Csx/Nkx2-5 Is Required for Homeostasis and Survival of Cardiac Myocytes in the Adult Heart*

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Csx/Nkx2-5, which is essential for cardiac development of the embryo, is abundantly expressed in the adult heart. We here examined the role of Csx/Nkx2-5 in the adult heart using two kinds of transgenic mice. Transgenic mice that overexpress a dominant negative mutant of Csx/Nkx2-5 (DN-TG mice) showed degeneration of cardiac myocytes and impairment of cardiac function. Doxorubicin induced more marked cardiac dysfunction in DN-TG mice and less in transgenic mice that overexpress wild type Csx/Nkx2-5 (WT-TG mice) compared with non-transgenic mice. Doxorubicin induced cardiomyocyte apoptosis, and the number of apoptotic cardiomyocytes was high in the order of DN-TG mice, non-transgenic mice, and WT-TG mice. Overexpression of the dominant negative mutant of Csx/Nkx2-5 induced apoptosis in cultured cardiomyocytes, while expression of wild type Csx/Nkx2-5 protected cardiomyocytes from doxorubicin-induced apoptotic death. These results suggest that Csx/Nkx2-5 plays a critical role in maintaining highly differentiated cardiac phenotype and in protecting the heart from stresses including doxorubicin.

The cardiac homeobox gene Csx/Nkx2-5 starts to be expressed at embryonic day 7.5 in the heart primordia of mice (1, 2), and targeted disruption of murine Csx/Nkx2-5 results in embryonic lethality (3). Many mutations in human Csx/Nkx2-5 have been reported to cause a variety of congenital heart diseases and atrioventricular conduction delay (4, 5). These observations indicate that Csx/Nkx2-5 plays a critical role in cardiac morphogenesis and contributes to diverse cardiac developmental pathways at the embryonic stage.

Knockout experiments have suggested that many genes such as Hand1, myocyte enhancer factor-2, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), cardiac α-actin, cardiac ankyrin repeat protein (CARP), N-myc, and MSX2 are genetically located downstream of Csx/Nkx2-5 at the embryonic stage (2, 6–8). Although Csx/Nkx2-5 continues to be expressed in the adult heart (1, 2), the function of Csx/Nkx2-5 in the later stage of development is unknown because of embryonic lethality of null mutant mice (3, 8). In our recently generated transgenic mice that overexpress human Csx/Nkx2-5 (WT-TG mice), mRNA levels of many cardiac genes such as ANP, BNP, CARP, and sarcolemmal calcium ATPase 2 (SERCA2) genes were up-regulated (9). These results suggest that Csx/Nkx2-5 regulates expression of cardiac-specific genes also in the adult heart. However, because there was no difference in phenotype between WT-TG and non-transgenic (non-TG) mice (9), the significance of these gene up-regulations remains unknown. Csx/Nkx2-5 itself is also differentially regulated by different stimuli. In response to hypertrophic stimuli such as isoproterenol, phenylephrine, and pressure overload, Csx/Nkx2-5 is up-regulated (10, 11), whereas it is down-regulated by treatment with doxorubicin (DOX) (12). These results suggest that Csx/Nkx2-5 has certain roles in the adult heart. In this study, we generated transgenic mice that overexpress a dominant negative mutant of human Csx/Nkx2-5 (DN-TG mice) under the control of α-myosin heavy chain (α-MHC) promoter and examined the role of Csx/Nkx2-5 in the adult heart.

EXPERIMENTAL PROCEDURES

Animal Models—Human Csx/Nkx2-5 LP mutant (LP mutant) cDNA created by substituting a proline for a highly conserved leucine in the homeodomain of human Csx/Nkx2-5 (13) was subcloned into the α-MHC promoter-containing expression vector (14). The linearized DNA was injected into pronuclei of eggs from BDF1 mice, and the eggs were transferred into the oviducts of pseudopregnant ICR mice. The transgene was identified by PCR with transgene-specific primers and by Southern blot analysis. Three independent lines of DN-TG mice were obtained, and they showed the same results. We used 12-week-old heterozygous mice for analysis. A single dose of 20 mg/kg DOX was injected intraperitoneally. Mice were sacrificed 24 h after DOX injection. Protocols were approved by the Institutional Animal Care and Use...
Committee of the University of Chiba.

