High expression levels and localization of Sox5 in dilated cardiomyopathy

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Abstract. Dilated cardiomyopathy (DCM) is a disease that can lead to heart expansion and severe heart failure, but the specific pathogenesis remains unclear. Sox5 is a member of the Sox family with a key role in cardiac function. However, the role of Sox5 in DCM remains unclear. In the present study, wild-type mice were intraperitoneally injected with doxorubicin (Dox) to induce DCM, and heart specimens from human patients with DCM were used to investigate the preliminary role of Sox5 in DCM. The present study demonstrated that, compared with control human hearts, the hearts of patients with DCM exhibited high expression levels of Sox5 and activation of the wnt/β-catenin pathway. This result was consistent with Dox-induced DCM in mice. Furthermore, in Dox-treated mice, apoptosis was activated during the development of DCM. Inflammation and collagen deposition also increased in DCM mice. The results of the present study indicate that Sox5 may be associated with the development of DCM. Sox5 may be a novel potential factor that regulates DCM.

Introduction

Dilated cardiomyopathy (DCM) is characterized by left ventricular dilation, which is associated with systolic and diastolic dysfunction (1). Schultheiss *et al* (2) reported that the prevalence of DCM in Minnesota, USA, is estimated at 1 case per 250 people. In 2010, the estimated mortality rate associated with cardiomyopathy was 5.9 deaths per 100,000 global population (2). The mechanism of DCM is complex, and there is evidence to indicate that inflammatory reactions and cardiac remodeling play a key role in compensatory repair following heart failure (3,4). Furthermore, cardiomyocyte apoptosis occurs during the inflammatory process in early heart failure and compensatory ventricular remodeling (5-9). Doxorubicin (Dox) is an anthracycline derivative that is an effective treatment for a number of soft and solid types of human malignancy. However, a number of studies have used doxorubicin to induce DCM in mice (10,11).

Sox5, a member of the Sox family of transcription factors, has a key function in the regulation of embryonic development and determination of cell fate (12). Previous studies have demonstrated that Sox5 can promote cell proliferation in gastric and lung cancer, as well as glioma and breast tumors (13-16). In addition, Sox5 has also been demonstrated to regulate cartilage formation (17). Previous studies have demonstrated that Sox5 is associated with the electrocardiographic PR interval, a higher resting heart rate, atrial fibrillation and left ventricular mass (18-21). These results indicate that Sox5 may play a key role in cardiac function (22). However, to the best of our knowledge, the role of Sox5 in DCM has not previously been identified.

In the present study, wild-type mice were intraperitoneally injected with Dox to induce DCM, and heart specimens from human patients with DCM were used to investigate the role of Sox5 in DCM. Furthermore, these results indicate that Sox5 is upregulated in DCM and may be involved in the progression of DCM by modulating wnt-1/β-catenin signaling. To our best knowledge, the present study provides the first evidence for an association between Sox5 expression levels and DCM. This may provide a new target for interventions in patients with DCM.

Materials and methods

Human tissue. The present study was performed at the Department of Thoracic and Cardiovascular Surgery, Nanjing First Hospital, Nanjing Medical University (Nanjing, China). Left ventricular tissues of heart transplant recipients with DCM were collected, and mismatched left ventricular tissues were collected from donors as the normal control group from the Nanjing first hospital from 2013 to 2019 (normal: Male: Female, 2:1, Age, 51±2 years; DCM: Male: Female, 1:2, age, 60±5 years). Patients with rheumatic heart disease, infectious endocarditis, inflammatory disease, underlying genetic syndromes and other causes of DCM were excluded. The left ventricular tissues were cut into two pieces (section thickness, 4 mm): One was fixed in 4% formalin at room temperature for

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12 h and embedded in paraffin, and the other was frozen in liquid nitrogen at -196˚C. The protocols of all human studies were approved by the Ethics Committee of Nanjing First Hospital and were performed in accordance with the relevant guidelines and regulations. All patients provided informed written consent. The study was performed according to the Declaration of Helsinki (2000).

Animals and experimental protocols. C57BL/6 mice (male; 8 weeks old; 22-27 g; n=12) were obtained from the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China). Animals received humane care and the mice experiments in the present study were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (approval no. SYXK2016-0006). The mice were randomly assigned to two groups (n=6): Sham or DCM group and kept in pathogen-free conditions with a 12/12 h light/dark cycle, 25 ˚C, with *ad libitum* access to food and water. In the DCM group, each mouse was injected with a cumulative dose of 25 mg/kg doxorubicin (Sigma-Aldrich; Merck KGaA) via five intraperitoneal (i.p.) injections (5 mg/kg i.p.) over 30 days at regular intervals. The sham group received the same volume of sterile isotonic saline at the same time points. All mice were sacrificed under anesthesia, and the hearts were immediately harvested.

