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Regulatory Factors Involved in Cardiogenesis

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Abstract—Determination of factors involved in cardiogenesis is fundamental to our understanding of heart growth and development. There are several families of transcription factors that play a role in cardiomyogenesis including the Nkx2, GATA, and MEF2 families. In this review, we describe factors implicated in development of cardiac tissues followed by a description of interactions among them.

Key words: cardiogenesis, factors

Cardiogenic lineages originate from paired regions of anterior lateral mesoderm, the cardiac crescent, soon after gastrulation and develop into parallel cardiac primordia that fuse to form the primitive heart tube along the ventral midline of the embryo. Subsequent events of looping, chamber maturation, and alignment with the vasculature give rise to the mature multi-chambered heart [1]. Several recent studies have revealed cis-regulatory elements that direct cardiac transcription in the developmental events. However, although the complex network of myogenic regulatory protein in skeletal muscle is well established, less is known about the mechanisms involved in cardiac tissue development. There are several families of transcription factors that play a role in cardiomyogenesis including the Nkx2, GATA, and MEF2 families, but the entire regulatory cascades have yet to be fully delineated. In this paper, we will review the transcription factors involved in cardiomyogenesis and their combinatorial interactions.

FACTORS INVOLVED IN CARDIOGENESIS

1. NK-2 homeodomain protein. *NK-2* homeobox genes are highly conserved across vertebrate species and are expressed in early cardiac progenitor cells. These genes encode DNA binding proteins containing a 60 amino acid helix–turn–helix motif related to homeobox-containing (*HOX*) genes that regulate early embryonic patterning. The overlapping expression patterns of these genes have led to the concept of an "Nkx code", in which cell fates are specified by unique combinations of these NK-homeodomain proteins. NK-homeodomain factors bind DNA and recognize novel NKE sequence elements 5′-NAAGTG-3′. Intact NKEs are required for transcription of a variety of vertebrate target genes, including *cardiac* α-*actin* and *atrial natriuretic factor (ANF)* [2]. Although these related *Nkx* genes are expressed in mammals in overlapping patterns, *Nkx2-5* appears to play a unique function in cardiac development. *Nkx2-5* (also called *Csx*) is the earliest known marker of vertebrate heart development expressed very early in development (by day E 8.5) preceding that of the other cardiac-specific genes [3].

Inactivation of the *Nkx2-5* homeobox gene in the mouse results in the failure of looping morphogenesis. Although mutant embryos are able to form a primitive heart tube and express cardiac specific genes including myosin, they die during mid-gestation and the heart fails to loop normally, possibly as a result of abnormal muscle growth. Because characterization of the role of *Nkx2-5* in early vertebrate heart formation has been complicated by a potential functional redundancy of the *Nkx* homologs; single knockouts have not been completely informative [4]. Inability to form cardiac muscle because of the loss of Nkx2-5 activity is also accompanied by the loss of endogenous expression of cardiomyoblast genes, such as *GATA-4* and *myocyte enhancer factor 2 (MEF2)*. Furthermore, there were no significant changes in genes expressed during mesoderm induction, such as *Brachyury T*, *Wnt5b*, and *Wnt3a*, or in genes expressed in the skeletal muscle pathway, such as *Mox1*, *Pax1*, *MyoD*, and *myogenin* [5].

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Optimal *Nkx2-5* activity requires physical interactions with other cardiac-restricted factors, such as GATA-4 and serum response factor (SRF) [6]. However, the GATA factors may also antagonize *Nkx2-5* transcription, restricting the domain of *Nkx2-5* expression within the heart field. In addition, the MADS-box containing MEF2 proteins can interact and synergize with GATA factors, similar to the cooperative interactions between MEF2 proteins and the myogenic basic helix–loop–helix factors (bHLH) in skeletal muscle.

