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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 *Csx/Nkx2.5* might result in a different phenotype (reviewed in Harvey, 1996; Tanaka et al., 1998). As for the genes downstream of *Csx/Nkx2.5*, it has only been reported that expression of the *ventricular specific myosin light chain 2* gene (*MLC2V)*, *HAND1* and the *cardiac ankyrin repeat protein* (*CARP)* was downregulated in homozygous mutant embryos for *Nkx2.5* (Biben and Harvey, 1997; Lyons et al., 1995; Zou et al., 1997). Identification of more downstream target genes of *Csx/Nkx2.5* will lead to a better understanding of the mechanisms by which *Csx/Nkx2.5* regulates cardiac development.

To determine the role of *Csx/Nkx2.5*, we generated and analyzed mutant mice completely null for *Csx/Nkx2.5*. We demonstrate that *Csx/Nkx2.5* regulates, either directly or indirectly, expression of several downstream target genes, especially transcription factors shown to be essential for 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 *Not*I 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 (1.0×10^7) 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 embryos and mice. Results of PCR assay were confirmed by Southern blot analysis. The primers used for detection of the wild-type allele were 5′-CAGCAACTTCGTGAACTTTGGC-3′ and 5′-AACATAAATACGGGTGGGTGGG-3′. Primers 5′-GCAGCCTC-TGTTCCACATACACTTC-3′ and 5′-AACATAAATACGGGTG-GGTGGG-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 ^{35}S-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 pyrophosphate, 1× 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 0.1× 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 *Pst*I-*Eco*RV fragment of *N-cadherin* was used as a 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 *Pst*I-*Eco*RV fragment of *MEF2C* was used as a probe. Full-length cDNA clones for *Msx1* and *Msx2* were kind gifts from Richard L. Maas (Brigham and Women's Hospital, Boston, MA). Probes for *NF-1*, *BMP-4*, *TGFβ-1*, *fibronectin* 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 PBSMT (3% skim milk and 0.1% Triton X-100 in PBS), and incubated with 10 μg/ml of anti-PECAM antibody, MEC13.3, in PBSMT 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 10^6 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, liters 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

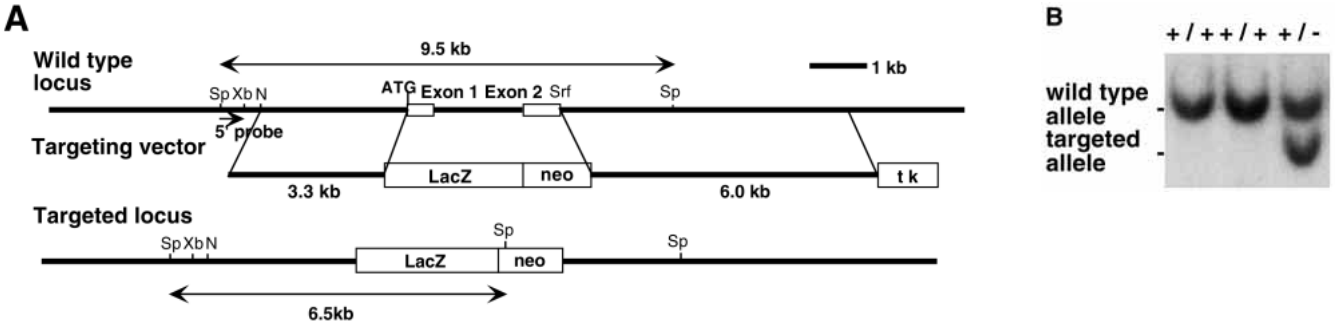

Fig. 1. Gene targeting of *Csx/Nkx2.5*. (A) The organization of the *Csx/Nkx2.5* gene and the structure of the targeting vector are shown. The 5′ probe (a *Spe*I–*Xba*I fragment) was used for Southern blot analysis. Sp, *Spe*I; Xb, *Xba*I; N, *Not*I; Srf, *Srf*I. (B) Genotyping of ES cell clones. Genomic DNA was digested with *Spe*I 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

