This Review is part of a thematic series on Genetics of Cardiovascular Development, which includes the following articles:

Transcriptional Regulation of Vertebrate Cardiac Morphogenesis

Early Signals in Cardiac Development
Morphology and Morphogenesis
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Development of Specialized Cells Within the Heart
Development Gone Awry: Congenital Heart Disease

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Transcriptional Regulation of Vertebrate Cardiac Morphogenesis

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Abstract—Transcription factors can regulate the expression of other genes in a tissue-specific and quantitative manner and are thus major regulators of embryonic developmental processes. Several transcription factors that regulate cardiac genes specifically have been described, and the recent discovery that dominant inherited transcription factor mutations cause congenital heart defects in humans has brought direct medical relevance to the study of cardiac transcription factors in heart development. Although this field of study is extensive, several major gaps in our knowledge of the transcriptional control of heart development still exist. This review will concentrate on recent developments in the field of cardiac transcription factors and their roles in heart formation. (Circ Res. 2002;90:509-519.)

Key Words: transcription factors • gene expression • heart development • embryo • congenital heart defects

The development of the vertebrate heart can be considered an additive process, in which additional layers of complexity have been added throughout the evolution of a simple structure (linear heart tube) in the form of modular elements (atria, ventricles, septa, and valves).1–3 Each modular element confers an added capacity to the vertebrate heart and can be identified as individual structures patterned in a precise manner. An understanding of the individual modular steps in cardiac morphogenesis is particularly relevant to congenital heart disease, which usually involves defects in specific structural components of the developing heart. Organ formation requires the precise integration of cell type–specific gene expression and morphological development; both are intertwined in their regulation by transcription factors. Although many transcription factors have been described as regulators of cardiac-specific gene expression, the transcriptional regulation of cardiac morphogenesis is still not well explored. For a transcription factor to be considered directly involved in heart development, it must be expressed in developing heart tissues and exert an influence on processes that impact the morphogenesis of the developing heart. The present review will concentrate on recent developments in the field of cardiac transcription factors and their roles in heart formation, which are summarized in Figure 1.

Transcriptional Regulation of Major Morphogenetic Events in Heart Development

Cardiogenesis and Cardiac Differentiation

The initiation of cardiac differentiation has been the topic of vigorous investigation.1,3 However, no single transcription factor that is responsible for the differentiation of lateral plate mesoderm into cardiac cells has been identified. Much excitement was generated on the discovery of the tinman
gene in the fruit fly *Drosophila melanogaster*, which in this organism is required, although not sufficient, for the generation of cardiac cells.\(^4\) The *tinman* gene encodes an NK-class homeodomain-containing transcription factor; the discovery of similar molecules in vertebrates, notably, the predomi-
nantly cardiac gene Nksx2-5 (also known as *Csx*), has added to the appealing notion of a cardiogenic transcription factor.\(^4,5\)

Generation of mice lacking Nksx2-5 did not result in mice lacking a heart but, instead, pointed to important roles for this transcription factor in the differentiation and morphogenesis of the early developing heart.\(^6,7\) In mice, Nksx2-5 is required for an aspect of terminal differentiation of cardiac myocytes that includes, in large part, the establishment or maintenance of these processes are listed below each stage. See text for further

details. ao indicates aorta; a, atrium; la, left atrium; lv, left

ventricle; ra, right atrium; rv, right ventricle; ol, outflow tract; sv, sinus venosus; and pa, pulmonary artery.

**Figure 1.** Summary of mouse heart development. Five major stages of heart development are shown: (1) cardiac crescent formation at embryonic day (E) 7.5; (2) formation of the linear heart tube at E8; (3) looping and the initiation of chamber morphogenesis at E8.5 to E9.5; (4) chamber formation; and (5) chamber maturation and septation and valve formation. The transcription factors involved or suspected of involvement in these processes are listed below each stage. See text for further details. ao indicates aorta; a, atrium; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle; ol, outflow tract; sv, sinus venosus; and pa, pulmonary artery.

replacement studies have shown that vertebrate NK proteins cannot functionally replace tinman function in cardiogenesis in *Drosophila*, although their homeodomains are functionally interchangeable.\(^15,16\) Therefore, divergence of gene function has occurred in the NK family of transcription factors, resulting in roles that are distinct and perhaps more specific than their *Drosophila* counterparts.