Echocardiographic evaluation. Mice were anesthetized using 1.5-2.0% isoflurane by inhalation, and echocardiography was performed using a Vevo2100 (VisualSonics, Inc.) ultrasound with a 30 MHz linear array ultrasound transducer. The left ventricular internal diameter in diastole (LVIDd), left ventricular internal diameter in systole (LVIDs), left ventricular ejection fraction (EF) and fractional shortening (FS) were measured from M-mode tracings with a sweep speed of 50 mm/s at the mid-papillary muscle level. The systole and diastole phases corresponded to the smallest and largest LV diameters, respectively. Echocardiographic measurements were taken in triplicate in M-mode from >3 individual mice per group.

Hematoxylin-eosin (HE) and Masson's staining. Mouse hearts were removed, immediately immersed in 4% neutral phosphate-buffered paraformaldehyde for 12 h at room temperature, embedded in paraffin, and sectioned (4 μm-thick). Sections were stained with HE or Masson's trichrome at the room temperature for 5 min and were observed to identify morphological changes and fibrosis in the myocardium under a light microscope.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from mouse heart tissues using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Equal quantities of RNA (1 μg) were transcribed into cDNA using the PrimeScript™ RT Reagent kit with gDNA Eraser (Takara). Quantitative TaqMan PCR assays were performed with SYBR Premix Ex TaqTM II (Takara) using the Applied Biosystems 7500 Real Time PCR System. The thermocycling conditions were as follows: 95˚C for 15 min, followed by 40 cycles (94˚C for 15 sec; 60˚C for 32 sec; 72˚C for 60 sec). All data were normalized to the level of GAPDH and are expressed as the fold increase relative to expression levels in a sham control mouse. Primers used for quantitative real-time PCR were as follows: Atriopeptin (ANP): Forward, 5'-AAGAACCTGCTAGACCACCTGGAG-3' and reverse, 5'-TGCTTCCTCAGTCTGCTCACTCAG-3'; brain natriuretic peptide (BNP): Forward, 5'-GGAAGTCCTAGCCAGTCT CCAGAG-3' and reverse, 5'-GCCTTGGTCCTTCAAGAG CTGTC-3'; collagen 1: Forward, 5'-TGGTCCTGCTGGTCC TGCTG-3' and reverse, 5'-CTGTCACCTTGTTCGCCTGTC TC-3'; Collagen 3: Forward, 5'-TCTCCTGGTGCTGCTGGT CAC-3' and reverse, 5'-TCCATGTGGTCCAACTGGTCC TC-3'; TNF-α: Forward, 5'-ACGGCATGGATCTCAAAG AC-3' and reverse, 5'-AGATAGCAAATCGGCTGACG-3'; TGF-β1: Forward, 5'-GCAACAATTCCTGGCGTTACC TTG-3' and reverse, 5'-CAGCCACTGCCGTACAACTCC-3'; IL-6: Forward, 5'-ACAACGATGATGCACTTGCAGA-3' and reverse, 5'-GATGAATTGGATGGTCTTGGTC-3'; and IL-10: Forward, 5'-GCTCTTACTGACTGGCATGAG-3' and reverse, 5'-CGCAGCTCTAGGAGCATGTG-3'.

Immunohistochemical (IHC) staining. Human and mouse heart tissues collected for morphological analysis were prepared as 4-μm-thick serial paraffin-embedded sections and rehydrated in graded alcohol for 1 h at room temperature. The sections were treated with 3% hydrogen peroxide for 15 min at 37˚C to block endogenous peroxidase activity and incubated in goat serum at room temperature for 1.5 h (OriGene Technologies, Inc.) to prevent non-specific binding of antibodies. The sections were then incubated separately for 14 h at 4˚C with antibodies against Sox5 (anti-human,1:100; cat. no. 26041; Abcam; anti-mouse,1:100; cat. no. 13216-1-AP; Proteintech) and wnt-1 (1:100; cat. no. 15251; Abcam) and then with goat anti-rabbit IgG (cat. no. KIT-5004; MXB) for 1 h at 37˚C in a humidified box. The signal of each antibody was developed using diaminobenzidine (DAB; OriGene Technologies, Inc.) substrate. The sections were counterstained with hematoxylin at room temperature for 6 min, and images were captured using a ZEISS microscope with an A1 camera. The IHC results were evaluated based on the Fromowitz semi-quantitative analysis score, which evaluated the brown chromogen intensity (range, 0-7). The average score of each slice, as determined by two independent observers, was used for later comparisons.

TUNEL staining. Frozen mice ventricular tissues embedded in optical cutting temperature compound were cut into 4 μm-thick sections and fixed in 4% paraformaldehyde at room temperature for 16 min. The TUNEL assay was performed according to the instructions of the *in situ* apoptosis detection kit (Roche Diagnostics (Shanghai) Co., Ltd.) and examined using a fluorescence microscope. Images were captured using Olympus BX-51 light microscope (magnification, x200). Only nuclei that were clearly located in cardiac myocytes were considered. The apoptotic index was calculated as the percentage of the number of TUNEL-positively stained nuclei to the number of DAPI- stained nuclei from 8 random fields.

