



HHS Public Access

Author manuscript

Cold Spring Harb Perspect Biol. Author manuscript; available in PMC 2022 April 01.

Published in final edited form as:

Cold Spring Harb Perspect Biol. ; 13(4): . doi:10.1101/cshperspect.a036764.

In vivo and in vitro genetic models of congenital heart disease

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Abstract

Congenital cardiovascular malformations represent the most common type of birth defect and encompass a spectrum of anomalies that range from mild to severe. The etiology of congenital heart disease (CHD) is becoming increasingly defined based upon prior epidemiologic studies that supported the importance of genetic contributors and technological advances of the human genome. These have led to the discovery of a growing number of disease-contributing genetic abnormalities in individuals affected by CHD. The ever-growing population of adult CHD survivors, which are the result of reductions in mortality from CHD during childhood, and this newfound genetic knowledge have led to important questions regarding recurrence risks, the mechanisms by which these defects occur, the potential for novel approaches for prevention and the prediction of long-term cardiovascular morbidity in adult CHD survivors. Here, we will review the current status of genetic models that accurately model human CHD as they provide an important tool to answer these questions and test novel therapeutic strategies.

Keywords

congenital heart disease; animal models; cellular models; genetics

Introduction

Congenital heart disease (CHD) affects 2–3% of newborn infants when including cases of isolated bicuspid aortic valve (BAV) (Pierpont et al. 2018). The impact of this most common type of birth defects is increasing with tremendous improvements in the clinical management of individuals affected by CHD. This improved survival has resulted in a growing population of adult CHD survivors who now outnumber children with CHD. Unfortunately, these adult survivors often suffer from co-morbid conditions and are at risk for neurodevelopmental and cognitive deficits, cardiac arrhythmias, and ventricular dysfunction which contribute to their early mortality (Russell et al. 2018). Epidemiologic studies investigating the etiology of CHD have implicated both genetic and environmental

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factors. With recent advancement in next generation sequencing technologies, a genetic abnormality, defined as chromosomal aneuploidy or a pathogenic chromosomal copy number or single gene variant, is able to be detected in approximately 1/3 of CHD cases (Akhirrome et al. 2017). While the majority of CHD cases remain unexplained, the discoveries of these genetic contributors have led to the development of genetic models of CHD.

In vivo and *in vitro* model systems are important tools to understand disease pathogenesis and progression as well as to investigate its underlying mechanisms. In addition, these models can serve to test preclinical therapeutic interventions. Accordingly, the best models recapitulate the anatomy and physiology of the disease being studied in humans and are also cost-effective and easy to generate and manipulate. Another aspect of a translational genetic model is that the genetic abnormality has a strong and consistent association with the disease phenotype in humans. In the fields of cardiac developmental biology and CHD, numerous animal models from *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish), *Xenopus laevis* (frog), *Gallus gallus* (chick) to small (mice and rats) and large mammals (sheep, dogs and pigs) have been utilized along with *in vitro* cell culture models.

Each of these models have their own benefits and limitations. As the focus of this review is the development of genetic models of human CHD, we have emphasized the role of murine models which are amenable to reverse genetics, share a high degree of sequence conservation to humans and have similar stages of cardiac morphogenesis along with adult cardiac structures and physiology. In addition, the field has amassed over 25 years of experience studying cardiac development and disease in mouse models. The murine model is not without its limitations primarily related to the time involved to generate and characterize a new mouse, which is both time-consuming and expensive. In addition, the models of CHD in mice often result in perinatal lethality limiting our ability to study long-term cardiovascular sequelae from the genetic abnormality. Alternatively, zebrafish, a lower species vertebrate model, is quite popular due to its low cost of housing, easily available genetic tools, the embryos lack of dependence upon a functional cardiovascular system and their optical transparency which allows for more detailed analysis of cellular mechanisms. However, the utility of relatively primitive two-chambered heart of the zebrafish is limiting for studying more complex structural abnormalities found in human CHD (Bakkers 2011). Similarly, the fruit fly has similar benefits in regards to being high throughput and lower cost but again is limited by its primitive cardiac structure (Zhu et al. 2017b). *In vitro* non-cardiac cell-based models have been used to study potentially pathogenic sequence variants identified in affected individuals with CHD, but have numerous limitations in regards to the biologic relevance of these observations. More recently, patient-specific induced pluripotent stem cells (iPSCs) have begun to be investigated as a model to study human CHD (Mital 2016; Mercer and Evans 2017). Somewhat similar to the weaknesses related to zebrafish and fruit fly in regards to modeling the structural and morphogenetic aspects of CHD, these models are the most genetically similar and may allow for the investigation of the most relevant molecular mechanisms.

In this chapter, we discuss the genetic models of CHD that are linked to well-established monogenic causes of CHD with a focus on the murine model system where these CHD

subtypes can morphologically be recapitulated. We have divided these models according to subtypes of CHD including cardiac septation defects, heart valve malformations and conotruncal heart defects and discussed a subset of these models in more depth to highlight the insights that can be obtained. We also discuss the development of *in vivo* genetic models of more complex CHD, which are unlikely to be monogenic in etiology, and look forward to the use of other model systems in lower species and *in vitro* modeling using iPSCs.

Murine models of cardiac septation defects

Formation of a four-chambered heart with its separate pulmonary and systemic circulations requires the proper development of atrial and ventricular septa. Defects of cardiac septation are amongst the most common forms of CHD and include atrial septal defects (ASD), ventricular septal defects (VSD) and atrioventricular septal defects (AVSD) (Figure 1). Many ASD and VSD are small and close spontaneously, while hemodynamically significant ASD and VSD and all AVSD require cardiac intervention. Genetic abnormalities associated with cardiac septation defects have been well-described and include pathogenic variants in single genes. These findings have been the basis for the generation of numerous mouse models, which have provided important insights into disease mechanisms (Table). Studies in families with autosomal dominant inherited CHD have linked pathogenic sequence variants in the cardiac transcription factors, *TBX5*, *NKX2-5* and *GATA4*, with cardiac septation defects in humans (reviewed in Pierpont et al. 2018).

TBX5

Pathogenic sequence variants in *TBX5* have been linked to Holt-Oram syndrome, which is characterized by upper limb and cardiac abnormalities (Basson et al. 1997; Li et al. 1997; Basson et al. 1999). Cardiac defects are found in 75–85% of individuals with Holt-Oram syndrome including ASD (to the point of common atrium), VSD, and defects in atrioventricular conduction that can progress to complete heart block (Basson et al. 1994; Newbury-Ecob et al. 1996; Bruneau et al. 1999). The majority of heterozygous pathogenic variants result in a loss-of-function allele and this has led to the successful development of a murine model for Holt-Oram syndrome which recapitulates the majority of human disease phenotypes.

Mice heterozygous for a *Tbx5* mutant allele, which deletes exon 3 that harbors the T-box domain, was generated by breeding *Tbx5^{lox/+}* from Black Swiss background to mice with a constitutively active Cre recombinase (*EIIa^{Cre}* from 129SvJ strain) (Bruneau et al. 2001). The resultant *Tbx5^{del/+}* progeny were viable but displayed a 40% perinatal lethality. This perinatal lethality was increased in *Tbx5^{del/+}*, when the *Tbx5^{lox/+}* mice were from 129SvEv background indicating a role for genetic modifiers. The surviving adult *Tbx5^{del/+}* mice from a mixed background (Black Swiss and 129SvJ) demonstrated large ASD along with evidence of varying degrees of atrioventricular block. Interestingly, VSD were found only in *Tbx5^{del/+}* embryos or neonatal mice, likely representing the reason for observed perinatal mortality. Another publication demonstrated that complete deletion of *Tbx5* from endocardial and endocardial-derived cells utilizing *Tie2^{Cre}* mice resulted in secundum ASD while endocardial-specific heterozygous *Tbx5* (*eTbx5^{+/-}*) deletion demonstrated a 65%

incidence of patent foramen ovale (PFO), suggesting that PFO is an attenuated manifestation of ASD (Nadeau et al. 2010).

