The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development

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SUMMARY

Csx/Nkx2.5 is a vertebrate homeobox gene with a sequence homology to the Drosophila tinman, which is required for the dorsal mesoderm specification. Recently, heterozygous mutations of this gene were found to cause human congenital heart disease (Schott, J.-J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E. and Seidman, J. G. (1998) Science 281, 108-111). To investigate the functions of Csx/Nkx2.5 in cardiac and extracardiac development in the vertebrate, we have generated and analyzed mutant mice completely null for Csx/Nkx2.5. Homozygous null embryos showed arrest of cardiac development after looping and poor development of blood vessels. Moreover, there were severe defects in vascular formation and hematopoiesis in the mutant yolk sac. Interestingly, TUNEL staining and PCNA staining showed neither enhanced apoptosis nor reduced cell proliferation in the mutant myocardium. In situ hybridization studies demonstrated that, among 20 candidate genes examined, expression of ANF, BNP, MLC2V, N-myc, MEF2C, HAND1 and Msx2 was disturbed in the mutant heart. Moreover, in the heart of adult chimeric mice generated from Csx/Nkx2.5 null ES cells, there were almost no ES cell-derived cardiac myocytes, while there were substantial contributions of Csx/Nkx2.5-deficient cells in other organs. Whole-mount β-gal staining of chimeric embryos showed that more than 20% contribution of Csx/Nkx2.5-deficient cells in the heart arrested cardiac development. These results indicate that (1) the complete null mutation of Csx/Nkx2.5 did not abolish initial heart looping, (2) there was no enhanced apoptosis or defective cell cycle entry in Csx/Nkx2.5 null cardiac myocytes, (3) Csx/Nkx2.5 regulates expression of several essential transcription factors in the developing heart, (4) Csx/Nkx2.5 is required for later differentiation of cardiac myocytes, (5) Csx/Nkx2.5 null cells exert dominant interfering effects on cardiac development, and (6) there were severe defects in yolk sac angiogenesis and hematopoiesis in the Csx/Nkx2.5 null embryos.

Key words: Csx/Nkx2.5, Cardiac development, Transcription factor, Gene expression, Vasculogenesis, Mouse, Heart

INTRODUCTION

The heart is the first organ to form during embryogenesis and derives from the anterior portion of the lateral plate mesoderm. Induced by signals from the underlying endoderm, splanchnic mesodermal cells ventral to the pericardial coelom become specified to a cardiac fate and differentiate into bilateral precardiac mesoderm. In the mouse, by 8.0 days post coitus (d.p.c.), as the embryo folds laterally, the bilateral heart primordia migrate to the ventral midline and fuse with each other to form a single heart tube, the primitive heart tube. The straight heart tube then undergoes looping and septation. By 9.5 d.p.c., the atrial portion shifts dorsally and to the left, and boundaries between the common atrium, the primitive ventricle (the future left ventricle) and the bulbus cordis (the future right ventricle) become prominent, principally due to formation of endocardial cushion. Septation in the common atrium begins around 10.5 d.p.c. All of the cardiac valves form by 13.0 d.p.c. and the embryonic heart acquires a definitive four-chamber structure by 14.0 d.p.c. (Kaufman, 1992).

Although heart formation has been well described morphologically, relatively little was known about molecular mechanisms underlying this process. Recently several candidate genes for cardiogenesis have been cloned and their functions have been analyzed in vivo by gene targeting technique (reviewed in Lyons, 1996; Olson and Srivastava, 1996; Rossant, 1996; Tanaka et al., 1998). In homozygous mutant mice for GATA4, the bilateral heart primordia did not fuse, resulting in the lack of the primitive heart tube (Kuo et al., 1997; Molkentin et al., 1997). Inactivation of MEF2C or HAND2 (dHAND) arrested heart formation at the looping stage. Moreover, a targeted mutation of each of these genes resulted in the absence of the future right ventricle (Lin et al., 1997; Srivastava et al., 1997). Disruption of HAND1 (eHAND)
resulted in the arrest of cardiac looping and defects in extraembryonic mesodermal development (Firulli et al., 1998; Riley et al., 1998). A null mutation for TEF-1 or N-myc caused poor development of the ventricular myocardium (Charron et al., 1992; Chen et al., 1994; Moens et al., 1993; Stanton et al., 1992). However, the precise mechanisms whereby inactivation of these genes affects heart formation remain to be elucidated and targeted disruption of any of these genes did not prevent induction of the heart cell lineage.

In Drosophila, the homeobox gene tinman is expressed in the dorsal vessel, an insect equivalent of the vertebrate heart (Bodmer et al., 1990). Csx (or Nkx2.5) is a murine homolog of tinman, and is expressed in the heart primordia and in the myocardium throughout development (Komuro and Izumo, 1993; Lints et al., 1993). Mutant mice with an insertion mutation of Nkx2.5 were embryonically lethal due to the absence of cardiac looping (Lyons et al., 1995). Because mutations in tinman gene causes complete lack of dorsal vessel formation (Bodmer 1993; Azpiazu and Frasch 1993), a question arose as to whether a complete null mutation of formation (Bodmer 1993; Azpiazu and Frasch 1993), a tinman mutation of Nkx2.5 will lead to a better understanding of the mechanisms by which tinman regulates cardiac development. Moreover, chimera analysis indicated that Csx/Nkx2.5-expressing cardiac myocytes are required for cardiac development in a dose-sensitive manner.

