The developmental genetics of congenital heart disease

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Congenital heart disease is the leading cause of infant morbidity in the Western world, but only in the past ten years has its aetiology been understood. Recent studies have uncovered the genetic basis for some common forms of the disease and provide new insight into how the heart develops and how dysregulation of heart development leads to disease.

Congenital heart disease usually refers to abnormalities in the heart's structure or function that arise before birth. They occur often and in many forms. Congenital heart diseases are found in 19–75 of every 1,000 live births, depending on which types of defect are included[1], and the incidence is higher if fetuses that do not survive to term are included[2]. This number excludes cardiomyopathies, conduction-system disease and laterality defects, which, although inherited and present at birth, are considered separately because of their distinct clinical presentation. A clear picture of how the heart forms is crucial for understanding the genesis of congenital heart disease, because dysregulation of heart development is at the root of the disease. This review focuses on genetic studies over the past ten years that have pinpointed the causes of inherited congenital heart diseases. Together with recent insight into how the heart normally develops, these studies have considerably improved the understanding of congenital heart diseases.

The clinical picture

Congenital heart diseases affect most parts of the heart (Fig. 1) and can be classified into three broad categories: cyanotic heart disease, left-sided obstruction defects and septation defects. Infants with cyanotic heart disease appear blue as a result of the mixing of oxygenated and deoxygenated blood. Defects that can contribute to this condition include transposition of the great arteries (TGA), tetralogy of Fallot (TOF), tricuspid atresia, pulmonary atresia, Ebstein's anomaly of the tricuspid valve, double outlet right ventricle (DORV), persistent truncus arteriosus (PTA) and total anomalous pulmonary venous connection. Left-sided obstructive lesions, the second main type of congenital heart disease, include hypoplastic left heart syndrome (HLHS), mitral stenosis, aortic stenosis, aortic coarctation and interrupted aortic arch (IAA). Septation defects, the third main type of congenital heart disease, can affect septation of the atria (atrial septation defects, ASDs), septation of the ventricles (ventricular septal defects, VSDs) or formation of structures in the central part of the heart (atrioventricular septal defects, AVSDs). Other types of congenital defect that do not fit neatly into the three main categories are bicuspid aortic valve (BAV) and patent ductus arteriosus (PDA). The most common congenital heart disease is BAV, and septation defects are the next most common.

Mortality and morbidity vary with the severity of the congenital heart disease and can be serious. The multiple surgeries needed to correct many of the anatomical defects can be debilitating, and quality of life is often greatly compromised. Children with congenital heart disease frequently develop neurological disorders, even if the child has not undergone surgery, indicating an important secondary effect of congenital heart diseases *in utero*[3]. It is therefore crucial to understand the effects of congenital heart diseases on prenatal and postnatal physiology.

Although the major underlying defects that cause congenital heart disease are thought to be mutations in regulators of heart development during embryogenesis[4], epidemiological data also point to environmental influences[5]. For example, prenatal exposure to angiotensin-converting-enzyme inhibitors increases the risk of several congenital malformations, including those that cause heart diseases[6]. However, these epidemiological studies have mostly suggested risk rather than pinpointing the underlying disease mechanisms.

A genetic component for congenital heart diseases was initially implicated by their recurrence in families, and by studies showing an association of congenital heart diseases with inherited microdeletion syndromes, in which a chromosomal region containing many genes is deleted. But until ten years ago, aside from these microdeletion syndromes, little was known about the genetics of congenital heart diseases. Indeed, geneticists and clinicians debated whether congenital heart diseases could be caused by a single-gene defect. Confounding these discussions were cases in which different members of one family might have anatomically distinct defects — for example, one member with an ASD, one with TOF and one with a VSD. These apparently discordant clinical phenotypes arising within one family were difficult to rationalize. In addition, mild or intermediate ('forme-fruste') defects, such as atrial septal aneurysms, are sometimes either discounted or not diagnosed, and thus the pattern of genetic inheritance of congenital heart diseases is often not clear.

New concepts in heart development

Congenital heart diseases arise from abnormal heart development during embryogenesis, so understanding how the heart forms normally is important (Fig. 2). The regulatory mechanisms involved in establishing the early heart and regulating its morphogenesis have been studied extensively[7,8]. The earliest cardiac progenitors arise from lateral plate mesoderm, controlled by a cascade of interacting transcription factors. Additional inputs come from secreted molecules, such as fibroblast growth factors, bone morphogenetic proteins, Wnt proteins and others[8].

