Current Insights into Genetics of Congenital Heart Diseases: GATA and T-box Cardiac Transcription Factors as the Hotspot Pathogenesis

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The molecular mechanisms underlying spatiotemporal regulation of transcription during the cardiac development remain to be fully understood. The association of huge number of modifier genes, contribution of multiple cell types and progenitors, and complex form of 3D-structural morphology needs to be clarified. The intense investigations of cardiogenesis using in vivo models have revealed a cluster of transcription factors which have essential roles for cardiac development including GATA family of zinc finger proteins, GATA4, 5, and 6; T-box factors, including Tbx1, Tbx2, Tbx3, Tbx5, Tbx18, and Tbx20; the homeodomain protein Nkx2.5; MEF2 factors; the Hand family of bHLH transcription factors, dHand and eHand; and the Lim-homeodomain protein Isl1. These essential cardiac transcription factors interact each other and form the complex core transcriptional circuit to regulate the framework of cardiac development to establish the basic morphology of the heart. In this review, we focus on GATA and T-box transcription factors as the hotspot pathogenesis of congenital heart diseases with developmental background obtained from transgenic animal studies.

Keywords: mutation, second heart field, cardiac neural crest, stem cells, outflow tract defect

Introduction

Congenital heart defects (CHD) occur in nearly 1% of all live births and are the major cause of infant mortality and morbidity. Additionally, approximately 3 per 1,000 live births will require some intervention during the first year of life. Although the recent progress of comprehensive genetic studies could provide us a subset of etiologies of CHDs, the underlying pathogenesis of many CHDs has not been fully uncovered. The formation of the heart proceeds by sequential gene regulatory steps that dictate cell fates and organize specialized cell types into complex 3-dimensional units of structure and function. In order to explore the pathology of CHD, an approach focusing on the individual modular steps in cardiovascular morphogenesis is important, since most CHDs result from defective malformation in specific structural components of the developing heart and vessels. Series of studies about the disease modeling using transgenic animals provided us the insights into the cardiogenesis via regulation of essential cardiac transcription factors. Furthermore, human genetic studies revealed that the mutations of these cardiac transcription factors are frequently found in patients with familial CHDs. Among the cardiac transcription factors, members of the GATA family of zinc finger transcription factors and T-box transcription factors are thought as the core regulatory molecules for cardiogenesis and, to date, these factors are common genetic causes of various CHDs.

Developmental Origins of the Heart

Current concept for developmental origins of the heart is shown in Fig. 1. Cells derived from the anterior lateral plate mesoderm form a crescent shape, called as the “first heart field (FHF),” at approximately embryonic (E) day 7.5 in the mouse embryo, corresponding to week 2 of human gestation. By E8.0 in mice, or 3 weeks in humans, the FHF cells form a primitive heart tube, con-
sisting of an interior layer of endocardial cells, an exterior layer of myocardial cells, and the cardiac jelly, the extracellular matrix for reciprocal signaling between the two layers. The FHF eventually contributes exclusively to the left ventricle and a part of atria.

In addition to the FHF, the second source of cardiac progenitor cells, termed the "second heart field (SHF)" lies medially to the cardiac crescent. During the primitive heart tube formation, the heart tube derived from the FHF may provide a scaffold upon which cells from the SHF migrate into both the arterial and venous poles of the heart tube. Upon rightward looping of the heart tube, SHF cells cross the pharyngeal mesoderm into the anterior and posterior portions, populating a large portion of the outflow tract (OFT) of the heart, the future right ventricle, and a part of atria.

Cardiac neural crest cells (cNCCs) originate from the dorsal neural tube between the mid-otic placode and the caudal boundary of the third somite. After they delaminate from the dorsal neural tube, cNCCs migrate into the caudal pharyngeal arches, and the OFT where they aggregate and form the OFT septum to separate the truncus arteriosus into two great arteries. cNCC is also localized to pharyngeal arches 3, 4 and 6, which give rise to the future great vessels. Many signaling pathways are involved in the migration and the condensation of cNCC, including reciprocal signaling between cNCC and the SHF that are essential for the development of the OFT and the aortic arch.

The fourth lineage of cardiac precursor cells is derived from the proepicardium (PE). The PE develops from the coelomic mesothelium that overlays the liver bud. During PE growth and epicardial formation, some PE/epicardial cells undergo an epithelial-mesenchymal transformation and are moved into the subepicardial space, giving rise to the precursors of the coronary vessels and connective tissue cells.

