CHAPTER 1

Molecular Basis of Cardiac Development

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THE HEART FIELDS AND HEART TUBE FORMATION

The heart begins simply as a bilateral field within the lateral plate mesoderm (Figure 1.1, Table 1.1). As the early embryo undergoes formation of the gut-tube, these bilateral fields migrate toward the midline, where the cranial-most aspect of the fields will fuse ventrally to form the outer curvature of the heart tube. These fields will continue to migrate together, with more of the heart fields contributing to the forming tube, until the dorsal aspect of the heart fields fuse to form a closed tube. The initial contributors to the heart tube are known as the first heart field. The first heart field gives rise to the left ventricle, with some contributions to the atria and the right ventricle. Additional heart field progenitors from the lateral plate mesoderm continue to add to the arterial and venous poles of the heart tube; these later-adding cells are known as the second heart field. The second heart field gives rise to most of the right ventricle and atria, the most distal myocardium that surrounds the aorta and pulmonary artery, and the most proximal smooth muscle that contributes to the tunica media of the great arteries.

Signaling Pathways in Heart Field Specification

The Wnt Pathway

The Wnt family includes the canonical pathway, the non-canonical pathway (also known as the planar cell polarity pathway), and the Wnt/calcium pathway (Figure 1.2). Both the canonical and non-canonical pathways have well-established roles in heart field specification. Temporal waves of canonical and non-canonical Wnt signaling play distinct roles during cardiac specification and morphogenesis. As the heart field forms from the primitive streak, Wnt3a is expressed in the primitive streak and serves as a repulsive cue to the forming heart. Experiments performed in Xenopus, due to its ease of manipulation and genetic tractability, have shown that the early repression of Wnt signaling in the Xenopus animal cap (i.e., in the ectodermal roof of the blastocele prior to heart field specification) via Dkk-1 and Crescent is necessary for initiation of transcription of cardiac transcription factors Nkx2.5 and Tbx5 and myocardial-specific proteins troponin I and myosin heavy chain α. However, later in cardiac development, canonical Wnt signaling in embryonic mice at E8.75 promotes Nkx2.5, Islet1, and Baf60c within the entire heart. Due to the genetic similarity between Xenopus and mouse, these differences likely reflect different temporal requirements for Wnt signaling as opposed to species-specific differences. In the second heart field, non-canonical Wnts 5a and 11, which act through the non-canonical planar cell polarity pathway, are expressed slightly later in development and co-operatively repress the canonical Wnt pathway while also promoting expression of Islet1 and Hand2, whose expression serves to ‘mark’ the heart field; as such, these genes are commonly referred to as cardiac markers. Both the repression of the canonical Wnts and the induction of the heart field markers require β-catenin in the second heart field. Wnt5a and Wnt11 also promote proliferation within this progenitor region. After the heart tube forms, Wnt-stabilized β-catenin is necessary in the Islet1-expressing second heart field cells to maintain their progenitor status. Loss of either β-catenin or Wnt signaling in the second heart field leads to second heart field defects, including right ventricular and outflow tract defects. Even if Wnt signaling is lost under cells expressing one of the first markers of differentiated cardiomyocytes, Mesp1, second heart field proliferation is decreased, and Islet-1
expression is down-regulated. Converversely, overexpressing β-catenin under the Mesp1 promoter expands the Islet-1-positive second heart field and promotes proliferation. Later, Wnt5a specifically acts upstream of the disheveled/planar cell polarity pathway to regulate the addition of the second heart field to the arterial pole. In addition, Wnt signaling also promotes bone morphogenetic protein (BMP) 4 and the non-canonical Wnt 11, which promote myocardial differentiation. Thus, the Wnt pathway is critical for inducing heart field formation, maintaining progenitor status and promoting myocardial differentiation.

**Retinoic Acid**

One of the earliest required signaling pathways is the retinoic acid pathway. RALDH2, the enzyme that

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**TABLE 1.1** Major Developmental Time Points in Humans and Common Experimental Models

<table>
<thead>
<tr>
<th>Milestone</th>
<th>Human</th>
<th>Mouse</th>
<th>Chick</th>
<th>Xenopus</th>
<th>Zebrafish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart is specified</td>
<td></td>
<td>E7.25</td>
<td>HH 3</td>
<td>Stage 15</td>
<td>8 somites</td>
</tr>
<tr>
<td>Heart tube forms</td>
<td>~CS 9/10 (2 mm)</td>
<td>E8</td>
<td>HH 9</td>
<td>Stage 33</td>
<td>24 hpf</td>
</tr>
<tr>
<td>Heart displays regular contractions</td>
<td>~CS 9/10</td>
<td>E8</td>
<td>HH 7*</td>
<td>Stage 35</td>
<td>24 hpf</td>
</tr>
<tr>
<td>Heart begins looping</td>
<td>CS 13</td>
<td>E8.5</td>
<td>HH 9+/10-</td>
<td>Stage 35</td>
<td>30 hpf</td>
</tr>
<tr>
<td>AV cushions begin forming</td>
<td>CS 17</td>
<td>E9.5</td>
<td>HH 12/13-</td>
<td>Stage 44</td>
<td>48 hpf</td>
</tr>
<tr>
<td>OFT cushions begin forming</td>
<td>CS 15/16</td>
<td>E10.5</td>
<td>HH 12/13-</td>
<td>Stage 39/40</td>
<td>–</td>
</tr>
<tr>
<td>Outflow tract is septated</td>
<td>CS 17</td>
<td>E13.5</td>
<td>HH 34</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Atria are septated</td>
<td>CS 18†</td>
<td>E14.5</td>
<td>HH 46</td>
<td>Stage 46</td>
<td>–</td>
</tr>
<tr>
<td>Ventricles are septated</td>
<td>CS 22</td>
<td>E13.5</td>
<td>HH 34</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The major stages in cardiovascular development are presented for humans and the most commonly used animal models.

*Because the heart tube begins forming as a trough that then closes dorsally, contractions are observed prior to the pinching off of the fully formed heart tube.

†The foramen ovale is still open at this stage. This fenestration is closed at the stages listed for the other species. CS, Carnegie stage; E, embryonic day; HH, Hamburger-Hamilton stage; Stage, Nieuwkoop and Faber stage; hpf, hours post-fertilization. See cited references for more details.
synthesizes retinoic acid, is restricted within the lateral plate mesoderm to a region nearest to the heart field.\textsuperscript{13,14} Expression of RALDH2 progresses in a cranial–caudal direction during heart field induction and heart tube formation and establishes the posterior boundary of the heart field.\textsuperscript{15} Within the lateral plate mesoderm, retinoic acid plays an inhibitory role, where it acts both directly and indirectly to restrict cardiac transcription factors Nkx2.5 and FoxF1 to the anterior lateral plate mesoderm, Hand1 to the anterior and middle of the lateral plate mesoderm, and Sal1 to the posterior lateral plate mesoderm.\textsuperscript{13,16,17} Retinoic acid further represses GATAs 4, 5, and 6.\textsuperscript{18} This inhibitory role is required to limit the size of the heart field, and fate-mapping studies in the zebrafish, another experimental model that is valued for its genetic similarity to the mouse and ease of studying embryonic development,\textsuperscript{19} have demonstrated that zebrafish embryos with decreased levels of retinoic acid exhibit an increased number of Nkx2.5-positive cells.\textsuperscript{17} Conversely, exposing either zebrafish or \textit{Xenopus} embryos to increasing levels of retinoic acid specifically leads to a reduced number of cardiomyocytes.\textsuperscript{16,17} In chick embryos, which physically develop more similar to humans as compared with mice but lack the ability to manipulate the genome as in mice,\textsuperscript{20} antagonizing retinoic acid signaling promotes the ventricular myocardial fate at the expense of the atria.\textsuperscript{15} Together, these studies suggest that retinoic acid plays two major roles in the early heart field. First, retinoic acid generally restricts the expression of heart field markers to limit the size of the heart field. Then, it specifically promotes a ‘posterior’ fate within the heart field, which affects the venous precursors that will give rise to the atria.