Recent findings have clarified the origin of cardiac precursors and their regulation. Discovery of a 'second' heart field (SHF) led to a rethinking of the origin and patterning of the embryonic heart[9]. The SHF is medial and dorsal to the early differentiating cardiomyocytes that comprise the 'cardiac crescent', and gives rise to a large portion of the heart, including the outflow tract, right ventricle and most of the atria (Fig. 2a). The SHF is further subdivided into a number of lineage

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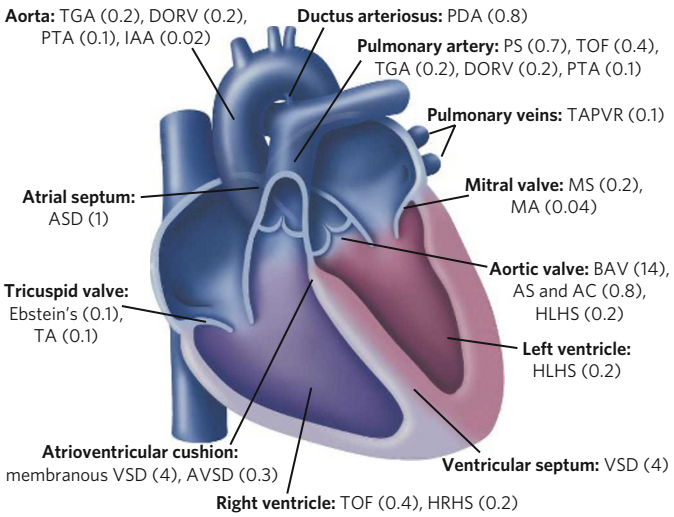

Figure 1 | Congenital heart defects. This diagram of the adult heart illustrates the structures that are affected by congenital heart diseases, with the estimated incidence of each disease per 1,000 live births indicated in parentheses. AC, aortic coarctation; AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; DORV, double outlet right ventricle; Ebstein's, Ebstein's anomaly of the tricuspid valve; HLHS, hypoplastic left heart syndrome; HRHS, hypoplastic right heart; IAA, interrupted aortic arch; MA, mitral atresia; MS, mitral stenosis; PDA, patent ductus arteriosus; PS, pulmonary artery stenosis; PTA, persistent truncus arteriosus; TA, tricuspid atresia; TAPVR, total anomalous pulmonary venous return; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect. (Image courtesy of F. Yeung, University of Toronto, Canada.)

pools[9], which contribute either to anterior structures (such as the outflow tract) or posterior components (such as the atria). These findings help explain how mutations associated with congenital heart disease can, by affecting only specific cell lineages within the SHF, result in defects in specific heart structures.

Progress has also been made in understanding how the pool of undifferentiated cardiac precursors that contribute to the SHF arises and how their further development is regulated. Intriguingly, the cardiovascular lineages — myocardial, endocardial and smooth muscle — all derive from common precursors that sequentially branch off as specialized cell types[10–12]. This strategy is similar to that used by the haematopoietic system. Regulation of the expansion and allocation of the early heart precursors has been attributed, in large part, to the Wnt family of secreted molecules[13]. However, which Wnts are important and where they signal from have yet to be determined.

An important principle in heart development is that regulation of different cell lineages must be tightly controlled so that the correct lineage differentiates at the correct time and in the correct location. Recent work in zebrafish has shown that a key level of regulation might be the active repression of the cardiac programme in anterior lateral plate mesoderm adjacent to heart precursors, by imposition of a haematopoietic and endocardial programme[14]. Heart-field size in zebrafish is negatively controlled by retinoic acid[15] and is thus influenced by both cell-type-specific determinants and broad patterning cues. In the SHF in mice, the transcription factor NKX2-5 limits the expansion of cardiac progenitors and promotes their differentiation potential: in mice lacking NKX2-5, early overproduction of progenitor cells is followed by impaired proliferation of SHF cells, resulting in a smaller outflow tract and right ventricle[16].