Materials and Methods

Gene targeting

A Csx/Nkx2.5 genomic clone was isolated from a mouse 129Sv genomic library. A 2.8 kb of upstream fragment containing 5’ flanking and 5’ untranslated sequences of Csx/Nkx2.5 was fused to the bacterial lacZ gene. This fragment was ligated into pPNT (Tybulewicz et al., 1991) as the 5’ arm and a 6.0 kb of downstream fragment containing 3’ untranslated and 3’ flanking sequences of Csx/Nkx2.5 were used as the 3’ arm. The targeting vector consisted of 2.8 kb of upstream sequences, the bacterial lacZ gene, a neomycin resistance gene (neo) under the control of the phosphoglycerol kinase (PGK) promoter, 6.0 kb of downstream sequences and a thymidine kinase gene under the control of the PGK promoter. The targeting vector was linearized with NotI for transfection.

AK7 ES cells (Soriano, 1997) from 129Sv strain were cultured on mouse embryonic fibroblast (MEF) feeder layers in high glucose Dulbecco’s modified Eagle medium containing 15% fetal calf serum and 10^3 U/ml of leukemia inhibitory factor (LIF). Cells (1.0x10^7) were electroporated with 30 μg of the targeting vector in 800 μl of phosphate-buffered saline (PBS) at 230 V and 500 μF (Bio Rad).

Electroporated ES cells were cultured on neomycin-resistant MEF feeders with 300 μg/ml of G418 and 2 μM of gancyclovir for 7 days. 91 drug-resistant colonies were picked up and half of each colony was frozen at ~80°C and the other half was expanded for DNA extraction.

Southern blot analysis was performed using the 5’ probe. Four ES cell clones were found to contain the correctly targeted event at the Csx/Nkx2.5 locus. Three clones with homologous recombination were injected into blastocysts from C57BL/6j mice at the Transgenic Core Facility of the University of Michigan. Male chimeras were bred with female C57BL/6j mice to test for germline transmission.

Genotyping of progeny

DNA was isolated from tail biopsies of weaned mice or yolk sacs of embryos. Polymerase chain reaction (PCR) was performed to genotype embryonic ES cells and mice. Results of PCR assay were confirmed by Southern blot analysis. The primers used for detection of the wild-type allele were 5’-CAACATCTCTGGAACCTGGC-3’ and 5’-AACATAATACCAGGTGGGTG-3’. Primers 5’-GCAGCCTC-TGTCCCAATAACTGTTCC-3’ and 5’-AACATAATACCGGTG-3’ were used to detect the targeted allele.

Scanning electron microgram

Embryos were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through graded ethanol and xylene, dried with critical point-drying apparatus and observed with an AMRAY 1000A scanning electron microscope (AMRAY, MA).

In situ hybridization

Embryos were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through graded ethanol and xylene and embedded in paraffin wax. Sections of 5 μm thickness were cut and after treatment with proteinase K (20 μg/ml at room temperature for 7.5 minutes), they were hybridized with 35S-CTP labeled riboprobe at 55°C overnight in 50% formamide, 0.3 M sodium chloride, 20 mM Tris-HCl, 5 mM EDTA, 10 mM sodium pyrophosphosphate, 1x Denhardt, 10% dextran sulfate and 0.5 mg/ml yeast RNA. After hybridization, they were treated with 20 μg/ml of RNase A at 37°C for 30 minutes, washed (final washing was 0.1x SSC at 65°C) and dehydrated through graded ethanol, and emulsion autoradiography was performed (Sato et al., 1995). Probes for α-cardiac actin (Sassoon et al., 1988), myosin light chain 2V (Miller-Hance et al., 1993) and atrial natriuretic factor (Miller-Hance et al., 1993) were kindly provided by Gary E. Lyons (University of Wisconsin Medical School, Madison, IL). A cDNA clone for N-cadherin was a kind gift from Masatoshi Takeichi (Kyoto University, Kyoto, Japan). A PstI-EcoRV fragment of N-cadherin was used as a probe probe. Probes for HAND1 (eHAND) (Srivastava et al., 1997), HAND2 (dHAND) (Srivastava et al., 1997), GATA4 (Molkentin et al., 1997) and MEF2C were kindly provided by Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, TX). A PstI-EcoRV fragment of MEF2C was used as a probe. Full-length cDNA clones for Mxsl and Mxc2 were kind gifts from Richard L. Maas (Brigham and Women’s Hospital, Boston, MA). Probes for NF-1, BMP-4, TGFβ-1, frbnecin and erbB4 were kindly provided by Neal G. Copeland (Frederick Cancer Research and Development Center, Frederick, MD), Brigid L. M. Hogan (Vanderbilt University, Nashville, TN), Harold L. Moses (Vanderbilt University, Nashville, TN), Elizabeth L. George (Brigham and Women’s Hospital, Boston, MA) and Greg Lemke (Salk Institute, La Jolla, CA), respectively. A 336 bp cDNA fragment 5’ of the homeodomain of Csx/Nkx2.5 and a 1.4 kb cDNA fragment of TEF-1 (Chen et al., 1994) were used as probes for Csx/Nkx2.5 and TEF-1, respectively. Probes for brain natriuretic peptide (nucleotides 520-751) and N-myc (nucleotides 3214-3744) were synthesized by RT-PCR using mouse heart mRNA and mouse 14.5 d.p.c. embryo mRNA as template, respectively. The identity of these PCR-generated probes was confirmed by DNA sequencing.
Whole-mount immunohistochemistry