### Transcription Factors in Heart Field Specification

Several well-known cardiogenic transcription factors are expressed in the early heart fields and play a role in driving lateral plate mesoderm to a cardiac fate. In the first heart field these transcription factors include but are not limited to Nkx2-5; GATAs 4, 5, and 6; and Tbx5. The transcription factor kernel required for driving mesoderm to myocardium has been minimally defined as the transcription factors GATA4 and Tbx5 and the chromatin remodeling subunit Baf60c.\textsuperscript{21} Furthermore, Tbx5 genetically interacts with Baf60c in cardiac morphogenesis.\textsuperscript{22} These findings have begun to elucidate the interplay between chromatin remodeling factors and cardiogenic transcription factors during the progressive differentiation of mesoderm to cardiac progenitor to cardiomyocyte.

The homeobox transcription factor Nkx2.5 is perhaps the best-known cardiac inducer. Nkx2.5 is expressed in the first and second heart fields. During normal development, Nkx2.5 becomes turned off in the first heart field, allowing these cells to begin differentiating while maintaining the second heart field in a progenitor state.\textsuperscript{23} During this process, Nkx2.5 represses the transcription of numerous cardiac progenitor markers, including Islet1, Mef2c, and Tgfβ2.\textsuperscript{23} In addition to repressing cardiac progenitor markers, Nkx2.5 also represses the BMP and fibroblast growth factor (FGF) pathways, which promote differentiation into myocardium and inhibit proliferation.\textsuperscript{23–25} This field of cells is the first to differentiate into functional myocardium and express muscle-specific markers such as the myosin light chain protein MLC3F\textsuperscript{26} while concomitantly down-regulating cardiac
progenitor transcription factors such as Islet1. In the later differentiating second heart field, these pathways must be down-regulated to allow for sufficient proliferation within the progenitor pool, and proliferation in this region is essential for normal development of the arterial pole. In addition, Mef2c is required to allow the transition from specification to myocardial differentiation.

Congenital Heart Disease (CHD) Resulting from Heart Field Defects: Cardia Bifida

A severe and uniformly lethal heart defect associated with the early heart fields is a failure of the bilateral heart fields to migrate and fuse at the midline, leading to two separate hearts, a condition known as cardia bifida. Though the previously mentioned signaling pathways and transcription factors are crucial for heart formation, loss of only a few specific factors can result in cardia bifida. These factors are described in detail, and Table 1.2 provides the Online Mendelian Inheritance of Man entry numbers if information on available genetic testing for these genes (as well as additional CHD-related genes presented later in the chapter) is of further interest.

Loss of GATA4 is perhaps the best-known cause of cardia bifida. The GATA4 homozygous null mouse embryo fails to form a heart tube. Furthermore, siRNA knockdown of GATA4 in the chick heart fields results in the formation of bilateral heart tubes, and this effect is caused by a down-regulation of N-cadherin. Similarly, blocking N-cadherin in chick embryos inhibits fusion of the heart fields but does not halt heart development, resulting in bilateral heart tubes. However, although the N-cadherin homozygous null embryo displays heart failure, as evidenced by pericardial effusion, a single heart tube is present. Thus, species-specific differences in the cadherins may underlie the contribution of N-cadherin to GATA4-mediated cardia bifida.

Loss of BMP or Nodal signaling can also result in cardia bifida, and this effect may also occur through the GATA transcription factors. In Xenopus, loss of BMP signaling through injection of mRNA encoding the BMP antagonist Smad6 or a mutated truncated BMP receptor leads to cardia bifida. In these embryos, the heart fields are also specified but fail to fuse at the midline. Consistent with BMP’s previously mentioned role in promoting myocardial differentiation, these embryos also show fewer cardiomyocytes compared with untreated control embryos. Similarly, the zebrafish swirl and one-eyed pinhead mutants, which lack Bmp2b and Cripto, respectively, display reduced Nkx2.5 expression, which can be rescued by the ectopic expression of GATA5. Of these two zebrafish mutants, only the one-eyed pinhead mutant presents with cardia bifida though, and genetic redundancy in the zebrafish may explain why the zebrafish swirl mutant has a less severe phenotype than the Smad6-injected Xenopus embryo. Perhaps unsurprisingly, cardia bifida is almost always incompatible with life. One case report, however, has described an infant who survived with cardia bifida in which each half heart showed characteristics of both ventricles but a single atrium. Each half heart also had its own truncus and sinus venosus, which may have been sufficient in the intrauterine environment.

**TABLE 1.2 Developmental Stages of Heart Formation and their Associated Congenital Heart Defects**

<table>
<thead>
<tr>
<th>Developmental Process</th>
<th>Transcription Factors and Genes Involved</th>
<th>Related Human Diseases</th>
<th>OMIM Entry #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart field specification and heart tube formation</td>
<td>GATA4, Sma6, Nodal</td>
<td>Cardia bifida</td>
<td>600576, 602931*</td>
</tr>
<tr>
<td>Establishment of laterality</td>
<td>Pitx2, FoxC1</td>
<td>Axenfeld-Rieger syndrome</td>
<td>601090</td>
</tr>
<tr>
<td>Conduction system</td>
<td>Pitx2c</td>
<td>Atrial fibrillation</td>
<td>601542</td>
</tr>
<tr>
<td>Valve formation</td>
<td>Fibrillin-1, Elastin, Fibrillin-4, TGFβ</td>
<td>Mitral valve prolapse</td>
<td>134797, 130160, 604633</td>
</tr>
<tr>
<td>Outflow tract formation</td>
<td>Tbx1, Lhx2, Tcf21, β-catenin, Neurphilin-1</td>
<td>DiGeorge syndrome</td>
<td>602054, 603759*</td>
</tr>
<tr>
<td>Chamber septation</td>
<td>GATA4, Nkx2.5, Tbx5</td>
<td>Atrial septal defects</td>
<td>600576, 600584, 606061, 601620</td>
</tr>
</tbody>
</table>

During some steps of development, specific genes have been definitively shown to cause certain congenital heart defects in humans. These developmental steps, along with their associated genes and defects, are presented herein. More information can be obtained from the Online Mendelian Inheritance in Man database (www.omim.org), which includes clinical resources, such as whether a genetic test is available to screen for known mutations.

*No genetic test is yet available.

**LOOPING AND LATERALITY**

The human body is asymmetric about the vertical midline, and the heart is among the visceral organs with left/right asymmetry. Left/right asymmetry is established early during embryonic development; left/right gene expression differences are observed in the lateral plate mesoderm from which cardiac progenitors emerge. The molecular mechanisms that dictate the left/right asymmetry of the heart during its morphogenesis are unknown; however, the mechanisms that underlie the
initial asymmetry-breaking events in the early embryo have been described.

**Left/Right Determination, Cilia, and Signaling**

The cilium, a subcellular organelle that protrudes from the cellular surface, plays an important role in left/right determination. In the gastrulating embryo, rotating cilia in Hensen’s node generate leftward flow that breaks left/right symmetry and establishes differential signaling and gene expression on the left and right sides of the embryo. In the heart field, the cilia initially express FGF receptors. These receptors respond to FGF, which induces them to move Sonic hedgehog and retinoic acid leftward, which results in the release of calcium. Despite this purported leftward movement, though, the asymmetrical expression of Sonic hedgehog and retinoic acid is controversial, with some but not all studies showing asymmetrical expression in the node. These signaling events limit the expression of Nodal, Pitx2c, and Lefty2 to the left side of the embryo. The side-specific expression of these transcriptional regulators activates a gene regulatory network on the left distinct from that on the right, causing sidedness of morphogenesis. The link from the left/right determination transcriptional kernel to the cardiac progenitor transcriptional kernel may be direct. Nkx2.5, a progenitor transcription factor, interacts with the N-terminal end of transcription factor Pitx2c, part of the left-determination transcriptional network. This specific interaction is required for normal heart looping, and antagonizing Pitx2c in the left side of the heart, where it is normally expressed, leads to randomized heart looping.