The role of transcription factors in heart development is well established[7,8], but less is known about the role of factors that modify the structure of chromatin; that is, the fibres of DNA and proteins (known as histones) that make up chromosomes and whose packaging can restrict or allow gene activation. BAF60C (also known as SMARCD3), a subunit of the Swi/Snf-like chromatin-remodelling complex BAF, physically links cardiac transcription factors to the BAF complex. Loss of BAF60C results in severe defects in cardiac morphogenesis and impaired activation of a subset of cardiac genes[17]. Interestingly, a partial reduction in BAF60C levels leads to more-restricted defects in outflow tract formation, suggesting that regulation of the dosage of chromatin-remodelling complexes is crucial for normal heart development[17]. Whereas BAF complexes alter the structure of chromatin, other chromatin-remodelling proteins modify histones, and these proteins are also important for heart formation. The muscle-restricted histone methyltransferase SMYD1 (also known as BOP) is a crucial regulator of cardiac chamber growth and differentiation[18]. With regard to the heart, histone deacetylases have mostly been characterized as having a role in hypertrophy, but they are also important in heart development[19].

Transcription-factor interactions

Human genetic studies have identified numerous genes that are responsible for inherited and sporadic congenital heart diseases. Most of these genes encode transcription factors that regulate specific events in heart development, such as ventricular septation or outflow tract morphogenesis (Fig. 3). The first identified single-gene mutation giving rise to an inherited congenital heart disease was in the T-box transcription factor gene *TBX5*, the causative gene in Holt–Oram syndrome (HOS)[20,21]. HOS predominantly includes ASDs, VSDs and conduction-system defects. Soon after this first discovery, mutations in *NKX2-5* were identified in families with inherited ASDs and atrioventricular block[22], and *NKX2-5* mutations were also found in families with diverse congenital heart-disease lesions, including VSDs, Ebstein's anomaly and TOF[23]. These results provided the insight that haploinsufficiency of a developmentally important transcription factor is at the root of disease and could explain the characteristic dominant pattern of disease inheritance. The importance of transcription-factor dosage was confirmed by using mouse models[24,25] (Fig. 4 and Box 1). An important finding from this work is that TBX5 and NKX2-5 interact physically and synergistically to activate their downstream targets[25,26], providing insight into how mutations altering either of these proteins affect cardiac gene expression and lead to disease.

The importance of interacting transcription factors was further emphasized by studies showing that mutations in the zinc-finger transcription-factor-encoding gene *GATA4* cause inherited septation defects[27]. GATA4, long studied as a regulator of cardiac gene expression, physically interacts with NKX2-5 (refs 7, 8). Defective interactions between GATA4 and NKX2-5, and between GATA4 and TBX5, might underlie congenital heart diseases caused by *GATA4* mutations. Thus, on the basis of positional cloning in three types of congenital heart disease with overlapping defects, three interacting cardiac transcription factors were identified as dosage-sensitive regulators of heart formation.

In mouse chromosome-engineering studies, another transcription factor gene, *Tbx1*, was pinpointed as the likely single-gene culprit in 22q11 microdeletion syndrome (also known as DiGeorge syndrome), which is characterized by congenital heart diseases such as TOF, PTA and IAA[28,29]. This conclusion was supported by the identification of *TBX1* missense mutations in patients with features of 22q11 microdeletion syndrome but without a microdeletion[30]. *Tbx1* is expressed in the SHF and is important for its normal expansion[31,32]. Other genes within the 22q11 critical region probably also contribute to the syndrome. Indeed, a deficiency in one such gene, *Crkl*, results in similar defects in a mouse model and exacerbates deletion of *Tbx1* (refs 33, 34).

The known network of interacting cardiac transcription factors has continued to grow in size and complexity with the identification of the Spalt-family gene *SALL4* as the causative gene in Okihiro syndrome — which includes congenital heart diseases and limb defects almost identical to those in HOS[35,36] — and the identification of *TBX20* mutations in families with ASDs, VSDs, valve defects and impaired chamber growth[37]. SALL4 interacts physically and genetically with TBX5 to pattern the interventricular septum in a mouse model[38]. Whereas TBX5 and SALL4 can function together either to repress or to activate gene expression (depending on the target gene), TBX5, GATA4 and NKX2-5 function together

