Building a heart: Implications for congenital heart disease

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Although impressive advances have been made regarding the diagnosis and treatment of congenital heart disease (CHD), it remains the leading noninfectious cause of death in infants. The heart appears to be particularly susceptible to developmental malformations, reflected in the nearly 1% of live births affected by CHD. Inclusion of bicuspid aortic valve that often results in adult-onset aortic valve stenosis doubles the incidence of CHD to 2% of the general population. The number of cases and types of CHD observed clinically represent only a small fraction of the cardiac anomalies that can occur because most are incompatible with intrauterine circulation and are estimated to be the cause of 10% of first-trimester miscarriages.[1] Although the etiology of CHD remains poorly understood, it is clear that the complex process of heart development requires a combination of hemodynamic forces and morphogenic events that are exquisitely sensitive to mild perturbations. In the last several years, elucidation of numerous genes involved in cardiogenesis with the use of human and animal models has provided insight into the genetic pathogenesis of CHD.

The morphologic features of CHD have been carefully described and categorized based on the specific regions of the heart that are affected. It has long been observed that infants born with CHD typically have isolated cardiovascular defects affecting only one chamber, septum, or valve of the heart. These findings suggest that relatively independent molecular developmental programs might exist for each specific region of the heart. In this summary we will review aspects of cardiac morphogenesis that are relevant to CHD, describe animal model systems used to study heart development, and provide examples of genes that have regionally restricted effects on the cardiovascular system.

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MORPHOGENESIS OF CARDIOVASCULAR SYSTEM

A functioning cardiovascular system is essential by the end of the third week of gestation to satisfy the nutritional requirements of the developing human embryo. Beginning soon after gastrulation, cardiac progenitor cells within the anterior-lateral plate mesoderm become committed to a cardiogenic fate in response to an inducing signal emanating from the adjacent endoderm.[2] The specific signaling molecules responsible for this commitment are unknown, but members of the transforming growth factor β (TGF-β) family are necessary for this step.[2] In addition, recent studies have shown that inhibition of a signaling molecule, Wnt, in the anterior-lateral mesoderm is necessary to create a "permissive" environment for cardiogenesis.[3,4] The bilaterally symmetric heart primordia migrate to the midline and fuse to form a single beating heart tube (Figure 1). The straight heart tube has an outer myocardium and an inner endocardium that is separated by an extracellular matrix (ECM) called the cardiac jelly. The linear heart tube is patterned along the anterior-posterior axis to form the future regions of the 4-chambered heart. Rightward looping of the heart tube converts the anterior-posterior polarity to a left-right polarity. The ventricular chambers mature by ballooning from the outer curvature of the looped heart while the inner curvature undergoes extensive remodeling to align the inflow and outflow portions of the heart with the appropriate ventricular chambers. Further septation and remodeling eventually lead to the 4-chambered heart.

Another major cell type that contributes to the development of the heart is a population of migratory neural crest cells known as the cardiac neural crest (Figure 1). These neural crest cells arise from the neural tube and populate the aortic sac, where they are necessary for the proper septation of the truncus arteriosus into the aorta and pulmonary artery and formation of the semilunar valves and superior portion of the ventricular septum. Cardiac neural crest cells also populate the bilaterally symmetric aortic arch arteries, where they are necessary for proper remodeling of the aortic arch arteries into a left aortic arch with normal branching of