**Sidedness of Cardiac Structures**

Cardiac left/right asymmetry is first noticeable during cardiac looping, during which the ventricles undergo characteristic D-handed looping with great fidelity. This rightward looping displaces the ventral midline of the heart towards the right and establishes the outer curvature of the heart tube. The formerly dorsal midline is then rotated to become the inner curvature of the heart tube. From this looped position, the outflow and inflow tracts converge, and the ventricular bend becomes displaced ventrally. Finally, as the outflow tract is separated to form the aorta and pulmonary artery, the aorta is wedged between the pulmonary artery and the atrio-ventricular valves.

Left/right determination can go awry in distinct ways with predictable results. The entire cascade can be inverted, resulting in normal but mirror image development of the embryo and heart. Consistent with this hypothesis, the inv mutant mice display situs inversus, and in this model, Pitx2c is selectively expressed on the right side instead of the left. The left-sided program can be disrupted, resulting in hearts with both sides developing as ‘right,’ or right isomerism. Work in mouse embryos suggests that Pitx2c is required for turning off the ‘right-sided’ developmental program on the left side. In Pitx2c homozygous null embryos, both atria display right-sided structures. Within the mature heart, left-sided structures include the left atrium with pulmonary veins, whereas right-sided structures include the right atrium with sinoatrial node. In Pitx2c mutant embryos, the sinoatrial node is duplicated on both sides, and valve and septation defects consistent with right atrial isomerism are observed. The requirement for Pitx2c in structural morphogenesis of the heart may come after heart looping: removing Pitx2c specifically from differentiated myocardium causes right atrial isomerism, including an atrial septal defect and the duplicated sinoatrial node. Thus, Pitx2c is required in the mesoderm for left/right determination and later in the myocardium to repress the ‘right-sided’ developmental program, including sinoatrial node formation within the left atrium.

The right-sided program can also be disrupted, resulting in hearts developing with two ‘left’ sides or left atrial isomerism. Left atrial isomerism is observed in embryos lacking Sonic hedgehog. Sonic hedgehog null mice display isomerism of the left atrial appendages and other malformations that are frequently observed in humans with left isomerism. In these embryos, Pitx2c expression is observed bilaterally, consistent with the instructive role of this transcription factor for left-sided structures. Consistent with the Pitx2c expression pattern in the Sonic hedgehog null mouse, the heart fails to loop in mice lacking hedgehog signaling. Finally, the left/right determination process can be randomized. Mice that lack cilia show randomized expression of left/right determination transcription factors, sidedness, cardiac looping, and sidedness of specific cardiac structures.

**CHD from Abnormal Left/Right Determination: Heterotaxy Syndrome and Abnormal Left/Right Specification**

Defects in left/right determination are common in humans and are grouped into the heterotaxy syndrome. The observed cardiovascular phenotypes of humans with this syndrome are broad, as predicted from the above discussion. The molecular genetics of heterotaxy syndrome are under current investigation, and rapid progress is being made given the large number of predicted candidate genes identified from animal model studies. In particular and consistent with the importance of cilia in left/right determination, cilia gene mutations have been identified in several heterotaxy syndrome patients, causing a range of cardiac structural abnormalities from
situs inversus to left or right isomerisms. Furthermore, proper cardiac looping is required to align the segments of the heart for subsequent septum morphogenesis. Mis-looping would be expected to cause cardiac structural abnormalities in addition to structural isomerisms. Consistent with this hypothesis, cardiac septal defects are commonly observed in patients with heterotaxy syndrome. For example, patients with Axenfeld-Rieger syndrome display a variety of congenital heart defects, including atrial and ventricular septal defects, mitral and tricuspid defects such as prolapse and stenosis, small left ventricular outflow tract, and stenotic or bicuspid aortic and pulmonary valves. An estimated 40–60% of these patients have mutations in Pitx2 or FoxC1.

**CHAMBER SPECIFICATION**

Cardiac Chamber Versus Non-Chamber Developmental Programs

Historically, the tubular heart was thought to be composed of segments that were each progenitor structures for the components of the mature cardiac form. Thus, from posterior to anterior, the linear tube was thought to possess domains that generated the atria, the left ventricle, the right ventricle, and outflow tract myocardium. However, recent molecular developmental studies have generated overwhelming evidence for a new model: the tubular heart is the progenitor structure primarily of the atrioventricular canal and atrioventricular node myocardium in the mature heart. The cardiac chambers, including the atria positioned dorsally and the ventricles positioned ventrally, form as rapidly proliferating structures that balloon off the primary heart tube. Thus, understanding the building blocks of the developing heart has progressed from trying to understand segmentation of the linear heart tube (with little progress) to understanding the regulation of chamber formation off the primary heart tube. The portion of the heart tube that does not enter the ‘chamber’ program becomes fated to the atrioventricular canal, such as atrioventricular node myocardium, and defines the region of myocardium that encourages adjacent endocardium to enter the endocardial cushion and cardiac valve program. Thus, this ‘ballooning’ model defines chamber versus non-chamber fates. Because non-chamber myocardium becomes fated to the atrioventricular conduction system and determines the location of the atrioventricular valves, this model integrates chamber morphogenesis with the cardiac conduction system and cardiac valve development. BMP signaling and T-box transcription factors play fundamental roles in chamber versus non-chamber determination.

Within the heart tube, the myocardium that will be retained in the atrioventricular canal is molecularly defined by expression of BMP2, Tbx2, and Tbx3 (Figure 1.3). BMP2 is required for the development of the atrioventricular canal and to prevent its conversion to chamber myocardial program. BMP2 induces the expression of Tbx2 and Tbx3, which act as transcriptional repressors. Tbx2 and Tbx3 directly interact with and repress chamber-specific genes such as Nppa (also known as ANF) and Cx40. Tbx2 and Tbx3 share redundant functions, and mice that lack at least three Tbx2/Tbx3 alleles do not form atrioventricular cushions, possibly due to reduced levels of BMP2.

In contrast to the dominance of T-box transcriptional repressors Tbx2 and Tbx3 in the atrioventricular canal, the T-box transcriptional activators Tbx20 and Tbx5 dominate in cardiac chamber myocardial development. Tbx20 and Tbx5 activate the chamber myocardial

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**FIGURE 1.3** Chamber versus non-chamber myocardium. Myocardium that is fated to contribute to the chambers is restricted based on interactions between BMP2, a series of Tbx genes, and Hey1 and 2. In the atrioventricular canal and outflow tract, BMP2 induces Tbx2 and 3, which repress chamber myocardium-specific genes. In contrast, chamber-specific Tbx20 expression inhibits BMP signaling, thus eliminating this inhibition and promoting myocardial differentiation. In addition atrial-specific Hey1 and ventricular-specific Hey2 further inhibit BMP2 from interacting with Tbx2 and 3, providing additional feedback to demarcate the chamber myocardium from the developing valves.
program, including networks for rapid proliferation and differentiation into strongly contractile myocardium, promoting Myc-induced myocardial specification and proliferation.\textsuperscript{65–69} Tbx20 also prevents regions that are fated for cardiac chamber development from expressing the ‘atrioventricular canal’ program by interacting with Smad1/5 to inhibit BMP signaling and repress Tbx2 expression within chamber myocardium.\textsuperscript{70} Additionally, atrial-specific Hey1 and ventricular-specific Hey2 also suppress Tbx2 and BMP2, thus reinforcing the boundaries between chamber myocardium and developing valves.\textsuperscript{71,72} Importantly, if the atrioventricular region is not defined, then patterning in the atria can also be disrupted, resulting in ventricular-specific markers (such as Nppa) extending into the atria.\textsuperscript{73}

