(3):435–445. [PubMed: 23801492]
- Thomas PS, Kim J, Nunez S, Glogauer M, Kaartinen V. Neural crest cell-specific deletion of Rac1 results in defective cell-matrix interactions and severe craniofacial and cardiovascular malformations. *Dev Biol.* 2010; 340(2):613–625. [PubMed: 20184871]
- Tobita K, Keller BB. Right and left ventricular wall deformation patterns in normal and left heart hypoplasia chick embryos. *Am J Physiol Heart Circ Physiol.* 2000; 279(3):H959–969. [PubMed: 10993756]
- Tomita H, Connuck DM, Leatherbury L, Kirby ML. Relation of early hemodynamic changes to final cardiac phenotype and survival after neural crest ablation in chick embryos. *Circulation.* 1991; 84(3):1289–1295. [PubMed: 1884453]
- Turner KN, Schachner M, Anderson RB. Cell adhesion molecule L1 affects the rate of differentiation of enteric neurons in the developing gut. *Dev Dyn.* 2009; 238(3):708–715. [PubMed: 19235728]
- Twal WO, Zile MH. Retinoic acid reverses ethanol-induced cardiovascular abnormalities in quail embryos. *Alcohol Clin Exp Res.* 1997; 21(6):1137–1143. [PubMed: 9309328]
- van Bueren KL, Papangeli I, Rochais F, Pearce K, Roberts C, Calmont A, Szumska D, Kelly RG, Bhattacharya S, Scambler PJ. Hes1 expression is reduced in Tbx1 null cells and is required for the development of structures affected in 22q11 deletion syndrome. *Dev Biol.* 2010; 340(2):369–380. [PubMed: 20122914]
- van den Hoff MJ, Moorman AF. Cardiac neural crest: the holy grail of cardiac abnormalities? *Cardiovasc Res.* 2000; 47(2):212–216. [PubMed: 10946058]
- van den Hoff MJ, Moorman AF, Ruijter JM, Lamers WH, Bennington RW, Markwald RR, Wessels A. Myocardialization of the cardiac outflow tract. *Dev Biol.* 1999; 212(2):477–490. [PubMed: 10433836]
- van Gelder MM, van Rooij IA, Miller RK, Zielhuis GA, de Jong-van den Berg LT, Roeleveld N. Teratogenic mechanisms of medical drugs. *Hum Reprod Update.* 2010; 16(4):378–394. [PubMed: 20061329]
- Van Mierop LH, Kutsche LM. Cardiovascular anomalies in DiGeorge syndrome and importance of neural crest as a possible pathogenetic factor. *Am J Cardiol.* 1986; 58(1):133–137. [PubMed: 3728313]