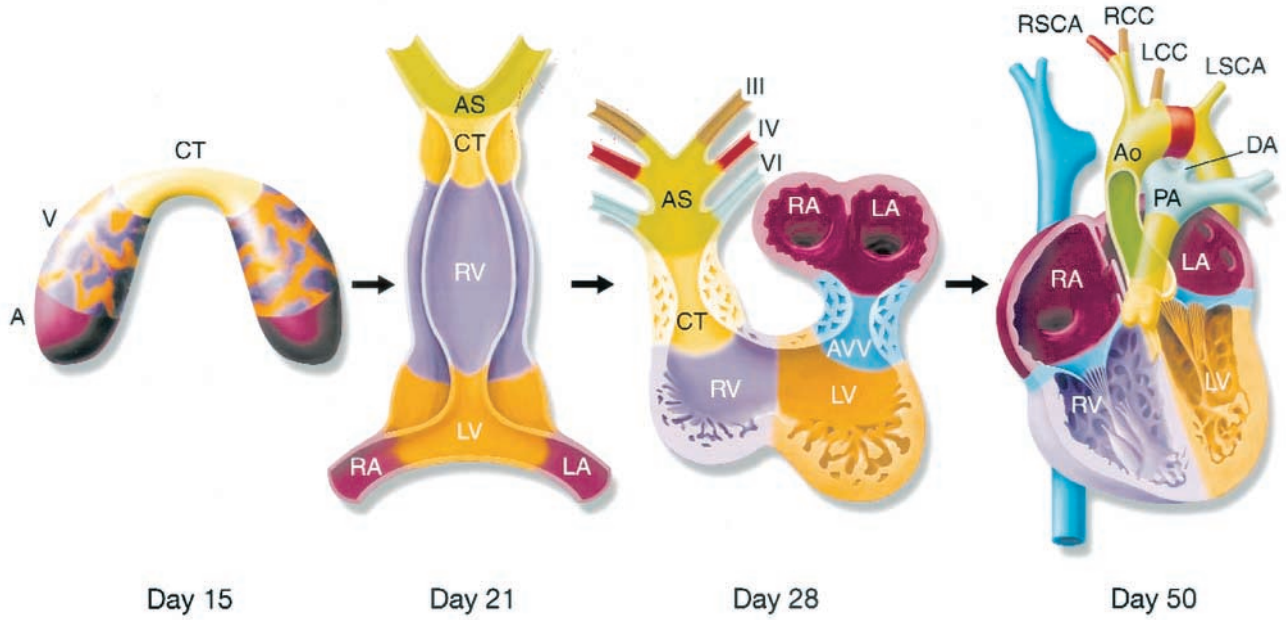

Figure 1. Schema of cardiac morphogenesis. Illustrations depict cardiac development with color coding of morphologically related regions, seen from a ventral view. Cardiogenic precursors form a crescent (Day 15 panel) that is specified to form specific segments of the linear heart tube, which is patterned 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 symmetric aortic arch arteries (III, IV, and VI) and aortic sac *(AS)*, which together contribute to specific segments of the mature aortic arch, also color-coded. Mesenchymal cells form the cardiac valves from the conotruncal *(CT)* and atrioventricular valve *(AVV)* segments. Corresponding days of human embryonic development are indicated. *RV,* Right ventricle; *LV,* left ventricle; *RA,* right atrium; *LA,* left atrium; *PA,* pulmonary artery; *Ao,* aorta; *DA,* ductus arteriosus; *RSCA,* right subclavian artery; *RCC,* right common carotid; *LCC,* left common carotid; *LSCA,* left subclavian artery. (Reproduced with permission from Srivastava D, Olson EN. A genetic blueprint for cardiac development. Nature 2000;407:221-6).

the head and neck vessels. Each aortic arch artery contributes to a specific segment of the mature arch, as indicated in Figure 1.

Initially, formation of the human heart was described primarily on an anatomic level. The observation that cardiac genetic pathways are highly conserved across diverse species from flies to human beings has allowed the use of model systems to explain the molecular mechanisms involved in heart formation. The fruit fly *Drosophila*, which has a primitive linear heart tube known as a dorsal vessel, has been used to discover genes involved in early cardiac determination events. *Drosophila* has the advantages of having a rapid breeding time and a simple genome. Most importantly, its DNA can be chemically mutated in a random fashion. Subsequently, by searching for flies with abnormal hearts and identifying the responsible mutations (reverse genetics), genes that are associated with specific developmental defects can be identified. Similarly, zebra fish can also be studied with the use of chemical mutagenesis, phenotype analysis, and reverse genetics but have the advantage of being vertebrates with 2-chambered hearts. In addition, a functioning circulatory system is not necessary until the late stages of zebrafish development, allowing visualization of defects while the fish are still alive. To study 4-chambered hearts, efforts have focused on chick and mouse model systems. The chick has easily accessible embryos that make it useful for surgical and molecular manipulation. However, the chick system is limited because true genetic studies are not possible. Mice, which have a cardiovascular system nearly identical to that of human beings, are mammals and do allow elegant in vivo genetic manipulation. With the use of direct gene targeting, mouse models for some types of CHD have been generated. Each model system has unique advantages, and each has provided important insights into the development of the human heart. Together, the phylogenetic and developmental observations suggest a modular development of the heart with highly conserved mechanisms throughout evolution.

