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ORIGINAL PAPER

Role of Nkx2.5 in H_2O_2-induced Nsd1 suppression

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Abstract

Nuclear receptor–binding SET domain–containing protein 1 (Nsd1) acts as a histone lysine methyltransferase, and its role in oxidative stress–related abnormal embryonic heart development remains poorly understood. In the present study, H_2O_2 decreased the expression of Nsd1 and NK2 transcription factor related locus 5 (Nkx2.5). We further focused on Nkx2.5 modulating the transcription of *Nsd1* in response to H_2O_2. Luciferase activity analysis indicated that a regulatory region from − 646 to − 282 is essential for the basal transcriptional activity, in which, an a Nkx2.5-binding element (NKE) was identified at − 412/− 406 of the *Nsd1* promoter by electrophoresis mobility shift assay and a chromatin immunoprecipitation assay. H_2O_2 obviously reduced the p646-luc promoter activity, and the depletion of Nkx2.5 expression weakened H_2O_2 inhibition on the p646-luc promoter. The overexpression of Nkx2.5 increase Nsd1 p646-luc promoter activity, but did not affected p646-luc-mut. Furthermore, overexpression and depletion of Nkx2.5 led to the increase and decrease of Nsd1 protein and mRNA levels. These data indicated that H_2O_2-induced Nsd1 suppression resulted from the decrease of Nkx2.5 expression through the NKE element.

Keywords Embryonic development · Nuclear receptor–binding SET domain–containing protein 1 · NK2 transcription factor related locus 5 · Oxidative stress · Hydrogen peroxide

Introduction

The heart is the first functional organ to form in developing embryos (van Weerd et al. 2011). Cardiogenesis is sensitive to oxidative stress, which is often associated with cardiovascular malformation, as oxidative stress regulates key transcription factors that influence cell signaling pathways involved in proliferation, differentiation, and apoptosis (Dennery 2007). A considerable number of studies have reported the response mechanisms to oxidative stress in embryonic development, for example, hyperoxia-induced oxidative stress activates NF-κB to protect the neonatal lung from acute injury (Yang et al. 2004); oxidative stress regulates the expression of AP-1 in rat conceptus (Ozolins and Hales 1997); oxidative stress impairs antioxidant gene expression in the fetal liver correlated with loss of Nrf1 function (Chen et al. 2003). Therefore, oxidative stress stimulates a wide variety of transcription factors implicated in embryonic development.

The cardiac-specific homeobox protein Nkx2.5 is essential for development of the heart, and mutations in the Nkx2.5 gene have been identified in patients and families with some of the most common forms of cardiac malformations and arrhythmias, including septal defects and abnormal conduction system (McCulley and Black 2012). Nkx2.5 is primarily known as a critical regulator of the expression of genes related to cardiac development and thus is critical for cardiogenesis. For instance, Nkx2.5 controls expression of eHand during murine heart development (Biben and Harvey 1997); Nkx2.5 and GATA4 could synergistically activate expression of ANF (Durocher et al. 1997); Nkx2.5 activates D-mef2 during Drosophila heart development (Gajewski et al. 1997); it has been reported that expression of the ventricular-specific myosin light chain 2 gene (MLC2V) and the cardiac ankyrin repeat protein (CARP) was downregulated in homozygous mutant embryos for Nkx2.5(Lyons et al. 1995; Zou et al. 1997). Recent studies have focused on the cardiac transcription network modulated by Nkx2.5 and histone modifications (Schlesinger et al. 2011). One example is Wolf–Hirschhorn syndrome candidate 1(WHSC1), a H3K36me3-specific histone methyltransferase, which negatively modulates the

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transcriptional activity of Nkx2.5 in congenital heart malformations (Nimura et al. 2009). Another example is SIRT1, an NAD-dependent histone deacetylase, which acts as a direct transcriptional target of Nkx2.5 and maintains cardiomyocyte homeostasis and survival (Vaquero et al. 2004; Zheng et al. 2013); in addition, Jarid2, a catalytically inactive jumonji family histone demethylase, is regulated by Nkx2.5 during outflow tract morphogenesis (Lei et al. 2016; Barth et al. 2010). Furthermore, the literature demonstrated that Nkx2.5 binds to the SIRT1 and Jarid2 promoter via the Nkx2.5-binding elements (NKE). Overall, many lines of evidence have suggested that Nkx2.5 and histone modification co-participate in influencing cardiogenesis, but the mechanism of this pathway has not yet been established.Nsd1 (nuclear receptor–binding SET domain protein 1) belongs to a family of mammalian histone lysine methyltransferases that is important in multiple aspects of development and disease (Berdasco et al. 2009; Choufani et al. 2015; Morishita and di 2011) and TFII-I can bind to the Nsd1 promoter through the consensus sequence BRGATTRBR during embryonic development (Makeyev and Bayarsaihan 2011). Although several histone methyltransferase–knock out mouse studies and genetic studies of human congenital heart disease patients have revealed the importance of histone modifications during heart development (Nimura et al. 2009; Delgado-Olguín et al. 2012), the pathogenic effects of Nsd1 in response to oxidative stress on heart defects remains unclear. We hypothesized that Nsd1 expression in response to oxidative stress is regulated by Nkx2.5 transcription factor.

In the present study, we aimed to determine the molecular mechanisms underlying the regulation of Nkx2.5 on Nsd1 expression in response to oxidative stress. We treated rat H9C2 cells with H_2O_2, which is an inducer of oxidative stress, and found that H_2O_2 inhibited Nsd1 expression by decreasing Nkx2.5 levels. Importantly, we showed that Nkx2.5 interacted with NKE2 (Nkx2.5 response element) to regulate the transactivation of the Nsd1 promoter. Thus, the binding affinity of Nkx2.5 and the transcription activity of Nsd1 were influenced by NKE2 in response to H_2O_2-induced oxidative stress.

Materials and methods

Experimental animals

Twelve- to 16-week-old three female and one male Sprague-Dawley rats weighing 200–250 g were obtained from the Animal Care Committee of Shengjing Hospital. Rats were fed with standard mouse chow, provided water ad libitum, and bred under a 12-h light/12-h dark cycle system. All experiments conformed to the guide for the care and use of laboratory animals and were approved by the Ethics Committee of Shengjing Hospital (Ethic approval no. 2017PS216K, Shenyang, China). In the morning of the second day, after mating between the male and female rats, microscopy examination of female vaginal secretions was performed and pregnancy confirmed, referred to as day 0 of pregnancy. Pregnant female rats were randomly injected with either H_2O_2 at 1.133 mL/kg body weight daily (Sigma-Aldrich) or saline from gestational day (GD) 9.0 to GD 19.0. On GD 19.0, pregnant dams were euthanized by isoflurane inhalation and the pups were delivered via cesarean section. The weights of the fetal hearts were measured.