Myocardin is a cardiac-specific transcription factor that may be important for the early differentiation of cardiac cells.\(^17\) Powerful in vitro activation of several cardiac gene promoters via serum response factor (SRF)-binding sites has been identified as a major function of myocardin. Experiments in frog embryos with the use of a dominant-negative myocardin molecule indicate that it may be necessary for early stages of cardiac differentiation, including high-level expression of Nksx2-5.\(^17\) These data point to an important role for myocardin in activating early cardiac gene expression, but the mechanism of its promoter specificity and its endogenous in vivo role remain to be defined.

Beyond the initiation of cardiogenesis, the progressive differentiation of precardiac cells is also under a cardiac-restricted transcriptional program. The GATA family of zinc finger–containing transcription factors appears to be potentially critical in this regard. Three GATA family genes have been identified as being expressed in the developing heart: gata4, gata5, and gata6.\(^18\) Gene deletion in the mouse has not been as informative as one would have predicted, mainly because of the roles of GATA factors outside the heart in early embryogenesis and perhaps because of genetic redundancy.\(^19–22\) The early endodermal defect in gata4 knockouts and the peri-implantation lethality of gata6 knockouts preclude analysis of the role of these GATA factors in further steps in myocardial differentiation, and gata5-deficient mice have no cardiac phenotype. Nevertheless, GATA family members have been implicated as key regulators of cardiogenesis in several model systems.\(^23–29\) In P19 embryonal carcinoma cells induced to differentiate into cardiocytes, gata4 or gata6 antisense oligonucleotides cause an arrest in cardiac differentiation and apoptotic death,\(^23,24,27\) and the potential of gata4-null (gata4\(^{-/-}\)) embryonic stem cells to undergo cardiac differentiation is reduced.\(^25\) The gata4\(^{-/-}\) embryonic stem cells can contribute to embryonic hearts in chimeric mice, but it not clear whether these cells are normally differentiated or have normal function.\(^25\) Furthermore, the presence of GATA5 and GATA6 in these cells may partially compensate for the lack of GATA4.\(^27,29\) GATA factor function has been well conserved throughout evolution: in *Drosophila*, the gata4 homologue pannier is required for normal proliferation of cardiogenic precursors,\(^28\) and in zebrafish, the *faust* mutation, in which cardi bifida and impaired cardiac differentiation occurs, is caused by mutations in GATA5.\(^29\)

**Migration of Cardiac Precursors**

A conserved transcriptional pathway involving GATA family zinc-finger transcription factors is important in the move-
ments of the paired progenitor pools that coalesce to form the linear heart tube.\(^19,20,29\) The involvement of GATA4 (in mouse) and GATA5 (in zebrafish) in this respect is primarily
in controlling normal formation of the endoderm underlying the myocardial precursors. In the situation in which GATA4/5 function is reduced, endodermal cells do not normally differentiate, their ventral migration is inhibited, and this prevents the concomitant movement of myocardial cells, leading to cardiomyopathy. A mix-like paired-class homeodomain transcription factor has also been described as being responsible for the impaired endodermal differentiation observed in the zebrafish bone-and-clyde (bon) cardiomyopathy mutants. 30

The basic helix-loop-helix (bHLH) transcription factor MesP1 has been shown to be required for an earlier step in the migration of cardiac precursors. In the absence of MesP1, mesodermal cells fated to become cardiac myocytes fail to migrate normally out of the primitive streak during gastrulation and, consequently, fall behind the morphogenetic movements of the rest of the embryo, resulting in complete or partial cardiomyopathy. 31 Mice lacking both MesP1 and its related gene, MesP2, have a complete block in migration (and perhaps differentiation) of the mesoderm from the primitive streak, resulting in a complete lack of cardiac and other mesodermal derivatives. 32 Interestingly, analysis of chimeric embryos created with the use of MesP1/MesP2 double-knockout embryonic stem cells reveals a specific cell-autonomous role for these transcription factors in ventricular, but not atrial, formation. This suggests an early lineage difference between atrial and ventricular cardiac precursors, as has been shown by lineage-tracing experiments in zebrafish 33 and chick embryos. 34, 35

**Regulation of Chamber-Specific Gene Expression**

Various promoter elements have been identified that restrict the expression of genes to the atrial or ventricular compartments of transgenic mouse hearts or to other subdivisions of the developing heart. However, the transcription factors responsible for the anatomic restriction of these control elements have not been identified; therefore, very few regulators of chamber-specific gene expression have been identified to date. Each chamber has specific biochemical and physiological properties that are important for heart function; thus, establishing and maintaining chamber-specific gene expression is crucial for heart formation and function.

