Cardiac Developmental Biology: From Flies to Humans

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Abstract: The heart is the first organ to form during embryogenesis, and heart formation is essential for subsequent embryonic development. Since the identification of a cardiac-restricted transcription factor *Csx/Nkx-2.5* in the early 1990s, extensive studies on cardiac development have been done in various species ranging from flies to hu-

mans. Molecular dissection of regulatory pathways that control multiple steps of cardiogenesis will not only advance our understanding of cardiac development and congenital heart diseases, but will also provide an important clue to novel therapeutic strategies for heart diseases. [The Japanese Journal of Physiology 55: 245–254, 2005]

The heart is the first organ to form during embryonic development, and the supplementation of oxygen and nutrients via the vascular system following heart formation is critical to embryogenesis. Classical embryology, in which morphological studies or tissue transplantation experiments were predominantly performed, has explored the precise timing and location of organogenesis and the interactions between different tissues or germ layers that are required for the proper formation of specific organs. However, little information has been provided by such studies regarding molecules or signaling pathways that regulate specific organ development during embryogenesis. The identification of a cardiac-restricted transcription factor *Csx/Nkx-2.5* more than a decade ago prompted a number of researchers to investigate the molecular mechanisms of cardiac development, leading to a great advance in our understanding of the signaling molecules, transcription factors, and growth factors that participate in multiple steps of cardiogenesis.

Cardiogenic Transcription Factors

Tinman: a *Drosophila* cardiac-restricted transcription factor. In the late 1980s, the MyoD family of basic helix-loop-helix transcription factors were identified as critical master regulators of skeletal muscle differentiation [1]. Although cardiac and skel-

etal muscle cells share some common features in terms of contractile protein expression, all the efforts to isolate MyoD-like transcription factors in the heart were unsuccessful. Meanwhile, a *Drosophila* gene called *NK-4/msh-2* was reported to be expressed predominantly in the heart [2]. *Drosophila* heart is formed on the dorsal side of the embryo by the fusion of a pair of heart primordia (cardiac mesoderm) that originates from the dorsal most part of the mesoderm. Unlike mammalian heart, the mature fly heart is a tube-like structure (also called “dorsal vessel”) in which cardiac cells form the inner layer and pericardial cells form the outer layer of the tubular structure (Fig. 1). *Drosophila* has an open circulatory system, and the dorsal vessel pumps the hemolymph from tail-to-head orientation by rhythmical contraction. *NK-4/msh-2* is expressed in the entire mesoderm in the early stage of development, and subsequently its expression becomes restricted to cardiac mesoderm and the heart. Furthermore, it was reported in 1993 that loss-of-function mutation of *NK-4/msh-2* results in loss of heart formation, indicating that *NK-4/msh-2* is a cardiac-restricted transcription factor that is essential for *Drosophila* heart development [3, 4]. *NK-4/msh-2* gene was therefore re-named *tinman* after a character in *The Wizard of Oz* that has no heart.

***Csx/Nkx-2.5*: a vertebrate Tinman homolog.** Following the characterization of *tinman* as an essen-

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Fig. 1. *Drosophila* heart development. *Drosophila* heart precursor cells derive from the dorsal part of the mesoderm, and two heart primordia fuse in the dorsal midline to form fly heart (dorsal vessel). Cardiac cells (red) constitute the inner layer and pericardial cells (green) constitute the outer layer of the heart, respectively. A, anterior; P, posterior; D, dorsal; V, ventral.