NKX2-5

Disease-causing sequence variants in *NKX2-5* were the first reported cause of non-syndromic CHD by studying 4 kindred with autosomal dominant disease (Schott et al. 1998). The predominant phenotype in affected family members was a secundum ASD along with atrioventricular conduction abnormalities. Since then, a range of cardiac abnormalities, including VSD, tetralogy of Fallot (TOF), pulmonary valve atresia, subvalvular aortic stenosis, and Ebstein's anomaly have been correlated with heterozygous *NKX2-5* mutations (Stallmeyer et al. 2010; Ellesoe et al. 2016). Heterozygous *Nkx2-5* knockout mice displayed a spectrum of cardiac developmental phenotypes that are dependent upon the genetic background. In the FVB/N and 129/Sv backgrounds, *Nkx2-5*^{+/-} mice have PFO, atrial septal aneurysms, and BAV with aortic stenosis (Biben et al. 2000). While *Nkx2-5*^{+/-} mice in C57Bl/6 background demonstrate a high incidence (~40%) of secundum ASD and both muscular and membranous VSD (Figure 1, D, E, G, H) (Winston et al. 2010). Additional mice harboring specific human disease-associated variants in *Nkx2-5* have also been reported and they display a spectrum of cardiac phenotypes which mimic human disease (Ashraf et al. 2014; Chowdhury et al. 2015; Zakariyah et al. 2017).

GATA4

A similar human genetics approach used for *TBX5* and *NKX2-5* led to the discovery that heterozygous mutations in *GATA4* caused cardiac septation defects, primarily secundum ASD and membranous VSD (Garg et al. 2003). Multiple disease-causing, likely loss-of-function genetic variants in *GATA4*, have been reported for a range of cardiac defects including AVSD, pulmonic valve stenosis, and TOF (Okubo et al. 2004; Hirayama-Yamada et al. 2005; Sarkozy et al. 2005; Nemer et al. 2006; Schluterman et al. 2007). Mice harboring a mutant *Gata4* allele (*Gata4*^{ex2/WT}), was generated in which the start codon and almost half of the coding region is deleted (Rajagopal et al. 2007). While mice heterozygous for this allele display no cardiac phenotypes in a mixed genetic background, when bred into C57Bl6 background display ~50% perinatal lethality. Analysis of *Gata4*^{ex2/WT} embryonic hearts identified CHD in 76% including AVSD; primum and secundum ASD; inlet, membranous and muscular VSD; and right ventricular hypoplasia (Figure 1 B, C, F, I). The incidence of CHD decreased to 30% when this mutant allele was backcrossed into FVB genetic background. Mice harboring a human CHD-segregating mutation (G296S) in *GATA4* have also been reported and display a PFO in 80% of adult mice along with stenosis of the aortic and pulmonic valve at a lower penetrance (Misra et al. 2012; LaHaye et al. 2019).

In combination, these models of a common form of CHD have provided important insights for *in vivo* modeling of human CHD. First, the mouse model recapitulates structural malformations found in humans. Second, these models demonstrated that one genetic mutation could result in multiple cardiac abnormalities, a septation defect and atrioventricular block with mutations in *NKX2-5* or *TBX5* and a septation defect and pulmonary valve stenosis with a *GATA4* mutation. Another important point was the variability of phenotypes found in these murine models with alterations in the genetic

background supporting the role of modifier genes. Finally, an interesting observation that was suggested is that PFO, which is present in 10–25% of the population (Fisher et al. 1995; Homma et al. 2016), may represent a mild form of ASD and therefore may also have genetic contributors.

Murine models of cardiac valve malformations

Proper development of heart valves is important for unidirectional blood flow in humans and other species with chambered hearts. Congenital valve malformations are the most frequent type of CHD and bicuspid aortic valve (BAV) is the most common with a population prevalence of 1.3% (Verma and Siu 2014). In BAV, there is improper septation or fusion of two of the three aortic leaflets (right coronary, left coronary and non-coronary) (Figure 2) and is associated with the development of adult-onset complications including aortic valve stenosis (AVS) and regurgitation, calcific aortic valve disease (CAVD) and aortopathy. BAV can be subdivided into three morphologic categories, R-L (fusion of right and left coronary leaflets) which is most common in humans, R-NC (fusion of right and non-coronary) and L-NC (fusion of left and non-coronary). In addition, congenital stenosis of the aortic or pulmonic valve are common types of CHD. For each of these conditions, genetic etiologies in humans have been described and murine models developed (Table).

NOTCH1

By studying multi-generational families with autosomal dominant inherited cardiac disease, pathologic loss-of-function sequence variants in *NOTCH1* were the first reported genetic cause of BAV and its associated aortic disease. Since then, other groups have also identified different *NOTCH1* mutations that cause BAV, other forms of left-sided CHD and TOF (McBride et al. 2008; Kerstjens-Frederikse et al. 2016; Page et al. 2019). Mice heterozygous for *Notch1* display minimal aortic disease with a low incidence of BAV and mild CAVD (Nigam and Srivastava 2009; Nus et al. 2011; Bosse et al. 2013). The BAV phenotype is more penetrant with complete deletion of *Notch1* from valve endothelial cells (after endocardial to mesenchymal transformation) using *Nfatc1^{enCre}* as these mice display a ~30% incidence of R-L BAV by one group (Figure 2C, D) but other investigators reported both R-L and R-NC BAV (MacGrogan et al. 2016; Wang et al. 2017). In addition, the analysis of *Notch1^{+/-}* mice lacking telomerase activity by genetic deletion of *Terc* (telomerase RNA component; *mTR*) displayed robust CAVD with associated AVS (Theodoris et al. 2017). Similarly, the generation of *Notch1^{+/-}* mice in the setting of complete deletion of endothelial nitric oxide synthase (*Nos3^{-/-}*) resulted in high incidence of BAV with associated AVS and regurgitation and molecular changes consistent with CAVD (Bosse et al. 2013). Interestingly, *Nos3^{-/-};Notch1^{+/-}* mice display significant perinatal lethality which was due to TOF-like phenotypes (Koenig et al. 2016). The role of genetic background in murine models has also been seen with *Notch1^{+/-}* mice, where dilation of the aortic root is seen when backcrossed into a 129SvEv strain (Koenig et al. 2017).

GATA5 and GATA6

In addition to *NOTCH1*, *GATA5*, a member of the *GATA* family of transcription factors, has also been implicated in BAV where predicted damaging sequence variants in *GATA5* have been identified in affected individuals by several investigators (Padang et al. 2012; Bonachea et al. 2014; Shi et al. 2014). Genetic deletion of *Gata5* in mice led to a partially penetrant R-NC BAV phenotype (25%) with associated valve stenosis (Figure 2E–F) (Laforest et al. 2011). Further studies revealed that endothelial cell-specific deletion of *Gata5* utilizing *Tie2-Cre* in a mixed genetic background (129SV/C57Bl6) recapitulated the R-NC BAV (21%) phenotype of *Gata5*^{-/-} mice. Of note, BAV identified in humans with *GATA5* variants had both R-NC and R-L BAV. Similar to *NOTCH1*, disease-causing variants in *GATA5* have been reported in a spectrum of CHD (Jiang et al. 2013; Wei et al. 2013; Kassab et al. 2016).

