

binding transcriptional activator (Kim & Nirenberg, 1989; Harvey, 1996). Four NK homeobox genes were originally identified (*NK1*, *NK2*, *NK3*, and *NK4*) in *Drosophila*, and thereafter, the NK homeobox gene family was classified into 2 subfamilies, NK-1 (*NK1* and its homologues) and NK-2 (*NK2*, *NK3*, *NK4*, and their homologues) class gene families (reviewed in Harvey, 1996).

In *Drosophila*, *NK4* (*tinman*) is expressed in presumptive mesoderm (Bodmer et al., 1990) and is required for cell fate specification of the dorsal vessel, the equivalent of the vertebrate heart, and visceral muscle (Azpiazu & Frasch, 1993; Bodmer, 1993). *Csx/Nkx2-5* was identified as a potential mammalian homologue of *tinman* (Komuro & Izumo, 1993; Lints et al., 1993). “*Csx*” is the acronym of cardiac-specific homeobox and represents its distinctive biological significance in myocardial cell lineage (Komuro & Izumo, 1993). “*Nkx2-5*” has originated from a taxonomical standpoint that it is the fifth vertebrate gene identified in the NK-2 homeobox gene family (Lints et al., 1993). Currently, both names are widely adopted, and a double name “*Csx/Nkx2-5*” is used in this paper. Tinman-related proteins have been identified in various species in vertebrates from *Xenopus* to humans (Komuro & Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994; Schultheiss et al., 1995; Chen & Fishman, 1996; Shiojima et al., 1996). These proteins commonly have highly conserved structure composed of the N-terminal TN domain, the homeodomain, and the NK-2-specific domain (NK2-SD), located just C-terminal to the homeodomain (reviewed in Harvey, 1996; Fig. 1). The homeodomain of *Csx/Nkx2-5* has a helix-turn-helix motif that binds to the specific consensus DNA sequence 5' T(C/T)AAGTG 3' (Chen & Schwartz, 1995). The 5' T(C/T)AAGTG 3' core is unique compared with the typical 5' TAAT 3' core for binding of homeoproteins, and its unique binding specificity is determined by the tyrosine residue at position 54 within the third helix of the homeodomain, characteristic of the NK-2 class homeobox gene family (reviewed in Harvey, 1996). The NK2-SD functions to mask the transcriptional activity in *in vitro* reporter assays. The NK2-SD is proline rich and may operate as an

interface of protein–protein interaction. The function of the TN domain remains unclear.

## 2.2. Involvement of *Csx/Nkx2-5* in cardiac development

As expected as a homologue of *tinman*, *Csx/Nkx2-5* is highly expressed in the early heart progenitor cells in both primary and secondary heart fields during murine embryogenesis and continues to be expressed at a high level in the heart through adulthood (Komuro & Izumo, 1993; Lints et al., 1993; Kasahara et al., 1998; Stanley et al., 2002). Especially, a transient elevation of *Csx/Nkx2-5* expression is observed in specialized myocardial conduction cells during the period of conduction system formation, suggesting a significant role of *Csx/Nkx2-5* in the development of the conduction system (Thomas et al., 2001).

The developmental role of *Csx/Nkx2-5* has been extensively investigated. In *Xenopus* embryos, the overexpression of either *XNkx2-5* or *XNkx2-3* caused an increase in heart size due to increased myocardial cell number, namely hyperplasia (Cleaver et al., 1996). Similarly, the injection of *Nkx2-5* into zebrafish embryos led to large hyperplastic hearts, and higher doses of *Nkx2-5* induced cells in ectopic locations to express a cardiac gene, although these cells did not beat (Chen & Fishman, 1996). These gain-of-function analyses indicate an important role of *Nkx2-5* in cardiogenic program, whereas it is unclear whether myocardial hyperplasia in embryos injected with *Nkx2-5* resulted from an increase in the proliferative activity of cardiac cells or from commitment of ectopic cells into cardiac cell fate. In contrast, transgenic overexpressing of *Csx/Nkx2-5* under the control of the cytomegalovirus enhancer/chicken  $\beta$ -actin promoter did not alter the heart size in adults (Takimoto et al., 2000). Although the heart sizes during embryogenesis were not investigated in *Csx/Nkx2-5* transgenic mice, the effects of *Csx/Nkx2-5* on the heart size might be dose and stage dependent and, more probably, variable according to the organisms.

Loss-of-function analyses by gene targeting in mice have undermined a distinctive role of *Csx/Nkx2-5* in regulating cardiogenesis. Three kinds of *Csx/Nkx2-5*-deficient mice

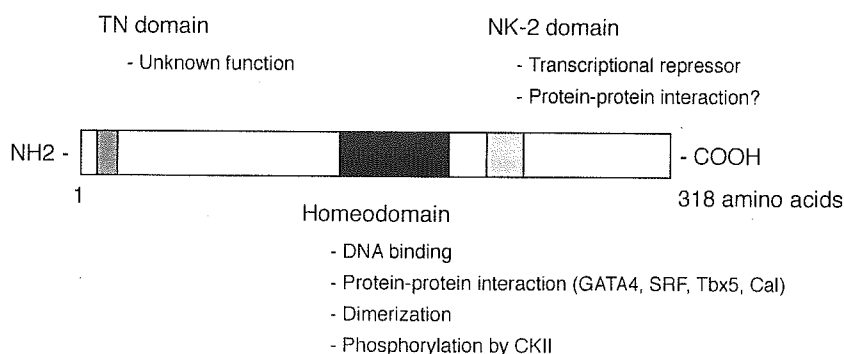


Fig. 1. Protein structure and functional domains of *Csx/Nkx2-5*. The homeodomain is indicated by a black box, TN domain with a dark grey box, and NK-2 domain with a light grey box.

harboring a different allele have been generated, and the phenotypes with regard to cardiogenesis were essentially the same (Lyons et al., 1995; Tanaka et al., 1999a; Biben et al., 2000). *Csx/Nkx2-5*-deficient mice were lethal around E9–10 due to the arrested looping morphogenesis of the heart tube and growth retardation (Lyons et al., 1995; Tanaka et al., 1999a). In contrast to *tinman*-mutant flies, commitment to cardiac lineage was not compromised in *Csx/Nkx2-5*-deficient embryos, because beating cardiomyocytes were present in normally formed linear heart tube. In vertebrates, there is a possibility that other NK-2 homeobox transcription factors are functionally redundant with *Csx/Nkx2-5* in adopting a cardiac cell fate. This idea is supported by the observation that *Csx/Nkx2-5*-related genes have been identified in embryonic hearts of flogs, zebrafish, chickens, and mice, which share similar and overlapping, but not identical, expression pattern with *Nkx2-5* (Tonissen et al., 1994; Evans et al., 1995; Buchberger et al., 1996; Lee et al., 1996; Brand et al., 1997; Reecy et al., 1997; Biben et al., 1998; Newman & Krieg, 1998; Newman et al., 2000). In zebrafish, the expression of *Nkx2-7* is first observed in cardiac mesodermal precursors and precedes those of *Nkx2-5* and *Nkx2-3*, suggesting a role of *Nkx2-7* in regulating *Nkx2-5* and *Nkx2-3* expressions (Lee et al., 1996). With regard to functional redundancy, the coinjection of dominantly acting repressor plasmids for *XNkx2-5* and *XNkx2-3* synergistically abrogated heart formation and cardiac gene expression in *Xenopus* embryos (Fu et al., 1998). Similar results were obtained in experiments using cultured P19 cells, which are derivatives of mouse embryonal carcinoma cells and undergo myocardial differentiation under treatment of dimethyl sulfoxide (DMSO). The expression of a dominant-negative mutant of *Csx/Nkx2-5*, consisting of *Csx/Nkx2-5* homeodomain fused to the repressor domain of *engrailed*, prevented the commitment of mesodermal cells into cardiac lineage during early P19 differentiation (Jamali et al., 2001b). Whereas murine *Nkx2-6*, a closely related gene to *Csx/Nkx2-5*, is redundantly expressed in the sinus venosa at E8.5 and in the outflow tract at E9.5 (Biben et al., 1998), no cardiac abnormalities were observed in *Nkx2-6*-deficient embryos (Tanaka et al., 2000). In *Csx/Nkx2-5* and *Nkx2-6* double mutants, atrial growth was significantly retarded with alterations in cardiac gene expression in atria (Tanaka et al., 2001), but atrial growth retardation is supposed to be secondary to the abnormal development of neighboring pharyngeal tissues because *Nkx2-6* is not expressed in atria. It remains to be determined how *Csx/Nkx2-5* in combination with other NK-2 transcription factors functions to induce the cardiogenic program. Alternatively, the deployment of *tinman* and *Csx/Nkx2-5* in cardiogenic program may have evolutionally diverged in spite of their close relationships. The introduction of *Csx/Nkx2-5* into the *Drosophila* germline failed to rescue the *tinman*-mutant phenotype, although the homeodomains were functionally interchangeable in this rescue assay (Park et al., 1998a; Ranganayakulu et al., 1998).

Whereas commitment to cardiac lineage was not hampered, the expression of several cardiac genes in the heart of *Csx/Nkx2-5*-deficient embryos (including *myosin light chain 2v* [*MLC2v*], *atrial natriuretic peptide* [*ANP*], *brain natriuretic peptide* [*BNP*], *cardiac ankyrin repeat protein* [*CARP*], *MEF2-C*, *eHAND/HAND1*, *N-myc*, *Iroquois homeobox gene 4* [*Irx4*], and *homeodomain-only protein* [*HOP*]) was reduced (Lyons et al., 1995; Biben & Harvey, 1997; Zou et al., 1997; Tanaka et al., 1999a; Bruneau et al., 2000; Shin et al., 2002; Table 1). These data indicate that *Csx/Nkx2-5* plays a crucial role in the transcriptional regulation of several sets of cardiac-specific genes, which are essential for embryonic myocardium to differentiate beyond the stage of looping morphogenesis. The reduced expression of chamber-restricted transcription factors such as *eHAND/HAND1* (Biben & Harvey, 1997) and *Irx4* (Bruneau et al., 2000) in *Csx/Nkx2-5*-deficient embryos implies that *Csx/Nkx2-5* reigns over the hierarchical cascade of cardiac transcription factors.

*dHAND/HAND2* and *eHAND/HAND1* are basic helix-loop-helix transcription factors that have distinctive roles in cardiac and extraembryonic development (reviewed in Srivastava, 1999). The expression of *eHAND* is predominant in the left ventricle and is excluded from the right ventricle. Analysis of *eHAND*-null mice defined an essential role of *eHAND* in myocardial differentiation of the left ventricle (Firulli et al., 1998; Riley et al., 1998; Riley et al., 2000). In contrast, the expression of *dHAND* is restricted to the right ventricle, and the development of the right ventricle was selectively compromised in *dHAND*-null embryos (Srivastava et al., 1997). In embryos deficient for both *Csx/Nkx2-5* and *dHAND*, only a single atrial chamber

Table 1  
Altered gene expressions in *Csx/Nkx2-5*-deficient hearts

Genes	Gene product
Down-regulated genes	
<i>MEF2C</i>	Transcription factor
<i>eHAND/HAND1</i>	Transcription factor
<i>Irx 4</i>	Transcription factor
<i>N-myc</i>	Transcription factor
<i>Myocardin</i>	Transcriptional coactivator
<i>CARP</i>	Transcriptional corepressor, titin-binding protein
<i>HOP</i>	Homeoprotein
<i>MLC2v</i>	Contractile protein
<i>ANP</i>	Natriuretic peptide
<i>BNP</i>	Natriuretic peptide
<i>Connexin 40</i>	Structural protein in gap junction
<i>minK</i>	Ion channel
<i>Chisel</i>	Undetermined
Up-regulated genes	
<i>BMP-10</i>	Morphogenic protein
<i>Sarcoplipin</i>	Inhibitor for sarcoplasmic reticulum calcium-ATPase
<i>Hcni</i>	Ion channel

*ANP*, atrial natriuretic peptide; *BNP*, brain natriuretic peptide; *CARP*, cardiac ankyrin repeat protein; *HOP*, homeodomain-only protein; *Irx4*, Iroquois homeobox gene 4; *MLC2v*, myosin light chain 2v; *MEF2C*, myocyte enhancer factor 2-C.

was formed with complete ventricular dysgenesis (Yamagishi et al., 2001). The importance of *Csx/Nkx2-5* in combination with *dHAND* in ventricular formation was strengthened by the observation that the expression of the ventricular-specific homeobox gene *Irx4* was completely abrogated in double mutants for *Csx/Nkx2-5* and *dHAND*.