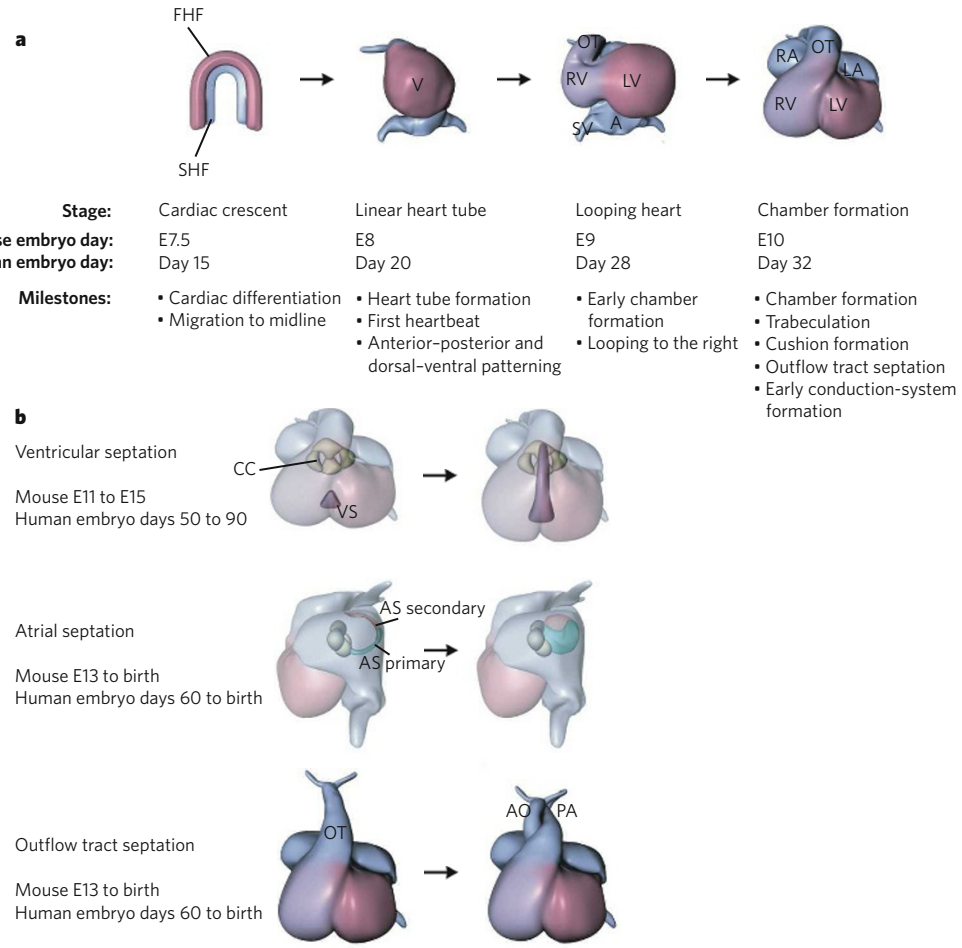

Figure 2 | Heart development. **a**, Early steps in heart development. Diagrams of heart development are shown in ventral views. At the earliest stages of heart formation (cardiac crescent), two pools of cardiac precursors exist. The first heart field (FHF) contributes to the left ventricle (LV), and the second heart field (SHF) contributes to the right ventricle (RV) and later to the outflow tract (OT), sinus venosus (SV), and left and right atria (LA and RA, respectively). V, ventricle. **b**, Maturation of the heart. The cardiac cushions (CC) will give rise to the atrioventricular valves. The ventricular septum (VS) arises from myocardium from the left and right ventricles. Atrial septation (AS) occurs by the growth of two septa: the primary septum (green) and the secondary septum (pink). Outflow tract septation separates the common outflow tract (OT) into the aorta (AO, connected to the left ventricle) and the pulmonary artery (PA, connected to the right ventricle). (An interactive version of the figure can be found at http://pie.med.utoronto.ca/HTBG/index.htm.) (Images courtesy of F. Yeung, University of Toronto, Canada.)

only to activate genes. The overlapping expression patterns and complex interactions of these transcription factors allow fine regulation of cardiac gene expression and morphogenesis[38].

Mutations in *TFAP2B*, which encodes the transcription factor activating enhancer-binding protein-2β (AP2β) and is expressed by neural crest cells, have been linked to PDA in families with Char syndrome, implying that regulation of neural-crest function is important for normal ductus closure[39]. However, the function of AP2β in heart development is unknown. Also, mutations in the gene encoding thyroid-hormone-receptor-associated protein 2 (THRAP2) — a subunit of the mediator complex, which is essential for transcriptional activation — have been reported in both a family with TGA and in sporadic cases of TGA[40], but little is known about this gene or how it functions in outflow tract development.