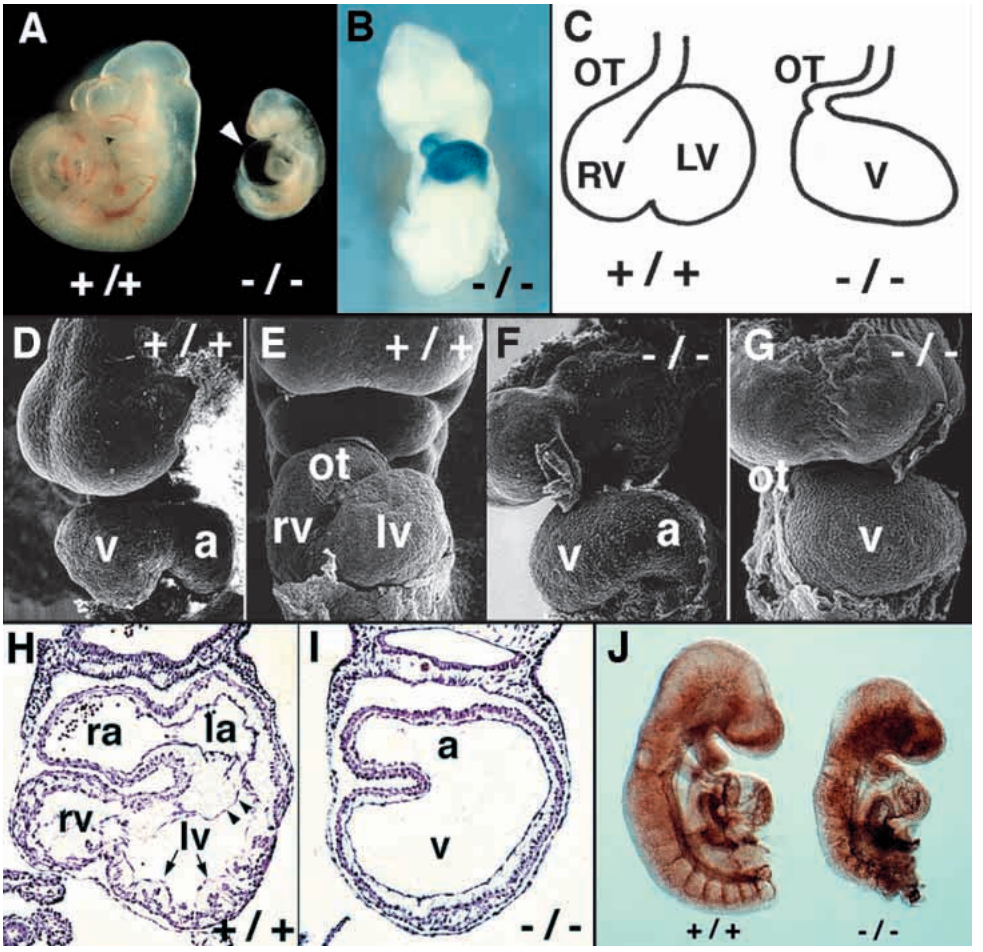

Fig. 2. Morphological analysis of homozygous *Csx/Nkx2.5* null embryos. (A) Wild-type (+/+) and homozygous mutant (–/–) embryos at 10.5 d.p.c. The arrowhead shows the pericardium of the mutant embryo. (B) Whole-mount β-gal staining of a homozygous mutant embryo at 9.5 d.p.c. Note that the beginning of the outflow tract is located on the right side. (C) Schematic representation of wild-type (+/+) and homozygous mutant (–/–) hearts at 9.5 d.p.c. RV, right ventricle; LV, left ventricle; V, single ventricle. (D-G) Scanning electron micrograms of wild-type (D,E) and homozygous mutant (F,G) embryos at 9.5 d.p.c. Left lateral views (D,F) and ventral views (E,G). The pericardium was removed to show heart structures. The atrioventricular canal was wide open and the bulboventicular sulcus did not form in the homozygous mutant embryo. a, atrium; v, ventricle; rv, right ventricle; lv, left ventricle; ot, outflow tract. (H,I) H&E-stained transverse sections of wild-type (H) and homozygous mutant (I) embryos at 9.5 d.p.c. Note the trabeculation (arrows) and endocardial cushion formation (arrowheads) in the wild-type embryo. (J) Whole-mount PECAM staining of wild-type and homozygous mutant embryos at 9.5 d.p.c. Note poor development of intersomitic arteries, dorsal aorta and pharyngeal arch arteries in the mutant embryo.