During the late stage of cardiogenesis, *Csx/Nkx2-5* plays an important role in lineage specification and maturation of ventricular cardiomyocytes. To analyze the effects of loss of *Csx/Nkx2-5* beyond the midgestation when the conventional *Csx/Nkx2-5* knockout mice die, ventricular-restricted *Csx/Nkx2-5* knockout mice were generated (Pashmforoush et al., 2004). Ventricular-specific deletion was achieved by *MLC2v*-Cre-mediated recombination (Hirota et al., 1999) of the floxed *Csx/Nkx2-5* allele. *Csx/Nkx2-5*-deficient hearts showed hypertrabeculation and noncompaction of myocardium at birth, and mice exhibited progressive heart failure characterized by chamber dilatation with extensive trabecular formation. Microarray analysis revealed aberrant expressions of a number of genes, including the potential downstream targets in *Csx/Nkx2-5*-deficient hearts (Table 1). The up-regulation of bone morphogenic protein (BMP)-10 appears to be mechanistically important, because the overexpression of BMP-10 induced similar hypertrabeculation. In addition, aberrant expressions of *sarcolipin* and *HCN1* were observed in ventricular cardiomyocytes of *Csx/Nkx2-5*-deficient mice, whereas *sarcolipin* and *HCN1* are normally expressed in the atria and conduction system, respectively.

### 2.3. Regulatory pathways upstream of *Csx/Nkx2-5*

Insomuch as *Csx/Nkx2-5* is one of the earliest markers for cardiogenic cells and plays a crucial role in cardiac development, it is important to delineate regulatory pathways that control *Csx/Nkx2-5* expression during cardiogenesis. In *Drosophila*, signaling cascades by Decapentaplegic (Dpp) and Wingless (Wg) are involved in the induction of *tinman* expression and the formation of dorsal vessel (Staehling-Hampton et al., 1994; Frasch, 1995; Wu et al., 1995).

Dpp is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily and is closely related to vertebrate BMP-2 or BMP-4 (reviewed in Hogan, 1996). Dpp is secreted in the dorsal ectoderm overlying the mesoderm, where *tinman* is expressed, and functions as an inductive signaling to restrict and maintain *tinman* expression in dorsal mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). In chick embryos, the implantation of BMP-2-soaked beads induced ectopic expression of *Csx/Nkx2-5* and *GATA-4*, and the administration of soluble BMP-2 or BMP-4 conferred competence to undergo cardiogenesis on explants from noncardiogenic anterior mesoderm (Schultheiss et al., 1997). Furthermore, the injection of dominant-negative BMP receptors in *Xenopus* embryos resulted in the reduction of heart formation (Shi et al., 2000), and mice deficient for *BMP-2* or *BMP-4* exhibited various degrees of

cardiac defects (Winnier et al., 1995; Zhang & Bradley, 1996). The essential role of BMP signaling in the induction of *Csx/Nkx2-5* expression and subsequent normal cardiac differentiation was confirmed by experiments using P19CL6 cells, which were derived from mouse P19 embryonal carcinoma cells (Monzen et al., 1999). In adherent culture with 1% DMSO, P19CL6 cell efficiently differentiate into spontaneously beating cardiomyocytes and exhibit the biological features recapturing embryonic cardiogenesis *in vivo*. In contrast, P19CL6 cells constitutively expressing BMP antagonist noggin (P19CL6-noggin) did not differentiate into beating cardiomyocytes, and expressions of cardiac transcription factors, including *Csx/Nkx2-5*, were not induced, even when cultured with DMSO. Similar results were observed in experiments using aggregated P19 cells (Jamali et al., 2001a). Cardiogenic activities in P19CL6-noggin were restored by the overexpression of BMP-2 or BMP signaling mediators (Yamaguchi et al., 1995; Heldin et al., 1997), TGF- $\beta$ -kinase 1 (TAK1), or Smads (Monzen et al., 1999, 2001).

The formation of dorsal vessel in *Drosophila* also requires segment polarity gene *wg*, which is expressed in posterior stripe-shaped parasegments of the ectoderm (Wu et al., 1995). The inductive Wg signaling restricts and maintains *tinman* expression in cardiac mesoderm (Wu et al., 1995; Park et al., 1996) via the canonical pathway involving *disheveled*, *zeste-white3/shaggy* (*glycogen synthase kinase-3*  $\beta$  homolog), *armadillo* ( $\beta$ -catenin homologue), and *pangolin* (*T-cell factor* homolog; Park et al., 1996, 1998b). In contrast to the essential role of *Drosophila* Wg pathway, an inhibitory role of the canonical Wnt pathway in cardiogenesis was unraveled in vertebrates (Marvin et al., 2001; Schneider & Mercola, 2001; Tzahor & Lassar, 2001). In *Xenopus*, the ectopic expression of endogenous Wnt antagonists, *dickkopf-1* and *crench*, induced cardiac differentiation in non-precordial mesoderm, while the overexpression of Wnt3A or Wnt8 blocked cardiogenesis in precordial mesoderm (Schneider & Mercola, 2001). Likewise, experiments using explants from chick embryos demonstrated that inhibitory effects of cardiogenesis by the neural tube were mimicked by Wnt1 or Wnt3A and were surmounted by a secreted Wnt antagonist, Frzb (Tzahor & Lassar, 2001). In addition, *dickkopf-1* or *crench* conferred cardiogenic response on normally blood-forming posterior mesoderm by antagonizing Wnt pathways, and reciprocally, the ectopic expression of Wnt proteins in precordial mesoderm induced blood cell formation (Marvin et al., 2001). Inconsistent with the antagonistic role in *Xenopus* and chick cardiogenesis, the canonical Wnt pathway is required for cardiac differentiation of mouse P19CL6 cells (Nakamura et al., 2003). The inductive or repressive effects observed in these experiments represent the complicated nature of the canonical Wnt signaling pathway, which might cause differences according to species, cell types, and stages of differentiation. To be more complex, cardiogenesis appears to

be dependent on noncanonical Wnt pathways mediating signals independently of  $\beta$ -catenin (Vecman et al., 2003). In *Xenopus* and quail embryos, quail mesodermal cell line QCE-6, mouse P19 cells, and embryonic stem cells, Wnt11 promoted cardiogenesis possibly by activating Jun N-terminal kinase (Eisenberg et al., 1997; Eisenberg & Eisenberg, 1999; Pandur et al., 2002; Terami et al., 2004). Therefore, multiple Wnt pathways may operate both positively and negatively in a complex manner to induce cardiogenic program.

The fibroblast growth factor (FGF) family is another factor that functions to promote cardiogenesis. In chick embryos, endodermally expressed FGF-8 contributed to the expression of *Csx/Nkx2-5* and *MEF2-C* in precardiac mesoderm, and ectopic FGF-8 expression expanded the expression of these cardiac transcription factors where BMP signaling was present (Alsan & Schultheiss, 2002). However, downstream mediators of FGF signaling that regulate cardiogenesis remain to be precisely elucidated. A recent study using mouse P19CL6 cells indicated that phosphatidylinositol 3 (PI3)-kinase was essential for the early stage of *Csx/Nkx2-5* and *GATA-4* activation and subsequent cardiac differentiation (Naito et al., 2003). Whereas PI3-kinase mediates various signaling pathways evoked by extracellular stimuli, it might be possible that PI3-kinase functions downstream to FGF signaling in inducing cardiogenic program.

In an attempt to decode the mechanisms how *Csx/Nkx2-5* expression is activated in cardiac mesoderm, regulatory *cis*-elements of *Csx/Nkx2-5* that direct cardiac-specific expression have been analyzed by the transgenic approach (Searcy et al., 1998; Lien et al., 1999; Reecy et al., 1999; Tanaka et al., 1999b). Within the mouse *Csx/Nkx2-5* locus, multiple independent enhancers and repressors are presumed to modulate *Csx/Nkx2-5* expression in distinct regions of embryonic hearts (reviewed in Schwartz & Olson, 1999). For example, 14 kb of 5'-flanking sequence directed *LacZ* reporter expression in cardiac crescent and in outflow tract, interatrial groove, atrioventricular canal, and right and left ventricles (Tanaka et al., 1999b). A series of deletion analyses mapped a distal cardiac enhancer (activating region 1, AR1), which is located about 9 kb upstream of the gene and consists of 2 positive regulatory regions with an intervening negative regulatory region (Lien et al., 1999). This distal enhancer recapitulated the authentic *Csx/Nkx2-5* expression until the looping morphogenesis, but thereafter, the enhancer activity was restricted to the right ventricle. About 3 kb upstream of the gene, another cardiac enhancer (activating region 2, AR2) is located, and this enhancer contributes to a reporter gene expression in cardiac crescent, primitive heart tube, and outflow tract (Searcy et al., 1998). Additionally, a proximal enhancer (activating region 3, AR3) was identified about 5 kb upstream of the gene, which drives gene expression in outflow tract (Reecy et al., 1999), and an enhancer for expression in right ventricle is located 6 kb downstream of the gene (Tanaka et al., 1999b).

A next important step for understanding the regulatory mechanisms of *Csx/Nkx2-5* expression is to identify *trans*-acting factors that bind to these *cis*-elements. The distal enhancer AR1 contains several binding sites for GATA transcription factors, and one of the GATA-binding sites is responsible for cardiac expression (Lien et al., 1999). Likewise, multiple GATA-binding sites are present within the proximal enhancer AR2, and the mutation of two of these GATA sites abolished enhancer activity in the heart (Searcy et al., 1998). Inasmuch as AR1 and AR2 direct *Csx/Nkx2-5* expression in cardiac crescent, GATA transcription factors may be critically involved in the initiation of *Csx/Nkx2-5* expression. However, during the early period of cardiogenesis, GATA transcription factors are expressed in visceral endoderm, as well as in precardiac mesoderm (reviewed in Molkenin, 2000), and it is likely that additional signaling pathways may be required for the initial activation of *Csx/Nkx2-5* in the correct region. Specific knockdown of GATA-6 in *Xenopus* and zebrafish embryos by injection of antisense morpholino also indicated that GATA-6 functions to maintain, but not to initiate *Csx/Nkx2-5* expression (Peterkin et al., 2003).