Cell culture and regents

The H9C2 cell line was purchased from the Shanghai Institution of Cellular Biology of Chinese Academy of Sciences (Shanghai, China). H9C2 cells were maintained in DMEM, supplemented with 10% (vol/vol) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in 5% CO_2/95% air. H_2O_2 treatment was performed at 0.25 mmol/L, 0.5 mmol/L, and 1 mmol/L for 1 h and 0.25 mmol/L and 0.5 mmol/L for 2 h. The dual luciferase reporter assay system, pGL3-basic, and pRL-TK were obtained from Promega (Madison, WI, USA). Restriction endonuclease, T4 DNA ligase, T4 polynucleotide kinase, Taq polymerase, and PrimeSTAR Max DNA Polymerase were purchased from TaKaRa Biotech (TaKaRa, Dalian, China). Non-immune rabbit IgG (sc-2027) and protein A/G-agarose beads (sc-2003) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against Nsd1 (ab70732) were obtained from Abcam (Cambridge, UK). Antibodies against β-tubulin (10094-1-AP) and Nkx2.5 (13921-1-AP) were obtained from Proteintech Group (Chicago, IL, USA). Peroxidase-conjugated AffiniPure goat anti-Rabbit IgG (ZB-2301) and peroxidase-conjugated AffiniPure goat anti-Mouse IgG (ZB-2305) were obtained from ZSGBBIO (Beijing, China).

Western blot analysis

Total protein was extracted from either treated (or untreated) H9C2 cells or prepared by homogenizing the frozen tissues, and protein concentrations were determined. Equal amounts of protein were subjected to 10% (wt/vol) SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF, Bio-Rad, Hercules, USA) membranes. The membranes were blocked with 5% (wt/vol) nonfat dry milk in TBS containing 0.1% Tween-20 and incubated with primary antibody specific for Nsd1 (1:200), Nkx2.5 (1:600), β-tubulin (1:1000) in 1% (wt/vol) BSA overnight at 4 °C. After washing for 15 min, the membranes were incubated

with horseradish peroxidase-conjugated secondary antibodies (1:5000) at room temperature for 2 h. The final detection reactions were visualize using ECL plus chemiluminescence reagent (Biotool, Houston, USA) and analyzed with ECL chemiluminescence detection system (Bio-Rad).

RNA isolation and real-time PCR

Total RNA was extracted from treated (or untreated) H9C2 cells using a TRIzol reagent (TaKara, Dalian, China) and reverse transcribed into cDNA using the reverse transcription reagent kit (Promega). Quantitive real-time PCR (qRT-PCR) was performed using TaKara SYBR Premix Ex TaqII (RR820) according to the manufacturer's protocol on the Applied Biosystems 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, USA). The sequences of the primers (Table 1) were for Nsd1 and Gapdh. The cycling variables were set as follows: 95 °C for10 min, followed by 30 cycles of 95 °C (15 s), 60 °C (30 s), and 70 °C (30 s). Cycle threshold values (Ct) were analyzed by SDS2.4 software (Applied Biosystems, Foster City, USA), and relative quantifications of Nsd1 expression were determined using the comparative Ct method with the Gapdh transcription as an internal control.

Plasmid construction

A series of truncated Nsd1 promoter luciferase reporter vectors were constructed. The promoter fragment from −950 to +161 of the Nsd1 gene (GenBank: NC_005116.4) was obtained by PCR using rat genomic DNA as the template. The amplified product was cloned into the pGL3-basic vector to generate pNsd1-Luc (Table 2). Full-length cDNA encoding rat Nkx2.5 was amplified by PCR in Table 3 containing BamH I and EcoRI sites (underlined) and subcloned into the pcDNA3.1 expression vector. Site-directed mutagenesis was performed, 5′-TGGA AACTTCCATGTCTCCTTCTTTAACCTGAGG-3′ 5′-AAGAAGGAGACATGGAAGTTTCCAGATGCAAA GT-3′, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic primers were synthesized and annealed. All constructs were confirmed to have no coding frame shift in the luciferase gene using sequencing.

Table 1 Nucleotide sequences for primers used in real-time PCR

Name	Forward and reverse primers
Nsd1	F: 5′-GCAGGATTTGTTCTGATCCAT-3′ R: 5′-CTTTCTGTTTCCCTCTTTTCCT-3′
Gapdh	F: 5′-TGCACCACCAACTGCTTAGC-3′ R: 5′-GGCATGGACTGTGGTCATGAG-3′

Table 2 Nucleotide sequences for primers used in Nsd1 promoter

Name	Forward and reverse primers
Nsd1	F1: 5′-CTGAATGGAGGGGATGGC-3′ (−950) F2: 5′-GGAAGAACCTTAGCGTGAT-3′ (−646) F3: 5′-AATGGGCTGTGGGTCTTG-3′ (−282) F4: 5′-TACTAAGCCTCTGGAGGAATGG-3′ (−52) R: 5′-TCCTGGGTTTGCTTCACG-3′

Transient transfection and luciferase assays

H9C2 cells were sub-cultured into 24-well plates, grown to 70–80% confluence after 24 h, and transiently transfected using jetPRIME transfection reagent (Polyplus-transfection, France) with 0.5 μg of pNsd1-lucplasmid and 0.01 μg of pRL-TK encoding for Renilla luciferase, which was used to normalize transfection efficiency; 24 h after transfection, the cells were harvested and the firefly and Renilla luciferase activities were measured consecutively using the dual luciferase reporter assay system with a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). In the co-transfection experiments, 0.5 μg of reporter construct was transfected into cells with 0.5 μg of expression vector encoding Nkx2.5 or 20 pmol Nkx2.5-specific siRNAs, and pcDNA3.1 or negative control siRNA were used as negative controls. The sequences of siRNAs used were shown in Table 4 and the cells were harvested at 48 h after transfection. Cells were incubated with 0.5 mmol/L H_2O_2 for an additional 2 h in some experiments.

