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Developmental paradigms in heart disease: insights from *tinman*

Owen W J Prall[1], David A Elliott[1] and Richard P Harvey[1,2]

Congenital heart disease is a significant cause of morbidity and mortality in humans, and gene mutations that underlie some of these anomalies are now being described. The NKX2.5 gene, which encodes a homeobox transcription factor, was initially discovered in mice through its similarity to the tinman gene of the fruitfly Drosophila. Tinman is required for formation of the dorsal pulsatile vessel or 'heart' of the fly. Tinman and NKX2.5 share structural and functional features, and in mice the gene is required for normal cardiac looping and differentiation of chamber myocardium. Humans with heterozygous mutations in the NKX2.5 gene generally have a disorder involving progressive atrio–ventricular conduction block and atrial septal defect, although sometimes other abnormalities including tetralogy of Fallot. The TBX5 gene, which encodes another cardiac transcription factor that collaborates with NKX2.5, is also an important cardiac disease gene, with heterozygous mutations responsible for Holt–Oram (hand/heart) syndrome. These contributions to human pathology underscore the relevance of studying biological phenomena in lower organisms, and examination of other genes acting in this and associated pathways will expand our knowledge of congenital abnormalities and disease predisposition, and improve genetic counseling.

Keywords: ASD; AV block; congenital cardiac disease; heart development; homeobox gene; NKX2.5; TBX5; tetralogy of Fallot; tinman.

Introduction

Congenital heart disease (CHD) is an important cause of morbidity and mortality in humans. It is estimated that 0.8% of live births are complicated by such malformations and cardiac defects are over-represented in stillborn babies by 10-fold (1). Genetic etiologies have been long suspected in many such malformations and specific mutations are now being identified, providing clues to mechanisms that control both normal and pathological cardiac development. One of the most common congenital cardiac abnormalities is atrial septal defect (ASD), occurring either alone or in combination with other abnormalities, and accounting for approximately 10% of all cardiac malformations. Some ASDs (and conduction defects) and tetralogy of Fallot (TOF) have been recently linked to mutations in the gene *NKX2.5*, also known as *CSX*, which encodes a cardiac transcriptional regulatory protein (see below). This was a boon for developmental biologists, who had previously identified a critical role for homologous genes during cardiac development in organisms as diverse as fruitflies, frogs and mice. Similar links between CHD and genes that collaborate with *NKX2.5* begin to define a major genetic pathway in heart formation, and validate the investigation of cardiac development in model organisms.

Using lesser organisms to study developmental and pathological processes relevant to human biology is not a novel idea, but the application of modern molecular and genetic techniques to comparative analyses has provided some stunning findings. For example, the morphological differences between the compound eye of insects and the mammalian eye led biologists to believe that they must have evolved independently, and this notion became a classic textbook example of 'convergent evolution'. The discovery that eye morphogenesis in organisms as diverse as flies and humans is controlled in common by members of the *homeobox* gene family (*Pax6* in vertebrates and *eyeless* in fruitflies) was a strong argument against this dogma (2), and implied that some sort of photosensitive organ had already developed in their shared evolutionary ancestor. Thus, a major axiom underscoring recent molecular studies is that genes and gene functions that are involved in important biological processes are likely

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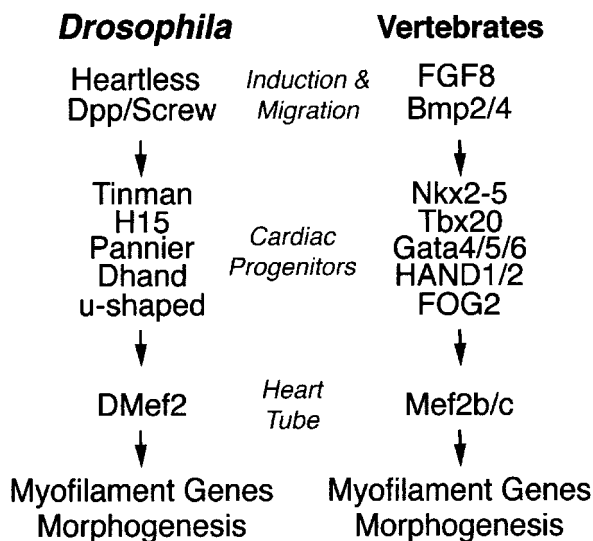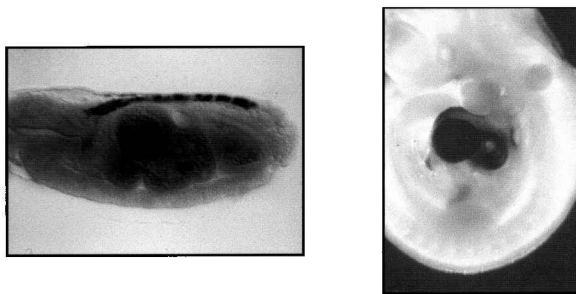
to be conserved in evolution. This has enabled researchers to define disease-causing genes in humans by studying other model organisms. Indeed, naturally occurring mutations of *Pax6* were shown to be a cause of aniridia in humans, and in the field of cancer research seminal contributions have come from the study of yeast, worms, fruitflies and frogs. Such results would no doubt have startled the *Drosophila* biologists who described a lack of eyes in what turned out to be *eyeless*/*Pax6* mutant flies as long ago as 1915.