Whole-mount immunohistochemistry of mouse embryos with anti-PECAM antibody (Pharmingen, CA) was performed according to Schlaeger et al. (1995). Briefly, embryos were fixed in 4% paraformaldehyde overnight at 4°C, blocked in PBS (3% skim milk and 0.1% Triton X-100 in PBS), and incubated with 10 μg/ml of anti-PECAM antibody, MEC13.3, in PBS at 4°C overnight. Next day, the embryos were washed and incubated with alkaline-phosphatase-conjugated goat anti-rat IgG (Kirkegaard and Perry Laboratory MD, 1:100 dilution) at 4°C overnight. After washing, the embryos were incubated in NBT/BCIP and postfixed in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS.

TUNEL staining and immunohistochemistry

Paraffin sections of embryos were dewaxed and rehydrated, treated with proteinase K (20 μg/ml) at room temperature for 7 minutes and incubated with TUNEL mixture (Boehringer Mannheim) according to the manufacturer’s protocol. Samples were analyzed under a fluorescent microscope.

For PCNA staining, paraffin sections of embryos were dewaxed and rehydrated, incubated with 1.0 μg/ml of anti-PCNA antibody (Santa Cruz, CA) at 4°C overnight in PBS with 5% goat serum, 0.2% Tween 20 and 0.1% bovine serum albumin. After incubation, tissue sections were washed and incubated with biotinylated anti-mouse IgG antibody and ABC reagent (Vector Laboratories, CA). Peroxidase was detected with 3,3′-diaminobenzidine.

RT-PCR

Total RNA was extracted from hearts dissected from wild-type or homozygous mutant embryos at 9.5 d.p.c. with TRI zol (Gibco BRL) and was treated with RNase-free DNase I. First-strand cDNA synthesis was performed using 250 ng of total RNA with AMV reverse transcriptase (Promega) and random primers. Five reverse transcription products were pooled and 5-fold serial dilutions were used for PCR reaction as template. PCR cycles were as follows: 94°C for 5 minutes, 20 cycles of 94°C for 30 seconds, optimal annealing temperature for each primer set for 30 seconds and 72°C for 30 seconds. PCR products were electrophoresed on 2% agarose gel, transferred to nylon membrane and hybridized with radiolabeled probes between 5′ and 3′ primers. Sequences of primers are available upon request.

Isolation of double knock-out ES cell lines and chimera analysis

The heterozygous ES cell clone that had transmitted the targeted allele through the germline was plated at 105 cells per 90-mm plate. 8 hours later, 1.0 mg/ml of G418 was added to the culture media. After 8 days of incubation, 20 drug-resistant colonies were picked up, expanded and genotyped by Southern blotting (Mortensen et al., 1992). Twelve clones were homozygous for the targeted allele. Chimeric mice were generated by injection of ES cell lines that were homozygous for the Csx/Nkx2.5 null allele into blastocysts from C57BL/6J mice at the Transgenic Core Facility of Beth Israel Deaconess Medical Center. Whole-mount β-gal staining was performed according to Schlaeger et al. (1995). Percentage of lacZ-positive cells in the heart was estimated visually. Glucose phosphate isomerase (GPI) assay was performed as described by Nagy and Rossant (1993). As for the analysis of the heart of adult chimeric mice, three small different parts of the heart were used together for GPI assay and the rest of the heart was used for sectioning and the subsequent β-gal staining.

RESULTS

Generation of mutant mice completely null for Csx/Nkx2.5

To determine the biological roles of Csx/Nkx2.5 during embryogenesis, we generated mutant mice completely null for Csx/Nkx2.5. The targeting vector was designed such that, after homologous recombination, the lacZ gene and PGK-neo would be inserted into the Csx/Nkx2.5 locus and the entire Csx/Nkx2.5 coding sequence would be deleted, generating a null allele for Csx/Nkx2.5 (Fig. 1A). AK-7 ES cells were electroporated with the targeting vector and after positive-negative selection, Southern blot analysis identified four independent clones that were correctly targeted at the Csx/Nkx2.5 locus (Fig. 1B). None of them showed an additional integration of the targeting vector when hybridized with the neo probe (data not shown). Three clones were injected into blastocysts from C57BL/6J and one clone transmitted the targeted allele through the germline.