tial gene for fly heart formation, a murine homolog of *Drosophila tinman* was isolated in 1993 independently by two groups and named *Csx* (cardiac-specific homeobox) or *Nkx-2.5* (hereafter referred to as *Csx/Nkx-2.5*) [5, 6]. In vertebrates, two heart primordia are situated in the anterior lateral mesoderm (also called cardiac crescent), and they fuse at the midline on the ventral side to form a linear heart tube as a result of lateral-to-ventral folding of the embryo. Subsequently, the linear heart tube undergoes rightward looping and atrial/ventricular septa are formed, leading to the formation of a four-chambered heart (Fig. 2). The expression of *Csx/Nkx-2.5* starts to be observed in the anterior lateral mesoderm at embryonic day 7.5 and is maintained at high levels exclusively in cardiac muscle cells throughout life. The isolation of *Csx/Nkx-2.5* genes from fish, frog, chick, and human revealed that the expression pattern and amino acid sequences of *Csx/Nkx-2.5* are highly conserved in evolution [7–10]. Based on this cardiac-restricted expression pattern and its homology with *Drosophila tinman*, *Csx/Nkx-2.5* was expected to be an essential gene for vertebrate heart formation. Indeed, targeted disruption of *Csx/Nkx-2.5* resulted in embryonic lethality because of the arrested development of the heart. Detailed analyses of the hearts of mutant mice revealed that heart development was arrested at the looping stage, associated with left ventricular hypoplasia [11, 12]. These data, on one hand, indicate that *Csx/Nkx-2.5* is essential for vertebrate heart development. On the other hand, however, unlike *tinman* mutant in flies, differentiation of mesodermal cells into cardiomyocytes was not greatly affected (except for several genes such as *myosin light chain 2v*, whose expression was reduced in *Csx/Nkx-2.5* mutant mice). Because several *tinman*-

related genes besides *Csx/Nkx-2.5* have been identified in vertebrate genomes, the phenotypic differences between *tinman* mutant flies (no heart formation) and *Csx/Nkx-2.5* mutant mice (abnormal morphogenesis of the heart) may be due to genetic redundancy among these *tinman*-related genes in mice. Supporting this notion is the finding that injection of *Xenopus* embryos with a dominant-negative form of *Csx/Nkx-2.5* (which is expected to inhibit the functions of several *tinman*-related genes including *Csx/Nkx-2.5*) results in a complete loss of heart formation [13]. Another possibility is that *tinman* and *Csx/Nkx-2.5* diverged in terms of their functional roles in cardiogenesis during evolution, because expression of *Csx/Nkx-2.5* in *tinman* mutant flies failed to rescue the defective cardiac development [14, 15].

GATA-4/5/6 and *Drosophila* pannier. GATA-4/5/6 belong to a subfamily of zinc-finger containing GATA transcription factors and are expressed predominantly in the heart and gut [16]. Like *Csx/Nkx-2.5*, *GATA-4* starts to be expressed in cardiac mesoderm at around E7.5 and continues to be expressed in the heart at high levels. The identification of functional GATA binding sites in the regulatory region of several cardiac-specific genes have strongly suggested the essential roles of GATA-4/5/6 in heart development and cardiac-specific gene expression. However, targeted disruption of mouse *GATA-4* gene resulted in embryonic lethality and abnormal cardiac morphogenesis (cardia bifida: two hearts on both sides of the embryo) because of a defect in ventral fusion of the embryo [17, 18]. In these mutants, most of cardiac-specific genes were expressed regardless of the abnormal morphology of the heart. *GATA-5* knockout mice exhibited no cardiac defects [19], and *GATA-6* null mice die during early em-

Fig. 2. Vertebrate heart development. Heart precursor cells are derived from the anterior lateral mesoderm (cardiac mesoderm), and two heart primordia fuse in the ventral midline to form the linear heart tube. At this stage, cardiac chamber specificity is already determined along the anteri-

or-posterior axis, where the future left ventricle and atria are situated in the posterior part of the linear heart tube. After rightward looping and septum formation, a four-chambered heart is formed. Ao, aorta; PA, pulmonary artery; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

bryonic development (E5.5–7.5), making it difficult to evaluate the role of GATA-6 during heart development [20,21]. Of note, zebrafish with mutations for *GATA-5* gene exhibit similar phenotypes with mouse *GATA-4* knockouts (i.e., cardia bifida) as a result of defective endoderm formation [22], suggesting that there was a reversal of the roles of GATA-4 and GATA-5 between fish and mice during evolution.