- Verberne ME, Gittenberger-de Groot AC, Poelmann RE. Lineage and development of the parasympathetic nervous system of the embryonic chick heart. *Anat Embryol (Berl)*. 1998; 198(3):171–184. [PubMed: 9764972]
- Vermot J, Forouhar AS, Liebling M, Wu D, Plummer D, Gharib M, Fraser SE. Reversing blood flows act through *klf2a* to ensure normal valvulogenesis in the developing heart. *PLoS biology*. 2009; 7(11):e1000246. [PubMed: 19924233]
- Vitelli F, Taddei I, Morishima M, Meyers EN, Lindsay EA, Baldini A. A genetic link between *Tbx1* and fibroblast growth factor signaling. *Development*. 2002; 129(19):4605–4611. [PubMed: 12223416]
- Vittorini S, Storti S, Parri MS, Cerillo AG, Clerico A. SERCA2a, phospholamban, sarcolipin, and ryanodine receptors gene expression in children with congenital heart defects. *Mol Med*. 2007; 13(1–2):105–111. [PubMed: 17515962]
- Waldo K, Miyagawa-Tomita S, Kumiski D, Kirby ML. Cardiac neural crest cells provide new insight into septation of the cardiac outflow tract: aortic sac to ventricular septal closure. *Dev Biol*. 1998; 196(2):129–144. [PubMed: 9576827]
- Waldo K, Zdanowicz M, Burch J, Kumiski DH, Stadt HA, Godt RE, Creazzo TL, Kirby ML. A novel role for cardiac neural crest in heart development. *J Clin Invest*. 1999; 103(11):1499–1507. [PubMed: 10359559]
- Waldo KL, Kumiski D, Kirby ML. Cardiac neural crest is essential for the persistence rather than the formation of an arch artery. *Dev Dyn*. 1996; 205(3):281–292. [PubMed: 8850564]
- Walker MB, Trainor PA. Craniofacial malformations: intrinsic vs extrinsic neural crest cell defects in Treacher Collins and 22q11 deletion syndromes. *Clin Genet*. 2006; 69(6):471–479. [PubMed: 16712696]
- Walker RK, Cousins VM, Umoh NA, Jeffress MA, Taghipour D, Al-Rubaiee M, Haddad GE. The good, the bad, and the ugly with alcohol use and abuse on the heart. *Alcohol Clin Exp Res*. 2013; 37(8):1253–1260. [PubMed: 23527963]
- Waller BR 3rd, McQuinn T, Phelps AL, Markwald RR, Lo CW, Thompson RP, Wessels A. Conotruncal anomalies in the trisomy 16 mouse: an immunohistochemical analysis with emphasis on the involvement of the neural crest. *Anat Rec*. 2000; 260(3):279–293. [PubMed: 11066038]
- Wang L, Sun H, Pan B, Zhu J, Huang G, Huang X, Tian J. Inhibition of histone acetylation by curcumin reduces alcohol-induced expression of heart development-related transcription factors in cardiac progenitor cells. *Biochem Biophys Res Commun*. 2012; 424(3):593–596. [PubMed: 22776204]
- Wang YT, Gu S, Ma P, Watanabe M, Rollins AM, Jenkins MW. Optical stimulation enables paced electrophysiological studies in embryonic hearts. *Biomed Opt Express*. 2014; 5(4):1000–1013. [PubMed: 24761284]
- Watanabe M, Chuck ET, Rothenberg F, Rosenbaum DS. Developmental transitions in cardiac conduction. *Novartis Found Symp*. 2003; 250:68–75. discussion 76–69, 276–279. [PubMed: 12956324]
- Watanabe Y, Miyagawa-Tomita S, Vincent SD, Kelly RG, Moon AM, Buckingham ME. Role of mesodermal FGF8 and FGF10 overlaps in the development of the arterial pole of the heart and pharyngeal arch arteries. *Circ Res*. 2010; 106(3):495–503. [PubMed: 20035084]
- Webster WS, Germain MA, Lipson A, Walsh D. Alcohol and congenital heart defects: an experimental study in mice. *Cardiovasc Res*. 1984; 18(6):335–338. [PubMed: 6744353]
- Wilson RD, Davies G, Desilets V, Reid GJ, Summers A, Wyatt P, Young D. The use of folic acid for the prevention of neural tube defects and other congenital anomalies. *J Obstet Gynaecol Can*. 2003; 25(11):959–973. [PubMed: 14608448]
- Wilson SE, Cudd TA. Focus on: the use of animal models for the study of fetal alcohol spectrum disorders. *Alcohol Res Health*. 2011; 34(1):92–98. [PubMed: 23580046]
- Wurdak H, Ittner LM, Lang KS, Leveen P, Suter U, Fischer JA, Karlsson S, Born W, Sommer L. Inactivation of TGFβ signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome. *Genes Dev*. 2005; 19(5):530–535. [PubMed: 15741317]

- Wurdak H, Ittner LM, Sommer L. DiGeorge syndrome and pharyngeal apparatus development. *Bioessays*. 2006; 28(11):1078–1086. [PubMed: 17041894]
- Xu H, Morishima M, Wylie JN, Schwartz RJ, Bruneau BG, Lindsay EA, Baldini A. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. *Development*. 2004; 131(13):3217–3227. [PubMed: 15175244]
- Ya J, van den Hoff MJ, de Boer PA, Tesink-Taekema S, Franco D, Moorman AF, Lamers WH. Normal development of the outflow tract in the rat. *Circ Res*. 1998; 82(4):464–472. [PubMed: 9506707]
- Yeaney NK, He M, Tang N, Malouf AT, O’Riordan MA, Lemmon V, Bearer CF. Ethanol inhibits L1 cell adhesion molecule tyrosine phosphorylation and dephosphorylation and activation of pp60(src). *J Neurochem*. 2009; 110(3):779–790. [PubMed: 19457108]
- Yelbuz TM, Waldo KL, Kumiski DH, Stadt HA, Wolfe RR, Leatherbury L, Kirby ML. Shortened outflow tract leads to altered cardiac looping after neural crest ablation. *Circulation*. 2002; 106(4):504–510. [PubMed: 12135953]
- Yelbuz TM, Waldo KL, Zhang X, Zdanowicz M, Parker J, Creazzo TL, Johnson GA, Kirby ML. Myocardial volume and organization are changed by failure of addition of secondary heart field myocardium to the cardiac outflow tract. *Dev Dyn*. 2003; 228(2):152–160. [PubMed: 14517987]
- Yelin R, Yelin D, Oh WY, Yun SH, Boudoux C, Vakoc BJ, Bouma BE, Tearney GJ. Multimodality optical imaging of embryonic heart microstructure. *J Biomed Opt*. 2007; 12(6):064021. [PubMed: 18163837]
- Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P. Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A*. 1997; 94(8):3789–3794. [PubMed: 9108056]
- Zenker M, Buheitel G, Rauch R, Koenig R, Bosse K, Kress W, Tietze HU, Doerr HG, Hofbeck M, Singer H, Reis A, Rauch A. Genotype-phenotype correlations in Noonan syndrome. *J Pediatr*. 2004; 144(3):368–374. [PubMed: 15001945]
- Zhang L. Prenatal hypoxia and cardiac programming. *Journal of the Society for Gynecologic Investigation*. 2005; 12(1):2–13. [PubMed: 15629664]
- Zhang LF, Gui YH, Wang YX, Jiang Q, Song HY. Effect of Tbx1 knock-down on cardiac performance in zebrafish. *Chin Med J (Engl)*. 2010; 123(9):1182–1189. [PubMed: 20529560]
- Zile MH. Vitamin a requirement for early cardiovascular morphogenesis specification in the vertebrate embryo: insights from the avian embryo. *Exp Biol Med (Maywood)*. 2004; 229(7):598–606. [PubMed: 15229353]

