Effect of Mental Stress on Coronary Flow Velocity Reserve in Healthy Men

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The effect of mental stress on coronary flow velocity reserve (CFVR) was examined in healthy men using transthoracic Doppler echocardiography. In the mental stress group (n = 31), CFVR was significantly reduced at 15 (to 3.3 ± 0.8, p <0.001) and 30 (to 3.7 ± 0.8, p <0.01) minutes after mental stress testing, compared with before mental stress (4.3 ± 0.9), whereas it did not change in each of 3 measurements in control subjects (n = 10). Mental stress impaired coronary circulation even after a certain interval after the stress. **(Am J Cardiol 2005;96:137–140)**

It has been reported that mental stress may induce myocardial ischemia,[1,2] whereas cardiovascular events sometimes occur after a certain interval after mental stress.[3,4] However, the change in coronary circulation after mental stress is unknown. Coronary flow velocity reserve (CFVR), partly endothelium dependent, has been considered a useful physiologic index of coronary microcirculation.[5] Recent developments in transthoracic Doppler echocardiography (TTDE) can provide noninvasive measurements of CFVR in the left anterior descending (LAD) coronary artery.[6–8] This study assessed the response of coronary circulation after mental stress using successive measurements of CFVR by TTDE in healthy men.

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We studied 44 healthy men (mean age 28.6 ± 6.4 years, range 20 to 49). We included only men in this study, because CFVR could be influenced by menstrual status in women.[9] None of subjects had a history of hyperlipidemia, hypertension, or diabetes mellitus or evidence of left ventricular wall motion abnormalities or left ventricular hypertrophy. Informed consent to the protocol, which had been approved by the ethics committee of our hospital, was obtained from all subjects. The 44 volunteers were randomly divided into 2 groups of 34 men with mental stress (the mental stress group) and 10 men without mental stress (the control group). Blood samples were taken immediately before the assessment of CFVR for the determination of the serum concentrations of blood sugar, total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol.

The method of measurement of CFVR with TTDE has been described previously.[5,7–9] Echocardiographic examinations were performed with an Acuson Sequoia 512 (Siemens Medical Solutions USA, Inc., Mountain View, California) using a high-frequency transducer (5 to 7 MHz). For color Doppler echocardiography, the velocity range was set at 11 to 17 cm/s. The acoustic window was around the midclavicular line in the fourth and fifth intercostal spaces in the left lateral decubitus position. After the lower portion of the interventricular sulcus had been located in the long-axis cross-sections, the ultrasound beam was rotated laterally, visualizing the distal portion of the LAD coronary artery under color flow mapping guidance. Blood flow velocity was measured by pulse-wave Doppler echocardiography, using a sample volume (1.5 to 2.0 mm) placed on the color signal in the distal LAD coronary artery. Adenosine triphosphate was administered by intravenous infusion (140 μg/kg/min) for 2 minutes to record spectral Doppler signals during hyperemia. The electrocardiogram and heart rate were monitored continuously during the examination. Blood pressure was recorded at baseline and every minute after the intravenous infusion of adenosine triphosphate. The rate–pressure product was calculated as systolic blood pressure × heart rate. Although we tried to align the ultrasound beam direction with distal LAD coronary artery flow in as parallel a manner as possible, angle correction was needed in each examination because of incident Doppler angle (mean angle 41°, range 25° to 54°). All studies were continuously recorded on S-VHS videotape, and clips of the stopped frame were also stored digitally on a magneto-optical disk (230 MB) for off-line analysis. Each study was analyzed by 2 experienced investigators blinded to the other data. Measurements of mean diastolic flow velocity were performed off-line by contouring the spectral Doppler signals, using the integrated evaluation program in the ultra-

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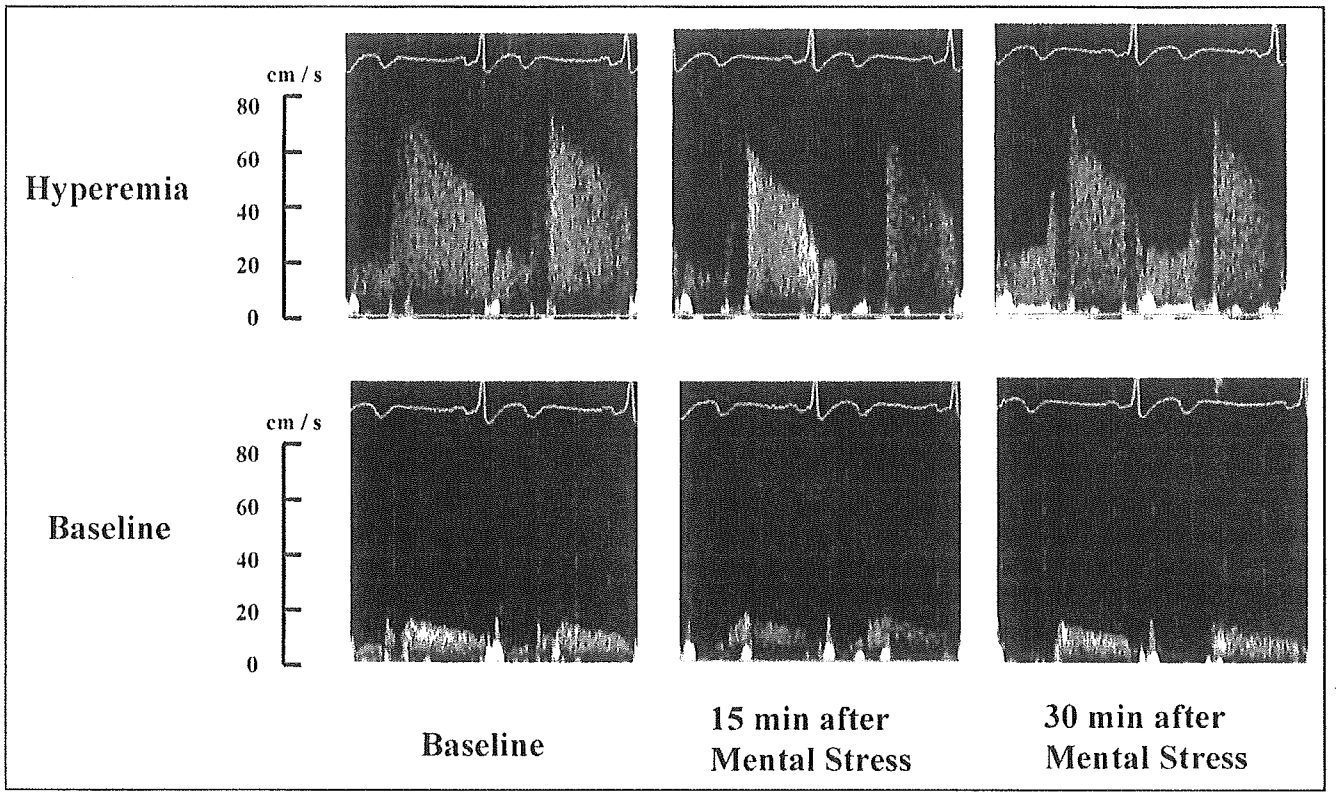

Figure 1. Coronary flow velocity profiles at baseline and hyperemia obtained from 1 patient in the mental stress group. Hyperemic coronary blood flow velocity at 15 minutes after mental stress was significantly less than before stress testing. *Bottom*, coronary flow velocity profile at baseline. *Top*, coronary flow velocity profile at hyperemic condition.

sound system. Each variable was averaged over 3 consecutive cycles. CFVR was calculated as the ratio of hyperemic to basal mean diastolic flow velocity.