Cardiac development in insects and vertebrates

Research performed over the last decade indicates that the human transcription factor gene *NKX2.5* and related genes are strongly conserved structurally and functionally throughout evolution, and furthermore that they control heart development in insects and all vertebrates studied, including frogs, zebrafish, mice and humans. The heart of the fruitfly, *Drosophila*,


Drosophila   **Vertebrates**

Drosophila		Vertebrates
Heartless Dpp/Screw	*Induction & Migration*	FGF8 Bmp2/4
↓		↓
Tinman H15 Pannier Dhand u-shaped	*Cardiac Progenitors*	Nkx2-5 Tbx20 Gata4/5/6 HAND1/2 FOG2
↓		↓
DMef2	*Heart Tube*	Mef2b/c
↓		↓
Myofilament Genes Morphogenesis		Myofilament Genes Morphogenesis

Figure 1. Conserved genes in the NKX2.5 pathway in flies and mice. Panels show *in situ* hybridizations for *tinman* in the fruitfly larva (left, supplied by R Bodmer) and for *Nkx2-5* in the mouse embryo (right). The *NKX2.5* pathway with genes of analogous function is listed for each organism, along with the approximate stage of expression.

often referred to as the dorsal vessel, is a midline structure composed of an inner layer of muscular 'cardial' cells and an outer layer of non-contractile 'pericardial' cells (Fig. 1). The tube pumps peristaltically, and the presence of valvular structures ensures that the insect blood (hemolymph) is pumped efficiently throughout the open (vessel-less) cavities of its body. Thus, while anatomically simple, the dorsal vessel possesses functional similarities to vertebrate hearts. Furthermore, its developmental origins and mode of contraction resembles that of the embryonic mammalian heart. Indeed, the cardial cells of the fly heart express myofilament proteins ((3) and references within) as well as muscle transcription factors similar to those expressed in the vertebrate heart (see below).

In fly embryos, the heart develops from the dorsal region of the nascent mesodermal germ cell layer. The expression of a gene called *tinman* – the fly orthologue of *NKX2.5* – is initially expressed widely in mesoderm but expression becomes restricted to the muscle and pericardial cells of the future heart, as well as to muscle cells of the gut and dorsal body wall ((3) and references within). Genetic analysis reveals that *tinman* is indeed critical for heart and gut muscle development since flies which lack the *tinman* gene fail to develop these organs or any trace of their precursor cells (3). However, *tinman* alone is not sufficient for formation of a heart-like structure. This process requires signaling from other germ layers of the embryo by way of secreted proteins such as *decapentaplegic* (encoding a bone morphogenetic protein (BMP) (Fig. 1) membrane-bound signaling ligands such as *wingless* (a Wnt family member) and ligand receptors such as *heartless* (a fibroblast growth
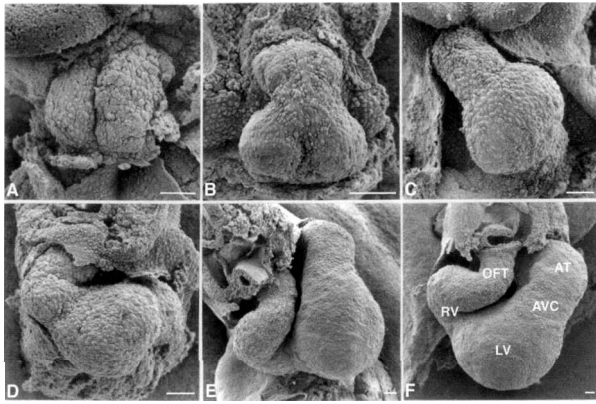
Figure 2. Mouse cardiac development. Scanning electron microscope images of the developing mouse heart (ventral aspect). *A, B.* Paired cardiac precursor cells migrate to the anterior midline of the embryo (*A*) and fuse to form a linear tube. *C-F.* The heart tube then loops to the right with a pronounced cranial movement of the atria, and expansion of the working myocardium of the ventricular chambers. The resulting structure then bears some resemblance to the morphology of a mature heart with the future atria (AT), atrioventricular canal (AVC), left ventricle (LV), right ventricle (RV) and outflow tract (OFT) clearly identifiable. Scale bars are included in each panel (all 50 µm). Reproduced and adapted from Ref. 54 with permission from Cambridge University Press.