In *Drosophila*, a GATA family of transcription factor called Pannier is expressed in cardiac mesoderm and the heart. A loss-of-function mutation of *pannier* results in the lack of cardiac cells, whereas overexpression of Pannier increases the number of cardiac cells. Co-expression of Tinman and Pannier further enhances the effects of Pannier overexpression. Functional substitution of Pannier by mouse GATA-4 was also demonstrated [23]. These findings indicate that *pannier* is an essential gene for heart development in flies and suggest that its function is related to vertebrate *GATA-4* gene. They also suggest the existence of a synergistic interaction between Tinman and Pannier. Likewise, physical interaction and synergistic action on downstream target genes have been reported on *Csx/Nkx-2.5* and *GATA-4* [24–28]. Besides these physical and synergistic interactions, there are upstream-downstream genetic relationships between Tinman/*Csx/Nkx-2.5* and Pannier/*GATA-4/5/6*: (i)

pannier is a direct target of Tinman, and *pannier* expression is induced by Tinman [29]; (ii) *Csx/Nkx-2.5* promoter contains functional GATA binding sites and is transactivated by GATA-4/5/6 [30, 31]; and (iii) *GATA-6* promoter, on the other hand, is transactivated by *Csx/Nkx-2.5* [32, 33]. These genetic relationships between *Csx/Nkx-2.5* and GATA family of transcription factors presumably provide a framework for mutually reinforcing regulatory network that maintains the expression of cardiogenic transcription factors once mesodermal cells are committed to a cardiac lineage.

D-MEF2 and MEF2C. MEF2 belongs to a family of MADS-box containing transcription factors. Mammalian genomes contain four *MEF2* genes (*MEF2A-D*), and *Drosophila* has a single *MEF2* gene, *D-mef2*. During the early stage of embryogenesis, *D-mef2* is expressed in the entire mesoderm, and subsequently its expression is restricted to somatic and visceral muscle precursor cells. In the heart, *D-mef2* is expressed in cardiac cells in the later stage of development [34, 35]. *D-mef2* gene is a direct target of Tinman and Pannier, and the expression of *D-mef2* in cardiac cells is regulated by these factors [29, 36]. In *D-mef2* mutant flies, although a heart-like structure is formed, the differentiation and maturation of cardiac cells are disturbed, and the expressions of contractile proteins in the heart are missing [37]. Thus D-MEF2 controls cardiomyo-

cyte terminal differentiation in *Drosophila* heart.

In mice, targeted disruption of *MEF2C* results in embryonic lethality because of right ventricular hypoplasia [38]. In these animals, the expressions of several contractile protein genes are reduced, suggesting that MEF2C plays a similar role in mice with D-MEF2 in *Drosophila* in the control of cardiomyocyte terminal differentiation. In contrast, *MEF2A* mutant mice exhibit sudden cardiac death resulting from mitochondrial defects [39], and *MEF2B* mutant mice exhibit no major phenotypes [40]. Since the expression patterns of *MEF2A-D* are grossly overlapping and DNA binding affinity and specificity to MEF2 elements are similar among these factors, the precise mechanisms by which deletion of different MEF2 factors have divergent effects are now unclear. Although *MEF2C* expression is reduced in *Csx/Nkx-2.5* knockout mice [12], it is also unclear whether *MEF2* genes are direct downstream targets of *Csx/Nkx-2.5* and/or GATA factors in mice, as is the case in flies.

SRF, myocardin, and Hop. Serum response factor (SRF) is another MADS-box containing transcription factor that binds to the core sequence of a serum response element/CAR_G box (CC[A/T]₆GG). Although SRF is not a cardiac-restricted transcription factor, functional CAR_G elements have been identified in several cardiac-specific genes. Further, SRF is preferentially expressed in cardiac mesoderm during the early stage of embryogenesis [41]. It also physically interacts with *Csx/Nkx-2.5* or GATA-4 and coordinately regulates downstream target genes [42, 43]. These findings have suggested important roles for SRF in heart development and cardiac-specific gene expression. Indeed, cardiac muscle cell-specific SRF knockout resulted in embryonic lethality because of abnormal cardiac morphogenesis associated with reduced expression of *Csx/Nkx-2.5* and *GATA-4*, indicating that SRF is also required for normal heart development [44]. Myocardin and Hop were identified as SRF co-factors, and myocardin positively regulates SRF-dependent transcription, whereas Hop negatively regulates it [45–47]. Overexpression of dominant-negative myocardin in *Xenopus* results in a loss of heart formation, whereas myocardin knockout mice exhibit no cardiac phenotype [45, 48]. This apparent discrepancy is presumably due to the genetic redundancies between myocardin and myocardin-related transcription factors in the heart [49].