Figure 1. Potential consequences of NCC depletion

During normal development (left side) cardiac neural crest (CNC) cells from otic placode (Oto) to somite 3 (S3) travel from the CNC ventrolaterally to arrive at the circumpharyngeal ridge with a subset entering the branchial (pharyngeal) arches and a population enwrapping the aortic arch 3, 4, and 6 endothelial cells and contributing to remodeling of the aortic arch arteries as well as contributing to the media of these arteries as they differentiate into their asymmetric final structure. A few enter the outflow tract (OFT) and are responsible for the formation of the aortic-pulmonary septum. When the CNC are compromised (right side; e.g., undergo extensive cell death after ethanol treatment), few neural crest cells arrive at the circumpharyngeal ridge, few populate the branchial arches, few contribute to the walls of the aortic arch arteries, and fewer than normal enter the heart for outflow tract (OFT) septation. With the failure of the interaction between neural crest and developing heart forming fields, the function of the heart becomes abnormal and could contribute to the development of CHDs. The figures at the top represent neural crest at stage 9–10, the stage ablations are usually conducted, and the bottom figures represent neural crest cell migrations that occur from stages 12 to 27. [Modified from Figure 11.4 (Kirby, 2007) by permission of Oxford University Press, USA. www.oup.com]

Figure 2. Juxtaposition of neural crest with heart forming mesoderm

Neural crest cell derivatives (NCC) are adjacent to heart forming mesoderm and in position to intercept and process signaling molecules from the foregut endoderm and/or endothelium/endocardium. AA=aortic arch, AS=aortic sac, End=endoderm, Ect=ectoderm, OFT=outflow tract, PA=pharyngeal (branchial) arch, Ph=pharynx. Adapted from (Kirby, 2007) and figures of an E9.5 mouse embryo section in (Kelly and Buckingham, 2002).

Figure 3. Connections

Ethanol exposure at gastrulation/neurulation stages causes CNCC abnormalities that lead to poor heart function. The resulting biomechanical forces fail to induce proper endocardial cushion development that is necessary for proper cardiac development including valve leaflet formation. Potential direct effects may contribute to abnormal functional parameters (boxes with ?). Direct effects of ethanol on cardiomyocytes and their precursors may directly affect abnormal heart function. Ethanol could also directly affect endocardial cells and other cell types that contribute to valve leaflet differentiation.

Table 1

Cardiac neural crest cell events superimposed on other important developmental processes in avian embryo development

<u>Normal Developmental Steps in Avian Development</u>	
Stage 9–10	tubular heart starts to beat
Stage 10	CNCCs migrate from the neural tube ventrally and laterally
Stage 12	CNCCs arrive at the circumpharyngeal ridge and pause
Stage 12/13	mesenchymal cells appear in the AV and OFT cushions
Stage 12/13	NCCs undergo cell death in rhombomeres 3–5 just cranial to the otic placode NCCs [(Cartwright et al., 1998) reviewed in (Graham et al., 1996)] undergo cell death at region centered on rhombomeres 3–5 (equivalent to region of otic placode and cranial)
Stage 13	CNCCs populate first the pharyngeal arches 3,4 and finally the 6th (Kuratani and Kirby, 1992)
Stage 14	CNCCs migrate into the caudal, ventral pharynx CNCCs In pharyngeal (branchial) arch 3 are in intimate contact with endothelium of the future aortic arch artery 3 (Bockman et al., 1989; Kuratani and Kirby, 1992; Waldo et al., 1996)
Stage 16	atrial septation begins
Stage 25/26	A subset of CNCCs enter the OFT aortic sac and distal conus (Waldo et al., 1998) chick-chimera studies OFT septation begins
Stages 28–38	thinning of valve leaflets (Guzman et al., 2010)
Stage 30–32	ventricular septation
Stages 28–32	Transition from immature base-to-apex conduction to immature apex-to-base conduction (Chuck et al., 1997; Chuck et al., 2004; Gurjarpadhye et al., 2007; Watanabe et al., 2003)
Stage 34–35	Septation is complete Common (His) bundle of the ventricular conduction system matures to its compact, isolated, organized structure (Gurjarpadhye et al., 2007)
Stage 36	AV Valve leaflets exhibit layers, atrialis, spongiosa, fibroso, ventricularis
Stage 29–40	Tricuspid valves leaflets are clearly trilaminar

