Molecular Regulation of Cardiomyocyte Differentiation

Sharon L. Paige, Karolina Plonowska, Adele Xu, Sean M. Wu

Abstract: The heart is the first organ to form during embryonic development. Given the complex nature of cardiac differentiation and morphogenesis, it is not surprising that some form of congenital heart disease is present in ≈1 percent of newborns. The molecular determinants of heart development have received much attention over the past several decades. This has been driven in large part by an interest in understanding the causes of congenital heart disease coupled with the potential of using knowledge from developmental biology to generate functional cells and tissues that could be used for regenerative medicine purposes. In this review, we highlight the critical signaling pathways and transcription factor networks that regulate cardiomyocyte lineage specification in both in vivo and in vitro models. Special focus will be given to epigenetic regulators that drive the commitment of cardiomyogenic cells from nascent mesoderm and their differentiation into chamber-specific myocytes, as well as regulation of myocardial trabeculation. (Circ Res. 2015;116:341-353. DOI: 10.1161/CIRCRESAHA.116.302752)

Key Words: cell differentiation ■ embryonic development ■ epigenomics ■ myocytes, cardiac ■ organogenesis ■ transcription factors

Molecular regulation of heart formation has fascinated biologists for more than a century. Driven by both the need for greater understanding of the causes of congenital heart disease and its potential in regenerative medicine, investigators in cardiac developmental biology have devoted significant efforts in recent years to address the fundamental mechanisms that underlie the complex patterning of the developing heart. In this review, we aim to summarize succinctly the critical regulators of this process. In particular, we focus on interactions between signaling molecules and transcription factor networks during cardiomyocyte lineage commitment and myocardial chamber specification. Furthermore, we highlight recent articles in this area and draw particular attention to epigenetic factors that play a role in these processes. For a detailed discussion of previous studies on this topic, we recommend several excellent reviews that address myocardial development in different model organisms, including mouse, fish, frogs, and chick.1,2 We hope this article will inform and inspire greater interest in cardiac development among investigators in cardiovascular biology and spur new collaborations between basic and clinical scientists interested in congenital heart disease.

Overview of Morphological Development

The following overview is intended to orient the reader with respect to major morphological processes that will be discussed in further detail in subsequent sections. The heart is formed from bilateral heart fields that are established during the gastrulation process (Figure 1). The heart field precursors migrate anteriorly and laterally from the primitive streak to form the anterolateral plate mesoderm that undergoes rapid migration toward the midline. Subsequent fusion at the midline, led by closure of the foregut,3 gives rise to the linear heart tube. Notably, elongation of the heart tube occurs primarily via addition of second heart field (SHF)-derived cardiomyocytes to both poles, producing an embryonic organ that consists of the truncus arteriosus most cranially followed by the bulbus cordis, ventricle, atrium, and sinus venosus. It is at this stage that the heart becomes functional as blood is pumped from the venous pole to the arterial pole.4 The heart’s ability to generate blood flow at this stage is critical to the continuation of organogenesis and to other processes in embryogenesis that respond to hemodynamic signals.5-9 The process of rightward looping occurs in conjunction with the addition of the SHF cells to both poles of the linear heart tube. Concurrently, the developing atria migrate and become cranially positioned relative to the ventricles. Complex remodeling, selective proliferation, myocardial subpopulation specification, septation, and valve development then result in the formation of the 4-chambered heart.

Specification of Cardiac Mesoderm and Formation of the Cardiac Crescent

During gastrulation, the 3 primary germ layers are formed, with mesoderm sandwiched between ventral endoderm and dorsal ectoderm. Myocardial precursor cells expressing the T-box

DOI: 10.1161/CIRCRESAHA.116.302752
transcription factor T (Brachyury) migrate from the posterior epiblast through the anterior portion of the primitive streak. These cells subsequently move laterally and, as the embryonic coelom forms, will occupy bilateral regions of lateral splanchnic mesoderm that is adjacent to underlying endoderm.10 The population of cardiac progenitor cells then converges at the midline and organizes into a crescent, with the peak of the crescent located cranially and the tails of the crescent extending caudally. Picrocadiogenic signals from underlying foregut endoderm and anteriocardiac signals from midline structures and the neural plate determine the precise size and shape of the heart fields (Figure 2).10

A cascade of transcription factor activation ultimately results in cardiac specification and differentiation. During the primitive streak stage of mouse embryonic development, the T-box transcription factor Eomesodermin (Eomes) activates the basic helix–loop–helix transcription factor mesoderm posterior 1 (Mesp1).11 Mesp1 expression at the start of gastrulation represents the first known molecular step toward cardiogenesis. Mesp1 and family member Mesp2 are the earliest markers of cardiovascular specification in the developing embryo.12 The mechanism by which Mesp1 promotes cardiac specification is multifaceted in that Mesp1 drives expression of cardiac transcription factors while repressing genes that maintain pluripotency.14 As cardiac precursors migrate away from the primitive streak to form the cardiac crescent, they downregulate Mesp1 and Mesp2 while activating other transcription factor networks that drive cardiac specification.