Interestingly another member of the GATA transcription factor family has also been implicated in BAV. While mutations in *GATA6* are implicated as a cause of truncus arteriosus (TA) and identified in patients with septal defects and TOF, murine models display a BAV phenotype (Kodo et al. 2009; Maitra et al. 2010). Mice haploinsufficient for *Gata6* display 56% incidence of BAV in males and 27% incidence in females due to R-L fusion. Further analysis revealed that second heart field (SHF) specific deletion of *Gata6* utilizing *Isl1-Cre* mice can recapitulate the BAV phenotype indicating SHF-specific role for *Gata6* in valve development (Gharibeh et al. 2018). The conotruncal heart defects identified in humans are only found in *Gata4*^{+/-}; *Gata5*^{+/-} and *Gata5*^{+/-}; *Gata6*^{+/-} murine embryos, as they display double outlet right ventricle (DORV) and overriding aorta (Laforest and Nemer 2011).

These mouse models of BAV provided important tools to gain insight into the disease. First, generation of murine models of R-L and R-NC BAV imply that these phenotypes have distinct embryologic origins and clinical observations have suggested that they have unique long term co-morbidities (Mahadevia et al. 2014). These murine models also display the common complications of BAV, which include CAVD and aortopathy. Accordingly, the models will serve as important tools that can be used to study the mechanisms of disease progression and potentially test novel therapies. A final observation is the genotype-phenotype discrepancy in leaflet fusion phenotype found in mouse models versus humans with corresponding genetic mutations.

Murine models of conotruncal and aortic arch artery defects

Malformations of the cardiac outflow tract and aortic arch include a range of CHD from TOF, DORV, TA to interrupted aortic arch (IAA) (Figure 3). The etiology for majority of conotruncal and aortic arch defects remains unknown, however, a known genetic risk factor is 22q11 microdeletion, which accounts for approximately 12% of conotruncal heart defects (Momma 2010).

22q11 Deletion Syndrome/TBX1/CRKL

Approximately 75% of patients with 22q11 deletion syndrome (22q11DS), who commonly harbor a ~3Mb heterozygous deletion of chromosome 22q11.2, are born with CHD,

primarily conotruncal heart defects and aortic arch anomalies (Yamagishi and Srivastava 2003). Identifying the causative gene for CHD associated with 22q11DS was an important step in the investigation of genetic and developmental basis for CHD. Although molecular genetic studies revealed that >40 genes resided in the crucial 22q11.2 locus, direct sequencing failed to detect mutations in any genes in patients with 22q11DS (Lindsay 2001). Therefore, mouse models for 22q11DS were generated by creating orthologous chromosomal deletions. By using this genetically engineered mouse (*Df1*), which harbored a heterozygous deletion of the orthologous 22q11 region, investigators were successful in reproducing aortic arch abnormalities and some conotruncal heart defects in *Df1*/+ mice that recapitulated those in human 22q11DS (Lindsay et al. 1999). Subsequent studies demonstrated that pathogenic variants in *TBX1*, a gene located in chromosome 22q11.2, as the cause of cardiovascular defects that occur in 22q11DS (Lindsay et al. 2001; Merscher et al. 2001).

Heterozygosity of *Tbx1* caused aortic arch anomalies, whereas homozygosity of *Tbx1* led to most of the phenotypes observed in 22q11DS, including not only conotruncal and aortic arch defects but also thymic hypoplasia, parathyroid hypoplasia, cleft palate and abnormal facial features that are characteristic of this syndrome (Hu et al. 2004; Zhang and Baldini 2008). Several animal models with mutant *Tbx1* alleles have also been generated (Table). Homozygous *Tbx1* null mouse embryos are associated with 100% neonatal lethality with cardiovascular defects including conotruncal heart defects. However, mice harboring hypomorphic alleles of *Tbx1* that express varying levels of Tbx1 display a range of defects including TOF, DORV, TA and IAA (Figure 3D–J) (Hu et al. 2004; Zhang and Baldini 2008).

CRKL, a gene encoding CRK like proto-oncogene, adaptor protein also maps within the commonly deleted region for 22q11DS. Mice homozygous for a targeted null mutation at *Crkl* locus (*Crkl*^{-/-}) showed abnormal aortic arteries and malalignment of the outflow tract including IAA, overriding aorta and DORV (Figure 3A–C) (Guris et al. 2001). While *TBX1* is considered to be the major genetic determinant for several phenotypes associated with 22q11DS, *CRKL* may also play a role (Guris et al. 2006; Moon et al. 2006).

Moreover, novel therapeutic strategies have been proposed using *Tbx1* mutant mouse models. Canonical *Wnt/β-catenin* has major roles in cardiac outflow tract development that may act upstream of *Tbx1*. By gene expression profiling, *Tbx1* and *β-catenin* were found to have opposing effects on anterior heart field (AHF) differentiation and consistent with this, genetic rescue experiments between *Tbx1* and *β-catenin* in the AHF found that TA observed in *Tbx1* conditional null embryos was partially rescued by loss of one allele of the *β-catenin* gene in the *Mef2c-AHF-Cre* domain (Racedo et al. 2017). While these genetic approaches are important, it is difficult to translate them to the clinical setting. A recent study using a high throughput screening approach identified vitamin B12 as a molecule capable of enhancing *Tbx1* gene expression (Lania et al. 2016). Using *Tbx1*^{lacZ} mice as a readout, vitamin B12 was shown to increase expression of *Tbx1* *in vivo* and partially rescue the *Tbx1* haploinsufficient phenotype.

FOXC1, FOXC2, FOXH1 (Forkhead transcription factors)

The forkhead transcription factors *Foxc1*, *Foxc2* and *Foxh1* have been shown to recognize and bind to cis-regulatory elements in *Tbx1* promoter and directly promote *Tbx1* expression (Maeda et al. 2006). Mutations in *FOXC1*, *FOXC2* and *FOXH1* were identified in patients with TOF (Roessler et al. 2008; Topf et al. 2014). Mice null for *Foxc1* have coarctation of the aorta and aortic and pulmonary valve dysplasia as well as IAA type B while *Foxc2*-null mice have IAA type A and type B (Winnier et al. 1999). In addition, *Foxc1* and *Foxc2* compound mutant mice also show coarctation of the aorta, aortic and pulmonary valve dysplasia, IAA type A and B (Winnier et al. 1999). Mice deficient for *Foxh1*, which mediates Nodal signaling, demonstrate defects in anterior-posterior patterning and in node formation (Yamamoto et al. 2001). Conditional deletion of *Foxh1* in mice manifests severe conotruncal heart defects including transposition of the great arteries (TGA) and DORV (Yamamoto et al. 2003).

These studies demonstrate the utility of mouse models to generate models of CHD that involve malformations of multiple cardiovascular structures including cardiac septa, valves, and great vessels. In addition, they show the value of genetically engineered murine models to identify the CHD-causing genes within a contiguous gene deletion syndrome or pathologic copy number variation (CNV). Lastly, they show how models can be used to define molecular mechanisms which are then used to identify novel genetic or pharmacologic therapies that may ameliorate the severity of the CHD phenotype.