In *Drosophila*, the mesodermal expression of *tinman* is dependent on the Dpp response element that contains binding sites for Medea, a homologue of Smad4 (Xu et al., 1998). The requirement of a Smad-binding site for cardiac-specific *Csx/Nkx2-5* expression is evolutionally conserved among fruit fly, chicken, mouse, and human (Liberatore et al., 2002; Lien et al., 2002). Interestingly, the indispensable Smad-binding site is located adjacent to the 2 essential GATA-binding sites within AR2 (Searcy et al., 1998; Liberatore et al., 2002; Lien et al., 2002). A recent paper reported that a novel enhancer containing clustered repeats of GATA-binding sites and Smad-binding sites was located about 6kb upstream of the gene (Brown et al., 2004). This composite enhancer is also required for *Csx/Nkx2-5* expression in cardiac crescent, and GATA-4 and Smad4 form a complex upon the enhancer in differentiated P19CL6 cells. These results suggest that BMP signaling may induce cardiac-specific *Csx/Nkx2-5* expression through the activation of the composite enhancer by Smads in collaboration with GATA-4. The characterization of 3'-flanking sequence of chick *Csx/Nkx2-5* identified a unique enhancer (cardiac activating region 3, CAR3). CAR3 contains a triad of binding sites for GATA factors, Smads, and YY-1 and directs reporter gene expression in both the primary and secondary heart fields (Lee et al., 2004). BMP-mediated activation of CAR3 is dependent on the interaction of Smads and YY-1, which enables the up-regulation of the transcriptional activity of YY-1.

Despite the evidence that BMP signaling modulates cardiac-specific *Csx/Nkx2-5* expression through interactions involving Smads, GATA factors, and YY-1, the transcriptional regulation of *Csx/Nkx2-5* appears too complicated to be fully deciphered. *Csx/Nkx2-5* regulatory regions contain multiple positive and negative enhancers with numerous

binding sites for known transcriptional regulators (Searcy et al., 1998; Lien et al., 1999; Reecy et al., 1999; Schwartz & Olson, 1999; Tanaka et al., 1999b; Shiojima et al., 2000; Liberatore et al., 2002; Lien et al., 2002). Especially, it remains unknown how *Csx/Nkx2-5* expression is regulated in response to Wnt canonical and noncanonical pathways. In addition to the genetic regulation, the transcription of *Csx/Nkx2-5* may be regulated epigenetically, as evidenced by the results that *Csx/Nkx2-5* expression was initiated but not sufficiently sustained in mice deficient for *Plycomb* group gene *Rae 28* that maintains transcriptional state by controlling chromatin structure (Shirai et al., 2002).

#### 2.4. Transcriptional regulation of downstream target genes by *Csx/Nkx2-5*

In search for the direct downstream target genes of *Csx/Nkx2-5*, the promoter activities of potential candidate genes were extensively explored. Among the cardiac genes examined, the promoter activity of *ANP* gene was found to be under the control of *Csx/Nkx2-5* (Durocher et al., 1996; Lee et al., 1998; Shiojima et al., 1999). *Csx/Nkx2-5* strongly transactivated the *ANP* promoter, and the transactivation of the *ANP* promoter was dependent on the *Csx/Nkx2-5* DNA-binding site located within the *ANP* promoter. In addition to *ANP*, direct downstream targets for *Csx/Nkx2-5* have been identified, such as *cardiac  $\alpha$ -actin* (Chen & Schwartz, 1996), *CARP* (Zou et al., 1997), *A1 adenosine receptor* (Rivkees et al., 1999), *calreticulin* (Guo et al., 2001), *connexin40* (Bruneau et al., 2001), *sodium-calcium exchanger-1* (Muller et al., 2002), *endothelin-converting enzyme-1* (Funke-Kaiser et al., 2003), *HOP* (Chen et al., 2002; Shin et al., 2002), *myocardin* (Ueyama et al., 2003a), *Csm* (Ueyama et al., 2003b), and *MEF2-C* (von Both et al., 2004; Table 2). These target genes encode important structural proteins and transcriptional regulators that confer features characteristic of cardiomyocytes, supporting a functional role of *Csx/Nkx2-5* in the transcriptional regulation of a cardiac gene program. The left-sided expression of *Pitx2*, a *bicoid*-type homeobox transcription factor responsible for left–right asymmetric morphogenesis, is controlled under a left side-specific enhancer element containing a *Csx/Nkx2-5*-binding site (Shiratori et al., 2001). This regulatory element is especially required for the maintenance of left-sided *Pitx2* expression. Based on the largely overlapping but partly different expression patterns of *Csx/Nkx2-5* and *Pitx2*, it is likely that *Pitx2* expression is also regulated by *Csx/Nkx2-5* and its related proteins.

The transcriptional activity of *Csx/Nkx2-5* is exquisitely regulated by post-translational modification. First, *Csx/Nkx2-5* forms a homodimer on its DNA-binding sites (Kasahara et al., 2001a; Fig. 1). Dimerization stabilizes the binding of *Csx/Nkx2-5* to DNA, whereas it is elusive whether dimerization of *Csx/Nkx2-5* is stochastically formed or induced by some regulatory mechanisms. Second, *Csx/Nkx2-5* is phosphorylated at the conserved serine

Table 2  
Direct downstream target genes for *Csx/Nkx2-5*

Genes	Gene product	References
<i>ANP</i>	Natriuretic peptide	Durocher et al., 1996 Lee et al., 1998 Shiojima et al., 1999
<i>Cardiac <math>\alpha</math>-actin</i>	Contractile protein	Chen & Schwartz, 1996
<i>MEF-2C</i>	Transcription factor	von Both et al., 2004
<i>A1 adenosine receptor</i>	Adenosine receptor	Rivkees et al., 1999
<i>Calreticulin</i>	Calcium-binding protein	Guo et al., 2001
<i>Sodium-calcium exchanger 1</i>	Ion exchanger	Muller et al., 2002
<i>Connexin 40</i>	Structural protein in gap junction	Bruneau et al., 2001
<i>Endothelin-converting enzyme-1</i>	Endothelin-converting enzyme	Funke-Kaiser et al., 2003
<i>HOP</i>	Homeoprotein	Chen et al., 2002 Shin et al., 2002
<i>Myocardin</i>	Transcriptional coactivator	Ueyama et al., 2003a
<i>CARP</i>	Transcriptional corepressor, titin-binding protein	Zou et al., 1997
<i>Csm</i>	RNA helicase	Ueyama et al., 2003b

*ANP*, atrial natriuretic peptide; *CARP*, cardiac ankyrin repeat protein; *HOP*, homeodomain-only protein; *MEF-2C*, myocyte enhancer factor 2-C.

residue within the homeodomain by casein kinase II (CKII; Kasahara & Izumo, 1999; Fig. 1). The DNA-binding affinity of *Csx/Nkx2-5* is increased by CKII phosphorylation, but it remains unclear whether the phosphorylation of *Csx/Nkx2-5* by CKII has some implications in biological contexts. Third, *Csx/Nkx2-5* interacts with other transcriptional regulators. The transcriptional activity of *Csx/Nkx2-5* is modulated through physical interaction with other transcription factors such as GATA-4 (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998; Rivkees et al., 1999; Shiojima et al., 1999), serum response factor (SRF; Chen & Schwartz, 1996), Tbx-5 (Bruneau et al., 2001; Hiroi et al., 2001), Tbx-2 (Habets et al., 2002), Tbx-20 (Stennard et al., 2003), dHAND/HAND2 (Thattaliyath et al., 2002), and Foxh1 (von Both et al., 2004; Table 3). Fourth, the transcriptional activity of *Csx/Nkx2-5* is antagonized when the binding of *Csx/Nkx2-5* to its cognate DNA sequence is interfered by COUP-TF1 (Guo et al., 2001) or Hmx1 (Amendt et al., 1999). The antagonistic roles of *Csx/Nkx2-5* and COUP-TF1 are implicated in the transcriptional regulation of *calreticulin* gene, whereas the significance of antagonism between *Csx/Nkx2-5* and Hmx remains unclear.

With regard to protein–protein interactions, the composition of protein complexes consisting of transcription factors and cofactors is thought to be the key determinant of transcriptional specificity and intensity. The interaction between *Csx/Nkx2-5* and GATA-4 has been well characterized. The overexpression of both *Csx/Nkx2-5* and GATA-4 induced much stronger transactivation of the luciferase construct containing the 300-bp 5'-flanking region of the rat *ANP* gene (*ANP(300)-luc*) than that induced by the expression of *Csx/Nkx2-5* alone (Shiojima et al., 1999).

Table 3  
Protein–protein interactions involving Csx/Nkx2-5

Cofactors	Target promoters	References
GATA4	+ <i>ANP</i>	Durocher et al., 1997 Lee et al., 1998 Sepulveda et al., 1998 Shiojima et al., 1999
	+ <i>CARP</i> + <i>A1 adenosine receptor</i>	Kuo et al., 1997 Rivkees et al., 1999
SRF	+ <i>cardiac <math>\alpha</math>-actin</i>	Chen & Schwartz, 1996
Tbx5	+ <i>ANP</i>	Bruneau et al., 2001 Hiroi et al., 2001
	+ <i>Connexin 40</i>	Bruneau et al., 2001
Tbx20	+ <i>ANP</i>	Stennard et al., 2003
Tbx2	– <i>ANP</i>	Habets et al., 2002
dHAND/HAND2	+ <i>ANP</i>	Thattaliyath et al., 2002
Foxh1	+ <i>MEF-2C</i>	von Both et al., 2004
Cal	+ <i>ANP</i>	Akazawa et al., 2004

*ANP*, atrial natriuretic peptide; *CARP*, cardiac ankyrin repeat protein; *MEF-2C*, myocyte enhancer factor 2, SRF, serum response factor. Up-regulation is denoted as '+' and down-regulation as '–'.