Among the cardiogenic factors expressed in endoderm and mesoderm are Hedgehog, bone morphogenetic proteins,15 fibroblast growth factors, and noncanonical Wnt/JNK.17 Canonical Wnt ligands, including Wnt1 and Wnt5a, secreted from the neural tube inhibit cardiac mesoderm specification,18 as do bone morphogenetic protein antagonists noggin and chordin secreted from the notochord. Canonical Wnt/β-catenin inhibitors, including Dkk-1 and crescent, are also secreted by the endoderm underlying cardiac mesoderm and serve to counteract the inhibitory Wnt signals emanating from the neural plate.19,20 Interestingly, selective elimination of canonical Wnt/β-catenin signaling in endoderm via deletion of β-catenin results in the formation of ectopic cardiac tissue in overlying mesoderm.21 The mechanism by which inhibition of Wnt/β-catenin signaling in endoderm induces cardiac specification is thought to be mediated by activation of the homeodomain transcription factor Hex in endoderm.22 Notably, Hex is associated with induction of cardiac transcription factors, such as Nkx2-5 and Tbx5, but does not directly activate expression of cardiac contractile genes, such as those encoding myofilament proteins.23

**First and Second Heart Fields**
The earliest cells to express Mesp1 form the first heart field (FHF), which makes up the cardiac crescent. A later wave of Mesp1 produces cardiac progenitors of the SHF that are derived from pharyngeal mesoderm and lie anterior and medial to the cells of the FHF.22–26 Clonal analysis in mice suggests that these progenitor cells are committed to the FHF or SHF early, likely before the onset of Mesp1 expression.27 While cells of the FHF differentiate and proliferate during the formation of the linear heart tube, the cells of the SHF retain their undifferentiated myocardial progenitor identity and become positioned dorsally relative to the heart tube.28 Lineage tracing studies in both chick and mouse embryos have demonstrated that the cells of the FHF contribute primarily to the left ventricle with small contributions to the atria, whereas the SHF will form the right ventricle, outflow tract, atria, and inflow myocardium (Figure 3).24,25,29–32

Within the SHF, progenitor cells that will give rise to the right ventricle and outflow tract are considered anterior SHF, whereas precursors of the atria and inflow tract are termed posterior SHF.33 SHF progenitors demonstrate increased proliferation and delayed differentiation compared with their FHF counterparts34 and differentiate as they contribute to both the venous and arterial poles of the linear heart tube, promoting its elongation.

The FHF cells are identified by the expression of Tbx5 and the first wave of Nkx2-5.35,36 Recently, early expression of ion channel gene HCN4 was also reported to be FHF-specific, leading to identification of the atrioventricular node and parts of the early conduction system as FHF-derived structures.37,38 Of note, early FHF-specific HCN4 expression is distinct from later expression in the cardiac conduction system and sinoatrial node.37 The SHF is marked by expression of the LIM (Lin11, Isl1, and Mec3) homeodomain transcription factor Islet1 (Isl1).31,35,39 Recently, expression of Tbx1 in the SHF was shown to coordinate contributions from the SHF to the 2 poles of the linear heart tube.40 Other genes expressed in SHF progenitor cells include Prdm1, Pitx2, Six1, Fgf8, and Fgf10.31–33,34,42 As cardiac differentiation progresses during addition of SHF cells to the linear heart tube, these genes will be downregulated as the next wave of cardiac transcription factors becomes activated, including Nkx2-5, Gata4, and Mef2c.41,42 A summary of the key transcription factor interactions that regulate FHF and SHF differentiation is shown in Figure 4.

In both human and mouse embryonic stem cell models, Isl1-expressing cells have been shown to give rise to cardiomyocytes, as well as smooth muscle cells and endothelium (Figure 3).39,43 The existence of a multipotent Isl1+ progenitor cell in vivo is postulated from the contribution of Isl1-expressing SHF to the smooth muscle cells of the great vessels (aorta and pulmonary trunk) and endothelial cells of the proximal coronary arteries. However, limited data exist to support the diversification of cardiovascular lineage cells from a single Isl1+ cell. Nevertheless, a population of Isl1+ progenitors has been identified in both mouse and human hearts in the fetal and early postnatal stages.43,44 Reportedly, these cells represent residual SHF progenitor cells, given the significant decline in the number of these cells after early fetal development and the absence of these cells in the adult heart. The potential that these cells may
participate in myocardial injury repair has motivated many investigators to examine the presence of Isll⁺ cells in the heart after infarction. Thus far, no reactivation of Isll⁺ cells or their contribution to new cardiomyocyte formation has been found.