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,

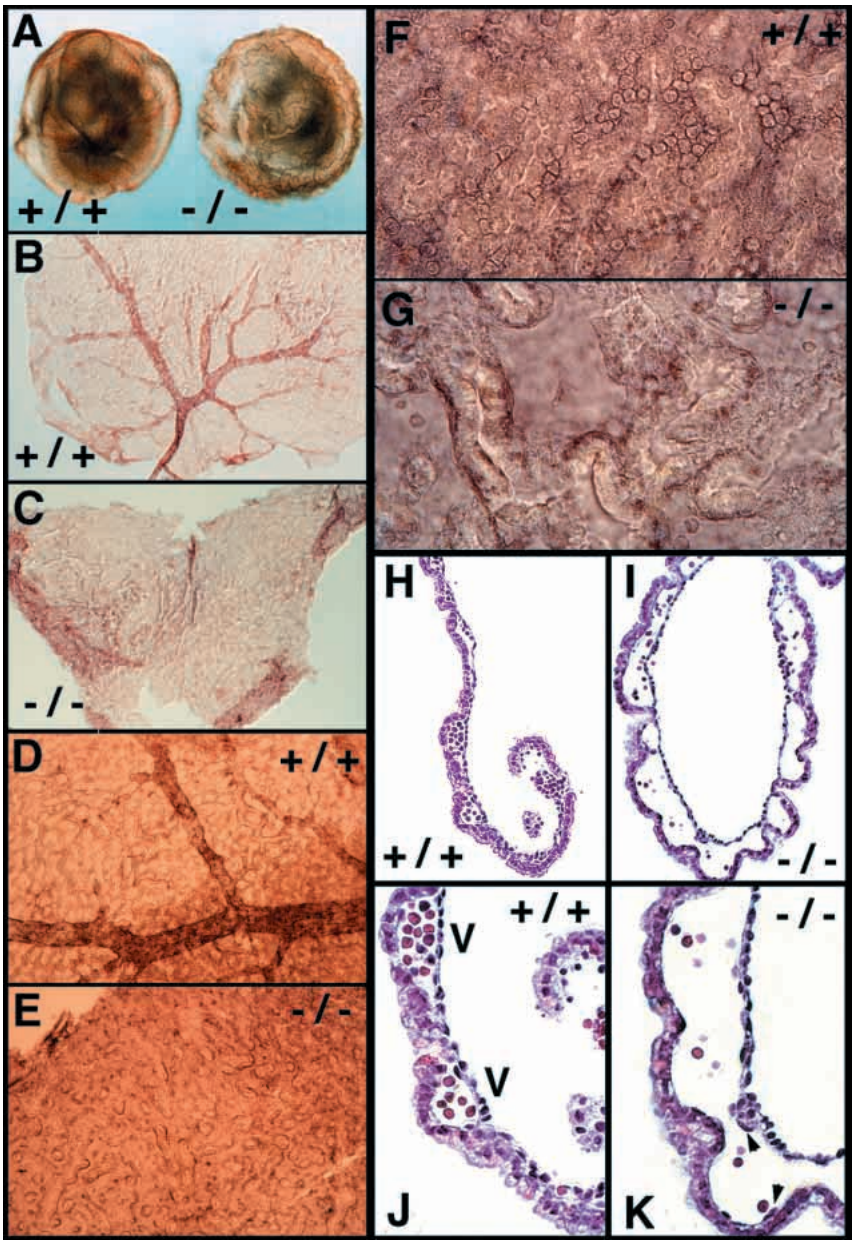

Fig. 3. Morphological analysis of yolk sacs of wild-type (+/+) and homozygous mutant (–/–) embryos at 9.5 d.p.c. (A) Wild-type (+/+) and homozygous mutant (–/–) yolk sacs at 9.5 d.p.c. There were excessive folds on the surface and no large vitelline vessels could be seen in the mutant yolk sac. (B,C) 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,E, ×200; F,G, ×400) of the same wild-type (D,F) and homozygous mutant (E,G) yolk sacs. A fine network of small vessels filled with blood cells could be observed in the wild-type yolk sac. Only enlarged channels with few blood cells could be seen 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. Vascular channels containing blood cells could be observed in the wild-type yolk sac. Some endothelial cells were present in the mutant yolk sac (arrowheads). However, they did not form defined vascular channels.

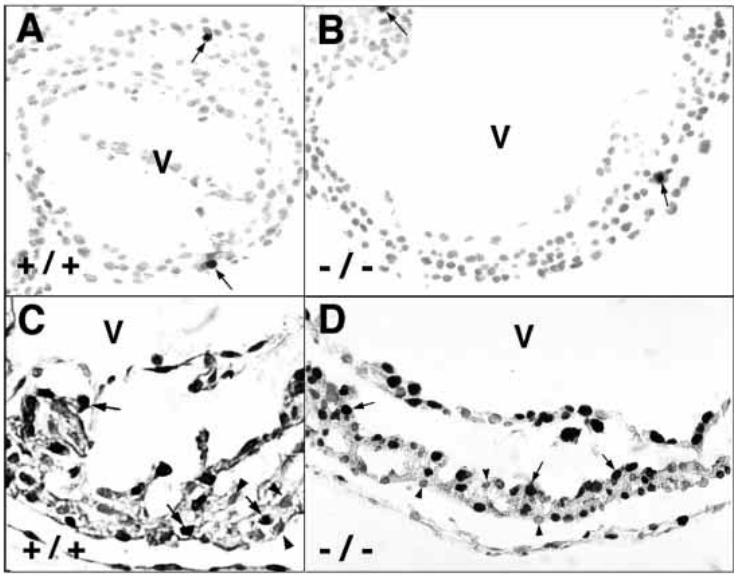