Cell Culture, DNA Transfection, and Reporter Gene Assay—CL6 cells were cultured as described previously (15). To induce differentiation, 1% dimethyl sulfoxide (Me$_2$SO) was added to the growth medium (differentiation medium). The plasmid containing human wild type (WT) Csx/Nkx2-5 or LP mutant was transfected into CL6 cells by the lipofection method (TX reagents, Promega) as described previously (15). Stable transformants were selected with 800 μg of neomycin/ml, and six independent cell lines were isolated. We transfected WT Csx/Nkx2-5 and/or LP mutant into COS-7 cells and cardiomyocytes of neonatal rats by the standard calcium phosphate method. The luciferase activity of the 300-bp ANP promoter containing the reporter gene was measured 48 h after transfection with a Berthold Lumat LB9501 luminometer as described previously (16).

Physiological and Analytical—Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). Cardiac function was evaluated with echocardiography (Image Point HX, Hewlett Packard) using a 10-MHz transducer as described previously (9). Left ventricular (LV) dimension, wall thickness, and percent fractional shortening (%FS) were obtained from M-mode images of the left ventricle. The quantitative measurements represent consensus estimates by two different investigators (H. Toko and E. Takimoto), and interobserver variability was less than 10%. Arterial blood pressure and cardiac rate were measured by tail cuff method.

Histological Analysis—Four-μm-thick paraffin sections were stained with hematoxylin-eosin and van Gieson. For the detection of apoptotic cells, TUNEL and immunohistochemical analysis to detect active caspase-3, which is one of the critical enzymes to induce apoptosis, were performed with an in situ apoptosis detection kit (Takara Syuzo) and with anti-active caspase-3 polyclonal antibody (Promega), respectively, according to the suppliers’ instructions. For electron microscopic analysis, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. All of the samples were coded and scored in a blind fashion as described previously (17). We examined five hearts of each group, and 10 pictures were randomly taken from each heart. Samples were coded and scored independently by two different investigators (H. Toko and M. Sakamoto) using a scale of 0–4 (score 0, normal; score 1, early degenerative alterations in some cells, i.e. loss of parallel orientation, swelling of mitochondria, and cell vesiculation; score 2, advanced degenerative changes, i.e. intracytoplasmic inclusions, loss of myofilaments, separation of intercalated discs, and nuclear modifications; score 3, myofibrillar atrophy and loss of contractile elements; score 4, myofiber degeneration accompanied by myositis.).

Northern Blot Analysis—Total RNA was extracted by the acid guanidine method (RNaZol B, TEL-TEST), and Northern blot analysis was performed as described previously (9).

Western Blot Analysis—The plasmids expressing Myc-tagged WT Csx/Nkx2-5, hemagglutinin-tagged WT Csx/Nkx2-5, and GATA-4 cDNAs were transiently transfected into COS-7 cells, and 48 h after transfection, whole cell extracts were prepared for immunoprecipitation/Western blot analysis as described previously (14). Western blot analysis was also performed with anti-Bcl-2 and anti-Fas/ligand monoclonal antibodies (Transduction Laboratories) as described previously (14). Hybridizing bands were visualized using an ECL detecting kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed using double-stranded oligonucleotides corresponding to the thyroid transcription factor-1 (TTF-1) binding sequence as described previously (14).

Immunofluorescent Cytchemistry—The plasmids expressing Myc-tagged WT Csx/Nkx2-5 and Csx/Nkx2-5 LP cDNAs were transfected into the cultured cardiomyocytes of neonatal rats plated on a cover glass. The cells transfected with LP mutant and WT Csx/Nkx2-5 were marked by anti-Myc monoclonal antibody and an anti-mouse IgG conjugated to rhodamine. To detect apoptotic cells, 50 μl of TUNEL reaction mixture containing both terminal deoxynucleotidyltransferase and fluorescein isothiocyanate-conjugated dUTP was added to each sample.