Murine models of complex congenital heart defects

The development of murine models which mimic the complex forms of CHD have been more difficult to develop. Most success has been obtained with heterotaxy syndrome, which is linked to mutation of genes involved in cilia morphogenesis. A classic example of this is the genetic association with mutations in *ZIC3*, which encodes a gene downstream of the left-right signaling pathway, in patients with X-linked heterotaxy (D'Alessandro et al. 2013). Consistent with the human phenotype mice, null for *Zic3* and those harboring a hypomorphic allele show the spectrum of left-right patterning cardiac defects including TGA, single ventricle/DORV, AVSD and abnormal systemic venous connections along with other neural tube and skeletal malformations (Purandare et al. 2002; Haaning et al. 2013). Numerous genetic murine models of left-right patterning defects have also been reported that recapitulate human disease (Pierpont et al. 2018).

Another major subtype of left-sided human CHD is hypoplastic left heart syndrome (HLHS). HLHS is a severe form of CHD that accounts for 4–8% of all CHD, and is characterized by hypoplasia of the left ventricle, aorta, and other left-sided cardiac structures including atresia or stenosis of both the aortic and mitral valves (Figure 4). In spite of significant advances in the management of HLHS, mortality and morbidity remain significant, and its etiology is not well understood. Although genetic syndromes such as Turner and Jacobsen syndrome are associated with HLHS, role of individual genes are unknown. While mouse knockouts of *Hand1* and *Hand2*, transcription factors expressed in developing ventricles, have provided critical insights in ventricular morphogenesis, they were unable to replicate the HLHS phenotype (McFadden et al. 2005). As no major genetic

driver for HLHS has yet been identified, no monogenetic animal model for HLHS exists to study its molecular basis and an oligogenic etiology has been proposed (McBride et al. 2005).

A recent study described HLHS in mutant mice and the identification of genes causing HLHS (Liu et al. 2017). Mutations in *Sap130*, which encodes Sin3A-associated protein in the histone deacetylase complex, and *Pcdha9* which encodes the cell-adhesion protein protocadherinA9, were validated by CRISPR-Cas9 genome editing in mice, revealing digenic etiology for HLHS. They also identified one individual with HLHS who harbored *SAP130* and *PCDHA13* mutations. 26% of *Sap130^{m/m};Pcdha9^{m/m}* mutant mice exhibit phenotypes associated with HLHS where *Sap130* mediates left ventricular hypoplasia, and *Pcdha9* increases penetrance of aortic valve abnormalities, suggesting synergistic interactions between *Sap130* and *Pcdha9* mutations resulting in HLHS (Figure 4). Of note, a subset of the embryos displayed DORV with hypoplastic left ventricle as opposed to the strict morphologic definition of HLHS. These studies raise the question if digenic or oligogenic murine models will need to be developed to accurately recapitulate some of these more complex forms of CHD.

Other approaches for genetic modeling of congenital heart disease

Rapid advancements in next generation sequencing approaches have aided in the discovery of novel etiologies for CHD. The NHLBI-funded Pediatric Cardiac Genomics Consortium (PCGC) and an international study led by the Wellcome Trust have performed whole exome sequencing of nearly 5,000 individuals with CHD (Sifrim et al. 2016; Jin 2017). These studies have uncovered numerous potential CHD-causing genes which are estimated to be ~400, which does not incorporate the potential different variants within each gene that may have differential effects. Accordingly, the sheer number of variants requires genetic models that allow for a more high throughput analysis. Along these lines, the zebrafish and the fruit fly have emerged as *in vivo* and iPSCs as *in vitro* models, and the details of each are discussed below.

Zebrafish and Fruit Fly

The zebrafish (*Danio rerio*) has emerged as an important vertebrate model to study cardiovascular disease. In the two-chambered zebrafish heart, deoxygenated blood flows through single atrium and ventricle, which pumps blood through the gills for oxygenation. Despite the anatomic differences between the two-chambered zebrafish and four-chambered mammalian heart, several studies have indicated similar gene regulatory networks driving cardiogenesis (Asnani and Peterson 2014). Eighty-two percent of human disease related genes (listed in OMIM) have at least one orthologue in zebrafish and these are well-suited for genetic manipulation as well as morpholino-induced gene knockdown to simulate human cardiac disease (Howe et al. 2013). In addition, zebrafish larvae are transparent, which allows easy visualization of the heart and vasculature during development (Brown et al. 2016). The zebrafish model has already been utilized to model cardiac phenotypes associated with genes implicated in non-syndromic (e.g. *GATA4*, *GATA6*, *MYH6*, *NKX2-5*, and *TBX20*) and syndromic CHD (e.g. *PTPN11*, *NIPBL*, *CHD7*, *TBX1*, and *TBX5*)

(reviewed in (Grant et al. 2017)). While the exact cardiac morphologic phenotypes are not often recapitulated, these high throughput studies highlight the ability for this model to screen for potential disease genes and genetic variants.

A relatively simple organism, the fruit fly (*Drosophila melanogaster*), is also being utilized in genetic studies. Although it is difficult to directly compare fly developmental defects to specific CHD subtypes, this can serve as a testing platform for disease-associated genes identified from patient genomic sequencing. Functional homologs of 75% of human disease associated genes are also represented in the fly genome (Reiter et al. 2001). In a recent study, a cardiac-specific, 4XHand-Gal4 driver, was combined with fly lines carrying UAS-Gene-IR RNAi based silencing constructs to examine cardiac structure and function for fly homologs. One hundred and thirty-four candidate disease genes published by the PCGC and 52% of these homologs demonstrated their involvement in cardiac development and function in flies (Zhu et al. 2017a). In addition, the *Drosophila* model system may be useful to test for gene-gene interactions that are important for heart formation which may be useful in attempts to define the oligogenic nature of complex CHD (as discussed above) (Vogler and Bodmer 2015).

Induced pluripotent stem cells (iPSC)

Various studies based on transgenic mouse models have verified genes involved in cardiac development. However, complete deletion of gene is often required to generate cardiac phenotypes in mouse model, which does not reflect the pathogenic point mutation and haploinsufficiency commonly observed in human CHD (Musunuru et al. 2018). Human-induced pluripotent stem cells (hiPSC) provide a unique *in vitro* platform to study human cells as they can be differentiated and expanded into multiple somatic cell types. When hiPSCs are derived from patient, they not only carry the exact pathogenic mutation in the human genome but also the genetic background, which may serve as a modifier of the CHD phenotype. These properties have uniquely qualified the hiPSCs model to study human disease in a dish (Mital 2016). Additionally, introduction and/or correction of genetic variants is possible with the genome editing tools which allows researchers to generate new disease models and determine therapeutic strategies for individuals. Initial progress has been made utilizing hiPSCs for comprehensive understanding of cardiomyopathies, arrhythmias, vascular disorders, along with metabolic risk factors for ischemic heart disease (Musunuru et al. 2018).

As discussed above, mutations in *GATA4* and *NOTCH1* have been linked to cardiac septal defects and aortic valve disease, respectively. In both cases, patient derived iPSCs have been used to define the underlying molecular mechanism for these diseases. Generation and analysis of iPSC-derived cardiomyocytes from patients with heterozygous *GATA4 G296S* missense mutation demonstrated that the mutant cells displayed decreased contractility and abnormal calcium handling along with dysregulation of Shh signaling, a pathway implicated in cardiac septation that was recently shown to be downstream of Gata4 in mice (Hoffmann et al. 2009; Ang et al. 2016b; Zhou et al. 2017). They also used multiple genomic approaches to investigate the underlying mechanisms. As anticipated, they found a disruption of the cooperative regulation of a cardiac gene program mediated by Gata4 and

Tbx5 but surprisingly also noted an upregulation of an endocardial/endothelial gene program, demonstrating the multitude of cellular effects from a single point mutation. A similar approach was used to investigate *NOTCH1* mutations, where iPSC-derived endothelial cells from patients with *NOTCH1* mutations were examined. Using an *in vitro* model of vascular shear stress, and these mutant cells failed to activate anti-osteogenic, anti-inflammatory, and anti-oxidant pathways that were induced in control cells (Theodoris et al. 2015). This observation was consistent with observed susceptibility towards calcification of the BAV. Supravalvular aortic and pulmonary artery stenosis can occur due to mutations in *ELN* (elastin) or as part of Williams-Beuren syndrome, a contiguous gene deletion syndrome at chromosome 7q11.23 where *ELN* resides. Disease-causing mutations in *ELN* were modeled using hiPSC-derived smooth muscle cells (SMCs) and demonstrated less differentiated SMCs, with impaired formation of actin filaments and vascular tubes, along with increased proliferation rates (Ge et al. 2012; Kinnear et al. 2013). The generation of these cellular models for *NOTCH1* and *ELN* mutation-mediated disease have resulted in a novel tool to screen for novel pharmacologic therapies.

