A genetic blueprint for cardiac development

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Congenital heart disease is the leading non-infectious cause of death in children. It is becoming increasingly clear that many cardiac abnormalities once thought to have multifactorial aetiologies are attributable to mutations in developmental control genes. The consequences of these mutations can be manifest at birth as life-threatening cardiac malformations or later as more subtle cardiac abnormalities. Understanding the genetic underpinnings of cardiac development has important implications not only for understanding congenital heart disease, but also for the possibility of cardiac repair through genetic reprogramming of non-cardiac cells to a cardiogenic fate.

Formation of the heart involves a wondrous and precisely orchestrated series of molecular and morphogenetic events, and even subtle perturbation of this process can have catastrophic consequences in the form of congenital heart disease (CHD). The course of heart formation in the embryo has been known for decades but, until recently, we have known little about the genes that control its developmental programme. Insight has come from studies in vertebrate and invertebrate model organisms that have identified cardiac developmental control genes, which can now be investigated as possible culprits in human CHD. Of equal importance has been the mapping and identification of human CHD genes (see Box 1, Table 1). Insights into the structure–function relationships of the encoded proteins *in vivo* have been gained by correlation of particular amino-acid mutations with the associated cardiac malformations. Most genetic studies in model organisms involve homozygous loss-of-function mutations, which frequently result in severe phenotypes and are lethal in early embryogenesis, and so this approach identifies essential genes that act at critical steps in cardiac development. CHD in humans is, however, primarily a disease of haploinsufficiency, often involving more subtle phenotypes with variable penetrance, which are evident at birth or later. Thus, the two approaches yield different but complementary insights.

Formation of an organ as complex as the heart, with its many integrated structures and cell types, must involve a myriad of genes, many (or most) of which are not cardiac specific. Nevertheless, it is useful to begin thinking about the developmental events of heart formation in the context of genetic networks. Here we outline the beginnings of a genetic blueprint for heart development, and consider how perturbations of cardiac developmental control genes may result in different forms of heart disease in humans.

Specification of cardiac cell fate

Studies in model organisms have revealed an evolutionarily conserved programme of heart development, triggered by specific signalling molecules and mediated by tissue-specific transcription factors. This programme controls the genesis of cardiomyocytes from mesodermal stem cells and the subsequent activation of genes responsible for cardiac contractility and morphogenesis. Cardiomyocytes originate in the anterior lateral mesoderm soon after gastrulation. They are produced in response to protein factors, including bone morphogenetic proteins, that are secreted from adjacent endoderm[1]. Cardiogenic signals, which seem to be at least partially conserved across species, activate expression of the homeobox gene *tinman* in flies and the related gene *Nkx2.5* in vertebrates, the earliest molecular markers of the cardiac lineage[2]. Tinman is necessary for specification of the cardiac lineage and directly activates transcription of the *Mef2* gene, which encodes a transcription factor that controls myocyte differentiation[3]. Tinman and Nkx2.5 cooperate with zinc-finger transcription factors of the GATA family to activate cardiac gene expression[4]; these two classes of cardiac transcription factors also regulate each others' expression through mutually reinforcing positive feedback loops[5].

In contrast to *tinman* in flies, *Nkx2.5* is not essential for specification of the cardiac lineage in mice. Thus, other genes may share related functions with *Nkx2.5*, or cardiogenesis in flies and vertebrates may differ in its dependence on this family of homeobox genes. Functional redundancy between *Nkx2.5* and other cardiac-expressed homeobox genes in vertebrates is suggested by the ability of dominant-negative versions of Nkx2.5 to block cardiogenesis in frog and zebrafish embryos[6,7].