In a more recent study, the expression of 20 candidate genes was examined by *in situ* hybridization in mice completely null for *Nkx2-5*, revealing a down-regulation in the expression of the *ANF*, *brain natriuretic peptide (BNP)*, *MLC2V*, *N-Myc*, *Msx2*, *eHAND*, and *MEF2C* genes in the mutant heart [7]. However, the latter study detected no change in the expression of *GATA-4*, *cardiac* α*-actin*, and *BMP4* in the mutant mice and no effect on the early formation of cardiomyocytes. It is likely that many murine homologs of *Nkx2-5* have overlapping and compensatory functions and thus, deletion of any one of these genes may not suffice to completely block cardiomyocyte differentiation [8]. *Nkx2-5* expression can up-regulate *BMP4* transcripts in aggregated P19 cells. The presence of BMP in the endoderm contacting the heart field promotes cardiomyogenesis by regulating the expression of *Nkx2-5* and *GATA-4* [9]. Loss of *Nkx2-5* activity was shown to result in a down-regulation of *BMP4* transcript level, which suggest that BMP4 and *Nkx2-5* function in a complex positive regulatory loop [10].

The transcriptional regulation of *Nkx2-5* expression is complex. A series of reports indicate that chamber-specific expression is regulated by distinct enhancer sequences. This suggests that chamber-specific upstream factors that mediate *Nkx2-5* expression exist in a region-specific fashion. Within 23 kb of DNA surrounding the *Nkx2-5* gene, seven different activating regions and three repressor regions were identified. Some *Nkx2-5* enhancers are active in the same regions of the heart and some direct expression patterns that demarcate distinct subpopulations of cardiomyocytes within cardiac compartments, but none can account for the complete expression pattern of the gene during embryonic development and after birth. The protein-coding region of *Nkx2-5* is contained in two exons, which can be spliced to either of two alternative exons (1a and 1b). Most transcripts contain only two 3′ coding exons and encode a protein of 318 amino acids [11].

2. The GATA family of transcription factors. The GATA factors are a family of transcriptional regulators that are expressed in a tissue-restricted manner. There are six GATA transcription factors, each of them containing a highly conserved DNA binding domain consisting of two zinc fingers of the motif Cys-X_2-Cys-X_{17}-Cys-X_2-Cys that directs binding to the nucleotide sequence element (A/T)GATA(A/G). Based on their expression patterns, the GATA proteins have been divided into two subfamilies, GATA-1, -2, and -3 and GATA-4, -5, and -6. The mouse *GATA-4*, *-5*, and *-6* genes encode proteins of 48, 42, and 45 kD, respectively. GATA-4, -5, and -6 are homologous in their amino acid sequence with 90% homology, particularly in the DNA binding domain, and form a distinct subclass of GATA factors. The proteins are more divergent outside of the zinc finger region with 45% homology in the N-terminal domain and 35% homology in the C-terminal domain. Consistent with a conserved specialized function for GATA factors the amino acid sequence of each protein is highly conserved among species. GATA-4, -5, and -6 have been implicated as important regulators of gene expression in heart, liver, gonads, gut epithelium, and lungs [12]. GATA factors also regulate developmental expression of the cardiac transcription factor Nkx2-5, suggesting the existence of the reinforcing transcriptional regulatory circuit between Nkx2-5 and GATA factors in the heart [13]. Furthermore, the *Drosophila* GATA factor, *Pannier*, regulates expression of *MEF-2* gene in cardioblasts extending the role of GATA factors as regulators of heart gene expression to include invertebrates [14].

Collectively, the studies discussed above indicate that GATA factors are important regulators of both structural and regulatory genes in the heart. Targeted disruption of the *GATA-4*, *-5*, and *-6* genes in the mouse has revealed phenotypes consistent with their individual expression patterns. Mice null for *GATA-4* die between embryonic day 8 and 9 because of defects in heart morphogenesis and ventral closure of the foregut. Specifically, *GATA-4* null mice demonstrate *cardia bifida* because of ineffective ventral fusion of the lateral aspects of the embryo and the subsequent formation of the foregut. Mice null for *GATA-5* have the same pattern of expression. *GATA-6* null mice die during early embryonic development (embryonic day 5.5-7.5) because of defects in visceral endoderm function and subsequent extra-embryonic development, a phenotype that is consistent with the expression pattern of *GATA-6* in the embryonic primitive endoderm [15].

The nuclear localization and transcriptional activation domains of GATA-4 are present within the basic domain adjacent to the C-terminal finger (amino acids 251-324), whereas two separate transcriptional activation domains are present within the N-terminus of the protein. These transcriptional activation domains are partially conserved in GATA-5 and -6 suggesting a similar mechanism of transcriptional activation within the GATA-4, -5, and -6 subfamily [16].

