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Review

Insights into the genetic basis of congenital heart disease

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Abstract. Cardiovascular malformations are the most common type of birth defect and result in significant mortality worldwide. The etiology for the majority of these anomalies remains unknown. Advances in the characterization of the molecular pathways critical for normal cardiac development have led to the identification of numerous genes necessary for this complex morphogenetic process. This work has aided the discovery of an increasing number of single genes being implicated as the cause of human cardiovascular malformations. This review summarizes normal cardiac development and outlines the recent discoveries of the genetic causes of congenital heart disease.

Keywords. Congenital heart defects, genetics, cardiac development.

Introduction

Congenital heart disease (CHD) is the most common type of birth defect, and is the leading non-infectious cause of death in the first of year of life [1]. CHD affects millions of children worldwide, among them approximately 36,000 children each year in the United States alone [2]. The mortality of children with CHD has decreased with advances in medical and surgical management, but the reported incidence of nearly 1% has remained unchanged [3].

The etiology of CHD is multifactorial, with environmental and genetic factors playing important roles. Environmental insults during fetal development are known to increase the risk of CHD and include viral infections with rubella, exposure to chemical teratogens such as retinoic acid and lithium, and maternal diseases that include diabetes and systemic lupus erythematosus. The influence of genetic factors is demonstrated by the association of CHD with chromosomal abnormalities such as Trisomy 21 and microdeletion of chromosome 22q11. Although cardiac malformations may occur in the setting of multiple birth defects as part of a syndrome, most are found as isolated defects and are 'non-syndromic'. Familial cases have been described for nearly each subset of cardiac malformations, suggesting primary genetic etiologies for a subset of non-syndromic CHD. The majority of individuals with CHD have no family history and are 'sporadic' cases, but even for these seemingly sporadic cases of CHD, epidemiologic studies have demonstrated an increased recurrence risk for cardiac malformations in subsequent pregnancies, supporting the existence of genetic predispositions [4].

The stages of cardiac embryogenesis have been well described for several decades, and in recent years molecular and developmental biologists have begun to elucidate the molecular pathways that regulate cardiac development. This newly acquired knowledge of genes coordinating the wide variety of cardiac morphological processes has resulted in the identification of genetic causes of a variety of cardiovascular diseases, including structural cardiac malformations, cardiomyopathies and conduction system disturbances. This review will focus on the recent discoveries of genetic etiologies of structural CHD that occur when cardiac development is perturbed.

Morphogenesis of cardiovascular system

During human development, the major part of organogenesis is completed during the first trimester of pregnancy, after which further maturation and growth predominate. The heart is the first organ to form, with the earliest recognizable cardiac structure evident at day 15 of gestation when these cardiac progenitor cells have been specified and are organized in the shape of a crescent (Fig. 1a). At 3 weeks of gestation, these bilaterally symmetric heart primordial cells migrate to the midline and fuse to form a single linear heart tube which has an inner endothelial lining surrounded by an outer myocardial cell layer (Fig. 1b). At this stage, a functioning cardiovascular system is essential in order to meet the nutritional requirements of the developing human embryo, and it is concomitant with the onset of rhythmic heartbeats. The primitive heart then undergoes rightward looping, positioning the atria, or inflow chambers, above the ventricles, or outflow chambers (Fig. 1c). During the sixth and seventh weeks of gestation, cardiac septa develop to divide the heart into four distinct chambers, and the common outflow tract, or truncus arteriosus, is septated into the aorta and pulmonary artery, resulting in divided pulmonary and systemic circulations. Extensive valvar remodeling and ventricular growth then occur to ultimately achieve the mature developed heart (Fig. 1d).