This result suggests that Csx/Nkx2-5 and GATA-4 synergistically activate the *ANP* gene. Deletion and mutation analyses revealed that the Csx/Nkx2-5-binding site, located at –250 bp in the *ANP* promoter, was responsible for transcriptional activation by Csx/Nkx2-5. Although the GATA-binding site was located at –280 bp, adjacent to the Csx/Nkx2-5-binding site, GATA-4 alone induced no significant activation of the ANP(300)-luc construct. Furthermore, the deletion of the GATA-binding site at –280 bp had little effect on the synergistic transactivation of the *ANP* promoter between Csx/Nkx2-5 and GATA-4. Based on the result that the deletion of the Csx/Nkx2-5-binding site at –250 bp abolished the cooperative activation of the *ANP* promoter between Csx/Nkx2-5 and GATA-4, this synergistic transactivation is dependent on the binding of Csx/Nkx2-5 to the Csx/Nkx2-5-binding site but not on binding of GATA-4 to the GATA-binding site. Co-immunoprecipitation and GST pull-down assay experiments showed that Csx/Nkx2-5 and GATA-4 directly interact with each other both in vitro and in vivo via the homeodomain of Csx/Nkx2-5 and the zinc-finger domain of GATA-4 (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998; Shiojima et al., 1999). With regard to the essential DNA target sites of Csx/Nkx2-5 and GATA-4 in the *ANP* promoter, contradictory results have been reported that may be, in part, attributed to the different cell types and experimental conditions. Durocher et al. (1997) demonstrated that Csx/Nkx2-5 and GATA-4 cooperated in activating the rat *ANP* promoter containing the –135 bp region and that synergistic transactivation requires both of the cognate binding sites located within this region. Lee et al. (1998) showed that synergism on transcriptional activation of the rat *ANP* promoter containing the –638 bp occurred after introducing various mutations of all the Csx/Nkx2-5 sites and GATA-binding sites within this region. Shiojima et al. (1999) confirmed

that Csx/Nkx2-5 and GATA-4 worked in concert with each other to activate the artificial promoter containing 4 tandemly arrayed Csx/Nkx2-5-binding sites independently of binding of GATA-4 to DNA. Consistently, transcriptional cooperativity between Csx/Nkx2-5 and GATA-4 independent of GATA4–DNA binding was also elicited from analyzing the effects of these factors on activation of *cardiac  $\alpha$ -actin* promoter (Sepulveda et al., 1998). With regard to the transcriptional regulation of the *cardiac  $\alpha$ -actin* gene, Csx/Nkx2-5 potentiates promoter activation by interacting with SRF via the homeodomain of Csx/Nkx2-5 and the MADS box of SRF, and this cooperation is dependent on binding of SRF and its cognate DNA sequence, but the binding of Csx/Nkx2-5 and DNA is not necessary as well (Chen & Schwartz, 1996). Likewise, the expression of *CARP* gene was shown to be controlled by cooperative regulation between Csx/Nkx2-5 and GATA-4, which was dependent on GATA4–DNA binding but not on Csx/Nkx2-5–DNA binding (Kuo et al., 1997). It is likely that the increasing number of cardiac-specific genes will be found to be under the control of Csx/Nkx2-5 in collaboration with GATA-4, like *A1 adenosine receptor* gene (Rivkees et al., 1999). Significant roles of transcriptional synergy between Csx/Nkx2-5 and GATA-4 during cardiomyocytes differentiation have been demonstrated by experiments using P19CL6 cells. Simultaneous overexpression of Csx/Nkx2-5 and GATA-4 restored normal cardiomyocyte differentiation in P19CL6 cells stably expression the BMP antagonist noggin, whereas the overexpression of Csx/Nkx2-5 or GATA-4 alone did not (Monzen et al., 1999).

The interaction between Csx/Nkx2-5 and Tbx-5 has attracted much attention, especially because common cardiac malformations are caused by haploinsufficiency of each gene. Tbx-5 is a T-box transcription factor (reviewed in Smith, 1999), and heterozygous mutations of *TBX-5* in humans cause Holt-Oram syndrome (HOS) characterized by upper limb malformations and a spectrum of cardiac malformations, including atrial septal defect (ASD), ventricular septal defect (VSD), and tetralogy of Fallot (TOF), frequently accompanied by cardiac conduction defect (Basson et al., 1997; Li et al., 1997). Csx/Nkx2-5 and Tbx-5 interact with each other via the homeodomain of Csx/Nkx2-5 and the N-terminal domain and T-box of Tbx-5 and collaborate to activate the promoters of *ANP* and *connexin 40*, a gap junction gene (Bruneau et al., 2001; Hiroi et al., 2001). The region between –270 and –240 bp of the *ANP* promoter is important for transactivation by Tbx-5, and a half-side of a palindromic T (brachyury)-binding site flanked by a Csx/Nkx2-5-binding site is located within this region. Csx/Nkx2-5 and Tbx-5 form a ternary complex together with this tandemly arrayed cognate DNA sequence. Interestingly, the Tbx-5 harboring G80R mutation, which causes severe cardiac defects in HOS, exhibits significantly weak synergism on promoter activation of the *ANP* gene, compared with the wild-type Tbx-5 and the Tbx-5 harboring

R237Q mutation, which rarely causes cardiac defects in HOS (Hiroi et al., 2001). Furthermore, the stable over-expression of wild-type Tbx-5 promotes cardiac differentiation in P19CL6 cells with the up-regulation of several cardiac genes such as *Csx/Nkx2-5*, *GATA-4*, *MEF2-C*, *ANP*, *CARP*, and *MLC2v*, whereas the overexpression of the G80R mutant of Tbx-5 attenuates cardiac differentiation with markedly reduced expression of these cardiac genes. The effects of the haploinsufficiency of Tbx-5 on cardiac differentiation were analyzed in a genetically engineered murine model (Bruneau et al., 2001). In *Tbx-5* heterozygous mice, cardiac malformations resembling those in HOS are observed, and the expression levels of *ANP* and *connexin 40* were significantly reduced. Cooperative regulation of several cardiac genes by *Csx/Nkx2-5* and Tbx-5 accounts for the overlapping cardiac defects caused by mutations of these transcription factors.

Recently, a unique model in the transcriptional regulation of the *ANP* gene was proposed. T-box transcription factor, Tbx-2, functions as a transcriptional repressor for *ANP* expression by displacing Tbx-5 and forming a complex with *Csx/Nkx2-5* in the region of atrioventricular canal, inner curvature, outflow tract, and inflow tract, where *Tbx-2* is expressed and *ANP* expression is exclusively absent (Habets et al., 2002). It is intriguing that the transcriptional activity of *Csx/Nkx2-5* is modulated positively and negatively by its respective binding partner, Tbx-5 or Tbx-2, in a region-specific manner.

To further understand the combinatorial mechanisms of how *Csx/Nkx2-5* regulates transcription of its distinct target genes, we screened a heart cDNA library by the yeast 2-hybrid system using *Csx/Nkx2-5* as a bait and isolated several factors that interact with *Csx/Nkx2-5* and modulate *Csx/Nkx2-5*-induced gene expression (Hiroi et al., 2001; Akazawa et al., 2004). One of the coactivating factors was a novel LIM domain-containing protein, which we named *Cal* for “CSX-associated LIM protein” (Akazawa et al., 2004). *Cal* is a member of Zyxin family of LIM proteins that commonly share 3 tandem LIM domains located at the C-terminus and a leucine-rich sequence that matches the consensus sequence for nuclear export signal (NES). The LIM domain is a double-zinc finger motif and functions as a module for protein–protein interactions. Consistently, the LIM domains of *Cal* are required for binding to the homeodomain of *Csx/Nkx2-5*. *Cal* itself has the transcription-promoting activity and activates the *ANP* promoter by forming a complex with *Csx/Nkx2-5*. Interestingly, *Cal* traffics out of the nucleus by NES-dependent mechanisms and traffics into the nucleus in response to an increase of intracellular calcium concentration. Recently, a function of LIM proteins linking cytoplasmic signals and nuclear gene expression has attracted much attention (Kadrmas & Beckerle, 2004). The dynamic intracellular shuttling of *Cal* appears to have implications in cardiogenetic gene program, because the nuclear expression of *Cal* promotes cardiac differentiation of P19CL6 cells in vitro.

## 2.5. Congenital heart diseases associated with *CSX/NKX2-5* mutations

In humans, mutations in *CSX/NKX2-5* gene provided the first evidence that the genetic factors are etiologically crucial in nonsyndromic congenital heart diseases (CHD). Linkage analyses were performed in 4 families having a high incidence of ASD and progressive conduction disturbance with an autosomal dominant trait, and the disease locus proved to be mapped to chromosome 5q34–q35 (Schott et al., 1998), where *CSX/NKX2-5* is located (Shiojima et al., 1995; Turbay et al., 1996). The sequencing of *CSX/NKX2-5* exons in affected individuals identified 3 kinds of mutations: 1 nonsense mutation (Gln170ter, type A) and 1 missense mutation (Thr178Met, type B) located within the homeodomain, and 1 nonsense mutation (Gln198ter, type C) located C-terminal to the homeodomain. To date, more than 10 disease-related mutations in *CSX/NKX2-5* have been documented in patients with a spectrum of CHD (Schott et al., 1998; Benson et al., 1999; Hosoda et al., 1999; Goldmuntz et al., 2001; Ikeda et al., 2002; Watanabe et al., 2002; Elliott et al., 2003; McElhinney et al., 2003; Reamon-Buettner & Borlak, 2004; Reamon-Buettner et al., 2004; Fig. 2). The most common phenotypes are secundum ASD and AV conduction disturbance, but other cardiac anomalies have been reported, such as VSD, TOF, double-outlet right ventricle (DORV), tricuspid valve abnormalities including Ebstein’s anomaly, and hypoplastic left heart syndrome. Estimation by cohort studies indicated that *CSX/NKX2-5* mutations were relatively rare among all the CHD patients unselected for a family history or AV conduction disturbance (Elliott et al., 2003; McElhinney et al., 2003). For example, a screening of 102 ASD and 25 patent foramen ovale patients (13 with positive family

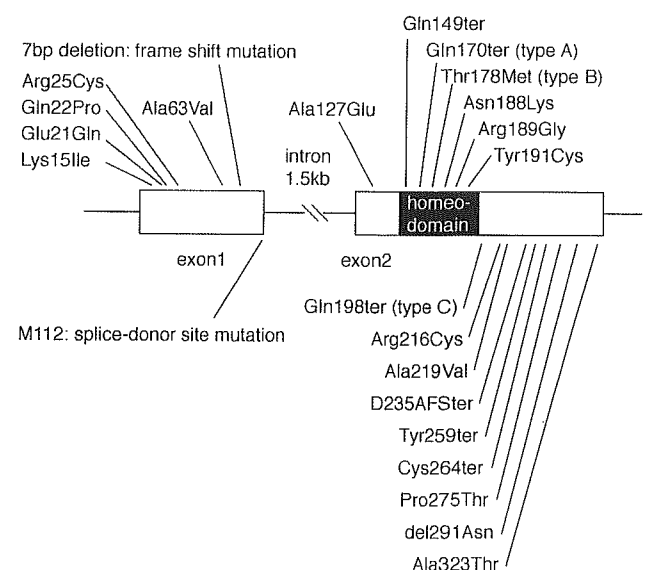


Fig. 2. Schematic presentation of the *CSX/NKX2-5* gene and the locations of the mutations. Two exons (open boxes) of *CSX/NKX2-5* are separated by a 1.5-kb intron. The homeodomain is denoted as a black box.

history and 5 with AV block) revealed only 1 *CSX/NKX2-5* mutation in a familial ASD patient without AV block (Elliott et al., 2003). Another study enrolled CHD patients prospectively without regard for family history and estimated the frequency of *CSX/NKX2-5* mutations to be ~3% (18 out of 608 probands; McElhinney et al., 2003). Likewise, a cohort study estimated that *CSX/NKX2-5* mutations were present in ~4% of nonsyndromic TOF patients (Goldmuntz et al., 2001). The genotype–phenotype correlations in patients with *CSX/NKX2-5* remain to be precisely determined, but it was reported that most of the mutations identified in sporadic CHD were missense situated outside the homeodomain and not associated with AV conduction disturbance (McElhinney et al., 2003). However, in the majority of patients with familial ASD, *CSX/NKX2-5* mutations are situated within or around the homeodomain and associated with AV block (Schott et al., 1998; Benson et al., 1999; Hosoda et al., 1999). Therefore, the identification of *CSX/NKX2-5* mutations in familial ASD patients is of clinical relevance, because such patients are frequently associated with progressive AV block (Schott et al., 1998; Hosoda et al., 1999) and the prediction of the requirement for pacemaker implantation may be feasible.