Morphogenesis and looping of the heart tube

Soon after their specification, cardiac muscle cells converge along the ventral midline of the embryo to form a beating

Table 1 Human mutations implicated in congenital heart disease

Disease	Chromosome locus	Gene
Atrial septal defect	5q34	*NKX2.5*
Atrial septal defect, ventricular septal defect	12q24	*TBX5*
Pulmonary stenosis; tetralogy of Fallot	20p12	*JAGGED-1*
Patent ductus arteriosus	6p12	*TFAP2B*
Supravalvar aortic stenosis	7q11	*Elastin*
Aortic aneurysm	15q21	*Fibrillin*
Tetralogy of Fallot; persistent truncus arteriosus; interrupted aortic arch	22q11	?

Box 1
Human genetics in congenital heart disease

We do not yet understand the causes of most complex genetic traits in humans, including congenital heart disease (CHD). For CHD, however, the study of chromosomal disorders and autosomal dominant syndromes, and the genetic linkage analysis of rare pedigrees with milder forms of CHD, have both been informative. A paradigm of variable penetrance and phenotype is emerging, even with single-gene defects, suggesting an important role for secondary factors in CHD.

The 22q11 deletion syndrome provides a way in to understanding the molecular basis of many cardiac neural crest defects. Deletion of a 3-megabase (Mb) region of 22q11 on one copy of chromosome 22 is the most common genetic deletion known in humans and produces DiGeorge or velocardiofacial syndrome[42]. This involves neural crest abnormalities including conotruncal and aortic arch defects, cleft palate and hypoplasia of the thymus and parathyroid glands. It is likely that one or more of the approximately 30 known genes in the 3-Mb 22q11 region is involved in neural crest development. The genetic complexity of this syndrome has, however, precluded definitive identification of the critical gene(s) in this region. Efforts to engineer syntenic chromosome deletions in mice have partially modelled the 22q11 deletion syndrome and are likely to contribute to the identification of the causal gene(s)[43]. This approach, combined with careful dissection of molecular pathways regulating neural crest-derived cells, should uncover the underlying cause of numerous CHDs[44].

Recent genetic studies of the cardiac transcription factors NKX2.5 and TBX5 exemplify the synergy between human genetics and studies of model organisms in understanding the aetiology of human CHDs. Many point mutations have been identified in *NKX2.5* in families with atrial septal defects and cardiac conduction abnormalities[45]. Sporadic mutations of *NKX2.5* have also been found in patients with tetralogy of Fallot, an outflow tract alignment defect, or tricuspid valve anomalies[46]. How these mutations result in cardiac defects is unknown, but the linkage of particular loss-of-function mutations with particular abnormalities suggests that different parts of NKX2.5 function in different developmental decisions in the heart.

Holt–Oram syndrome is characterized by abnormalities in the heart (atrial and ventricular septal defects) and limbs, and is due to mutations in *TBX5* (refs 47, 48). Intriguingly, mutations responsible for heart and limb defects respectively are clustered in different regions of the protein, suggesting that TBX5 engages different downstream genes or cofactors in the different tissues, and that the distinction again depends on unique structural motifs in the protein[49]. The association of limb and heart defects in Holt–Oram and other syndromes suggests a commonality in developmental control mechanisms for these structures, which are both derived from the lateral mesoderm.

Recent genetic studies of Alagille syndrome revealed a critical role for a well-studied developmental pathway involving the transmembrane receptor, Notch. Alagille syndrome is an autosomal dominant disorder characterized by biliary atresia (absence of biliary ducts) and cardiac defects, typically pulmonary artery stenosis and tetralogy of Fallot. It is caused by mutations in Jagged-1, a ligand for the Notch receptor[50,51]. Isolated pulmonary stenosis or tetralogy of Fallot have also been associated with Jagged-1 mutations[52]. The Notch signalling pathway is involved in decisions about cell fate and differentiation throughout the embryo, but has only recently been implicated in cardiovascular development. Whether other mediators of Notch signalling, including the gridlock/Hairy-related transcription factor proteins (see main text), are involved in a similar fashion is under investigation.