Formation of this complex cardiovascular structure requires the contribution of numerous cell types in a precise and coordinated manner. The majority of cardiac myocytes arise from the 'primary' heart field, or the original population of cells that reside in the anterior lateral plate mesoderm that have become committed to a cardiogenic fate in response to a signal emanating from the adjacent endoderm [5]. Additional cellular contributions arise from the secondary, or anterior, heart field, which was described recently [6–8]. The secondary heart field comprises mesodermal cells caudal to the outflow tract of the heart that perhaps arise from the pharyngeal arch mesenchyme. During looping, these cells migrate to contribute to the outflow tract and right ventricle. Other well-known contributors are the cardiac neural crest cells, which initially reside in the neural tube between the midotic placode and the third somite. These cells migrate into the aortic sac to septate the truncus arteriosus and populate the bilaterally symmetric aortic arch arteries, where they are necessary for proper remodeling of these vessels into the mature left aortic arch with its normal branching of the head and neck vessels [9]. Lastly, similar to other organs in the developing embryo, factors responsible for establishment of left-right (L-R) asymmetry are critical for normal cardiac development [10, 11].

This intricate process of cardiac morphogenesis, with its multiple cellular contributors, is controlled by a set of highly conserved molecular pathways. Studies using diverse species, from flies to mice, have led to the identification of many genes critical for normal cardiac development. With this knowledge in hand, investigators have begun to identify genetic etiologies of human CHD and are

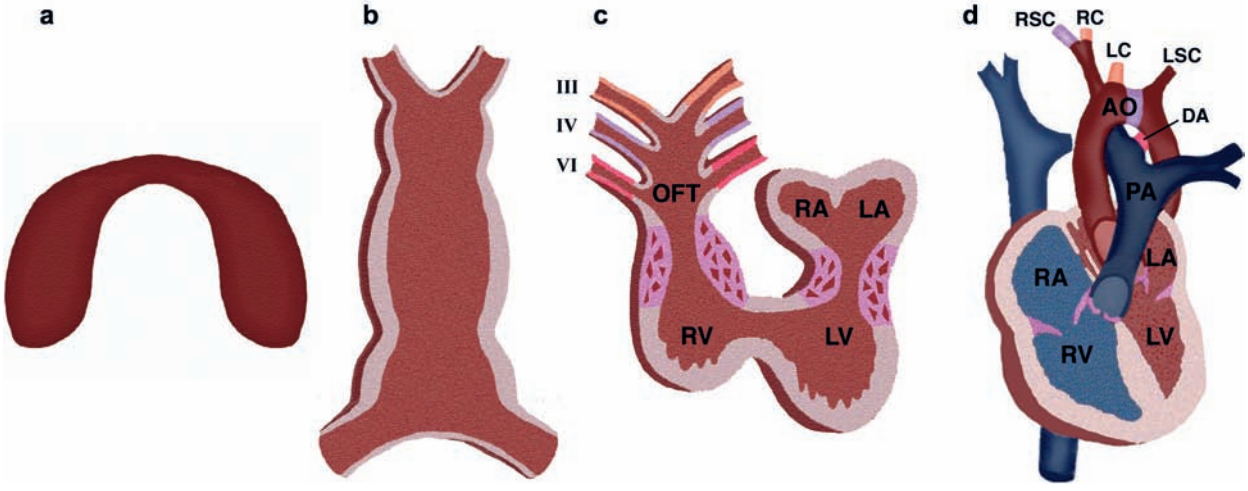

Figure 1. Schematic of stages of cardiac morphogenesis. (*a*) Cardiac crescent is shown at day 15. (*b*) Mesodermal cells fuse to form the linear heart tube by day 21. (*c*) By day 28, the rightward heart looping occurs and bilaterally symmetric aortic arch arteries (III, IV and VI) are seen arising from outflow tract. (*d*) Significant remodeling of the inner curvature and growth of the ventricular chambers then occurs to result in the maturely developed heart with partitioned systemic (red) and pulmonary circulations (blue) by day 50 to birth. The endocardial cushions (pink) develop and transform into the atrioventricular (tricuspid and mitral) and semilunar (pulmonary and aortic) valves, while the aortic arch arteries (III, orange; IV, purple; VI, red) are patterned to contribute the normal left aortic arch and its branches. Timing of stages is in days of human embryonic development. AO, aorta; DA, ductus arteriosus; LA, left atrium; LC, left carotid artery; LV, left ventricle; LSC, left subclavian artery; OFT, outflow tract; PA, pulmonary artery; RA, right atrium; RC, right carotid artery; RSC, right subclavian artery; RV, right ventricle.

beginning to dissect the mechanisms behind these malformations.