EARLY CARDIAC DIFFERENTIATION AND HEART TUBE FORMATION

The early steps of midline cardiac tube formation involve a complex array of transcriptional programs with much redundancy in vertebrates. As a result, *Drosophila* has been particularly useful in understanding early aspects of cardiac differentiation and heart tube formation because of the relatively simpler genome. A gene that abolishes formation of the *Drosophila* heart is known as *tinman* on the basis of the *Wizard of Oz* character who wished "he only had a heart."[5] However, tinman, a homeodomain-containing transcription factor, has many orthologues in vertebrates.[6] Unlike in *Drosophila*, mice lacking the closest tinman orthologue, Nkx2.5, form a heart tube but fail to progress much beyond that stage.[7,8] Disruption of two Nkx family members in frog hearts prevents cardiac formation altogether, providing evidence for genetic redundancy in vertebrates.[9,10]

DEFECTS OF CARDIAC LOOPING AND LEFT-RIGHT ASYMMETRY

Once initial formation of a midline heart tube is complete, the heart responds to a complex cascade of left-right asymmetric signals that result in rightward looping of the heart tube, the direction of which is conserved in all species studied to date. Proper folding of the heart tube is necessary to align the atrial chambers with their appropriate ventricles and the right and left ventricles with the pulmonary artery and aorta, respectively. The atrioventricular (AV) septum, which divides the common AV canal into a right and left AV orifice, moves to the right to position the AV septum over the ventricular septum. Simultaneously, the conotruncus septates into the aorta and pulmonary artery and moves to the left so that the conotruncal septum is positioned over the ventricular septum (Figure 2). This movement converts the 2-chambered heart into a 4-chambered heart. Abnormalities in the direction and process of cardiac looping underlie a variety of CHD types.

Arrest or incomplete movement of the AV septum or conotruncus may result in malalignment of the inflow and outflow tracts (Figure 2). When the AV septum fails to shift to the right, it results in both AV orifices emptying into the left ventricle (double-inlet left ventricle), whereas failure of the conotruncus to shift to the left results in both the aorta and pulmonary artery arising from the right ventricle (double-outlet right ventricle). Fog-2 is a zinc finger protein that may play a role in this process. Deletion of *Fog-2* in mice results in embryos that have a single AV valve that empties into the left ventricle, in addition to pulmonic valve stenosis and absence of the coronary vasculature.[11,12] The morphologic defects in *Fog-2* mutants are likely due to improper folding of the heart tube resulting in malalignment of the inflow and outflow tracts. Such defects in folding may be due to failure of myocardialization, a process in which myocardial cells evacuate the inner curvature of the heart and migrate into the cushions.

Several cascades of signaling molecules that regulate left-right asymmetry have been identified and provide a framework in which to consider human left-right defects. Asymmetric expression of Sonic hedgehog (Shh) leads to expression of the TGF-β members nodal and lefty in the left lateral plate mesoderm.[13] The left-sided expression of nodal induces rightward looping of the straight heart tube. In the right lateral mesoderm, Shh and nodal are inhibited by an activin-/receptor-mediated pathway. Conversely, the snail-related zinc finger transcription factor is expressed in the right lateral mesoderm and is repressed by Shh on the left.[14] Ultimately, the activin- and nodal-dependent pathways result in expression of a transcription factor, Pitx2, on the left side of visceral organs.[15] The asymmetric expression of Pitx2 is sufficient for the establishment of left-right asymmetry in the heart, lungs, and gut.

Recent studies have revealed how the initial asymmetry of molecules such as Shh might be established. Henson's node contains ciliary processes that beat in a vortical fashion, creating a leftward movement of morphogens around the node.[16] In mice homozygous for the inversus viscerum (*iv*) mutation, left-right orientation of the heart and viscera is randomized.[17] The *iv* gene encodes for left-right dynein that might act as a force-generating component in cilia that are present in the node.[18,19] Mice with situs inversus totalis (*inv*) have complete reversal of left-right asymmetry, but the function of the *inv* gene remains unknown.