Electrophoretic mobility shift assay

Nuclear protein extracts from H9C2 cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific Inc., USA), and the electrophoretic mobility shift assay (EMSA) was performed using LightShift Chemiluminescent EMSA Kit (Thermo Scientific) following the manufacturer's protocols described. Binding reactions were performed in a volume of 20 μl containing approximately 20-fmol Biotin-end-labeled double-stranded probes, 10 μg of nuclear extracts, 1 μg/μL of poly (dI/dC), 1% NP-40, 100 mM $MgCl_2$, 50% (vol/vol) Glycerol, and 1× binding buffer. For competition

Table 3 Nucleotide sequences for primers used in Nkx2.5 cDNA

Name	Forward and reverse primers
Nkx2.5	F: 5′-CGGGATCCATGTTCCCCAGCCC TGCGCTCAC-3′ R: 5′-CCGGAATTCTACCAGGCTCGGA TGCCGTGCA-3′

Table 4 Nucleotide sequences for primers used in Nkx2.5 siRNAs

Name	Forward and reverse primers
Nkx2.5	F1: 5′-GCCCUUCUCAGUCAAAGACTT-3′ R1: 5′-GUCUUUGACUGAGAAGGGCTT-3′
siRNAs	F2: 5′-GGGCGGAUAAGAAAGAGCUTT-3′ R2: 5′-AGCUCUUUCUUAUCCGCCCTT-3′ F3: 5′-GCUUCAAGCAACAGCGGUATT-3′ R3: 5′-UACCGCUGUUGCUUGAAGCTT-3′

assays, a 100-fold molar excess of unlabeled oligonucleotides was added into the binding reaction mixture. To confirm the binding of Nkx2.5 to the NKE2 element of Nsd1, nuclear extracts were pre-incubated with 1 μg of Nkx2.5 antibody for 20 min at 4 °C before the probes were added. After incubation at room temperature for 30 min, the reaction mixtures were loaded on to a 6% (wt/vol) polyacrylamide gel and separated at 120 V in 0.5 Tris-borate-EDTA (TBE) for 1 h, and then transferred onto Hybond N+ (Amersham Biosciences UK Limited) membranes and detected signal using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

Chromatin-immunoprecipitation

H9C2 cells were cultured for chromatin-immunoprecipitation (ChIP) assays. ChIP assays were performed as described previously. The lysate was incubated with anti-Nkx2.5 antibody or with non-immune rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, USA) as a negative control at 4 °C overnight. The DNA product was analyzed by PCR with forward primers 5′-CGGT CTCCAAGGGATAGG-3′ and reverse primer 5′-CAAG ACCCACAGCCCATTA-3′. The product is a 232 bp fragment of the Nsd1 promoter including the NKE2 element site. All of the shown data represent the average of at least three independent ChIP experiments.

Statistical analysis

Data are expressed as the mean ± SEM from three experiments and analyzed by SPSS (Version 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). Student's independent two-tailed test or one-way ANOVA followed by S–N–K test was

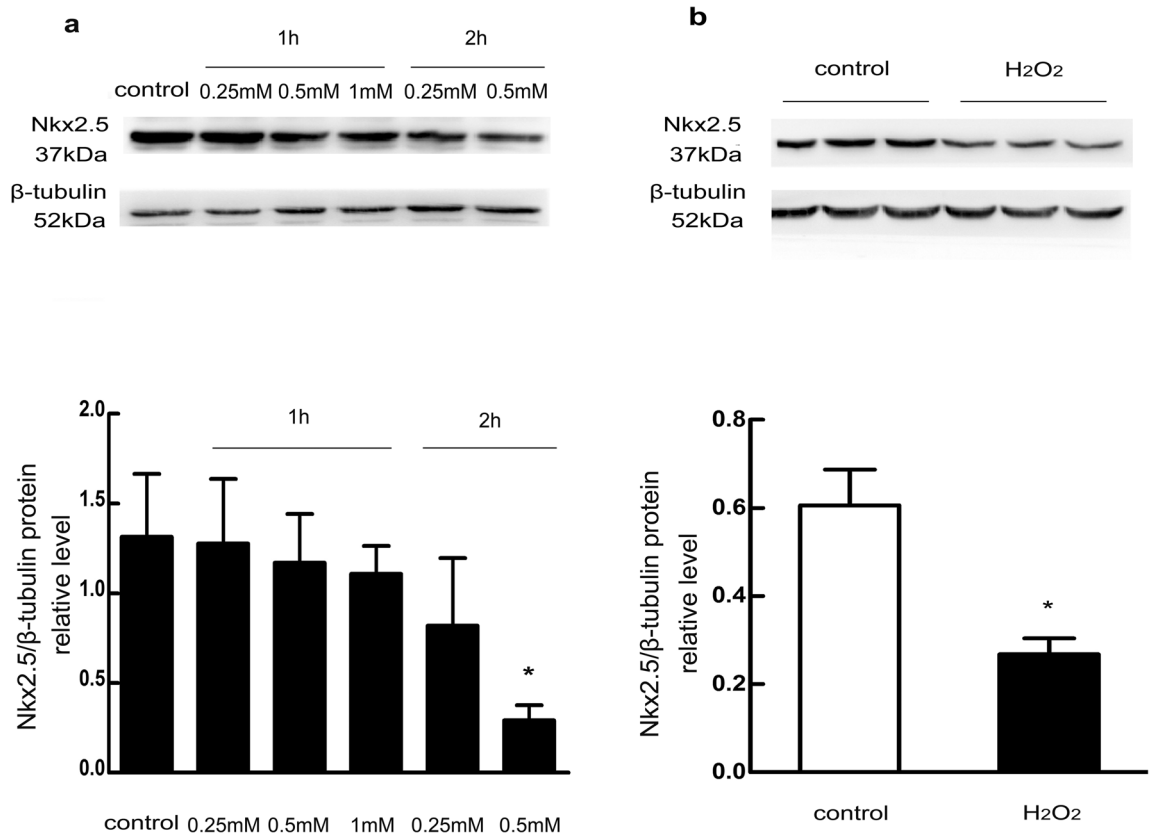

Fig. 1 H_2O_2 inhibits Nkx2.5 expression not only in H9C2 cells but also in fetal rat heart. (a) H9C2 cells were treated with H_2O_2 in the condition of 0.25 mmol/L for 1 h, 0.5 mmol/L for 1 h, 1 mmol/L for 1 h, 0.25 mmol/L for 2 h, and 0.5 mmol/L for 2 h. (b) Fetal rat hearts were isolated from pregnant rats which were injected intraperitoneally with either H_2O_2 at 1.133 mL/kg body weight or saline. $n = 3$ for each group. Nkx2.5 protein was detected by western blot, and β-tubulin was used as total extracts loading controls. All experiments are performed three times independently. $^*p < 0.05$ versus untreated value of the respective group