Fig. 4. TUNEL staining and PCNA staining of wild-type (+/+) and homozygous mutant (–/–) embryos at 9.5 d.p.c. (A,B) TUNEL staining of transverse sections of wild-type (A) and homozygous mutant (B) ventricles. The arrows show TUNEL-positive cardiac myocytes. (C,D) PCNA staining of transverse sections of wild-type (C) and homozygous mutant (D) ventricles. The arrows and arrowheads show PCNA-positive and PCNA-negative cardiac myocytes, respectively.

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. 6I,J) 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

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

	% agouti				
ES cells	>75%	50-75%	25-50%	<25%	Total
Csx/Nkx2.5 +/–	9	4	3	3	19
Csx/Nkx2.5 –/–	0	0	5	9	14

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.

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

% Csx/Nkx2.5 –/– cells in the heart	0%	<5%	5-15%	20-30%	30-60%	>75%	Absorbed	Total
No. of embryos at 10.5 d.p.c.	18	8	4	3	7	8	2	50
Growth retardation and pericardial effusion	–	–	–	+	+	+	N/A	

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.

% Csx/Nkx2.5 –/– cells in the heart	0%	<5%	5-15%	20-30%	30-60%	>75%	Absorbed	Total
No. of embryos at 13.5 d.p.c.	10	7	5	0	0	0	29	51
Growth retardation and pericardial effusion	–	–	–	N/A	N/A	N/A	N/A	

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.