**Atrial Versus Ventricular Identity**

Hey family transcription repressors act to maintain chamber identity and define atrial versus ventricular identity. Independent of the Notch pathway, atrial-restricted Hesr1 (Hey1) and ventricular-restricted Hesr2 (Hey2) both suppress Tbx2, which is expressed between these two genes to define the atrioventricular canal and thus separate the ventricles from the atria.\textsuperscript{71} Hey2 expression in ventricular compact myocardium inhibits atrial gene expression and thus atrial identity.\textsuperscript{74} Hey2 also promotes proliferation within the compact myocardium.\textsuperscript{74} However, trabecular ventricular myocardium is unaffected by Hey2, based on BMP10 expression in the Hey2 homozygous null embryo. Similar to the effect of Tbx20 overexpression in differentiated cardiomyocytes, Hey2 knockout results in up-regulated Tbx5 and Cx40.\textsuperscript{74}

**VENTRICULAR SEPTATION AND MYOCARDIAL PATTERNING**

To septate the ventricles, a muscular septum grows from the apex of the ventricles toward the atrioventricular canal (Figure 1.4).\textsuperscript{75} A membranous septum forms from the cushion tissue of the atrioventricular mesenchymal septum and bridges the gap between the atrioventricular canal and the crest of the ventricular muscular septum.\textsuperscript{76} Tbx5 and Tbx20 are crucial within the ventricles for placement of the muscular ventricular septum, independent of their role in chamber versus non-chamber determination. In Hamburger-Hamilton stage (HH) 30 chick embryos, Tbx5 is restricted to the left ventricle, and Tbx20 is restricted to the right ventricle.\textsuperscript{68} When Tbx5 is ectopically expressed throughout both ventricles, both ventricles express left-sided markers, such as ANF.\textsuperscript{68} The ventricular septum fails to form in these embryos.\textsuperscript{68} Furthermore, if Tbx5 is misexpressed on the other side of the Tbx20-positive right ventricle (i.e., within the outflow tract), a second ventricular septum-like structure appears at the juxtaposition of these two transcription factors.\textsuperscript{68} Thus, a boundary at the edge of Tbx5 expression appears essential for patterning the placement of the ventricular septum.

Patterning across the myocardial wall is also essential for proper cardiac function. For example, the ventricles possess an outer layer of highly contractile compact myocardium, and an inner layer of poorly contractile trabeculated myocardium.\textsuperscript{77} Neuregulin signaling is required for the proper trans-myocardial wall patterning. The neuregulin-1 homozygous null embryo forms chambers that are poorly differentiated.\textsuperscript{78} Neuregulin-1 promotes exit from the cell cycle and cardiac differentiation and acts through the ERK intracellular pathway.\textsuperscript{78} The neuregulin-1 knockout mouse has poor trabeculation and a thin compact myocardial layer.\textsuperscript{78} Furthermore, most cardiac transcription factors are down-regulated, including Hand1 and Cited1, in this mouse, and the conduction system prematurely switches from base-to-apex to apex-to-base activation,\textsuperscript{78} suggesting that the conduction system myocardium develops early and at the expense of the working ventricular myocardium. In addition to Cited2’s early role in establishing left/right asymmetry, knocking out Cited2 under the Nkx2.5 promoter leads to incomplete ventricular septation and a thin compact myocardial layer.\textsuperscript{79} Cited2 also binds to the vascular endothelial growth factor (VEGF)A promoter, thus promoting coronary vessel formation within the compact myocardium.\textsuperscript{79} Transcription factor Tbx20 promotes

**FIGURE 1.4** Chamber septation. To form the ventricular septum, the muscular component of ventricular septum elongates toward the atrioventricular canal. From the atrioventricular (AV) canal, a membranous septum forms from the cushion tissue and closes that gap between the atrioventricular canal and the muscular ventricular septum. To form the atrial septum, the septum primum grows from the cranial aspect of the common atrium toward the atrioventricular cushions. Once this septum fuses with the atrioventricular cushions, the cranial aspect detaches. This fenestration is closed by a fold in the dorsal atrial myocardium termed the septum secundum, which forms to the right of the septum primum.
the transcription of BMP10, which is expressed and required for the formation of the trabeculated myocardium. BMP10 in turn promotes Tbx20. When Tbx20 is overexpressed under the early expressing Nkx2.5 promoter, Tbx5 is reduced, but ventricular chamber markers are present, and myocardial proliferation is reduced. In contrast, when Tbx20 is overexpressed under the later-expressing βMYHC promoter, Tbx2 is repressed, and cardiac conduction-specific factors (e.g., Tbx5, Cx40, and Cx43) are induced; furthermore, cardiomyocyte proliferation is increased, resulting in a thickened compact myocardium. These results are consistent with Tbx20 playing multiple roles during myocardial differentiation.

CONDUCTION SYSTEM DEVELOPMENT

In the primitive heart tube, all the cardiomyocytes are electrically coupled, and contractions proceed in peristaltic waves from the inflow to outflow. However, in the four-chambered heart, the conduction system paces and orients the contractions of the atria and ventricles to maximize cardiac output and efficiency. In the mature heart, an impulse initiates at the sinoatrial node at the junction of the right atrium and superior vena cava and activates electrically coupled atrial myocardium, causing the atria to contract. The impulse is rapidly transmitted to the atrioventricular node, in the atrioventricular junctional complex. The atrioventricular node is the only route of electrical continuity between the atria and ventricles and is a slowly propagating structure. The atrioventricular node slows the signal prior to its transmission to the ventricles, providing a delay to allow atrial contraction and ventricular filling. From the atrioventricular node, the impulse travels into the rapidly conducting ventricular conduction system. The impulse travels anteriorly in the atrioventricular bundle (or bundle of His), through the bundle branches coursing down each side of the interventricular septum, and ramifies into a network of Purkinje fibers at the apex of the heart. The Purkinje network activates surrounding ventricular myocardium, which triggers ventricular contraction and provides for the observed apex-to-base direction of ventricular contraction.

The Atrial Nodal Conduction System

The model of chamber specification described above provides a coherent model for the localization and specification of the atrial components of the conduction system, the sinoatrial and atrioventricular nodes. Both of these conduction components are specified early and are present in the primary heart tube. Primary heart tube myocardium is poorly contractile, poorly coupled, and autonomously depolarizing. These cellular characteristics describe the precise characteristics required in the mature sinoatrial and atrioventricular nodal myocardium. Thus, primary myocardium with the desired characteristics is retained in the atrial nodes, highlighting the myocardial derivation of the atrial conduction system, and the ‘chamber’ myocardial program is repressed in these cells, allowing chamber positioning to retain the nodes in the appropriate locations. In addition to repressing a conversion to chamber myocardium, the T-box transcription factor repressors Tbx3 and Tbx2 actively promote the cellular characteristics of the nodal conduction system.

Early in conduction system development (HH 17 in chick), a transient sinoatrial node is observed on the left side of the sinus venosus. Consistent with an incompletely differentiated phenotype, this region is negative for Nkx2.5 and differentiated marker TnI and positive for heart field marker Islet1, Tbx18, and conduction system marker hyperpolarization activated cyclic-nucleotide gated channel (HCN). Within a day, the sinoatrial markers in the chick are present on the right side and remain transiently expressed on the left side of the sinus venosus. By HH 22, TnI has been up-regulated and will remain expressed in the mature sinoatrial node. In addition to its earlier roles, Pitx2c expressed by TnT-positive cardiomyocytes will repress sinoatrial node formation on the left side of the sinus venosus. By HH 35, the definitive sinoatrial node is present at the junction of the superior vena cava and the right atrium, and it almost exclusively activates the right atrium first. Only a small percentage of hearts with left-sided sinoatrial node maintain pace-making ability. Gap junctions and desmosomes are rare in the sinoatrial node, leading to slow conductance. Of the connexins, connexin 30.2 and connexin 45 have been identified in sinoatrial node, and the gap junctions that these connexins form exhibit slow conductance.