Apart from monogenic diseases, hiPSC studies are also important to delineate complex CHD caused by multiple genetic and environmental factors, such as HLHS. Accordingly, investigators have generated iPSC derived-cardiomyocytes from patients with HLHS and found that these cells display a diminished yield of cardiomyocytes, a reduced beating rate, disorganized sarcomeres and sarcoplasmic reticulum along with downregulation of NOTCH signaling components, which were interestingly rescued by nitric oxide stimulation (Jiang et al. 2014; Hrstka et al. 2017; Yang et al. 2017). Together these observations show the advantages of iPSCs as an *in vitro* model to understand cellular events leading to morphogenetic defects along with generating an important tool to investigate new therapies. In addition, iPSC cells may serve as a promising strategy for the functional examination of the predicted disease-causing variants in the non-coding genome that are identified from whole genome sequencing approaches in the CHD population.

Concluding Remarks

With the sequencing of human genome and technological advances in genome analysis, there has been the discovery of numerous monogenic causes of non-syndromic and syndromic CHD. While cardiac defects span a spectrum of malformations involving the four-chambered heart, genetic manipulation of mice has been successful in recapitulating these phenotypes from septation defects, valve abnormalities to conotruncal heart defects. These models have served to define disease mechanisms and have also allowed for a better understanding of genotype-phenotype correlations and the importance of modifiers that result in phenotypic variability. In addition, novel gene-environment effects have been discovered where maternal exercise is able to rescue the ASD phenotype in *Nkx2-5* haploinsufficient mice and *Notch1* haploinsufficient embryos exposed to maternal diabetes display a highly penetrant VSD phenotype (Schulkey et al. 2015; Basu et al. 2017). Also for mice that survive to adulthood, they allow for investigation into the role of cardiac developmental genes in adult-onset cardiovascular complications.

The recent large-scale sequencing efforts in human CHD have also demonstrated its complex genetic architecture. These studies have frequently identified likely pathogenic CNV and rare and predicted pathogenic sequence variants in children with CHD. For the majority of cases, the functional significance of the proposed candidate genes and genetic variants in causing CHD is unknown. Accordingly, analysis of this large number of genetic abnormalities is not amenable using the murine model system, which has been the gold standard. Therefore, it will be important to use additional *in vivo* (fruit fly and zebrafish) and *in vitro* (iPSC) model systems to functionally characterize these new potentially pathologic variants. Ultimately as the field moves forward, it may be necessary to generate larger animal models of CHD (i.e. pig) using gene-editing techniques (Ryu et al. 2018) as this will allow for surgical correction of these defects and long term studies to better understand the mechanisms of disease found in adult CHD survivors.

Acknowledgements

The authors thank Dr. Madhumita Basu for critical review of the manuscript. V.G. is supported by funding from the Abigail Wexner Research Institute at Nationwide Children's Hospital and grants from the National Institutes of Health (R01HL121797 and R01HL132801). J.Y. is supported by funding from the Japan Heart Foundation/Bayer Yahukin Research Grant Abroad.

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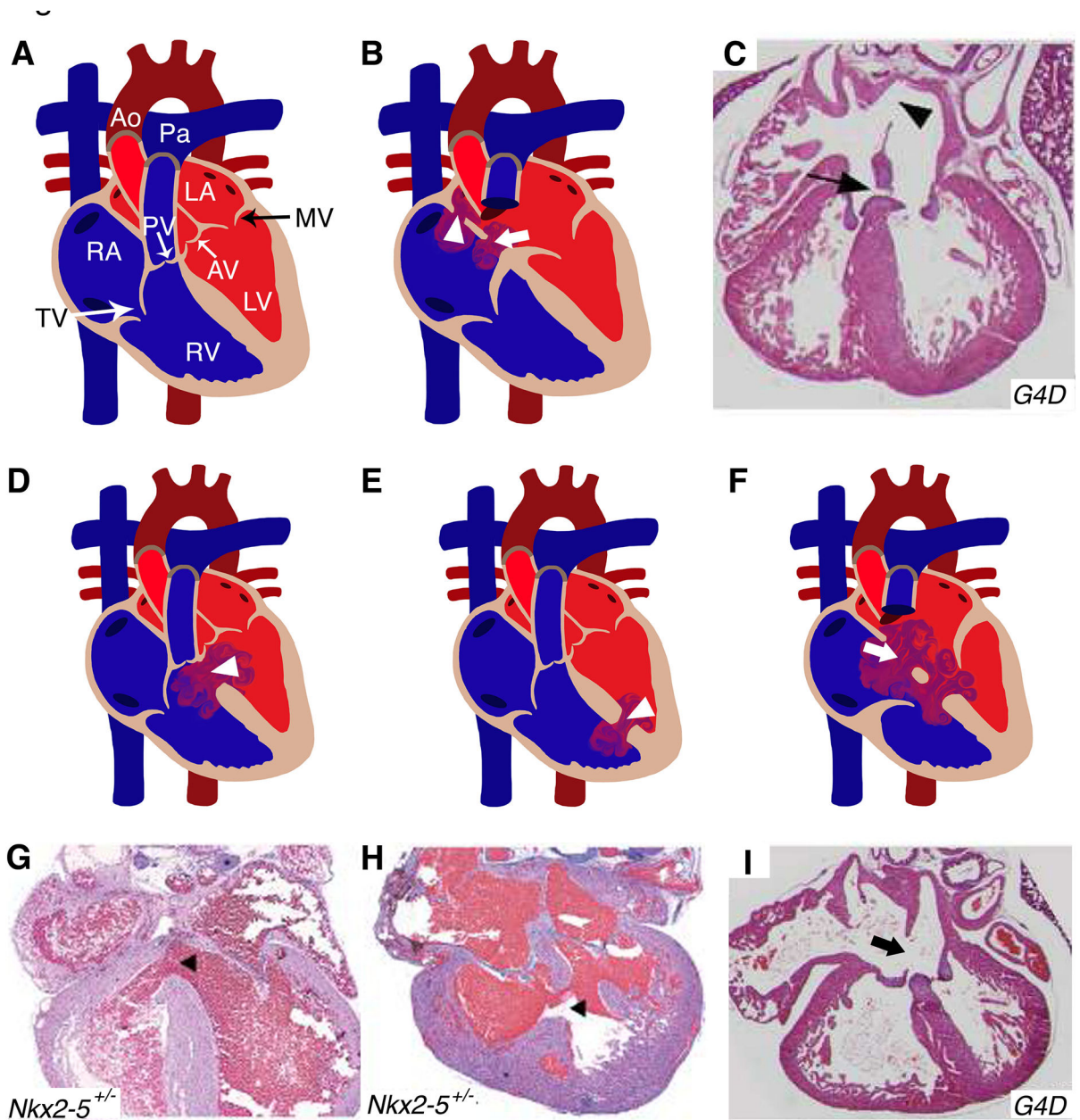