The characteristic delayed differentiation and continued proliferation of SHF progenitors are regulated by fibroblast growth factor, Hedgehog, and canonical Wnt/β-catenin signaling pathways. Fgf8 has been identified as a necessary signaling molecule for SHF deployment as evidenced by the observation that conditional ablation of Fgf8 in mesoderm results in the formation of hearts that are without a right ventricle or outflow tract. Other fibroblast growth factor family members involved in SHF development include Fgf10⁴⁷ and Fgf3.⁴⁸ Shh is expressed in adjacent pharyngeal endoderm and is required for SHF proliferation.⁴⁹ Interestingly, Shh has been shown to activate a set of forkhead-containing transcription factors, including Foxa2, Foxc1, and Foxc2, that then activate Tbx1.⁵⁰,⁵¹ Tbx1 in turn is critical for activation of Fgf3, Fgf8, and Fgf10 and for suppression of Mef2c and serum response factor.⁴⁶,⁴⁷,⁵²–⁵⁴ Canonical Wnt/β-catenin pathways activate proliferation in SHF cells in mouse embryos and also promote the maintenance of multipotency of Isll⁺ progenitors derived from embryonic stem cells.⁴⁵,⁵⁵

Figure 1. Overview of heart development. The cardiac crescent consisting of the first heart field (FHF) and second heart field (SHF) cardiac progenitors is established during late gastrulation. The subsequent proliferation and differentiation of cells in the FHF lead to the formation of a linear heart tube, giving rise primarily to the left ventricle and a portion of the atria. The cardiac progenitors in the anterior SHF contribute to the right ventricle and the outflow tract, whereas the posterior SHF cells give rise to the atria and the inflow tract. Extension and rightward looping of the linear heart tube allow cranial positioning of the atria with respect to the ventricles. Remodeling events modulate chamber formation, septation, and valve development, resulting in the formation of the 4-chambered heart. Transcription factors that regulate each stage of heart development are listed. FHF and its derivatives are shown in orange. SHF and its derivatives are shown in blue. LA indicates left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle; and SV, sinus venosus.

Figure 2. Regulation of cardiac mesoderm specification. Shown is a cross-section of an E7.5 mouse embryo detailing the signaling pathways that regulate cardiac specification within splanchnic mesoderm. Factors secreted by the adjacent endoderm that support cardiac mesoderm specification include fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and sonic hedgehog (Shh). In addition, noncanonical Wnt ligands, such as Wnt11, expressed in splanchnic mesoderm also promote cardiac differentiation. Conversely, canonical Wnt ligands, including Wnt1, Wnt3a, and Wnt8, secreted from the overlying neuroectoderm, as well as BMP antagonists noggin and chordin secreted from the notochord inhibit cardiac mesoderm specification, thereby limiting the size of the cardiogenic fields.
Neural Crest and Proepicardial Contributions

Other cell lineages also contribute significantly to heart development alongside the FHF and SHF. Classically, the postotic neural crest has been described to give rise to a population of cells, known as the cardiac neural crest, that migrate from the neural tube in a dorsolateral fashion to reach the caudal pharyngeal arches from where they continue on to migrate into the cardiac outflow tract.56 These cells are responsible for establishing the aorticopulmonary septum, which divides the outflow tract into the aorta and pulmonary artery, and give rise to the neural tube in a dorsolateral fashion to reach the caudal pharyngeal arches from where they continue on to migrate into the cardiac outflow tract.56 These cells are responsible for establishing the aorticopulmonary septum, which divides the outflow tract into the aorta and pulmonary artery, and give rise...
to the smooth muscle cells in the tunica media of both vessels in the distal region of the arterial pole.\textsuperscript{29,57} Recently, Arima et al.\textsuperscript{34} have demonstrated that the preotic neural crest also contributes to the conotruncal region and interventricular septum. In addition, Escot et al.\textsuperscript{50} have identified stromal cell–derived factor 1 as a chemotactic agent driving the cardiac neural crest cell migration toward the pharyngeal arches. Furthermore, Holler et al.\textsuperscript{60} observed a marked reduction in the neural crest–derived cells found in the outflow tract after the loss of Hand2 expression in murine embryos. Ablation of these cells results in persistent truncus arteriosus, arterial pole alignment defects, and aberrant myocardial calcium transients stemming from elevated Fgf8 expression, which normally seems to be modulated by the cardiac neural crest cells.\textsuperscript{41} In addition, cardiac neural crest will contribute to the autonomic and sensory innervation of the heart. Anomalies involving the cardiac neural crest cells are of clinical interest because they are responsible for a multitude of human cardiocraniofacial defects, such as DiGeorge syndrome and velocardiofacial syndrome, which are associated with the loss of Tbx1 in the context of 22q11 deletion.\textsuperscript{61} The deployment of cardiac neural crest cells in a coordinated fashion and their dependence on signaling interactions SHF cells are highlighted by the requirement of Jagged-1/Notch signaling in the SHF cells to regulate Fgf8 expression that is essential for cardiac neural crest cell migration and endothelial–mesenchymal transition. The exogenous replacement of Fgf8 seems to rescue the developmental defect in endocardial cushion explant assays.\textsuperscript{63,64}