factor (FGF) receptor). The precise role of *tinman* in relation to these other genes is unknown, although *tinman* is thought to occupy a primary position in a genetic hierarchy controlling the expression of a transcriptional program rendering embryonic meso-dermal cells competent to differentiate into heart and gut muscle cells in collaboration with other signals.

In vertebrates, the gross morphological changes that accompany cardiac development are well described and proceed with reference to defined anterior/posterior, dorsal/ventral and left/right axes. This progression creates the familiar four-chambered adult heart in which two atrio-ventricular pumps function in series but are fused anatomically. In the early vertebrate embryo, bilateral groups of cells in the left and right lateral mesoderm give rise to the principle cardiac tissue types, namely myocardium, endocardium and pericardium (4). These precursor cells move to the ventral midline, fuse to form firstly a crescent-shaped epithelium (the cardiac crescent) then a linear heart tube. This tube then undergoes a process called 'looping morphogenesis' (Fig. 2). Looping is the first departure from bilateral symmetry in the embryo, and the direction of looping, always to the right in normal embryogenesis, is guided by a molecular/genetic pathway determining other left/right differences in the embryo (5). Looping brings about a left/right rearrangement of the ventricles as well as a dorso-cranial shift in the position of the atrial chambers, events required for proper alignment

of the chamber primordia with each other, and with valve precursors and the great vessels. During loop-ing, many patterning processes occur. One key event is the specification of 'working myocardium' at the outer curvature of the looping heart tube (6). Working myocardium is a specialized muscle type, expressing unique cytoskeleton and conduction proteins, and is the force-generating component of the heart cham-bers. The formation of specialized chamber muscle is part of a larger set of patterning processes that divide the developing heart into distinct regions or building blocks: the inflow tract or sinus venosus; common atrium; atrioventricular canal (AVC); primitive ven-tricle; and conotruncus or outflow tract (Fig. 2). These building blocks roughly correspond to their mature counterparts in the adult heart. The myocar-dium of the inner curvature, AVC and outflow tract contribute little to the overall mass of the heart or to the working myocardium of chambers. However, these regions control the formation of endocardial cushions, precursors of valves and aortico-pulmonary septa, and contribute to the nodal and His-bundle elements of the conduction system. Further remodel-ing of the looping heart includes septation of the common atrium and ventricle, as well as thickening and trabeculation of the ventricles.

The genetic control of these morphogenetic pro-cesses is beginning to be elucidated. As in the fly, *NKX2.5* is essential for correct cardiogenesis in mammals. The mouse *NKX2.5* gene (*Nkx2-5*) was the first *tinman* orthologue to be identified in vertebrates (7, 8) and is expressed in the paired bilateral myocardial precursor cell populations of the mouse embryo. Its expression then continues in muscular layers of the heart throughout all stages of embryogenesis, and into postnatal and adult life. *NKX2.5*-related genes were subsequently isolated from many other vertebrate species including human, rat, chicken, zebrafish and frog. Frogs have at least three *NKX2.5* orthologues, and simultaneous inhibi-tion of these genes completely inhibits heart tube formation (9), whereas overexpression results in enlargement of the heart and hypertrophy of indivi-dual muscle cells (10). To gain further insights into *NKX2.5* function the genetic locus was disrupted in mice (11). Heterozygous mutant animals were viable and showed no gross abnormalities (but see below). Homozygous null *Nkx2-5* embryos developed hemo-dynamic insufficiency due to the failure of both cardiac looping and molecular differentiation of the ventricles, dying midway through gestation (11, 12). Interestingly, *Nkx2-5* was not essential for initial cardiomyocyte specification or formation of the linear heart tube, but rather for the patterning and differentiation of primitive myocytes into ventricular working myocardium. Gene expression analysis indicates that the ventricles of *Nkx2-5* mutant hearts

are blocked in a primitive myogenic state, similar to that found in the primary heart tube.