Patients with heterotaxia syndromes display randomization of the cardiac, pulmonary, and gastrointestinal situs, whereas patients with situs inversus totalis have a well-coordinated reversal of left-right asymmetry. Disruption of the signaling cascades on the left or right side of the embryo results in randomization of cardiac looping and often leads to bilateral right-sidedness (asplenia syndrome) or left-sidedness (polysplenia syndrome), respectively. In human beings, point mutations of several genes involved in the left-right signaling cascade have been identified including ZIC3, a zinc finger transcription factor, activin receptor IIB, and cryptic, a cofactor of nodal.[20]

DEFECTS OF ATRIAL AND VENTRICULAR DEVELOPMENT

Infants born with CHD provide evidence for chamber-specific molecular programs. For example, in hypo-

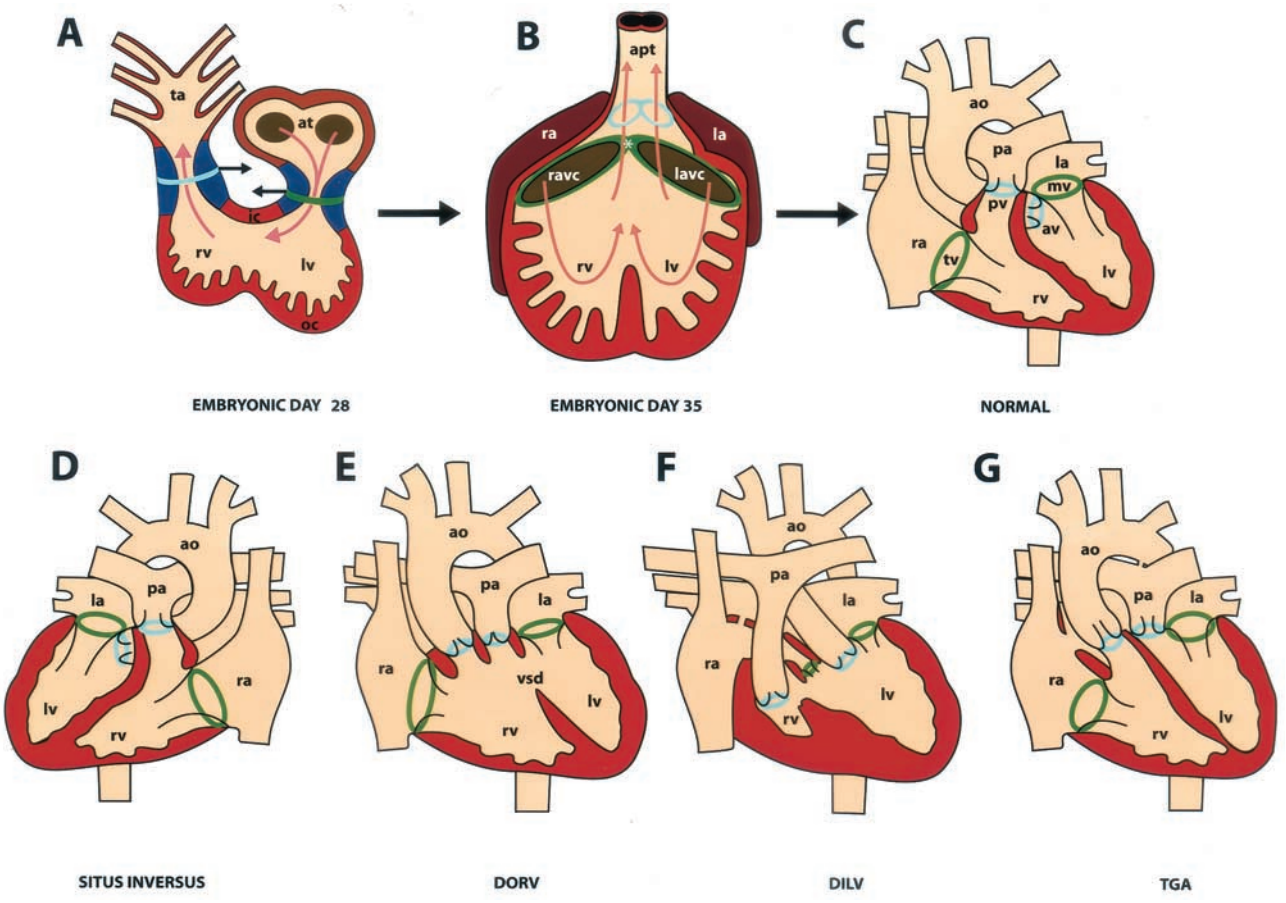

Figure 2. Normal and abnormal cardiac morphogenesis associated with left-right signaling. **A,** As the linear heart tube loops rightward with inner curvature *(ic)* remodeling and outer curvature *(oc)* proliferation, the endocardial cushions *(dark blue)* of the inflow *(green)* and outflow *(light blue)* tracts become adjacent to one another. Subsequently, the AV septum shifts to the right, and the aortopulmonary trunk shifts to the left. **B,** The inflow tract is divided into the right atrioventricular canal *(ravc)* and left atrioventricular canal *(lavc)* by the AV septum *(asterisk)*. The outflow tract, known as the truncus arteriosus *(ta)*, becomes the aortopulmonary trunk *(apt)* upon septation. **C,** Ultimately, the left atrium *(la)* and right atrium *(ra)* are aligned with the left ventricle *(lv)* and right ventricle *(rv)*, respectively. The left ventricle and right ventricle become aligned with the aorta *(ao)* and pulmonary artery *(pa)*, respectively, after 180° rotation of the great vessels. **D,** If the determinants of the left-right axis are coordinately reversed, then a condition known as situs inversus results. **E,** If the aortopulmonary trunk fails to shift to the left, then a condition known as double-outlet right ventricle *(DORV)* results, in which the right ventricle is aligned with both the aorta and pulmonary artery. **F,** Likewise, if the AV septum fails to shift to the right, both atria communicate with the left ventricle in a condition known as double-inlet left ventricle *(DILV)*. **G,** Transposition of the great arteries *(TGA)* results if the aortopulmonary trunk fails to twist, resulting in communication of the right ventricle with the aorta and the left ventricle with the pulmonary artery. (Reproduced with permission from Am J Med Genet 2000;97:271-9.)