Cells of the proepicardial organ migrate to cover the surface of the developing heart. Though little is known about the molecular drivers of this migration, studies in mouse embryos suggest that the interplay between α4-integrins and vascular adhesion molecule 1 is essential in maintaining the epicardial lining after embryonic day 11.\textsuperscript{55,66} Some of these cells undergo an epithelial-to-mesenchymal transition and invade the underlying myocardium. Del Monte et al.\textsuperscript{67} have shown that Notch1 activity may play a critical role in this process by promoting blood vessel progenitor migration through the compact myocardium. These cells then differentiate into cardiac fibroblasts and vascular smooth muscle cells of the coronary vessels.\textsuperscript{68,69} Specific subpopulations from within the proepicardium may also contribute to coronary endothelium.\textsuperscript{70} Interestingly, 2 studies report the differentiation of epicardial cells into cardiomyocytes\textsuperscript{71,72}; however, significant controversies exist in this area. For additional discussion, the readers are encouraged to review articles that cover these lineages in greater detail.\textsuperscript{73–76}

**Specification of Chamber Myocardium**

The cardiomyocytes that make up the linear heart tube are termed primary myocardium on the basis of their slow proliferation rate, limited automaticity, slow conduction velocity, and poor contractility.\textsuperscript{77,78} The cardiomyocytes that make up the outflow tract, inner curvature, atrioventricular canal, and sinus horns retain this primary phenotype. As atrial and ventricular cells selectively proliferate and differentiate, ballooning of the outer curvature of the heart tube occurs. The linear heart tube’s peristaltic pumping may drive this ballooning process because endocardial and myocardial cells alter their shape, size, and proliferation in response to mechanical stress from blood flow and contraction.\textsuperscript{79–81} The atrial and ventricular cells obtain a chamber myocardium identity, with associated fast conduction velocity, increased automaticity, increased contractility, and enhanced sarcomere organization.\textsuperscript{82}

Multiple transcription factors have been shown to regulate cardiac chamber morphogenesis, including the T-box transcription factors Tbx2, Tbx3, Tbx5, and Tbx20, as well as Nkx2-5, Gata4, Cited1, Irx4, Irx1/Irx3/Irx5, and Hand1 (Figure 5). Tbx5 and Tbx20 act in combination with Nkx2-5 and Gata4 to promote specification of chamber myocardium, which includes induction of atrial natriuretic factor (encoded by Nppa), gap junction proteins connexin 40 (Cx40) and connexin 43 (Cx43), as well as the cytoskeletal protein Chisel (encoded by Smpx).\textsuperscript{4,62,83} In addition, Tbx5 cooperates with Nkx2-5 to regulate the expression of the transcriptional repressor Id2, which contributes to patterning of the right and left ventricular bundle branches.\textsuperscript{84,85} The multiple roles of Tbx5 are consistent with the varied cardiac phenotypes observed in patients with Holt–Oram syndrome.\textsuperscript{86} In contrast, Tbx2 and Tbx3 can also interact with Nkx2-5 and Gata4 to repress chamber specification.\textsuperscript{87,88} Suppression of chamber specification by Tbx3 allows for the expression of the pacemaker channel Hcn4 in cells at the sinoatrial junction that will give rise to the sinoatrial node.\textsuperscript{75,89}