Western blotting analysis. Total protein samples (30 μg) were extracted from left ventricular tissues and separated by 5% SDS-PAGE. The proteins were transferred to PVDF membranes (EMD Millipore), which were washed twice

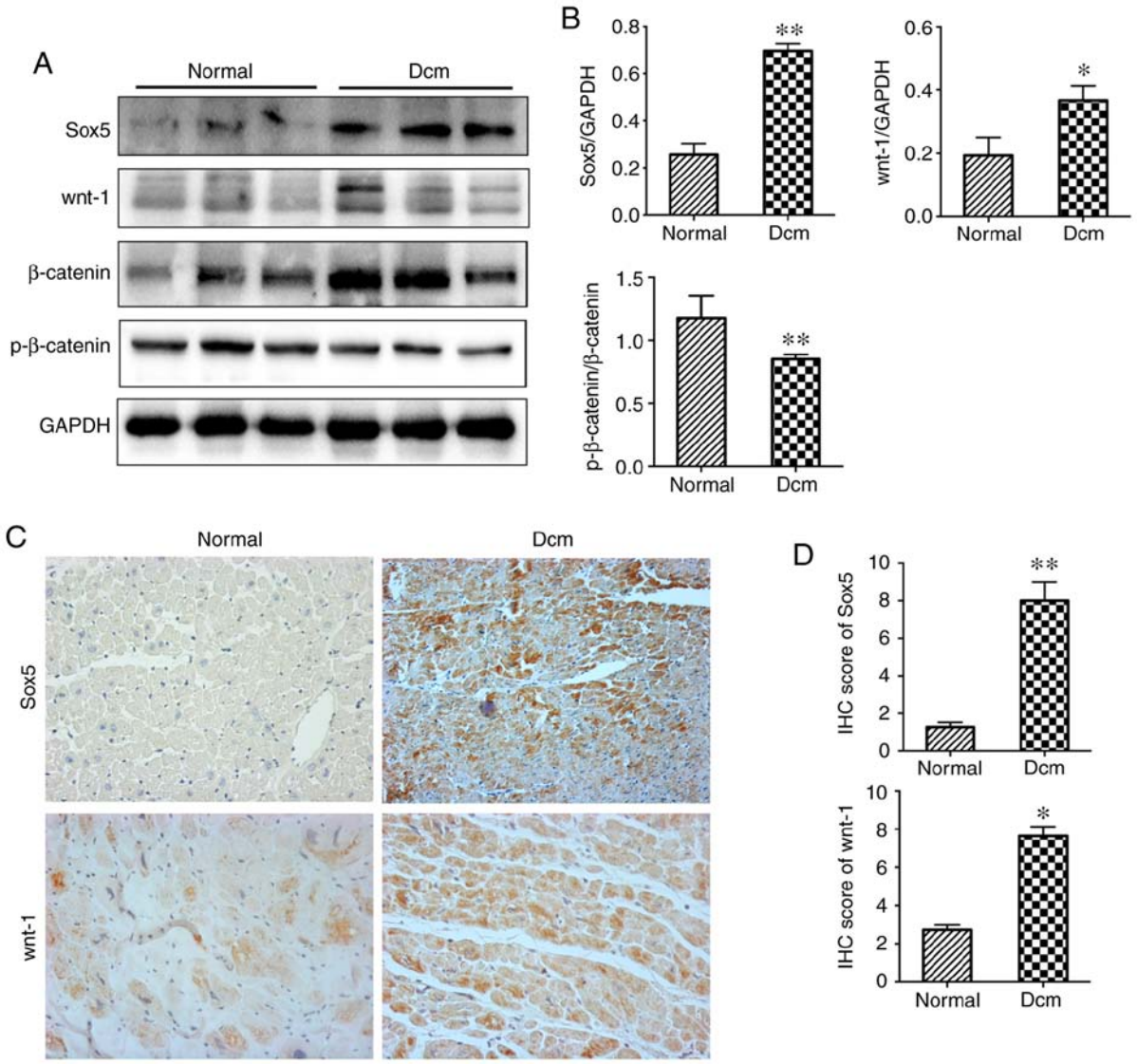

Figure 1. High expression levels of Sox5 and activation of the wnt/β-catenin pathway in the hearts of patients with DCM. (A) Representative images of western blots of Sox5 and wnt/β-catenin pathway-associated proteins (magnification, x200) in human hearts. (B) Quantitative analysis of western blots. (C) Representative images of IHC staining of Sox5 and wnt-1 (magnification, x400). (D) Quantitative analysis of IHC staining. n=6. *P<0.05; **P<0.01 vs. normal control. DCM, dilated cardiomyopathy; IHC, immunohistochemistry; WT, wild type; Dox, doxorubicin; p, phosphorylated.