tv, tricuspid valve; mv, mitral valve; av, aortic valve; pv, pulmonary valve; rsd, ventricular septal defect.

plastic right ventricle conditions, only the right ventricle does not develop properly whereas the left ventricle and both atria have relatively normal structure and function. Although disruption of blood flow–induced growth may play an etiologic role in some conditions, growing evidence suggests that region-specific regulatory pathways may directly contribute to chamber-specific growth defects. In support of this notion, several transcription factors have been shown to be expressed in a chamber-specific pattern. Two members of the basic helix-loop-helix family of transcription factors, dHAND and eHAND (deciduum/extraembryonic membrane, heart,

autonomic nervous system, neural crest–derived tissues), are predominantly expressed in the right and left ventricles, respectively.[21,22] Deletion of the *dHAND* gene in mice results in hypoplasia of the right ventricle, providing evidence that a single gene can ablate an entire chamber.[21] dHAND appears to regulate survival of ventricular cells, although the downstream targets of *dHAND* that regulate right ventricular survival remain to be identified. Remarkably, in the absence of both *HAND* genes, a heart still forms in the mouse, but it is entirely composed of atrial cells, providing further evidence for modular construction of the heart.[23] The function of the HAND proteins appears to be conserved across species such as zebra fish, which have only one ventricular chamber and only one HAND protein (dHAND)[24] but also require HAND function for ventricular formation.[25] Recent evidence for epigenetic regulation of chamber formation has come from analysis of a muscle-specific protein involved in chromatin remodeling called m-Bop. Similar to dHAND, m-Bop is also required for right ventricular development and, in fact, is necessary for activation of the *dHAND* gene.[26]

The ECM also plays a critical role in right ventricular development, as two ECM proteins are necessary for proper right ventricular development. Versican, a chondroitin sulfate proteoglycan, and hyaluronan synthase 2 (Has2) are expressed in the endocardial cushions and in the ventricular myocardium. When *versican* or *Has2* is disrupted in mice, the right ventricle is hypoplastic whereas the left ventricle is less affected.[27,28] The mechanism by which perturbation of ECM proteins results in right ventricular hypoplasia is being explored.