Char syndrome is an autosomal dominant trait characterized by patent ductus arteriosus (PDA), hand anomalies and facial dysmorphism. Genetic linkage and candidate gene analysis in families with this syndrome revealed dominant-negative mutations in the transcription factor TFAP2B, which is expressed in the neural crest[41]. An understanding of ductal regulation through the actions of TFAP2B may lead to insight into the aetiology of isolated cases of PDA, which account for 10% of all CHD.

Analyses of families with supravalvar aortic stenosis (SVAS) and Marfan's syndrome, both autosomal dominant in transmission, have helped understand aortic disease. They are both caused by mutations in structural proteins of connective tissues. SVAS, which is characteristic of William's syndrome, is caused by mutations in elastin, although the pathogenetic mechanism remains unknown[53]. Mutations in fibrillin result in Marfan's syndrome, which is characterized by progressive ascending aortic aneurysms (dilations)[54].

linear heart tube composed of distinct myocardial and endocardial layers separated by an extracellular matrix. Studies in mice and fish have revealed an essential role for GATA transcription factors in this process[8–10]. In all vertebrates, the linear heart tube undergoes rightward looping, which is essential for proper orientation of the pulmonary (right) and systemic (left) ventricles, and for alignment of the heart chambers with the vasculature. The molecular mechanisms governing cardiac looping remain unknown, but identification of genes differentially expressed along the outer and inner curvatures of the looped heart tube suggests that intrinsic properties of the two surfaces may underlie this critical morphogenetic event.

The direction of cardiac looping is determined by an asymmetric axial signalling system that also affects the position of the lungs, liver, spleen and gut (see ref. 11 for a review). Before organ formation begins, this signalling cascade directs the asymmetrical expression of Sonic hedgehog and Nodal, a member of the transforming growth factor-β (TGF-β) family, in the lateral mesoderm. Interpretation of left–right signals is mediated in part by the transcription factor Ptx2, which is expressed along the left side of developing organs, including the early heart tube. Mouse models of left–right defects demonstrate absent, bilaterally symmetrical, or reversed Nodal and Ptx2 expression. In humans, mirror-image reversal of left–right asymmetry is often associated with normal organogenesis. But a discordance of cardiac, pulmonary and visceral asymmetry (heterotaxy syndrome) reflects a lack of coordinated left–right signalling and is universally associated with defects in organogenesis. The common association of human cardiac alignment defects with abnormalities in left–right asymmetry points to intersecting pathways that regulate the direction and process of cardiac looping, and highlights the clinical significance of this area of study.

Segmentation and growth of cardiac chambers

Although individual cardiac chambers do not become morphologically distinguishable until after cardiac looping, their cell fates seem to be genetically programmed much earlier. The linear heart tube is segmentally patterned along the anterior–posterior axis into precursors of the aortic sac, conotruncus (outflow tract), pulmonary and systemic ventricles, and atria (Fig. 1). Each cardiac chamber differs in its morphological and contractile properties and its patterns of gene expression. How chamber identities are established is unknown, but this is likely to involve a combinatorial code of transcription factors, both cardiac-specific and more widely expressed (Fig. 2).

In addition, a subset of ventricular cardiomyocytes surrounding the developing coronary arteries differentiates in response to arterial