in TBS with Tween-20 (1:1,000; Promega Corporation) for 10 min each and blocked with TBST containing 5% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at 4˚C. The membranes were incubated with the following primary antibodies in TBST with Tween plus 5% BSA overnight at 4˚C: Anti-sox5 (anti-human, 1:100; cat. no. 26041; Abcam; anti-mouse, 1:100; cat. no. 13216-1-AP; ProteinTech Group, Inc.)), anti-wnt-1 (1:1,000; cat. no. 15251; Abcam), anti-β-catenin [1:1,000; cat. no. 8480s; Cell Signaling Technology, Inc. (CST)], anti-phosphorylated β-catenin (1:1,000; cat. no. 4176s; CST), anti-bax (1:1,000; cat. no. 2772s; CST), anti-cleaved-caspase3 (1:1,000; cat. no. 9661s; CST), anti-caspase 9 (1:1,000; cat. no. 9508T, CST) and horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-GAPDH (1:5,000; cat. no. KC-5G5; Kangchen BioTech Co., Ltd.).. The PVDF membranes were washed with TBST (Tween-20, 1:1,000; Promega Corporation) three times for 10 min each. Subsequently, the PVDF membranes were incubated with goat anti-mouse IgG/HRP (1:5,000, cat. no. bs-0296G-HRP; BIOSS), goat anti-rabbit IgG or an HRP-conjugated antibody (1:5,000; cat. no. 7074P2; Cell Signaling Technology, Inc.) at room temperature for 1 h. Specific proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) and images were captured using a Chemi Scope system (3300 Mini; Clinx Science Instruments Co., Ltd.). The results were analyzed using Chemi Analysis Software (Clinx Science Instruments Co., Ltd.) by semi-quantifying the mean gray value of each blot. All results are representative of at least three independent experiments.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Differences among groups were assessed using analysis of variance followed by Tukey's post-hoc test. Comparisons between two groups were performed using paired Student's t test. All statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