Iroquois homeobox gene 4 (Irx4) is a member of the Iroquois family of homeodomain-containing transcription factor genes, which have been implicated in patterning events in *Drosophila*. Expression of *Irx4* is restricted at all stages of development to the ventricular myocardium in all species examined, 8, 36, 37 although it is more concentrated in the outer curvature of the myocardium, which will give rise to the ballooning chambers. 38 *Irx4* expression is reduced in mice lacking Nkx2-5 or *dHand*, in which ventricular differentiation is compromised. 8 However, *Irx4* expression is unaffected in *Mef2c- or Rxrα-deficient mice, which also have defective ventricular differentiation, indicating specificity of the regulation of *Irx4* by Nkx2-5 and *dHand*. 8

Experiments in avian embryos have demonstrated that Irx4 is involved in the positive and negative regulation of chamber-specific myosin heavy chain gene expression in the ventricular myocardium. 36, 39 Irx4 does not appear to be a global regulator of ventricle-specific gene expression, inasmuch as mice with a targeted disruption of *Irx4* have only a partial disturbance of ventricle-specific gene expression, including decreased *eHand* expression in the embryonic heart and atrial natriuretic factor (ANF) derepression in the ventricles after birth. 40 The expression of additional Irx genes in the developing heart suggests the possibility of genetic redundancy. 40, 41 However, the analysis of the atrium-specific *slow myosin heavy chain 3 (SmHC3)* promoter, which is derepressed in *Irx4*–/– embryonic ventricles, shows that Irx4 does play a role in chamber-specific gene expression in the ventricles. 40 *SmHC3* is the quail homologue of the chicken *AMHC1* gene, which is repressed by Irx4 in chicken ventricular myocardium. 36 The regulatory elements of the *SmHC3* gene are functional in quail and mouse 42–44 and, in both species, are derepressed in the ventricles in the absence of Irx4. 36, 39, 40 Therefore, the transcriptional elements controlling the chamber specificity of this promoter are functional in mammals and are under the control of Irx4. This implies that endogenous mammalian chamber-specific genes may also be under the control of Irx4, but additional factors add complexity and redundancy to this regulation. In fact, Irx4 does not bind directly to the *SmHC3* promoter elements required for ventricular repression and, instead, may act via interaction with a retinoic acid receptor (RAR)/vitamin D receptor complex of proteins. 39 *Irx4*-deficient mice have impaired cardiac function and develop cardiomyopathy, 40 underscoring the importance of maintaining normal chamber-specific gene expression for proper cardiac function.

*Hey2* (also known as HRT2, CHF1, and Hesr1) is a ventricle-specific transcription factor related to the Hairy family of bHLH transcription factors. 45–48 On the basis of its expression pattern and its potential as a transducer of signals downstream from notch signaling, *Hey2* is an attractive candidate for being a regulator of ventricle-specific morphogenesis or gene expression. *Hey2* was also cloned as the gene mutated in gridlock, a zebrafish mutant that has aortic coarctation. 49 Mice lacking *Hey2* do not have aortic coarctation, but they develop a severe postnatal cardiomyopathy (M. Gessler, PhD, written communication, October 2001).

**Regulation of Chamber Morphogenesis**

As with chamber-specific gene expression, each chamber of the heart arises independently from an initially genetically patterned but morphologically primitive early heart tube. The expression patterns and roles of the bHLH transcription factors *dHand* (Hand2) and *eHand* (Hand1) and the T-box transcription factor *Tbx5* illustrate the correlation between chamber-restricted roles for factors expressed in a specific domain of the developing heart.

*dHand* and *eHand* exhibit complementary patterns of expression in the developing mouse heart, with *dHand* more strongly expressed in the right (pulmonary) ventricle (RV) than in the left (systemic) ventricle (LV) and *eHand* predominately restricted to the LV. This restriction of expression follows a very dynamic expression pattern throughout various segments of the early linear and looping heart tube. 50–53 In mammals, both *Hand* genes appear to be required for normal growth of the developing myocardium of the chamber in...
which they are restricted: the LV, in the case of eHand, and the RV, in the case of dHand. The role of eHand in LV development is not clear except for the extraembryonic lethality caused by extraembryonic deletion. The RV and outflow tract are initially formed in dHand-null embryos, but subsequently, the RV precursor undergoes dramatic apoptosis, impairing the expansion of this segment and resulting in the absence of the RV. The chamber restriction of Hand gene function is not conserved in the chicken heart.