Characterization of the 5′-regulatory regions of numerous genes has demonstrated that GATA-4, -5, and -6 factors also interact with the DNA sequence element containing a core GATA motif. Many studies indicate that although GATA-4, -5, and -6 each bind a GATA or GATA-like sequence element, their individual affinities

for various promoters might depend on flanking nucleotide sequences or even on interactions with cofactors and other transcription factors.

GATA-4 can be detected in the precardiac mesoderm as early as 7.0-7.5 day at the late primitive streak stage, where it precedes the expression of the earliest cardiac differentiation markers, such as contractile protein genes and natriuretic peptide genes. In the mesoderm, *GATA-4* expression is confined to the cardiogenic crescents, on each side of the embryo; *GATA-4* transcripts are also detected in the visceral endoderm. This expression pattern is very similar to that of *Nkx2-5* and coincides with the heart-forming region in mouse and chicken. At later stages, *GATA-4* transcripts are detected throughout the myocardium and endocardium where they are present at high level in the postnatal heart [17]. Thus, *GATA-4* is an early marker of the cardiac cells important at various stages of cardiogenesis that regulates expression of a number of cardiac genes such as *α-myosin heavy chain*, *cardiac troponin-C*, *ANF and BNP*, *cardiac troponin-I*, *sodium/calcium exchanger*, *cardiac restricted ankyrin repeat protein*, *A1 adenosine receptor*, *m2 muscarinic receptor*, and *the myosin light chain 1/3* [18].

GATA-4 expression was knocked-down by an antisense strategy in the pluripotent P19 embryonal carcinoma cells which provide a cellular model of inducible cardiac differentiation. In *GATA-4(−/−)* lines, terminal cardiac differentiation could not be achieved and massive apoptosis of precardiac cells was observed, suggesting that *GATA-4* is a mediator of survival, proliferation, and differentiation signals. Mice lacking *GATA-4* fail to develop linear heart tube and die *in utero* by day 9. The exact mechanism underlying the role of GATA-4 during early cardiac development remains unclear. In fact, both the *in vivo* and *in vitro* studies suggest that GATA-4 is required for proliferation and migration of cardiac cells or for early mesoderm–endoderm interactions. These possibilities are not exclusive as GATA-4 may be involved at more than one developmental stage [19]. Complementary gain-of-function studies revealed another function for GATA-4 in cardiogenesis. Experiments carried out in P19 cells where *GATA-4* was stably overexpressed revealed that *GATA-4* markedly potentiates cardiogenesis as evidenced by the earlier appearance and persistence of beating cardiac cells. These results suggest that GATA-4 can potentiate cardiogenesis by recruiting more cells to the cardiogenic field. A recent study suggests that early expression of *GATA-4* may even serve to maintain precursor cells in a cardiac competent stage.

GATA regulatory elements have been identified in a wide array of cardiac-expressed genes suggesting its important role in regulation of the global gene expression in the heart. In addition, GATA-4 has been implicated as a regulator of inducible gene expression in cardiac myocytes in response to hypertrophic stimulation. GATA binding sites have been shown to mediate the hypertrophic responsiveness of the *ANF*, *BNP*, *13-MHC* gene promoters in cultured cardiomyocytes [20]. Specifically, analysis of the *β-myosin heavy chain* promoter in aortic-banded rats (pressure overload) revealed a proximal GATA binding site that directed hypertrophy-responsive gene expression. In a similar approach, GATA-4 was implicated as a regulator of *angiotensin type-1A receptor* promoter in response to pressure overload stimulation in the adult rat heart. In cultured neonatal cardiomyocytes electrical pacing-induced hypertrophy was associated with a significant increase in *GATA-4* mRNA suggesting a mechanism of regulation whereby the total *GATA-4* content is up-regulated during hypertrophy. Alternatively, *GATA-4* transcriptional activity might also be regulated by phosphorylation mediated by extracellular signal-regulated kinase in response to hypertrophic agonist administration. Finally, *GATA-5* was shown to mediate leukemia inhibitory factor (LIF)-induced expression of the β-myosin heavy chain in cultured cardiomyocytes.