It is a challenging issue to be solved why specific mutations of *CSX/NKX2-5* cause various types of CHD with a dominant inheritance. Some reports illustrated the molecular basis of mutant *CSX/NKX2-5* proteins (Kasahara et al., 2000; Zhu et al., 2000; Monzen et al., 2002). The transcriptional activities of most *CSX/NKX2-5* mutant proteins with mutations in the homeodomain are impaired due to reduced DNA-binding affinity, and haploinsufficiency is postulated to be the underlying mechanism. A *CSX/NKX2-5* mutant with a nonsense mutation in the homeodomain, Gln170ter, type A, was not localized in the nucleus but distributed diffusely within the cell, and failed to transactivate the *ANP* promoter (Zhu et al., 2000). Altered subcellular localization was observed in a mutant harboring a point mutation at the intron-splicing donor site (Kasahara et al., 2000). Another kind of *CSX/NKX2-5* mutant harboring a missense mutation located in the homeodomain, Thr178Met, type B, was localized normally in the nucleus but showed weaker DNA-binding affinity and milder promoter activation than wild-type *CSX/NKX2-5* did (Zhu et al., 2000). The co-transfection of wild-type *CSX/NKX2-5* together with type A or B mutant revealed that these mutant proteins somehow inhibited promoter activation by wild-type *CSX/NKX2-5* (Zhu et al., 2000). Therefore, these mutations may affect the transcriptional activity of *CSX/NKX2-5*, in part, by a dominant-negative manner, as well as by haploinsufficiency. In contrast, *CSX/NKX2-5* mutants with mutations located C-terminal to the homeodomain showed intact DNA-binding activity to the monomeric binding site but significantly decreased DNA-binding activity to the dimeric binding sites (Kasahara et al., 2000). Therefore, the mutations outside the homeodomain may disturb the ability to form dimers on the dimeric sites. Interestingly, a *CSX/NKX2-5* mutant with

nonsense mutation in the homeodomain, Gln198ter, type C, enhanced transcriptional activity (Kasahara et al., 2000; Zhu et al., 2000), although the overexpression of this mutant induced apoptosis in cultured cardiomyocytes of neonatal rats (Zhu et al., 2000). In P19CL6 cardiomyogenic cells, the stable overexpression of *CSX/NKX2-5* promoted cardiomyocyte differentiation not only by enhancing expressions of cardiac-specific genes but also by preventing stress-induced apoptotic cell death (Monzen et al., 2002). On the other hand, P19CL6 cells overexpressing *CSX/NKX2-5* type C mutant showed lower expression levels of antiapoptotic proteins and higher expression levels of apoptosis-promoting proteins and were vulnerable to cytotoxic insults such as exposure to H<sub>2</sub>O<sub>2</sub> or nutrition deprivation (Monzen et al., 2002). Therefore, the function of *CSX/NKX2-5* to prevent stress-induced cardiomyocyte apoptosis may be hampered by type C mutation. Recently, a novel deletion frameshift mutation in *CSX/NKX2-5* was reported in patients with familial ASD and AV block (Watanabe et al., 2002). An unusual finding in this case was that the proband exhibited heterotaxia as well as ASD. Whereas the penetrance was extremely low, it is possible that the *CSX/NKX2-5* mutation might predispose to situs abnormalities because maintenance of left-sided expression of *Pitx2* is controlled under an enhancer element containing a *Csx/Nkx2-5*-binding site (Shiratori et al., 2001).

Physiological and anatomical reevaluation of mice heterozygous for *Csx/Nkx2-5* null mutation demonstrated that the effects of *Csx/Nkx2-5* haploinsufficiency in mice were not intense and were influenced by genetic backgrounds (Biben et al., 2000). Only 5 cases of ASD were found out of 425 *Csx/Nkx2-5*-heterozygous mice, although the frequency of patent foramen ovale and septal aneurysm were increased. Septal dysmorphogenesis was most frequent in the 129/Sv strain, and in particular, 17% of *Csx/Nkx2-5*-heterozygous mice exhibited ASD. With regard to AV conduction disturbance, Biben et al. (2000) reported that only mild prolongation of P–R interval was observed in female *Csx/Nkx2-5*-heterozygous mice. However, a recent study using electrophysiological recordings with higher resolution indicated that the cardiac conduction system was more defective than imagined in *Csx/Nkx2-5*-heterozygous mice (Jay et al., 2004). Jay et al. (2004) reported that the conduction system was hypocellular in the absence of *Csx/Nkx2-5* and that *Csx/Nkx2-5* haploinsufficiency was associated with conduction and electrophysiological abnormalities. The essential role of *Csx/Nkx2-5* in the formation and maturation of the conduction system was further confirmed by the observation that ventricular-restricted deletion of *Csx/Nkx2-5* resulted in the formation of hypoplastic AV node and atrophic conduction system. Transgenic mice overexpressing human *CSX/NKX2-5* with a DNA-nonbinding missense mutation in the homeodomain (Ile183Pro) in the hearts exhibited AV conduction defect accompanied by reduced expression levels of gap junction proteins, connexin 40 and 43 (Kasahara et al.,



2001b). Connexin 40 is a major constituent protein of gap junction in the specialized conduction cells, and the loss of *connexin 40* predisposed mice to cardiac conduction abnormalities (Kirchhoff et al., 1998; Simon et al., 1998). In addition, the promoter of *connexin 40* is directly transactivated by *Csx/Nkx2-5* together with *Tbx-5* (Bruneau et al., 2001). These results suggest that *Csx/Nkx2-5* plays an essential role in the differentiation of specialized cells in the conduction system in part through transcriptional regulation of gap junction proteins, although the possibility cannot be ruled out that the phenotype of *Csx/Nkx2-5* (Ile183Pro) transgenic mice might be influenced by a hypomorphic gain-of-function effect because the endogenous *Csx/Nkx2-5* expression was increased. To make the matters more complicated, a recent immunohistochemical analysis revealed that the number of connexin 40-positive gap junctions per individual Purkinje cells was not significantly altered in *Csx/Nkx2-5*-heterozygous mice (Jay et al., 2004), whereas connexin 40 was almost absent in the conduction system of ventricular-restricted *Csx/Nkx2-5*-deficient mice (Pashmforoush et al., 2004). Further investigations will be required for the elucidation of the molecular and cellular mechanism how *Csx/Nkx2-5* regulates proper development of cardiac conduction system.

Unequal phenotypes of *Csx/Nkx2-5* heterozygotes between in mice and in humans may arise from the differences in genetic effects of *Csx/Nkx2-5* mutations or the intrinsic differences in hemodynamic status. Importantly, studies in mice and in humans highlighted the phenotypic variations of *Csx/Nkx2-5* mutants, which suggested that the effects of *Csx/Nkx2-5* mutations might be influenced by genetic modifiers and environmental factors. Of course, it is possible that some human *CSX/NKX2-5* mutant proteins operate in a dominant-negative manner. To elucidate the function of *CSX/NKX2-5* mutant proteins in vivo, a sophisticated approach will be required, such as the generation of genetically engineered mice in which a specific *CSX/NKX2-5* mutation is knocked in. The genotype–phenotype relationship of human *CSX/NKX2-5* mutations contributed to understanding the etiologies of human cardiac abnormalities and provided a clue to undermining the role of *Csx/Nkx2-5* in cardiac morphogenesis and conduction system formation.

## 2.6. Role of *Csx/Nkx2-5* in postnatal hearts

In contrast to the essential role of *Csx/Nkx2-5* during embryogenesis, its functional role in the postnatal heart has not been fully determined. *Csx/Nkx2-5* is expressed in the adult heart (Komuro & Izumo, 1993; Shiojima et al., 1996; Kasahara et al., 1998), and notably, its expression is up-regulated in hypertrophied hearts. Banding of the feline pulmonary artery induces right ventricular hypertrophy and up-regulated the expression of *Csx/Nkx2-5* and its downstream target genes, such as *ANP* and *cardiac  $\alpha$ -actin*

(Thompson et al., 1998). In phenylephrine- or isoproterenol-mediated hypertrophic hearts, the expression of *Csx/Nkx2-5* is increased as well as fetal genes such as *ANP* and  $\beta$ -myosin heavy chain (*MHC*), and immediate-early genes such as *c-fos*, *c-jun*, and *Egr-1* (Saadane et al., 1999). The up-regulation of *Csx/Nkx2-5* expression in pressure overload-induced and agonist-induced hypertrophic hearts indicates a potential role of *Csx/Nkx2-5* in the generation of cardiac hypertrophy.

However, transgenic mice overexpressing *Csx/Nkx2-5* under the control of the cytomegalovirus enhancer/chicken  $\beta$ -actin promoter exhibited normal-sized hearts (Takimoto et al., 2000). The expression levels of cardiac genes such as *ANP*, *BNP*, *CARP*, and *MLC2v* were increased in the hearts of *Csx/Nkx2-5* transgenic mice. These results suggest that *Csx/Nkx2-5* is not sufficient for the generation of cardiac hypertrophy but that *Csx/Nkx2-5* controls cardiac gene program in adult hearts as well as in embryonic hearts. As mentioned in the previous section, the transcriptional activity of *Csx/Nkx2-5* is modulated through physical interaction with other transcription factors such as *GATA4* (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998; Shiojima et al., 1999) and *SRF* (Chen & Schwartz, 1996), which are implicated in the generation of cardiac hypertrophy (reviewed in Akazawa & Komuro, 2003a). Cooperative transactivation of the *ANP* promoter by *Csx/Nkx2-5* and *Cal* was enhanced when *Cal* protein was targeted into the nucleus (Akazawa et al., 2004). The nuclear translocation of *Cal* in response to an increase in intracellular calcium prompted us to speculate that *Cal* may play a certain role in the generation of cardiac hypertrophy by modulating the transcriptional activity of *Csx/Nkx2-5*. Combinatorial regulation involving *Csx/Nkx2-5* and its coactivators might be necessary for the generation of cardiac hypertrophy, although it is still speculative.

In addition, transcriptional regulation by *Csx/Nkx2-5* in postnatal hearts is profoundly involved in maintaining the highly differentiated properties and in protecting the heart from cytotoxic stress. We generated transgenic mice overexpressing a dominant-negative human *CSX/NKX2-5 LP* mutant, in which a highly conserved leucine in the homeodomain was replaced by a proline, under the control of  $\alpha$ -*MHC* promoter (Toko et al., 2002). The homeoproteins harboring the identical mutation have been used to interfere with the biological activities of *Xenopus* homeoproteins such as *Mix.1* (Mead et al., 1996), *Xvent-2* (Onichtchouk et al., 1998), *XNkx2-3*, and *XNkx2-5* (Grow & Krieg, 1998). The *CSX/NKX2-5 LP* mutant also acts as a dominant-negative repressor because it inhibited *CSX/NKX2-5*-induced promoter activation in a dose-dependent manner and inhibited cardiac differentiation of *P19CL6* cells (Toko et al., 2002). In dominant-negative *CSX/NKX2-5* transgenic mice, cardiac function was deteriorated and degenerative changes of cardiomyocytes were apparent. Furthermore, after the injection of doxorubicin, dominant-negative *CSX/*

NKX2-5 transgenic mice showed more severe cardiac dysfunction in association with a significant increase in cardiomyocyte apoptosis, while doxorubicin-induced myocardial damage was mild in transgenic mice overexpressing the wild-type of CSX/NKX2-5. These results indicate a cardioprotective role of Csx/Nkx2-5 in postnatal hearts.