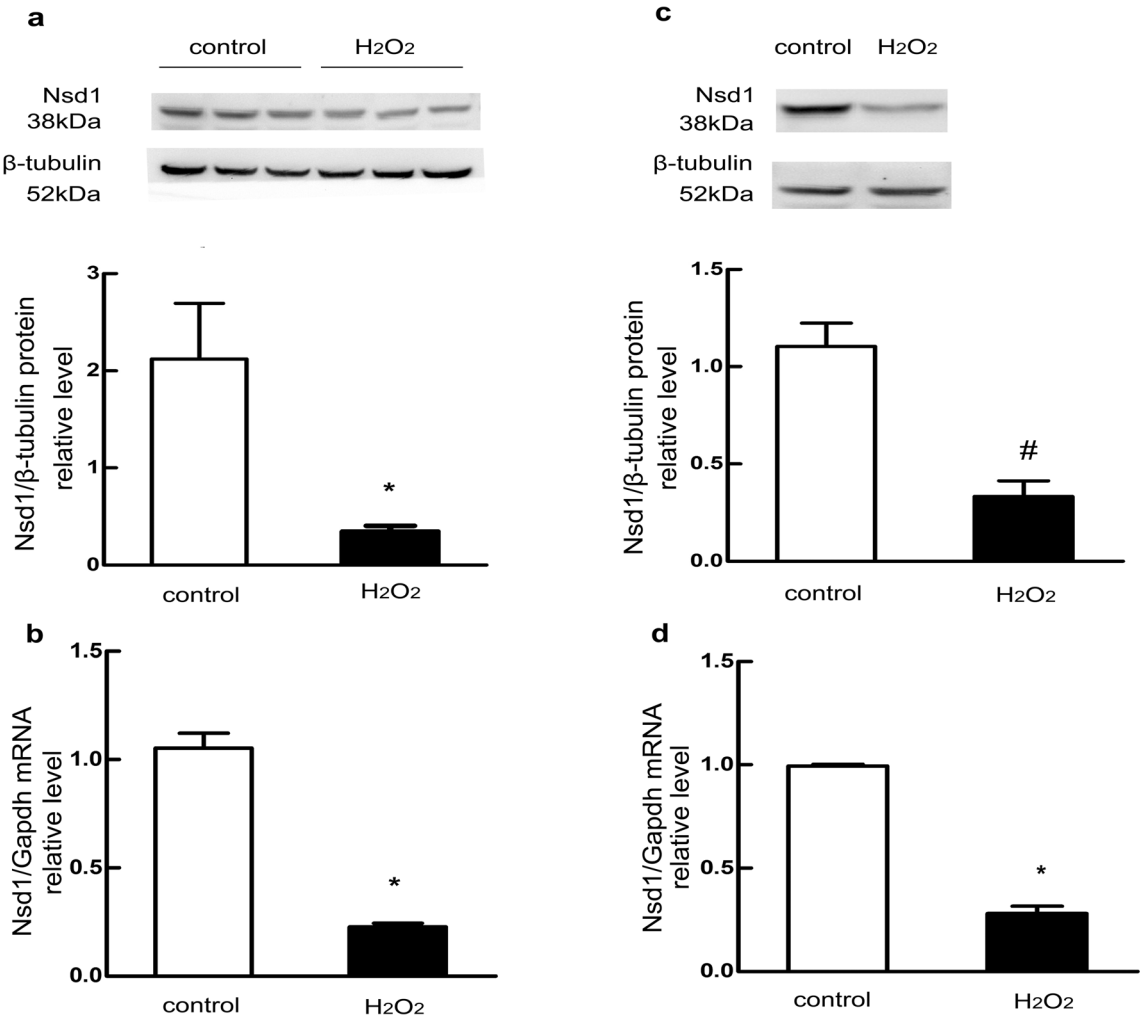

Fig. 2 H_2O_2 inhibits Nsd1 expression in fetal rat heart and H9C2 cells. Pregnant rats were injected intraperitoneally with either H_2O_2 (H_2O_2 group) or saline (control group) randomly. $n = 3$ for each group. (a) The protein expression of Nsd1 in fetal rat heart analyzed by Western blot. (b) The Nsd1 mRNA in fetal rat heart tested by qRT-PCR. H9C2 cells were treated (or untreated) with H_2O_2 (0.5 mmol/L for 2 h). (c) The protein expression of Nsd1 in H9C2 cells analyzed by Western blot. (d) qRT-PCR was performed to determine the Nsd1 mRNA level in H9C2 cells using the comparative Ct method. All experiments are performed three times independently. $^{*}p < 0.05$ versus untreated value of the respective group; $^{\#}p < 0.01$ versus values of control group

used for statistical analysis. A p value of < 0.05 was considered statistically significant.

Results

Suppression of the Nkx2.5 protein by H_2O_2 in H9C2 cells and fetal rat hearts

To gain an insight into the expression of Nkx2.5 in heart development in response to oxidative stress, we used H_2O_2 as an inducer of oxidative stress and examined the expression of Nkx2.5. H9C2 cells were treated with H_2O_2 at different concentrations, and Western blot analysis demonstrated that Nkx2.5 expression decreased in response to H_2O_2 in a dose-dependent manner (Fig. 1a), and the most significant decrease was with 0.5 mmol/L H_2O_2 for 2 h, which was thus used for the subsequent cell experiments. In addition, pregnant rats were injected intraperitoneally with H_2O_2 on day 9 of gestation, and fetal rat hearts were isolated from pregnant rats on day 19 of gestation. Western blot analysis showed that Nkx2.5 expression decreased by twofold in H_2O_2-treated rats compared with saline-treated controls (Fig. 1b). These results indicate that H_2O_2 inhibits Nkx2.5 expression in vitro and in vivo.

H_2O_2 inhibits Nsd1 expression in fetal rat hearts and H9C2 cells

To investigate the expression of Nsd1 in response to H_2O_2, we detected mRNA and protein levels of Nsd1 in fetal rat hearts from pregnant rats exposed to H_2O_2. As shown in Fig. 2a, b,