Hand1/eHand and Hand2/dHand. Hand1/eHand and Hand2/dHand belong to a family of basic helix-loop-helix transcription factors and are ex-

pressed in cardiac mesoderm during embryogenesis. Subsequently, the expression of *Hand1* becomes restricted to a future left ventricle, whereas that of *Hand2* becomes restricted to a future right ventricle [50]. Targeted disruption of *Hand2* results in embryonic lethality resulting from right ventricular hypoplasia that is similar to *MEF2C* knockout phenotype [51]. *Hand1* deletion results in early embryonic lethality because of placental defect, making it difficult to explore the role of Hand1 in cardiac development [52, 53]. However, *Hand1* null embryos obtained by tetraploid rescue to bypass the placental defect exhibit a cardiac phenotype similar to that of *Csx/Nkx-2.5* knockout mice (left ventricular hypoplasia) [53]. Consistent with this, *Hand1* expression is reduced in *Csx/Nkx-2.5* mutant mice [12]. Furthermore, combined deletion of *Hand2* and *Csx/Nkx-2.5* results in hypoplasia of both ventricles [54]. Taken together, these findings suggest that *Hand* genes are important for ventricular chamber specification and that Hand1 and Hand2 play critical roles in the formation of left and right ventricles, respectively. Cooperative regulations of target genes through physical interactions between Hand2 and *Csx/Nkx-2.5* or GATA-4 have also been reported [55, 56], suggesting the existence of complex protein-protein interactions among multiple transcription factors in the regulation of cardiac development and cardiac-specific gene expression.

Tbx5 and Tbx20. *Tbx5* belongs to a family of T-box transcription factors and was originally identified as a gene mutated in Holt-Oram syndrome with congenital cardiac defects and/or limb deformity [57, 58]. *Tbx5* is expressed in the entire cardiac mesoderm during the early stage of development, and subsequently its expression becomes restricted to the posterior region of the heart (i.e., left ventricle and atria) [59]. Targeted disruption of *Tbx5* in mice results in hypoplasia of the region where *Tbx5* is normally expressed [60], indicating that *Tbx5* is critical to the formation of the “posterior” part of the heart. Although the expressions of *Csx/Nkx-2.5* and *GATA-4* are reduced in *Tbx5* mutant mice, it is unclear whether these genes are direct targets of *Tbx5*. *Tbx5* physically interacts with *Csx/Nkx-2.5* and synergistically transactivates downstream target genes [60, 61]. *Tbx20* is another member of T-box transcription factors that is expressed in cardiac mesoderm and developing heart. At the stage when *Tbx5* expression is restricted to the posterior part of the heart, *Tbx20* is still expressed throughout the entire heart tube, including the outflow tract [62]. Targeted disruption of *Tbx20* results in embryonic

Fig. 3. Induction of *Csx/Nkx-2.5* expression by combined BMP and Wnt signals. During early chick embryogenesis, BMP is expressed in primitive streak and endoderm/ectoderm in the lateral side of the embryo. At the same developmental stage, Wnt inhibitors are expressed in the anterior part, whereas Wnts are expressed in the

posterior part of the embryo, respectively. Because BMP induces and Wnt inhibits *Csx/Nkx-2.5* expression, the expression domain of *Csx/Nkx-2.5* is restricted to the anterior lateral mesoderm where BMP and Wnt inhibitors are co-expressed.

lethality with perturbed cardiac morphogenesis [63–66]. Of note, *Tbx2* is ectopically expressed in the entire heart-forming region in *Tbx20* null mice. *Tbx2* is also a member of the T-box family normally expressed prominently in the atrioventricular (AV) canal, where it has been proposed to repress chamber-specific genes. These findings suggest that *Tbx20* represses another transcriptional repressor *Tbx2* to induce normal chamber specification and differentiation. Consistent with this notion, *Tbx5* activates whereas *Tbx20* represses cardiac-specific promoters such as *atrial natriuretic peptide* gene [62].