Figure 1. Murine models of cardiac septation defects:

(A) Illustration depicts a cross-sectional view of a normal and mature four chambered heart. (B) Location of primum (arrowhead) and secundum (arrow) ASD in human heart diagram which has been recapitulated in *Gata4*^{ex2/WT} (*G4D*) mouse model (C). (D, E) Schematics display similar features of perimembranous and muscular VSDs (arrowhead) seen in human CHD patients were also detected in *Nkx2-5*^{+/-} mice (G, H). (F) The diagram represents AVSD phenotype with single valve (arrow) noted in humans, resembled by the *G4D* murine model (I). RA: right atrium; RV: right ventricle; LA: left atrium; LV: left ventricle; MV: mitral valve; AV: aortic valve; PV: pulmonary valve; TV: tricuspid valve; Ao: Aorta; Pa: Pulmonary artery; ASD: atrial septal defect; VSD: ventricular septal defect; AVSD:

atrioventricular septal defect. (C, F, I) and (G, H) have been adapted from Rajagopal et al. 2007 and Winston et al. 2010 with permission.

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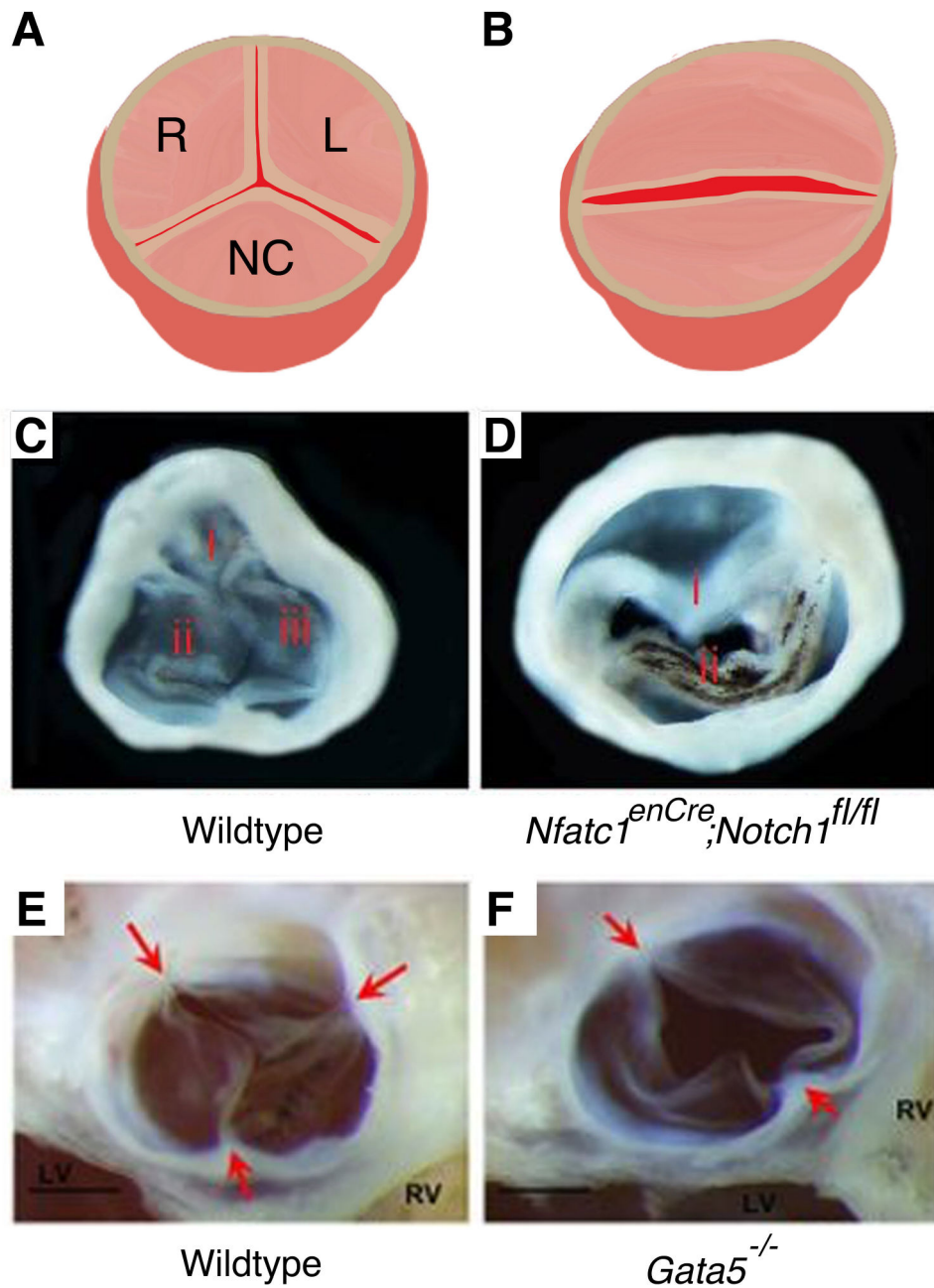


Figure 2. Mouse models for aortic valve malformations:

Illustrations represent a tricuspid aortic valve (A) and a bicuspid aortic valve, BAV (B). Right (R), left (L) and noncoronary (NC) leaflets are labelled. BAV were demonstrated in endothelial specific deletion of *Notch1* (*Nfatc1^{enCre}; Notch1^{fl/fl}*) (D) and in *Gata5^{-/-}* (F) mice compared to normal three leaflet structure found in wildtype mice (C, E). (C, D) and (E, F) have been adapted from Wang et al. 2017 and Laforest et al. 2011 with permission.