Cardiac septation defects

Defects of cardiac septation are the most common type of CHD, accounting for nearly 50% of all CHD [1]. Cardiac septal defects (CSDs) are categorized depending on their location in the heart. Atrial and ventricular septal defects are communications between only the right and left atria or ventricles, respectively, while atrioventricular septal defects are a defect of the atrioventricular septum that result in communication between the atria and ventricles and a lack of division of the common atrioventricular valve. If unrepaired, CSDs can cause pulmonary overcirculation, leading to pulmonary vascular disease, atrial enlargement predisposing to atrial arrhythmias, ventricular dilation and ultimately decreased life expectancy. These type of defects are most commonly seen in genetic syndromes, especially those with significant chromosomal aberrations such as Trisomy 21, but the specific genes involved have remained elusive.

In humans, genetic linkage analysis of large families with autosomal forms of CHD has led to the identification of three transcription factors that play an important role in cardiac septation defects. *TBX5* encodes a transcription factor and is mutated in individuals with Holt-Oram syndrome, which is characterized by atrial and ventricular septal defects along with upper limb anomalies [12, 13]. This T-box transcription factor is highly expressed in the atrial and ventricular septum, and targeted deletion of *Tbx5* in mice results in embryonic lethality in homozygous-null embryos. Heterozygote mice recapitulate the human malformations, allowing investigators to dissect the molecular pathways regulated by Tbx5 using this mouse model of human disease [14, 15].

Although CSDs are common in syndromic forms of CHD, they are most commonly seen as isolated defects without other birth anomalies. The first discovery of a genetic cause of non-syndromic CHD was mutations in the transcription factor, NKX2.5. These were found by studying several large families with autosomal dominant CSDs and cardiac conduction abnormalities in the form of complete heart block using a positional cloning approach [16]. Subsequently, investigators identified *NKX2.5* mutations in individuals with other forms of CHD such as tetralogy of Fallot and tricuspid valve abnormalities, supporting a role for this gene in diverse cardiac morphogenetic processes [17, 18]. *NKX2.5* encodes a transcription factor that is critical for cardiac development in *Drosophila*, where its orthologue is necessary for formation of the dorsal vessel [19], and in mice, where targeted disruption results in embryonic lethality and cardiac failure at the heart looping stage [20, 21]. Atrial septal abnormalities have been identified in mice heterozygous for *NKX2.5* consistent with the human phenotype [22]. More recently, studies in genetically manipulated mice have demonstrated the importance of NKX2.5 in the cardiac conduction system. *Nkx2.5* heterozygote and homozygous-null mice have hypoplastic or absent atrioventricular (AV) nodes, respectively, and examination of mice with a ventricular-restricted knockout of *Nkx2.5* demonstrated progressive loss of this AV nodal conduction tissue, leading to complete heart block. These studies demonstrated the dual role of *NKX2.5* in cardiac disease, both in cardiac formation and in the maintenance of the cardiac conduction system with aging [23, 24].