[Stages are according to Hamburger and Hamilton, (Hamburger and Hamilton, 1992)][steps in chick embryo cardiogenesis are from (Martinsen, 2005)]

Table 2

Neural crest ablation and function.

Model	NC ablation method	stage	assay	Outcome for NC ablated embryos	reference
chick	microcautery (mc) needle	st 18	doppler ultrasound, pressure sensor	>dorsal aortic blood flow velocity; <systolic and diastolic blood pressure	(Stewart et al., 1986)
chick	mc needle	st 18	microcine	<contractility, <emptying of bulbus cordis, altered blood flow in aortic arch arteries	(Leatherbury et al., 1990b)
chick	mc needle	st 18	Pressure sensor	Increased wall stress	(Leatherbury et al., 1990a)
chick	mc needle	st 18	microcine	<SF and EF	(Leatherbury et al., 1991)
chick	mc needle	st 18	microcine	output	(Tomita et al., 1991)
chick	laser	dy 15 (st 41)	force transducer	<twitch force in trabeculae, <SR CICR	(Nosek et al., 1997)
chick	mc needle & laser	st 14 & 18	optical imaging	<calcium transient in inf and outflow, rescued with neural crest replacement	(Waldo et al., 1999)
zebrafish	laser	48 hpf	videocine	< SV, EF, & CO	(Li et al., 2003)
chick	laser	dy 11 (st 37) & dy 15 (st 41)	patch clamp	<channel opening event and mean open channel probability	(Nichols and Creazzo, 2005)
chick	needle	st 30 & 34/35	optical mapping	Immature activation sequence	(Gurjarpadhye et al., 2007)
mouse	PO-Cre transgene mediated deletion of Bmp1a	E11.5 & 12.5	doppler imaging	60% of E11.5 had reversed/retrograde flow, HR and contractile function were normal, 15% had arrhythmias	(Nomura-Kitabayashi et al., 2009)

* The incubation conditions of these studies ranged from 37–38°C in temperature and 70–97% in humidity. < = reduced, >=increased.

** The lasers used for ablation were pulsed nitrogen/dye lasers with 337nm wavelength or 445 with a pulsed energy of 300

*** Stages of ablation were generally between stage 8–11.

**** Survival rates were not reported in most papers and varied widely when reported.

SV=stroke volume, EF=ejection fraction, CO=cardiac output

Table 3

Ethanol exposure and cardiac function

Model	Stage of analysis	Function change after ethanol exposure	Reference
Chicken, chronic exp	Stage 19–20* & 22–23	Video-cinematography, decreased contractility, increased heart rate	(Ruckman et al., 1988)
Chicken, chronic exp starting at Stage 19 (late looping stage)	Stage 19+10 hrs= stage 20–21	Video-cinematography, decreased cardiac output and stroke volume	(Bruyere and Stith, 1994)
Xenopus laevis, chronic exp	Stage 48 (post-morphogenesis, tadpole stage)	OCT detected circulation abnormalities	(Yelin et al., 2007)
Zebrafish, chronic exp	hpf 54, 72 (post-morphogenesis)	Heart rate reduced, reduced to adrenergic agonists, carbachol	(Dlugos and Rabin, 2010)
Mouse in utero, single exp at gastrulation*	15.5 dpc** (post-septation)	Echocardiography, regurgitation at AV and aortic valve	(Serrano et al., 2010)
Quail, ex ovo, single exp at gastrulation***	Stage 19**** (late looping stage)	DOCT Doppler, retrograde flow at vitelline vein was increased and “shoulder” disappeared	(Karunamuni et al., 2014)

* For mice the dose was applied at 6.5–7 dpc.

** Mouse embryo stage dpc (days post coitum) with E0.5 or 0.5 dpc being noon on the day of vaginal plug ascertainment,

*** Ethanol doses varied but for avians, the single dose of ethanol was applied at stage 4.

**** Quail embryo stages were determined using Hamburger and Hamilton criteria (Hamburger and Hamilton, 1992)

In vitro=embryos were cultured off the yolk

In ovo=embryos were cultured with intact yolk

In utero=embryos were intact in the anesthetized mouse

DOCT=Doppler optical coherence tomography

OCT=optical coherence tomography