Long intergenic non-coding RNA-p21 mediates cardiac senescence via the Wnt/β-catenin signaling pathway in doxorubicin-induced cardiotoxicity

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Abstract. Doxorubicin (Dox)-induced cardiotoxicity has been a well-known phenomenon to clinicians and scientists for decades. It has been confirmed that Dox-dependent cardiotoxicity is accompanied by cardiac cellular senescence. However, the molecular mechanisms underlying Dox cardiotoxicity remains to be fully elucidated. Long non-coding (lnc) RNAs regulate gene transcription and the fate of post-transcriptional mRNA, which affects a broad range of age-associated physiological and pathological conditions, including cardiovascular disease and cellular senescence. However, the functional role of lncRNAs in Dox-induced cardiac cellular senescence remains largely unknown. Using the reverse transcription-quantitative polymerase chain reaction method, the present study indicated that long intergenic non-coding (linc) RNA-p21 was highly expressed in Dox-treated HL-1 murine cardiomyocytes. Dox-induced cardiac senescence was accompanied by decreased cellular proliferation and viability, increased expression of p53 and p16, and decreased telomere length and telomerase activity, while these effects were relieved by silencing endogenous lincRNA-p21. We found that lincRNA-p21 interacted with β-catenin and that silencing β-catenin abolished the anti-senescent effect of lincRNA-p21 silencing. It was observed that modulating lincRNA-p21 to exert an anti-senescent effect was dependent on decreasing oxidant stress. To conclude, the present findings suggest that lincRNA-p21 may be involved in Dox-associated cardiac cellular senescence and that silencing lincRNA-p21 effectively protects against Dox cardiotoxicity by regulating the Wnt/β-catenin signaling pathway and decreasing oxidant stress. Furthermore, modulating lincRNA-p21 may have cardioprotective potential in patients with cancer receiving Dox treatment.

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Introduction

Doxorubicin (Dox) is one of the most widely used antineoplastic drugs. Its anticancer effects are believed to occur through the inhibition of topoisomerase enzyme and subsequent blockage of DNA resealing during cell replication (1). Despite its highly beneficial effects against cancer, the clinical use of Dox has been confined by its drawback of cardiotoxicity (2). Risk of Dox cardiotoxicity increases with both treatment concentration and duration, and delayed onset of cardiomyopathy can often occur years after initial treatment (3). The mechanism for Dox-induced cardiotoxicity is controversial, and several hypotheses have been proposed. One of the important mechanisms inducing cardiotoxicity is Dox induction of cardiomyocyte senescence (4,5). Dox treatment enhanced secretion of senescence-associated cytokines and augmented the DNA damage-response signaling cascade, thus inducing cardiomyocyte senescence (5,6). Therefore, there is an urgent need to find an efficient way to ameliorate Dox-induced cardiomyocyte senescence in order to prevent future cardiac complications.

Non-coding RNAs are non-protein-coding transcripts that function as regulators of RNA molecules. Long non-coding (lnc) RNAs are non-coding RNAs with at least 200 nucleotides (7) that have been reported to impact a broad spectrum of biological processes including development, differentiation, cell division, apoptosis, cellular senescence, diseases and disorders, thus regulating the complexity of the organism as a whole (8,9). LncRNA expression patterns are tissue- and stage-specific, suggesting their considerable importance in controlling different biological functions, cellular senescence in particular (10). Among lncRNAs, long intergenic non-coding (linc) RNA-p21 is closely related to cellular senescence. Recent research has shown that lincRNA-p21, which interacts with β-catenin, promoted cellular senescence (11). Also, lincRNA-p21 has been identified as a regulator of p53

expression, and reciprocally p53 can regulate the expression of lincRNA-p21, which plays a major role in pro-senescence networks (12). At the same time, lincRNA-p21 is a key regulator of age-related heart diseases such as coronary artery disease (13). However, there has been no research on the biological function of lncRNAs in Dox-related cardiotoxicity, so in this study we explored the involvement of lincRNA-p21 in cardiomyocyte senescence in the Dox-induced cardiotoxic process.

LncRNAs have been proposed to act in transvia several mechanisms ranging from repression of gene transcriptional networks to regulation of mRNA translation and protein stability (14). The Wnt/β-catenin signaling pathway is closely associated with ageing-associated impairments in cardiac regeneration and function (15). A previous study found that the canonical Wnt signaling pathway was involved in the senescence process of cardiac stem cells (CSCs) (16). As a post-transcriptional inhibitor of translation, β-catenin was initially identified as a direct transcriptional target of lincRNA-p21 (11). Moreover, lincRNA-p21 inhibited β-catenin signaling, thereby attenuating viability, self-renewal and glycolysis of colorectal cancer stem cells (17). In addition, in the Dox-induced dilated cardiomyopathy model, β-catenin signaling was apparently inhibited by Dox (18). Based on the role of lincRNA-p21-regulated β-catenin signaling and the inhibitory effect of Dox on β-catenin signaling, the present study aimed to determine if modulating lincRNA-p21 could activate β-catenin signaling and relieve Dox-related cardiotoxicity.