Tbx5 is expressed initially throughout the cardiac mesoderm in its earliest stages, but its expression pattern is rapidly refined, first as a posterior-anterior gradient in the linear heart tube, until mid gestation, when it is restricted to the atria and LV. Tbx5 mRNA levels decrease in the LV during subsequent stages of development, such that by late gestation and adulthood, low levels of Tbx5 transcripts can be detected equivalently in both LV and RV in mice and humans (Hatcher et al. and author’s unpublished data, 2001). Lack of Tbx5 results in severely hypoplastic atria and LV, with RV and outflow tract growth remaining intact. Overall, ventricular differentiation is impaired in Tbx5-deficient embryos, including decreased expression of the ventricle-specific genes Mlc2v, Irx4, and Hey2. This is perhaps due to early pleiotropic effects of the absence of Tbx5 on cardiac differentiation, including decreased Gata4 and Nkx2-5 expression. Consistent with these observations, Tbx5 has been shown to accelerate cardiac differentiation of P19C16 cell lines, including increased Nkx2-5 expression, and inhibition of Tbx5 in Xenopus embryos also leads to hypoplasia of cardiac tissues and decreased Nkx2-5 mRNA levels.

Transgenic overexpression/misexpression of Tbx5 throughout developing mouse, chicken, or Xenopus hearts results in thinned and hypoproliferative ventricular myocardium, which is somewhat reminiscent of what is observed in Tbx5-deficient embryos, albeit less dramatic. These apparently conflicting results compare favorably with parallel observations obtained with another T-box factor, Tbx1, and apparently conflicting results compare favorably with parallel experiments should be interpreted with caution, and more refined, first as a posterior-anterior gradient in the linear heart tube, until mid gestation, when it is restricted to the atria and LV. Tbx5 mRNA levels decrease in the LV during subsequent stages of development, such that by late gestation and adulthood, low levels of Tbx5 transcripts can be detected equivalently in both LV and RV in mice and humans (Hatcher et al. and author’s unpublished data, 2001). Lack of Tbx5 results in severely hypoplastic atria and LV, with RV and outflow tract growth remaining intact. Overall, ventricular differentiation is impaired in Tbx5-deficient embryos, including decreased expression of the ventricle-specific genes Mlc2v, Irx4, and Hey2. This is perhaps due to early pleiotropic effects of the absence of Tbx5 on cardiac differentiation, including decreased Gata4 and Nkx2-5 expression. Consistent with these observations, Tbx5 has been shown to accelerate cardiac differentiation of P19C16 cell lines, including increased Nkx2-5 expression, and inhibition of Tbx5 in Xenopus embryos also leads to hypoplasia of cardiac tissues and decreased Nkx2-5 mRNA levels.

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In lower vertebrates, such as frogs and fish, a single cardiac Hand gene exists and is responsible for the morphogenesis of the single ventricle found in these species. Similarly, Tbx5 is expressed throughout the single ventricle of frogs and fish and appears to be involved in the morphogenesis of the entire heart in these species. This implies that with the addition of pulmonary circulation and the acquisition of an RV chamber, duplication and specialization of Hand and Tbx5 gene expression and function occurred to regulate chamber-specific morphogenesis. This was presumably accompanied by restricted expression of these genes to specific segments of the developing heart.

A perhaps unexpectedly specific role for the MADS-box transcription factor Mef2c in ventricular growth has been elucidated from the generation of mice lacking Mef2c, in which cardiogenesis is initiated but in which the segments of the heart corresponding to both LV and RV are severely hypoplastic. However, ventricle-specific gene expression appears normal in Mef2c−/− embryos. The orphan nuclear receptor transcription factor Coup-tfII is important for the normal growth of the atrial and sinus venosa precursors, although this is perhaps secondary to its role in endocardial differentiation.

Chamber Maturation and Septation
Maturation of the heart into fully functional trabeculated chambers and septation of the atria and ventricles from one another and between their left and right sides are important processes that require precise integration of growth and differentiation signals. Defects in these processes account for the majority of congenital heart malformations in humans, including atrial and ventricular septal defects (ASDs and VSDs, respectively); tetralogy of Fallot (TOF), common atrioventricular canal, and double-outlet right ventricle. Genetic analysis of inherited cardiac septation defects has shown that dosage-sensitive redeployment or sustained function of transcription factors required for early cardiogenesis (e.g., Nkx2-5 and Tbx5) is a major factor in septal morphogenesis.