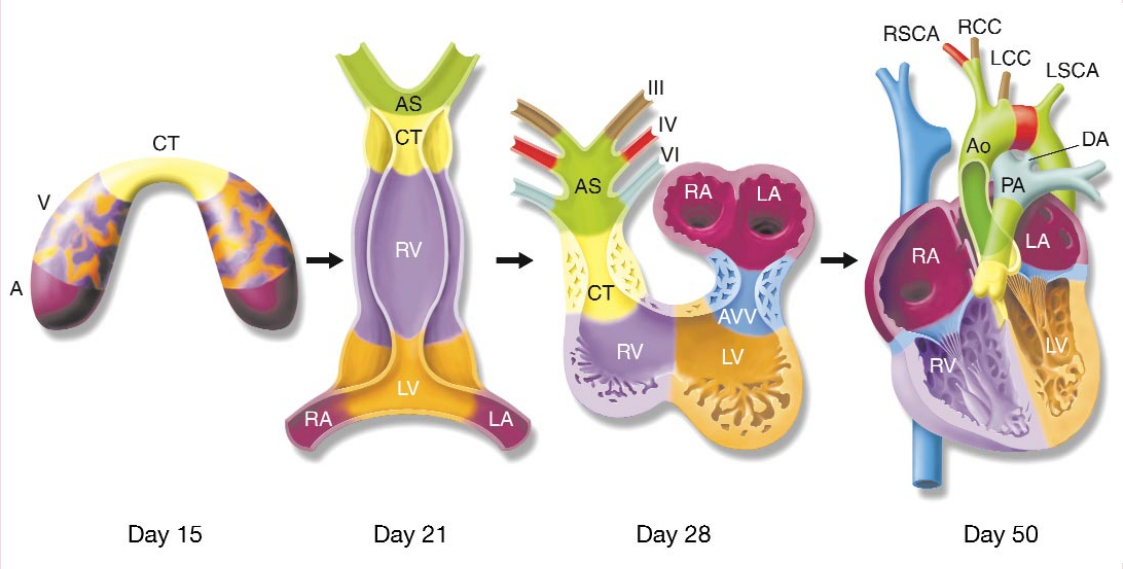

Figure 1 Schematic of cardiac morphogenesis. Illustrations depict cardiac development with colour coding of morphologically related regions, seen from a ventral view. Cardiogenic precursors form a crescent (left-most panel) that is specified to form specific segments of the linear heart tube, which is patterned along the anterior–posterior axis to form the various regions and chambers of the looped and mature heart. Each cardiac chamber balloons out from the outer curvature of the looped heart tube in a segmental fashion. Neural crest cells populate the bilaterally symmetrical aortic arch arteries (III, IV and VI) and aortic sac (AS) that together contribute to specific segments of the mature aortic arch, also colour coded. Mesenchymal cells form the cardiac valves from the conotruncal (CT) and atrioventricular valve (AVV) segments. Corresponding days of human embryonic development are indicated. A, atrium; Ao, aorta; DA, ductus arteriosus; LA, left atrium; LCC, left common carotid; LSCA, left subclavian artery; LV, left ventricle; PA, pulmonary artery; RA, right atrium; RCC, right common carotid; RSCA, right subclavian artery; RV, right ventricle; V, ventricle.

endothelin-1 (ET-1) signalling into tracts of cells that form the cardiac conduction system[12] that regulates heartbeat. The genes regulated by ET-1 that are responsible for this developmental decision are unknown, but are likely to be relevant to cardiac conduction disorders in humans.

Ventricles

Intriguingly, many CHDs affect only a particular segment of the heart, consistent with the notion that each segment develops relatively independently. One of the most severe forms of CHD involves hypoplasia (underdevelopment) of the right or left ventricle. Studies in model organisms have begun to reveal a genetic basis for ventricular development (Fig. 2). The related basic helix–loop–helix (bHLH) transcription factors dHAND/HAND2 and eHAND/HAND1 are expressed predominantly in the primitive right and left ventricular segments, respectively, during mouse heart development[13,14]. Deletion of *dHAND* in mice results in hypoplasia of the right ventricular segment[14]. eHAND has also been implicated in left ventricular development, although early placental defects of *eHAND* mutant mice precluded a detailed analysis of its role in the heart[15,16]. Mice lacking Nkx2.5 also show lethal defects in ventricular morphogenesis and fail to express eHAND in the heart, suggesting that eHAND may act downstream of Nkx2.5 to control left ventricular development[17]. The ventricular-specific homeobox gene *Irx4* is dependent on dHAND and Nkx2.5 for expression, and is sufficient, when misexpressed in the atria, to activate ventricle-specific gene expression[18,19]. In the zebrafish, which has a single ventricle, only one *HAND* gene (*dHAND*) has been identified, mutation of which abolishes the ventricular segment of the heart[20]. Mice lacking the transcription factor MEF2C, normally expressed throughout the atrial and ventricular chambers, also show hypoplasia of the right and left ventricles, resulting in their early demise in embryogenesis[21]. The cardiac defects in *MEF2C* mutant embryos suggest that MEF2C might be a necessary cofactor for one or more ventricular-restricted regulatory proteins. Whether members of such ventricular developmental pathways are disrupted in human syndromes of right or left ventricular hypoplasia remains to be determined.