All studies were performed in the morning in a quiet, air-conditioned room (22°C to 25°C). During this study, the room lights were dimmed, background noise was maximally reduced, and subjects were encouraged to relax. After 30 minutes of rest, a baseline measurement of CFVR was performed by TTDE immediately before the mental stress test. For mental stress, subjects were forced to add as quickly and accurately as possible using the Uchida-Krapelin psychodiagnostic test for 5 minutes.[10] A metronome was used as an additional distractor. At 15 and 30 minutes of quiet rest after the test, CFVR was assessed in the same manner. The 15-minute interval was chosen on the basis of previous reports[2] that all hemodynamic changes returned to baseline levels at 15 minutes after mental stress testing. In the control group, after 30 minutes of rest, CFVR was measured 3 times at 15-minute intervals by TTDE.

Data are shown as mean ± SD. Baseline characteristics in the 2 groups were compared by the unpaired Student's *t* test. Echocardiographic and hemodynamic variables during adenosine triphosphate infusion in the 2 groups were evaluated by repeated-measures analysis of variance, testing for stress effect, adenosine triphosphate effect, and interaction. Fisher's protested least-significant difference test was used for the post hoc test. A p value <0.05 was considered statistically significant.

Adequate spectral Doppler recordings of coronary flow for assessing CFVR were obtained in 41 (31 in the mental stress group and 10 in the control group) of the 44 study subjects (93%) (Figure 1). The clinical characteristics of the 2 study groups are listed in Table 1. There were no differences between these 2 groups in terms of age, body mass index, smoking status, lipid profile (total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein

Table 1
Clinical characteristics

Characteristic	Control (n = 10)	Mental Stress (n = 31)
Age (yrs)	29.0 ± 9.2	27.8 ± 5.5
Men	10 (100%)	31 (100%)
Body mass index	23.8 ± 1.9	22.8 ± 2.0
Smoking	2 (20%)	7 (22.5%)
Total cholesterol (mg/dl)	182 ± 38	187 ± 33
Low-density lipoprotein cholesterol (mg/dl)	107 ± 35	113 ± 34
High-density lipoprotein cholesterol (mg/dl)	64 ± 16	58 ± 14
Triglycerides (mg/dl)	128 ± 97	116 ± 85
Blood sugar (mg/dl)	101 ± 14	100 ± 17

Data are presented as mean ± SD.

cholesterol, triglycerides), and blood sugar. None of the subjects experienced any symptoms or presented any electrocardiographic changes during the mental stress test or adenosine triphosphate administration.

Table 2 lists the hemodynamic data at the measurements of CFVR, before and 15 and 30 minutes after mental stress. Mental stress induced significant increases in heart rate, systolic blood pressure, diastolic blood pressure, and the rate–pressure product immediately after mental stress, and these parameters returned to baseline values just before the CFVR measurements after mental stress. No significant differences were observed in hemodynamic data in each of the measurements of CFVR in the same group and between the 2 groups.

In the control group, mean diastolic flow velocity at baseline and CFVR were similar in each of the 3 measurements (Figure 2 and Table 3). In the mental stress group, there was no difference in mean diastolic flow velocity at baseline before and 15 and 30 minutes after mental stress. However, repeated-measures analysis of variance showed a significant interaction in mean diastolic flow velocity at 15 minutes after the stress test between the 2 groups over adenosine triphosphate ($p = 0.02$). Furthermore, there was a significant group effect and interaction in CFVR in the 3 measurements ($p < 0.001$). CFVR significantly decreased at 15 (to 3.3 ± 0.8, $p < 0.001$) and 30 (to 3.7 ± 0.8, $p < 0.01$) minutes after mental stress, compared with before mental stress (4.3 ± 0.9). No significant correlations were observed among decreased CVFR and increased parameters. such as heart rate, blood pressure, and the rate–pressure product during mental stress. Inter- and intraobserver variabilities for measurement of Doppler velocity recording were 5.0% and 3.9%, respectively.

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We have demonstrated for the first time that CFVR was reduced even after a very brief period of mental stress in healthy men without coronary risk factors, even after blood pressure and heart rate returned to baseline levels. This abnormal response of coronary circulation to mental stress may indicate the mechanism by which repetitive mental stress induces coronary circulatory dysfunction, leading to ischemic events in subclinical subjects.

In this study, we measured CFVR with TTDE as an index of coronary microcirculation, which is widely accepted as a surrogate for coronary flow reserve.[5] Although coronary flow reserve is ideal for assessing the function of coronary microcirculation, coronary flow velocity change, but not coronary flow volume, is measured in the epicardial coronary artery. However, it is reported that changes in coronary flow velocity during drug-induced hyperemia closely reflect changes in coronary blood flow.[11] Recent studies have found a good correlation between CFVR assessed with Doppler guidewire and coronary flow assessed by perfusion scintigraphy and positron emission tomography.[12,13] Moreover, the assessment of CFVR with TTDE,

Table 2
Changes in hemodynamics

Variable	Before		During Mental Stress	15 Minutes After		30 Minutes After	
	Baseline	Hyperemia		Baseline	Hyperemia	Baseline	Hyperemia
Mental stress group (n = 31)							
Heart rate (beats/min)	65.5 ± 10.1	70.0 ± 12.4	70.3 ± 10.5*	63.3 ± 9.0	67.0 ± 12.7	62.9 ± 10.4	66.1 ± 12.0
Systolic blood pressure (mm Hg)	115.7 ± 16.0	111.5 ± 15.4	132.1 ± 17.8†	113.3 ± 25.2	112.3 ± 17.0	115.5 ± 165	113.0 ± 16.0
Diastolic blood pressure (mm Hg)	63.2 ± 13.5	56.4 ± 13.8	73.5 ± 12.3†	61.9 ± 12.5	58.4 ± 14.0	61.3 ± 14.8	59.3 ± 14.4
Rate–pressure product (mm Hg × beats/min)	7,666.0 ± 1,958.0	7,872.9 ± 2,016.1	9,382.0 ± 2,418.8†	7,237.3 ± 2,220.1	7,619.1 ± 2,188.3	7,312.0 ± 1,810.7	7,569.1 ± 2,109.2
Control group (n = 10)							
Heart rate (beats/min)	65.2 ± 10.1	69.5 ± 11.1		64.1 ± 9.4	67.1 ± 12.7	65.0 ± 10.5	72.8 ± 16.2
Systolic blood pressure (mm Hg)	102.2 ± 7.5	101.8 ± 8.1		104.9 ± 13.2	103.7 ± 9.8	106.8 ± 11.8	105.3 ± 10.9
Diastolic blood pressure (mm Hg)	52.4 ± 11.3	48.8 ± 12.4		58.0 ± 21.2	50.7 ± 12.1	52.6 ± 12.1	50.6 ± 13.6
Rate–pressure product (mm Hg × beats/min)	6,695.9 ± 1359.7	7,120.7 ± 1,534.1		6,801.2 ± 1,733.0	7,024.4 ± 1,796.3	7,023.3 ± 1,805.6	7,770.5 ± 2,394.9

Values are presented as mean ± SD.
* $p < 0.01$ versus before mental stress; † $p < 0.001$ versus before mental stress.