Cardiogenic Growth Factors

Interactions between different tissues or germ layers play important roles in organ development during embryogenesis. Using *Csx/Nkx-2.5* as a marker of cells committed to cardiomyocytes, it has been shown that soluble factors secreted from adjacent endodermal or ectodermal tissues are critical to the commitment of mesodermal cells into a cardiac lineage.

Dpp and BMP. In flies, decapentaplegic (Dpp) has been identified as a soluble factor that is capable of inducing *tinman* expression in the mesoderm. Dpp belongs to a TGF- β superfamily of growth factors and is most closely related to vertebrate bone morphogenetic protein (BMP)-2 and BMP-4. In the early stage of *Drosophila* embryogenesis, *dpp* is expressed in the dorsal ectoderm adjacent to the *tinman* expression domain in dorsal cardiac mesoderm [67]. Dpp mutant flies fail to maintain *tinman* expression in the dorsal mesoderm, whereas ectopic expression of Dpp in the ventral ectoderm results in the expansion of the *tinman* expression domain [67], indicating that Dpp is required for proper *tinman* expression in dorsal cardiac mesoderm. In chick embryos, BMP-2 is expressed in the anterior lateral endoderm (Fig. 3). The ectopic

expression of BMP-2 in the anterior medial mesoderm (where *Csx/Nkx-2.5* is not normally expressed) induces ectopic *Csx/Nkx-2.5* expression [68]. These data suggest the existence of signaling mechanisms conserved between invertebrates and vertebrates by which Dpp/BMP induces *tinman/Csx/Nkx-2.5* expression in cardiac mesoderm. Among the signaling pathways downstream of BMP, Smad- and TAK1-dependent pathways have been implicated in the induction of *Csx/Nkx-2.5* by BMP [69, 70].

Wg/Wnt and Wnt inhibitors. Wingless (Wg) in flies and the closely related Wnt ligands in vertebrates comprise a large family of secreted factors and play important roles in multiple aspects of embryogenesis. *Drosophila* Wg is required for the induction of cardiac mesoderm, because elimination of Wg function for a short time period after gastrulation using temperature sensitive *wg* mutant flies results in a selective loss of heart precursor cells [71]. This effect of Wg on heart development is mediated by the canonical Wnt pathway that leads to the activation of β -catenin/TCF-dependent transcription [72]. However, the regulation of heart development by Wnt signaling is rather complicated in vertebrates. In chick embryos, Wnt inhibitors expressed in the anterior part of the embryo are required for heart formation by suppressing canonical Wnt activity in the posterior part of the embryo [73]. Wnt signals from neural tube have also been shown to block heart formation in the anterior paraxial mesoderm [74]. In *Xenopus* embryos, ectopic expression of Wnt antagonist induces ectopic heart formation in non-precordial mesoderm, while expression of Wnt ligands inhibits heart formation [75]. Based on these findings, it was proposed that Wnt signals are negative regulators of cardiac development and that *Csx/Nkx-2.5* is expressed in the region where BMP and Wnt inhibitors are co-expressed (Fig. 3). In contrast to these observations, non-canonical Wnt signals activated by

Table 1. Roles of cardiogenic transcription factors during embryogenesis as revealed by gene targeting.

Transcription factor	Roles of cardiogenic transcription factors during embryogenesis
Csx/Nkx-2.5	Heart development, pharyngeal endoderm development
GATA-4/5/6	Ventral fusion of the embryo, visceral endoderm formation
MEF-2C	Heart development, vascular development
SRF	Primitive streak formation, heart and skeletal muscle development
Hand	Heart development, limb development, autonomic nervous system formation
Tbx5	Heart development, limb development

Wnt-11 are required for heart formation in *Xenopus* [76], and canonical Wnt signals are essential for in vitro differentiation of P19CL6 cells into cardiomyocytes [77, 78]. Taken together, although it is difficult to reconcile these apparently paradoxical observations in various species, one possible explanation is that Wg/Wnt signals at different developmental stages may have both positive and negative effects on cardiac development. The differential effects of canonical and non-canonical Wnt signals on heart development should also be taken into consideration.