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

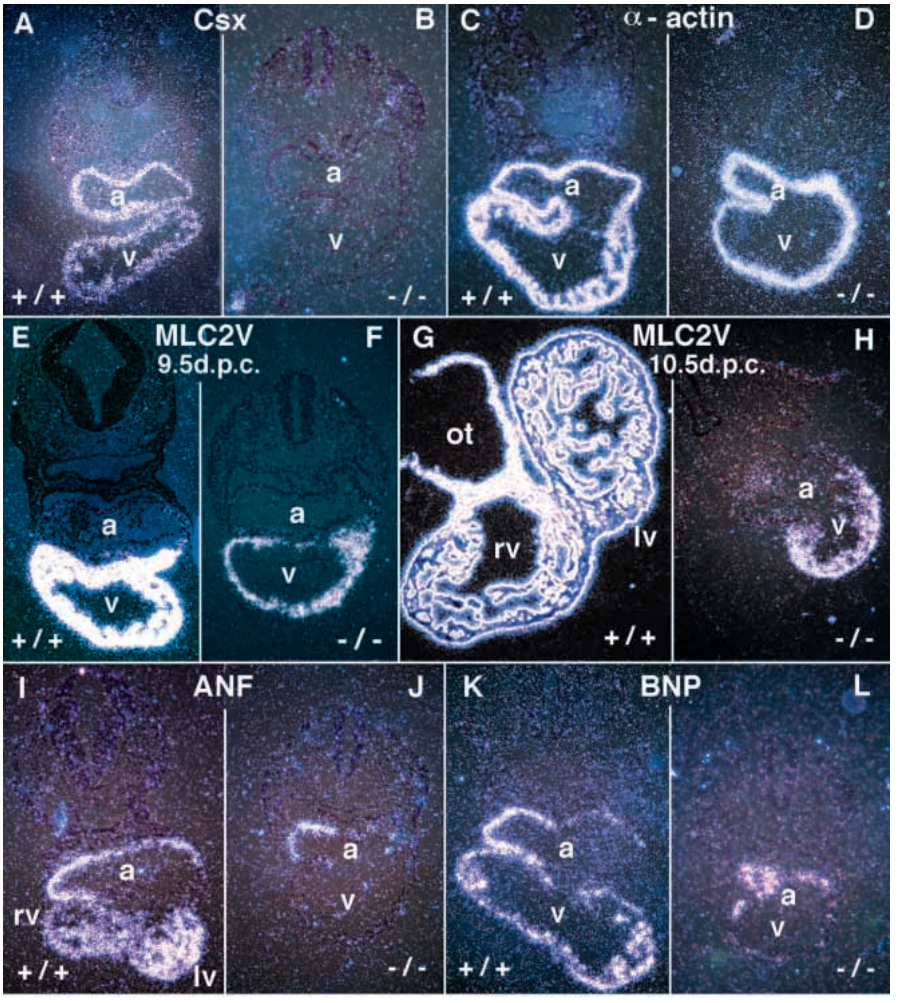

Fig. 5. In situ hybridization analysis of wild-type (+/+) and homozygous mutant (–/–) embryos at 9.5 d.p.c. (A-F,I-L) and at 10.5 d.p.c. (G,H). In situ hybridization using antisense cRNA probes for *Csx/Nkx2.5* (A,B), *α-cardiac actin* (C,D), *MLC2V* (E-H), *ANF* (I,J) and *BNP* (K,L). Expression of *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).