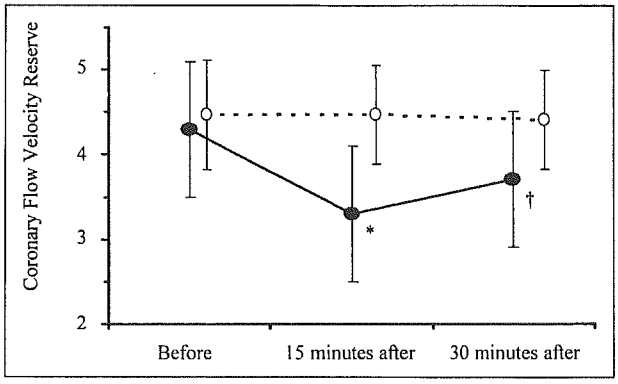

Figure 2. CFVR measurements in the control group *(open circles)* and the mental stress group *(closed circles)*. In the control group, CFVR did not change over 3 measurements. However, in the mental stress group, CFVR was significantly reduced at 15 and 30 minutes after mental stress. Data are presented as mean ± SD. *p <0.001 versus before mental stress; †p <0.01 versus before mental stress.

which has been confirmed to accurately reflect the results of invasive measurement by Doppler guidewire,[7] permits the rapid, reproducible, and totally noninvasive assessment of coronary blood flow at a small cost. Therefore, the noninvasive method we used in the present study permitted the serial evaluation of the physiologic response of coronary circulation after mental stress.

Recently, Ghiadoni et al[14] showed that mental stress resulted in prolonged endothelial dysfunction in brachial arteries for up to 4 hours in healthy subjects, although flow-mediated dilation in the brachial artery does not directly reflect the response of coronary circulation. One limitation of this study is that we did not assess the change of CFVR >30 minutes after mental stress, although this issue would be interesting. Further investigation is needed to address this issue.

Table 3
Coronary flow velocity measurements

Variable	Before	15 Minutes After	30 Minutes After
Mean diastolic flow velocity			
Mental stress group (n = 31)			
Baseline (cm/s)	19.9 ± 6.5	19.9 ± 6.5	21.6 ± 7.9
Hyperemia (cm/s)	83.8 ± 27.6	72.6 ± 28.7*	79.8 ± 27.3
Control group (n = 10)			
Baseline (cm/s)	17.8 ± 6.5	18.2 ± 6.5	19.1 ± 6.6
Hyperemia (cm/s)	79.2 ± 28.1	82.6 ± 29.3	83.5 ± 27.2
CFVR			
Mental stress group (n = 31)	4.3 ± 0.9	3.3 ± 0.8†	3.7 ± 0.8‡
Control group (n = 10)	4.5 ± 0.6	4.5 ± 0.6	4.4 ± 0.6

Values are presented as mean ± SD.
* p <0.05 versus before mental stress; † p <0.001 versus before mental stress; ‡ p <0.01 versus before mental stress.

1. Jiang W, Babyak M, Krantz DS, Waough RA, Coleman RE, Hanson MM, Frid DJ, McNulty S, Morris JJ, O'Connor CM, et al. Mental stress-induced myocardial ischemia and cardiac events. *JAMA* 1996; 275:1651–1656.
2. Schöder H, Silverman DH, Canpisi R, Karpman H, Phelps ME, Schelbert HR, Czernin J. Effect of mental stress on myocardial blood flow and vasomotion in patients with coronary artery disease. *J Nucl Med* 2000;41:11–16.
3. Leor J, Poole WK, Kloner RA. Sudden cardiac death triggered by an earthquake. *N Engl J Med* 1996;334:413–419.
4. Mwisel SR, Kutz I, Dayan KI. Effect of Iraqi missile war on incidence of acute myocardial infarction and sudden death in Israeli civilians. *Lancet* 1991;338:660–661.
5. Hirata K, Amudha K, Elina R, Hozumi T, Yoshikawa J, Homma S, Lang CC. Measurement of coronary vasomotor function: getting to the heart of the matter in cardiovascular research. *Clin Sci (London)* 2004;107:449–460.
6. Fusejima K. Noninvasive measurement of coronary artery blood flow using combined two-dimensional Doppler echocardiography. *J Am Coll Cardiol* 1987;10:1024–1031.
7. Hozumi T, Yoshida K, Ogata Y, Akasaka T, Asami Y, Takagi T, Morioka S. Noninvasive assessment of significant left anterior descending coronary artery stenosis by coronary flow velocity reserve with transthoracic color Doppler echocardiography. *Circulation* 1998; 97:1557–1562.
8. Daimon M, Watanabe H, Yamagishi H, Muro T, Akioka K, Hirata K, Takeuchi K, Yoshikawa J. Physiologic assessment of coronary artery stenosis by coronary flow reserve measurements with transthoracic Doppler echocardiography: comparison with exercise thallium-201 single-photon emission computed tomography. *J Am Coll Cardiol* 2001;37:1310–1315.
9. Hirata K, Shimada K, Watanabe H, Muro T, Yoshiyama M, Takeuchi K, Hozumi T, Yoshikawa J. Modulation of coronary flow velocity reserve by gender, menstrual cycle and hormone replacement therapy. *J Am Coll Cardiol* 2001;38:1879–1884.
10. Kashiwagi S, Yamada K. Evaluation of the Uchida-Krapelin psychodiagnostic test based on the Five-Factor Model of personality traits. *Shinrigaku Kenkyu* 1995;66:24–32.
11. Wilson RF, Laughlin DE, Ackell PH, Chilian WM, Holida MD, Hartley CJ, Armstrong ML, Marcus ML, White CW. Transluminal, subselective measurement of coronary artery blood flow velocity and vasodilator reserve in man. *Circulation* 1985;72:82–92.
12. Miller DD, Donohue TJ, Wolfford TL, Kern MJ, Bergmann SR. Assessment of blood flow distal to coronary artery stenoses: correlation between myocardial positron emission tomography and poststenotic intracoronary Doppler flow reserve. *Circulation* 1996;94:2447–2454.
13. Verberne HJ, Piek JJ, Van Liebergen RAM, Koch KT, Schroeder-Tanka JM, Van Royen EA. Functional assessment of coronary artery stenosis by Doppler derived absolute and relative coronary blood flow velocity reserve in comparison with (99m) Tc MIBI SPECT. *Heart* 1999;82:509–514.
14. Ghiadoni L, Donald AE, Cropley M, Nullen MJ, Oakley G, Taylor M, Conor GO, Betteridge J, Klein N, Steptoe A, et al. Mental stress induced transient endothelial dysfunction in humans. *Circulation* 2000;102:2473–2478.

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Cardiac transcription factor Csx/Nkx2-5: Its role in cardiac development and diseases

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Abstract

During the past decade, an emerging body of evidence has accumulated that cardiac transcription factors control a cardiac gene program and play a critical role in transcriptional regulation during cardiogenesis and during the adaptive process in adult hearts. Especially, an evolutionally conserved homeobox transcription factor Csx/Nkx2-5 has been in the forefront in the field of cardiac biology, providing molecular insights into the mechanisms of cardiac development and diseases. Csx/Nkx2-5 is indispensable for normal cardiac development, and mutations of the gene are associated with human congenital heart diseases (CHD). In the present review, the regulation of a cardiac gene program by Csx/Nkx2-5 is summarized, with an emphasis on its role in the cardiac development and diseases.

Keywords: Homeobox; Transcriptional regulation; Cardiac development; Congenital heart disease; Cardiac hypertrophy; Cardioprotection

Abbreviations: ANP, atrial natriuretic peptide; ASD, atrial septal defect; AV, atrioventricular; BMP, bone morphogenic protein; BNP, brain natriuretic peptide; CARP, cardiac ankyrin repeat protein; CHD, congenital heart diseases; CKII, casein kinase II; Dpp, decapentaplegic; DMSO, dimethyl sulfoxide; DORV, double-outlet right ventricle; FGF, fibroblast growth factor; HOP, homeodomain-only protein; Irx4, Iroquois homeobox gene 4; MEF2, myocyte enhancer factor 2; MHC, myosin heavy chain; MLC2v, myosin light chain 2v; NES, nuclear export signal; NK2-SD, NK-2-specific domain; PI3-kinase, phosphatidylinositol 3-kinase; Sca-1, stem cell antigen-1; SRF, serum response factor; TAK1, TGF-β-kinase 1; TGF-β, transforming growth factor-β; TOF, tetratology of Fallot; VSD, ventricular septal defect; Wg, wingless.