Morphological analysis of Csx/Nkx2.5 mutant mice

We have observed the Csx/Nkx2.5 mutant mice and embryos over five generations so far, and they showed the same phenotype. Heterozygous mutant mice grew normally and were fertile. From heterozygous crosses, no homozygous pups were born, indicating that homozygous mutants are embryonically lethal. Therefore, litters from heterozygous crosses were examined at 9.5, 10.5 and 11.5 d.p.c. At 11.5 d.p.c., no homozygous mutant embryos were observed. At 10.5 d.p.c., homozygous mutants were markedly growth-retarded as compared with wild-type litter mates and had massive pericardial effusion (Fig. 2A). At 9.5 d.p.c., genotypes of embryos showed Mendelian inheritance of the mutant allele, indicating that homozygous mutants die between 9.5 and 11.5

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**Fig. 1.** Gene targeting of Csx/Nkx2.5. (A) The organization of the Csx/Nkx2.5 gene and the structure of the targeting vector are shown. The 5′ probe (a SpeI-XbaI fragment) was used for Southern blot analysis. Sp, SpeI; Xb, XbaI; N, NorI; Srf, SrfI. (B) Genotyping of ES cell clones. Genomic DNA was digested with SpeI and analyzed by Southern blotting. Hybridization with the 5′ probe revealed the expected 9.5 kb and 6.5 kb fragments from the wild-type and targeted alleles, respectively.
d.p.c. At 9.5 d.p.c., the beginning of the outflow tract was always located on the right side, and the atrial region was located posteriorly and on the left side, indicating that the rightward looping of the heart tube occurred in homozygous mutant embryos (Fig. 2B,C). Similarities and differences in the phenotype of the null allele and that of the insertional allele (Lyons et al., 1995) are described in Discussion.

Scanning electron microscopy showed that the atrioventricular canal was still wide open (Fig. 2F) and a single ventricle was abruptly connected to a poorly developed outflow tract (Fig. 2G) in homozygous mutant embryos at 9.5 d.p.c. In contrast, in wild-type embryos, the atrioventricular canal had a narrow luminal diameter (Fig. 2D,H) and the ventricle (the future left ventricle), the bulbus cordis (the future right ventricle) and the outflow tract already formed at this stage (Fig. 2E).

Histological analysis showed that formation of trabeculae was very poor and endocardial cushion was absent in the mutant hearts (Fig. 2I). Interestingly, whole-mount staining with anti-PECAM antibody showed that blood vessels, such as intersomitic arteries, pharyngeal arch arteries and the dorsal aorta, were poorly developed in homozygous mutant embryos (Fig. 2J).

Moreover, yolk sacs of homozygous Csx/Nkx2.5 mutant embryos showed a striking difference. The mutant yolk sac had excessive folds on the surface and no large vitelline vessels could be seen (Fig. 3A). Whole-mount PECAM staining of wild-type yolk sacs at 9.5 d.p.c. showed formation of large vitelline vasculatures and a fine network of small vessels, which were filled with blood cells (Fig. 3B,D,F). In contrast, in the mutant yolk sac, no defined vasculatures could be seen (Fig. 3C). Only enlarged channels, which contain few blood cells, could be observed (Fig. 3E,G). Histological analysis showed that the endodermal and the mesodermal layers had close contacts with each other and that there were numerous red blood cells and blood islands in wild-type yolk sacs (Fig. 3H,J). There were also endothelial cells on both mesodermal and endodermal layers, forming blood vessels. However, the two layers were widely separated and few red blood cells were present in the yolk sac from Csx/Nkx2.5 homozygous mutant embryos (Fig. 3I,K). Some endothelial cells could be seen in the mutant yolk sac (Fig. 3K, arrowheads), but these cells did not form vascular channels.

**Growth of cardiac myocytes in Csx/Nkx2.5 null embryos**

In order to determine the cause of arrest of heart development in Csx/Nkx2.5 null mutant embryos, we tested whether enhanced apoptosis or reduced cell proliferation occurred in Csx/Nkx2.5 null embryos. Tissue sections from three wild-type and three homozygous mutant embryos at 9.5 d.p.c. were stained with TUNEL reagent. The rates of TUNEL-positive nuclei in the myocardium were similar between wild-types and...
homozygous mutants (one or two positive cells per section, Fig. 4A,B). No enhanced apoptosis in the myocardium could be seen in the homozygous mutant embryos. Next, tissue sections from three wild-type and three homozygous mutant embryos at 9.5 d.p.c. were stained for the expression of proliferating cell nuclear antigen (PCNA). In tissue sections from wild-type embryos, 47.8±3.6% of myocyte nuclei were stained positive, while 50.3±3.5% were positive in the myocardium in the homozygous mutant embryos, suggesting that Csx/Nkx2.5 null cardiac myocytes could enter cell cycle normally at this stage (Fig. 4C,D).

**Downstream target genes of Csx/Nkx2.5**

We next examined the expression of 20 different genes that are implicated in heart development by in situ hybridization in Csx/Nkx2.5 null mutant embryos. First, in situ hybridization with a Csx/Nkx2.5 probe showed that there were no detectable signals for Csx/Nkx2.5 in the mutant embryo, confirming that the mutant embryo was null for Csx/Nkx2.5 (Fig. 5A,B). Then, we examined expression of myofilament genes. Csx/Nkx2.5 and Serum Response Factor were shown to synergistically activate the expression of the α-cardiac actin promoter in transfected cells (Chen et al., 1996). However, expression of α-cardiac actin was not significantly disturbed in Csx/Nkx2.5 null mutant embryos (Fig. 5C,D). It was reported that the myosin light chain 2V (MLC 2V) gene was not expressed in the Nkx2.5 mutant heart except for a small population of cells on the dorsal side (Lyons et al., 1995). However, although it was downregulated compared to the wild-type embryo, MLC2V transcripts were readily detectable in the entire myocardium of the single ventricle of Csx/Nkx2.5 null mutant embryos at 9.5 d.p.c. (Fig. 5E,F). At 10.5 d.p.c., although homozygous mutant embryos had marked growth retardation and pericardial effusion, expression of MLC2V was still maintained in the entire ventricular myocardium (Fig. 5G,H). Expression of α-myosin heavy chain and β-myosin heavy chain was not affected in homozygous mutant embryos (data not shown).