Although the concept that transcription factors participate in a complex set of interactions has been important for understanding the regulation of cardiac gene expression, as well as the aetiology of congenital heart diseases and their patterns of inheritance, few downstream targets have been identified that might explain the precise cellular basis for congenital heart diseases. The main challenge now is to identify the specific targets and cellular mechanisms that are involved in congenital heart diseases downstream of the associated transcription factors.

Altered haemodynamics

Complex congenital heart diseases with an outflow tract defect, such as TOF, can be accompanied by 'accessory' congenital heart diseases, such as persistent right-sided aortic arch, which can markedly alter heart physiology. Because the heart functions during its morphogenesis, haemodynamic forces might participate in cardiac morphogenesis, providing an explanation for how a primary outflow tract defect can lead to secondary structural defects. In zebrafish, altering haemodynamics mechanically or genetically has profound consequences on heart morphology[41,42]. In mice, a recent study pinpointed altered haemodynamics as a key intermediate between altered outflow tract morphogenesis and signalling events in branchial-arch artery remodelling[43]. Effects on haemodynamics might also explain some puzzling genetic data regarding the presence of mutations in the gene *MYH6*, which encodes α-myosin heavy chain, in families with inherited ASD[44]. It is unclear how defects in a gene encoding a contractile protein cause ASDs, but altered haemodynamics during embryonic development is probably a crucial factor.

Signalling defects underlie valve disease

Several types of congenital heart disease involve valve defects of varying severity. Valve dysfunction might not be severe in the infant but can often progress during adulthood, requiring valve-replacement surgery in the adult. Cardiac valve formation relies on a complex interplay of signalling between the myocardium and the overlying endocardium, which undergoes an epithelial-to-mesenchymal transition[45]. Secreted proteins are important in this process[45], and mutations affecting signalling proteins and downstream pathways can lead to valve disease.

A notable example is the Notch signalling pathway. *NOTCH1* is expressed in the endocardium of the great vessels of the heart, where it is thought to be important for epithelial-to-mesenchymal transition and valve formation[46], and it is the causative gene in some cases of BAV[47]. Individuals with BAV can also have HLHS, aortic stenosis or other serious valvular anomalies; in many cases, these patients later develop aortic-valve calcification, a major indicator for valve replacement. Individuals with *NOTCH1* mutations have a similar spectrum of defects, including aortic stenosis, VSD, TOF and, in one patient, mitral atresia, DORV and hypoplastic left ventricle[47]. NOTCH1 also represses a bone-related pathway[47], which might explain calcifications in the cardiac valves of patients with *NOTCH1* mutations.

Alagille syndrome, which affects cardiac valves, can also result from defective Notch-pathway signalling. The causative gene in most patients

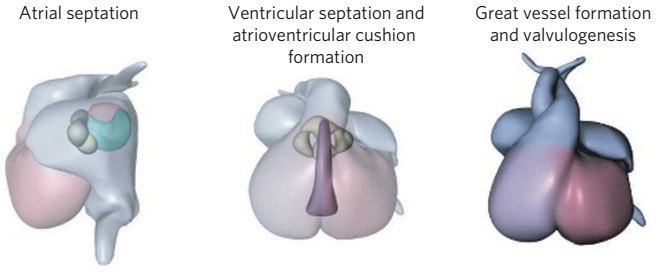

Figure 3 | Origin and genetic aetiology of congenital heart disease. Three major classes of developmental defects are indicated: defects in atrial septation, in ventricular or atrioventricular septation, and in the great vessels. The types of congenital heart disease that occur within each class are indicated, with the associated mutated genes listed. Genes for which mutations result in discrete congenital heart diseases are indicated in black; genes that are mutated in congenital heart diseases that are part of a wider syndrome (also involving defects that are not associated with congenital heart disease) are indicated in blue. *CRELD1*, cysteine-rich with epidermal-growth-factor-like domains 1; *KRAS*, ki-Ras; *PTPN11*, protein tyrosine phosphatase, non-receptor type 11; *SOS1*, son of sevenless homologue 1. (Images courtesy of F. Yeung, University of Toronto, Canada.)

with this syndrome, which includes pulmonary valvular stenosis and occasionally TOF, encodes the Notch ligand JAG1 (refs 48, 49). More recently, mutations in the gene *NOTCH2* have also been identified in families with Alagille syndrome[50], reinforcing the link between this disease and defective Notch-pathway signalling and demonstrating genetic heterogeneity of the disease.