T-box genes Tbx18 and Tbx3 are required for sinoatrial node formation. Tbx18 promotes the development of the ‘head’ of the sinoatrial node, whereas Tbx3 promotes the differentiation of the ‘tail’ that runs along the terminal crest. Similar to the molecular signaling required to define the regions of the heart tube that will form the atrioventricular cushions, Tbx3 plays a role in conduction system development by repressing atrial-specific genes such as Nppa. Tbx3 expression occurs as soon as atrial markers are expressed, and the Tbx3 homozygous null mouse has a small sinoatrial node as compared with wild-type mice. The sinoatrial node is likely derived from the Tbx18-positive region because these two Tbx genes regulate spatially distinct portions of the sinoatrial node.

Tbx2 and Tbx3 expression delineate the atrioventricular node. Whereas these genes are redundant in the atrioventricular canal for repressing the chamber myocardial
Conduction is observed in a base-to-apex pattern before the His Purkinje network has fully matured, highlighting the conductive properties of the early myocardium. Once the His Purkinje fibers have matured, electrical activation in the heart switches from a base-to-apex pattern (HH 25–28 in chick) to the mature to-base pattern (HH 33–36 in chick). Specific channel proteins provide both rapid depolarization and large conductance connectivity between the cells of the ventricular conduction system. The large gated sodium channel Nav1.5 is unregulated in these cells, providing rapid depolarization. The large conductance channel connexin 40 is also up-regulated specifically in the ventricular conduction system. Tbx5 directly transcriptionally activates expression of both of these genes, providing a direct link from specification to functional differentiation of the ventricular conduction system.96–98

Among its many roles in cardiac development,99 the vasoconstrictor peptide endothelin-1 promotes a conductive fate. Endothelin-1 induces both cardiac progenitors and cardiomyocytes to become conduction cells.100,101 In response to endothelin-1, Nkx2.5-positive cardiac progenitors express Hcn4 and connexin 45.101 Intriguingly, endothelin receptor type A is expressed within a subset of the heart field, specifically within the future atria and left ventricle.102 Thus, a subset of myocardial cells that are endothelin receptor type A-positive/MESP1-negative may give rise to the conduction system myocardium.

Atrial Fibrillation

Consistent with Pitx2c’s role in repressing the sinoatrial node fate within the left atrium, patients with reduced Pitx2c levels often develop atrial fibrillation.103 In mice, if Pitx2 is knocked out in the atria, the atrial chambers are enlarged but thin, and the mice exhibit atrioventricular node block.103 In addition, the atrioventricular node and the bundle branches have reduced insulation, which would further miscue contractions.103
1. MOLECULAR BASIS OF CARDIAC DEVELOPMENT

the semilunar valves, the outflow tract cushions also contribute to the aorticopulmonary septum (described below) and the outflow tract myocardium.104

Both sets of valves undergo similar development (Figure 1.6). Signals from the myocardium induce epicardial–mesenchymal transition (EMT). The lateral cushions develop later and are mostly populated by epicardially derived cells.222 In the outflow tract, the proximal left and right cushions are formed through EMT; cardiac neural crest-derived cells migrate from the pharyngeal arches into the outflow tract and form two prongs in the distal left and right outflow tract cushions.

Valve Specification and Growth

Once a subset of the myocardium has been restricted to surrounding a cardiac cushion, it begins secreting proteins between the myocardial and endocardial layers of the early heart tube. The proteoglycans that are secreted from the myocardium and released between the cell layers are called the cardiac jelly; the resulting swellings that arise from this deposition are known as the cushions.106,107 In the atrioventricular canal, myocardially secreted BMP2 activates the Tbx2/Msx2/Has2 pathways and induces a subset of endothelial cells to undergo epithelial–mesenchymal transition and migrate into the cardiac jelly.73,108 Removing BMP2 from atrioventricular canal myocardium inhibits EMT and ECM deposition into the cushions.73,108 Furthermore, Tbx2 and Tbx3 induce BMP2, suggesting that a feed-forward mechanism protects the region that will form the atrioventricular valves.61 If Tbx2 is misexpressed in the chamber myocardium under the Myh6 promoter, where it is normally absent, ECM is deposited between the compact and trabecular myocardium, thus expanding the cardiac jelly and leading to stenotic lumens.109 Of the numerous ECM enzymes, Tbx2 specifically induces HA synthetase via its T-box binding sites.109 Increased Tbx2 also increases TGFβ expression and Smad2 phosphorylation,109 suggesting that epithelial-mesenchymal transition may have also been increased. BMP signaling from the myocardium to the endocardium acts through canonical Smad transcription factors in induced endocardial epithelial-mesenchymal transition.110

Epithelial-mesenchymal transition establishes mesenchymal cells that populate the atrioventricular and outflow tract cushions. The transcription factor nuclear factor of activated T cells (Nfat)c1 acts downstream of VEGF via the MEK/mitogen-activated protein kinase 1 (ERK) pathway to promote proliferation of both the overlying endocardial cells and the mesenchymal cells within the cushions (Figure 1.7).111 When VEGF is down-regulated at the beginning of the ECM remodeling period (E14.5 in mouse),112 receptor activator of nuclear factor κB ligand (RANKL) expression increases and promotes Nfatc1 nuclear translation via the c-Jun NH2-terminal kinase (JNK) pathway to promote the expression of ECM remodeling enzyme cathepsin...
This switch from VEGF to RANKL expression is associated with a reduction in proliferation. The up-regulation of cathespin K expression is associated with increased expression of ECM proteins versican and periostin.

Once the cushions are populated, they begin remodeling and elongating. The rapid proliferation in the earlier cushions is attenuated by the ErbB1 signaling pathway. ErbB1 signaling also down-regulates signaling through the BMP pathway. In addition to reduced proliferation, this stage is also accompanied by the deposition of ECM layers. Three distinct layers are present in the valves: an elastin-rich layer on the side of the valve exposed to flow, a proteoglycan-rich spongiosa middle layer, and a fibrillar collagen-rich layer on the non-flow-exposed side. BMP2-induced Sox9 promotes the deposition of proteoglycans and cartilage differentiation, and FGF8-induced Scleraxis promotes the deposition of tenascin.

Although the signaling events that define where the outflow tract cushions will form are not as well defined as for the atrioventricular cushions, there are many similarities between atrioventricular canal and outflow tract cushion epithelial-mesenchymal transition and morphogenesis. The outflow tract cushions are formed through a similar series of myocardial secretions, swelling between the myocardial and endocardial layers, and subsequent population through epithelial-mesenchymal transition. Furthermore, Smad-mediated BMP signaling is required for outflow and atrioventricular canal cushion epithelial-mesenchymal transition. However, the outflow tract cushions are also populated by a subset of cardiac neural crest-derived cells, which play a role in outflow tract cushion morphogenesis and valve formation.

Mitral Valve Prolapse

To underscore the importance of the ECM in valve development, most of the genes that have been correlated with congenital valve defects include ECM proteins, such as collagens, tenascin, and elastin. Among the valve defects, mitral valve prolapse is the most common. In this defect, excess ECM deposition within the valve leaflets causes the leaflets to thicken or prolapse into the atrium. The valve leaflet cannot close properly, leading to regurgitation. This defect is associated with mutations in fibrillin-1, elastin, and fibulin-4. Disrupted TGFβ signaling can also lead to the development of mitral valve prolapse, as observed in patients with Loeys-Dietz syndrome.

ATRIAL SEPTATION

Septation of the cardiac inflow (atrial) and outflow (arterial) poles of the heart are later stages of cardiac morphogenesis. While the poles of the heart are structurally distinct, they are both dependent on late additions of cardiac progenitors from the second heart field for septation and morphogenesis.