Atria

Considerably less is known about the genetics of atrial development. The orphan nuclear receptor COUP-TFII is expressed in atrial precursors and is required for atrial, but not ventricular, growth[22]. Retinoid signalling has also been implicated in atrial specification and in the regulation of the atrioventricular border along the anterior–posterior axis of the heart tube[23]. How this border is established remains unclear. In Ebstein's anomaly in humans, the right atrioventricular valve is displaced inferiorly into the ventricle, resulting in 'atrialization' of ventricular myocardium. It will be interesting to determine whether the gene responsible for Ebstein's anomaly is involved in atrioventricular specification.

Growth

Proliferation of cardiomyocytes within each chamber is necessary to support the increasing haemodynamic load during embryonic development. Neuregulin growth factors, secreted from the endocardium, and their myocardial receptors ErbB2 and ErbB4, are required for development of trabeculae, the finger-like projections comprised of the ventricular cardiomyocytes[24]. Defects in ventricular trabeculation have also been observed in mice lacking angiogenic factors, such as vascular endothelial growth factor (VEGF)[25] and angiopoietin-1 (ref. 26), that are expressed in the endocardium. Understanding the mechanism of reciprocal signalling between endocardium and myocardium may provide clues to the regulation of myocardial cell proliferation during development. Harnessing this information to drive adult myocardial cells into a proliferative state remains one of the most important and challenging goals in this field.

Cardiac valve formation

Appropriate placement and function of cardiac valves is essential for division of the chambers and to ensure the unidirectional flow of blood through the heart. Early in development, septation of the cardiac tube into distinct chambers is achieved through regional swellings of extracellular matrix, known as cardiac cushions, that form the anlage of atrioventricular and ventriculoarterial valves (Fig. 1). Reciprocal signalling between the endocardial and myocardial cell layers in the cushion region, mediated in part by TGF-β family members, induces a transformation of endocardial cells into mesenchymal cells[27]. These migrate into the cushions and differentiate into the fibrous tissue of the valves; they are also involved in septation of the common atrioventricular canal into right- and left-sided