Signalling defects also underlie valve disease in Noonan syndrome, an inherited multifaceted disease that includes various heart defects, predominantly defective pulmonary valves and AVSDs but also hypertrophic cardiomyopathy. Mutations in a set of genes that encode proteins of the Ras–mitogen-activated protein-kinase signalling pathway (SHP2, ki-Ras, RAF1 and SOS1), which regulates multiple aspects of cellular function, cause Noonan syndrome and the related cardio-facio-cutaneous syndrome[51–55]. Most of the mutations that lead to these two syndromes are activating mutations, and study of a mouse model of Noonan syndrome led to the conclusion that overactive SHP2 signalling results in hyperproliferation of outflow tract cushions[56].

MicroRNA dysfunction

In the past few years, much excitement has resulted from a newly identified class of small non-coding RNAs called microRNAs (miRNAs). These small (21-nucleotide) RNAs modulate protein function by binding to target messenger RNA, resulting in repression of translation or in degradation of the target mRNA[57]. A number of miRNAs have recently been shown to function in the heart[58]. Potentially of most relevance to congenital heart disease, miR-1 was shown to be important in the embryonic development of the heart[59,60].

Two separate genes, *miR-1-1* and *miR-1-2*, encode miR-1. Both genes are expressed in the developing heart, and transgenic overexpression experiments have suggested that these genes might be involved in regulating cardiomyocyte proliferation[59]. Both genes are under the control of serum response factor, indicating that they are part of a developmental programme regulated by cardiac transcription factors[59]. It has been shown that miR-1 targets the cardiac transcription factor HAND2, which is implicated in the growth of the embryonic heart, as well as several other regulators of cardiac growth and development. A gene-targeting approach found that deletion of *miR-1-2* results in heart defects that include VSDs; surviving mice have conduction-system defects and increased cardiomyocyte proliferation[60]. Thus, dysregulation of miR-1 or other developmentally important miRNAs might result in congenital heart disease in humans.

The later consequences of embryonic defects

Individuals with congenital heart disease can suffer from secondary heart disease later in life, possibly as a result of corrective surgery during infancy. The sequelae are sometimes severe; for example, after closure of a septal defect, some patients can progress to heart failure[61]. With improved surgical outcomes for those with congenital heart disease, the number of adults with such diseases now exceeds the number of children. Thus, it has become imperative to understand the postnatal consequences of congenital heart diseases. Recent results suggest that these might be caused, at least in part, by the direct effects of mutations associated with congenital heart disease on postnatal heart morphology and function. For example, in a family with *GATA4* mutations, apart from having heart structural defects, some individuals developed dilated cardiomyopathy later in life[27]. Indeed, data from mouse models support a connection between *GATA4* mutations and adult cardiomyopathy[62]. Similarly, mutations in *TBX20* were identified in patients with cardiomyopathy[37], as well as in those with structural congenital heart diseases.

Mouse studies have also revealed roles for other congenital-heart- disease-associated genes in cardiac function. Studies of mice in which *Nkx2-5* had been deleted only in the ventricles suggest a role for this gene in the function of the postnatal conduction system and in myocardial structure, and examination of patients with *NKX2-5* mutations revealed that some had aspects of cardiomyopathy, as predicted from the mouse data[63]. A primary defect in cardiac relaxation has been identified in a mouse model of HOS. This defect results from impaired calcium cycling owing to reduced expression of the gene *Serca2*, which encodes a calcium-uptake pump. Patients with HOS also have diastolic dysfunction (Y. H. Zhou, A. O. Gramolini, M. A. Walsh, Y. Q. Zhou, C. Slorach, M. Friedberg, J. K. Takeuchi, H. Sun, R. M. Henkelman, P. H. Backx, A. N. Redington, D. H. MacLennan and B.G.B., unpublished observations). Thus, embryonic-patterning genes control structural components of the heart and can also have a separate role in heart function, for example by regulating *Serca2*. These genes can thus modulate important aspects of heart function that cause pathology in the postnatal heart when dysregulated. This concept has important implications for the clinical management of adults with congenital heart disease.