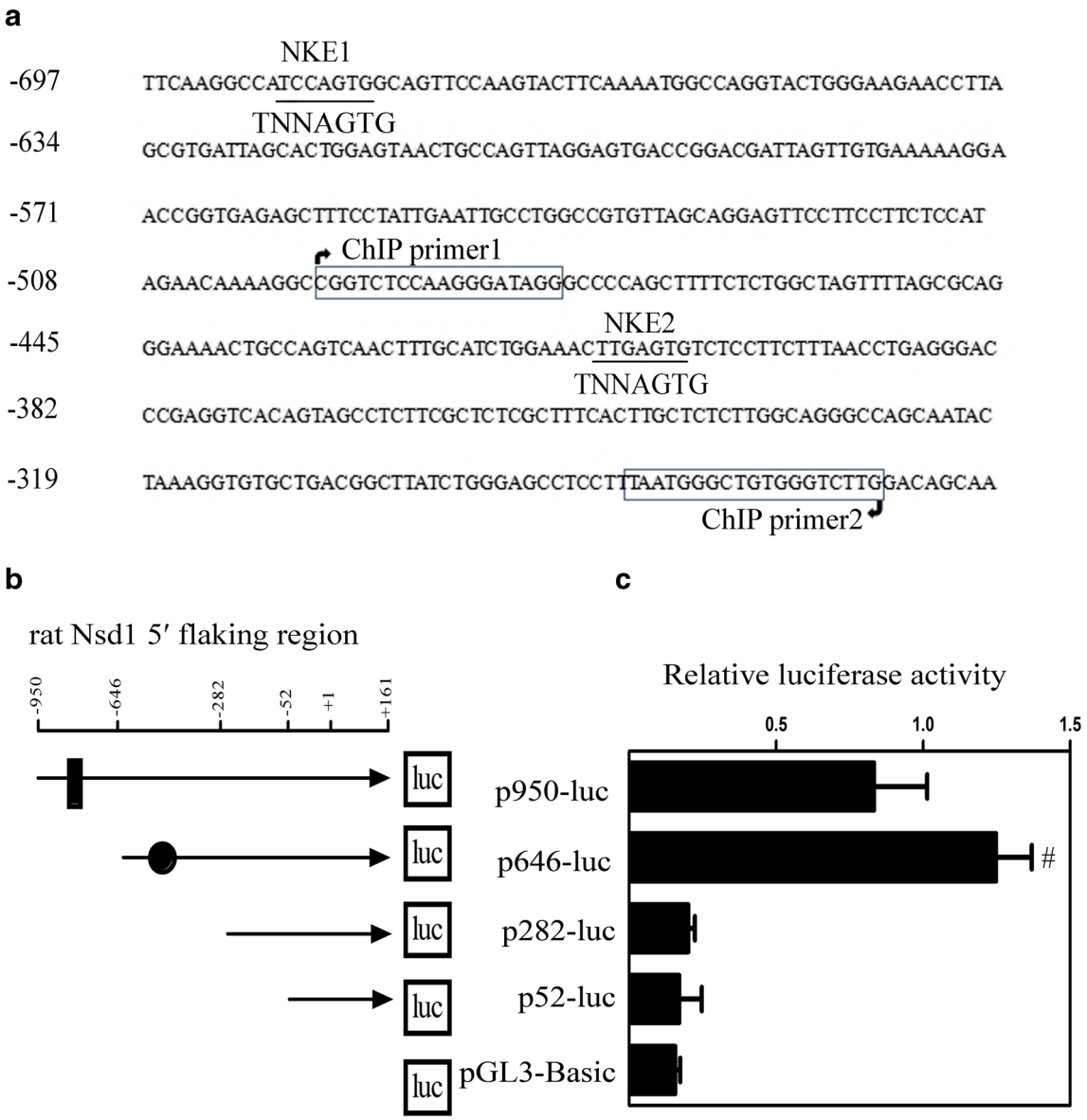

Fig. 3 Transient transfection analysis of the Nsd1 promoter/Luc constructs. (a) A 1111-bp fragment (−950 to +161) of the Nsd1 promoter according to sequence [GenBank: NC_005116.4]. Nucleotide sequences of identified and partial putative regulatory elements are underlined. The locations of the primers used for ChIP are boxed. (b) Schematic representation of a series of 5′-flanking deletions of the Nsd1 promoter luciferase reporter constructs. Numbering is defined relative to the transcription start site. "▌", "●" represented the identified NKE1 and the putative NKE2-responsive elements, respectively. (c) The basal activity of the corresponding constructs in H9C2 cells. Luciferase activities were measured and normalized to Renilla luciferase activities. The bar is the mean ± SEM from three independent experiments in duplicate for each construct

Western blot demonstrated that Nsd1 protein decreased by 84% in H_2O_2-treated fetal rat hearts compared with saline-treated controls, and qRT-PCR showed that Nsd1 mRNA in H_2O_2-treated fetal rat hearts also decreased by about 81% relative to controls. Furthermore, H9C2 cells were treated with 0.5 mmol/L H_2O_2 for 2 h, and Nsd1 protein and mRNA levels were reduced by 68% and 77%, respectively, as a result of H_2O_2 treatment (Fig. 2c, d). These data indicate that H_2O_2 suppresses Nsd1 expression in heart development partially via a transcriptional pathway.

Analysis of the rat *Nsd1* promoter

Since Nkx2.5 is a specific transcription factor in heart development, we considered the impact of Nkx2.5 on *Nsd1* transcription. The luciferase reporter gene was fused with an *Nsd1* promoter from −950 to +161, in which two potential NKEs with the 5′-TNNAGTG-3′ consensus-binding motif, NKE1 (−687/−681) and NKE2 (−412/−406), were predicted (Fig. 3a). Based on the luciferase assay of a series of truncated *Nsd1* promoters, we observed that p646-luc activity was