The GATA Family

The GATA family is one of the major groups of zinc finger superfamily of transcription factors and conserves two zinc fingers that are necessary for binding to the unique DNA sequence (A/T)GATA(A/G) and the interaction with other transcription factors. In the GATA family, GATA4/5/6 are known to contribute to the cardiac development. GATA4 is the most investigated member, which is the predominant transcript in cardiomyocytes at all stages. Mice lacking Gata4 show embryonic lethality by E10.5 due to abnormal ventral folding, failure of midline fusion of the heart primordia, and extensive endoderm defects. Decreasing expression of Gata4 leads to abnormal cardiac development with atrioventricular septal defect (AVSD), double outlet right ventricle (DORV), and hypoplasia of the ventricular myocardium in a dose-dependent manner.

Null mutations in Gata5 caused hypoplastic hearts and partially penetrant bicuspid aortic valves (BAV) formation. Gata5 is essential for the endocardial differentiation in a mouse cell line derived from cardiac mesoderm. In fact, expression pattern of Gata5
becomes restricted to the endocardium during development.\textsuperscript{14} Endocardial cell-specific inactivation of Gata5 led to BAV like null mutants.\textsuperscript{15}

Gata6 null mice die at E5.5–7.5 due to defects in extra-embryonic endoderm.\textsuperscript{15, 16} Other knockdown experiments showed that GATA6 is required for differentiation of the cardiac lineage during embryogenesis in Xenopus and zebrafish.\textsuperscript{17} These results suggest that GATA6 is required at the earliest stage of development among the GATA family of proteins.

To date, the implication of GATA6 and GATA4 in CHD, as well as their genetic interaction, has been suggested. Genetic disruption of Gata6 in mice results in early embryonic lethality due to defects of endodermal differentiation. Conditional inactivation of Gata6 specifically in cNCCs causes persistent truncus arteriosus (PTA), suggesting an essential role of Gata6 during OFT development.\textsuperscript{18} Mice that are compound heterozygous for Gata4 and Gata6 null alleles die in utero and exhibit a spectrum of CHD, including septal and OFT defects.\textsuperscript{19} It has also been shown that Gata6 and Gata4 regulate their expression each other during development and that Gata6 may function in concert with Gata4 to direct tissue-specific gene expression essential for formation of the mammalian heart.

Besides the interaction between GATA4 and GATA6, many of the combinatorial interactions between the GATA factors and other cardiac transcription factors have been defined. Numerous studies have shown that Gata4 and Nkx2.5 directly interact to regulate the expression of the ANF, α-cardiac actin and cardiac restricted ankyrin repeat protein (CARP).\textsuperscript{20–23} Serum response factor (SRF) can physically interact with Gata4/5/6 or Nkx2.5 to synergistically activate the ANF and/or α-cardiac actin genes in cardiomyocytes.\textsuperscript{24–27} Gata4 recruited a subfamily of the MADS-box, myocyte enhancer factor-2 (MEF2) proteins to the ANF, α-cardiac actin, α-myosin heavy chain and BNP promoter and interact to activate the promoter synergistically.\textsuperscript{28} Gata4 also co-operate the ANF promoter with one of the cardiac essential T-box factor, Tbx5 similar to Nkx2.5,\textsuperscript{29} suggesting that a complex consisting of Tbx5, Nkx2.5 and Gata4 may function in the regulation of a subset of cardiac genes. In the mouse, Gata5 is the predominant factor expressed with Tbx20 in the endocardium and it has been shown to regulate endocardial differentiation in vitro,\textsuperscript{14} suggesting that the regulatory relationship between Tbx20 and Gata5 may be important in endocardial development.\textsuperscript{30} In summary, combinatorial interactions with other transcription factors involved in cardiogenesis are regulated by spatio-temporal and gene specific manner. The cardiac GATA factors orchestrate the synergistic regulatory cascade as central core factors.

Garg et al. first reported GATA4 mutations in patients with CHD and they found disturbance of interaction of GATA4 mutant proteins with TBX5.\textsuperscript{29} This study implicates a cooperative role for GATA4 and TBX5 in cardiac septation. Further linkage analyses were used to demonstrate associations between GATA4 mutations and multiple cardiac defects including atrial septal defect (ASD) and ventricular septal defect (VSD). As for GATA5, several genetic analyses revealed the association of GATA5 mutation with spectrum of CHDs similar to GATA4 mutation.\textsuperscript{31, 32}