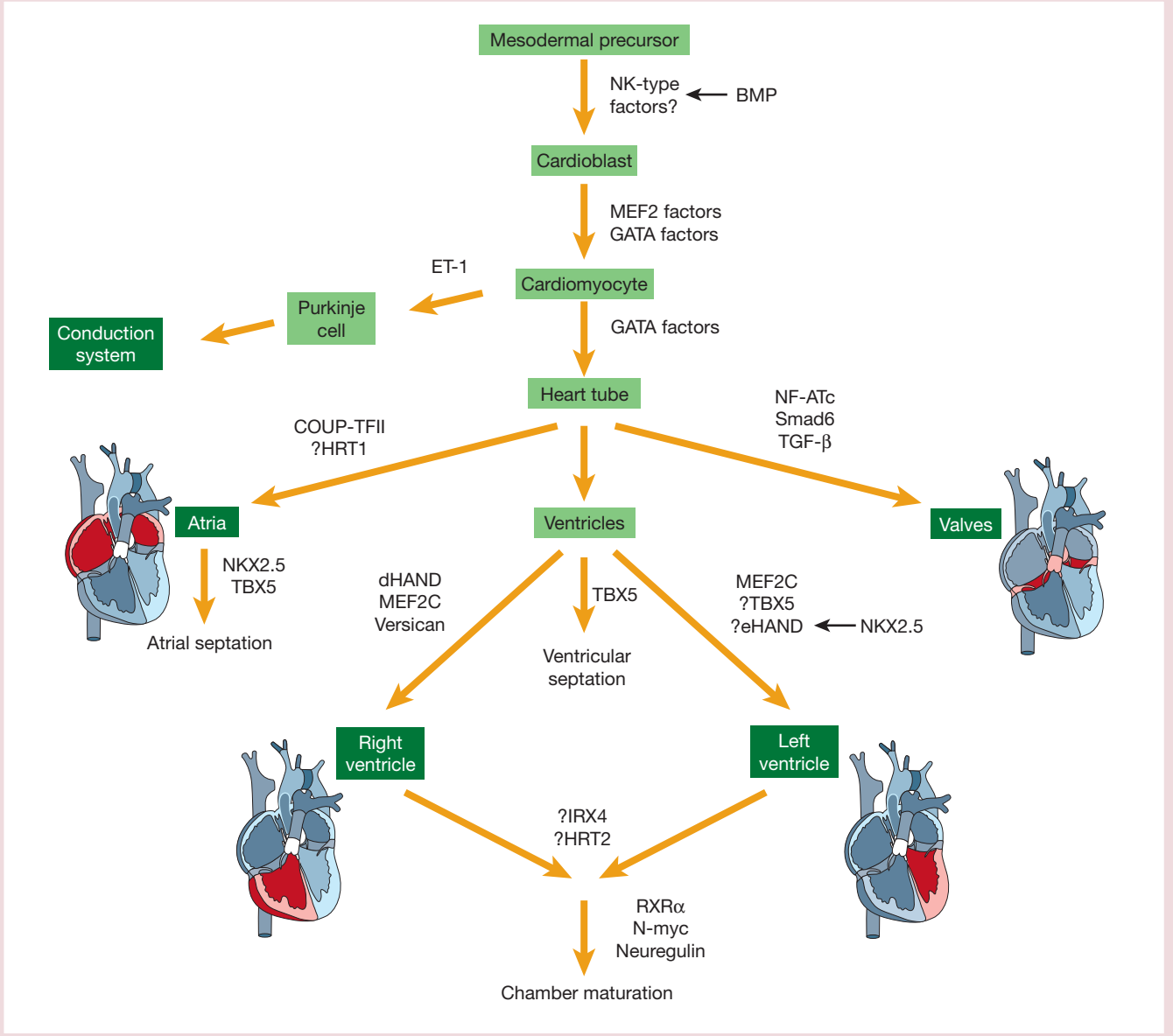

Figure 2 A genetic blueprint for heart development. This schematic shows particular steps in cardiac morphogenesis, focusing on mesodermal contributions. Genetic pathways leading to linear heart-tube formation are partly conserved between *Drosophila* and mouse. The formation of valves, ventricles, atria and the conduction system are under the control of groups of regulatory proteins that may act independently or in a common pathway. Cardiac regions affected by specific pathways are highlighted in dark green boxes with white type. Factors necessary for distinct steps during cardiogenesis in model organisms or humans are indicated beside the arrows. Regulatory factors suspected to have region-specific roles are indicated with a '?'. Cell types or regions of the heart are indicated in boxes. Distinct processes during cardiogenesis (atrial septation, ventricular septation and chamber maturation) are indicated.

orifices. Trisomy 21 (Down syndrome) in humans is commonly associated with incomplete septation of the atrioventricular valves, but the gene(s) on chromosome 21 responsible for valve development remains unknown. Abnormalities in extracellular matrix composition, production or reabsorption may underlie many such defects.