Atrial Septum Formation

From the second heart field, the Islet-positive/FGF10-negative posterior region gives rise to atrial myocardium. Once the common atrium has formed, a septum forms to divide the left and right atria. This septum is formed from three structures, from dorsal to ventral, the septum secundum, the septum primum, and the dorsal mesenchymal protrusion. The dorsal mesenchymal protrusion grows like a spine from dorsal to ventral along the posterior border of the common atrium. This structure is adjacent to the atrioventricular canal and participates in atrial and atrioventricular septation. The septum primum is first observed as a muscular crescent within the cranial aspect of the common atrium; this septum grows toward the atrioventricular canal. The mesenchymal cap of the septum will fuse with the cushions and detach from the cranial aspect of the atria, leaving open a fenestration known as the ostium secundum, or foramen ovale, to maintain communication between the atria. The septum secundum is a fold of the dorsal atrial myocardium, begins forming from the cranial aspect of the atria, and forms the roof of the foramen ovale. This septum will eventually fuse with the septum primum to close the foramen ovale and complete atrial septation after birth.
Atrial Septal Defects

Defects in any one of the atrial septum components can result in atrial septal defects (ASDs). ASDs therefore occur at different places in the atrial septum, with different names and pathogenesis. Defects in septum primum, the flap valve of the foramen ovale, cause ASDs of the secundum type. ¹²⁴ Defects in the septum secundum also are most likely to result in ASDs of the secundum type. ASDs of the secundum type occur in the dorsal portion of the atrial septum away from the atrioventricular canal. In contrast, defects of the dorsal mesenchymal protrusion cause ASDs of the primum type. ¹²⁴ This defect causes a deficiency of the atrial septum in the ventral portion of the atrial septum directly adjacent to the atrioventricular canal. ASDs of the primum type are therefore part of a class of atrioventricular septal defects and can include concomitant defects of the atrioventricular valves.

Human genetics has been successful in implicating several well-known cardiogenic transcription factors in ASD pathogenesis. Specifically, dominant mutations in GATA4, Nkx2.5, Tbx20, and Tbx5 can all cause atrial septal defects. ¹²⁵–¹³⁰ Loss of both copies of these essential genes results in early embryonic arrest with severe abnormalities, ¹⁹⁸,¹³¹,¹³² reflecting the early requirement of these genes in the heart field described earlier. Whereas early cardiac development appears immune to haploinsufficiency or a lack of a single copy of these transcriptional regulators, atrial septation is apparently more sensitive to transcription factor dose. The mechanistic link from transcription factor haploinsufficiency to failure of atrial septum morphogenesis has recently begun to emerge.

GATA4 and Secundum and Primum ASDs

The point mutation C839T affects the DNA-binding site of GATA4; this mutation is found in familial ASDs in combination with pulmonary stenosis. ¹²⁶ Mutations within the transactivation domain (TAD)-1 and the nuclear localization signal of GATA4 are also associated with familial ASD. ¹²⁵,¹²⁷ Missense mutations within GATA4 can affect both atrial and ventricular septation, in addition to pulmonary valve stenosis. ¹³³ Within the 3’ untranslated region of GATA4, additional mutations are also associated with ASD and atrioventricular septal defects. ¹³⁴ Furthermore, mutations in the GATA4 promoter that lead to either up- or down-regulation of GATA4 are both associated with ventricular septation defects (VSDs). ¹³⁵

Some GATA4 mutations are particularly detrimental because they disrupt interactions with other signaling pathways. Unlike the GATA4 S52F mutation, which affects the septum secundum to cause isolated ASD, the G296S and G303E mutations affect the septum primum, leading to ASD, common atrioventricular canal, and cleft mitral valve. ¹¹⁰ These mutations reduce the ability of GATA4 to interact with Smad4, the co-regulatory Smad that mediates BMP signaling. ¹¹⁰

Although GATA4 is the best-characterized GATA transcription factor in humans with congenital heart defects, studies in mice suggest that other GATA family members also have important roles. GATA3 mutations are associated with VSDs in the mouse. ¹³⁶ GATA4 and GATA6 are broadly expressed throughout the heart, and knocking out either gene singly results in early embryonic death. ²⁹,³⁰,¹³⁷ The GATA4/6 double homozygous null mouse does not form a heart at all. ¹³⁸ In contrast, GATA5 is restricted to the endocardium and endothelial cells. Mice that are doubly heterozygous for GATA4/5 or GATA5/6 show VSDs in combination with outflow tract defects. ¹³⁹ However, VSDs are not seen in isolation, ¹³⁹ suggesting that the GATA4 mutations observed in humans with isolated VSD may be the most crucial mutations from a human health perspective.

Nkx2.5 and ASDs

Dominant Nkx2.5 mutations have been associated with several forms of congenital heart defects, but the most frequent is ASDs. Among the identified mutations, many are within the homeobox domain or lead to truncated proteins. ¹²⁷,¹³⁰ Specific mutations include C568T, which is within the homeodomain, and C533T, which affects the homeodomain. ¹²⁷ The G325T mutation introduces a stop codon in exon 1 and was identified in a three-generation family in which five females had ASD with additional congenital defects (e.g., VSD, atrial fibrillation, and patent foramen ovale). ¹⁴⁰

The T-box Transcription Factors and ASDs

Heterozygous mutations in both Tbx5 and Tbx20 are associated with ASDs and VSDs. Dominant Tbx5 mutations cause Holt-Oram syndrome, which includes skeletal and heart defects. A mutation that leads to a premature stop codon (C408A) within Tbx5 has been correlated with familial ASD. ¹¹⁴ Similarly, mutations within the T-box domain (e.g., A79V and Y100C) have also been associated with ASD. ¹⁴² These patients are often affected by more than ASD, with the additional complications including pulmonary stenosis and bicuspid pulmonary valve. ¹⁴² Three additional mutations within the T-box domain (P96L, L102P, and T144I) are associated with atrioventricular septation defects and complex clinical phenotypes. ¹⁴² Similarly, mutations within the T-box domain and mutations that lead to protein truncations for Tbx20 are also associated with numerous congenital heart defects, including VSDs, ASDs, and mitral valve prolapse. ¹²⁸

The requirement for Tbx5 in atrial septation occurs in progenitor cells in the second heart field, not in the heart
itself.\textsuperscript{143} Signaling pathways such as Sonic hedgehog and Wnt signaling are also required for atrial septation.\textsuperscript{144,145} Hedgehog signaling is only present in the second heart field, and cells that receive hedgehog signaling in the second heart field migrate into the heart to form the atrial septum. These observations suggest that molecular processes in second heart field progenitor cells drive atrial septation, rather than molecular processes in the myocardium itself. Interestingly, all the genes genetically implicated in atrial septation, including GATA4 and Nkx2-5, are expressed in the second heart field.\textsuperscript{29,146} Molecular diversity generated early among cardiac progenitors is an organizer of structural cardiac morphogenesis that occurs many days later.