Mutations in a known molecular partner of Nkx2-5, *GATA4*, were identified as the genetic cause of non-syndromic atrial and ventricular septal defects without conduction disturbances by studying large pedigrees with familial CHD [25]. Subsequent studies identified *GATA4* mutations in other familial cases of cardiac septal defects [26–28]. GATA4 is a well-studied zinc finger transcription factor, as mice homozygous for the null allele of *Gata4* are embryonic lethal just prior to heart tube fusion [29, 30]. Although *Gata4* heterozygote mice are phenotypically normal, mice homozygous for a hypomorphic allele of *Gata4* display a spectrum of embryonic cardiac defects that include atrioventricular septal defects [31]. Interestingly, one of the *GATA4* mutations identified in the human familial cases of CHD was a missense mutation that disrupted a highly conserved glycine residue adjacent to the second zinc finger of GATA4, which is critical for protein-protein interactions. Biochemical analysis of this mutation led to the discovery of a novel biochemical interaction between Gata4 and Tbx5 [25]. This missense mutation in Gata4 specifically disrupted the Gata4-Tbx5 interaction while maintaining its ability to interact with Nkx2.5. In previous studies, Tbx5 had been shown to interact with Nkx2.5, demonstrating that all three transcription factors could physically interact *in vitro* [32]. In summary, a mutation in any of these three genes can result in human CSD and suggests that these three genes may work to direct common molecular pathways critical for cardiac septum formation. Consistent with this, mutations in *MYH6*, a downstream transcriptional target of GATA4 and TBX5, was implicated as a cause of human atrial septal defects [33].

Conotruncal and aortic arch artery defects

Defects of the cardiac outflow tract and aortic arch account for 20–30% of all CHD [1]. The 22q11 deletion syndrome (22q11del), which encompasses the DiGeorge, velo-cardio-facial and conotruncal anomaly face syndromes, has provided an entry point to study the molecular pathways critical for development of these defects.

22q11del is the most common human genetic deletion syndrome and the second most common genetic cause of CHD after Trisomy 21 [34]. Seventy-five percent of individuals with 22q11del have CHD in the form of persistent truncus arteriosus (lack of septation of the conotruncus into the aorta and pulmonary artery), tetralogy of Fallot (malposition of the infundibular septum, resulting in a ventricular septal defect, subvalvar and valvar pulmonary stenosis, and overriding aorta with associated right ventricular hypertrophy), interrupted aortic arch or double outlet right ventricle. In addition, they have other birth defects that result from maldevelopment of the pharyngeal arches and pouches such as cleft palate, dysmorphic facies, and thymic and parathyroid hypoplasia [35]. Approximately 90% of patients have a monoallelic deletion spanning ~3 Mb of chromosome 22q11 that contains nearly 30 genes. In an effort to identify the important genes in this locus, mouse models were generated that deleted syntenic portions of the commonly deleted region on 22q11 [36, 37]. Such approaches suggested that Tbx1, a transcription factor that is expressed in the pharyngeal arches [38, 39], was responsible for the predominant features of the 22q11del phenotype. This was demonstrated by generation of mice harboring a targeted deletion allele of *Tbx1*. Mice heterozygous for a *Tbx1*-null allele had fourth aortic arch artery anomalies, including interrupted aortic arch and anomalous right subclavian artery partially resembling the phenotype in humans who have monoallelic deletion of chromosome 22q11. *Tbx1*-null mice recapitulated the human 22q11del phenotype with the entire spectrum of defects, including cleft palate, thymic aplasia, ear anomalies and cardiac defects, suggesting that gene dosage was critical for phenotypic expression [40–42]. Subsequently, studies of mice with hypomorphic alleles of *Tbx1* demonstrated that decreasing levels of Tbx1 expression resulted in variability of the cardiac phenotype and a higher incidence of ear, palatal and thymic abnormalities [43, 44]. Lastly, investigators have found *TBX1* mutations in individuals with the 22q11del phenotype who do not have a detectable 22q11 microdeletion by fluorescent in situ hybridization (FISH), supporting the conclusion that haploinsufficiency of *TBX1* results in the majority of the phenotypic features seen with 22q11del [45]. It remains possible that other genes in the 22q11 locus, such as *Crkl*, a gene encoding a signaling adaptor protein that phenocopies the 22q11 del phenotype when mutated in mice, may function independently or in combination with Tbx1 to affect the phenotype seen in humans with the 22q11del [46].