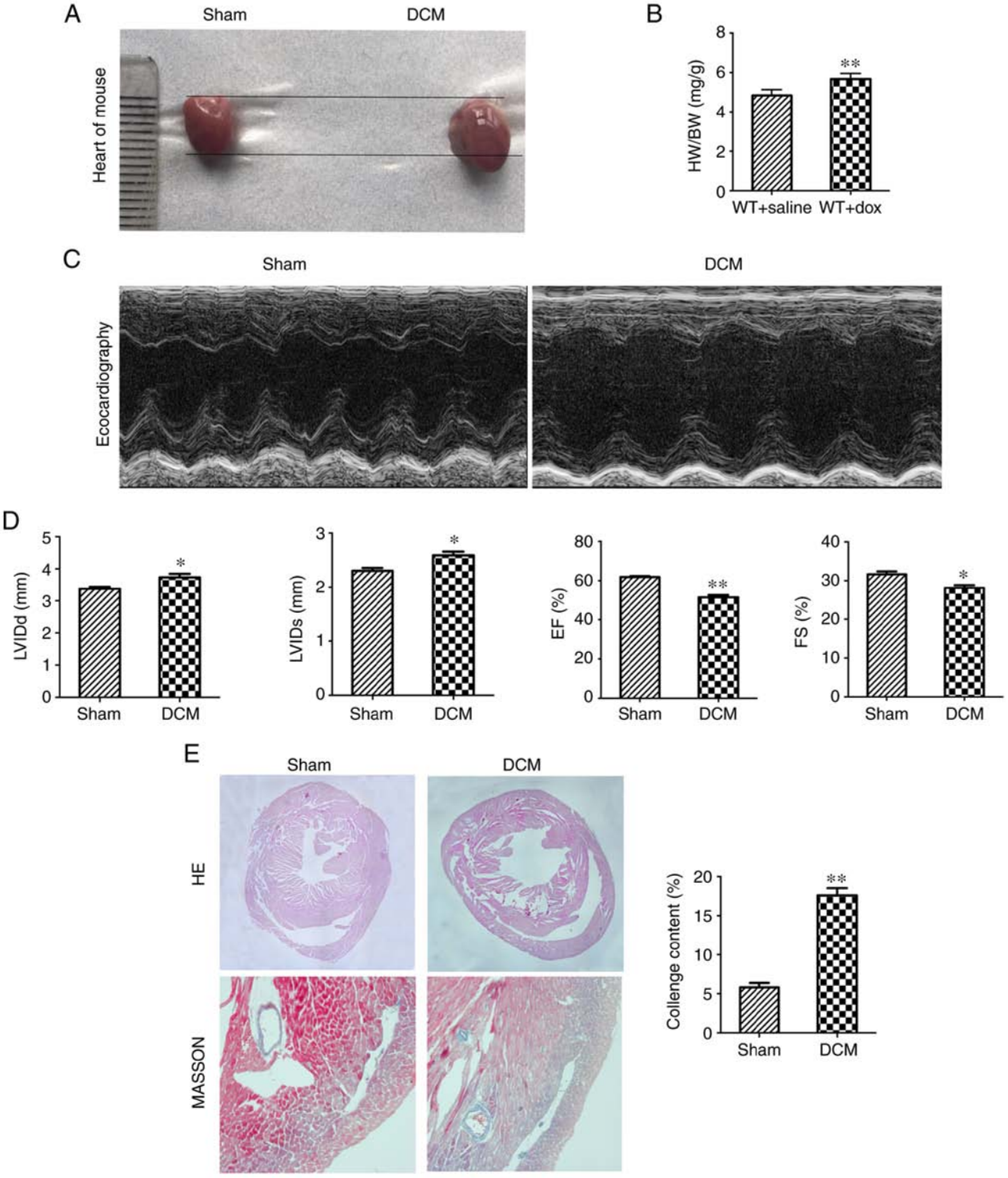

Figure 2. Dox-induced cardiac dilatation and left ventricular dysfunction in mice. (A) Representative images of the hearts of sham and DCM mice. (B) Analysis of heart/body weight. (C and D) Representative echocardiographs and echocardiographic parameters of WT mice following Dox treatment. (E) Representative images of HE and Masson's staining of tissues and analysis of collagen content. n=6. *P<0.05, **P<0.01 vs. sham group. Dox, doxorubicin; DCM, dilated cardiomyopathy; WT, wild type; HE, hematoxylin-eosin; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; EF, ejection fraction; FS, fractional shortening.

Result

Expression levels of Sox5 and activation of the wnt/β-catenin pathway in the hearts of patients with DCM. Myocardial tissue samples were selected from five patients with DCM. The mismatched left ventricular tissues of donors were collected as the normal control group. Western blotting analysis demonstrated that the expression level of Sox5 was increased in DCM heart tissue compared with the normal control heart tissue (Fig. 1A). Images of IHC staining demonstrated that the expression level (Fig. 1C) and localization of Sox5 was the same in cardiomyocytes in samples from both groups. The present