Recently, we screened mutations of cardiac transcription factors in non-syndromic patients with PTA, and identified 2 different GATA6 mutations in 2 probands, but not in 182 unrelated controls with no CHD.\textsuperscript{33} Our subsequent biological analyses revealed that the expression of a neurovascular guiding molecule, SEMA3C, and its receptor, PLXNA2, were directly regulated by GATA6, and that both GATA6 mutant proteins failed to transactivate these genes. Transgenic analysis further suggested that the expression of SEMA3C and PLXNA2 in the OFT was dependent on GATA transcription factors during heart development. Together, our data implicate mutations in GATA6 as novel genetic causes of CHD involving the OFT, as a result of disruption of the direct regulation of semaphorin–plexin signaling (Fig. 1). After our study, there have been series of reports on mutations in GATA6 associated with CHD. Maitra et al. identified 2 novel sequence variations in GATA6 (A178V and L198V) in 2 individuals with tetralogy of Fallot (TOF) or AVSD from the screening of 310 individuals with non-syndromic CHD.\textsuperscript{34} Biochemical analysis demonstrated that the GATA6 A178V mutant protein resulted in increased transactivation ability for cardiac genes compared with the wild-type. Lin et al. identified a missense mutation (S184N) in GATA6 in 2 individuals with TOF or ASD.\textsuperscript{35} Allen et al. reported GATA6 mutations in patients with CHD including VSD, ASD or TOF complicated with pancreatic agenesis as well as congenital biliary tract anomalies, gut developmental disorders, and additional endocrine abnormalities.\textsuperscript{36} In mouse
model, Gata6 and Gata4 contribute to the development of endoderm/mesoderm specification and differentiation.\textsuperscript{11, 37} Recent genetic analysis identified multiple GATA6 mutations in patients with CHD including TOF with pancreatic anomaly.\textsuperscript{38, 39} These findings implicate GATA6 in the development of multiple organ systems, including heart, especially the OFT and intracardiac septum, the biliary tract, gut, pituitary and thyroid, as well as the pancreas. Mutations of GATA genes associated with human CHD are summarized in Table 1.

### Table 1 Mutations reported in core cardiac transcription factors in patients with non-syndromic CHD

<table>
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<td>LVNC, DCM</td>
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The T-box Family

T-box factors belong to an evolutionary conserved family of transcription factors which function as repressors or activators. The spatiotemporal expression of T-box genes in the cardiogenic region modulates key steps for genesis of the cardiac components. These factors play an essential role in the development of cardiac progenitor cells as well as in the patterning of chambers. Tbx1/2/3/5/18/20 are the major factors that are associated with the heart development in mammals, birds, fish, and amphibians.

The expression program in the SHF appears to be modulated by T-box factors. Recent progress of transgenic mouse model revealed that a T-box transcription factor, Tbx1, is a major genetic determinant of cardiac and craniofacial disorders associated with Tbx1 is expressed in the SHF, but not in the FHF or cNCC. OFT defects, typically PTA and TOF, and aortic arch anomalies are highly associated with 22q11DS, as well as craniofacial defects including cleft palate. Tbx1 null mice phenocopy the 22q11DS phenotype. Tissue-specific disruption of Tbx1 in the expression domain of Nkx2.5 developed the single OFT with no evidence of an aortopulmonary septum, suggesting that loss of Tbx1 function in the SHF or pharyngeal endoderm may result in OFT defects.

The expression of Tbx1 in the SHF is directly regulated by forkhead-containing (Fox) transcription factors, Foxa2, Foxc1 and Foxc2. The Tbx1 hypomorphic mouse demonstrated that the development of the OFT was sensitive to Tbx1 dosage. In experiments using knockout mice, Foxc1 and Foxc2, and Foxh1 were involved in the development of the OFT, although Foxa2-null mice die too early in utero to study cardiac development. Foxc1 or Foxc2 null mice and compound heterozygous mice for Foxc1 and Foxc2 display aortic arch defects reminiscent of Tbx1 heterozygous mice. Like Tbx1 hypomorphic mice, they also display a reduction in the size of the OFT, suggesting a dose-dependent regulatory mechanism of Foxc1 and Foxc2 in the SHF. Moreover, embryos homozygous-null for both Foxc1 and Foxc2 display an absence of the OFT and right ventricle associated with downregulation of Tbx1 and its downstream effectors, Fgf8 and 10.

Tbx18 is expressed in progenitors that contribute to the PE and the mesenchyme that borders the inflow tract, specifically the sinus venosus. Coronary smooth muscles and cardiac fibroblasts are also derived from Tbx18 positive progenitors.

Tbx20 is expressed in a subset of SHF precursors and mice deficient for Tbx20 show outflow tract defects with short heart tubes, suggesting failure in the development of SHF progenitors. A protein–protein interaction between Tbx20 and Gata4 induces Nkx2.5 and Mef2c in the right ventricle and outflow tract, whereas these factors were severely downregulated in Tbx20 null embryos with an ectopic upregulation of Tbx2. Tbx2 represses the ventricular chamber specific markers via an interaction with Tbx and contributes to specify the non-chamber region including segmentation of atrioventricular boundary. These results suggest that Tbx20 contributes to the stable expression of chamber specific genes by inhibition of Tbx2. Tbx20 also plays a role for the proliferative expansion of the chambers via induction of Nmyc and thought to be a key factor for the development of embryonic myocardium.