Contents

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1. Introduction

The heart is the first functional organ in the developing embryos, and the appropriate delivery of oxygen and nutrients through the circulatory system is prerequisite for embryonic growth and survival. The formation of the heart involves a precisely coordinated process of cellular differentiation and integrated multicellular morphogenesis, and even a minute perturbation of this process gives rise to congenital heart diseases (CHD). The susceptibility of the heart to malformation is reflected by the high incidence of congenital heart disease (nearly 1% of live births; American Heart Association, 2003).

Although the morphological events of heart formation have been described for centuries, it is not until a decade ago that the genetic explorations for cardiac development have started. Cardiogenic progenitors become committed to cardiac lineage in the anterior lateral mesoderm (primary heart filed) of the late gastrulation embryos in response to inducing signals secreted from adjacent endoderm (reviewed in Olson & Srivastava, 1996; Fishman & Olson, 1997; Srivastava & Olson, 2000). These cardiogenic cells, clustering in a form of bilaterally symmetrical crescent, migrate and fuse at the anterior midline to form a linear heart tube. The linear heart tube initiates autonomous contraction and undergoes rightward looping morphogenesis to form a mature four-chambered heart in association with atrioventricular (AV) septations. Maturation of the heart also requires coordinated proliferation and differentiation of myocardium to form functional trabeculated chambers. At the AV canal, mitral and tricuspid valves originate from endocardial cushions, regional swellings forming as a consequence of epithelial–mesenchymal transformation of endocardial cells. Endocardial cushions also participate in the formation of the aortic and pulmonary valves. Migrating neural crest cells populate the outflow tract as well as aortic and pharyngeal arches. Recently, it is proposed that a part of cardiomyocytes in the outflow tract and, possibly, right ventricle is generated from a "secondary (anterior) heart field" situated in splanchnic mesoderm medial and adjacent to the primary heart field (reviewed in Kelly & Buckingham, 2002).

A novel paradigm for heart development originated from the discovery of the *tinman* gene in the fruit fly *Drosophila melanogaster*, which is required for the primitive heart formation in this organism (Azpiazu & Frasch, 1993; Bodmer, 1993). The *tinman* encodes a homeobox-containing transcription factor, and the identification of the *tinman*-related gene *Csx/Nkx2-5* (Komuro & Izumo, 1993; Lints et al., 1993) in mammals attracted much attention to the key regulatory roles of cardiac transcription factors in the intricate program of heart development. Cardiac transcription factors are essential transcriptional activators that are expressed predominantly in hearts and that regulate the expression of the cardiac genes encoding structural proteins or regulatory proteins characteristic of cardiomyocytes (reviewed in Bruneau, 2002). In vertebrates, cardiac transcription factors are represented by the homeobox transcription factor Csx/Nkx2-5, the GATA family transcription factors, and myocyte enhancer factor 2 (MEF2) transcription factors.

Recent data have suggested the significant role of these transcription factors in postnatal hearts as well (reviewed in Akazawa & Komuro, 2003a). Cardiomyocytes are highly differentiated and lose their ability to proliferate soon after birth. Thereafter, cardiomyocytes grow in cell size without cell division to adapt to a demand for an increased workload. In a variety of pathological conditions (e.g., hypertension, valvular disease, myocardial infarction, and cardiomyopathy) that impose overwork on the heart, postnatal cardiomyocytes undergo hypertrophic cell growth. Although cardiac hypertrophy is initially compensatory for an increased workload, the prolongation of this process leads to deleterious outcomes such as congestive heart failure, arrhythmia, and sudden death (Levy et al., 1990; Lorell & Carabello, 2000). Cellular responses characteristic of cardiac hypertrophy include accelerated synthesis of sarcomeric and structural proteins and reprogramming of the fetal cardiac genes (reviewed in Komuro & Yazaki, 1993; Sadoshima & Izumo, 1997). With regard to the transcriptional adaptation induced by hypertrophic stimulation, it is reasonable to assume that cardiac transcription factors play the leading part because they directly regulate a number of cardiac genes that are up-regulated in hypertrophied myocardium. Indeed, the transcriptional activities of GATA and MEF2 transcription factors are enhanced in response to hypertrophic stimulations and they function as essential effectors of divergent intracellular signaling pathways mediating hypertrophic features (reviewed in Akazawa & Komuro, 2003a). However, the role of Csx/Nkx2-5 in the adult hearts remains elusive. Csx/Nkx2-5 is up-regulated in response to hypertrophic stimulations and may have implications in the transcriptional regulation of the cardiac gene program in hypertrophied hearts. In addition, the role of Csx/Nkx2-5 may extend to maintenance of homeostasis in highly differentiated cardiomyocytes.

In line with the involvement in transcriptional regulation of myriad cardiac genes, both during cardiogenesis and during the adaptive process in response to hemodynamic stresses, aberrant expressions of *Csx/Nkx2-5* directly give rise to heart diseases both in mice and humans. This review comprehensively summarizes recent advances in understanding the role of Csx/Nkx2-5 in transcriptional regulation in the heart, especially focusing on its role in cardiac development and diseases.

2. Cardiac homeobox transcription factor Csx/Nkx2-5

2.1. NK-2 class homeobox transcription factor Csx/Nkx2-5

Csx/Nkx2-5 is a member of the NK homeobox gene family that is conserved in evolution and acts as a DNA-

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 medoserm (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 *β-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

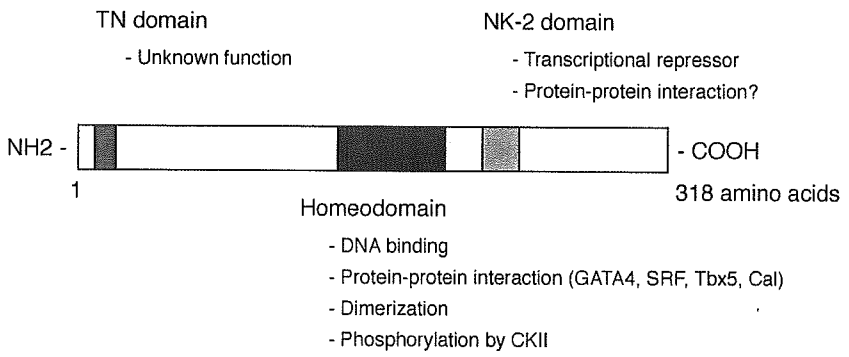

Fig. 1. Protein structure and functional domains of Csx/Nkx2-5. The homeodomain is indicated by a black box, TN domain with a dark grey box, and NK-2 domain with a light grey box.