Gene targeting in mice has revealed important roles for the NF-ATc and Smad6 transcription factors in the formation of cardiac valves. Mice lacking NF-ATc, a downstream mediator of signalling by the calcium-dependent protein phosphatase calcineurin, exhibit fatal defects in valve formation, reflecting a potential role for calcineurin in transduction of signals for valvulogenesis[28,29]. Smad6, which is implicated in the activation of gene expression in response to TGF-β signalling, is also expressed specifically in cardiac valve precursors, but disruption of *Smad6* leads to abnormally thickened, gelatinous valves[30]. Interestingly, most human valve defects, which are among the most common CHDs, result in stenotic (narrow) or incompetent valves that are often found to be thick and gelatinous on autopsy.

Vascular connections to the heart

During the evolutionary transition from aquatic to terrestrial organisms, separation of the pulmonary and systemic circulations into two parallel circuits became necessary. This was accomplished in part by cardiac neural crest cells that migrate from the neural folds into the pharyngeal arches and heart, where they have essential roles in septation of the single outflow of the heart (the truncus arteriosus) into the aorta and pulmonary artery, and in the formation of the conotruncal portion of the ventricular septum[31] (Fig. 1). After septation of the aorta and pulmonary artery, the vessels rotate in a twisting fashion to achieve their final connections with the left and right ventricles, respectively. Neural crest cells also contribute to the bilaterally symmetrical aortic arch arteries that arise from the aortic sac and undergo extensive remodelling, resulting in formation of the aorta,

ductus arteriosus, proximal subclavian, and carotid and pulmonary arteries (Fig. 1). Perturbation of neural crest cell specification or migration can result in life-threatening abnormalities of this region, which account for about 30% of congenital heart abnormalities in humans.

Mice lacking ET-1 or its G-protein-coupled receptor, ETA, have post-migratory cardiac neural crest defects, cleft palate and other craniofacial anomalies reminiscent of 22q11 deletion syndrome in humans[32,33] (see Box 1). dHAND and eHAND, normally expressed in the neural crest-derived pharyngeal and aortic arches, are downregulated in these structures in mice deficient in *ET-1* or *ETA*, suggesting that the HAND proteins are regulated by ET-1 signalling[34]. Consistent with this notion, *dHAND*-null mice also exhibit a severe defect in survival of pharyngeal and aortic arch mesenchyme[34]. Neuropilin-1, a semaphorin and VEGF receptor, is downregulated in *dHAND* mutants, and targeted mutation of *neuropilin-1* results in a phenotype similar to that of *ET-1* mutants. This suggests that ET-1, dHAND and neuropilin-1 may function in a common pathway regulating neural crest development[35,36].

Much like the heart itself, segments of the mature aortic arch are derived from distinct embryonic structures (aortic arches III, IV and VI, see Fig. 1) that develop relatively independently. Disruption of the Forkhead transcription factor Mfh1 causes hypoplasia of the fourth aortic arch artery in mice, resulting in absence of the transverse aortic arch[37]. This phenotype resembles the interruption of the aortic arch seen in humans. Mice lacking combinations of retinoic acid receptors (RAR/RXR) or the homeobox gene *pax3* also have a variety of outflow tract and aortic arch defects[38,39]. In zebrafish, mutation of the *gridlock* gene, which is expressed in the arch arteries and encodes a bHLH transcription factor related to the *Drosophila* protein Hairy, results in coarctation or narrowing of a particular region of the aorta, a defect commonly observed in humans[40]. This may reflect an essential role for *gridlock* in controlling the decision of an early vascular progenitor to adopt an arterial fate in a specific vascular segment. Finally, evidence for independent regulation of the sixth aortic arch artery comes from the third most common CHD — patent ductus arteriosus — in which the ductus arteriosus, an embryonic vessel connecting the aorta and pulmonary artery, fails to close after birth. Heterozygous mutations of the transcription factor TFAP2B can result in familial patent ductus arteriosus, suggesting a role for TFAP2B in governing closure of this vessel[41].