Recently, we performed genetic tests in left ventricular non-compaction (LVNC) cardiomyopathy patients and identified two independent TBX20 mutations from a family with LVNC and another isolated patient. LVNC has a unique structural phenotype including a characteristic deep and extensive hypertrabeculation of the LV. LVNC has been theorized to result from the arrest of compaction of the developing LV myocardium, as it passes through several distinct evolutionally conserved steps, and thought to have a potential as the good model to understand the developmental mechanism of myocardium. We generated patient-specific induced pluripotent stem cells (iPSCs) from the family members and induced cardiac differentiation. LVNC patient-specific iPSC-derived cardiomyocytes have decreased cell proliferation which prevents proper development of the embryonic heart and which is associated with abnormal upregulation of TGFβ signaling. Abnormal activation of TGFβ signaling in embryonic myocardium causes growth arrest of the developing heart in vivo. Functional disturbance of TBX20 prevents proper regulation of TGFβ signaling and contributes to the LVNC phenotype. Importantly, we showed that LVNC iPSCs could be rescued using drugs targeting TGFβ signaling or by genetic modification of the TBX20 mutation. These results clearly show the essential role of TBX20 in
embryonic myocardium growth and ventricular chamber development. This study suggests that iPSC technology is a powerful tool for uncovering the pathological mechanisms of CHD (Fig. 2).

Tbx5 is an essential factor for the morphological development of the heart as well as the development of the cardiac conduction system.\textsuperscript{62} Like the other core cardiac transcription factors, Tbx5 is involved in multiple transcription factor pathways and combinatorial interactions with other cardiac transcription factors. Tbx5 knockout mouse embryos have abnormal heart tube formation with hypoplastic atria.\textsuperscript{63} Mice with haploinsufficiency of Tbx5 have ASD with or without VSD, and atrioventricular (AV).\textsuperscript{64} Mice doubly heterozygous for Tbx5 and Gata4 have growth retardation and early neonatal lethality with AVSD and myocardial thinning, similar to the human GATA4 mutant phenotype.\textsuperscript{64} Interactions between Tbx5/20, Gata4 and Nkx2.5 have an important role in the activation of chamber specification and upregulation of the chamber-specific markers, including Nppa.\textsuperscript{65, 66}

Regarding the CHD in human, TBX1 mutations are associated with the cardiac phenotype of DiGeorge syndrome (DGS), including outflow tract and conotruncal defects as well as great vessel mis-patterning.\textsuperscript{41, 42} Mutations in TBX5 are linked to the Holt–Oram syndrome (HOS) characterized by forelimb and heart defects. The aberrant cardiac phenotype manifests in atrial and ventricular septal defects as well as conduction system disturbance.\textsuperscript{67} TBX20 mutations correlate with aberrant valvulogenesis and septal defects.\textsuperscript{68, 69} These mutations, however, can lead to either a gain or a loss of function, suggesting an important role of gene and protein dosage in regulating different aspects of chamber formation and subsequent function of the heart.\textsuperscript{68} Interestingly, TBX3 mutations are correlated with the ulnar-mammary syndrome (UMS) with rare evident cardiac defects only two case studies identified UMS patients with overt ventricular septal defects (VSD) as well as cardiac conduction disturbance\textsuperscript{70, 71}. This may be due to a functional redundancy with TBX2. To date, there is no report about the mutation in TBX2 gene, however, recent two studies showed that duplication or deletion of genomic region including TBX2

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Fig. 2 A new strategy for the exploration of pathological mechanisms underlying poorly understood developmental cardiovascular defects

The combination of multifaceted assessment including genetic screening, \textit{in vitro} modeling with induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), \textit{in vivo} modeling with transgenic mouse and observation with human heart tissue sample will provide us the solid evidence for the determination of the pathological feature of developmental cardiovascular defects.
gene may be associated with CHD in human\textsuperscript{72,73}. TBX3 may also be associated with the left ventricular mass in human\textsuperscript{24}. Mutations of T-box genes associated with human CHD are summarized in Table 1.

**Concluding Remarks**

Through the use of animal models combined with human genetic investigations, a molecular framework via core transcription factors has gradually uncovered the detailed steps of cardiovascular morphogenesis. Such advances lead to an approach to the etiology of “multifactorial” CHD in the post-genomic era. However, it is still challenging to use the resources derived from cardiac tissues or cells of patients with CHD and reveal molecular mechanisms underlying the pathogenesis of 3D-structural CHD in human. Recent progress of comprehensive analysis using deep-sequencing would provide more detailed network between core cardiac transcription factors and their effectors that influence the phenotype, and eventually lead to the preventive and/or regenerative medicine for CHD.

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**Conflicts of Interest**

The authors have no conflicts of interest to declare.

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