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*-dedicient 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	
MEF-2C	Transcription factor
eHAND/HAND1	Transcription factor
Irx 4	Transcription factor
N-myc	Transcription factor
Myocardin	Transcriptional coactivator
CARP	Transcriptional corepressor, titin-binding protein
HOP	Homeoprotein
MLC2v	Contractile protein
ANP	Natriuretic peptide
BNP	Natriuretic peptide
Connexin 40	Structural protein in gap junction
minK	Ion channel
Chisel	Undetermined
Up-regulated genes	
BMP-10	Morphogenic protein
Sarcolipin	Inhibitor for sarcoplasmic reticulum calcium-ATPase
HCN1	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; *MEF-2C*, 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-β (TGF-β) 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-β-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 β* homolog), *armadillo* (*β-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 *crescent*, induced cardiac differentiation in non-precardiac mesoderm, while the overexpression of Wnt3A or Wnt8 blocked cardiogenesis in precardiac 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 *crescent* conferred cardiogenic response on normally blood-forming posterior mesoderm by antagonizing Wnt pathways, and reciprocally, the ectopic expression of Wnt proteins in precardiac 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 β-catenin (Veeman 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 Molkentin, 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 α-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 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 transcriptionl 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 2
Direct downstream target genes for *Csx/Nkx2-5*

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

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

Cofactors	Target promoters	References
GATA4	+*ANP*	Durocher et al., 1997 Lee et al., 1998 Sepulveda et al., 1998 Shiojima et al., 1999
	+*CARP*	Kuo et al., 1997
	+*A1 adenosine receptor*	Rivkees et al., 1999
SRF	+*cardiac α-actin*	Chen & Schwartz, 1996
Tbx5	+*ANP*	Bruneau et al., 2001 Hiroi et al., 2001
	+*Connexin 40*	Bruneau et al., 2001
Tbx20	+*ANP*	Stennard et al., 2003
Tbx2	−*ANP*	Habets et al., 2002
dHAND/HAND2	+*ANP*	Thattaliyath et al., 2002
Foxh1	+*MEF-2C*	von Both et al., 2004
Cal	+*ANP*	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 transactivtion is dependent on the binding of Cscx/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 α-actin* promoter (Sepulveda et al., 1998). With regard to the transcriptional regulation of the *cardiac α-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 tetratology 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 overexpression 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 secumdum 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

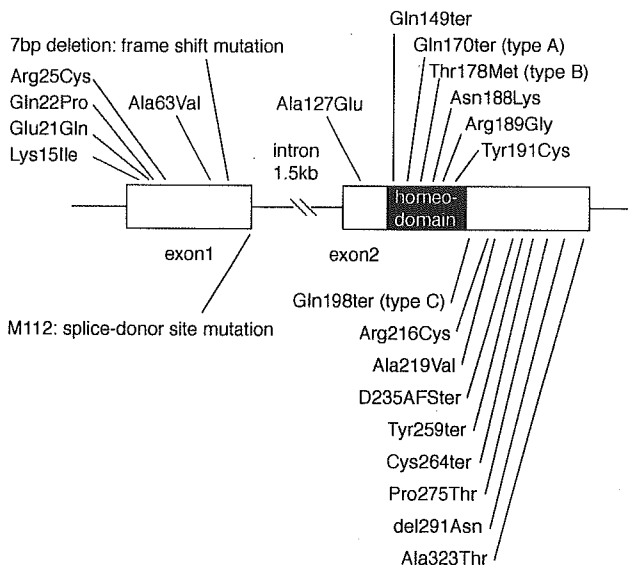

Fig. 2. Schematic presentation of the *CSX/NKX2-5* gene and the locations of the mutations. Two exons (open boxes) of *CSX/NKX2-5* are separated by a 1.5-kb intron. The homeodomain is denoted as a black box.

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 dimmers 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_2O_2 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*-heterogygous 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 α-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 β-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 *β-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 α-*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 α-*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

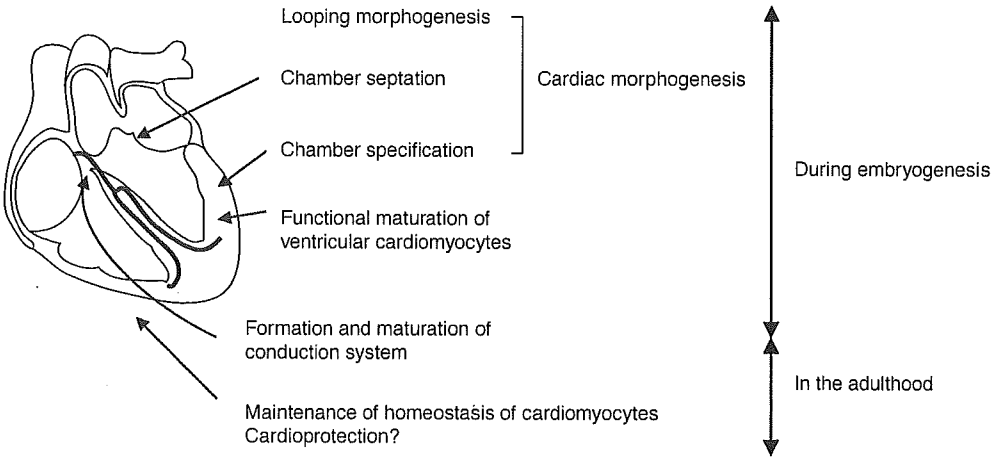

Fig. 3. Functional role of Csx/Nkx2-5 during embyrogenesis, and in adulthood, Csx/Nkx2-5 plays an important role in transcriptional regulation essential for normal cardiac development and homeostasis of postnatal hearts. Especially, Csx/Nkx2-5 is involved in cardiac morphogenesis, including rightward 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. Insomuch 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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References

Akazawa, H., & Komuro, I. (2003a). Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res 92*, 1079–1088.

Akazawa, H., & Komuro, I. (2003b). Too much Csx/Nkx2-5 is as bad as too little? *J Mol Cell Cardiol 35*, 227–229.

Akazawa, H., Kudoh, S., Mochizuki, N., Takekoshi, N., Takano, H., Nagai, T., et al. (2004). A novel LIM protein Cal promotes cardiac differentiation by association with CSX/NKX2-5. *J Cell Biol 164*, 395–405.

Alsan, B. H., & Schultheiss, T. M. (2002). Regulation of avian cardiogenesis by Fgf8 signaling. *Development 129*, 1935–1943.

Amendt, B. A., Sutherland, L. B., & Russo, A. F. (1999). Transcriptional antagonism between Hmx1 and Nkx2.5 for a shared DNA-binding site. *J Biol Chem 274*, 11635–11642.

American Heart Association. (2003). *Heart Disease and Stroke Statistics—2004 Update*. Dallas, TX: American Heart Association.

Azpiazu, N., & Frasch, M. (1993). Tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev 7*, 1325–1340.

Basson, C. T., Bachinsky, D. R., Lin, R. C., Levi, T., Elkins, J. A., Soults, J., et al. (1997). Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. *Nat Genet 15*, 30–35.

Benson, D. W., Silberbach, G. M., Kavanaugh-McHugh, A., Cottrill, C., Zhang, Y., Riggs, S., et al. (1999). Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. *J Clin Invest 104*, 1567–1573.

Biben, C., & Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. *Genes Dev 11*, 1357–1369.

Biben, C., Hatzistavrou, T., & Harvey, R. P. (1998). Expression of NK-2 class homeobox gene Nkx2-6 in foregut endoderm and heart. *Mech Dev 73*, 125–127.

Biben, C., Weber, R., Kesteven, S., Stanley, E., McDonald, L., & Elliott, D. A., et al. (2000). Cardiac septal and valvular dysmorphogenesis in mice heterozygous for mutations in the homeobox gene Nkx2-5. *Circ Res 87*, 888–895.

Bodmer, R. (1993). The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development 118*, 719–729.

Bodmer, R., Jan, L. Y., & Jan, Y. N. (1990). A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of *Drosophila*. *Development 110*, 661–669.

Brand, T., Andree, B., Schneider, A., Buchberger, A., & Arnold, H. H. (1997). Chicken NKx2-8, a novel homeobox gene expressed during early heart and foregut development. *Mech Dev 64*, 53–59.

Brown III, C. O., Chi, X., Garcia-Gras, E., Shirai, M., Feng, X. H., & Schwartz, R. J. (2004). The cardiac determination factor, Nkx2-5, is activated by mutual cofactors GATA-4 and Smad1/4 via a novel upstream enhancer. *J Biol Chem 279*, 10659–10669.