Regions of chamber versus primary myocardium are exquisitely regulated by overlapping regions of these various T-box transcription factors, leading to the proposed T-box code hypothesis.\textsuperscript{1} Whereas Nkx2-5 and Gata4 are highly expressed throughout the linear heart tube, the T-box transcription factors have more narrow expression patterns.\textsuperscript{78,83} As a result of retinoic acid signaling, Tbx5 is expressed in a graded fashion with peak expression caudally in the area of the inflow tract and primitive atrium with diminished expression in the primitive ventricle and absence in the outflow tract.\textsuperscript{56,60} Tbx2 and Tbx3 are expressed in the primary myocardium of the outflow tract, inner curvature, atrioventricular canal, and inflow tract, with Tbx2 expression extending more rostrally compared with Tbx3.\textsuperscript{78} Tbx2 has been shown to inhibit proliferation and suppress expression of chamber myocardium–specific genes, including Cx40, Cx43, Smpx, and Nppa.\textsuperscript{87,89} Notably, Tbx20 is broadly expressed throughout the linear heart tube, and deletion of Tbx20 in mice results in defects in chamber development, at least in part because of expansion of Tbx2 expression (Figure 5).\textsuperscript{72–75}

Within the chamber myocardium, each of the 4 chambers is marked by unique gene expression patterns. The mouse atria and ventricles express myosin light chain isoforms Mlc2a and Mlc2v, respectively.\textsuperscript{96} Both ventricles additionally express the homeobox gene Irx4, which activates ventricular myosin heavy chain 1 and suppresses the atrial myosin heavy chain 1 isoform in chick.\textsuperscript{88} In both mouse and chick, Tbx5 expression is absent in the right ventricle.\textsuperscript{88} In chick, the right ventricle is also distinguished by Tbx20 expression, which the left ventricle lacks.\textsuperscript{88} The basic helix–loop–helix transcription factors eHAND and dHAND are expressed in the systemic and pulmonary ventricles, respectively, even in situs inversus.
Figure 5. Specification of chamber myocardium. The specification of cells to the chamber myocardium is modulated by Tbx5 and Tbx20 in tandem with more broadly expressed factors Nkx2-5 and Gata4. Tbx2 and Tbx3 suppress the expression of chamber myocardium-specific genes, resulting in low proliferation rate, slow conduction velocity, and poor contractility characteristic to the primary myocardium. The primary myocardium phenotype becomes restricted to the atrioventricular canal (AVC) and proximal outflow tract (p-OFT). d-OFT indicates distal outflow tract; LA, left atrium; LV, left ventricle; and RV, right ventricle.

mouse embryos. Expression of the homeobox gene Pitx2 in the left atrium suppresses Shox2, thereby inhibiting genes associated with sinoatrial node development and preventing ectopic pacemaker specification.

### Trabeculation of the Ventricular Myocardium

Trabeculation and subsequent compaction of the ventricular myocardium facilitate septation, confer greater contractility and conductivity, and help establish the coronary circulation system in the developing heart. Paracrine signaling between the endocardium and the maturing myocardium is an essential driver for the formation of the organized muscular ridges lining the ventricular wall (Figure 6), a process initiated toward the end of cardiac looping.

Central to endocardium-to-myocardium regulation of trabeculation is the transmembrane receptor Notch1. When the extracellular domain of Notch1 binds to ligands, such as Delta4 and Jagged, proteolytic cleavage releases the Notch1 intracellular domain into the cytoplasm. Notch1 intracellular domain then associates with its transcriptional cofactor recombining binding protein suppressor and translocates to the nucleus. Notch1 intracellular domain/recombining binding protein suppressor upregulates the myocardial expression of Bmp10, which stimulates proliferation by decreasing the activity of cell cycle inhibitor P57 and by up-regulating Tbx20 and Hey2 activity. Because Notch1 is predominantly expressed in endocardial cells lining the base of trabeculae at E9.0, Notch activity therefore promotes the growth of the trabecular myocardium. Endothelial-specific ablation of a Notch1 intracellular domain inhibitor, Fkbp1a, accordingly results in hypertrabeculation. Curiously, inactivation of Mib1, an ubiquinase that promotes ligand binding and subsequent activation of Notch1, in the myocardium also leads to hypertrabeculation. In addition, Mib1-deficient mice have an abnormally thin compact myocardial zone. The apparently contradictory effects of Notch1 regulator manipulation suggest that Notch1 plays a complex role in spatio-temporal regulation of trabecular growth.