However, the apparently established notion that Csx/Nkx2-5 is cardioprotective is now open to question. Kasahara et al. (2003) generated transgenic mice overexpressing wild-type of murine *Csx/Nkx2-5* under the control of the  $\alpha$ -MHC promoter. Unexpectedly, the transgenic mice exhibited heart failure with AV conduction disturbance. Cardiomyocytes expressing the transgene showed sarcomere disorganization in association with down-regulation of *connexin 43* expression. Furthermore, the adenoviral delivery of Csx/Nkx2-5 in cultured postnatal cardiomyocytes induced the down-regulation of its potential target genes such as *connexins*, *ANP*, and *CARP*. From these results, the authors concluded that the overexpression of Csx/Nkx2-5 altered cardiac structure and function in postnatal hearts. It could be in principle possible that transcriptional activities switch from “promotive” to “repressive” according to the context of cell types. The contradictory effects of Csx/Nkx2-5 gain-of-function in the hearts might arise from the functional gaps of human CSX/NKX2-5 versus murine Csx/Nkx2-5, but it is likely that Csx/Nkx2-5 proteins in both species should share the fundamental properties in common. Alternatively, the phenotypes might be dependent on the expression levels of *Csx/Nkx2-5* transgenes (Akazawa & Komuro, 2003b). Of note, the overexpression of a *Csx/Nkx2-5* mutant with truncation of the C-terminus induced apoptosis in cultured rat neonatal cardiomyocytes, although this mutant functioned as a transcriptional activator of the *ANP* promoter (Zhu et al., 2000). It is speculated that the transcriptional activity of Csx/Nkx2-5 may be strictly regulated by the expression levels and be modulated both positively and

negatively by the interacting endogenous partners in a cell-type-specific fashion.

### 3. Conclusion and perspectives

In summary, Csx/Nkx2-5 plays a crucial role in transcriptional regulation essential for normal cardiac development and homeostasis of postnatal hearts (Fig. 3). Especially, Csx/Nkx2-5 is involved in cardiac morphogenesis, including rightward looping and subsequent chamber specification and septation and in functional maturation and maintenance of working myocardium and conduction system. Several mutations of human *CSX/NKX2-5* have been identified that cause nonsyndromic CHD occasionally associated with AV block. A number of downstream target genes have been identified, and transcriptional regulatory mechanisms whereby protein–protein interactions with other cofactors allow an intricate gene expression have been clarified.

However, our current knowledge just outlines the functional role of Csx/Nkx2-5 in the heart, and numerous issues remain to be solved to make our understandings to be satisfactory. Insomuch as Csx/Nkx2-5 is considered to be an early and specific marker for cardiogenic lineage, the elucidation of the pathways controlling *Csx/Nkx2-5* expression will provide novel insights into the molecular mechanism of vertebrate heart formation. In addition, the pathways enhancing *Csx/Nkx2-5* expression in response to hypertrophic stimulations should be clarified. With regard to transcriptional regulation by Csx/Nkx2-5, it is assumed that Csx/Nkx2-5 executes regulatory decisions in combination with ubiquitous and tissue-specific cofactors. It should be comprehensively determined how much the cooperative transcriptional regulation weighs and how the mutual interaction is regulated according to the stages, cell types, and extracellular stimulations. The elucidation of the

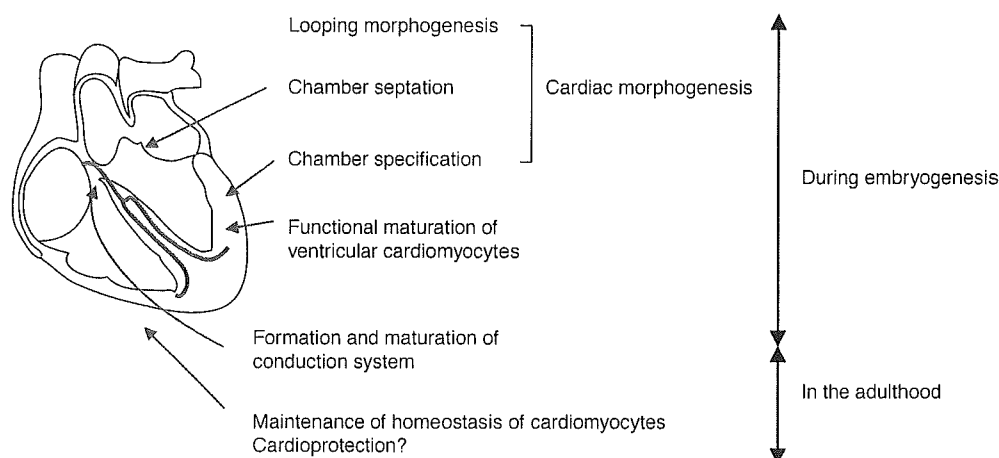


Fig. 3. Functional role of Csx/Nkx2-5 during embryogenesis, and in adulthood, Csx/Nkx2-5 plays an important role in transcriptional regulation essential for normal cardiac development and homeostasis of postnatal hearts. Especially, Csx/Nkx2-5 is involved in cardiac morphogenesis, including rightward looping and subsequent chamber specification and septation and in the functional maturation and maintenance of working myocardium and conduction system.

upstream and downstream framework involving transcriptional regulation by *Csx/Nkx2-5* will not only enrich our understanding of the intrinsic cardiogenic program, but also provide a helpful clue to make the ‘stem cell therapy’ to be more sophisticated. Recent discoveries of stem cells or progenitor cells with an ability to differentiate into cardiomyocytes have prompted researchers to try to repair the damaged heart by introducing the exogenous stem cells or by stimulating the endogenous stem cells, but these approaches have not obtained satisfactory results because of the low efficiency of cardiomyocytes differentiation (reviewed in Caplice & Deb, 2004; Mathur & Martin, 2004). It remains largely unknown whether the cardiogenic signaling pathways are similarly utilized during cardiomyocytes differentiation of adult stem cells or progenitor cells, but adult heart-derived cardiac progenitor cells expressing stem cell antigen-1 (Sca-1) was reported to differentiate in vitro in part depending on the BMP signals (Oh et al., 2003). Recently, we isolated Sca-1-positive stem cells from adult hearts and demonstrated that Sca-1-positive cells differentiated in vitro into beating cardiomyocytes when treated with oxytocin (Matsuura et al., 2004), which is also capable of inducing differentiation of P19 cells into cardiomyocytes (Paquin et al., 2002). A detailed understanding of transcriptional regulation involving *Csx/Nkx2-5* will contribute to enhance the efficacy of stem cell therapy by optimizing the strategy for stimulating the stem cells to highly differentiated cardiomyocytes.

Despite the accumulation of case reports presenting clinical features caused by specific *CSX/NKX2-5* mutations, the mechanisms linking each mutation to cardiac phenotypes remains obscure. Inasmuch as transcriptional activity of *Csx/Nkx2-5* is supposed to be subject to genetic modifiers in a complex manner, the creation and analysis of genetically engineered mouse models will be required for the clarification of the genotype–phenotype relationship in the individual *Csx/Nkx2-5* mutations. Further cumulative information regarding *CSX/NKX2-5* mutations will also facilitate the genetic counseling on affected individuals.

Finally, the postnatal role of *Csx/Nkx2-5* remains to be an open question, “to be cardioprotective or cardiotoxic”? A recent paper demonstrated that the dysregulation of contractile proteins and ion channels might contribute to cardiac dysfunction in *Csx/Nkx2-5*-deficient hearts, supporting the cardioprotective role of *Csx/Nkx2-5* that extends into maintenance of normal cardiac function throughout life (Pashmforoush et al., 2004). Then, is it possible to restore and maintain cardiac function in failing hearts by introducing *Csx/Nkx2-5* expression? Indeed, *Csx/Nkx2-5* may be a promising therapeutic target for cardiovascular diseases, but the researcher should be cautious because too much *Csx/Nkx2-5* is as bad as too little (Akazawa & Komuro, 2003b). In a near future, a high-throughput screen may identify a chemical compound that is capable of modulating the transcriptional activity of *Csx/Nkx2-5*. Further investigation will be required to understand the molecular basis of the

gene expression program by *Csx/Nkx2-5* and to target this molecule for therapeutic purposes.

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## Kir6.2-deficient mice are susceptible to stimulated ANP secretion: $K_{ATP}$ channel acts as a negative feedback mechanism?

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### Abstract

**Objective:** While atrial natriuretic peptide (ANP) has been shown to be released mainly from cardiac muscle cells in response to atrial distension, the regulatory mechanisms of ANP secretion are still not fully understood. We sought to determine whether the ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channel modulates the secretion of ANP, using mice with homozygous knockout of the Kir6.2 (a pore-forming subunit of cardiac  $K_{ATP}$  channel) gene.

**Methods:**  $K_{ATP}$  channel currents were recorded from isolated mouse atrial cells with patch-clamp techniques. Plasma ANP concentrations in anesthetized mice and ANP content and secretion in isolated atrial preparations were determined by radioimmunoassay. Action potentials were recorded from the isolated atria.

**Results:** Exposure to 2,4-dinitrophenol (100  $\mu$ M) evoked a glibenclamide-sensitive  $K_{ATP}$  channel current in atrial cells from wild-type (WT) but not Kir6.2 knockout (Kir6.2 KO) mice. Although there were no significant differences in the basal plasma ANP levels between WT and Kir6.2 KO mice, volume expansion caused a significant elevation of plasma ANP concentration in Kir6.2 KO but not WT mice with accompanying hypotension. When isolated left atria were stretched, ANP secreted into the bath from Kir6.2 KO atria was significantly higher than that from WT atria. Furthermore, stretching the atria from WT but not Kir6.2 KO mice significantly shortened the action potential duration. A hypotonic stretch of the membrane induced the glibenclamide-sensitive  $K_{ATP}$  channel current in atrial cells from WT but not Kir6.2 KO mice.

**Conclusions:** Kir6.2 is essential for the function of  $K_{ATP}$  channel in mouse atrial cells. Given that Kir6.2 KO mice are susceptible to stretch-induced secretion of ANP, our results suggest that  $K_{ATP}$  channels may act as a negative feedback mechanism for the control of ANP secretion. © 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

**Keywords:** Natriuretic peptide; K-ATP channel; Myocytes

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### 1. Introduction

ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels, originally discovered in cardiac muscle [1], are present in many tissues and play an important role in various cellular responses [2]. The molecular identity of sarcolemmal  $K_{ATP}$  channels is now known to be a hetero-octameric complex of four pore-

forming subunits (Kir6.x) and four sulfonylurea receptor regulatory subunits (SUR) [2]. Previous studies have shown that mutant mice lacking the Kir6.2 subunit of  $K_{ATP}$  channels (Kir6.2 KO mice) display obvious impairment of insulin response to glucose [3] and are susceptible to generalized seizures after brief hypoxia [4]. Our recent studies using the Kir6.2 KO mice have provided direct evidence that Kir6.2 forms the pore region of ventricular  $K_{ATP}$  channels [5] and the activation of  $K_{ATP}$  channels plays an important role in cardioprotection [6]. More recently, Kir6.2 KO mouse models highlight the importance of  $K_{ATP}$  channels in adaptation to stress beyond their role in cytoprotection [7].