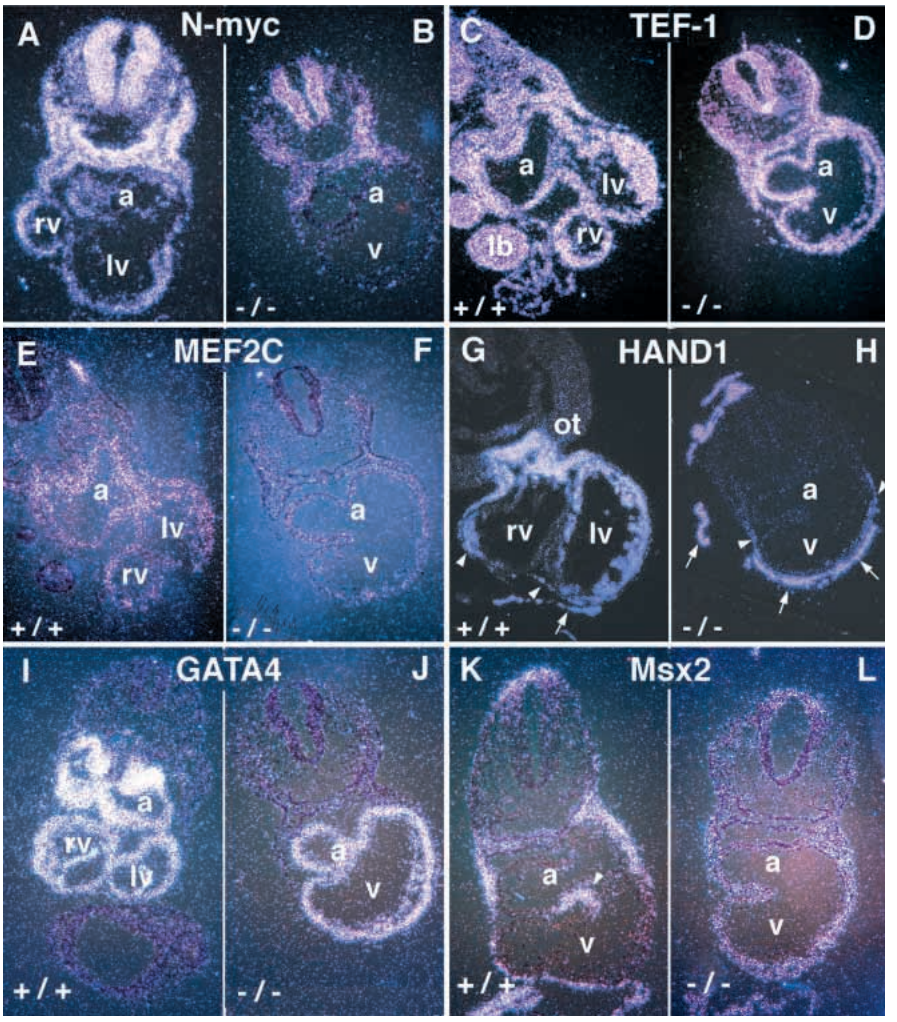

Fig. 6. In situ hybridization analysis of wild-type (+/+) and homozygous mutant (–/–) embryos at 9.5 d.p.c. In situ hybridization using antisense cRNA probes for *N-myc* (A,B), *TEF-1* (C,D), *MEF2C* (E,F), *HAND-1* (G,H), *GATA4* (I,J) and *Msx2* (K,L). Expression of *N-myc* and *HAND1* was abolished and *MEF2C* expression was downregulated in the mutant heart (B,F,H). Arrows and arrowheads in G,H show *HAND-1* expression in the amnion and the pericardium, respectively. Note that *Msx2* expression in the heart was normally localized to the atrioventricular canal (arrowhead in Fig. 6K). However, *Msx2* was expressed in the entire myocardium in the homozygous mutant embryo (L). lb, limb bud.