Table 2 Cardiac defects associated with gene mutations

Protein	Species	Cardiac defect
Signalling molecule		
Neuregulin	Mouse	Absent myocardial trabeculation
ErbB2, B4	Mouse	Absent myocardial trabeculation
Neurotrophin-3	Mouse	Outflow tract defects
Neuropilin-1	Mouse	Outflow tract and arch defects
Endothelin-1	Mouse	Outflow tract and arch defects
Jagged-1	Human	Pulmonary stenosis, outflow tract
TGF-β	Mouse	Cardiac valve defects
Endoglin	Human	Arteriovenous malformations
Cell adhesion molecule		
VCAM	Mouse	Epicardial dissolution
α4 integrin	Mouse	Epicardial dissolution
Versican	Mouse	Hypoplastic right ventricle
Ion channel		
HERG	Human	Long QT, Brugada syndrome
SCN5A	Human	Long QT syndrome
KVLQT1	Human	Long QT syndrome
MinK	Human	Long QT syndrome
Transcription factor		
GATA-4	Mouse	Cardiac bifida
GATA-5	Zebrafish	Cardiac bifida
Fog-2	Mouse	Cardiac alignment, coronary artery defects
MEF2C	Mouse	Hypoplastic right and left ventricles
dHAND	Mouse, zebrafish	Hypoplastic right ventricle, arch defects
COUP-TFII	Mouse	Hypoplastic atria
Nkx2.5	Mouse, Human	Looping, conduction defect, atrial septal defect
TBX5	Human	Ventricular septal defect, atrial septal defect
NF-ATc	Mouse	Absent valves
Smad6	Mouse	Thickened valves
Pax3	Mouse	Persistent truncus arteriosus
RXR/RAR	Mouse	Outflow tract, aortic arch, myocardial defects
Mfh1	Mouse	Interrupted aortic arch
Gridlock/HRT2	Zebrafish	Coarctation of aorta
NF-1	Mouse	Outflow tract defects, thin myocardium
TFAP2B	Human	Patent ductus arteriosus
N-Myc	Mouse	Thin myocardium
TEF-1	Mouse	Thin myocardium
WT-1	Mouse	Epicardial dissolution

Looking ahead

Given the complexity of cardiac development and the devastating consequences of even subtle perturbations in the process, it is not surprising that mutations in such a large number of genes can cause cardiac malformations (Table 2). The heterogeneity of CHDs associated with single-gene defects, as demonstrated for *NKX2.5* or *TBX5* mutations, makes mechanistic understanding of gene function challenging and points to the importance of modifier genes, environmental factors and genetic polymorphisms in determining the severity and type of CHD. Clinically, the prospect that secondary factors affect the ultimate phenotype of heterozygous genetic mutations provides hope that at least some types of heart defects might be prevented by modulating these cofactors.

Elucidation of the genetic networks and mechanisms underlying cardiac development also opens up the possibility of overcoming the inability of adult cardiomyocytes to divide, which is one of the major obstacles in cardiovascular medicine in adults. Genetic strategies for converting non-muscle cells to a cardiac cell fate through expression of developmental control genes that specify cardiac cell identity might eventually be used in cardiac regeneration or cell replacement.

Finally, the availability of complete genome sequences for humans and model organisms should revolutionize our understanding of cardiac development. Mapping of human CHD genes, once daunting, will be simplified, and genomic sequence profiling will enable genetic screening for mutations and polymorphisms in CHD genes. Comparative analysis of genes involved in cardiac development and disease in different species will enable human disease genes to be identified from their orthologues in other species, and vice versa. The identification of genes acting downstream in cardiac developmental hierarchies, which is a major challenge at present, will be made easier by the ability to profile gene expression in the normal and abnormal heart. As we enter the post-genomic era, the emerging genetic blueprint of heart development will enable us to understand the all too common complex genetic trait that is CHD. □

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