Our interpretation of this genetic 'knock-out' is that *Nkx2-5* is critical for the correct implementation or interpretation of anterior-posterior and dorsal-ventral signals that enable the simple linear heart tube to loop and form specialized chamber myocardium. *Nkx2-5* also appears to be required for correct deployment of the pathway governing left/right asymmetry (13). In this respect, *tinman* and *Nkx2-5* both maintain the ability of early cardiac cells to interpret external signals and to achieve a fully differentiated myogenic state. In contrast, *Nkx2-5* mutant mice develop a linear heart tube whereas *tinman*-mutant flies do not. Therefore, it is likely that the genetic pathways controlling cardiac specification have evolved to become more complex in mammals and thereby buffered against mutation. It is unlikely that cardiogenesis is controlled by a 'master regulatory gene', as in the case of the eye, and rather relies on a combination of transcription factors. It is also likely that during the evolution of diverse heart structures, *tinman* and *NKX2.5* have diverged functionally, since they contain both common and unique protein domains and since mouse *Nkx2-5* cannot rescue the cardiac lineage in *tinman*-mutant flies when expressed as a transgene (14, 15).

Heterozygous NKX2.5 mutations

The above studies in mice suggest that homozygous null mutations of human *NKX2.5* would be early embryonic lethal. However, heterozygous mutations in *NKX2.5* were described in families with progressive atrioventricular (AV) conduction block and secundum atrial septal defect (ASD) as their dominant features, following the discovery of a genetic linkage between the disease phenotype and DNA markers located at chromosome 5q35 (16, 17). *NKX2.5* mutant families also show a wide range of other cardiac abnormalities such as TOF, ventricular septal defects (VSDs), Ebstein's anomaly, subvalvular aortic stenosis and tricuspid valve abnormality, some of these without conduction defects (17). About 10 different mutations in *NKX2.5* have been found to date in families affected with progressive AV block. Most lie within the homeodomain, which is the DNA-binding domain of the NKX2.5 protein, and these are proposed to inactivate the protein. Other mutations lie outside of this domain and may interrupt transcriptional activation domains or those that interact with other transcription factors. Some of the mutations have been tested in vitro and are suggested to have dominant-negative effects in that they can inhibit the activity of the normal NKX2.5 protein (18, 19). This can be explained by the fact that

NKX2.5 can form homodimers (i.e. associate with another NKX2.5 protein) and/or heterodimers (i.e. associate with other transcription factors), and therefore mutant proteins may interact with, and inactivate, their wild-type partners. Direct confirmation that mutations in *NKX2.5* are causative for at least some aspects of the human phenotype was provided by transgenic experiments in mice, in which one mutant allele, after expression in the heart, led to complete AV conduction block shortly after birth (20). These mice also showed decreased expression of connexins 40 and 43, gap junction proteins which facilitate cardiac depolarization, and this may have contributed to the AV block. Furthermore, *NKX2.5* is expressed at relatively high levels in chick Purkinje fibers (21), and therefore the function of these cells may be particularly compromised following a reduction in NKX2.5 protein activity. The tight association of conduction disease with ASD in the families studied, and our findings that only a single *NKX2.5* mutation predicted to be deleterious was found within a cohort of 65 individuals with idiopathic secundum ASD (D Elliott, E Kirk and R Harvey, unpublished data), suggest that the described mutations are associated only with a rare familial syndrome of AV conduction defect plus ASD.

Four heterozygous *NKX2.5* mutations were recently reported in 6/114 non-syndromic TOF patients (22). One of these mutations was also reported in familial AV conduction block/ASD (16, 17), but the other three mutations were novel, not associated with conduction defects, and were not located within the homeodomain. This potential genotype-phenotype correlation suggests that separate domains of the normal NKX2.5 protein have distinct developmental roles in the formation of the atrial septum, conduction system and outflow tract septa. Major contributions are made to the developing outflow tract septa by neural crest cells that migrate through the pharyngeal region. Therefore, *NKX2.5* may function in outflow tract septation either through its involvement in looping morphogenesis, and therefore alignment of outflow tract structures, or its impact upon pharyngeal cell survival/differentiation (23), which may alter signaling to migrating neural crest cells.