Notch1 signaling also promotes neuregulin-1 (Nrg1) activity through EphpinB2. Notch1, 1 of the endothelial growth factors produced by the endocardium, binds to myocardial tyrosine kinase receptor ErbB4 and leads to its dimerization with ErbB2, thus activating signaling cascades modulating the cell growth and migration. Nrg1, ErbB4, and ErbB2 null mice die in utero around E9.5 to 10.5 because of embryonic heart failure, with histological analyses revealing ventricular chambers lacking trabeculae. Liu et al have identified ErbB2 as a dual factor in cardiomyocyte proliferation and directional delamination during trabecular establishment. Their assessment of the cardiac function in ErbB2 mutants revealed decreased ventricular cardiac contractility and bradycardia, supporting previous reports that Nrg1/ErbB2/4 modulation of trabeculae formation may play an important physiological role in cardiac contractility. In addition, high-resolution imaging of cardiomyocytes in zebrafish embryos suggests that ErbB2 contributes to robust formation of cardiomyocyte surface protrusions and contacts between non-neighboring cardiomyocytes, which may be required for initiating trabeculation.

Intriguingly, EphrinB2 expression is altered by shear stress in embryonic stem cells in vitro, suggesting that trabeculation may simultaneously contribute to cardiac contractility while responding to the consequential increase in blood flow. This interdependence between cardiogenesis, blood flow, and other embryonic processes presents challenges for experimental design.

Growth factor signaling from the myocardium to the endocardium also plays an essential role in sustaining trabeculation. Mice that lack expression of angiopoietin-1, the primary ligand produced in the myocardium for the endocardial Tie2 receptor, display ventricular morphology similar to that of the Nrg1/ErbB4 mutants with further aberrant phenotypes, such as defective angiogenesis, a less intricately folded immature endocardium, and embryonic lethality at E12.5. Vascular endothelial growth factor results in an attenuated compact zone and intrusions and contacts between non-neighboring cardiomyocytes, which may be required for initiating trabeculation.

Conversely, overexpression of vascular endothelial growth factor results in an attenuated compact zone and hypertrabeculation. The modes of communication between the endocardium and myocardium may be moderated or complemented by the cardiac jelly, the extracellular matrix between the 2 layers. Beginning at E7.5 in the normal embryo, expression of hyaluronic acid synthase-2 (Has2) contributes to the formation of
During E12.5 to E14.5, ADAMTS1 is highly expressed and eliminates cardiac jelly and terminates trabeculation. During this time, ADAMTS1 is facilitated by fibulin-1, another extracellular matrix protein that binds both ADAMTS1 and versican, and global deletion of ADAMTS1 or fibulin-1 results in hypertrabeculation. Endocardial-specific loss around E9.0 of Brg1, an ADAMTS1 inhibitor, allows ADAMTS1 to prematurely thin the cardiac jelly and terminates trabeculation.

The development of the cardiac conduction system provides an additional example of cardiac jelly–mediated endocardium-to-myocardium signaling in relation to trabeculation. Recent work suggests that endocardial signals promote Cx40 expression in the myocardium and that the cardiac jelly reduces the intensity of these molecular signals. This model is supported by the observation that the atrioventricular junction, where the endocardium and myocardium are separated by relatively thick cardiac jelly during valve formation, has low Cx40 expression and delayed conduction through the atrioventricular node. Endocardial promotion of connexin expression may also explain why the peripheral ventricular conduction system, marked by Cx40 in the mature heart, forms from trabecular cardiomyocytes closest to the endocardium.

To further support the interdependence of trabeculation and cardiac conduction system development, 3 recent reports have identified mutations in either ion channel HCN4 or ryanodine receptor RYR2 in comorbid cases of left ventricular noncompaction and certain familial arrhythmias. Whereas earlier surveys of left ventricular noncompaction cardiomyopathy patients suggested that the noncompact phenotype was arrhythmogenic, these reports motivate further investigation into the mechanisms by which HCN4 and RYR2, genes previously associated with arrhythmic disorders, may also perturb myocardial structure.

Epigenetic Regulation of Heart Development

Acting in concert with signaling pathways and transcription factors, several epigenetic factors play a critical role in modulating cardiac lineage specification and cardiac morphogenesis during development. Included in the category of epigenetic regulators are histone modifications, adenosine triphosphate (ATP)-dependent chromatin remodeling complexes, and DNA methylation. Each of these regulatory modes influences gene expression by modulating the accessibility of regulatory DNA sequences to DNA-binding proteins, such as transcription factors. As a result, epigenetic regulation adds an additional layer of complexity to the tight temporal and spatial control of cardiac gene expression during development.