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Although considerable advances have been made in recent years towards understanding the nature of ventricular  $K_{ATP}$  channels, the molecular identity and functional role of atrial  $K_{ATP}$  channels are poorly understood. Atrial distention causes release into the circulation of atrial natriuretic peptide (ANP), a hormone that plays a role in the regulation of cardiovascular homeostasis [8]. Since the gating of the atrial  $K_{ATP}$  channel is mechanosensitive [9], the relationship to the process of ANP secretion is a subject of considerable interest. Kim et al. [10] reported that the  $K_{ATP}$  channel blocker glibenclamide suppressed the stretch-stimulated ANP secretion from rat atria. On the other hand, conflicting observation was reported that the  $K_{ATP}$  channel blocker tolbutamide increased the release of ANP in neonatal rat atrial myocytes [11]. Moreover, Xu et al. [12] reported that the stretch-induced ANP secretion was inhibited by  $K_{ATP}$  channel openers. Thus, the results so far obtained by using  $K_{ATP}$  channel blockers and openers are not conclusive. In this study, we sought to determine whether atrial  $K_{ATP}$  channel regulates the secretion of ANP, using Kir6.2-deficient mice. The results show that Kir6.2 is essential for the function of atrial  $K_{ATP}$  channel and Kir6.2-deficient mice are susceptible to stretch-induced secretion of ANP.

## 2. Methods

### 2.1. Kir6.2-deficient mice

All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Chiba University. A mouse line deficient in the  $K_{ATP}$  channels was generated by targeted disruption of the gene coding for Kir6.2, as described previously [3]. C57BL/6 mice were used as control because they had been back-crossed to a C57BL/6 strain for five generations. Twelve- to fourteen-week-old Kir6.2-deficient mice or C57BL/6 control mice were used in this study.

### 2.2. Electrophysiology

Single atrial cells were enzymatically isolated by the method of Suzuki et al. [5] with some modifications. Single-channel and whole-cell membrane currents were recorded by the patch-clamp method as previously described [5,6]. Whole-cell current recordings were performed at 36 °C with nystatin in the pipette solution. Single atrial cells were superfused with HEPES–Tyrode's solution (in mM): NaCl 143, KCl 5.4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 0.33, glucose 5.5, and HEPES–NaOH buffer 10 (pH=7.4). The pipette solution contained (in mM): K-aspartate 110, KCl 20, MgCl<sub>2</sub> 1.0, CaCl<sub>2</sub> 1.0, EGTA 0.1, and HEPES–KOH buffer 5 (pH=7.4), with 250 µg/ml nystatin. A ramp-pulse protocol (–100 to +50 mV over 2.5 s, repeated at 30-s

intervals) was used to record the quasi-steady-state membrane current. For the experiments of hypotonic stretch of the membrane (Fig. 6), the isolated atrial cells were exposed to the low-chloride isotonic or hypotonic solution in order to minimize swelling-induced chloride current contamination [13]. Isotonic solution contained (in mM): Na-aspartate 80, KCl 5.4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 0.33, glucose 5.5, mannitol 119, and HEPES–NaOH buffer 10 (pH=7.4, ≈36 °C) with an osmolarity of 300 mosM/kg H<sub>2</sub>O. Hypotonic solution was prepared by simply omitting mannitol from the isotonic solution with the osmolality of 180 mosM/kg H<sub>2</sub>O. For single-channel recordings from the cell-attached patches, symmetrical high K<sup>+</sup> external and internal solutions (≈22 °C) were used. The external solution contained (in mM): KCl 140, EGTA 5, Na<sub>2</sub>–ATP 0.1, and HEPES–KOH buffer 5 (pH=7.4) and the pipette solution contained (in mM): KCl 140, MgCl<sub>2</sub> 1.8, CaCl<sub>2</sub> 1.8, and HEPES–KOH buffer 5 (pH=7.4). The current signals were digitized at 2 kHz for data analysis with pClamp software (Axon Instruments, Foster City, CA).

### 2.3. Volume expansion

The mice were anesthetized with urethane (1.5 g/kg, i.p.) and placed on a heating pad to maintain rectal temperatures at 37 °C. The left external jugular vein was cannulated with small polyethylene catheters for intravenous infusion. Volume expansion was performed by a modification of the method of Kishimoto et al. [14]. The lactated Ringer's solution containing 4.5% bovine serum albumin was infused at a rate of 200 µl/h/g (body weight) for 30 min. The rate of infusion was then lowered to 4.3 µl/h/g for another 30 min. In some experiments the initial infusion rate was increased from 200 to 400 µl/h/g for 30 min, after which the infusion was continued at a rate of 4.3 µl/h/g for 30 min. These infusions were carried out with a microinfusion pump (Harvard Apparatus, MA). At the end of the volume expansion, blood (700 µl) was drawn from the right carotid artery, collected into a tube containing EDTA and aprotinin, and centrifuged for 20 min at 4 °C. Plasma samples were stored at –80 °C until analysis by ANP radioimmunoassay.

### 2.4. Hemodynamic measurements

Arterial blood pressure was measured continuously in mice anesthetized with urethane. The right carotid artery was cannulated with small polyethylene catheters for the measurement of blood pressure via a pressure transducer. Heart rate was derived from the arterial blood pressure signal.

### 2.5. Isolated atrial preparations

Tissue bath preparations of left atria were prepared by a modification of the method described by Bilder et al. [15].

Briefly, left atria from WT and Kir6.2 KO mice were dissected free and placed in a 3-ml water jacketed tissue bath containing HEPES–Tyrode's solution (pH=7.4) gassed with 100% O<sub>2</sub> (37 °C). The edge of preparation was pinned to the rubber base of the tissue bath and electrically paced at a frequency of 5 Hz. After 10 min equilibration, the bath solution was discarded and 1 ml of fresh HEPES–Tyrode's solution was added to the bath. Atrial preparations were incubated in this solution with or without stretch stimulus. The atria connected to a string were stretched with a fixed resting tension of 0.5 g. After 10 min of incubation, the bath solution was collected into a tube containing EDTA and aprotinin and was stored at –80 °C until analysis by ANP radioimmunoassay.

### 2.6. ANP content of the atria

The hearts were rapidly removed from the anesthetized mice and the left atria were dissected out and immediately frozen in liquid nitrogen. The lysates were obtained from three preparations by homogenization in ice-cold buffer (0.1 mM acetic acid). The lysates were incubated for 8 min at 100 °C, kept on ice for 15 min and cleared by centrifugation at 66,000 × *g* for 20 min at 4 °C. Protein concentration was determined by the BCA protein assay kit (PIERCE, Rockford, IL).

### 2.7. ANP radioimmunoassay

The concentration of immunoreactive ANP was measured by commercially available radioimmunoassay, as described previously [16]. The threshold for detection of ANP was 1.5 pg/ml.

### 2.8. Action potential recordings

The preparations of left atria from WT and Kir6.2 KO mice were mounted in a recording chamber and perfused at a constant flow (5 ml/min) with Tyrode's solution (in mM): NaCl 125, KCl 4.0, CaCl<sub>2</sub> 2.7, MgCl<sub>2</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 1.8, glucose 5.5, and NaHCO<sub>3</sub> 25 and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> (37 °C). Action potentials were evoked by electrical field stimulation at 5 Hz (2-ms rectangular pulses at 2 × threshold intensity) and recorded by use of a 3 M KCl-filled microelectrode (tip resistance 10–20 MΩ). The edge of left atrial preparation was stretched with a fixed resting tension of 0.5 g. Transmembrane potential was recorded by a direct current preamplifier (MEZ-7200, Nihon Kohden, Tokyo, Japan) and digitized (PowerLab 2/20, ADInstruments, Castle Hill, Australia).

### 2.9. Drugs

The following drugs were used: nystatin (Wako Pure Chemicals, Osaka, Japan) and glibenclamide (Sigma-Aldrich Japan, Tokyo, Japan). Nystatin was dissolved in

methanol at a concentration of 10 mg/ml and added to the pipette solution at a concentration of 250 μg/ml just before experiments. Glibenclamide was dissolved in dimethyl sulfoxide as a stock solution of 10 mM and final concentration of dimethyl sulfoxide was less than 0.1%.

### 2.10. Statistics

All data are presented as mean ± SEM. Statistical comparisons were made with the use of Student's *t* test or ANOVA combined with Fisher post hoc test, as appropriate. A *p* value of less than 0.05 was considered significant.

## 3. Results

### 3.1. K<sub>ATP</sub> channel function in atrial cells

We first evaluated K<sub>ATP</sub> channel function in atrial cells isolated from wild-type (WT) and Kir6.2-deficient (Kir6.2 KO) mice. Whole-cell membrane currents were recorded using a ramp-pulse protocol (Fig. 1A and B). There were no significant differences in the density of the outward current at 0 mV between atrial cells isolated from WT (1.4 ± 0.1 pA/pF, *n*=6) and Kir6.2 KO mice (1.6 ± 0.1 pA/pF, *n*=6) in the control condition. In WT cells, metabolic inhibition with a glucose-free, 100 μM DNP-containing solution induced an outward current (9.1 ± 0.1 pA/pF at 0 mV, *n*=6), which, by virtue of its blockade by 1 μM glibenclamide (1.3 ± 0.1 pA/pF, *n*=6), was confirmed to be the K<sub>ATP</sub> current. However, such a membrane current change was not observed in Kir6.2 KO cells (1.6 ± 0.1 pA/pF after metabolic inhibition, *n*=6). When the atrial cells were exposed to a solution containing DNP (100 μM), single K<sub>ATP</sub> channel activity could be recorded from 25 of 25 cell-attached patches of 9 WT mice (Fig. 1C). The channel openings were inhibited by addition of 1 μM glibenclamide to the solution (data not shown). The linear slope conductance, obtained from the current–voltage relationship from –100 to –40 mV for the single channel current of WT cells, was 71.6 ± 1.7 pS (*n*=7). In contrast, opening of K<sub>ATP</sub> channels could not be recorded from any cell-attached patches of Kir6.2 KO cells (*n*=18). These results indicate that Kir6.2 is essential for the function of K<sub>ATP</sub> channel in atrial cells.

### 3.2. Effect of volume expansion on plasma ANP level

In anesthetized mice, there were no significant differences in the basal plasma ANP concentrations between WT (28.7 ± 3.0 pg/ml, *n*=7) and Kir6.2 KO mice (36.6 ± 4.9 pg/ml, *n*=6). After volume expansion (200 μl/h/g for 30 min and 4.3 μl/h/g for another 30 min), the plasma ANP level was increased to 31.8 ± 1.6 pg/ml (*n*=7) in WT mice, but this change was not statistically significant (*p*=0.385 vs. baseline). On the other hand, Kir6.2 KO mice reacted to