Dominant mutations in NKX2-5 have been found in patients with ASDs, VSDs, TOF, and Ebstein’s anomaly of the tricuspid valve, often accompanied by conduction disease. Nkx2-5 haploinsufficiency appears to be the major mechanism for these defects, on the basis of biochemical analysis of mutant proteins, although mice lacking one copy of Nkx2-5 do not have cardiac defects as severe as those caused by human mutations. Some missense mutations act as dominant inhibitory proteins, but in mice, their dominant-negative potential appears to be selective, resulting in atrioventricular block and heart failure. It is of great interest to determine why certain structures and not others are affected by dominant Nkx2-5 mutations and what is at the source of interfamily and intrafamily variation in expressivity of the mutations. These questions also apply to Tbx5 (see below).

The identification of Tbx5 mutations in Holt-Oram syndrome has also provided insight into cardiac septation. Holt-Oram syndrome is a rare inherited disease characterized mainly by upper limb and congenital heart defects. The cardiac malformations in this syndrome resemble those caused by NKK2-5 mutations: ASDs and VSDs, with occasional reports of TOF and often combined with conduction system disease (see summary). As with NKK2-5 mutations, haploinsufficiency of Tbx5 is thought to be at the root of Holt-Oram syndrome. This hypothesis is supported by the observation that a deletion of one copy of Tbx5 in the mouse faithfully reproduces Holt-Oram syndrome.

Missense mutations in Tbx5 have also been reported, and it is not clear whether these result in Tbx5 protein with altered function or no functional capacity. In patients with Tbx5 mutations, different mutations may confer distinct phenotypes: some mutations have been identified in patients with severe cardiac defects, whereas other mutations are associated with milder cardiac defects. Therefore, the
delineation of the functional defects caused by these mutations has important clinical implications. Functional studies of TBX5 missense mutations indicate that at least some may result in a nonfunctional protein, whereas others are likely to impart altered properties of an unknown nature.26,64,86 For example, binding of Tbx5 to its DNA-binding sites is abolished by some mutations, whereas others do not affect binding.80 The latter type of mutation may instead decrease the ability of Tbx5 to bind other proteins (such as Nkx2-5; see below) that may act in a complex to activate downstream targets.

A poorly understood aspect of chamber formation is the acquisition of left versus right identity. This is particularly evident in the atri, which are initially positioned in a left-right arrangement, unlike the ventricles, for which the left and right arise from an initial anteroposterior arrangement. Atrial identity is important for the proper alignment of septal and valve structures and for the normal connections of venae cavae and pulmonary veins; abnormalities in these processes lead to severe defects, such as common atrioventricular canal or total anomalous pulmonary venous return. A major player in establishing atrial identity is the paired homeodomain transcription factor Pitx2, inasmuch as mice lacking Pitx2 have a single large atrium with right atrial morphology, including abnormal connection of venae cavae and pulmonary veins.81–84 The resulting defects resemble complete atrioventricular septal defects, with a single atrioventricular valve, VSD, double outlet right ventricle, and a large ASD. Decreased dosage of Pitx2 leads to relatively normal chamber formation, but septal and valve defects occur, which are perhaps due to the misalignment of structures during development.81

The multi-type zinc finger transcription factor FOG-2 has been implicated in cardiac septation. FOG-2 is similar to the previously described mammalian FOG and Drosophila U-shaped proteins, which function as negative or positive regulators of transcription via interactions with GATA proteins.85 In Drosophila, U-shaped proteins negatively regulate cardiac cell numbers and repress a cardiac-specific promoter regulated by pannier (a GATA protein) and tinman (an Nkx2-5 homologue).85 In light of these data, it is somewhat surprising that the deletion of FOG-2 in mice leads not to increased cardiac cell proliferation but instead to ventricular and atrioseptal defects reminiscent of TOF,86,87 accompanied by a general failure of coronary vessel formation.88 Forced cardiomyocyte expression of FOG-2 in FOG-2–deficient mice rescues their cardiac and vascular defects, indicating that endogenous cardiac targets of FOG-2 signal the coronary vasculature.86 The implication from these findings is that defects in these intercellular signaling events are responsible for the morphological abnormalities regulated by FOG-2. This adds an additional level of complexity to the regulation of cardiac morphogenesis, and as such, it will be of great interest to identify the operative pathways.

Retinoic acid receptors (RARs) are ligand-activated transcription factors that have been implicated in many aspects of heart development, including ventricular maturation and cardiac septation.88–91 Mice lacking the RAR coreceptor RXRa have defective ventricular maturation, related to accelerated cardiomyocyte differentiation.88–90,92 Careful analysis of RXRa-deficient mice as well as mice with combinatorial deletions of several RAR genes has identified a role for RAR-dependent signaling in ventricular and outflow tract septation.90,91 It appears that the role of RXRa is not intrinsic to cardiomyocytes,93,94 and the specific cell types in which RXRa and RARs control heart development or the pathways regulated by these transcription factors have yet to be identified.