Another arch artery anomaly commonly observed is patent ductus arteriosus, the third most common form of CHD. The ductus arteriosus is derived from the sixth aortic arch artery, and is necessary for normal fetal life after ventricular and outflow tract septation. Soon after birth, the ductus arteriosus normally closes but in certain instances may remain patent, resulting in heart failure. Pedigree analysis of individuals with Char syndrome, characterized by patent ductus arteriosus, dysmorphic facies and digit anomalies, identified heterozygous mutations of the transcription factor *TFAP2β* in affected individuals [47]. These findings suggested a critical role for TFAP2β and its molecular partners or downstream targets in the normal closure of the ductus arteriosus after birth. Accordingly, mutations in *CITED-2*, a ubiquitously expressed transcriptional co-factor of TFAP2β, have been identified in children with CHD [48, 49].

Obstructive defects of the pulmonary artery and aorta

Defects that obstruct the outflow tracts of the heart, either the aorta or pulmonary artery, can vary in their location and severity and in the most extreme instances lead to hypoplasia of the corresponding ventricle. The first genetic etiology was identified by studying individuals with Williams syndrome that is characterized by supravalvar aortic stenosis and peripheral pulmonary artery stenosis. Other syndromic features include an elfin-like face, mental retardation, neonatal hypercalcemia and outgoing social skills, the so-called 'cocktail personality'. The genetic etiology was found to be a microdeletion on chromosome 7q11 where haploinsufficiency of the elastin gene, *ELN*, resulted in the cardiac defects [50]. Subsequent work identified point mutations in *ELN* in children with non-syndromic forms of supravalvar aortic stenosis [51, 52].

Thickened valve leaflets resulting in stenotic valves are a common form of CHD. In mouse models, the absence of *Ptpn11*, which encodes the protein tyrosine phosphatase Shp-2, results in dysplastic semilunar valves by its involvement in a Ras signaling pathway mediated by epidermal growth factor receptor [53]. The importance of *PTPN11* in congenital heart disease was shown by the identification of gain-of-function point mutations in *PTPN11* in patients with Noonan syndrome, whose phenotype commonly includes pulmonic stenosis, which is often due to a bicuspid valve [54]. Consistent with this, the genetic etiology of neurofibromatosis type 1, which is characterized by pulmonic valve stenosis along with café-au-lait spots and fibromatous tumors of the skin, is loss-of-function mutations in the gene *NF1* (neurofibromin) [55, 56]. Reduced NF1 results in increased Ras signaling and points to a common pathway for pulmonary valve thickening. These findings implicate other members of this signaling pathway as candidate genes for human valvar disease [57]. Human genetic studies have identified the gene responsible for Alagille syndrome, which is characterized by biliary atresia and right-sided heart defects ranging from mild pulmonary stenosis to tetralogy of Fallot. Affected individuals were found to have mutations or chromosomal deletions encompassing

JAGGED-1, a membrane-bound ligand that was originally identified in *Drosophila* [58, 59]. Subsequently, *JAGGED-1* mutations have been identified in patients with apparently isolated pulmonary stenosis or tetralogy of Fallot who did not meet criteria for Alagille syndrome, suggesting that haploinsufficiency of this gene may contribute to presumed non-syndromic CHD [60]. Jagged-1 is a ligand for the Notch1–4 family of transmembrane receptors, which are involved in embryonic patterning and cellular differentiation.

Recently, mutations in *NOTCH1* were identified as the etiology for aortic valve malformations in families with autosomal dominant aortic valve disease [61]. Linkage studies mapped the disease locus to chromosome 9q34 and subsequently identified a *NOTCH1* nonsense mutation in affected family members. This was supported by the discovery of a *NOTCH1* frameshift mutation in an unrelated family with similar aortic valve phenotype. The predominant phenotype of the affected family members was bicuspid aortic valve (BAV), the most common type of CHD with a prevalence of 1–2% in the population. BAVs are known to undergo premature calcification leading to aortic valve dysfunction that ultimately requires valve replacement. Interestingly, a subset of family members who harbored mutations in *NOTCH1* had trileaflet aortic valves but ultimately developed aortic valve calcification that required surgical valve replacement. These findings suggested that NOTCH1 signaling may be important for valvar calcification, and *in vitro* studies supported this hypothesis. This work also suggested that genetic mutations that cause defects in aortic valve development may also predispose to adult cardiovascular disease similar to the findings seen with *NKX2.5* mutations.