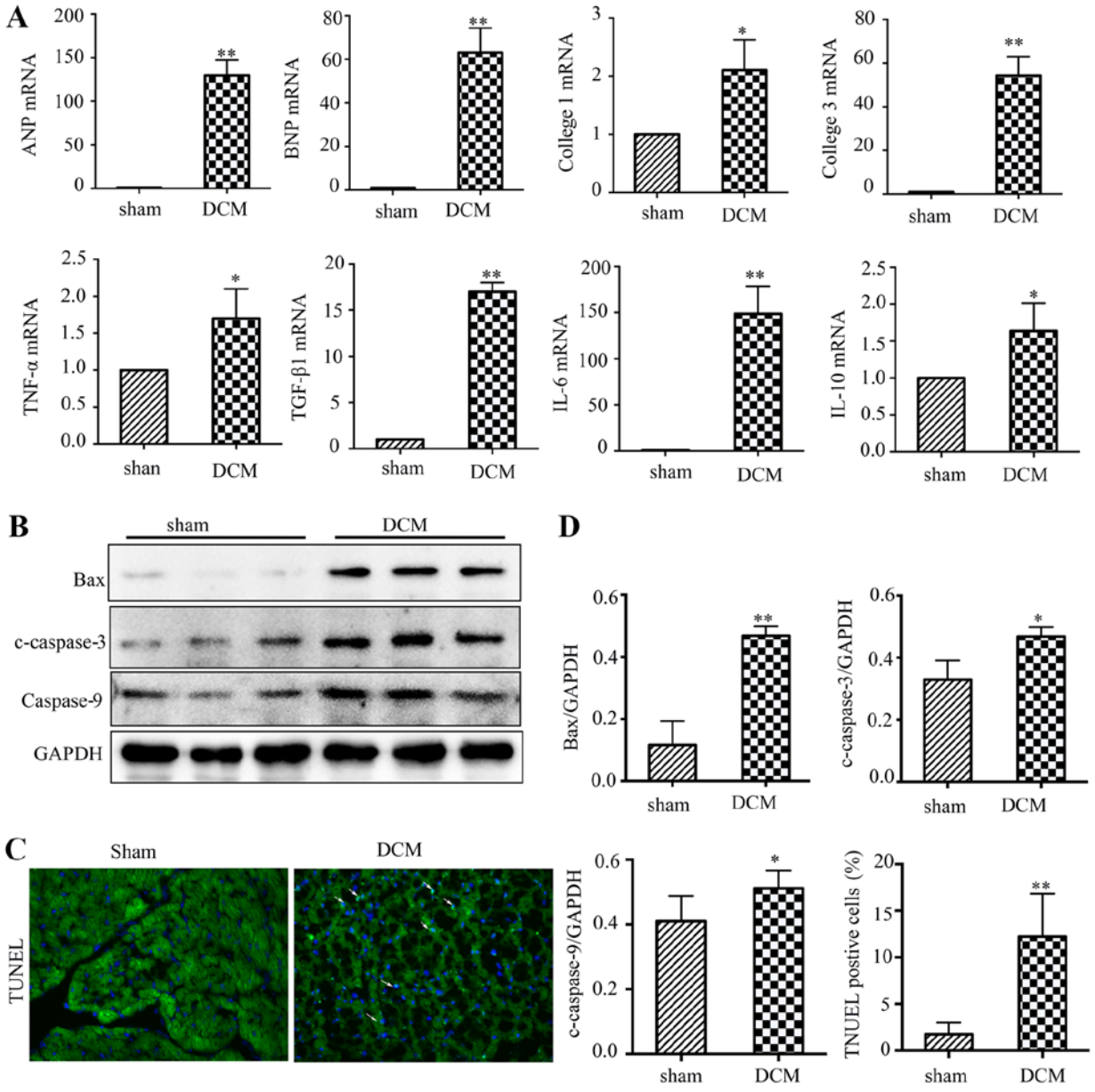

Figure 3. Inflammatory reactions, fibrosis and apoptosis are activated in the hearts of Dox-treated mice. (A) The relative mRNA levels of heart failure markers (ANP and BNP), fibrosis markers (collagen 1 and collagen 3), pro-inflammatory factors (IL-6 and TNF-α) and anti-inflammatory factors (TGF-β and IL-10). (B) Representative western blotting of Bax, c-caspase3 and caspase9 expression levels in heart tissues from WT mice following drug injection. (C) Representative images of TUNEL staining (magnification, x200) of heart tissues from WT mice following drug injection. (D) Quantitative analyses of western blots and TUNEL staining. (n=6). *P<0.05; **P<0.01 vs. sham group. Dox, doxorubicin; ANP, atriopeptin; BNP, brain natriuretic peptide; WT, wild type; DCM, dilated cardiomyopathy.

study also demonstrated that the expression levels of wnt-1 and β-catenin were increased in DCM hearts, while the expression level of p-β-catenin was decreased, as determined by western blotting (Fig. 1A). IHC demonstrated that the expression levels of wnt-1 were the same in both groups (Fig. 1C). The results demonstrated that Sox5 and wnt/β-catenin pathway-associated proteins are highly expressed in DCM.

Dox-induced cardiac dilatation and left ventricular dysfunction in mice. In order to further investigate the role of Sox5 in DCM, a mouse model of Dox-induced DCM was used. Gross anatomical analysis demonstrated that the hearts of mice treated with Dox were larger than those of mice treated with saline (Fig. 2A). Furthermore, the heart-to-body weight ratio of the sham group was lower than that of the DCM group (Fig. 2B). The EF and FS were notably decreased in the DCM group compared with the sham group; in contrast, the LVIDd and LVIDs were notably increased in the DCM group (Fig. 2C and D). HE staining of the hearts demonstrated that the sham group exhibited an increased myocardial thickness and decreased cardiac chamber diameter compared with those of the DCM group. Masson's trichrome staining demonstrated that the collagen content was notably elevated in the DCM group (Fig. 2E). These results demonstrated that mice treated with Dox exhibited pathological changes associated with DCM.

Inflammatory reactions, fibrosis and apoptosis were increased in the hearts of Dox-treated mice. DCM is associated with