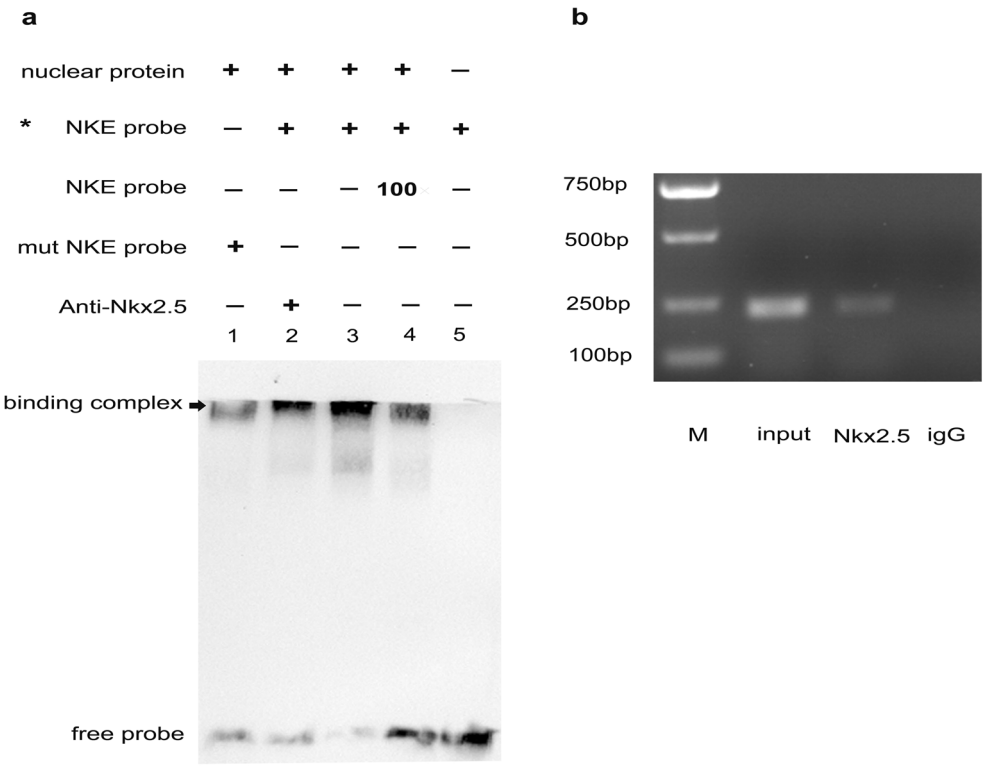

Fig. 4 Identification of Nkx2.5 bind to NKE2. (a) EMSA was performed with nuclear extracts from H9C2 cells, *NKE represents labeled probes, the arrow showed the position of binding complex, 100× represents the competition assays. (b) ChIP assays with Nkx2.5 antibody. Chromatin from H9C2 cells was immunoprecipitated with anti-Nkx2.5 antibody or non-immune IgG (negative control). Input was total chromatin DNA used in the PCR reaction as a positive control. M represented DL2000 DNA ladder. A 232 bp amplification product was observed after immunoprecipitating the chromatin with Nkx2.5 antibody, but not with non-immune IgG

higher relative to other constructs, where a putative NKE2-binding motif was present (Fig. 3b). Deletion of the 364 bp containing the putative NKE2 led to an obvious decrease of transcriptional activity (p646-luc vs. p282-luc), and further truncation of 230 bp showed basic transcriptional activity (Fig. 3c). These data suggest that the putative NKE2-binding motif is critical for transcriptional activity of the Nsd1 promoter.

Identification of Nkx2.5 binding to the NKE2 of the *Nsd1* promoter

To define the putative NKE2 element at −412/−406 of the *Nsd1* promoter, we performed an electrophoretic mobility shift assay (EMSA) using nuclear extracts from H9C2 cells. As shown in Fig. 4a, a DNA–protein–binding complex was formed (lane 3) and competed by 100-fold molar excess of unlabeled probe (lane 4). Moreover, the binding affinity of the NKE probe was much higher than that of the mutated NKE probe (lane 1). Further studies showed that an anti-Nkx2.5 antibody blocked binding (lane 2), demonstrating that there was specific binding of Nkx2.5 to the NKE2 of *Nsd1*. These results indicated that an NKE element is present at position −412/−406 of the *Nsd1* promoter.

Subsequently, we carried out a ChIP assay to confirm binding under in vivo conditions. After chromatin from H9C2 cells was immunoprecipitated with the Nkx2.5 antibody, we amplified the region −496 to −265 containing the NKE2 of *Nsd1* promoter and obtained a 232 bp fragment. The results, depicted in Fig. 4b, validated that the binding was specific, as the antibody against Nkx2.5 but not the non-immune IgG showed a detectable Nsd1 promoter fragment in H9C2 cells, which was in accordance with the results of the EMSA.

Nkx2.5 controls the transcription of the *Nsd1* promoter with NKE2 in response to H_2O_2

To determine the effect of Nkx2.5 on the *Nsd1* promoter transcriptional activity in response to H_2O_2, we used three Nkx2.5-specific siRNAs to block Nkx2.5 expression in H9C2 cells, and we showed that siRNA2 clearly reduced Nkx2.5 expression (Fig. 5a). siRNA2 was used in subsequent experiments. The p646-luc containing NKE2 was co-transfected with the siRNA2 into H9C2 cells that were untreated or treated with H_2O_2. Compared with the controls, H_2O_2 reduced 95% of the *Nsd1* promoter activity, and the depletion of Nkx2.5 expression resulted in a 76% decrease of the promoter activity, but reduced H_2O_2 inhibition on the promoter activity (Fig. 5b). These results imply that Nkx2.5 is involved in H_2O_2-induced transcriptional suppression of *Nsd1*.

To verify the function of NKE2 in the *Nsd1* promoter, we mutated NKE2 derived from the p646-luc construct to generate a p646-luc-mut. Overexpression of Nkx2.5 in H9C2 cells (Fig. 5c) significantly increased p646-luc promoter activity