We next examined expression of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) in Csx/Nkx2.5 null mutant embryos, since it has been shown that Csx/Nkx2.5 and GATA4 could directly and synergistically activate expression of these genes in vitro (Durocher et al., 1997; Lee et al., 1988). In wild-type embryos at 9.5 d.p.c., ANF was expressed both in the atrium and in the ventricle, and expression in the ventricle exceeded that in the atrium (Fig. 5I). In contrast, expression of ANF in the ventricle was abolished in homozygous mutant embryos.

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**Fig. 3.** Morphological analysis of yolk sacs of wild-type (+/+ ) and homozygous mutant (−/− ) embryos at 9.5 d.p.c. (A) Wild-type (+/+ ) and homozygous mutant (−/− ) yolk sacs at 9.5 d.p.c. There were excessive folds on the surface and no large vitelline vessels could be seen in the mutant yolk sac. (B,C) Whole-mount PECAM staining of wild-type (B) and homozygous mutant (C) yolk sacs. Note that there was no large vitelline vessel formation in the mutant yolk sac. (D-G) Higher magnification (D,F, ·200; E,G, ·400) of the same wild-type (B) and homozygous mutant (C) yolk sacs. Note that there was no large vitelline vessel formation in the mutant yolk sac. (H,I) Histological analysis of wild-type (H) and homozygous mutant (I) yolk sacs. Note the abnormal separation of the endodermal and mesodermal layers and few blood cells in the mutant yolk sac. (J,K) Higher magnification of the same wild-type (J) and homozygous mutant (K) yolk sacs. Note the abnormal separation of the endodermal and mesodermal layers and few blood cells in the mutant yolk sac.
whereas expression of ANF in the atrium was still maintained (Fig. 5J). Expression of BNP in the ventricle was more intense than that in the atrium in wild-type embryos (Fig. 5K). However, in homozygous mutant embryos, BNP expression in the ventricle was almost absent, while BNP was comparably expressed in the atrium (Fig. 5L). These results, together with the data from in vitro experiments (Durocher et al., 1997; Lee et al., 1998), indicate that expression of ANF and BNP in the ventricle is directly regulated by Csx/Nkx2.5, whereas expression of these genes in the atrium is not.

To examine a transcriptional cascade downstream of Csx/Nkx2.5, we further performed a series of in situ hybridizations using probes for transcription factors expressed in the heart. We first examined expression of the ubiquitous transcription factors N-myc and TEF-1, because inactivation of each gene resulted in poor development of ventricular myocardium, leading to cardiac lethality at 11-12 d.p.c. (Charron et al., 1992; Chen et al., 1994; Moens et al., 1993; Stanton et al., 1992). TEF-1 was ubiquitously expressed both in wild-type and homozygous mutant embryos (Fig. 6C,D). In contrast, N-myc transcripts could not be detected above the background in the heart of Csx/Nkx2.5 null mutant embryos, although expression of N-myc was maintained in the neural tube and pharyngeal arch mesenchyme (Fig. 6A,B).

Next, we examined expression levels of cardiac-specific transcription factors. At 9.5 d.p.c., MEF2C expression was significantly downregulated in the heart of homozygous mutant embryos (Fig. 6E,F). This was confirmed by a semiquantitative RT-PCR (see below). Moreover, while HAND1 was expressed in the outer curvature of the left and right ventricles in wild-type embryos (Fig. 6G), HAND1 expression in the myocardium was absent in homozygous mutant embryos (Fig. 6H). On the contrary, expression of GATA4 (Fig. 6J) and HAND-2 (data not shown) was not affected in homozygous mutants. Selective downregulation of N-myc, HAND1 and MEF2C suggested that Csx/Nkx2.5 might control later differentiation of cardiac myocytes through essential downstream transcription factors.

Absence of endocardial cushion is one of the main features of the mutant heart. Therefore, we examined expression of several genes that might play important roles in endocardial cushion formation. We performed in situ hybridization using probes for Msx1, Msx2, bone morphogenetic protein (BMP)-4, transforming growth factor (TGF) β-1 and fibronectin. In wild-type embryos, Msx2 was highly expressed in the pharyngeal arch mesenchyme and in the myocardium of the atrioventricular canal (Fig. 6K, arrowhead). Little expression of Msx2 was seen in other parts of the myocardium, although the expression in the pericardium was readily observed (Fig. 6K). However, in the mutant embryos, Msx2 was expressed in the myocardium more diffusely, albeit at lower levels, especially in the ventricle (Fig. 6L). These results suggested that Csx/Nkx2.5 seems to suppress expression of Msx2 in the ventricle. Expression of Msx1, BMP-4, TGFβ-1 and fibronectin was not affected in the mutant heart (data not shown).