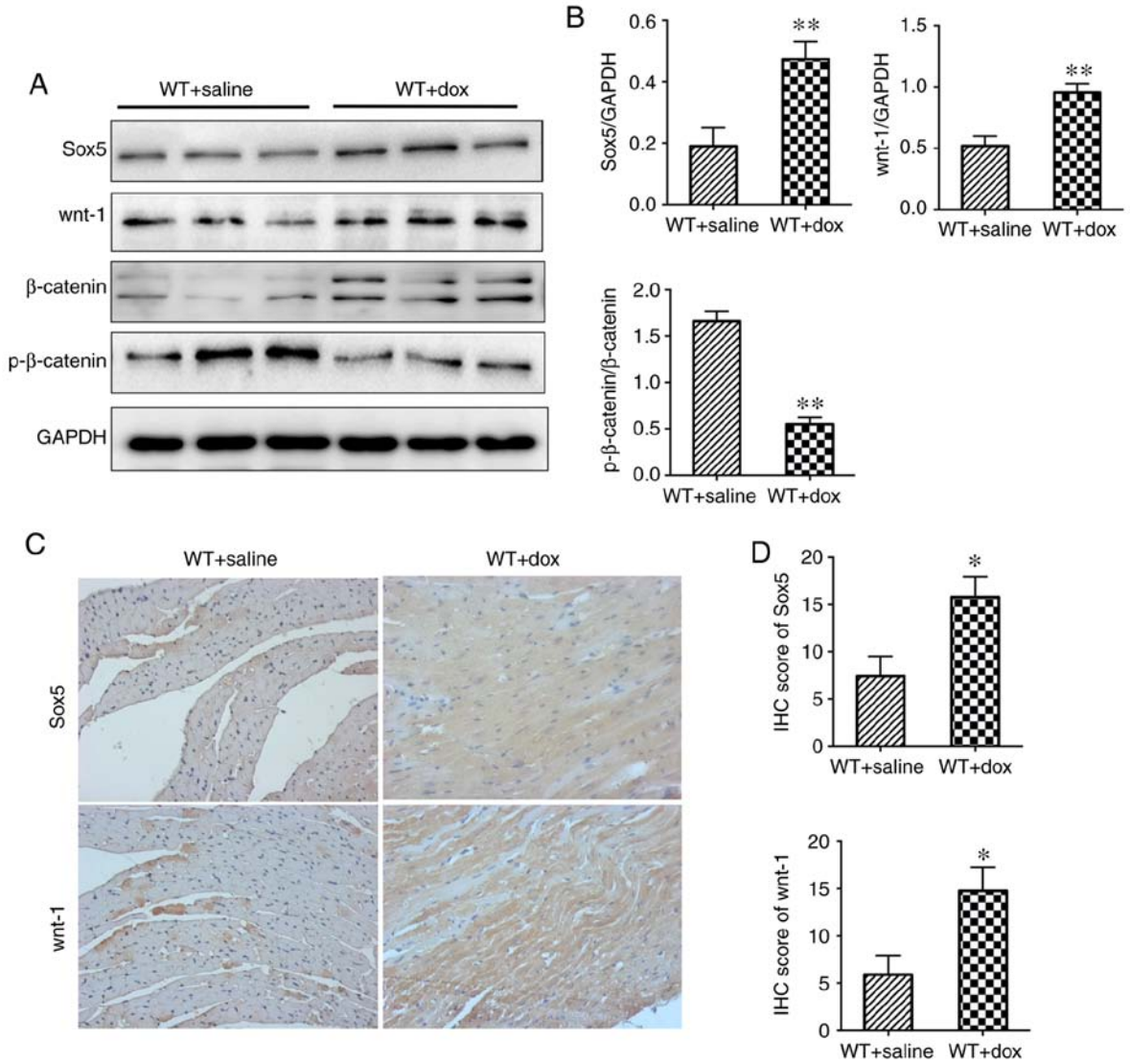

Figure 4. Increased expression levels of Sox5 and activation of the wnt/β-catenin pathway in hearts of Dox-induced mice. (A) Representative images of the western blotting of Sox5 and wnt/β-catenin pathway-associated proteins. (B) Quantitative analysis of western blots. (C) Representative images of IHC staining of Sox5 and wnt-1 (magnification, x400) in heart tissues from WT mice following drug injection. (D) Quantitative analysis of IHC staining. n=6. *P<0.05; **P<0.01 vs. sham group. Dox, doxorubicin; IHC, immunohistochemistry; WT, wild type.

acute myocardial inflammation and heart failure (1). RT-qPCR analysis of heart tissues demonstrated a notable increase in heart failure markers (ANP and BNP), pro-inflammatory factors (IL-6 and TNF-α) and anti-inflammatory factors (TGF-β and IL-10) in the DCM group compared with the sham group. The present study also found that the mRNA levels of fibrosis markers (collagen 1 and 3) were increased in the DCM group (Fig. 3A), which was consistent with the results of Masson's trichrome staining. In addition, the present study tested the expression levels of apoptosis-associated proteins in mouse heart tissues. The western blot results showed that the expression levels of pro-apoptotic genes, including Bax, cleaved-caspase3 and caspase 9, were increased in the DCM group. Furthermore, the number of TUNEL-positive nuclei was notably increased in the DCM group (Fig. 3B-D).

Increased expression levels of Sox5 and activation of the wnt/β-catenin pathway in the hearts of Dox-treated mice. High expression levels of Sox5 and activation of the wnt/β-catenin pathway have been reported in the hearts of human patients with DCM (Fig. 1). Western blotting and IHC analysis demonstrated increased expression levels of Sox5 and wnt-1 in the DCM group compared with the sham group (Fig. 4A and C). In addition, increased expression levels of β-catenin and decreased expression levels of p-β-catenin were exhibited in the DCM group (Fig. 4A). In conclusion, Sox5 is upregulated in DCM and may be involved in the development of DCM via modulation of Wnt-1/β-catenin signaling.

Discussion

DCM is a type of heart failure characterized by ventricular dilatation. In the absence of hypertension, coronary artery or valvular disease, DCM is the most significant and third most common cause of heart failure and treatment requires heart transplantation (23). However, the pathogenesis of DCM is not clear. Sox5 can downregulate the cyclin D1/cyclin-dependent kinase 4 complex to regulate cell