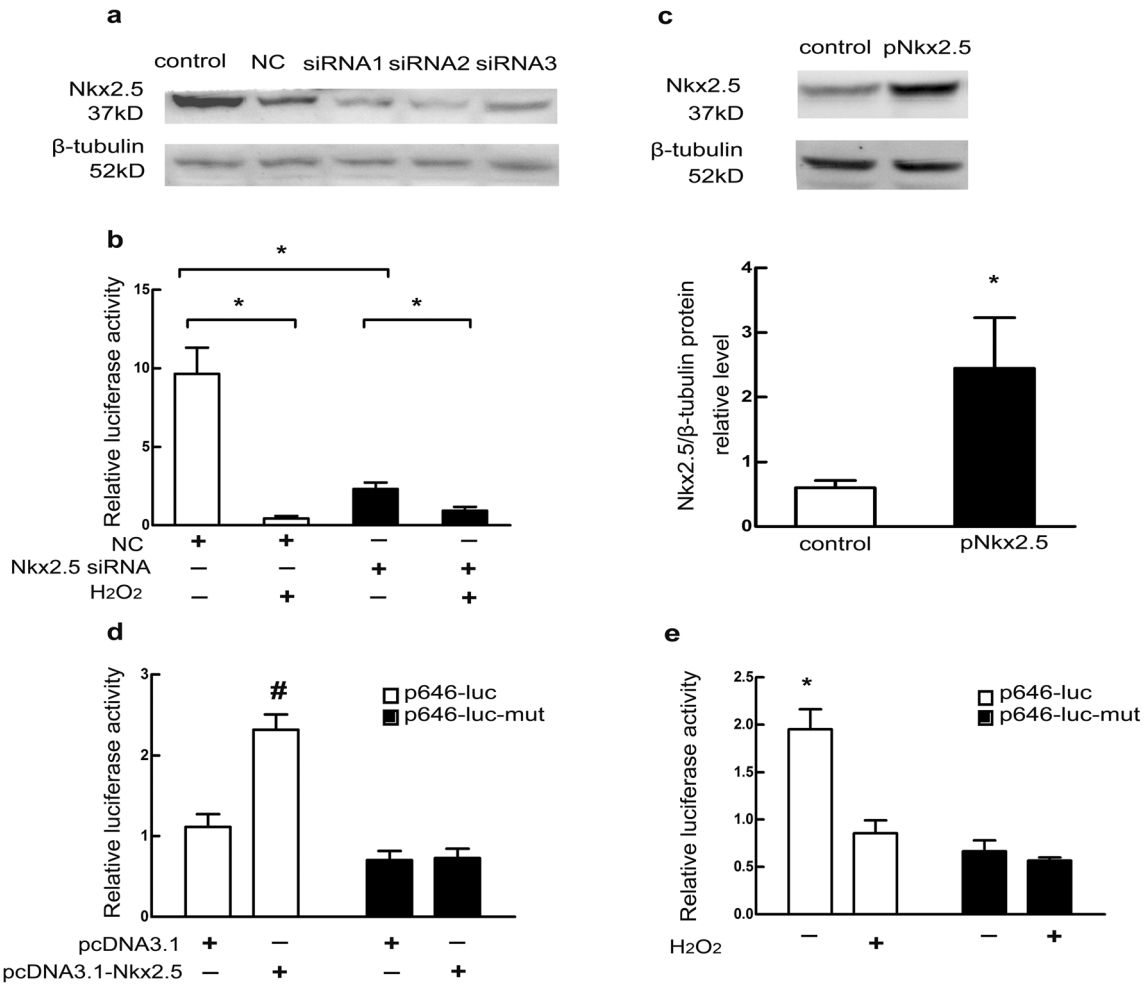

Fig. 5 Transient transfection analysis of the Nsd1 promoter/luc constructs. (a**)** Western blot assay shows knockdown of Nkx2.5 protein expression with siRNAs. Control and NC represent non-transfected cells and negative control–transfected cells, respectively. (b) The transcriptional activities of Nsd1 promoter in H9C2 cells were treated (or untreated) with H_2O_2 (0.5 mmol/L for 2 h) after luciferase reporter plasmids co-transfected with Nkx2.5 siRNA. (c) Western blot assay shows increase of Nkx2.5 protein expression in H9C2 cells with Nkx2.5 expression vector. Control represents transfected with pcDNA3.1 vector into cells. (d) Transcriptional effect of Nkx2.5 on the p646-luc and p646-luc-mut. (e) H_2O_2 on the p646-luc and p646-luc-mut. Histograms represent the mean ± SEM from three independent experiments. $^{*}p < 0.05$ compared with control. $^{\#}p < 0.01$ compared with pcDNA3.1 vector

but did not affect p646-luc-mut promoter activity (Fig. 5d). We then compared the promoter activity between p646-luc and p646-luc-mut in response to H_2O_2 (0.5 mmol/L for 2 h) and found a strong inhibition on the promoter activity of p646-luc but not on that p646-luc-mut (Fig. 5e). These data imply that Nkx2.5 is involved in H_2O_2-induced transcriptional suppression of *Nsd1* with the NKE2 element.

Nkx2.5 regulates endogenous Nsd1 expression

Finally, we investigated whether Nkx2.5 regulates the endogenous expression of Nsd1. Overexpression of Nkx2.5 increased the protein and mRNA expression of Nsd1 (Fig. 6a, b). Furthermore, the depleted Nkx2.5 expression by siRNA2 in H9C2 cells led to reduced protein and mRNA levels of Nsd1 (Fig. 6c, d), suggesting that Nkx2.5 plays a positive role in Nsd1 transcription. These results indicate that the reduction of Nkx2.5 by H_2O_2 could be partly attributed to the downregulation of Nsd1.

Discussion

In the present study, the NKE2 element in the *Nsd1* promoter was identified at position − 412/− 406, which was shown to be essential in regulating the transcriptional activity of the *Nsd1* promoter. Importantly, we succeeded in verifying one of the molecular mechanisms underlying Nkx2.5-regulated Nsd1 expression in response to H_2O_2.

Substantial evidence indicates that oxidative stress influences the development of the fetus (Al-Gubory et al. 2010; Dennery 2010). Recently, Niu et al. (2015) showed