Statistical Analysis—Data are shown as mean ± S.E. Multiple group comparison was performed by one-way analysis of variance followed by the Bonferroni procedure for comparison of means. Two-tailed Student’s t test was used to compare transgenic with non-transgenic specimens under identical conditions. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Dominant Negative Mutant of Csx/Nkx2-5—Human LP mutant was created by substituting a proline residue for a leucine residue in the homeodomain as described previously (13). Grows et al. (13) have reported that overexpression of Xenopus LP mutant inhibits heart development, suggesting that the LP mutant has dominant inhibitory function. In this study, we further examined the dominant inhibitory function of LP mutant using the ANP promoter. When co-transfected with ANP promoter containing the luciferase reporter gene into COS-7 cells, the LP mutant did not activate the ANP promoter. However, the LP mutant suppressed WT Csx/Nkx2-5-induced activation of the ANP promoter in a dose-dependent manner (Fig. 1A). The LP mutant also suppressed the synergistic activation of the ANP promoter induced by Csx/Nkx2-5 and GATA-4 (Fig. 1A). EMSA revealed that WT Csx/Nkx2-5, but not the LP mutant, bound to TTF-1 binding sequence, and the TTF-1 binding of WT Csx/Nkx2-5 was mildly but significantly inhibited by the LP mutant (Fig. 1B). These results suggest that a part of the dominant negative effects of the LP mutant is to inhibit the ability of the WT Csx/Nkx2-5 to bind DNA. Our and other groups have demonstrated that Csx/Nkx2-5 may interact with Csx/Nkx2-5 itself and GATA-4 (16, 18). Immunoprecipitation assay indicated that the LP mutant interacted with WT Csx/Nkx2-5 and with GATA-4 (Fig. 1C). These results suggest that the LP mutant suppresses Csx/Nkx2-5-induced activation of the ANP promoter possibly by sequestering associated protein.

CL6-LP Cell Line—The CL6 cell line, derived from P19 cells, is a useful in vitro model to study cardiomyocyte differentiation because CL6 cells differentiate into beating cardiomyocytes with high efficiency by treatment with 1% Me$_2$SO (15). To further examine the functions of the LP mutant, we isolated three permanent CL6 cell lines that overexpress WT Csx/Nkx2-5 (CL6-WT), LP mutant (CL6-LP), and the empty vector (CL6–/–). When cultured in growth medium, all of these cells grew well, and there was no difference in growth rate. When treated with 1% Me$_2$SO, ~80% of CL6–/– cells were differentiated into beating cardiomyocytes (positive for MF20) (Fig. 1D), and the spontaneous beating was first observed on day 10 after the initiation of the Me$_2$SO treatment. In contrast, more than 95% of CL6-WT cells were differentiated into beating cardiac myocytes, and the spontaneous beating was first observed on day 8–9, 1 or 2 days earlier than CL6–/– (Fig. 1D). In contrast, CL6-LP cells did not differentiate into beating cardiomyocytes until day 12, and less than 10% of CL6-LP cells were differentiated into MF20-positive beating cardiomyocytes on day 16 (Fig. 1D). These results suggest that overexpression of Csx/Nkx2-5 promotes cardiomyocyte differentiation of CL6 cells and that overexpression of the LP mutant inhibits the cardiomyocyte differentiation.

Physiological Characteristics of DN-TG Mice—We obtained three independent lines of transgenic mice that overexpressed human Csx/Nkx2-5 LP mutant under the control of α-MHC promoter (Fig. 2A). The transgene was abundantly expressed in the adult heart, and mRNA levels of LP mutant were much higher (>10-fold) than those of endogenous Csx/Nkx2-5 (Fig. 2B). Because the antibody against Csx/Nkx2-5 is not available at present, we estimated the abundance of Csx/Nkx2-5 proteins using EMSA. The band shift was observed when the extracts from the heart of WT-TG mice, but not of DN-TG or non-TG mice, were used (Fig. 2C). These results suggest that exogenous Csx/Nkx2-5 proteins are much higher than endogenous Csx/Nkx2-5.