Transcription enhancer factor-1 (TEF-1) is a simian virus 40–related protein that binds M-CAT binding sites in striated muscle genes and regulates the troponin T and α-skeletal actin genes in cardiac myocytes.95 A retroviral gene trap insertion into the mouse TEF1 gene resulted in embryonic lethality that was in part due to defects in maturation of the ventricular myocardium.96 It has not yet been determined which genes important for cardiac muscle maturation and growth are regulated by TEF-1. Mice deficient in the proto-oncogene transcription factor N-myc also have defective trabeculation and thinned ventricular myocardium.97 There is evidence of the PAS family of HILH transcription factors in cardiac gene expression in response to hypoxia; in hypoxia-inducible factor 1 knockouts in which placental defects have been rescued, abnormal cardiac maturation is observed, implying a direct (but undefined) role of hypoxia-inducible factor 1 in cardiac morphogenesis.98 These results may indicate a transcriptionally regulated sensitivity to oxygen levels in the developing heart, but this remains to be conclusively shown.

Deficiency of the SRY-family transcription factor Sox4 in the mouse results in a phenotype similar to common trunk, in which atrioventricular septal defects are the predominant feature.99 VSDs are also found in mice lacking the Nf-atc transcription factor, a protein that was anticipated to be important only in the immune system.100,101 Mice lacking the Tip2 coactivator CITED2 have a constellation of cardiac defects that include ASDs and VSDs, as well as outflow tract malformations, with the latter possibly being due to abnormalities in cardiac neural crest cells.102

Conduction System Formation

The specialized cells that compose the cardiac conduction system are formed from the differentiation of cardiac cells into specialized conduction cells.103 A role for Nkx2-5 and Tbx5 in the formation of a functional conduction system has been inferred from the atrioventricular node defects observed in humans and mice with dominant mutations in these transcription factors.61,66,71,72,75–77 The increased expression of Nkx2-5 in specialized conduction fibers compared with working myocardium perhaps explains the sensitivity of these cells to decreased Nkx2-5 dosage.104 The identification of connexin 40 (cx40) as a direct downstream target of Tbx5 and Nkx2-5 and the decreased expression of this gap junction protein in Tbx5-deficient mice and in mice expressing a mutant Nkx2-5 protein point to a specific role of Tbx5 and Nkx2-5 in regulating the genes involved in conduction system function, such as cx40.61,66

The zinc-finger transcription factor HF-1b has been shown to be critical in establishing conduction system identity. Mice...
lacking HF-1b have abnormal conduction system function, including sinoatrial, atrioventricular, and specialized conduction system deficiencies. Molecular analyses of these mice have determined that in the absence of HF-1b, the myocardium surrounding specialized conduction fibers adopts a “confused” identity, characterized by simultaneous and diffuse expression of genes normally confined to the conduction fibers or nonconduction fibers. Therefore, HF-1b appears to be required for the establishment of a molecular or physical distinction between conduction and nonconduction cells in the developing myocardium. It remains to be determined what the direct targets of HF-1b are and how this genetic pathway is established.

**Downstream Targets**

A major gap in our knowledge is identifying the specific targets of transcription factors that are responsible for the morphological or other events that they regulate. Several cardiac transcription factors have been identified on the basis of the presence of DNA-binding sites in promoter elements that confer cardiac-specific expression to the genes that they regulate. Similarly, potential downstream genes have been identified on the basis of their activation by a candidate transcription factor. However, few endogenous bona fide targets of cardiac transcription factors have been conclusively identified; these will be discussed below.

*ANF* and *cx40* have been identified as bona fide in vivo targets of Nkx2-5 and Tbx5. Delineation of the ANF regulatory elements has yielded considerable insight into the transcriptional regulation of a cardiac-specific gene (see reviews). Dose-dependent regulation of ANF by Tbx5 has been observed in vivo as well as in vitro, indicative of a fine regulation of gene expression by a transcription factor. Transcriptional activation of ANF by Nkx2-5 is dose dependent in a heterologous system, but only a complete lack of Nkx2-5 causes decreased ANF expression in vivo. Therefore, endogenous regulation of ANF is mainly dependent on Tbx5 dosage, with Nkx2-5 playing an important but less critical role (see Figure 2). However, as described below, interactions between these two transcription factors ensure full activation of ANF.