The critical role of *NKX2.5* in atrial septum and conduction system development was demonstrated directly in *Nkx2-5* knock-out mice where heterozygosity was found to confer ASD and milder forms of atrial septal dysmorphogenesis (represented predominantly by patent foramen ovale (PFO) and septal aneurysm), as well as a conduction defect (longer PR interval in females) (24). Interestingly, genetic background had a major influence on both the number and size of PFO observed in normal and *Nkx2-5* mutant mice, suggesting the presence of modifier genes for
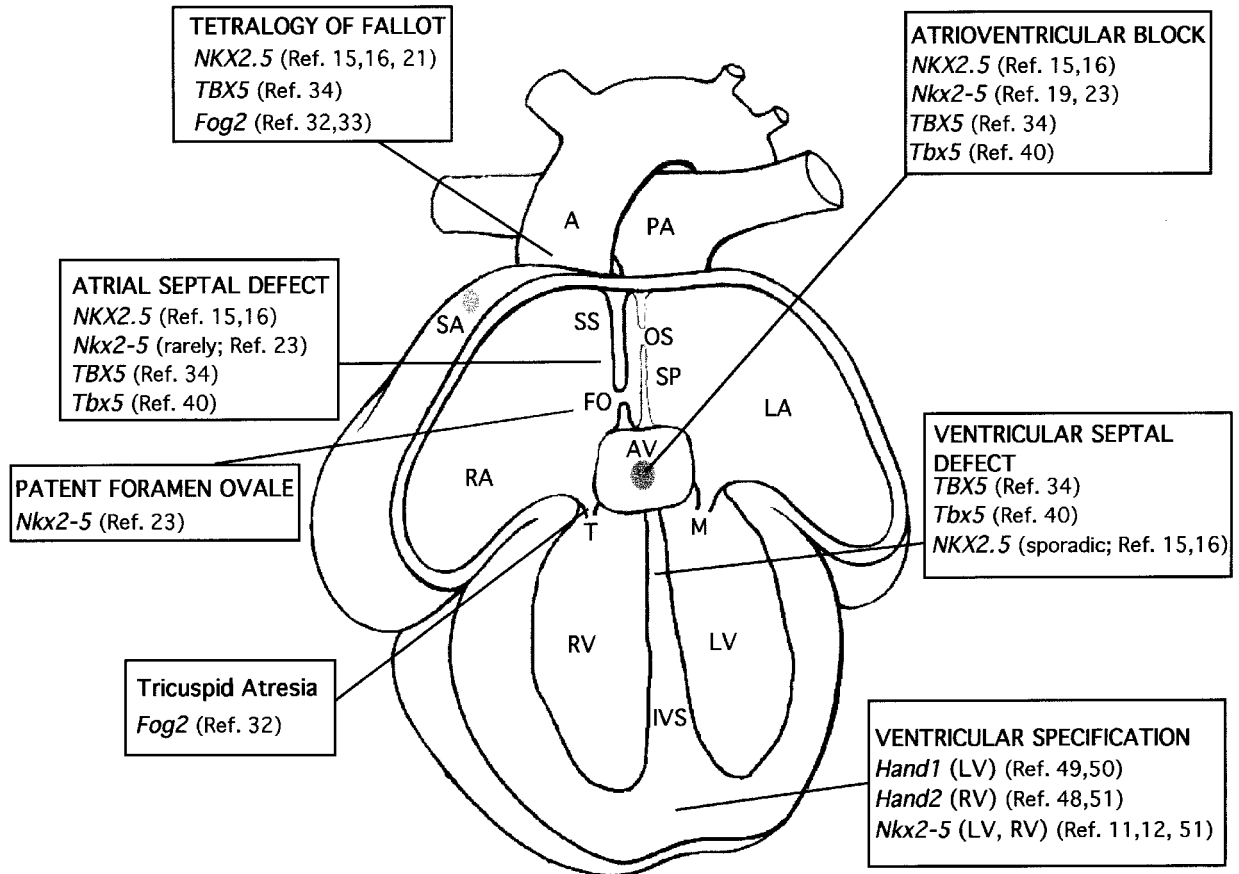
Figure 3. Predominant cardiac phenotypes associated with mutations in NKX2.5 pathway genes. Structures within the developed vertebrate heart that are affected by mutations in genes involved in the *NKX2.5* pathway are illustrated. Human genes are indicated in uppercase, mouse genes in lowercase. TOF = tetralogy of Fallot; ASD = atrial septal defect; PFO = patent foramen ovale; VSD = ventricular septal defect not associated with TOF; A = aorta; PA = pulmonary artery; SA = sino-atrial node; AV = atrio-ventricular node; SS = septum secundum; FO = foramen ovale; OS = ostium secundum; SP = septum primum; RA = right atrium; LA = left atrium; T = tricuspid valve; M = mitral valve; RV = right ventricle; LV = left ventricle; IVS = inter-ventricular septum.