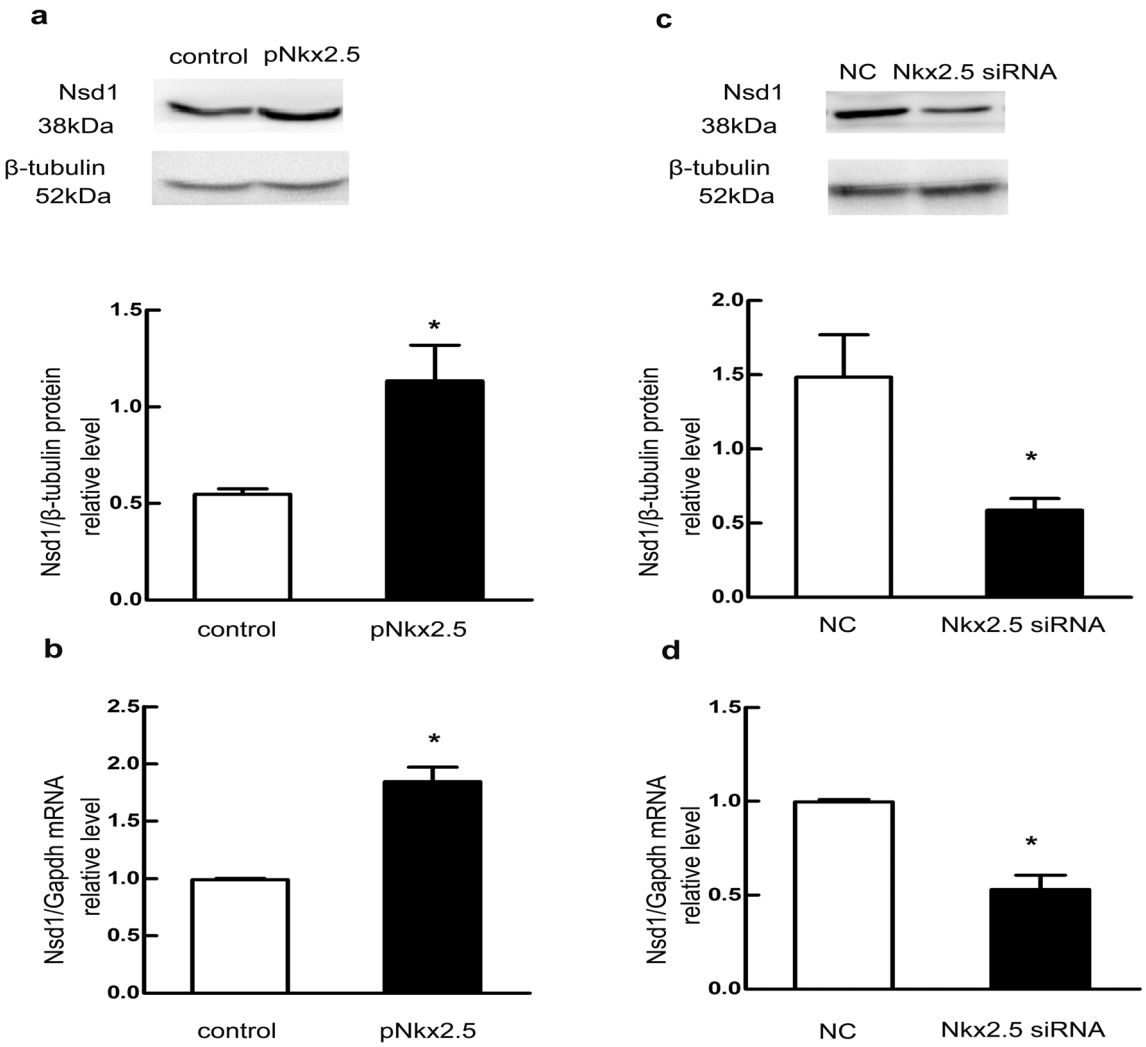

Fig. 6 The assay of Nsd1 mRNA and protein expression after either Nkx2.5 expression vector or Nkx2.5-specific siRNA was transfected into H9C2 cells. (a) Western blot and (b) qRT-PCR were performed to determine the Nsd1 protein level and mRNA expression with Nkx2.5 expression vector. (c) Western blot and (d) qRT-PCR were performed to determine the Nsd1 level by Nkx2.5-specific siRNA. Histogram represents the mean SEM from three independent experiments. *$p < 0.05$ compared with pcDNA3.1 vector, *$p < 0.05$ compared with NC

that oxidative stress alters global histone methylation; in addition, Khanal et al. (2015) indicated that the harmful effects of oxidative stress are associated not only with genetic mutations but also with epigenetic aberrations. Nsd1 functions as a histone lysine methyltransferase, but whether it participates in the mechanisms responsible for the development and progression of oxidative stress–related embryonic disease is unclear. To determine the association between Nsd1 and the teratogenic effects of oxidative stress in embryonic development, we showed that mRNA and protein levels of Nsd1 were suppressed in H9C2 cells, as well in fetal rat hearts, as a result of H_2O_2 treatment. We previously reported that H_2O_2 decreased the levels of Nsd1 mRNA and protein in Bel 7402 cells (Chu et al. 2014). There is evidence that cardiac transcription factor Nkx2.5 is a master regulator during heart development, and deletion of this gene results in the cardiomyocyte maintenance and failure of normal heart development (Kinnunen et al. 2015). To better understand the effect of H_2O_2 on Nkx2.5, we also investigated Nkx2.5 expression in response to H_2O_2 and found that Nkx2.5 decreased in both H9C2 cells and fetal rat hearts. Our results were in accordance with oxidative stress–induced reduced expression of Nkx2.5, due to treatment with a high salt concentration (Wang et al. 2015). Therefore, we considered whether Nkx2.5 regulated Nsd1 expression in heart development. We over-expressed Nkx2.5 in H9C2 cells, resulting in an increase of protein and mRNA levels of Nsd1. We then depleted Nkx2.5 expression by siRNA in H9C2 cells, leading to the suppression of Nsd1 protein and mRNA levels. These results indicate that Nkx2.5 plays a positive role in Nsd1 expression. Further studies are required to explore the mechanism by which Nkx2.5 mediates the transcriptional regulation of Nsd1 expression in response to H_2O_2.

To address the molecular mechanisms underlying the Nkx2.5 regulation of Nsd1 expression in response to H_2O_2, we first scanned the Nsd1 promoter region for possible Nkx2.5 cis-acting elements. The results showed two potential binding sites (NKE1 and NKE2) containing the Nkx2.5/Csx consensus-binding motif (5′-TNNAGTG-3′) (Chen and Schwartz 1995), which has been shown to be significant in the regulation of the collagen I gene in smooth muscle cells (Ponticos et al. 2004) and the a-SMA gene transcription in suppressing myofibroblast differentiation (Hu et al. 2010). We performed promoter truncation together with a luciferase activity assay, and showed that a regulatory region (−412/−406) of the *Nsd1* promoter was required for basal transcriptional activity. Of interest, after depletion of Nkx2.5, the p646-luc promoter activity decreased compared with the control, and the reduced p646-luc promoter activity in H_2O_2-treated cells was restored. This can be explained by the fact that H_2O_2 inhibited Nsd1 expression mainly via Nkx2.5-mediated transcription. We then focused on NKE2 and performed a luciferase assay and in vitro and in vivo DNA–protein binding assays, demonstrating that NKE2 may affect transcriptional activity and binding affinity of Nkx2.5 to the *Nsd1* promoter. When Nkx2.5 was overexpressed, the p646-luc activity was significantly enhanced ($p < 0.05$), implying that Nkx2.5 plays a positive role in Nsd1 transcription. There was no change in the mut-p646-luc promoter activity. Regarding H_2O_2 treatment, p646-luc promoter activity decreased more than the mut-p646-luc promoter activity. These findings suggest that H_2O_2 can suppress Nkx2.5 expression, resulting in the decrease of Nsd1 expression via binding of NKE2 in H9C2 cells. Furthermore, the data from the EMSA confirmed this binding affinity of Nkx2.5 to the NKE2 of the Nsd1 promoter. ChIP result also showed the binding of Nkx2.5 to the NKE2 of H9C2 cells. Therefore, we speculated that the interaction of NKE2 with Nkx2.5 could contribute to the H_2O_2-induced repressive effect on Nsd1 transcription.

In summary, we demonstrated that H_2O_2 inhibited Nsd1 expression via Nkx2.5-mediated transcription of the *Nsd1* promoter. Our findings suggest that Nkx2.5, as a transcription factor, may be a regulatory element connecting different signaling pathways in response to oxidative stress and that reduced Nsd1 expression could lead to the alteration of global H3K36 and H4K20 methylation, influencing cardiac development. We hypothesized that oxidative stress reduced Nsd1 expression through suppression of Nkx2.5, which may be one of the common pathways in oxidative stress–related developmental abnormalities.

Compliance with ethical standards

All experiments conformed to the guide for the care and use of laboratory animals and were approved by the Ethics Committee of Shengjing Hospital (Ethic approval no. 2017PS216K, Shenyang, China).

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