Myocyte enhancer binding factor 2 (MEF2) is another transcription factor that plays a critical role in ventricular development. Initially discovered in *Drosophila*, MEF2 has four orthologues in mammals that are expressed in precursors of the cardiac, skeletal, and smooth muscle lineages in vertebrates.[29,30] Targeted deletion of one of these, *MEF2C*, in mice results in hypoplasia of right and left ventricles but not of the atria.[31] The chamber-specific role of MEF2C, despite its homogenous expression in the heart, suggests that MEF2C is a necessary cofactor for other ventricular-restricted regulatory proteins.

ATRIAL AND VENTRICULAR SEPTATION DEFECTS

Defects of the atrial or ventricular septum are the most common types of CHD. Genetic linkage analyses of families with autosomal dominant inheritance of CHD have revealed a critical role for two transcription factors in the genesis of septal defects. In human beings, point mutations of *NKX2.5* cause familial atrial septal defects and conduction abnormalities, in addition to sporadic cases of a variety of other types of CHD such as tetralogy of Fallot and Ebstein's anomaly.[32,33] As described previously, Nkx2.5 is a homeodomain protein whose orthologue in *Drosophila*, tinman, is necessary for formation of the dorsal vessel.[5] Careful analysis of mice heterozygous for *Nkx2.5* has identified abnormalities of the atrial septum and the conduction system.[34] Analysis of the mutated gene products revealed important structure-function relationships of the Nkx2.5 protein,[35] but the mechanism of how *NKX2.5* mutations result in CHD remains unknown.

Tbx5 is a transcription factor that is mutated in individuals with the Holt-Oram syndrome, which is characterized by ventricular and atrial septal defects and limb anomalies.[36] Tbx5 is expressed highly in the septum and future left ventricular segment during mouse embryogenesis.[37] Targeted deletion of *Tbx5* in mice results in embryonic lethality in the homozygous state, whereas heterozygous mice have atrial and ventricular septal defects and limb anomalies.[38] Further studies in mice will likely elucidate how Tbx5 regulates ventricular and septal formation. Tbx5 and Nkx2.5 physically interact with one another to regulate common target genes, potentially explaining their common role in human septal and conduction defects.

DEFECTS IN VALVE DEVELOPMENT

Congenital abnormalities of the cardiac valves are commonly seen in infants and children. The cardiac valves develop from regional swellings of ECM, known as cardiac cushions. Reciprocal signaling between the endocardial and myocardial cell layers induces a transformation of endocardial cells into mesenchymal cells. Migration of these cells into the cushions and differentiation into the fibrous tissue of valves then occurs. These cells are also responsible for septation of the common AV canal into separate right- and left-sided orifices. Trisomy 21, or Down syndrome, is commonly associated with incomplete septation of the AV valves. A mouse model of trisomy 21 has been generated,[39] but to date, the responsible gene(s) on chromosome 21 remain unknown.

Nuclear factor of activated T cells c (NF-ATc) is a transcription factor that is needed for cytokine gene expression in activated lymphocytes and is controlled by a calcium-regulated phosphatase, calcineurin. In the heart, NF-ATc expression is restricted to the endocardium. By gene targeting in mice, NF-ATc is necessary for formation of the semilunar valves and, to some extent, the AV valves.[40,41] The stimulus for activation of NF-ATc in the heart and its downstream targets is under investigation and will likely lead to better understanding of cardiac valve formation.

Although lack of cardiac valve leaflets is a rare

cardiac anomaly, thickened valve leaflets resulting in stenotic valves are a common form of CHD. The Smad proteins are intracellular transcriptional mediators of signaling initiated by TGF-β ligands. *Smad6* is specifically expressed in the AV cushions and outflow tract during cardiogenesis and is a negative regulator of TGF-β signaling. Targeted disruption of *Smad6* in mice results in thickened and gelatinous AV and semilunar valves, similar to those observed in human disease.[42] In addition to *Smad6*, there are likely other genes in the TGF-β signaling pathway that, when mutated, result in the formation of stenotic and hyperplastic valves.