septal morphogenesis in some mouse strains. Therefore, the mouse *Nkx2-5* mutant model may be useful in mapping genes that interact genetically with *NKX2.5* during atrial septation. Given that the phenotype of *Nkx2-5* mutant mice is similar to, albeit milder, than that seen in humans, the human and mouse *NKX2.5* genes probably play similar roles in cardiogenesis (24). Furthermore, the changes in gene expression associated with the conduction disease in *NKX2.5* transgenic mice suggest that *NKX2.5* directly controls the transcription of conduction system genes. It remains to be determined, however, whether *NKX2.5* plays a direct role in atrial septation, or whether this defect is secondary to mild impairment of earlier embryonic processes, such as cardiac looping.

The NKX2.5 pathway and cardiac disease

NKX2.5 does not function in isolation but is part of a cellular biochemical pathway. The encoded protein is modified by phosphorylation and physically interacts with a set of other nuclear regulatory proteins in order to activate transcription from the promoters of target genes. Transcriptional regulation is a complex process involving site-specific recognition of DNA promoter elements in target genes by transcription factors, recruitment and activation of RNA polymerase complexes and modification of chromatin structure. It is likely that other proteins involved in the *NKX2.5* pathway will also have roles in human cardiac disease.

NKX2.5 can directly associate *in vitro* with the zinc finger family transcription factor GATA-4 (so called because it binds to the DNA sequence 'GATA') (25, 26) and co-expression of these two proteins leads to synergistic augmentation of transcription from some cardiac genes (26, 27). A related gene, *pannier*, is expressed in the fly heart (Fig. 1) where it activates cardiac genes synergistically with *tinman*, with ectopic expression of either *pannier* or *Gata-4* inducing overproduction of cardiac cells (28). Disruption of the mouse *Gata-4* locus revealed that the

gene was not required for cardiac cell specification, but rather for fusion of the paired cardiac progenitor pools ((29) and references within). Subsequent analysis revealed that this was not a defect in the cardiac precursor cells *per se*, but rather a deficiency in the underlying endoderm germ layer, which may provide a physical scaffold and secreted factors necessary for migration and fusion of precursor cells. The early lethality in this mutant precluded examination of the contribution of *Gata-4* to mature cardiac structures and any heterozygous phenotype may have been masked by expression of the highly similar *Gata-6* gene. Direct evidence that *Gata-4* is required for cardiogenesis, independent of its effects on endoderm, were provided by the study of mice with a homozygous *Gata-4* point mutation (*Gata-4$^{ki/ki}$*) that prevented its interaction with *Fog* cofactors (see below). These mice died between E11.5–13.5 and had hearts with large atrial and ventricular communications, common AV canal, double outlet right ventricle and virtually absent coronary vessels (30). Tantalizing evidence that *GATA-4* may also be involved in formation of the atrial septa in humans comes from the study of patients with deletions of the *GATA-4* genetic locus (8p23.1) (31, 32), which is associated with a spectrum of CHD. Atrial septal anomalies (secundum ASD or PFO) were evident in 3/4 (31, 32) and 1/1 (31, 32) patients with hemizygosity for *GATA-4*. However CHD (including combinations of ASD, VSD, pulmonary stenosis and other lesions) can occur with deletions of 8p23.1 that do not involve gross deletion of GATA-4 (32) and therefore other CHD-linked genes may be involved at this locus. Sequencing analysis of *GATA-4* for point mutations in patients with congenital atrial defects has not yet been reported.