Collectively, these various reports implicate GATA transcription factors as important regulators of hypertrophy-associated gene expression in cardiomyocytes. Evidence suggests that both transcriptional and post-transcriptional mechanisms are involved in augmenting GATA-4 potency during hypertrophy in cardiac myocytes. Despite these positive accounts implicating GATA factors in hypertrophic signaling, direct experimental evidence linking *GATA* transcriptional activity to the initiation and/or maintenance of cardiac hypertrophy has not been demonstrated. Ad*GATA-4* or Ad*GATA-6* infection in cultured cardiomyocytes or mild overexpression of *GATA-4* in mouse heart were each sufficient to induce a hypertrophic response [21]. *GATA-4* transgenic mice demonstrated a slowly progressing cardiac hypertrophy characterized by a gradual increase in heart to body weight ratio over 8 months of age. However, phenotypic hypertrophy was preceded by increased expression of the hypertrophy responsive *ANF* and *BNP* genes as early as 2 months of age. These results suggest that *GATA-4* overexpression might directly stimulate pre-hypertrophic expression of *ANF* and *BNP* genes which are each well characterized GATA transcriptional targets or simply that the molecular program for hypertrophy precedes phenotypic hypertrophy by 4 months in these mice.

GATA-6 could also be a regulator of the cardiogenic field. Injection of *GATA-6* mRNA in gastrulating embryos resulted in a transient block of cardiac differentiation and enhanced proliferation of cardioblasts; after the decay of the injected GATA-6 mRNA, cardiomyocytes resumed differentiation to generate an enlarged heart [22]. Collectively, these genetic manipulations are consistent with critical roles of GATA factors in the developing heart.

3. MEF2 family of transcription factors. The MEF2 family belongs to the MADS-box family of transcriptional regulators. MEF2 factors contain a 56-amino-acid

MADS-box domain and an adjacent 29-amino-acid MEF2 domain, which mediate homo- and heterodimerization and DNA binding. Within the MADS-box, MEF2 factors share homology at several invariant residues with other members of the MADS-box family of transcription factors [23]. These conserved residues are important for DNA sequence recognition. The MEF2 domain is unique to MEF2 factors, but other MADS-box proteins contain domains with analogous functions. MEF2 has been shown to play a pivotal role in morphogenesis and myogenesis of skeletal, cardiac, and smooth muscle cells. MEF2 was originally identified as a DNA-binding protein from skeletal muscle cells that recognized a conserved A/T-rich DNA sequence in the muscle creatine kinase (MCK) enhancer [24]. The MEF-binding site has subsequently been identified in the control regions of nearly all skeletal and cardiac muscle genes.

In vertebrates, there are four *MEF-2* genes, referred to as *MEF2A*, *-B*, *-C*, and *-D*, that are located on different chromosomes. The vertebrate *MEF2* gene products share about 50% amino acid identity overall and about 95% similarity throughout the highly conserved MADS-box and MEF2 domain whereas they are divergent in their C-terminal regions [25]. MEF2 factors from invertebrates are also highly homologous to the vertebrate factors in the MADS-box and MEF2 domain. MEF2 binds $YTA(A/T)_4TAR$. MEF2A, -C, and -D have the same DNA binding specificity whereas MEF2B binds the MEF2 consensus sequence with reduced affinity relative to the other members [26]. In vertebrates, *MEF2* transcripts are highly enriched in developing muscle cell lineages during embryogenesis. In mouse and chick *MEF2C* is the first of the *MEF2* genes to be expressed with transcripts appearing initially in mesodermal precursors that give rise to the heart. Soon thereafter transcripts for the other *MEF2C* genes are expressed in the developing myocardium. Null *MEF2C* embryos appear normal until about embryonic day 9 when they begin to show retarded growth and pericardial effusion indicative of cardiac insufficiency. In contrast to normal embryos in which the heart tube initiates rightward looping to form the right ventricular chamber beginning at about embryonic day 8.5, the heart tubes of mutant embryos do not loop and the future right ventricular region fails to form [27].

It is interesting to note that the morphogenic defects in the hearts of *MEF2C* mutant embryos are similar to those seen in mice lacking the bHLH transcription factor dHAND. Whether the partial phenocopies of cardiac defects in *MEF2C* and *dHAND* mutants reflect cooperative interactions between the products of these genes, as occurs between MEF2 and myogenic bHLH factors, is under investigation [28].