The DN-TG mice were apparently healthy and fertile, and there were no significant differences in body weight, heart weight, and blood pressure among DN-TG mice, non-TG mice,
FIG. 1. Dominant negative mutant of Csx/Nkx2-5. A, transcriptional activity of Csx/Nkx2-5 LP mutant. COS-7 cells were transfected with 0.2 μg of the 300-bp ANP promoter containing the luciferase reporter plasmid (ANP (300)-luc) and various amounts of the LP mutant, WT Csx/Nkx2-5, and/or GATA-4. The luciferase activity was normalized to the β-galactosidase activity for each sample. The activity was presented as fold relative to the activity of the ANP promoter alone (=1). Values are the mean ± S.E. of data from three independent experiments performed in triplicate. *, p < 0.05. B, DNA binding activity of Csx/Nkx2-5 LP mutant. The DNA binding activity of Csx/Nkx2-5 proteins was examined by EMSA using TTF-1 binding sequences. The cDNAs of WT Csx/Nkx2-5 and the LP mutant were transfected into COS-7 cells, and nuclear extracts were prepared after 48 h. A 32P-labeled oligonucleotide probe corresponding to the TTF-1 binding sequences was incubated with the nuclear extracts and subjected to electrophoresis on a 5% polyacrylamide gel. The binding affinity of the WT Csx/Nkx2-5 protein was reduced by the presence of unlabeled TTF-1. The TTF-1 binding sequences bound strongly to WT Csx/Nkx2-5 but not to the LP mutant (left panel). To elucidate whether the LP mutant inhibited the DNA binding activity of WT Csx/Nkx2-5, the cDNAs of WT Csx/Nkx2-5 and the LP mutant were co-transfected into COS-7 cells, and EMSA was performed using nuclear extracts. The DNA binding of WT Csx/Nkx2-5 was mildly but significantly reduced by co-transfection of the LP mutant (right panel). FP indicates free probes. *, p < 0.01. C, association of Csx/Nkx2-5 LP mutant with WT Csx/Nkx2-5 and GATA-4. The plasmids expressing Myc-tagged WT Csx/Nkx2-5 (myc-WT) and LP mutant (myc-LP), hemagglutinin-tagged WT Csx/Nkx2-5 (HA-WT), and GATA-4 cDNAs were transfected into COS-7 cells. GATA-4 was immunoprecipitated (IP) with anti-GATA-4 antibody, and the immune complex was subjected to SDS-PAGE and immunoblotted (blot) with anti-Myc antibody (top) or with anti-GATA-4 antibody (bottom). (−), empty vector. D, CL6 cell lines. We isolated three permanent CL6 cell lines that overexpress WT Csx/Nkx2-5 (CL6-WT), LP mutant (CL6-LP), and empty vector (CL6-−). Cardiomyocyte differentiation from these CL6 cells was examined using anti-sarcomeric myosin heavy chain (MF20) (top). Hoechst33342 DNA staining showed that there were equal numbers of cells (bottom).
Csx inhibition of WT-TG mice (0/H11006 (0.875 electron microscopic (EM) scores were higher in DN-TG mice not in the hearts of non-TG mice or WT-TG mice (Fig. 4 B). The of cardiac myofilaments and an increase in the number of analysis revealed that DOX induced cytoplasmic vacuolization and microscopic analysis (data not shown), electromicroscopic anal-

The murine α-MHC promoter and human growth hormone (hGH) poly(A). The LP mutant was created by substituting proline (P) for leucine in the non-TG mice. In WT-TG mice, LV dimension and cardiac function induces degenerative changes of the adult heart. and WT-TG mice (data not shown). In echocardiograms, however, an increase in LV end-systolic dimension and a decrease in %FS was observed in DN-TG mice but not in non-TG or WT-TG mice (Fig. 3).

Histological Analysis—Although there was no significant difference in macroscopic morphology among the three groups, light microscopic analysis revealed that interstitial fibrosis was increased in DN-TG mice compared with non-TG mice and WT-TG mice (Fig. 4A) (percent fibrosis: non-TG mice, 0.75 ± 0.12%; WT-TG mice, 0.42 ± 0.59%; DN-TG mice, 6.71 ± 1.33%; p < 0.01, non-TG mice versus DN-TG mice; p < 0.05, WT-TG mice versus DN-TG mice). In electronmicroscopic analysis, a loss of cardiac myofilaments and an increase in the number of mitochondria were observed in the heart of DN-TG mice but not in the hearts of non-TG mice or WT-TG mice (Fig. 4B). The electron microscopic (EM) scores were higher in DN-TG mice (0.875 ± 0.149) than in non-TG mice (0.062 ± 0.062) and WT-TG mice (0 ± 0) (Fig. 4C). These results suggest that inhibition of Csx/Nkx2-5 function induces degenerative changes of the adult heart.