**ARTERIAL POLE MATURATION**

After ASDs and VSDs, arterial pole defects are the next most prevalent group of congenital heart defect.\textsuperscript{147} The arterial pole, more commonly called the outflow tract, connects the right ventricle to the pharyngeal arch arteries (in avians and mammals) or the branchial arch arteries (in fish and *Xenopus*). The arterial pole is derived from the second heart field and is added after the primitive heart tube has formed.\textsuperscript{24,148,149} The mesodermal second heart field cells are added through a combination of active migration into the outflow tract and the movement of the outflow tract caudally across the pharyngeal arches.\textsuperscript{150} The pharyngeal arches are a bilateral series of arches with mesenchymal cores. Initially bilateral arteries will form in arches 3, 4, and 6; these bilateral pharyngeal arch arteries will remodel, yielding the great arteries (i.e., the aorta and pulmonary artery), the carotid arteries, and the subclavian arteries.\textsuperscript{151}

![FIGURE 1.8](https://via.placeholder.com/150)

**Arterial Pole Septation**

In species with divided circulation, the arterial pole must be septated into the aorta and the pulmonary artery, which lead to the body and the lungs, respectively. This septation is initiated by the addition of cardiac neural crest-derived cells (Figure 1.8). These cells arise from the neuroepithelium at the border of the ectoderm, migrate through the circumpharyngeal ridge, and invade the outflow tract between the myocardial and endocardial layers.\textsuperscript{152} As the cardiac neural crest derivatives migrate into the outflow tract, they migrate in two prongs, following a spiral pattern through the outflow tract.\textsuperscript{153} At the distal end of these prongs is a ‘shelf’ that begins dividing the outflow tract into systemic and pulmonary flow. This shelf elongates into the distal outflow tract, following the spiraling prongs, and septates the distal outflow tract. The remainder of the outflow tract is septated by the ‘zippering’ of the proximal outflow tract cushions. In this proximal region, the outflow tract cushions are brought into close proximity, and the endocardium breaks down. The sub-endocardial cardiac neural crest-derived cells thus form the seam between the pulmonary and aortic outlets at this level of the outflow tract.\textsuperscript{155,156}

The spiral, in combination with apoptosis predominantly at the base of the aorta,\textsuperscript{154} helps rotate the outflow tract with respect to the ventricles. This rotation is critical for alignment of the aorta with the left ventricle and the pulmonary artery with the right ventricle.\textsuperscript{155} Recent evidence suggests that this rotation is formed in part by asymmetrical contributions from the second heart field, with Nkx2.5-positive second heart field progenitors adding preferentially to the pulmonary side of the outflow tract to help drive this side of the outflow tract more ventrally.\textsuperscript{156} The cardiac neural crest derivatives
populate distinct swellings in the outflow tract, termed the outflow tract cushions, that will remodel to form the semilunar valves of the aorta and pulmonary artery and the outflow tract septum.153

Additional Cardiac Neural Crest Contributions within the Arterial Pole

In addition to populating the pharyngeal arches and outflow tract cushions and septating the outflow tract, the cardiac neural crest derivatives also provide the smooth muscle of the great arteries and differentiate into the cardiac ganglia.157,158 This smooth muscle abuts the smooth muscle derived from the second heart field,24 and the seam between these two sources of smooth muscle is a common location of aortic dissection.150

Signaling in the Pharyngeal Arches and the Arterial Pole

Signaling pathways that are crucial for outflow tract development include Sonic hedgehog, which promotes proliferation within the second heart field progenitors,27 and BMP, which promotes myocardial differentiation once the progenitors enter the outflow tract.23,24 In addition, T-box and GATA transcription factors are also crucial in this region.139,159

Signaling between the second heart field and the migrating cardiac neural crest derivatives is essential for both populations to properly add to the outflow tract. Transcription factor Tbx3 is expressed in the pharyngeal endothelium and the cardiac neural crest derivatives.160 However, its loss is accompanied by a failure of the second heart field to add to the outflow tract, as evidenced by abnormal heart looping beginning at E9.5 in the mouse.160 Sonic hedgehog expression is reduced in the pharyngeal endoderm of the Tbx3 homozygous null embryo at E9.5,160 which would lead to reduced second heart field proliferation and thus fewer cells to migrate into the outflow tract.27 In addition, the Tbx3 homozygous null also displays reduced BMP4 expression in the outflow tract at E9.5, suggesting that myocardial differentiation is impaired for any second heart field cells that successfully migrate to the outflow tract.160 Tbx3 also has a direct effect on the pharyngeal arches, with a delay in the formation of pharyngeal arch 3 in the Tbx3 homozygous null embryo.160 Despite its expression in the cardiac neural crest derivatives, these cells deploy normally to and septate the outflow tract of the Tbx3 homozygous null embryo.160 However, the reduced contribution of the second heart field results in a shortened outflow tract and double outlet right ventricle, both with and without transposition of the arteries.160

Additional interplay is observed between Tbx1 and the TGFβ/BMP superfamily. Tbx1 can suppress BMP4 signaling via binding to Smad1 and preventing its interaction with co-activator Smad4.161 Furthermore, if BMP4 is conditionally knocked out under the control of the Tbx1 promoter, embryos display pharyngeal arch artery remodeling defects; increased apoptosis within the outflow tract cushions; and a range of arterial pole defects, including persistent truncus arteriosus and interrupted aortic arch type B.162 Additionally, Tbx1 genetically interacts with the repressor Smad7. Smad7 inhibits both branches of the TGFβ/BMP superfamily and is expressed in the outflow tract during cardiac neural crest migration.163 Tbx1;Smad7 double heterozygous mice display reduced cardiac neural crest-derived smooth muscle and decreased extracellular matrix in the pharyngeal arches, and the fourth pharyngeal arch artery fails to open in most of these embryos.163

In addition to its role in ventricular septation, GATA3 is expressed in the outflow tract and atrioventricular cushions.136 GATA3 homozygous null embryos show delayed arch artery formation, with the third arch partially forming by E9.5.136 By E10.5, the arches are hypoplastic, and the cardiac neural crest derivatives do not spiral into the outflow tract, which leads to outflow tract septation defects.136 GATA3 homozygous null embryos that survive to E15.5 display persistent truncus arteriosus (i.e., an unseptated outflow tract) or double outlet right ventricle, and both of these defects occur in combination with VSDs.136

The migratory cardiac neural crest cells are sensitive to Notch signaling, with either excessive or down-regulated levels of Notch activity in the migratory cardiac neural crest, reducing their ability to migrate into the pharyngeal arches and outflow tract cushions.164 Excessive Notch signaling results in persistent truncus arteriosus, and down-regulated Notch signaling leads to double outlet right ventricle.164 Both persistent truncus arteriosus and double outlet right ventricle result from abnormal patterning of the pharyngeal arch arteries, resulting from the absence of developing arteries that do not form cardiac neural crest-derived smooth muscle.164

DiGeorge Syndrome

The cardiac phenotype of DiGeorge syndrome (also known as 22q11.2 syndrome and velo-cardio-facial syndrome) is one of the most well-studied arterial pole anomalies observed in humans. DiGeorge syndrome includes craniofacial anomalies, thymus and parathyroid defects, and cardiac anomalies such as tetralogy of Fallot. This syndrome is widely attributed to a 3 Mb deletion on chromosome 22. However, this potential deletion can be observed in the absence of the syndrome,165 and this deletion does not account for all DiGeorge patients.166,167
Within the 3 Mb deletion, the most promising candidate gene is Tbx1. Mice lacking either one or two copies of Tbx1 and mice that lack Tbx1 expression in the pharyngeal endoderm recapitulate the DiGeorge syndrome phenotype, including cardiac anomalies such as double outlet right ventricle, persistent truncus arteriosus, and interrupted aortic arch, and these defects are more severe in the homozygous null mice. Furthermore, many of these same defects are also observed in the Tbx1-overexpressing mouse, thus supporting the hypothesis that the dosage of Tbx1 is critical for normal cardiac development. Consistent with the controversy about the relationship between the chromosomal deletion on chromosome 22 and the phenotype, however, contradictory reports also suggest that Tbx1 mutations may or may not be responsible for the phenotype. A recent analysis of 664 patients with DiGeorge syndrome including cardiac defects shows that mutations in Tbx1 were not enriched compared with patients with DiGeorge syndrome in the absence of cardiac defects.

Other transcription factors interact with Tbx1 in the pharyngeal mesoderm, including the LIM domain-containing Lhx2 and Tcf21. Lhx2 is expressed within the pharyngeal arch mesenchyme and is down-regulated in the Tbx1 homozygous-null embryo. In turn, Tcf21 is down-regulated in the Lhx2 homozygous-null embryo, setting up a hierarchy in which Tbx1 induces Lhx2, which then induces Tcf21. Tcf21 binds to the regulatory elements of Myh5 and promotes muscle differentiation in the pharyngeal arches. Both the Lhx2 and the Tcf21 homozygous null embryos display the cardiovascular phenotypes associated with DiGeorge syndrome, including tetralogy of Fallot and double outlet right ventricle.