Covalent modifications to histone proteins alter DNA–histone interactions, resulting in the establishment of looser or more accessible versus tighter or more restricted chromatin. Histone acetylation primarily promotes chromatin accessibility, thereby leading to transcriptional activation. The specific role of histone acetyltransferases in heart development is still unclear. However, mice that carry a global loss-of-function mutation in the histone acetyltransferase gene p300 die between E12.5 and E15.5, possibly because of the development of severe heart defects. Interestingly, this effect may be because of acetylation and activation of Gata4 by p300. In addition, mice globally lacking the histone acetyltransferase Moz show aortic arch abnormalities and ventricular septal defects similar to those found in 22q11 deletion syndrome because of decreased acetylation and therefore transcription of Tbx1. In contrast to histone acetyltransferases, histone deacetylases remove acetyl groups from histones, resulting in the formation of condensed chromatin and transcriptional repression. Cardiac-specific loss of either Hdac1 or HDac2 has
no significant effect, whereas double mutants lacking both Hdac1 and Hdac2 in myocardium die in the neonatal period because of cardiac arrhythmias and dilated cardiomyopathy.\textsuperscript{143} The deacetylase activity of these epigenetic factors may not be limited to histone modification. For example, Hdac2 is thought to limit myocardial proliferation via a partnership with Hopx (homeodomain-only protein) that results in deacetylation of Gata4, thereby decreasing its transcriptional activity.\textsuperscript{144,145} Similarly, Hdac3 may suppress Tbx5 activity by altering acetylation of Tbx5 residues.\textsuperscript{146}

In contrast to histone acetylation, the effect of histone methylation is highly residue-specific, with methylation of histone H3 at lysines 4, 36, and 79 leading to gene activation, whereas methylation at lysines 9 and 27 generally results in gene silencing. Both histone methyltransferases and demethylases have been shown to play critical roles in cardiac development, and de novo mutations in genes associated with regulation of histone H3 methylation at lysines 4 and lysine 27 have been detected in excess in children with nonfamilial congenital heart disease.\textsuperscript{147} Loss of the histone methyltransferase Smyd1 results in right ventricular hypoplasia, possibly because of diminished expression of key cardiac transcription factors Hand2 and Irx4.\textsuperscript{148} Jarid2, a member of the Jumonji family of histone demethylases, is a key regulator of cardiac trabeculation (discussed in the preceding section) via repression of endocardial Notch and Nrg1.\textsuperscript{149}

ATP-dependent chromatin remodeling complexes harness energy from ATP hydrolysis to alter nucleosome packaging and thus DNA accessibility.\textsuperscript{140} Of the 4 families of ATP-dependent chromatin remodelers, the family best studied in cardiac development is the switching defective/sucrose nonfermenting family. The Brg1/BRM-associated factor complex contains 12 subunits, including the ATPase subunit encoded by either brahma (Brm) or brahma-related gene 1 (Brg1).\textsuperscript{150} Brg1 has been shown to genetically interact with several cardiac transcription factors, including Nkx2-5, Gata4, Tbx5, and Tbx20.\textsuperscript{151,152} Mice that are globally haploinsufficient for Brg1 show a wide range of mild cardiac defects, including mild septal defects and aberrant conduction. Interestingly, mice with both haploinsufficiency for Brg1, as well as haploinsufficiency for Nkx2-5, Tbx5, or Tbx20, show more severe and often lethal cardiac defects than single mutants alone.\textsuperscript{153-155} Brg1 also promotes myocardial proliferation through stimulation of Bmp10, and the thin myocardium phenotype observed in the absence of Brg1 can be rescued with Bmp10.\textsuperscript{156} Also of note, murine Brg1 activates fetal β-myosin heavy chain while repressing adult α-myosin heavy chain, thereby maintaining a fetal phenotype during development.\textsuperscript{157} Finally, perhaps the most striking evidence demonstrating a central role for chromatin remodeling in cardiomyocyte lineage specification is the observation that overexpression of Tbx5, Gata4, and the Brg1/BRM-associated factor subunit Baf60c can promote transdifferentiation of noncardiac mesoderm into cardiac tissue.\textsuperscript{157}

DNA methylation involves the addition of a methyl group to cytosine in CpG islands, which are segments of DNA with a high frequency of cytosine followed immediately by guanine. Notably, DNA methylation patterns are wiped clean at the time of conception and are gradually reintroduced during embryogenesis. Recent genome-wide analysis of DNA methylation patterns in developing mouse hearts demonstrated overall stability in the amount of DNA methylation between E11.5 and E14.5.\textsuperscript{158} However, <1% of methylation sites did show differential methylation patterns between the 2 time-points, of which about two-thirds were more highly methylated at E14.5.\textsuperscript{158} Cardiac regulatory genes were highly enriched in this subset, including signaling molecules such as Wnt2 and Fgf2, as well as transcription factors such as Gata6 and Mef2c.\textsuperscript{158} Among those sites with the greatest amount of differential methylation, half also showed corresponding changes in the expression of the associated gene.\textsuperscript{158}