Effects of DOX on Cardiac Function and Histology—To highlight the effect of loss of Csx/Nkx2-5 on the adult heart, we injected a cardiotoxic agent, DOX, into these mice. After DOX injection, an enlargement of the LV dimension and a decrease of %FS were observed in non-TG mice and DN-TG mice. Depression of %FS was more prominent in DN-TG mice than in non-TG mice. In WT-TG mice, LV dimension and cardiac function were not significantly changed even after administration of DOX (Fig. 3). Furthermore, although there were no histological changes in all groups of mice after DOX treatment in light microscopic analysis (data not shown), electronmicroscopic analysis revealed that DOX induced cytoplasmic vacuolization and myofibrillar loss of cardiomyocytes in both non-TG mice and DN-TG mice, and these ultrastructural changes were more prominent in DN-TG mice than in non-TG mice. In contrast, these ultrastructural changes were barely detectable in the ventricle of WT-TG mice (Fig. 4B). DOX induced an increase of the EM score in non-TG mice (0.489 ± 0.139) and DN-TG mice (1.270 ± 0.104) but not in WT-TG mice (0.116 ± 0.044), and EM scores were increased more in DN-TG mice than in non-TG mice (Fig. 4C). These results suggest that Csx/Nkx2-5 protects the heart from DOX-induced impairment of myocardium.

Induction of Apoptosis by DOX—Apoptosis of cardiac myocytes has been reported to be a cause of cardiac dysfunction (19). We thus examined whether DOX induced apoptotic cell death in the hearts of these mice using TUNEL analysis and anti-active caspase-3 antibody. TUNEL- and active caspase-3-positive cardiomyocytes were barely detectable in the heart of all groups of mice without DOX treatment. DOX increased the number of TUNEL- and active caspase-3-positive cells in non-TG mice and DN-TG mice but not in WT-TG mice, and positive cells were more abundant in DN-TG mice than in non-TG mice (Fig. 5A). These results suggest that Csx/Nkx2-5 inhibits DOX-induced cardiomyocyte apoptosis.

To further clarify the protective role of Csx/Nkx2-5, we trans-
fected the cDNAs of WT Csx/Nkx2-5 and LP mutant into the cultured cardiomyocytes and examined cell death after DOX treatment for 24 h. ~70% of LP mutant-transfected cells were TUNEL-positive, while only ~10% of WT Csx/Nkx2-5-transfected cells were TUNEL-positive (Fig. 5B). Furthermore, when differentiated CL6 cell lines were exposed to DOX for 24 h, the number of surviving cells was much lower in CL6-LP than in CL6-WT. In contrast, the number of surviving cells of CL6-WT was more than that of CL6-(−) (Fig. 5C). These results suggest
that Csx/Nkx2-5 also inhibits DOX-induced cardiomyocyte apoptosis in vitro.

Cardiac Gene Expression—We examined expression of cardiac genes such as ANP, BNP, CARP, and SERCA2. As we reported previously, all of these genes were up-regulated in WT-TG mice (9). There was no significant difference in mRNA levels of these genes between the DN-TG and non-TG mice (Fig. 6A). We next examined mRNA levels of these genes after DOX injection. mRNA levels were all down-regulated by DOX injection in all of these mice (Fig. 6A). It is noteworthy that the suppressed mRNA levels of all these genes in WT-TG mice were comparable to or still higher than basal mRNA levels of these genes in non-TG mice; however, mRNA levels in DN-TG mice were lower than those in non-TG mice after DOX injection (Fig. 6A). These results suggest that DOX specifically inhibits the transcription of cardiac genes and that Csx/Nkx2-5 prevents DOX-induced suppression of gene expression.

Expression of Death-related Proteins—To get insights into the mechanism by which Csx/Nkx2-5 protects cardiomyocytes from DOX, some death-related proteins were examined after DOX injection using Western blot analysis. There was no significant difference in the basal protein levels of an anti-apoptotic protein, Bcl-2, among non-TG, WT-TG, and DN-TG mice. Following DOX injection, Bcl-2 protein levels were increased most markedly in DN-TG mice and moderately in non-TG mice but not changed in WT-TG mice (Fig. 6B). There was no significant difference in protein levels of Fas/ligand in all of these mice before or after DOX injection (Fig. 6B).

DISCUSSION

In the present study, we examined the role of Csx/Nkx2-5 in the adult heart using two transgenic mice that express WT Csx/Nkx2-5 and Csx/Nkx2-5 LP mutant. We obtained the following results: (i) DN-TG mice showed impaired cardiac function and cardiomyocyte degeneration; (ii) DOX induced impairment of cardiac function and loss of myofilaments in DN-TG and non-TG mice, and the degree was more prominent in DN-TG mice than in non-TG mice; and (iii) DOX-induced cardiomyocyte apoptosis was enhanced by overexpression of LP mutant and suppressed by overexpression of WT Csx/Nkx2-5 in vivo and in vitro.