The regulation of *MEF2* expression occurs primarily at the level of mRNA accumulation during embryonic development. The vertebrate *MEF2* genes contain large 5′ noncoding regions with multiple alternatively spliced exons and large introns. Because of this complexity, no cis-acting regulatory sequences have yet been described for any vertebrate *MEF2* gene. However, progress has been made toward defining the regulatory elements that control *MEF2* transcription in *Drosophila*. The *D-MEF2* gene contains at least a dozen independent enhancers within 12 kb upstream of the gene each of which directs transcription in a unique temporospatial pattern during development. The integration of transcriptional inputs from these different elements is required for the complete pattern of *D-MEF2* expression throughout embryonic and adult muscle development [29].

Two *D-MEF2* enhancers have been characterized in detail. A cardiac-specific enhancer that directs transcription in cardiac precursor cells and in the heart-like organ of the embryo, known as the dorsal vessel, is located about 6 kb upstream of the gene. This enhancer contains two binding sites for the cardiac homeodomain protein *tinman* that is required for *Drosophila* heart formation. Mutation of these binding sites abolishes enhancer activity and ectopic expression of *tinman* under control of a heat shock promoter results in activation of this enhancer outside the mesoderm. These results suggest that part of *tinman*'s function in cardiac myogenesis is to activate *D-MEF2*.

4. Cardiotrophin-1. Cardiotrophin-1 (CT-1) is a member of the IL-6 family of cytokines discovered as a factor that can induce hypertrophy of cardiac myocytes both *in vitro* and *in vivo* [30]. Subsequently, CT-1 has been shown to have a wide variety of different effects on cardiac and non-cardiac cells including the ability to stimulate the survival of both cardiac and neuronal cells. CT-1 is expressed at high levels in the myocardium during the course of cardiogenesis and promotes the proliferation and survival of embryonic cardiomyocytes [31]. CT-1 may therefore represent a candidate cytokine to activate gp130 during cardiac development. Like all members of the IL-6 family that induce their biological effects through the shared signaling subunit, gp130, CT-1 receptor contains a common protein chain known as gp130 also. In the adult mouse and human expression of CT-1 is found in a variety of different tissues. However, during mouse embryonic development *CT-1* is first detected in the primitive heart tube at day 8.5, whereas other tissues do not show significant expression at this time. *CT-1* is expressed in atrial and ventricular muscle of the developing heart but not in endocardium. Although the developing heart remains the predominant site of *CT-1* expression until E10.5, other organs begin to display expression of *CT-1* at later developmental stages; still, the expression of *CT-1* remains high in the heart [32].

CT-1 is likely to play a key role in the normal development of the heart and in particular in ensuring that the myocardium develops to a normal thickness. Deletion of the gp130 receptor for CT-1 results in hypoplastic ventricular myocardium. The continued expression of CT-1 in

the adult heart suggests that it continues to have some functions following the completion of cardiac development [33]. One is that CT-1 has a cardioprotective effect. It was observed that the treatment with CT-1 was able to enhance the survival of neonatal rat cardiac myocytes cultured in serum-free medium. Moreover, it was shown that pretreatment with CT-1 was able to protect cultured neonatal cardiac myocytes against subsequent exposure to either elevated temperature (heat shock) or simulated ischemia/hypoxia. Therefore, it is possible that the activation of p42/p44 MAPK by CT-1 leads to the activation of NF-IL-6, which in turn induces the synthesis of the protective heat shock proteins hsp70 and hsp90 whose overexpression has previously been shown to protect cardiac myocytes against both thermal and ischemic stress [34].

CT-1 has another effect on the heart. It has been shown that intravenous administration of CT-1 to rat results in a significantly elevated cardiac output and heart rate as well as decreased mean arterial pressure and systemic vascular resistance. As no alteration was observed in stroke volume, it is likely that the increased cardiac output was secondary to the increased heart rate. Interestingly, both the depressor effect of CT-1 on blood pressure and the tachycardic effect could be significantly reduced by treatment with a nitric oxide synthase inhibitor, suggesting that the hemodynamic effects of CT-1 may be mediated by nitric oxide [35].