Bruneau, B. G. (2002). Transcriptional regulation of vertebrate cardiac morphogenesis. *Circ Res 90*, 509–519.

Bruneau, B. G., Bao, Z. Z., Tanaka, M., Schott, J. J., Izumo, S., Cepko, C. L., et al. (2000). Cardiac expression of the ventricle-specific homeobox gene Irx4 is modulated by Nkx2-5 and dHand. *Dev Biol 217*, 266–277.

Bruneau, B. G., Nemer, G., Charron, F., Robitaille, L., Caron, S., Conner, D. A., et al. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell 106*, 709–721.

Buchberger, A., Pabst, O., Brand, T., Seidl, K., & Arnold, H. H. (1996). Chick NKx-2.3 represents a novel family member of vertebrate homologues to the *Drosophila* homeobox gene tinman: differential expression of cNKx-2.3 and cNKx-2.5 during heart and gut development. *Mech Dev 56*, 151–163.

Caplice, N. M., & Deb, A. (2004). Myocardial-cell: the science, the clinic and the future. *Nat Clinic Pract Cardiovasc Med 1*, 90–95.

Chen, J. N., & Fishman, M. C. (1996). Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. *Development 122*, 3809–3816.

Chen, C. Y., & Schwartz, R. J. (1995). Identification of novel DNA binding targets and regulatory domains of a murine tinman homeodomain factor, nkx-2.5. *J Biol Chem 270*, 15628–15633.

Chen, C. Y., & Schwartz, R. J. (1996). Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. *Mol Cell Biol 16*, 6372–6384.

Chen, F., Kook, H., Milewski, R., Gitler, A. D., Lu, M. M., Li, J., et al. (2002). Hop is an unusual homeobox gene that modulates cardiac development. *Cell 110*, 713–723.

Cleaver, O. B., Patterson, K. D., & Krieg, P. A. (1996). Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in *Xenopus* embryos results in myocardial hyperplasia. *Development 122*, 3549–3556.

Durocher, D., Chen, C. Y., Ardati, A., Schwartz, R. J., & Nemer, M. (1996). The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. *Mol Cell Biol 16*, 4648–4655.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., & Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J 16*, 5687–5696.

Eisenberg, C. A., & Eisenberg, L. M. (1999). WNT11 promotes cardiac tissue formation of early mesoderm. *Dev Dyn 216*, 45–58.

Eisenberg, C. A., Gourdie, R. G., & Eisenberg, L. M. (1997). Wnt-11 is expressed in early avian mesoderm and required for the differentiation of the quail mesoderm cell line QCE-6. *Development 124*, 525–536.

Elliott, D. A., Kirk, E. P., Yeoh, T., Chandar, S., McKenzie, F., Taylor, P., et al. (2003). Cardiac homeobox gene NKX2-5 mutations and congenital heart disease: associations with atrial septal defect and hypoplastic left heart syndrome. *J Am Coll Cardiol 41*, 2072–2076.

Evans, S. M., Yan, W., Murillo, M. P., Ponce, J., & Papalopulu, N. (1995). tinman, a *Drosophila* homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrates: XNkx-2.3, a second vertebrate homologue of tinman. *Development 121*, 3889–3899.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D., & Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet 18*, 266–270.

Fishman, M. C., & Olson, E. N. (1997). Parsing the heart: genetic modules for organ assembly. *Cell 91*, 153–156.

Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature 374*, 464–467.

Fu, Y., Yan, W., Mohun, T. J., & Evans, S. M. (1998). Vertebrate tinman homologues XNkx2-3 and XNkx2-5 are required for heart formation in a functionally redundant manner. *Development 125*, 4439–4449.

Funke-Kaiser, H., Lemmer, J., Langsdorff, C. V., Thomas, A., Kovacevic, S. D., Strasdat, M., et al. (2003). Endothelin-converting enzyme-1 (ECE-1) is a downstream target of the homeobox transcription factor Nkx2-5. *FASEB J 17*, 1487–1489.

Goldmuntz, E., Geiger, E., & Benson, D. W. (2001). NKX2.5 mutations in patients with tetralogy of fallot. *Circulation 104*, 2565–2568.

Grow, M. W., & Krieg, P. A. (1998). Tinman function is essential for vertebrate heart development: elimination of cardiac differentiation by dominant inhibitory mutants of the tinman-related genes, XNkx2-3 and XNkx2-5. *Dev Biol 204*, 187–196.

Guo, L., Lynch, J., Nakamura, K., Fliegel, L., Kasahara, H., Izumo, S., et al. (2001). COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene during cardiac development. *J Biol Chem 276*, 2797–2801.

Habets, P. E., Moorman, A. F., Clout, D. E., van Roon, M. A., Lingbeek, M., van Lohuizen, M., et al. (2002). Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev 16*, 1234–1246.

Harvey, R. P. (1996). NK-2 homeobox genes and heart development. *Dev Biol 178*, 203–216.

Heldin, C. H., Miyazono, K., & ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature 390*, 465–471.

Hiroi, Y., Kudoh, S., Monzen, K., Ikeda, Y., Yazaki, Y., Nagai, R., et al. (2001). Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet 28*, 276–280.

Hirota, H., Chen, J., Betz, U. A., Rajewsky, K., Gu, Y., Ross Jr., J., et al. (1999). Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. *Cell 97*, 189–198.

Hogan, B. L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev 10*, 1580–1594.

Hosoda, T., Komuro, I., Shiojima, I., Hiroi, Y., Harada, M., Murakawa, Y., et al. (1999). Familial atrial septal defect and atrioventricular conduction disturbance associated with a point mutation in the cardiac homeobox gene CSX/NKX2-5 in a Japanese patient. *Jpn Circ J 63*, 425–426.

Ikeda, Y., Hiroi, Y., Hosoda, T., Utsunomiya, T., Matsuo, S., Ito, T., et al. (2002). Novel point mutation in the cardiac transcription factor CSX/NKX2.5 associated with congenital heart disease. *Circ J 66*, 561–563.

Jamali, M., Karamboulas, C., Rogerson, P. J., & Skerjanc, I. S. (2001a). BMP signaling regulates Nkx2-5 activity during cardiomyogenesis. *FEBS Lett 509*, 126–130.

Jamali, M., Rogerson, P. J., Wilton, S., & Skerjanc, I. S. (2001b). Nkx2-5 activity is essential for cardiomyogenesis. *J Biol Chem 276*, 42252–42258.

Jay, P. Y., Harris, B. S., Maguire, C. T., Buerger, A., Wakimoto, H., Tanaka, M., et al. (2004). Nkx2-5 mutation causes anatomic hypoplasia of the cardiac conduction system. *J Clin Invest 113*, 1130–1137.

Kadrmas, J. L., & Beckerle, M. C. (2004). The LIM domain: from the cytoskeleton to the nucleus. *Nat Rev Mol Cell Biol 5*, 920–931.

Kasahara, H., & Izumo, S. (1999). Identification of the in vivo casein kinase II phosphorylation site within the homeodomain of the cardiac tissue-specifying homeobox gene product Csx/Nkx2.5. *Mol Cell Biol 19*, 526–536.

Kasahara, H., Bartunkova, S., Schinke, M., Tanaka, M., & Izumo, S. (1998). Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. *Circ Res 82*, 936–946.

Kasahara, H., Lee, B., Schott, J. J., Benson, D. W., , Seidman, J. G., et al. (2000). Loss of function and inhibitory effects of human CSX/NKX2.5 homeoprotein mutations associated with congenital heart disease. *J Clin Invest 106*, 299–308.