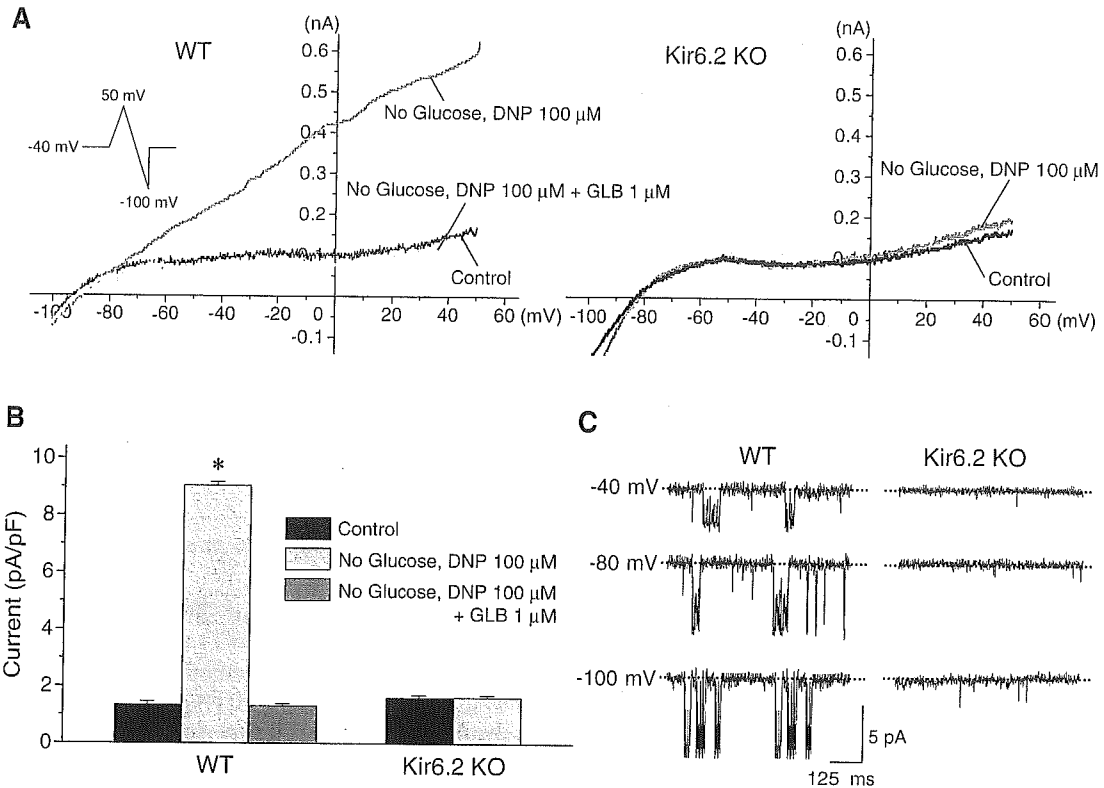


Fig. 1. (A) Effect of metabolic inhibition with a glucose-free, DNP-containing (100  $\mu\text{M}$ ) solution and co-application of glibenclamide (GLB, 1  $\mu\text{M}$ ) on the nystatin-perforated whole-cell membrane currents recorded from atrial cells of WT (left) and Kir6.2 KO mice (right). (B) Current densities at 0 mV from control ( $n=6$  of WT,  $n=6$  of Kir6.2 KO, black bar), DNP-treated ( $n=6$  of WT,  $n=6$  of Kir6.2 KO, grey bar) and DNP+GLB ( $n=6$  of WT, dark grey bar) mice of atrial cells are summarized. Values are mean  $\pm$  SEM. \* $p < 0.01$  vs. control. (C) Single-channel current recordings in the cell-attached mode by addition of DNP (100  $\mu\text{M}$ ) from atrial cells of WT (left) and Kir6.2 KO mice (right). The test potential is indicated on the left.

volume expansion and the plasma ANP concentration significantly increased to  $73.5 \pm 13.4$  pg/ml ( $n=5$ ,  $p=0.021$  vs. baseline). The elevation of plasma ANP levels observed in Kir6.2 KO mice was notably higher than that observed in WT mice ( $p=0.004$ , Fig. 2). When the initial infusion rate was increased from 200 to 400  $\mu\text{l/h/g}$  for 30 min (then the rate of infusion was lowered to 4.3  $\mu\text{l/h/g}$  for 30 min) to give a severe volume expansion, the plasma ANP concentration in WT mice significantly increased to  $49.8 \pm 8.2$  pg/ml ( $n=4$ ,  $p=0.024$  vs. baseline). This value was not significantly different from that in Kir6.2 KO mice ( $53.9 \pm 4.9$  pg/ml,  $n=4$ ). These results indicate that Kir6.2 KO mice are susceptible to the ANP secretion by volume expansion.

### 3.3. Hemodynamic effects of volume expansion

There were no significant differences in the basal values of mean arterial pressure (MAP) between WT and Kir6.2 KO mice (Fig. 3). The values of MAP at 30 min of volume expansion were similarly increased in both WT ( $112.0 \pm 4.1\%$  of baseline,  $n=4$ ) and Kir6.2 KO mice ( $111.3 \pm 2.3\%$  of baseline,  $n=4$ ). In WT mice, MAP returned to baseline

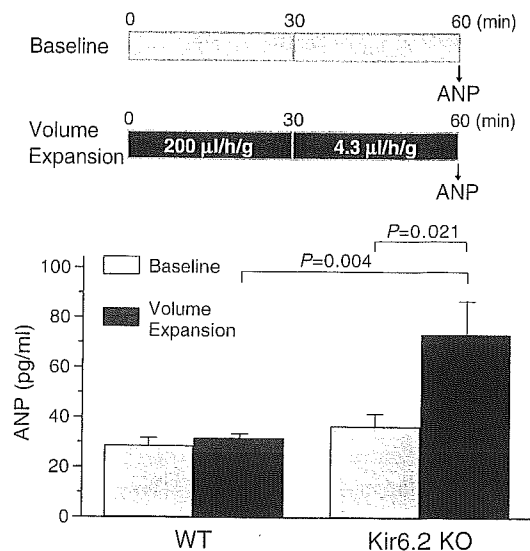


Fig. 2. Effect of volume expansion on plasma ANP levels of WT and Kir6.2 KO mice. Top panel shows the experimental protocol. Bottom panel shows summarized data for plasma ANP concentration. Blood samples were drawn at baseline ( $n=7$  of WT,  $n=6$  of Kir6.2 KO, grey bar) and after volume expansion ( $n=7$  of WT,  $n=5$  of Kir6.2 KO, black bar). Values are mean  $\pm$  SEM.

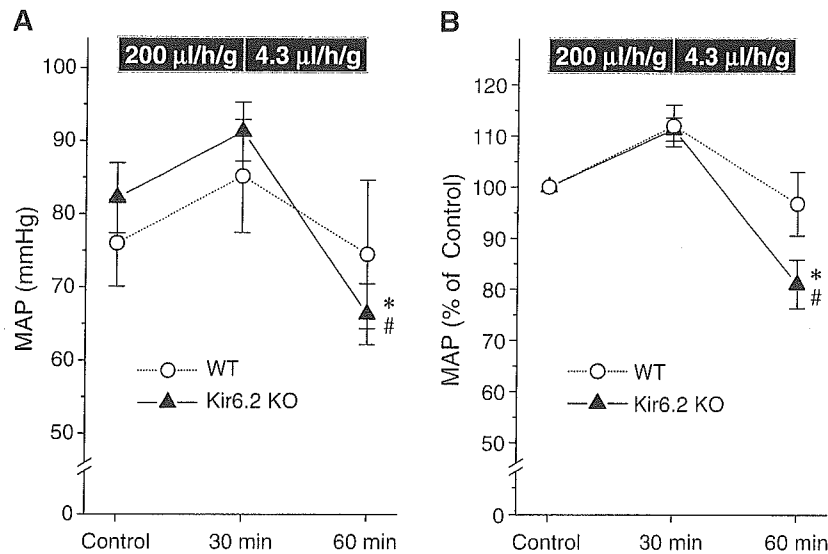


Fig. 3. Effect of volume expansion on mean arterial pressure (MAP) in anesthetized mice. (A) Changes of MAP before (baseline) and after 30 and 60 min of volume expansion. (B) MAPs are given as a percentage of the value measured at baseline. Data represent mean  $\pm$  SEM of 4 experiments for each group. \* $p < 0.05$  vs. baseline, # $p < 0.05$  vs. 30 min.

levels after 60 min of volume expansion ( $96.7 \pm 6.2\%$  of baseline,  $n = 4$ ). In contrast, 60 min of volume expansion in the Kir6.2 KO mice produced significant decrease in MAP ( $81.0 \pm 4.7\%$  of baseline,  $n = 4$ ,  $p < 0.05$ ). There were no significant differences in the basal values of heart rate between WT ( $654 \pm 31$  bpm,  $n = 7$ ) and Kir6.2 KO mice ( $642 \pm 50$  bpm,  $n = 5$ ). Volume expansion did not alter the heart rate in both WT ( $689 \pm 25$  bpm,  $n = 7$ ) and Kir6.2 KO mice ( $644 \pm 50$  bpm,  $n = 5$ ).

### 3.4. ANP secretion from isolated atria

Isolated mouse atrial preparations were used to study the direct effect of mechanical stretch on ANP release. As shown in Fig. 4, in most cases the ANP concentration did

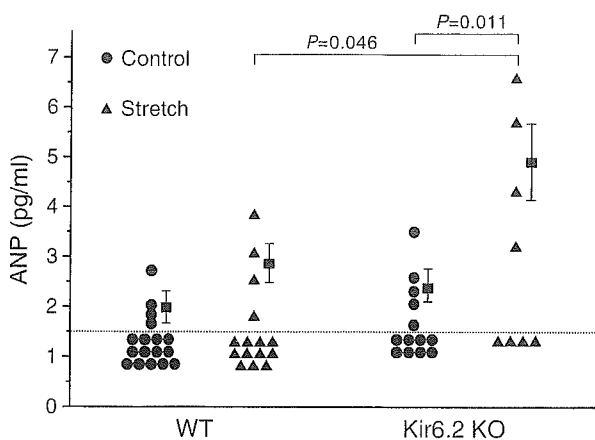


Fig. 4. Effects of mechanical stretch on ANP secretion in isolated atria from WT and Kir6.2 KO mice. The threshold for detection of ANP (1.5 pg/ml) is represented as dotted line. Each symbol represents data from individual preparations. Closed squares indicate the mean and the vertical bars indicate SEM.

not exceed the threshold for detection ( $\geq 1.5$  pg/ml). We therefore repeated the experiment until the ANP secretion was detected in 4 or 5 of the preparations in each group. In control (non-stretch) conditions, ANP secretion was detected in 4 of 17 atria from WT and in 5 of 13 atria from Kir6.2 KO, respectively. The mean ANP concentration in each of 4 or 5 preparations (WT:  $2.2 \pm 0.2$  pg/ml, Kir6.2 KO:  $2.8 \pm 0.4$  pg/ml) was not statistically significant. After mechanical stretch for 10 min, ANP secretion was detected in 4 of 15 atria from WT. In Kir6.2 KO atria, ANP secretion was detected in 4 of 8 preparations and the mean ANP concentration ( $4.9 \pm 0.7$  pg/ml) was notably higher than that observed in WT atria ( $2.4 \pm 0.3$  pg/ml,  $p = 0.046$ ).

ANP content of atria obtained from WT and Kir6.2 KO mice was  $0.64 \pm 0.30$  ng/mg protein and  $0.74 \pm 0.23$  ng/mg protein, respectively; these values did not reach statistical significance ( $n = 3$ ,  $p = 0.696$ ).

### 3.5. Action potentials in isolated atria

Representative recordings of action potentials before and after 5 min of stretch are shown in Fig. 5A and B. The action potential duration (APD) in the WT atrium was shortened at 5 min of stretch, while the APD remained unaltered in the Kir6.2 KO atrium. As summarized in Fig. 5C, stretching the WT atria significantly shortened the APD measured at 90% repolarization (APD<sub>90</sub>) to  $71.8 \pm 3.4\%$  of control ( $n = 4$ ,  $p = 0.004$ ). Stretch-induced shortening of APD<sub>90</sub> was not observed in Kir6.2 KO atria ( $98.6 \pm 0.7\%$ ,  $n = 4$ ).

### 3.6. Hypotonic stretch-induced $K_{ATP}$ current

To determine whether  $K_{ATP}$  channel currents could be mechanically induced, the nystatin-perforated patch was