It has been reported that LP mutant has dominant negative effects (13). Luciferase assay revealed that the LP mutant used in our experiments inhibited WT Csx/Nkx2-5-induced activation of the ANP promoter in a dose-dependent manner. EMSA revealed that the DNA binding of WT Csx/Nkx2-5 was mildly but significantly inhibited by the LP mutant. These results suggest that a part of the dominant negative effects of the LP mutant is to inhibit the ability of the WT Csx/Nkx2-5 to bind DNA. Our and other groups have reported that Csx/Nkx2-5 and GATA-4 display synergistic transactivation of the ANP promoter (16, 18). Luciferase assay revealed that the LP mutant inhibited the synergistic activation of ANP by WT Csx/Nkx2-5 and GATA-4, and immunoprecipitation assay showed that the LP mutant interacted with WT Csx/Nkx2-5 and GATA-4. Previous studies indicated that Csx/Nkx2-5 and GATA-4 synergistic action required the interaction of the two factors (16). Since the LP mutant partially inhibited the DNA binding of Csx/Nkx2-5, there should be other mechanisms by which the LP mutant inhibits the function of WT Csx/Nkx2-5. A possible mechanism of dominant negative effects of the LP mutant is consumption of Csx/Nkx2-5-associated proteins including GATA-4.

In Xenopus, injection of RNA of the LP mutant of Csx/Nkx2-5 suppressed normal heart formation (13). In our present study, the CL6 cells that express the LP mutant did not well differentiate into cardiomyocytes. Following these observations and
results, we generated the transgenic mice that overexpress the dominant negative mutant of Csx/Nkx2-5 to clarify the role of Csx/Nkx2-5 in the adult heart. Unlike the previous reports showing that Csx/Nkx2-5 mutations cause human congenital heart diseases and atrioventricular conduction delay (4, 5), the DN-TG mice had no congenital heart diseases. The later expression of the LP mutant, which is driven by the o-MHC promoter, may be a cause of the lack of congenital heart diseases and atrioventricular conduction delay in the transgenic mice.

DN-TG mice showed impaired contractile function and some histological abnormalities. These results suggest that inhibition of Csx/Nkx2-5 function impairs the integrity of highly differentiated cardiomyocytes, which may lead to cardiac dysfunction. Injection of Csx/Nkx2-5 mRNA into oocytes of Xenopus and Zebrafish induces enlargement of hearts and ectopic hearts, respectively (20, 21). We have reported that transgenic mice that overexpress Csx/Nkx2-5 show up-regulation of some cardiac genes including ANP, BNP, and CARP (9). These results suggest that Csx/Nkx2-5 functions as a transcriptional regulator in adult hearts as well as in embryonic hearts.

DOX has been known to have severe cardiotoxic effects. After DOX injection, cardiac function of DN-TG mice was markedly impaired, while WT-TG mice showed only slight impairment of cardiac function. Ultrastructural abnormalities induced by DOX were also more prominent in DN-TG mice than in WT-TG mice.