Summary

As our understanding of the molecular regulation of cardiac development continues to improve, so will our ability to clarify the determinants of congenital heart disease and our capacity to generate cells for regenerative medicine therapies. In recent years, the identification of key transcription factors and their requirement in distinct stages of cardiomyogenesis has helped lay the groundwork for determining the precise molecular mechanisms involved in commitment of cardiac progenitor cells from their mesodermal precursors, chamber-specific cardiomyocytes from heart field progenitors, and trabecular versus compact myocardium from chamber myocytes. Furthermore, the elucidation of critical epigenetic modifiers that regulate expression of cardiac genes in a spatiotemporal manner has helped reveal essential mechanisms that modify chromatin at specific locations for transcription factors to execute their designated roles during development.

Future Perspectives

To realize the tremendous potential made by our cumulative investment in cardiac development biology in recent decades to identify the pathogenic mechanisms of congenital heart diseases, such as hypoplastic left heart syndrome, transposition of great vessels, double outlet right ventricle, and ventricular noncompaction, further clarification is needed on several key unresolved issues. For example, with regards to the understanding of heart field precursor commitment, we need greater insight into the differences in epigenetic regulation between FHF and SHF cells because they may explain the differences in the response to failure between the right and the left ventricle. Specifically, the lack of requirement for Isil expression in FHF cells and the differential effects of fibroblast growth factor and bone morphogenetic protein signaling in SHF cells suggest that the biology of right ventricular cardiomyocytes at the single-cell level may be different from those in the left ventricle.

In the formation of chamber-specific cardiomyocytes, the distinct expression of T-box and Hand transcription factors along the linear and looping heart tube suggests differences in epigenetic regulation between atrial versus ventricular, as well as right versus left ventricular myocyte development. It would be of great value to elucidate the gene expression and epigenetic profile of all chamber myocyte precursors during the linear heart tube stage of development at the single-cell level. This level of resolution could help us determine the key regulators at the earliest steps of chamber myocyte formation and their link to chamber-specific malformations.
Finally, in the context of trabecular versus compact myocardium formation for chamber-specific myocytes, we need a greater understanding of the role of specific signaling pathway and biomechanical influences on myocardial maturation. Identifying the answers to these questions may hold the key to our ability to accurately predict and diagnose congenital heart diseases and treat them in utero using novel interventional or molecular therapies. Indeed, one of the most significant barriers to translate pluripotent stem cell–derived cardiomyocytes into regenerative therapies is the inability of these cells to reach full maturity both in vitro and in vivo after transplantation. Despite significant efforts to enhance the maturation of these cells by pacing, stretching, and coculturing with nonmyocytes, they remain largely immature. A better understanding of the key factors that regulate normal cardiomyocyte maturation during embryonic development should help guide, conceptually, the designing of new approaches to enhance the maturation of pluripotent stem cell–derived cardiomyocytes in vitro. If we are successful in this endeavor, the potential for screening and discovering new drugs using pluripotent stem cell–based disease models to treat defective signaling pathways in congenital heart disease could be significantly increased.

In summary, we think that although major strides have been made in recent years to unravel the molecular underpinnings of cardiomyogenic lineage commitment, expansion, and maturation, there remain significant gaps in our knowledge that await further investigation. With the continuous advancements in stem cell and genome-editing technology, as well as detailed mechanistic analysis using model organisms, we shall be able to identify and pinpoint key regulators of cardiomyogenesis and devise approaches to correct their functional deficiency before the development of disease.

Acknowledgments

We apologize to colleagues whose work we could not cite in this brief review article. We thank Dr Anthony Sturzu for article critique. This article was funded in part by the National Institutes of Health (NIH)/National Heart, Lung, and Blood Institute Progenitor Cell Research Institute at Stanford to S.M. Wu.

Disclosures

None.

References

350 Circulation Research

January 16, 2015


Wiese C, Clout DE, Papaioannou VE, Brown NA, Harvey RP, Moorman AF, etc.


Molecular Regulation of Cardiomyocyte Differentiation
Sharon L. Paige, Karolina Plonowska, Adele Xu and Sean M. Wu

Circ Res. 2015;116:341-353
doi: 10.1161/CIRCRESAHA.116.302752
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/116/2/341

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at: http://circres.ahajournals.org/subscriptions/