FGF and FGF receptors. *Drosophila* has two fibroblast growth factor (FGF) receptors, and one is called Heartless. It can be seen from this nomenclature that heart formation is defective in *heartless* mutant flies [79, 80]. This phenotype, however, is thought to be due to a defect in migration of mesodermal cells. In fish, *fgf8* loss-of-function mutation results in a severe perturbation of heart development with reduced expressions of *Csx/Nkx-2.5* and *GATA-4*, suggesting that FGF signals are directly involved in zebrafish heart formation [81]. In chick embryos, FGF8 is expressed in the endoderm adjacent to the cardiac mesoderm. Expression of FGF8 can induce ectopic *Csx/Nkx-2.5* expression lateral to the heart field [82], suggesting that FGF signaling is capable of inducing heart formation in association with BMP signaling. Whether FGF signaling is required for cardiogenesis awaits further investigations.

Human Heart Diseases and Cardiogenic Transcription Factors

Several cardiogenic transcription factors have been implicated in human congenital heart diseases. Linkage analysis and candidate gene approach have identified human *CSX/NKX-2.5* as a causative gene for some forms of autosomal dominant familial heart diseases. More than 10 disease-related mutations in human *CSX/NKX-2.5* gene have been reported, and clinical manifestations of patients with these mutations

Fig. 4. Cardiogenic transcription factors and growth factors. Each step of heart formation during embryogenesis is shown by boxes, and transcription factors and growth factors implicated in distinct steps during cardiogenesis are indicated beside the arrows.

are rather divergent, including atrial septal defect, AV block, tetralogy of Fallot, ventricular septal defect, and hypertrophic cardiomyopathy [83–92]. Even in members of the same family with identical point mutations, the phenotypes are sometimes divergent, suggesting the existence of some modifier genes. Ventricular myocyte-specific *Csx/Nkx-2.5* deletion in mice results in hyper-trabeculation with AV conduction defect, and upregulation of BMP-10 in these animals appears to

be the underlying mechanism for accelerated trabecular formation [93]. As mentioned earlier, *TBX5* is a causative gene for Holt-Oram syndrome in humans, and cooperative transcriptional regulation between *Tbx5* and *Csx/Nkx-2.5* has been reported. Some Holt-Oram patients exhibit minimal cardiac anomaly with severe limb deformity, while others have extensive cardiac anomaly with minimal limb phenotype. Of note, *TBX5* mutants associated with the former group retain cooperative interactions with *CSX/NKX-2.5*, whereas those associated with the latter group lack such synergistic interactions [61], suggesting that the disruption of *TBX5-CSX/NKX-2.5* interactions may contribute to cardiac defects in Holt-Oram syndrome patients. Mutations in human *GATA-4* gene have also been identified as the cause of congenital heart diseases, including atrial septal defect [94]. Introduction of disease mutations in *GATA-4* results in decreased DNA binding, decreased transcriptional activity, and, notably, loss of cooperative interactions with *TBX5* [94]. Collectively, these observations demonstrate the critical roles of cardiogenic transcription factors in later stages of cardiac development. Given the essential roles of these transcription factors in the early stage of heart development, these findings indicate that cardiogenic transcription factors such as *Csx/Nkx-2.5*, *GATA-4*, and *Tbx5* play critical roles at multiple steps in cardiac development. They also suggest that physical interactions and transcriptional co-operations between these transcription factors are essential for normal heart development in vivo.

Summary and Conclusion

Since the identification of *Csx/Nkx-2.5* as a cardiac-restricted transcription factor, there was a great advancement in our understanding of individual molecules implicated in cardiac development and cardiomyocyte differentiation, and several transcription factors and soluble factors that are critical to heart development have been identified (Fig. 4 and Table 1). However, we still do not know how these players cooperatively regulate multiple steps of cardiogenesis. Moreover, accumulating evidence suggests that the heart has substantial regenerative capacity, in part through the contribution of resident or bone marrow-derived stem cell population. It is highly conceivable that insights gained from cardiac developmental biology will also provide valuable information on the regulatory mechanisms of stem cell-based cardiac regeneration.

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