Kasahara, H., Usheva, A., Ueyama, T., Aoki, H., Horikoshi, N., & Izumo, S. (2001a). Characterization of homo- and heterodimerization of cardiac Csx/Nkx2.5 homeoprotein. *J Biol Chem 276*, 4570–4580.

Kasahara, H., Wakimoto, H., Liu, M., Maguire, C. T., Converso, K. L., Shioi, T., et al. (2001b). Progressive atrioventricular conduction defects

and heart failure in mice expressing a mutant Csx/Nkx2.5 homeoprotein. *J Clin Invest 108*, 189–201.

Kasahara, H., Ueyama, T., Wakimoto, H., Liu, M. K., Maguire, C. T., Converso, K. L., et al. (2003). Nkx2.5 homeoprotein regulates expression of gap junction protein connexin 43 and sarcomere organization in postnatal cardiomyocytes. *J Mol Cell Cardiol 35*, 243–256.

Kelly, R. G., & Buckingham, M. E. (2002). The anterior heart-forming field: voyage to the arterial pole of the heart. *Trends Genet 18*, 210–216.

Kim, Y., & Nirenberg, M. (1989). *Drosophila* NK-homeobox genes. *Proc Natl Acad Sci U S A 86*, 7716–7720.

Kirchhoff, S., Nelles, E., Hagendorff, A., Kruger, O., Traub, O., & Willecke, K. (1998). Reduced cardiac conduction velocity and predisposition to arrhythmias in connexin40-deficient mice. *Curr Biol 8*, 299–302.

Komuro, I., & Izumo, S. (1993). Csx: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci U S A 90*, 8145–8149.

Komuro, I., & Yazaki, Y. (1993). Control of cardiac gene expression by mechanical stress. *Annu Rev Physiol 55*, 55–75.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., et al. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev 11*, 1048–1060.

Lee, K. H., Xu, Q., & Breitbart, R. E. (1996). A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. *Dev Biol 180*, 722–731.

Lee, Y., Shioi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E., et al. (1998). The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol Cell Biol 18*, 3120–3129.

Lee, K. H., Evans, S., Ruan, T. Y., & Lassar, A. B. (2004). SMAD-mediated modulation of YY1 activity regulates the BMP response and cardiac-specific expression of a GATA4/5/6-dependent chick Nkx2.5 enhancer. *Development 131*, 4709–4723.

Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B., & Castelli, W. P. (1990). Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med 322*, 1561–1566.

Li, Q. Y., Newbury-Ecob, R. A., Terrett, J. A., Wilson, D. I., Curtis, A. R., Yi, C. H., et al. (1997). Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat Genet 15*, 21–29.

Liberatore, C. M., Searcy-Schrick, R. D., Vincent, E. B., & Yutzey, K. E. (2002). Nkx-2.5 gene induction in mice is mediated by a Smad consensus regulatory region. *Dev Biol 244*, 243–256.

Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A., & Olson, E. N. (1999). Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development 126*, 75–84.

Lien, C. L., McAnally, J., Richardson, J. A., & Olson, E. N. (2002). Cardiac-specific activity of an Nkx2-5 enhancer requires an evolutionarily conserved Smad binding site. *Dev Biol 244*, 257–266.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., & Harvey, R. P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development 119*, 419–431.

Lorell, B. H., & Carabello, B. A. (2000). Left ventricular hypertrophy: pathogenesis, detection, and prognosis. *Circulation 102*, 470–479.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., et al. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev 9*, 1654–1666.

Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M., & Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev 15*, 316–327.

Mathur, A., & Martin, J. F. (2004). Stem cells and repair of the heart. *Lancet 364*, 183–192.

Matsuura, K., Nagai, T., Nishigaki, N., Oyama, T., Nishi, J., Wada, H., et al. (2004). Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem 279*, 11384–11391.

McElhinney, D. B., Geiger, E., Blinder, J., Woodrow Benson, D., & Goldmuntz, E. (2003). NKX2.5 mutations in patients with congenital heart disease. *J Am Coll Cardiol 42*, 1650–1655.

Mead, P. E., Brivanlou, I. H., Kelley, C. M., & Zon, L. I. (1996). BMP-4-responsive regulation of dorsal–ventral patterning by the homeobox protein mix 1. *Nature 382*, 357–360.

Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem 275*, 38949–38952.

Monzen, K., Shiojima, I., Hiroi, Y., Kudoh, S., Oka, T., Takimoto, E., et al. (1999). Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase kinase kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. *Mol Cell Biol 19*, 7096–7105.

Monzen, K., Hiroi, Y., Kudoh, S., Akazawa, H., Oka, T., Takimoto, E., et al. (2001). Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation. *J Cell Biol 153*, 687–698.

Monzen, K., Zhu, W., Kasai, H., Hiroi, Y., Hosoda, T., Akazawa, H., et al. (2002). Dual effects of the homeobox transcription factor Csx/Nkx2-5 on cardiomyocytes. *Biochem Biophys Res Commun 298*, 493–500.

Muller, J. G., Thompson, J. T., Edmonson, A. M., Rackley, M. S., Kasahara, H., Izumo, S., et al. (2002). Differential regulation of the cardiac sodium calcium exchanger promoter in adult and neonatal cardiomyocytes by Nkx2.5 and serum response factor. *J Mol Cell Cardiol 34*, 807–821.

Naito, A. T., Tominaga, A., Oyamada, M., Oyamada, Y., Shiraishi, I., Monzen, K., et al. (2003). Early stage-specific inhibitions of cardiomyocyte differentiation and expression of Csx/Nkx-2.5 and GATA-4 by phosphatidylinositol 3-kinase inhibitor LY294002. *Exp Cell Res 291*, 56–69.

Nakamura, T., Sano, M., Songyang, Z., & Schneider, M. D. (2003). A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. *Proc Natl Acad Sci U S A 100*, 5834–5839.

Newman, C. S., & Krieg, P. A. (1998). Tinman-related genes expressed during heart development in *Xenopus*. *Dev Genet 22*, 230–238.

Newman, C. S., Reecy, J., Grow, M. W., Ni, K., Boettger, T., Kessel, M., et al. (2000). Transient cardiac expression of the tinman-family homeobox gene, XNkx2-10. *Mech Dev 91*, 369–373.

Oh, H., Bradfute, S. B., Gallardo, T. D., Nakamura, T., Gaussin, V., Mishina, Y., et al. (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A 100*, 12313–12318.

Olson, E. N., & Srivastava, D. (1996). Molecular pathways controlling heart development. *Science 272*, 671–676.

Onichtchouk, D., Glinka, A., & Niehrs, C. (1998). Requirement for Xvent-1 and Xvent-2 gene function in dorsoventral patterning of *Xenopus* mesoderm. *Development 125*, 1447–1456.

Pandur, P., Lasche, M., Eisenberg, L. M., & Kuhl, M. (2002). Wnt-11 activation of a noncanonical Wnt signalling pathway is required for cardiogenesis. *Nature 418*, 636–641.

Paquin, J., Danalache, B. A., Jankowski, M., McCann, S. M., & Gutkowska, J. (2002). Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. *Proc Natl Acad Sci U S A 99*, 9550–9555.

Park, M., Wu, X., Golden, K., Axelrod, J. D., & Bodmer, R. (1996). The wingless signaling pathway is directly involved in *Drosophila* heart development. *Dev Biol 177*, 104–116.

Park, M., Lewis, C., Turbay, D., Chung, A., Chen, J. N., Evans, S., et al. (1998a). Differential rescue of visceral and cardiac defects in *Drosophila* by vertebrate tinman-related genes. *Proc Natl Acad Sci U S A 95*, 9366–9371.