proliferation and apoptosis (24,25). It can also upregulate the protein expression levels of N-cadherin, vimentin and fibronectin to promote fibrosis (17). Sox5 is expressed in numerous human tissues, including the testis, liver, lung, fetal brain and heart (26). Liu *et al* (27) demonstrated that overexpression of Sox5 can promote inflammatory responses and reverse microRNA-193a-3p mimic-mediated decrease in chondrocyte apoptosis in human osteoarthritis. However, studies have demonstrated that inhibiting Sox5 can promote apoptosis in rheumatoid arthritis and lung cancer (24,25). Axelsson *et al* (28) demonstrated that Sox5 protein is present both in the nucleus and in the cytosol of pancreatic sections. Stolt *et al* (29) demonstrated that the transcription factor Sox5 modulates Sox10 function during melanocyte development and that Sox5 is expressed in the nuclei of murine melanocytes. In the present study, Sox5 was highly expressed in DCM myocardial tissue compared with normal myocardial tissue, and histological manifestations, such as severe inflammatory responses, collagen deposition and apoptosis, were increased in DCM. Therefore, it was hypothesized that Sox5 may be a protective factor in DCM, and its specific role will be further studied in Sox5-knockout mice. Previous studies have indicated that Sox5 plays a key role in heart function (18-21). In the present study, western blotting analysis demonstrated a notable increase in the expression level of Sox5 in the hearts of patients with DCM; this was confirmed via IHC. To the best of our knowledge, these results are the first to demonstrate that Sox5 expression level is associated with DCM.

Dox is associated with dose-dependent cardiotoxicity, which can progress to heart failure. Administration of Dox at doses >1 mg/kg results in a decreased survival rate and classic signs of DCM (30). In order to study the effect of Sox5 on dilated cardiomyopathy *in vivo*, the present study constructed a mouse model of DCM via i.p. injection of Dox into wild-type mice. In the present study, the hearts of mice treated with Dox were larger, and the heart-to-body weight ratio of the DCM group was higher than that of the sham group. The body weight of mice treated with Dox was significantly decreased compared with that of mice treated with saline. In addition, following Dox treatment, the mice became thin and responded poorly. Furthermore, the mRNA levels of heart failure markers (ANP and BNP) were significantly increased, as determined by PCR, which indicated that mice treated with Dox experienced heart failure. Moreover, the Dox-treated mice exhibited pathological manifestations of DCM. HE and Masson's trichrome staining demonstrated that Dox-treated mice exhibited dilated ventricles, disordered arrangement of cardiac cells, collagen deposition and fibrosis. The echocardiography results indicated that Dox-treated mice exhibited an increased ventricular inner diameter, a decreased ejection fraction and heart failure consistent with DCM.

Inflammation is involved in the pathogenesis of DCM. DCM is associated with inflammation, as documented by increased levels of inflammatory cytokines, such as IL-6, IL-10 and TNF-α (31). PCR analysis of the expression levels of pro-inflammatory factors in the DCM group were consistent with this. Collagen deposition and ventricular remodeling, which maintain the function of the heart, are important during the progression of DCM (32,33). Consistent with the Masson's trichrome staining results, RT-qPCR demonstrated that the expression levels of collagen (collagen 1 and collagen 3) were increased in the DCM group compared with the sham group. Apoptosis is a form of programmed cell death controlled by numerous genes that maintains the stability of the internal environment (34). Previous studies have demonstrated that cell apoptosis promotes the progression of Dox-induced DCM and may be the molecular mechanism underlying DCM (35). The cysteine aspartate protein (caspase) family participates in the regulation of cell apoptosis. Caspase-9, which is an initiator of apoptosis, triggers an executioner caspase (caspase 3 or 7), ensuring continuity of the process (34). In the present study, TUNEL staining and analysis of Bax, cleaved-caspase3 and caspase9 expression levels using western blotting demonstrated a notable increase in apoptosis in the DCM group compared with the sham group.

Wnt proteins are a family of secreted cysteine-rich glycoproteins involved in a number of cellular processes, including proliferation, differentiation, senescence and apoptosis. The wnt/β-catenin pathway has been demonstrated to play a role in DCM (36). In previous studies, Sox5 has been demonstrated to regulate the wnt/β-catenin pathway (37,38). β-catenin is the key effector of the wnt/β-catenin pathway and functions as a transcriptional co-activator that is critical for target gene expression level. In the absence of wnt ligands, β-catenin is bound by the scaffold protein Axin, which facilitates its phosphorylation by glycogen synthase kinase 3-b via a destruction complex. When wnt ligands bind to the Frizzled and low-density lipoprotein receptor 5/6 complex, the β-catenin destruction complex becomes dysfunctional (37,38). However, the mechanism of this process is not fully understood. When the wnt/β-catenin pathway is activated, the expression level of β-catenin in cytosol increases, and β-catenin translocates to the nucleus to form a complex with the transcription factor TCF/LEF, leading to the activation of target genes (39,40). The present study demonstrated that the expression levels of wnt/β-catenin pathway-associated proteins were altered in DCM. In light of the expression level pattern of Sox5 and the reported association between the wnt/β-catenin pathway and Sox5, it was hypothesized that sox5 may be associated with the wnt/β-catenin pathway during the development of DCM.

In conclusion, the present study demonstrated that Sox5 may be associated with acute inflammation, apoptosis, collagen deposition and ventricular remodeling during the development of DCM.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL designed the experiment and wrote the manuscript. BJ constructed the mouse model. YC, LY and YX performed mechanism research and molecular biological detection. WC and ZQ conceptualized the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nanjing First Hospital (approval no. KY20190404-03-KS-01) and were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

All patients provided informed written consent.

Competing interests

The authors declare that they have no competing interests.

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