Heart looping was arrested in N-cadherin knock-out mice just at the same stage as in Csx/Nkx2.5 null mutant mice (Radice et al., 1997). Disruption of erbB4 (Gassmann et al., 1995) or NF-1 (Brannan et al., 1994) were reported to result in poor trabeculation. We therefore examined expression of N-cadherin, erbB4 and NF-1 by in situ hybridization. However, these genes were normally expressed in the mutant heart (data not shown).

In order to confirm the results of in situ hybridization, we performed semiquantitative RT-PCR using RNA extracted from wild-type and homozygous mutant embryos at 9.5 d.p.c. (Fig. 7). MLC2V expression was downregulated, but transcripts were still detectable. Transcripts for ANF, BNP, HAND-1 and HAND-2 were expressed in the mutant heart, whereas expression of ANF and BNP was still detectable in the atrium (Fig. 7).

### Table 1. Summary of adult chimeric mice generated from Csx/Nkx2.5+/- ES cells and Csx/Nkx2.5+/- ES cells

<table>
<thead>
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<th>% agouti</th>
<th>75%</th>
<th>50-75%</th>
<th>25-50%</th>
<th>&lt;25%</th>
<th>Total</th>
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<tr>
<td>Csx/Nkx2.5 +/-</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Csx/Nkx2.5 +/-</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>9</td>
<td>14</td>
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Blastocyst injection of Csx/Nkx2.5+/- ES cells generated adult chimeric mice only with less than 50% contribution of ES cells in the coat color.
N-myc were severely reduced, but that of α-actin was equivalent, consistent with the in situ hybridization data. MEF2C expression was decreased by approximately 50%. There were no significant changes in the overall expression level of Msx2 between wild-type and homozygous mutant hearts (Fig. 7, bottom), probably due to the high levels of localized expression in the wild-type heart versus the diffuse, lower levels of expression in the mutant.

**In vivo analysis using Csx/Nkx2.5-deficient ES cells**

In order to determine the fate of Csx/Nkx2.5-deficient cells in the adult animals, we performed a chimeric analysis using ES cells.

### Table 2. Summary of chimeric embryos generated from Csx/Nkx2.5−/− ES cells at 10.5 d.p.c.

<table>
<thead>
<tr>
<th>% Csx/Nkx2.5−/− cells in the heart</th>
<th>0%</th>
<th>&lt;5%</th>
<th>5-15%</th>
<th>20-30%</th>
<th>30-60%</th>
<th>&gt;75%</th>
<th>Absorbed</th>
<th>Total</th>
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<tr>
<td>No. of embryos at 10.5 d.p.c.</td>
<td>18</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>8</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Growth retardation and pericardial effusion</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
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Chimeric embryos with more than 20% contribution of Csx/Nkx2.5-deficient cells in the heart showed growth retardation and pericardial effusion.

N/A, not applicable.

### Table 3. Summary of chimeric embryos generated from Csx/Nkx2.5−/− ES cells at 13.5 d.p.c.

<table>
<thead>
<tr>
<th>% Csx/Nkx2.5−/− cells in the heart</th>
<th>0%</th>
<th>&lt;5%</th>
<th>5-15%</th>
<th>20-30%</th>
<th>30-60%</th>
<th>&gt;75%</th>
<th>Absorbed</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of embryos at 13.5 d.p.c.</td>
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<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>51</td>
</tr>
<tr>
<td>Growth retardation and pericardial effusion</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>N/A</td>
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At 13.5 d.p.c., we only observed chimeric embryos with less than 15% contribution of Csx/Nkx2.5-deficient cells in the heart.

N/A, not applicable.

**Fig. 5.** In situ hybridization analysis of wild-type (+/+) and homozygous mutant (−/−) embryos at 9.5 d.p.c. (A-F,I-L) and at 10.5 d.p.c. (G,H). In situ hybridization using antisense cRNA probes for Csx/Nkx2.5 (A,B), α-cardiac actin (C,D), MLC2V (E-H), ANF (I,J) and BNP (K,L). Expression of MLC2V was downregulated, but was detectable in the entire ventricle of the homozygous mutant embryos (E-H). Note that ANF expression was abolished and BNP expression was severely downregulated in the mutant ventricle (J,L).
cell lines with a homozygous null mutation (“double knock-out”) for Csx/Nkx2.5. We obtained 12 independent double knock-out ES cell lines by incubating ES cells with a heterozygous mutation for Csx/Nkx2.5 at a high concentration of G418 (Fig. 8A). Blastocyst injection of double knock-out ES cells generated only chimeric mice with less than 50% contribution of ES cells in their coat color, in contrast to greater than 75% contribution often observed when the heterozygous ES cells were used (Table 1). We analyzed five adult chimeric mice generated from double knock-out ES cells (25-50% contribution of ES cells in their coat color, Table 1) by β-gal staining and GPI assay. Very few lacZ-positive cells (0-2 colonies per cross-section) could be observed in serial sections of the hearts of two mice (Fig. 8B,C), and no lacZ-positive cells were found in the hearts of the remaining three mice. GPI assay indicated that there were substantial contributions of Csx/Nkx2.5-deficient cells in other adult organs, whereas they are barely detectable in the heart (Fig. 9). There were faint GPI-AA bands in the hearts of two mice (No.1 and 3 in Fig. 9), but there were no lacZ-positive cells in the hearts of these mice. Because Csx/Nkx2.5 is cardiomyocyte-specific, these GPI-AA bands were likely to originate from non-myocyte cells in the heart, such as vascular endothelial cells, smooth muscle cells and fibroblasts.