Park, M., Venkatesh, T. V., & Bodmer, R. (1998b). Dual role for the zeste-white3/shaggyencoded kinase in mesoderm and heart development of *Drosophila*. *Dev Genet 22*, 201–211.

Pashmforoush, M., Lu, J. T., Chen, H., Amand, T. S., Kondo, R., Pradervand, S., et al. (2004). Nkx2-5 pathways and congenital heart disease; loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. *Cell 117*, 373–386.

Peterkin, T., Gibson, A., & Patient, R. (2003). GATA-6 maintains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. *EMBO J 22*, 4260–4273.

Ranganayakulu, G., Elliott, D. A., Harvey, R. P., & Olson, E. N. (1998). Divergent roles for NK-2 class homeobox genes in cardiogenesis in flies and mice. *Development 125*, 3037–3048.

Reamon-Buettner, S. M., & Borlak, J. (2004). Somatic NKX2-5 mutations as a novel mechanism of disease in complex congenital heart disease. *J Med Genet 41*, 684–690.

Reamon-Buettner, S. M., Hecker, H., Spanel-Borowski, K., Craatz, S., Kuenzel, E., & Borlak, J. (2004). Novel NKX2-5 mutations in diseased heart tissues of patients with cardiac malformations. *Am J Pathol 164*, 2117–2125.

Reecy, J. M., Yamada, M., Cummings, K., Sosic, D., Chen, C. Y., Eichele, G., et al. (1997). Chicken Nkx-2.8: a novel homeobox gene expressed in early heart progenitor cells and pharyngeal pouch-2 and -3 endoderm. *Dev Biol 188*, 295–311.

Reecy, J. M., Li, X., Yamada, M., DeMayo, F. J., Newman, C. S., Harvey, R. P., et al. (1999). Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. *Development 126*, 839–849.

Riley, P., Anson-Cartwright, L., & Cross, J. C. (1998). The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet 18*, 271–275.

Riley, P. R., Gertsenstein, M., Dawson, K., & Cross, J. C. (2000). Early exclusion of hand1-deficient cells from distinct regions of the left ventricular myocardium in chimeric mouse embryos. *Dev Biol 227*, 156–168.

Rivkees, S. A., Chen, M., Kulkarni, J., Browne, J., & Zhao, Z. (1999). Characterization of the murine A1 adenosine receptor promoter, potent regulation by GATA-4 and Nkx2.5. *J Biol Chem 274*, 14204–14209.

Saadane, N., Alpert, L., & Chalifour, L. E. (1999). Expression of immediate early genes, GATA-4, and Nkx-2.5 in adrenergic-induced cardiac hypertrophy and during regression in adult mice. *Br J Pharmacol 127*, 1165–1176.

Sadoshima, J., & Izumo, S. (1997). The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol 59*, 551–571.

Schneider, V. A., & Mercola, M. (2001). Wnt antagonism initiates cardiogenesis in *Xenopus laevis*. *Genes Dev 15*, 304–315.

Schott, J. J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., et al. (1998). Congenital heart disease caused by mutations in the transcription factor NKX2-5. *Science 281*, 108–111.

Schultheiss, T. M., Xydas, S., & Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development 121*, 4203–4214.

Schultheiss, T. M., Burch, J. B., & Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev 11*, 451–462.

Schwartz, R. J., & Olson, E. N. (1999). Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. *Development 126*, 4187–4192.

Searcy, R. D., Vincent, E. B., Liberatore, C. M., & Yutzey, K. E. (1998). A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. *Development 125*, 4461–4470.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., & Schwartz, R. J. (1998). GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol Cell Biol 18*, 3405–3415.

Shi, Y., Katsev, S., Cai, C., & Evans, S. (2000). BMP signaling is required for heart formation in vertebrates. *Dev Biol 224*, 226–237.

Shin, C. H., Liu, Z. P., Passier, R., Zhang, C. L., Wang, D. Z., Harris, T. M., et al. (2002). Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. *Cell 110*, 725–735.

Shiojima, I., Komuro, I., Inazawa, J., Nakahori, Y., Matsushita, I., Abe, T., et al. (1995). Assignment of cardiac homeobox gene CSX to human chromosome 5q34. *Genomics 27*, 204–206.

Shiojima, I., Komuro, I., Mizuno, T., Aikawa, R., Akazawa, H., Oka, T., et al. (1996). Molecular cloning and characterization of human cardiac homeobox gene CSX1. *Circ Res 79*, 920–929.

Shiojima, I., Komuro, I., Oka, T., Hiroi, Y., Mizuno, T., Takimoto, E., et al. (1999). Context-dependent transcriptional cooperation mediated by cardiac transcription factors Csx/Nkx- 2.5 and GATA-4. *J Biol Chem 274*, 8231–8239.

Shiojima, I., Oka, T., Hiroi, Y., Nagai, R., Yazaki, Y., & Komuro, I. (2000). Transcriptional regulation of human cardiac homeobox gene CSX1. *Biochem Biophys Res Commun 272*, 749–757.

Shirai, M., Osugi, T., Koga, H., Kaji, Y., Takimoto, E., Komuro, I., et al. (2002). The Polycomb-group gene Rae28 sustains Nkx2.5/Csx expression and is essential for cardiac morphogenesis. *J Clin Invest 110*, 177–184.

Shiratori, H., Sakuma, R., Watanabe, M., Hashiguchi, H., Mochida, K., Sakai, Y., et al. (2001). Two-step regulation of left–right asymmetric expression of Pitx2: initiation by nodal signaling and maintenance by Nkx2. *Mol Cell 7*, 137–149.

Simon, A. M., Goodenough, D. A., & Paul, D. L. (1998). Mice lacking connexin40 have cardiac conduction abnormalities characteristic of atrioventricular block and bundle branch block. *Curr Biol 8*, 295–298.

Smith, J. (1999). T-box genes: what they do and how they do it. *Trends Genet 15*, 154–158.

Srivastava, D. (1999). HAND proteins: molecular mediators of cardiac development and congenital heart disease. *Trends Cardiovasc Med 9*, 11–18.

Srivastava, D., & Olson, E. N. (2000). A genetic blueprint for cardiac development. *Nature 407*, 221–226.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D., & Olson, E. N. (1997). Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat Genet 16*, 154–160.

Staehling-Hampton, K., Hoffmann, F. M., Baylies, M. K., Rushton, E., & Bate, M. (1994). Dpp induces mesodermal gene expression in *Drosophila*. *Nature 372*, 783–786.

Stanley, E. G., Biben, C., Elefanty, A., Barnett, L., Koentgen, F., Robb, L., et al. (2002). Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3′UTR-ires-Cre allele of the homeobox gene Nkx2-5. *Int J Dev Biol 46*, 431–439.

Stennard, F. A., Costa, M. W., Elliott, D. A., Rankin, S., Haast, S. J., Lai, D., et al. (2003). Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev Biol 262*, 206–224.

Takimoto, E., Mizuno, T., Terasaki, F., Shimoyama, M., Honda, H., Shiojima, I., et al. (2000). Up-regulation of natriuretic peptides in the ventricle of Csx/Nkx2-5 transgenic mice. *Biochem Biophys Res Commun 270*, 1074–1079.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N., & Izumo, S. (1999a). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development 126*, 1269–1280.

Tanaka, M., Wechsler, S. B., Lee, I. W., Yamasaki, N., Lawitts, J. A., & Izumo, S. (1999b). Complex modular cis-acting elements regulate expression of the cardiac specifying homeobox gene Csx/Nkx2.5. *Development 126*, 1439–1450.

Tanaka, M., Yamasaki, N., & Izumo, S. (2000). Phenotypic characterization of the murine Nkx2.6 homeobox gene by gene targeting. *Mol Cell Biol 20*, 2874–2879.