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,

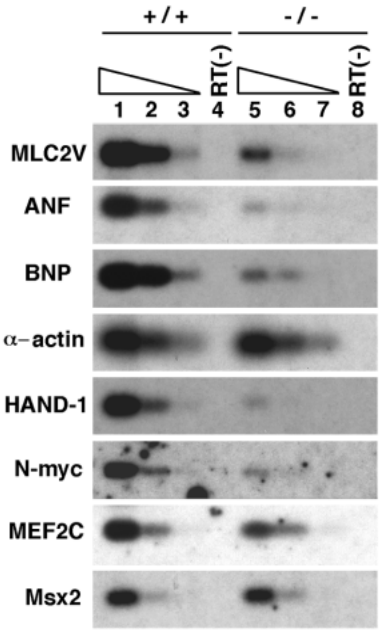

Fig. 7. Semiquantitative RT-PCR analysis. 5-fold serial dilutions (lanes 1-3 and lanes 5-7) of pooled RT products were used for subsequent PCR amplification. Lanes 4 and 8, RT reaction was performed without reverse transcriptase. +/+, wild-type embryonic hearts at 9.5 d.p.c. –/–, homozygous mutant embryonic hearts at 9.5 d.p.c.

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

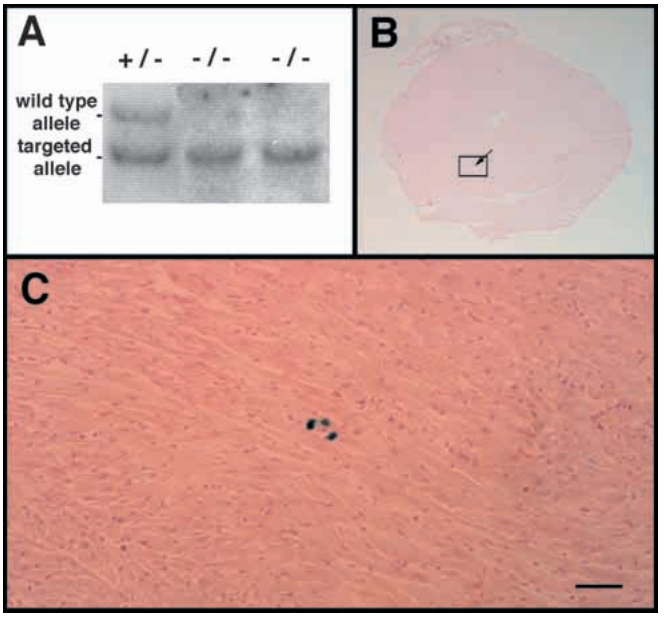

Fig. 8. β-gal staining of an adult chimeric heart generated from *Csx/Nkx2.5*–/– ES cells. (A) Southern blot analysis. +/–, a targeted ES cell line heterozygous for *Csx/Nkx2.5*; –/–, targeted ES cell lines homozygous for *Csx/Nkx2.5*. (B) β-gal staining of a cross-section of the heart from a chimeric mouse generated from mutant ES cells homozygous for *Csx/Nkx2.5*. (3 months old). The arrow points to *lacZ*-positive cells. (C) Higher magnification (×200) of the same section. Note that there are only a few *lacZ*-positive cells in the entire section of the heart. Bar, 100 μm.

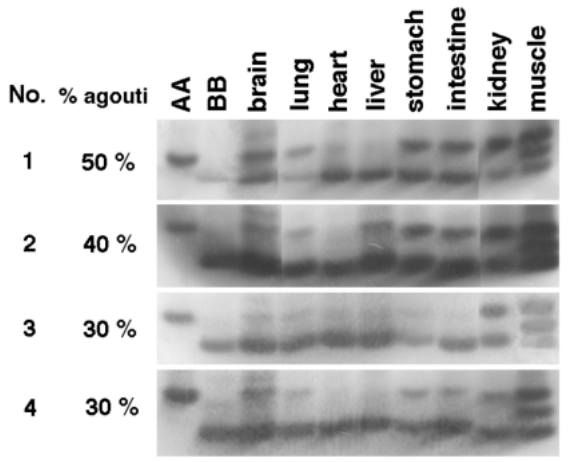

Fig. 9. GPI analysis of adult chimeric mice generated from*Csx/Nkx2.5–/–* ES cells. GPI isoenzyme analysis of various tissues taken from four chimeric mice (3 months old) generated from *Csx/Nkx2.5*-deficient ES cells indicated that *Csx/Nkx2.5*-deficient cells could contribute substantially in organs except for the heart. AA, liver taken from a 129 mouse. BB, liver taken from a C57BL mouse. Skeletal muscle has a third band (AB) due to fusion of wild-type (BB) and *Csx/Nkx2.5*-deficient (AA) cells.