Both Tbx5 and Nkx2-5 interact with specific binding sites in the cx40 promoter and activate the cx40 gene directly. Cx40 transcription is exquisitely sensitive to Tbx5 dosage, inasmuch as a 50% decrease in Tbx5 leads to an almost complete elimination of cx40 transcription in vivo in the mouse heart. This implies that the occupancy of multiple sites in the cx40 promoter by Tbx5 is the primary mechanism for the regulation of this gene, and a decreased Tbx5 dosage results in a nonlinear response of the transcriptional apparatus at this locus. Therefore, critical levels of Tbx5 in specific locations during heart development act as an on/off switch of gene expression via this mechanism (see Figure 2).

The cardiac ankyrin repeat protein (CARP) and Pitx2 genes have also been identified as bona fide targets of Nkx2-5. The CARP gene was found to be downregulated in Nkx2.5−/− embryos; direct binding and activation of the CARP promoter has been demonstrated, providing evidence that Nkx2-5 directly regulates CARP in vivo. Pitx2 is also under the control of complex regulatory elements that contain an Nkx-binding site required for late cardiac expression; it is likely that Nkx2-5 is the transcription factor involved in this regulation.

GATA4 and other GATA factors have been shown to be potentially important for the regulation of multiple cardiac genes, including ANF, several contractile protein genes, *gata6*, Nkx2-5, and *dHand*. The promoters for these genes have functional GATA binding sites that are required for fully active transcription in vitro. In the case of Nkx2-5, gata6, dHand, and *SM22α*, the requirement of GATA-dependent regulation has been shown to be relevant in vivo, on the basis of transgenic analysis of regulatory elements.

Cardiac-specific regulation of transcription by Mef2 proteins has been shown in vivo and in vitro for the desmin gene. Embryonic cardiac expression of the SM22α and skeletal actin genes are regulated in vivo predominantly under the control of CarG boxes, which are bound by SRF, and, presumably, myocardin. These genes are expressed in other muscle types (skeletal or smooth muscle); therefore, the MEF2- and SRF-binding sites are perhaps required for muscle-specific expression rather than cardiac-specific expression. Multimerized Mef2-binding sites or CarG boxes can drive muscle-specific gene expression by themselves; therefore, additional factors interacting with these proteins, such as myocardin, Nkx2-5, or GATA4 (see below), must be required to add cardiac specificity to Mef2- and SRF-dependent transcriptional regulation in the developing heart.

**Combinatorial Interactions Between Transcription Factors in Heart Formation**

**Genetic Interactions**

Interaction between the zinc-finger protein GATA4 and its binding partner, FOG-2, is important for cardiac septation.
Physical interaction between GATA4 and FOG2 results in the repression of GATA4-dependent transcription, although synergistic enhancement of transcription has also been described. In Drosophila, U-shaped and mammalian FOG proteins repress the activity of pannier, a GATA4 homologue, in dorsal vessel formation. Mice with an engineered mutation in GATA4, designed to abolish GATA-FOG interactions, have phenotypes similar to those found in FOG-2–deficient mice. However, the GATA4 missense mutation engineered to disrupt GATA-FOG interactions also leads to impaired outflow tract septation, resulting in mice with double-outlet right ventricle, a phenotype not observed in FOG-2–deficient mice. This implies that other factors bind to GATA4 at this site or that this mutation cripples GATA4 in an undefined manner.

Mice lacking both Nxk2-5 and dHand do not develop a ventricle. mice have decreased eHand expression, and eHand is expressed in the developing LV and is presumably required for LV growth. Therefore, creating mice lacking both dHand and Nxk2-5 would result in a complete lack of Hand gene expression in the developing heart. Nkx2-5/dHand double-null embryos do not form a ventricle, although a small remnant of ventricular tissue can be identified by the expression of ventricle-specific genes. An interaction between Nxk2-5 and dHand has also been deduced from the regulation of the Irx4 gene: mice lacking either transcription factor have greatly reduced, although detectable, Irx4 transcripts, whereas mice lacking both Nxk2-5 and dHand have no detectable Irx4 expression.

**Physical Interactions**

Investigation of candidate binding sites and the transcription factors that bind them have led to the identification of multiple physical interactions between factors on specific regulatory elements. However, caution is warranted in interpreting these results, because in many cases, it is not clear whether the interactions are a consequence of DNA bridging. Interactions relevant to cardiac development are discussed below.