As well as its normal expression in the heart, *CT-1* expression has also been shown to be up-regulated in a variety of pathological states where it may contribute to ongoing disease process. For instance, augmented expression of *CT-1* has been detected in the ventricle of genetically hypertensive rats where it may contribute to the ongoing hypertrophic response. Similarly, overexpression of both *CT-1* and *gp130* has been reported in the rat ventricle following myocardial infarction and during experimental acute Chagas' cardiomyopathy. In addition to cell enlargement, the factor was active in two other assays of hypertrophy, namely, the incorporation of myosin light chain 2 into sarcomeric units and the induction of ANP secretion [36].

Several genes stimulated by treatment of cardiac cells with CT-1 have now been identified. One of these, *ANP*, was shown to be transcriptionally induced by CT-1 treatment. Similarly, BNP induced in response to the hypertrophic agent endothelin-1 (ET-1) is also induced by treatment with CT-1. Moreover, it was shown that this factor is able to induce the expression of genes encoding the protective heat shock proteins hsp70 and hsp90 [37]. Although a number of genes induced by CT-1 have been defined, it is of interest that in no case has the CT-1 response element been mapped in the gene promoter. It is possible that in some cases CT-1 may induce enhanced mRNA and protein levels by post-transcriptional processes, although it should be noted that at least in the case of the *ANP* gene transcription regulation has apparently been demonstrated by nuclear run-on assays.

As with all members of the IL-6 family, binding of CT-1 to its receptor sets off a cascade of signaling processes. These signaling pathways result in the activation of at least two types of cellular transcription factors that can subsequently activate the expression of specific target genes. Thus, following binding of CT-1 to its receptor activation of the p42/p44 MAPK enzymes is observed and this results in threonine phosphorylation of the NF-IL-6 (C/EBP$^{\beta}$) transcription factor allowing it to activate gene transcription. Similarly, activation of Jak tyrosine kinases results in tyrosine phosphorylation of the STAT-3 transcription factor resulting in its dimerization and transport to the nucleus where it can activate its target genes. It is likely that CT-1 achieves its effect via a combination of these two signaling pathways [38].

The fact that CT-1 can have a protective effect in the isolated heart suggests its possible usefulness as a protective agent during heart reperfusion after surgery [39]. Clearly, any therapeutic use of CT-1 would be greatly facilitated if its protective effect could be separated from the potentially damaging hypertrophic activity that was the original cause of *CT-1* identification. Progress towards this end would be greatly facilitated by an understanding of the signaling pathways that are involved in the ability of CT-1 to stimulate hypertrophy and to produce protection. Most interestingly, current evidence indicates that there are two signaling pathways for CT-1; one is used predominantly to induce hypertrophy whilst the other is involved in the protective effect. It was shown that PD98059, an inhibitor of the p42/p44 MAPK pathway, was able to block the protective effect of CT-1 whilst having no effect on its ability to induce hypertrophy. Similarly, a dominant negative mutant of the MAPKK1, an upstream activator of p42/p44 MAPK, was able to block the protective effect of CT-1 without affecting the ability to induce hypertrophy [40].

5. dHAND and eHAND. A growing number of known transcription factors are expressed in chamber-specific patterns and are likely to be responsible for the specification of chamber identity. The basic helix–loop–helix transcription factors dHAND and eHAND are expressed predominantly in right and left ventricles during mouse development. Targeted mutations of *dHAND* and *eHAND* in mice have revealed novel pathways of organogenesis in mesodermal and neural crest derivatives. *dHAND* mutants exhibit hypoplasia of the right ventricle, branchial arches, and aortic arch arteries. The distinct nature of cardiac defects in *dHAND* mutants provides an entry into dissecting molecular pathways governing morphogenesis of specific components of the heart. Mice lacking *dHAND* have a hypoplastic right ventricle and abnormal development of vessels arising from the heart and cell death of craniofacial precursors [41].