To examine how mutant cardiomyocytes became selected against during development, we analyzed 50 chimeric embryos generated from Csx/Nkx2.5-deficient ES cells at 10.5 d.p.c. (Table 2). Unexpectedly, whole-mount β-gal staining demonstrated that chimeric embryos with more than 30-40% contribution of Csx/Nkx2.5-deficient cells in the heart had severe growth retardation and massive pericardial effusion (Fig. 10A). Histological analysis showed almost no trabeculation and no endocardial cushion formation in these chimeric embryos (data not shown). The phenotype of these embryos at 10.5 d.p.c. were indistinguishable from that of the germline homozygous mutant embryos. Interestingly, chimeric embryos with 20-30% contribution of Csx/Nkx2.5-deficient cells in the heart showed a milder phenotype than homozygous mutant embryos. They showed moderate growth retardation (Fig. 10B) and less pericardial effusion (Fig. 10C). Histological analysis demonstrated that the ventricle did not show septation or endocardial cushion formation, but had some degrees of trabeculation (Fig. 10D). Less than 15% contribution of Csx/Nkx2.5-deficient cells in the heart did not significantly affect heart formation at 10.5 d.p.c. (Fig. 10E,F). These results suggest that Csx/Nkx2.5-deficient cells have a dominant interfering effect on cardiac development.

When additional 51 chimeric embryos were analyzed at 13.5 d.p.c., we only found embryos with less than 15% contribution of Csx/Nkx2.5-deficient cells in the heart (Table 3). This result,
together with existence of many absorbed embryos, indicated that chimeric embryos with more than 20% contribution of Csx/Nkx2.5-deficient cells in the heart were lethal between 10.5 and 13.5 d.p.c. All embryos with less than 15% contribution of Csx/Nkx2.5-deficient cells in the heart appeared normal at 13.5 d.p.c.

**DISCUSSION**

**Phenotypes of Csx/Nkx2.5 null mutant embryos**

In this study, we created mice carrying a null mutation in the Csx/Nkx2.5 locus and analyzed functions of this gene during embryogenesis. First, we could demonstrate that the null mutation did not eliminate the heart cell lineage, implying that the function of Csx/Nkx2.5 is different from that of tinman, which specifies the precardiac and midgut mesoderm.

The overall phenotype of the null mutant mice was similar to that of the previously reported mutant mice with an insertion mutation of Nkx2.5 (Lyons et al., 1995). However, there were some distinct differences in cardiac morphology between these two mutant mice. In the previous paper, it was described that the mutant hearts were often biased toward left and clearly devoid of the dextraloop (Lyons et al., 1995). However, in null mutant embryos, the proximal part of the outflow tract was always situated on the right side, and the atrium and the atrioventricular junction on the left, clearly demonstrating that the dextra looping was initiated. It was reported that the expression of MLC2V was abolished except for a small cluster of cells on the dorsal side of mutant hearts (Lyons et al., 1995). However, in the null mutant embryos, MLC2V was homogeneously expressed, albeit at lower levels in the ventricle, indicating that the ventricular specification is more advanced in the null mutant. Since the insertional allele is capable of coding for a truncated version of Csx/Nkx2.5 protein, it is possible that such protein might have ‘dominant’ negative effects through protein-protein interaction.

We could also demonstrate the extracardiac phenotype of Csx/Nkx2.5 null mutant mice. The yolk sac is the first site of hematopoiesis and the major source of blood cells in mouse embryos. Surprisingly, there was no defined vascular formation in the mutant yolk sac. Few blood cells could be seen between the two layers of the mutant yolk sac and homozygous null embryos were severely anemic. Some endothelial cells were present even in the mutant yolk sac, but they did not form vascular channels, indicating that further vasculogenesis and angiogenesis did not occur in the mutant yolk sac. Defects in the yolk sac vasculature have been observed in mutant embryos for other genes, such as BMP-4, TGFβ1, vascular endothelial growth factor (VEGF), tissue factor, arylhydrocarbon-receptor nuclear translocator (ARNT), fibronectin and HAND1 (Carmeliet et al., 1996a,b; Dickson et al., 1995; Ferrara et al., 1996; Firulli et al., 1998; George et al., 1997; Maltepe et al., 1997; Riley et al., 1998; Winnier et al., 1995). All of these genes are expressed in the yolk sac. However, we could not detect transcripts for Csx/Nkx2.5 in the yolk sac either by in situ hybridization or RT-PCR (data not shown). Furthermore, poor development of blood vessels, such as intersomitic arteries and pharyngeal arch arteries, was also observed in Csx/Nkx2.5 null mutant embryos. Csx/Nkx2.5 is not expressed in these blood vessels.
It is possible that this phenotype is secondary to circulatory failure. However, defects in vascular formation in the yolk sac were not described for mutant embryos homozygous for HAND2, although heart formation arrested at the same stage as in Csx/Nkx2.5 null mutant embryos (Srivastava et al., 1997). Therefore, the absence of vascular formation in the mutant yolk sac raises the possibility that there might be some secreted factor(s) from the myocardium or pharyngeal endoderm that is dependent on Csx/Nkx2.5. These factors might promote vasculogenesis and angiogenesis as well as hematopoiesis in the yolk sac.