Nxk2-5 physically interacts with Tbx5 and GATA4 to synergistically activate transcriptional target genes (eg, see Figure 2). The functional basis for synergistic activation of cardiac promoters by Nxk2-5/Tbx5 or Nxk2-5/GATA4 interactions has been shown to be based on stable ternary complexes composed of both proteins physically interacting with each other as well as the simultaneous binding of each protein to its cognate binding site. Other reports suggest that interaction between Nxk2-5 and GATA4 is independent of the binding of GATA4 to DNA and is sufficient for synergistic activation and that synergy can occur on multimerized Nkx-binding sites. Therefore, Nxk2-5 and GATA4 can activate transcription via unmasking of the activation domain of DNA-bound Nkx2-5 as well as recruitment of GATA4 by Nkx2-5 into a transcriptionally active complex. The Nxk2-5/Tbx5 interaction is very much relevant to congenital heart defects, because it provides a mechanism for the common cardiac phenotypes caused by haploinsufficiency of either transcription factor: disruption of
regulation of downstream targets. It follows that regulatory elements exist in the promoters of cardiac transcription factors to activate or repress them in the developing heart. Furthermore, modification of transcription factors from inactive to active proteins and vice versa is likely to play an important role in modulating transcriptional activity (eg, see Figure 3).

Analysis of the Nkx2-5 regulatory elements in transgenic mice has provided some insight into the events controlling Nkx2-5 transcription but has also raised a number of intriguing questions regarding the complex transcriptional modularity of the embryonic heart.111 Nkx2-5 genomic fragments driving the lacZ reporter gene in transgenic embryos express lacZ in the early cardiac crescent, as does endogenous Nkx2-5, but during later cardiac development, the expression controlled by the longest genomic fragments examined becomes rapidly restricted to the atria and a portion of the RV, and later expression occurs only in a segment of the outflow tract. Therefore, additional sequences are required for the continued expression of Nkx2-5 throughout the entire developing heart. Delineation of critical regulatory elements has identified multiple GATA-binding sites essential for cardiac expression of Nkx2-5 as well as autoregulatory Nkx-binding sites operative in vivo.111 Posttranslational modifications, acting either positively or negatively, are likely to account for an important component of the regulation of transcription factors during heart development. In skeletal and cardiac muscle, negative regulation of Mef2 function by histone deacetylases (HDACs) has been uncovered as an important mechanism modulating Mef2 activity.125 Repression in ventricular myocytes of the ANF gene by the Kruppel-related neuron-restrictive silencer factor (also known as RE-1 silencing transcription factor) operates by recruitment of HDACs.126 Therefore, HDAC-regulated repression of cardiac-specific gene expression may be widespread.

The E1A-binding protein p300 appears to be involved in the regulation of cardiac transcription during development via its interactions with multiple cardiac transcription factors: GATA4, GATA5, Mef2c, Mef2D, and tinman have all been uncovered as an important mechanism modulating Mef2 activity.125 Signal-dependent modification of transcriptional activity has been shown to be important in the regulation of GATA4 activity.132 Rho-like GTPases can phosphorylate GATA4 on specific residues via activation of the p38/mitogen-activated protein kinase pathway, which enhances the potency of this transcription factor.132 This dependent effect is likely to be essential for the remodeling of cardiomyocytes by growth factor stimulation122 but also may be very important in early heart development, inasmuch as Rho kinase expression is detected in the early developing heart, and inhibition of Rho kinase activity results in cardia bifida, a phenotype similar to that resulting from removing GATA4 activity.133 Nkx2-5 is also phosphorylated on its translocation to the nucleus, and this modification increases DNA binding and is important for transcriptional activation by Nkx2-5.134 Mef2c activity has been shown to be regulated by phosphorylation,135 and activity of a Mef2-dependent transgene in the heart is stimulated by calmodulin kinase activation.136 Which signaling pathways are operative in heart development that may influence Mef2 and Nkx2-5 phosphorylation is not known. These mechanisms that allow fine-tuning of activity independent of expression levels or location add an additional layer of complexity to the network of transcription factors that operate in the developing heart.

Conclusions

A number of complex transcriptional networks and interactions are involved in the morphogenesis of the developing vertebrate heart. The identities of crucial regulators involved in defined events in cardiogenesis are being uncovered at a rapid rate, but a number of critical questions remain. First and foremost, it is still not known which transcription factors are involved in the earliest differentiation of cardiac cells from the mesoderm. Second, the downstream pathways regulated by transcription factors responsible for key morphogenetic events are still largely unknown. Third, the concept of maintained function or redeployment of functions throughout various stages of development remains to be addressed in detail. The challenge for the future lies in defining pathways downstream from cardiac transcription factors and understanding the intersection of these pathways as the heart develops from a simple patterned structure into a complex multifunctional organ.

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