6. LIM proteins. The LIM domain is a zinc finger structure that is present in several types of proteins,

including homeodomain transcription factors, kinases and proteins that consist of several LIM domains. Proteins containing LIM domains have been discovered to play important roles in a variety of fundamental biological processes including cytoskeleton organization, cell lineage specification and organ development, and also for pathological functions such as oncogenesis leading to human disease. LIM proteins that are nuclear, cytoplasmic, or shuttled between both compartments have been identified in many different species. These proteins contain one, two, or multiple LIM domains and can be categorized into different classes according to their amino acid homologies. According to the structure, LIM proteins have been classified in several classes. LIM-hd proteins contain two conserved N-terminal LIM domains. In numerous instances, these proteins have been shown to play important roles in cell fate decision and organ development. Nuclear LIM-only proteins consist of two LIM domains and little else and are thought to act as molecular adapter molecules linking proteins of various types together. These proteins possess important biological roles in development and oncogenesis. Two LIM kinases, Lmk1(kiz) and Lmk2, display two N-terminal LIM proteins linked to a C-terminal kinase domain. Members of the cytoplasmic cysteine-rich proteins (CRPs) family are evolutionarily conserved proteins that have been implicated in the processes of cell proliferation and differentiation. The subfamily members, CRP1, CRP2, CRP3/MLP, and CRIP, consist primarily of characteristic LIM domains that are each linked to a short additional conserved motif. Mice which lack the *CRP3/MLP* gene show disturbances of the cytoarchitecture of cardiac myofibers and the normal semicrystalline arrays of contractile proteins fail to organize [42].

FHL2 is a heart-specific member of the LIM domain gene family. It is an early marker of cardiogenic cells and a cardiac-specific LIM protein in the adult. *FHL2* transcripts are present at embryonic day (E) 7.5 within the cardiac crescent in a pattern that resembles that of *Nkx2.5* mRNA. During later stages of cardiac development and in adult animals, *FHL2* expression is localized to the myocardium and is absent from endocardium, cardiac cushion, outflow tract, or coronary vasculature. The gene encoding FHL2 was disrupted by homologous recombination and knockout mice devoid of *FHL2* were found to undergo normal cardiovascular development. FHL2 is not required for normal cardiac development [43].

COMBINATORIAL INTERACTION AMONG GATA-4, Nkx2-5, AND OTHER TRANSCRIPTION FACTORS

GATA factors may have later functions during cardiac development and may be redundant with one another. Potential GATA binding sites have been found in many cardiac specific gene promoters and GATA-4 is capable of synergizing with other transcription factors such as Nkx2-5 and SRF to activate cardiac-specific gene expression [44]. GATA-4 and Nkx2-5 are essential for normal heart development as targeted disruption of these genes in mice leads to embryonic death due to cardiac morphogenetic defects. Interestingly, gain-of-function studies in zebra fish and *Xenopus* indicate that ectopic expression of *Nkx2-5* results in enhanced myocyte recruitment but is not sufficient to initiate cardiac gene expression or differentiation, suggesting that *Nkx2-5* acts in concert with other transcription factors to specify the cardiac phenotype [45, 46]. The fact that *GATA-4* and *Nkx2-5*, two of the earliest markers of precardiac cells, are essential for heart formation and that over-expression of either one alone cannot initiate cardiogenesis, yet enhances recruitment and differentiation of committed precursors, raised the possibility that these proteins may be mutual cofactors [47]. GATA-4 directly interacts with the transcription factor Nkx2-5 to regulate expression of the *ANF* and *cardiac* α*-actin* promoter. This interaction is mediated by the C-terminal zinc finger domain of GATA-4 and helix III of the homeodomain of Nkx2-5. At the level of the *ANF* promoter, GATA-4 and Nkx2-5 are mutual cofactors as coexpression of *GATA-4* and *Nkx2-5* resulted in synergistic activation of the *ANF* promoter in heterologous cells. The synergy that requires the DNA binding site for both factors involves physical interaction between Nkx2-5 and GATA-4 as evidenced *in vitro* and *in vivo*. This interaction maps to the C-terminal zinc finger of GATA-4 and the C-terminus extension of Nkx2-5. Similarly, a C-terminally extended homeodomain of Nkx2-5 is required for GATA-4 binding. Remarkably, the other myocardial GATA factor, GATA-6, is unable to substitute for GATA-4 in binding or functional interaction with Nkx2-5 [48]. Thus, the molecular interactions between specific members of the GATA zinc finger family and Nkx2-5, which appear to be evolutionary conserved, may impart functional specificity to GATA factors and provide cooperative cross-talk between two pathways that are critical for the early events of cardiogenesis. Functional and physical interactions between GATA-4 and Nkx2-5 were also observed on the *ANF* and α*-actin* promoters.