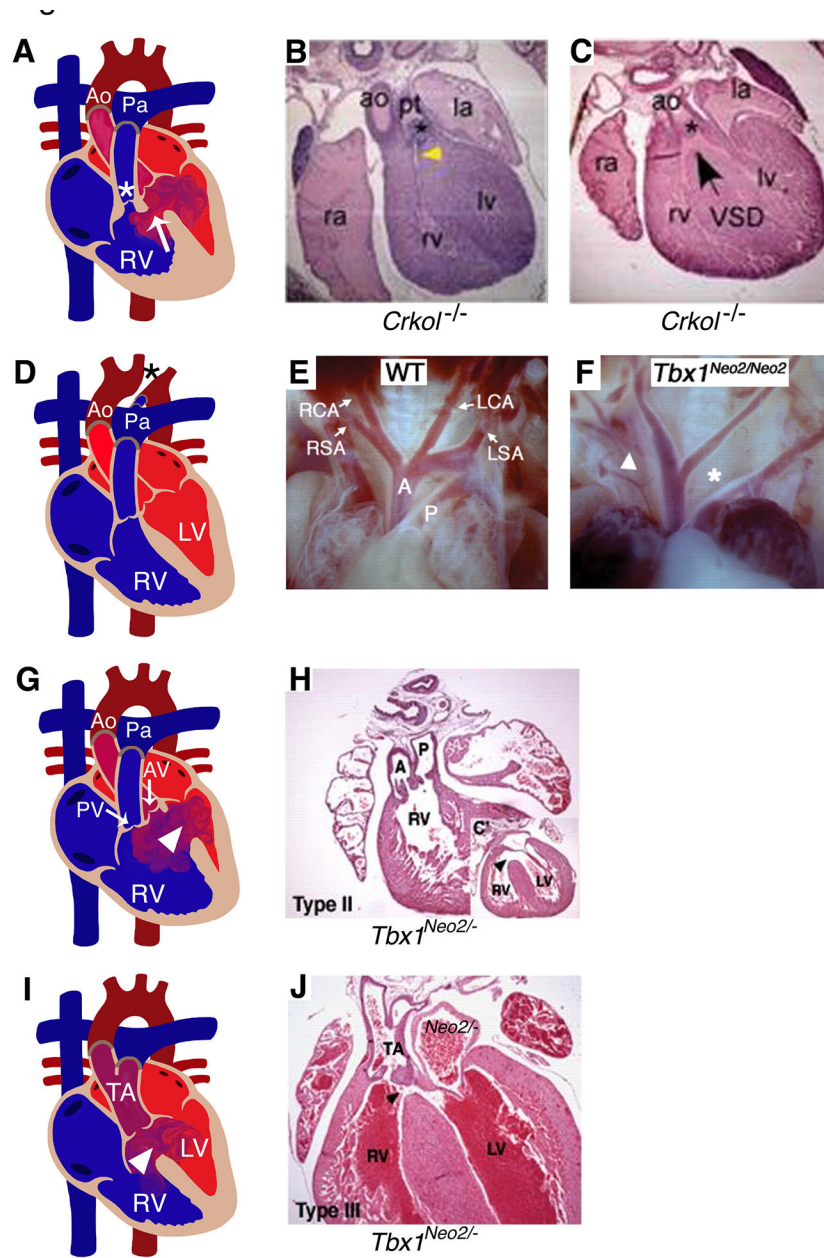


Figure 3. Murine models for conotruncal and aortic arch artery defects.

(A) Diagram illustrates TOF, characterized by right ventricular hypertrophy, VSD (arrow), stenosis of pulmonary valve and sub-valvar area (*) in human heart. Comparable TOF phenotype was observed in mouse model of *Crkol*^{-/-} at embryonic day 16.5 shown in B and C. (D, F) IAA (*) detected in human patients were recapitulated in *Tbx1*^{Neo2/Neo2} mouse model at E18.5 compared to wildtype mouse (E). (G, I) Representative diagram of a diseased heart display DORV and TA with VSD (arrow head) in human (G, I). Similar phenotypes were observed in *Tbx1*^{Neo2/Neo2} mice (H, J) at E18.5. RA: right atrium; RV: right ventricle; LA: left atrium; LV: left ventricle; AV: aortic valve; Ao/A: Aorta; Pa/P: Pulmonary artery; TOF: tetralogy of Fallot; IAA: Interrupted aortic arch; DORV: double

outlet right ventricle; TA: truncus arteriosus; VSD: ventricular septal defect. (E, F, H, J) have been adapted from Guris et al. 2001 and Zhang and Baldini 2008 with permission.

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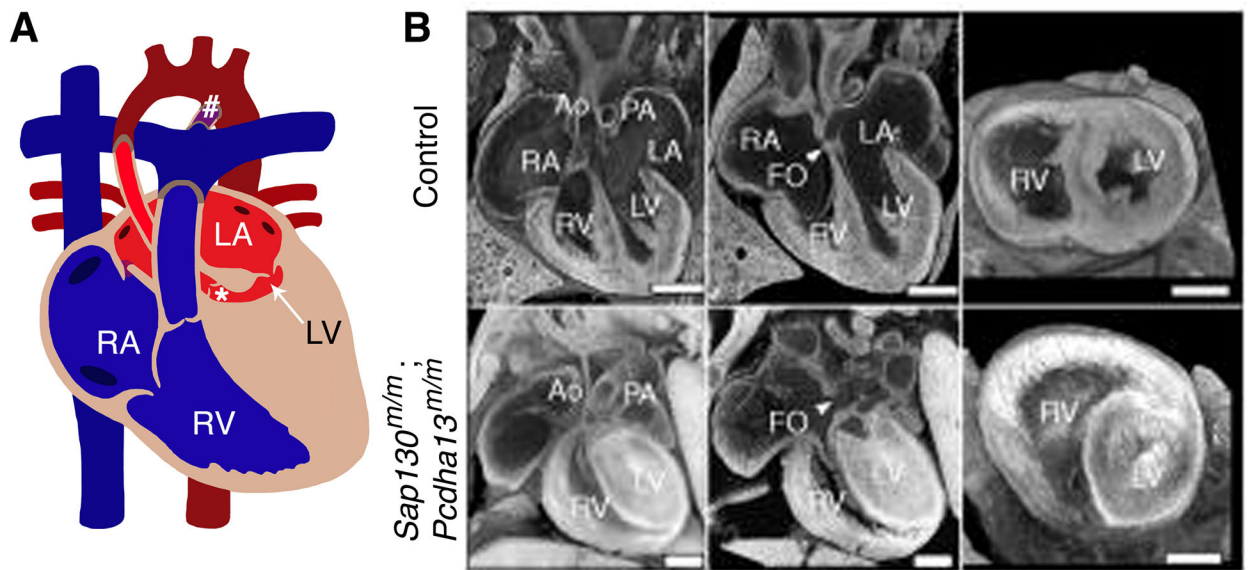


Figure 4. Models for hypoplastic left heart syndrome (HLHS)

(A) Diagram represents a heart with HLHS, characterized by small/hypoplastic left ventricle (arrow) and aorta (*) and PDA (#). B represents similar features of HLHS phenotype in mouse model of *Sap130^{m/m}; Pcdha13^{m/m}* compared to littermate controls at postnatal day 0 (P0). RA: right atrium; RV: right ventricle; LA: left atrium; LV: left ventricle; Ao: Aorta; Pa: Pulmonary artery; PDA: Patent ductus arteriosus; FO: foramen ovale. (B) has been adapted from Liu et al. 2017 with permission.

Table.

Murine models of congenital heart disease

Cardiac septation defects						
Gaie	OMIM #	human CHD phenotype	Syndrome with associated CHD	Mouse Genotype	Mouse phenotype	References
<i>ACVR1(ALK2)</i>	102576	AVSD		<i>Alk2^{fl/fl}, Tie2-Cre</i>	AVSD, VSD	(Wang et al. 2005)
<i>CITED2</i>	602937	ASD, VSD		<i>Cited2^{-/-}</i>	ASD, VSD, DORV, TA	(Bamforth et al. 2001)
<i>CREBBP</i>	600140	ASD, VSD, CoA, PS, BAV	Rubinstein-Taybi syndrome	<i>CBP^{+/-}</i>	ASD, VSD, BAV	(Oike et al. 1999)
<i>EP300</i>	602700	ASD, VSD, CoA, PS, BAV	Rubinstein-Taybi syndrome	<i>EP300^{+/AS}</i>	ASD, VSD	(Shikama et al. 2003)
<i>GATA4</i>	600576	ASD, PS, TOF, VSD, AVSD		<i>Gata ex2^{wt}</i>	ASD, VSD, AVSD	(Rajagopal et al. 2007)
				<i>Gata4^{G295Sski/wt}</i>	ASD, AS, PS	(Misra et al. 2012; LaHaye et al. 2019)
<i>KMT2D</i>	602113	AS, CoA, ASD, VSD, BAV, HLHS, TOF	Kabuki syndrome	<i>Kmt2^{fl/fl}, Mef2c-AHF-Cre</i>	VSD	(Ang et al. 2016a)
<i>NIPBL</i>	608667	ASD, VSD, PS	Comelia de Lange syndrome	<i>Nipbl^{+/-}</i>	ASD, VSD	(Kawauchi et al. 2009)
<i>NKX2-5</i>	600584	ASD, atrioventricular conduction delay, TOF, VSD, HLHS		<i>Nkx2.5^{+/-}</i>	ASD, PFO, VSD, AVSD, BAV, AS	(Biben et al. 2000; Winston et al. 2010)
				<i>Nkx2.5^{+/R52G}</i>	ASD, VSD, AVSD, Ebstein's anomaly, atrioventricular block, tricuspid valve atresia	(Ashraf et al. 2014; Chowdhury et al. 2015)
				<i>Nkx2.5^{R141C+}</i>	ASD, atrioventricular block, VSD	(Zakariyah et al. 2017)
<i>PTPN11</i>	176876	PS, AVSD, CoA, ASD, VSD, TOF, left ventricular outflow tract obstruction	Noonan syndrome	<i>Ptpn11^{D61G+}</i>	ASD, AVSD, DORV	(Araki et al. 2004)
<i>SHOC2</i>	602775	PS, AVSD, CoA, ASD, VSD, TOF	Noonan syndrome	<i>Sur-8^{fl}, Tie2-Cre</i>	VSD, DORV, TGA	(Yi et al. 2010)
<i>TBX5</i>	601620	ASD, VSD	Holt-Oram syndrome	<i>Tbx5^{5del+}</i>	ASD, atrioventricular block, VSD	(Bruneau et al. 2001)
				<i>Tbx5^{fllox/fllox}, Tie2-Cre</i>	ASD, PFO	(Nadeau et al. 2010)
	190685	ASD, VSD, AVSD, TOF	Down syndrome	<i>Tc1</i>	VSD, AVSD	(O'Doherty et al. 2005)
				<i>Dp(10)1Yey/+; Dp(16)1Yey/+; Dp(17)1Yey/+</i>	VSD, AVSD	(Yu et al. 2010)
				<i>Dp1Tyb Dp3Tyb</i>	VSD, AVSD, DORV	(Lana-Elola et al. 2016)
Cardiac valve malformations						