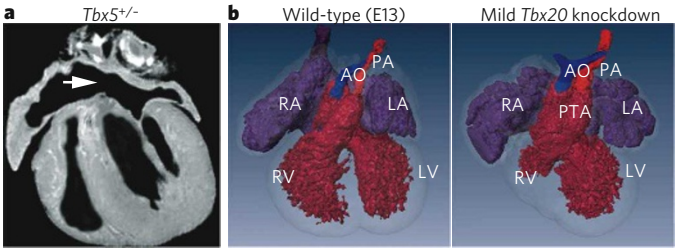

Figure 4 | Modelling human congenital heart diseases in mice, and dosage-dependent regulation of cardiac morphogenesis. a, Heart magnetic resonance imaging section of a mouse that is heterozygous for a *Tbx5* mutation, demonstrating an ASD (arrow), as also seen in humans heterozygous for a *TBX5* mutation. (Panel adapted, with permission, from ref. 25.) **b**, A partial (about 60%) reduction in TBX20 levels leads to a hypoplastic right ventricle and PTA, as also seen in humans heterozygous for a *TBX20* mutation. The outline of the heart is translucent white; the fill of the atria is purple; the fill of the ventricles and outflow tract is dark red; the aorta is blue; and the pulmonary artery is light red. E, day of embryonic development. (Panel adapted from ref. 65.)

Box 1 | The relevance of mouse models

Mice are often good models for studying human disease. But do mice carrying mutations in genes that are associated with congenital heart disease closely recapitulate the human conditions? The answer is yes for some such genes; for example, mice in which one allele of *Tbx5* has been deleted have heart defects that accurately mimic those found in patients with HOS who have a heterozygous *TBX5* mutation[25] (Fig. 4a). However, for other gene mutations that are associated with congenital heart disease, there are differences in the precise heart defects seen and in the sensitivity of the phenotype to gene dosage. For example, deletion of one allele of *Nkx2-5* in mice replicates only subtle aspects of human disease resulting from *NKX2-5* deficiency[24]. As another example, *Tbx20* deficiency in mice results in heart defects only if the dosage falls below 50% (refs 65, 66) (Fig. 4b), whereas in humans, *TBX20* mutations that are predicted to cause a 50% loss of function result in a range of structural defects[37]. Similar dosage effects on the severity of congenital heart disease can be found in mice with mutations affecting several signalling molecules, such as bone morphogenetic proteins. For example, an allelic series of *Bmp4*, in which different mutations result in varying amounts of protein being produced, shows the full spectrum of human atrioventricular canal defects, from primum ASD to complete AVSD[67], indicating that mutations affecting this pathway could cause human congenital heart disease (although this has not yet been verified). Differing sensitivities to gene dosage in mice and humans are probably an important factor in their differing manifestations of congenital heart disease. The basis of these differences in dosage sensitivity might be species-specific physiology — such as the shorter gestation time or faster heart rate of mice — or it might be influenced by genetic factors, as suggested by studies that demonstrate an effect of genetic background on congenital heart diseases[68]. Another difference between human and mouse models of congenital heart disease is that humans with defects such as PTA or DORV survive to term, whereas mice with these defects rarely do. Thus, mouse models are of considerable value for research on congenital heart diseases, but intrinsic differences between mouse and human physiology need to be carefully taken into account.

Future perspectives

The study of congenital heart diseases has come a long way since their description and classification. Improvements in *in utero* diagnosis and surgical techniques have considerably brightened the prospects for infants born with congenital heart diseases, but biological insights into this set of developmental diseases have been gained only recently. Identification of the causative genes in inherited forms of congenital heart disease has pointed towards specific pathways of disease and, in the process, provided considerable new knowledge about heart development.

Many issues remain to be addressed, however. Although transcriptional programmes that are impaired in individuals with congenital heart diseases are being identified, the mechanisms of how these deficiencies translate to a structural defect are unknown. For example, what cellular events are defective in the pathogenesis of TGA? A recent clue comes from the finding that WNT11, which signals downstream of the transcription factor PITX2, regulates morphogenesis of the outflow tract through transforming growth factor-β2, resulting in altered cell shape[64]. Another question is how epigenetic regulation coordinates these genetic programmes into a cohesive whole, and why a mechanism so sensitive to dosage perturbation has been maintained throughout evolution. Now that some of the genes involved in the main forms of congenital heart disease have been identified, a new challenge is to understand how common polymorphisms in these genes might cause subtle, yet more prevalent, disease. Finally, with the success of surgical interventions for many congenital heart diseases, what are the consequences of such diseases for adults, and how should these patients be evaluated and treated? With the current progress in understanding congenital heart diseases and heart development, the next ten years are likely to provide much clearer answers to these questions. ■

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