The transcriptional activities of GATA-4 and -6 proteins are modulated by interactions with yet other transcription factors including the Friend of GATA (Fog) proteins, which can repress GATA-4-dependent transcription from multiple cardiac-restricted promoters (33) and therefore may impact upon *NKX2.5*-dependent processes. Mouse *Fog2* is first expressed in the looping embryonic heart and expression is maintained in the adult heart. *Fog2*-deficient mice have been generated by two groups, reporting complex cardiac anomalies including tricuspid atresia, ASD and TOF, and reduced expression of the Nkx2-5 target gene *Hand1* (see below) (34, 35) (Fig. 3). Interestingly, in *Fog2*-deficient mice the coronary vasculature was also absent, despite formation of an intact epicardial layer, and this could be rescued by transgenic expression of *Fog2* in cardiomyocytes (35). These abnormalities are substantially similar to those in *Gata-4$^{ki/ki}$* mice, clearly implying roles for the GATA-4/Fog2 interaction in their etiology. Whether these features are related to the potential role of this

complex in regulating Nkx2-5 function is presently unclear, although the presence of ASD and reduced expression of *Hand1* in *NKX2.5*, *Gata-4*, *Gata-4$^{ki/ki}$* and *Fog2* mutants, and TOF in *NKX2.5* and *Fog2* mutants hint at a commonality in mechanism.

T-box transcription factors and cardiogenesis

T-box factors are conserved transcriptional regulators expressed in mesoderm and its derivatives, and are strongly implicated as critical regulators of early cell fate decisions. T-box genes expressed in the mammalian heart include *Tbx5* (36) and *Tbx20* (37, 38). *Tbx20* has orthologous genes expressed in the heart in zebrafish (*hrT*) and the fruitfly (*H15*) (39), suggesting a conserved role in cardiogenesis. *Tbx5* is expressed in a graded fashion across the myocardium, with highest levels in the posterior (sinoatrial) region (40). Indeed, evidence suggests a role in specification of atrial structures mediated by retinoic acid signaling, and overexpression of *Tbx5* in the ventricular chamber leads to chamber dysmorphogenesis and down-regulation of ventricle-specific genes (41).

Genetic and biochemical characterization of *TBX5* indicates that it may function within the *NKX2.5* pathway. While expression of mouse *Tbx-5* is unaffected in *Nkx2-5* null embryos (51 and C Biben and R Harvey, unpublished results), TBX5 and NKX2.5 proteins can associate with each other *in vitro* and synergize in activation of genes encoding atrial natriuretic factor (ANF) and connexin − 40 (42, 43). Humans with heterozygous mutations in *TBX5* have Holt-Oram syndrome (36, 44) which is characterized by forelimb abnormalities and abnormal cardiac electrophysiology (particularly atrioventricular conduction block), although other congenital cardiac abnormalities including ASD, VSD and TOF can be present. This spectrum of defects is reminiscent of patients with *NKX2.5* mutations, suggesting a role for *TBX5* in the *NKX2.5* pathway. This is strengthened by structure-function studies of various Holt-Oram *TBX5* mutations, some of which are associated with a predominance of cardiac over limb abnormalities and other mutations show the reverse association. A cardiac-type mutation in TBX5 did not activate the *ANF* promoter or show synergistic activation with NKX2.5 *in vitro*, whereas a limb-type mutation was able to activate the promoter to the same extent as wild-type TBX5 (45). The cardiac *TBX5* mutations are predicted to produce null or non-functioning proteins, and this is supported by heterozygous null mutation of *Tbx5* in mice (43). The spectrum of cardiac defects present in these mice mimics the human Holt-Oram syndrome, with first and second degree AV block, ASD, VSD and cardiac failure. Furthermore, decreased expression of *ANF*

and *connexin-40* genes in these mice provided strong evidence that the *NKX2.5* pathway was partially disrupted and may have contributed to the phenotype (43).

Transcriptional targets of the NKX2.5 pathway

Since *NKX-5* and its interacting genes can function as transcriptional regulators, it seems certain that loss of *NKX2.5* function would prevent the normal expression of a number of genes required for correct cardiac morphogenesis. A number of such genes have now been identified. Genes known to be down-regulated in *Nkx2-5* null mice include *atrial natriuretic factor (ANF), connexin-40, cardiac-restricted ankyrin repeat protein (CARP), myosin light chain-2v, chisel (Csl), Iroquois-4 (Irx4), smooth muscle protein-22 (SM22),* and *Hand1* ((46) and references within). In *in vitro* transcription assays, the promoters of the *ANF, CARP* and *connexin-40* genes can be directly activated by *Nkx2-5* (43, 47), providing a mechanism for their decreased expression. Some of the genes down-regulated in heterozygous or homozygous *Nkx2-5* mutant mice may contribute directly to their phenotype. Decreased expression of the *connexin-40* gene may contribute to the conduction defect in heterozygous mice, since this is the predominant gap junction protein expressed in the sino-atrial node, atria and His-Purkinje conduction system, and genetic deletion of *connexin-40* predisposes mice to AV block (48).