GATA-4 is also a target of mouse Nkx2-5 [49]. Nkx2-5 synergistically interacts with GATA-4 at cardiac gene promoters and enhancers containing their binding sites in close proximity. Similarly, the mouse *Nkx2-5* gene is controlled by two enhancers containing GATA-4/5/6 binding sites [50]. Together these studies suggest that Nkx2-5 and GATA proteins may participate in transcriptional regulatory loops in which expression of both factors is reinforced throughout cardiac muscle development. Data support the presence of this loop and furthermore indicate that a functional Nkx2-5 is essential for its proper formation resulting in stable expression of *GATA-4* and subsequent cardiomyogenesis [51]. Additionally, the loss

of GATA-4 function in P19 cells blocked cardiomyogenesis at the cardioblast stage. *Nkx2-5* and *MEF2C* expression was down-regulated but not lost, consistent with the regulatory loop where Nkx2-5 is upstream of *GATA-4* [52].

A vast array of GATA-4, -5, and -6 interacting proteins has been described, including both DNA binding factors and general transcriptional activators and repressors. It is likely that such a wide array of interacting factors reflects transcriptional mechanisms whereby tissue-specific gene expression is orchestrated across various mesodermal and endodermal cell types. In addition, Nkx 2-5, GATA-4 also physically interacts by way of the C-terminal zinc finger with nuclear factor of activated T-cell-c4 (NFATc4) and MEF-2 in the regulation of cardiac gene expression. Such results suggest a paradigm whereby GATA-4 regulates heart-specific gene expression through complexes with other heart-expressed transcription factors. In Sertoli cells, GATA-4 was shown to physically interact with the nuclear receptor SF-1 leading to transcriptional synergy on the *Mullerian inhibiting substance* gene promoter. Finally, GATA-4 and -6 were shown to physically interact with one another in cardiac myocytes suggesting heterodimerization between GATA factors [53].

It is uncertain how the C-terminal zinc finger domain of GATA-4 is capable of mediating interactions with such a broad array of disparate transcription factors, especially because this same protein domain makes direct nucleotide contacts within the major groove of DNA. Despite this concern, it is formally possible that GATA-4 directly interacts with each of the characterized transcription factors as part of a cell type-specific complex. However, it is also possible that GATA-4 exists as a heterogeneous pool consisting of only one or a few of these cofactors at a time. Alternatively, GATA-4 may exist as a large complex with other transcription factors through an indirect association with general regulators of transcription such as p300/CBP. Consistent with this hypothesis, GATA-5 and -6 were each shown to physically interact with p300 resulting in transcriptional synergy, and CBP was reported to stimulate transcription dependent on GATA-4 [54]. More recently, GATA-4 was shown to interact with the transcriptional modifying protein friend of GATA-2 (FOG-2). This interaction involves N-terminal zinc finger of GATA-4.

In addition, members of the MEF2 family as other MADS-box proteins interact with a variety of transcription factors to activate diverse programs of gene expression. The most extensively studied interactions of MEF2 factors are with the members of the MyoD family of skeletal muscle bHLH proteins. The apparent ability of MEF2 to control the transcription of genes involved in muscle differentiation and cell proliferation is reminiscent of the function of another MADS-box protein, SRF. A variety of muscle-specific genes contain essential SRF-binding sites in their control regions, and SRF is also required for activation of serum-inducible genes such as *c-fos*. Furthermore, the *D-MEF2* gene was shown to be a direct target of *tinman*, and the level of *MEF2C* in *Nkx2-5* null mice heart is down-regulated by ~50%, which indicates that functional Nkx2-5 is essential for *MEF2C* expression in mammalian cardiogenesis [55].

Cardiac growth and development is a highly organized sequence of events that requires the correct spatial and temporal expression of specific sets of genes leading to the development of the heart. Transcription factors have been shown to serve as master switches for regulating the process of cardiogenesis. The study of cardiac gene expression and delineation of the mechanisms that maintain cardiac myocytes in terminally differentiated state are essential for future attempts to repair or replace damaged myocardial tissue. Further analysis will reveal more of the mechanisms and factors involved in cardiogenesis and will help to define regulatory networks involved in the formation of the heart.

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