Csx/Nkx2.5 regulates expression of multiple target genes in the developing heart

What are the functions of Csx/Nkx2.5 in cardiac development? We first tested the effects of inactivation of Csx/Nkx2.5 on growth capacity of cardiac myocytes. An interesting observation was that Csx/Nkx2.5 null cardiac myocytes showed normal PCNA labeling at 9.5 d.p.c. The frequency of TUNEL-positive cardiac myocytes was also normal. These results indicated that there is no overt defect in the cell cycle entry or enhanced apoptosis in Csx/Nkx2.5 null cardiac myocytes at this stage.

We next examined expression of 20 candidate downstream genes in the mutant heart. Our results demonstrated that Csx/Nkx2.5 controls expression of ANF and BNP in the ventricle and that expression of these genes is differentially regulated in the atrium and in the ventricle. N-myc is expressed in the developing nervous system, kidney, limb buds and heart (Mugrauer et al., 1988). In the heart, N-myc transcripts can be observed mainly in the compact layer of the myocardium (Moens et al., 1993). N-myc expression in the heart was abolished in Csx/Nkx2.5 null mutant mice. This observation is intriguing, since homozygous mutant mice for N-myc showed poor development of the ventricular myocardium and the interventricular septum (Charron et al., 1992; Moens et al., 1993; Stanton et al., 1992). In agreement with the previous report (Biben et al., 1997), expression of HAND1 (eHAND) in the myocardium was abolished in Csx/Nkx2.5 null mutant hearts. On the contrary, expression of GATA4 and TEF1 was not affected in the mutant embryo, suggesting that they are not genetically downstream of Csx/Nkx2.5. Interestingly, a targeted mutation of HAND2 (dHAND) (Srivastava et al.) or N-cadherin...
(Radice et al.) caused the almost identical phenotype, yet dHAND or N-cadherin expression was not affected in Csx/Nkx2.5 null embryos.

Drosophila has one copy of MEF2 (D-mef2) and an upstream enhancer of D-mef2 contains tinman binding sites, which are essential for expression of D-mef2 in the cardiac cell lineage. Thus, D-mef2 seems to be a direct target gene of tinman (Gajewski et al., 1997). In Csx/Nkx2.5 null mutant hearts, expression of MEF2C was downregulated. In addition, expression of MEF2C in the myotome was also reduced in homozygous mutants (Fig. 6F), although Csx/Nkx2.5 is not expressed in the myotome. Slight growth retardation might have affected expression of MEF2C in the myotome of the mutant embryos at 9.5 d.p.c., since MEF2C expression becomes high in therostal somites between 9.0 and 9.5 d.p.c. (Edmondson et al., 1994).

In the chicken embryo, Msx2 expression is initially restricted to myocardial cells at the right AV junction and at later stages extends to the entire AV junction, the crest of the interventricular septum and coalescing trabeculae (Chan-Thomas et al., 1993). From this morphological coincidence, it has been thought that Msx2 might play roles in the formation of the cardiac conduction system as well as septal formation (Eisenberg and Markwald, 1995). Our study suggests that Csx/Nkx2.5 may inhibit expression of Msx2 in the embryonic ventricular wall. It is of interest that there are high incidences of atrial septal defect, ventricular septal defect and AV conduction defects in human mutations of CSX/NKX2.5 (Schott et al., 1998). It is not known whether MSX2 expression was misregulated in these patients.

Csx/Nkx2.5 expressing cardiac myocytes are required for cardiac development in a quantitatively sensitive manner

Chimeric analysis using Csx/Nkx2.5-deficient ES cell lines has demonstrated that Csx/Nkx2.5-deficient cardiomyocytes have dominant interfering effects on cardiac development. Interestingly, 10.5 d.p.c. embryos with more than 30-40% contribution of Csx/Nkx2.5-deficient cells in the heart showed the same phenotype as that of homozygous null mutant embryos. However, embryos with less contribution of Csx/Nkx2.5-deficient cells in the heart showed a milder phenotype. This result suggested that the certain number of cardiac myocytes expressing Csx/Nkx2.5 is essential for a proper cardiac morphogenesis. It is possible that there might be some secreted factor(s) downstream of Csx/Nkx2.5 that could promote survival or further differentiation of cardiac myocytes. Alternatively, it is also possible that Csx/Nkx2.5-deficient cardiac myocytes might secrete factor(s) that inhibit differentiation, survival or proliferation of cardiac myocytes if expression of these factor(s) were suppressed by Csx/Nkx2.5. Moreover, it was shown that Csx/Nkx2.5-deficient cells could differentiate into cardiac myocytes in the trabecular layer (Fig. 10D,F), suggesting that the absence of trabeculation in homozygous mutant embryos may result from a non-cell-autonomous function of Csx/Nkx2.5. Since the tinman family of genes are highly conserved throughout evolution, further elucidation of the function of Csx/Nkx2.5 is likely to provide a new insight into a general mechanism of heart development in diverse species.

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