Cardiac septation defects						
Gaie	OMIM #	human CHD phenotype	Syndrome with associated CHD	Mouse Genotype	Mouse phenotype	References
Gene	OMIM #	human CHD phenotype	Syndrome with associated CHD	Mouse Genotype	Mouse phenotype	References
<i>DCHS1</i>	603057	MVP		<i>Dchs1^{+/-}</i>	MVP	(Durst et al. 2015)
<i>GATA5</i>	611496	BAV		<i>Gata5^{-/-} Gata^{fl/fl}; Tie2-Cre</i>	BAV, AS	(Laforest et al. 2011)
<i>GATA6</i>	601656	TA, ASD, AVSD, TOF, BAV		<i>Gata6^{-/-} Gata6^{wu/fl}; Isll-Crs</i>	BAV	(Gharibeh et al. 2018)
<i>MATR3</i>	164015	BAV, CoA, PDA		<i>Matr3^{+/-}</i>	BAV, CoA, PDA, VSD, DORV	(Quintero-Rivera et al. 2015)
<i>NOTCH1</i>	190198*	BAV, AS, HLHS, TOF, PS, CAVD		<i>Notch1^{+/-}</i>	BAV, CAVD, aortic aneurysm	(Nigam and Srivastava 2009; Nus et al. 2011; Bosse et al. 2013; Koenig et al. 2017)
				<i>Notch1^{fl/fl}, Nfatc1-enCre</i>	BAV	(MacGrogan et al. 2016; Wang et al. 2017)
				<i>Notch1^{+/-} mTR^{G2}</i>	CAVD, AS	(Theodoris et al. 2017)
				<i>Nos3^{-/-}; Notch1^{+/-}</i>	BAV, AS, AR, CAVD, TOF	(Bosse et al. 2013; Koenig et al. 2016)
<i>SMAD6</i>	602931	BAV, AS, CoA		<i>Smad1^{-/-}</i>	cardiac valve hyperplasia	(Galvin et al. 2000)
Conotruncal and aortic arch artery defects						
Gene	OMIM #	human CHD phenotype	Syndrome with associated CHD	Mouse Genotype	Mouse phenotype	References
<i>CHD7</i>	608892	TOF, DORV, VSD, ASD, TA, PS, AS, MS, TS	CHARGE syndrome	<i>Chd7^{+/-}</i>	IAA, aortic arch defects	(Randall et al. 2009)
<i>CRKL</i>	602007	TOF, TA, IAA, VSD, aortic arch defects	22q11 deletion syndrome	<i>Crkl^{-/-}</i>	IAA, VSD, overriding aorta, DORV	(Guns et al. 2001)
<i>FOXC1</i>	601090	TOF		<i>Foxc1^{-/-}</i>	CoA, semilunar valve dysplasia, IAA, VSD	(Winnier et al. 1999)
<i>FOXC2</i>	602402	TOF		<i>Foxc2^{-/-}</i>	IAA, VSD	(Winnier et al. 1999)
<i>FOXH1</i>	603621	TOF, VSD		<i>Foxh1^{C/-}</i>	right isomerism, ASD, VSD, TGA, DORV	(Yamamoto et al. 2003)
<i>JAG1</i>	601920	TOF, PS, ASD, VSD	Allagille syndrome	<i>Jag1^{fl/fl}, Islet1-Cre Jag1^{fl/fl}, Mef2c-AHF-Cre</i>	DORV, PS, TA, ASD, VSD, aortic arch defects	(High et al. 2009)
<i>TBX1</i>	602054	TOF, TA, IAA, VSD, aortic arch defects	22q11 deletion syndrome	<i>Tbx1^{-/+}</i>	Aortic arch defects, VSD	(Lindsay et al. 1999)

Cardiac septation defects						
Gaie	OMIM #	human CHD phenotype	Syndrome with associated CHD	Mouse Genotype	Mouse phenotype	References
				<i>Tbx1</i> ^{Neo2/Neo}	TOF, TA, DORV, IAA, VSD, aortic arch defects	(Zhang and Baldini 2008)
				<i>Tbx1</i> ^{neo/neo}	TA, IAA, VSD, aortic arch defects	(Hu et al. 2004)
				<i>Tbx1</i> ^{+/-}	IAA, aortic arch defects	(Lindsay et al. 2001; Zhang et al. 2005)
<i>ZFPM2(FOG2)</i>	603693	TOF, DORV		<i>Fog2</i> ^{-/-}	TOF, ASD, VSD	(Tevosian et al. 2000; Pizzuti et al. 2003)
Other cardiac defects						
Gene	OMIM #	human CHD phenotype	Syndrome with associated CHD	Mouse Gmotype	Mouse phenotype	References
<i>ELN</i>	130160	SVAS	Williams-Beuren syndrome	<i>Eln</i> ^{+/-}	SVAS	(Li et al. 1998)
<i>FBN1</i>	134797	BAV, AR, MVP, aortic aneurysm, aortic dissection	Marfan syndrome	<i>Fbn1</i> ^{C1039G+}	MVP, aortic aneurysm	(Ng et al. 2004; Habashi et al. 2006)

AS, aortic valve stenosis; ASD, atrial septal defect; AR, aortic valve regurgitation; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CAVD, calcific aortic valve disease; CHD, congenital heart diseases; CoA, coarctation of aorta; DORV, double outlet right ventricle; HLHS, hypoplastic left heart syndrome; IAA, interrupted aortic arch; MS, mitral valve stenosis; MVP, mitral valve prolapse; PDA, patent ductus arteriosus; PFO, patent foremen ovale; PS, pulmonic valve stenosis; SVAS, supraaortic stenosis; TA, truncus arteriosus; TGA, transposition of great arteries; TOF, tetralogy of Fallot; TS tricuspid valve stenosis; VSD, ventricular septal defect.