either. 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*

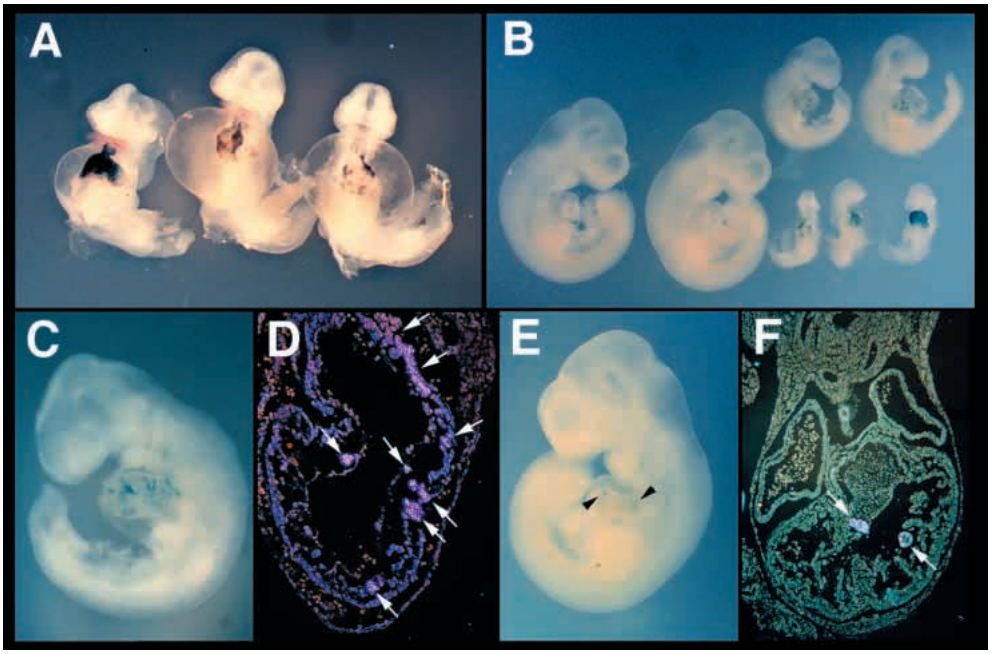

Fig. 10. β-gal staining of chimeric embryos generated from *Csx/Nkx2.5–/–* ES cells. (A) Whole-mount β-gal staining of chimeric embryos generated from *Csx/Nkx2.5–/–* ES cells at 10.5 d.p.c. The contribution of *Csx/Nkx2.5–/–* cells in the heart was greater than 90%, 40-50% and 30-40%, respectively (from the left). (B) Whole-mount β-gal staining of chimeric embryos generated from *Csx/Nkx2.5–/–* ES cells at 10.5 d.p.c. The contribution of *Csx/Nkx2.5–/–* cells in the heart was (from the left) 0%, less than 5%, 20-30% (the upper two embryos) and more than 30% (the lower three embryos), respectively. (C,D) Whole-mount β-gal staining (C) and a transverse section (D) of a chimeric embryo with 20-30% contribution of *Csx/Nkx2.5–/–* cells in the heart. The pink cells (arrows) show *lacZ*-positive cells (dark field). Note some degrees of trabeculation and contribution of *Csx/Nkx2.5–/–* cells in the trabecular layer. (E,F) Whole-mount β-gal staining (E) and a transverse section (F) of a chimeric embryo with less than 5% contribution of *Csx/Nkx2.5–/–* cells in the heart. The arrows show *lacZ*-positive cells (dark field). Note normal heart structure and the contribution of *Csx/Nkx2.5–/–* cells in the trabecular layer.

(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 the rostal 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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