Homozygous deletion of *Hand1* leads to a cardiac phenotype that closely resembles that present in *Nkx2-5* knock-out mice, and thus, down-regulation of *Hand1* in *Nkx2-5* mutants is likely to be a critical component of the phenotype. *Hand* genes encode members of the basic helix-loop-helix (bHLH) transcription factor family which are capable of binding to regions of DNA called E-boxes found in the promoter elements of many structural cardiac genes. The expression patterns of the *Hand* genes is reflected by their nomenclature; <u>h</u>eart, <u>a</u>utonomous <u>n</u>ervous system and <u>n</u>eural crest-<u>d</u>erived cell types. In the developing mouse heart *Hand1* and *Hand2* display restricted expression (49, 50). In the linear and early looping heart tube *Hand1* is expressed at the outer curvature of the presumptive left ventricle as well as in the outflow tract, then later at the outer curvature of the right ventricle. *Hand1* homozygous null embryos die at an early embryonic stage due to placental defects, precluding a detailed study of the cardiac phenotype. However, the placental defects can be rescued in chimaeric embryos in which the critical parts of the placenta can be formed from wild-type cells, while the embryo proper is formed by cells that are mutant for *Hand1* (51). In this situation, and as for *Nkx2-5* null embryos, a primitive heart tube forms but fails to undergo looping morphogenesis and chamber formation is blocked. Subsequently, another chimaeric study has demonstrated that cells that are mutant for *Hand1* contribute poorly to the expanding sections of the left ventricle (52).

Hand2 is expressed throughout the myocardial and endocardial layers of the linear heart tube but then becomes predominantly restricted to the future right ventricular 'segment' (50). Interestingly *Hand2* homozygous mutants initiate cardiac looping but the primitive right ventricle dies by apoptosis (50). *Hand* genes therefore appear to be responsible for the expansion and differentiation of entire functional units in the heart. Indeed, in mice doubly mutant for both the *Nkx2-5* and *Hand2* genes, neither *Hand1* nor *Hand2* are expressed and the entire ventricular region fails to form with only a rudimentary atrium remaining (53). This loss appears through the failure of ventricular expansion rather than specification of ventricular cells, and *Hand* genes may therefore control elements of cell cycle and/or cell death pathways in developing cardiomyocytes. In conclusion, while down-regulation of *Hand1* may be responsible for part of the ventricular phenotype of *Nkx2-5* null embryos, the contribution of *Hand1* to the atrial and conduction defects in mice and humans with heterozygous mutations in *NKX2.5* is unclear. There are presumably many other direct and indirect transcriptional targets of the *NKX2.5* pathway awaiting discovery which may contribute to these abnormalities.

Conclusions

Recent research has demonstrated a link between mutations in *NKX2.5* and CHD involving conduction defects, ASD and TOF. Mutations in TBX5, which directly interacts with NKX2.5, cause Holt-Oram syndrome which is also characterized by atrioventricular conduction block, and occasionally ASD and TOF. The discovery of multiple members of the *tinman*/*NKX2.5* pathway in organisms as diverse as flies and humans indicates that these are ancient genes for control of heart-like structures, and that much can be learnt about human cardiogenesis by studying other species. Mutations in other proteins active in the *NKX2.5* pathway are also likely to be involved in human CHD. The recent or imminent sequencing of the human, mouse, fly, worm and yeast genomes, and the development of systems for high throughput parallel data collection and analysis (e.g. cDNA microarrays for analysis of gene expression) will enable the identification of all genes involved in cardiac development. This expression 'blueprint' for cardiogenesis will provide a rational platform for

defining the causative and downstream genes under-pinning congenital and acquired cardiac disease, and has massive potential in diagnostics and therapeutics. Gene therapy is a legitimate goal for many human diseases. The targets of most drugs are the protein products of expressed genes, and therefore the development of new pharmaceuticals against specific gene targets will be one important outcome in the post-genome era. Whether modification of gene expression in the embryo to correct congenital defects will be feasible or safe remains to be determined. In the meantime, however, genetic screening of individuals at risk is an immediate benefit of these advances. In the case of familial heterozygous *NKX2.5* mutations, secundum ASD is now correctable without open-heart surgery if detected before irreversible damage occurs.

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