Left-right abnormalities (Heterotaxy syndrome)

Abnormal cardiac looping underlies a variety of CHD, as proper folding of the straight heart tube aligns the atrial chambers with their appropriate ventricles and the right and left ventricles with the pulmonary artery and aorta, respectively. Abnormalities in the process of cardiac looping are often observed in the setting of randomized L-R patterning of the heart, lungs and visceral organs. During development, the heart is the first organ to disturb the bilateral symmetry of the early embryo. Studies in several species led to the discovery of numerous signaling molecules that regulate L-R asymmetry and provide a framework in which to consider human L-R laterality defects. In the chick embryo, asymmetric expression of Sonic hedgehog (Shh) leads to expression of the transforming growth factor-β (TGF-β) members *Nodal* and *Lefty* in the left lateral plate mesoderm [62]. The nodal expression on the left-side developing embryo induces rightward looping of the straight heart tube. In the right lateral mesoderm, an activin receptor-mediated pathway inhibits *Shh* and *Nodal* expression. Conversely, the zinc finger transcription factor *Snail* is expressed in the right lateral mesoderm and is repressed by Shh on the left, resulting in unique gene expression profiles on the left and right of the embryo [63]. The activin and nodal-dependent pathways ultimately result in expression of the transcription factor Pitx2 on the left side of visceral organs, which is sufficient for the establishment of L-R asymmetry in the developing heart, lungs and gut [64].

Recent studies have revealed how the initial asymmetry of molecules such as Shh or Nodal might be established. Henson's node contains ciliary processes that beat in a vortical fashion, creating a leftward movement of morphogens around the node [65]. In mice homozygous for the *inversus viscerum* (*iv*) mutation, L-R orientation of the heart and viscera is randomized [66]. The *iv* gene encodes for L-R dynein, which acts as a force-generating component in cilia that are present in the node [67, 68], and this was shown in explanted mouse embryo experiments where reversal of nodal flow reversed L-R development [69]. Recent studies have demonstrated that Polycystin 2, a cation channel, senses nodal flow and establishes L-R asymmetry in the embryo via calcium signaling [70]. These findings are consistent with the findings of situs inversus in Kartagener syndrome, also known as immotile cilia syndrome, which is characterized by mutations in the dynein proteins (reviewed in [71]).

Patients with heterotaxy syndromes display randomization of the cardiac, pulmonary and gastrointestinal situs, while patients with situs inversus totalis have a well-coordinated reversal of L-R asymmetry. Disruption of the signaling cascades on the left or right side of the embryo result in randomization of cardiac looping, and often lead to bilateral right (aplenia syndrome) or left (polysplenia syndrome) sidedness, respectively. In humans, point mutations of several genes involved in the L-R signaling cascade have been identified in subjects with heterotaxy syndromes, including *ZIC3* (zinc finger protein of the cerebellum), *ACVR2B* (activin A receptor IIB) and *CRYPTIC*, a cofactor of nodal (reviewed in [72]).

Summary

Numerous genetic causes have been discovered for congenital cardiac malformations that are associated with genetic syndromes, and more recently positional cloning approaches have uncovered the genetic etiologies of familial cases of non-syndromic CHD. Despite these discoveries, the etiology for most isolated cases of non-syndromic CHD remains unknown. But it is likely that single-nucleotide polymorphisms and/or mutations of genes critical for cardiac morphogenesis are present in affected

individuals and provide a genetic predisposition for CHD. Additional screening of sporadic cases of CHD for mutations in these newly identified candidate genes will hopefully lead to genotype-phenotype correlations. Studies will also need to determine the potential role of environmental factors in the setting of these genetic variants that may be required to ultimately perturb normal cardiac development in this multifactorial disease. The continuing advances in the understanding of the molecular mechanisms of cardiac development will lead to a better understanding of the genetic basis of CHD and hopefully result in improved genetic counseling and care of affected individuals and their families.

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