CONOTRUNCAL AND AORTIC ARCH ARTERY DEVELOPMENT

Defects of the outflow tract, aortic arch, and ductus arteriosus account for 20% to 30% of all CHD,[43] and the 22q11 deletion syndrome (del22q11) has provided an entry point to study the molecular pathways critical for the generation of these defects. del22q11 is the most common human gene deletion syndrome and is the second most common genetic cause of CHD after trisomy 21.[44] Of individuals with the 22q11 deletion syndrome, 75% have conotruncal and aortic arch defects, which are derived from the cardiac neural crest, in addition to other neural crest–derived defects that include cleft palate, abnormal facial features, thymic hypoplasia, and hypoparathyroidism.[45-48] Of patients with this syndrome, 85% to 90% have a monoallelic microdeletion of chromosome 22q11 spanning approximately 3 megabases that contains nearly 30 genes.[49] Extensive human genetic analyses have failed to identify the critical genes for del22q11. In an effort to identify the important genes in this locus, mouse models were generated that deleted syntenic portions of the commonly deleted region on chromosome 22q11.[50-52] With the use of such approaches, Tbx1, a transcription factor that is expressed in the pharyngeal arches,[53,54] was identified as a likely candidate gene and was shown to cause fourth aortic arch artery anomalies (interrupted aortic arch and anomalous right subclavian artery) when haplo-insufficient in a subset of mice.[52,55] However, most other features of the syndrome have not been reproduced in heterozygous mice. Mice homozygous-null for Tbx1 display many features of del22q11, suggesting that Tbx1 plays an essential role in pharyngeal arch development.[56]

Numerous other genes involved in conotruncal and craniofacial development have been identified by targeted disruption studies in mice. Mice lacking endothelin 1 (ET-1) or its receptor, ET_A, have postmigratory neural crest defects reminiscent of del22q11.[57,58] dHAND and eHAND are downregulated in neural crest-derived tissues in ET-1– and ET_A-deficient mice, suggesting that the HAND transcription factors function downstream of this signaling cascade.[59] Targeted deletion of dHAND results in programmed cell death of the postmigratory neural crest cells, suggesting that dHAND is necessary for survival of these neural crest–derived cells. Neuropilin-1, a downstream target of dHAND, is expressed in neural crest–derived tissues, and targeted deletion of it in mice also results in a phenotype similar to del22q11.[60,61] Dissections of such molecular pathways in mice represent a promising approach to elucidate the bases for cardiovascular developmental defects.

The zebra fish mutant *gridlock* has no circulation to the posterior trunk and tail because of a blockage in the dorsal aorta where the bilateral aortae fuse.[62] This phenotype is similar to aortic coarctation in human beings. Positional cloning revealed mutations in a gene encoding a hairy-related transcription factor similar to the mammalian *HRT-2/Hey2* gene. This gene product appears to be involved in arterial versus venous specification during early development.[63] Aortic coarctation is known to have a high familial recurrence rate, and it will be interesting to determine whether mutations of *gridlock* are present in a subset of these affected patients.

Human genetic studies have identified the gene responsible for Alagille syndrome, which is characterized by biliary atresia and conotruncal defects. Mutations were identified in Jagged-1, a membrane-bound ligand that was originally identified in *Drosophila*.[64,65] *Jagged-1* mutations have since been identified in patients with isolated pulmonary stenosis or tetralogy of Fallot.[66] Jagged-1 is a ligand for the transmembrane receptor Notch, which is involved in embryonic patterning and cellular differentiation.

The ductus arteriosus is derived from the sixth aortic arch artery, and lack of ductal closure after birth results in patent ductus arteriosus, the third most common form of CHD. Pedigree analysis of individuals with familial patent ductus arteriosus identified heterozygous mutations of the transcription factor TFAP2B.[67] This suggests a critical role for this factor or its downstream targets in the normal closure of the ductus arteriosus after birth.

SUMMARY

The findings described here, with the use of multiple animal models, have elucidated some of the genes and molecular mechanisms involved in heart development. However, the etiology of CHD is complex and likely results from a combination of genetic and environmental influences. Genetic analysis in human beings with CHD has revealed point mutations in some of these critical genes. The identification of mutated genes in affected individuals will only be the first step, as it is becoming increasingly clear that similar genetic abnormalities re-

sult in a spectrum of phenotypes in human beings. These differences are likely the result of other genetic and environmental influences. Over the next decade, the challenge will be to identify environmental and epigenetic factors that result in CHD in the setting of appropriate genetic susceptibility. In this manner, genetic identification and subsequent environmental alteration could result in the prevention of some forms of CHD.

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