Furthermore, canonical Wnt signaling via β-catenin within the pharyngeal mesenchyme represses Tbx1, and conditionally knocking out the gene encoding β-catenin from the mesenchyme recapitulates the DiGeorge phenotype. This phenotype is caused in part by abnormal remodeling of the pharyngeal arch arteries and a failure of the cardiac neural crest to migrate into the arches. Interestingly, FGF8, a known chemoattractant for the cardiac neural crest, is up-regulated in these conditional knockout embryos, suggesting that the cardiac neural crest provides feedback that down-regulates FGF8 expression upon entering the pharyngeal arches and that this feedback is missing in the conditional knockout embryos.

As an alternative model for DiGeorge syndrome, the conditional knockout of neuropilin-1 under the endothelial Tie2 promoter also leads to a DiGeorge-like phenotype, including persistent truncus arteriosus. Neuropilin-1 is a coreceptor for VEGF-A and acts in conjunction with theplexins and semaphorins. Additionally, VEGF regulates Tbx1. When neuropilin-1 is conditionally knocked out in endothelial cells, the pharyngeal arch arteries are abnormal, with hypoplastic and completing missing arch arteries.

Together, these studies suggest the complex DiGeorge syndrome phenotype results from an equally complex signaling network. The Tbx1 pathway is crucial for the normal development of the arterial pole, and recent studies have begun addressing how other pathways, such as Sonic hedgehog and retinoic acid, exacerbate the DiGeorge phenotype. However, further work is required to better understand the genotype/phenotype discrepancies observed in patients with the 22q11.2 deletion without the syndrome or with the syndrome but without defects in Tbx1.

**EPICARDIAL AND CORONARY VASCULAR DEVELOPMENT**

**Epicardial Formation**

The epicardium is primarily derived from the pro-epicardial organ, a Tbx18-positive cluster of cells caudal to the sinus venosus. In the mouse, cells from the pro-epicardial organ began covering the heart, starting at the sinus venosus and migrating ventrally and toward the outflow tract to cover the heart. Despite expressing Tbx18, this transcription factor is not required for addition of the pro-epicardium to the heart. In addition, the epicardium of the distal outflow tract arises from a pro-epicardial organ-like structure in the pericardium adjacent to the outflow tract. Maintenance of the epicardium layer is dependent on α4-integrin, and both mice lacking α4-integrin and chick embryos with virus-mediated α4-integrin knockdown lose their epicardial layer. The epicardial cells appear to migrate across the heart but then lose their epicardial characteristics and invade the myocardium.

As the epicardium forms, it expresses RALDH2 and secretes factors such as PDGF-A, retinoic acid, and erythropoietin toward the myocardium. In cultured NIH3T3 cells, PDGF-A has a mitogenic effect, but the myocardium does not express receptor PDGF-R2. Furthermore, retinoic acid and erythropoietin have both pro-proliferative and pro-survival effects on cardiomyocytes grown using a slice culture method.

A subset of the epicardium undergoes epithelial–mesenchymal transition and invades the sub-epicardial space. These epicardial-derived cells differentiate into interstitial and perivascular fibroblasts, coronary smooth muscle cells, and coronary endothelial cells. Although the Snail transcription factors are crucial for epithelial–mesenchymal transition in regions such as the atrioventricular and outflow...
tract cushions, their role in epicardial-mesenchymal transition is less clear. Snail1 is expressed in the epicardial layer and is downstream of the epicardial-specific protein WT-1. Although epicardial-mesenchymal transition is disrupted in a conditional WT-1 knockdown mouse (under the GATA5 promoter), epicardial-mesenchymal transition is not disrupted when Snail1 is conditionally knocked out under control of either the WT-1 promoter or the Tbx18 promoter. Thus, although WT-1 promotes epicardial-mesenchymal transition, this effect does not occur through Snail1.

Tbx18 is expressed in the pro-epicardium and the epicardium but not in epicardial-derived cells after epicardial-mesenchymal transition. When Tbx18 expression is ectopically maintained in these epicardial-derived cells, no changes in phenotype are observed. However, when an activator form of Tbx18 is expressed constitutively, the compact myocardium thins, and the coronary plexus fails to fully penetrate the right ventricle. Both phenotypes are attributed to impaired epicardial signaling, and the epicardial-derived cells prematurely differentiate into smooth muscle in these mice. Thus, Tbx18 plays a role in repressing smooth muscle differentiation of epicardial-derived cells.

**Coronary Vascular Development**

Formation of the epicardium is closely followed by the appearance of the coronary vasculature, which follows a similar spatial pattern (Figure 1.9). Discrete patches of endothelial cells are first observed near the sinus venosus, and this plexus of endothelial cells spreads ventrally and toward the outflow tract. This spread occurs over a two- to three-day span in the chick and mouse embryo, and arterial- and venous-specific markers are already apparent during this process. Only after encompassing the heart do these endothelial cells connect to the aorta to provide blood flow to the heart. After flow initiates, the coronary vasculature begins remodeling to form larger, branched vessels that are invested with smooth muscle.

The venous and arterial coronary endothelial cells appear to have different origins. The majority of the sub-epicardial venous coronary endothelial cells derive from a subset of pro-epicardial cells that migrate through the sinus venosus, and the majority of the myocardially embedded arterial coronary endothelial cells arise from the endocardium.

The development of the coronary vasculature is intimately connected with the other layers of the heart. Failure of the epicardium to cover the heart inhibits coronary plexus formation. Furthermore, the myocardium fails to undergo trabecular compaction if the coronary plexus cannot supply the heart with nutrients, leading to thin ventricular walls. The Hey2 homozygous null mouse displays collapsed coronary veins throughout the ventricular myocardium, and smooth muscle recruitment to the larger vessels is impaired; subsequently, this mouse has thin ventricular walls.

**Coronary Atresia**

The coronary vasculature is clearly required for continued development. However, an assortment of coronary artery anomalies are observed in humans. These coronary artery anomalies include missing coronary stems (i.e., coronary atresia) and misplaced coronary stems. Although coronary atresia is linked with sudden death, particularly in young athletes, no genetic causes have been identified as of yet.

**CONCLUSIONS**

Generating a complex, four-chambered heart from a mesodermal field of cells requires the coordinated actions of numerous signaling pathways and gene regulatory networks. Hundreds of genes work together to induce, specify, and differentiate the different cardiac cell types; coordinate their migration; and regulate additional behaviors such as epithelial-mesenchymal transition. A single gene may affect a number of different steps, further complicating our ability to dissect how each stage of heart development is carried out. In addition, while genetic studies in mice often rely on knocking out a gene in its entirety, humans typically present with more subtle genetic mutations, such as...
the single nucleotide polymorphisms that have been associated with ASDs. From an analysis standpoint, a global knockout provides the easiest way to quickly determine whether and where a gene is important and what developmental processes go awry in its absence. However, complex genetic mouse crosses are allowing for the analysis of heart defects in response to a range of gene expression levels (e.g., Tbx1208), and these studies are becoming more prevalent. Even these detailed studies, though, may not fully recapitulate a defect observed in the clinical setting, where the defect may arise from a mutation within the exon that changes the resulting protein or within a promoter region, resulting in a normal protein with altered expression levels.

Over the past few decades, tremendous progress has been made identifying many genes important to cardiac development, from human genetics and animal models. We now have the challenge of understanding the precise developmental requirements of these genes and how they work cooperatively to achieve complex morphogenetic events. As our understanding of the molecular underpinnings of cardiac development improves further, the next major challenge will be to relate these genes to the etiology of congenital heart defects. Furthermore, how we translate this knowledge into better therapeutic strategies to prevent or correct congenital heart defects will pose an even greater, but highly significant, challenge.

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