**FIG. 4.** Histological analysis. A, light microscopic analysis. Histological analysis was performed on hearts from non-TG (top row), WT-TG (middle row), and DN-TG (bottom row) 12-week-old mice. Myofiber alignment was normal (middle column), but interstitial fibrosis (red, right column) was prominent in DN-TG mice. B, electron microscopic analysis before and after DOX injection. There was no significant difference between WT-TG and non-TG mice, but a loss of myofilaments and an increase in the mitochondria were observed in the ventricle of DN-TG mice. The injection of DOX (20 mg/kg) induced the cytoplasmic vacuolization and the myofibrillar loss of cardiac myocytes (arrow) in both non-TG mice and DN-TG mice, and these changes were more prominent in DN-TG mice. In contrast, there were few morphological changes in WT-TG mice even with DOX treatment. Bar = 1 μm. C, EM score. The EM scores were higher in DN-TG mice than in non-TG and WT-TG mice without DOX treatment. DOX induced an increase of EM score in non-TG mice and DN-TG mice but not in WT-TG mice, and EM scores were increased more in DN-TG mice than in non-TG mice. *, p < 0.01; †, p < 0.05. H.E., hematoxylin-eosin.
mice. These results indicate that Csx/Nkx2-5 protects hearts from the cardiotoxic effects of DOX. Because we have previously demonstrated that DOX induces apoptosis in cultured cardiomyocytes (22), we further examined cardiomyocyte apoptosis in these mice after DOX injection. TUNEL and immunohistochemical analysis using anti-active caspase-3 antibody in multiple sections at 24 h after injection of DOX. TUNEL-positive cells and active caspase-3-positive cells were counted out of 10,000 cells. There were few positive cells among the three groups before DOX injection. DOX significantly increased the number of apoptotic cardiomyocytes in non-TG mice and DN-TG mice but not in WT-TG mice. The positive cells were more abundant in DN-TG mice than in non-TG mice. *, p < 0.01. B, after transfection with Myc-tagged WT Csx/Nkx2-5 and LP mutant, cardiomyocytes were stimulated by 1 μM DOX for 24 h and stained with TUNEL (green) and anti-Myc antibody (red). (−), empty vector; WT, WT Csx/Nkx2-5; LP, LP mutant. C, the CL6 cell lines were treated with 1 μM DOX for 24 h in the absence of serum. The number of surviving cells was lower in CL6-LP than in CL6(−). In contrast, the number of surviving cells of CL6-WT was more than that of CL6(−).

Fig. 5. Apoptosis. A, DNA fragmentation was analyzed in situ with the TUNEL method, and activation of an apoptosis-related protein was analyzed with immunohistochemistry using anti-active caspase-3 antibody in multiple sections at 24 h after injection of DOX. TUNEL-positive cells and active caspase-3-positive cells were counted out of 10,000 cells. There were few positive cells among the three groups before DOX injection. DOX significantly increased the number of apoptotic cardiomyocytes in non-TG mice and DN-TG mice but not in WT-TG mice. The positive cells were more abundant in DN-TG mice than in non-TG mice. *, p < 0.01. B, after transfection with Myc-tagged WT Csx/Nkx2-5 and LP mutant, cardiomyocytes were stimulated by 1 μM DOX for 24 h and stained with TUNEL (green) and anti-Myc antibody (red). (−), empty vector; WT, WT Csx/Nkx2-5; LP, LP mutant. C, the CL6 cell lines were treated with 1 μM DOX for 24 h in the absence of serum. The number of surviving cells was lower in CL6-LP than in CL6(−). In contrast, the number of surviving cells of CL6-WT was more than that of CL6(−).

cardiac dysfunction, even a small rate of death might have eventually led to a cause of cardiac dysfunction over a longer time in this study. Several studies have also suggested the interaction of DOX with myofibrillar proteins as an etiology of DOX cardiotoxicity (23–25). Similarly, in the present study, the myofibrillar structure was shown to be damaged in a very early stage.

DOX induced expression of Bcl-2 in the three kinds of mice with different levels. The different expression levels of Bcl-2 after DOX injection among the three kinds of mice may reflect the different degrees of cardiac impairments, which may be dependent on the different expression of cardiac genes after
DOX injection. It has been reported that ANP and BNP are sensitive markers of cardiac impairments induced by DOX (26). In this study, DOX suppressed the transcription of ANP, BNP, CARP, and SERCA2 in non-TG mice. The inhibition was more prominent in DN-TG mice. In WT-TG mice, although mRNA levels of these genes were also down-regulated, the levels were comparable to or still higher than basal levels of these genes in non-TG mice. A previous study also demonstrated that transcription of cardiac genes is suppressed rapidly and selectively by DOX (25). In skeletal muscle, the inhibition of gene transcription by DOX has been linked to a reduction of transcription factor MyoD activity (27), suggesting that the transcriptional repression of many cardiac-specific genes by DOX may be due to the reduced activity of cardiac transcription factors. Recently it has been reported that cardiac transcription factors such as Csx/Nkx2-5, myocyte enhancer factor-2C, and dHAND were down-regulated by exposure of cultured cardiomyocytes to DOX (12). These observations and results suggest that Csx/Nkx2-5 protects heart from DOX through controlling transcriptional homeostasis of cardiac-specific genes. Further studies are necessary to elucidate whether overexpression of Csx/Nkx2-5 generally protects the heart from various stresses.

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