Senescence can be triggered by multiple mechanisms, including those resulting in the production of reactive oxygen species (ROS) and oxidative stress (19). Oxidative stress and generation of ROS are also important mediators of the cellular alterations caused by Dox exposure (20). Cardiac senescence induced by Dox correlates with increased generation of ROS and oxidative stress (21). LncRNAs have close relationships with oxidative stress, DNA damage and other types of cellular stress responses (22). With respect to lincRNA-p21, a recent report observed that UVB-induced apoptosis of keratinocytes involved increased lincRNA-p21 expression and ROS-associated DNA damage (23). Furthermore, endoplasmic reticulum stress induced by lincRNA-p21 has been suggested to account for its effects on apoptosis induction and inhibition of hepatocellular carcinoma cell proliferation (24). The current study explored whether suppressing lincRNA-p21 expression could attenuate oxidative stress to alleviate cellular senescence induced by Dox and thus exert a cardioprotective effect.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA), TRIzol® reagent was from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the Transcriptor First Strand cDNA Synthesis kit, FastStart Universal SYBR® Green Master (Rox) and X-tremeGENE HP DNA transfection reagent were purchased from Roche Diagnostics (Basel, Switzerland). Rabbit monoclonal antibodies against β-catenin and β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Small interfering RNAs (siRNAs) targeting lincRNA-p21 and β-catenin transcripts were purchased from Thermo Fisher Scientific, Inc. WST-1 Cell Proliferation and Cytotoxicity Assay kit, Mitochondrial Membrane Potential Assay kit with JC-1 and Reactive Oxygen Species Assay Kit were purchased from Beyotime Technology (Jiangsu, China). Superoxide Dismutase (SOD) Activity Colorimetric Assay and Lipid Peroxidation (Malondialdehyde; MDA) Assay kits were purchased from Abcam (Cambridge, UK). *N*-acetyl cysteine (NAC) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture and cell treatment. HL-1 murine cardiomyocytes were a kind gift from Dr William C. Claycomb. Cells were maintained in fibronectin-coated flasks supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine and maintained semi-confluent at all times. The treatment was carried out by exposing the cell culture to 5 μM Dox for short periods of time.

WST-1 proliferation assay. HL-1 cells were plated at a density of $1x10^5$ cells/well in a 96-well plate. The cells were analyzed at 0, 24, 48 and 72 h, using the WST-1 assay. In brief, the cells were incubated with WST-1 at a concentration of 10 nM for 2 h. The reaction product was quantified by measuring absorbance using an ELISA reader at 440 and 690 nm. Data were analyzed using absorbance analysis software.

MTT assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. Briefly, 300 μl of MTT reagent was added to each well 2 h prior to harvesting. The supernatant was then removed and cells were incubated with 400 μl of dimethylsulfoxide for 10 min. Absorbance at 540 nm was recorded using the ELISA plate reader. Three repeats were performed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression levels of several genes were analyzed with RT-qPCR. Briefly, total cellular RNA was isolated using TRIzol® reagent and reversed transcribed using the transcriptor First Stand cDNA Synthesis Kit according to the manufacturer's instructions. RT-qPCR was carried out using the Fast Start Universal SYBR Master and fluorescence quantitative PCR system. The quantification number of cycles (Cq) was set within the exponential phase of the PCR. The ΔCq value for each target gene was calculated by subtracting the Cq value of *GAPDH* (internal control) from the target gene. Relative gene expression levels were calculated by comparing the ΔCq values between control and experimental conditions for each target PCR using the following equation: Relative gene expression=$2^{-(\Delta Cq\ sample-\Delta Cq\ control)}$. The primer pairs used to detect the mRNA levels of target genes are listed in Table I.

Relative telomere length measurement. Relative telomere length quantification in HL-1 cells was performed using a qPCR approach based on a previously established method (25),

Table I. Primer sequences.

Genes	Sequences
LincRNA-p21	F: 5'-CCTGTCCACTCGCTTTC-3' R: 5'-GGAACTGGAGACGGAATGTC-3'
p53	F: 5'-GGATGCCCATGCTACAGAGGAGTCT-3' R: 5'-GTCTGAGTCAGGCCCCACTTTCTTG-3'
p16	F: 5'-TTGGCCCAAGAGCGGGGACA-3' R: 5'-GCGGGCTGAGGCCGGATTTA-3'
telomere length	F: 5'-TGAAAGTAGAGGATTGCCACTG-3' R: 5'-AGCCAGAACAGGAACGTAGC-3'
β-catenin	F: 5'-TAGTGTGACAAGCTGAGTATGCGA-3' R: 5'-CTGGAGCGTCTGATGAG-3'
GAPDH	F: 5'-GGAGCCAAAAGGGTCATCAT-3' R: 5'-GTGATGGCATGGACTGTGGT-3'
siRNA-LincRNA-p21	UGAAAAGAGCCGUGAGCUA
siRNA-β-catenin	CTCACTTGCAATAATTACAAA
siRNA-NT	CTCUCCGAACGUGUCACGUTT

LincRNA-p21, long intergenic noncoding RNA-p21; siRNA, small interfering RNA; siRNA-NT, non-target-specific small interfering RNA.

with *GAPDH* as the normalizing gene. The primer pairs used to detect telomere length are listed in Table I.

Relative telomerase activity measurement. Telomerase activity of HL-1 cells was examined using the Telo TAGGG Telomerase PCR ELISA PLUS kit according to the manufacturer's instructions. Cell lysates were centrifuged for 20 min at 4˚C and 3 μl of cell extract were used for each telomeric repeat PCR amplification reaction and 3 μl of inactivated cell lysate were used for Telomeric Repeat Amplification Protocol (TRAP) reactions according to the manufacturer's recommendations. Each TRAP reaction was performed with amplification of an internal control from the kit to validate the absence of a PCR inhibitor. Using the ELISA method, the amplified products were immobilized on streptavidin-coated microtiter plates via biotin-streptavidin interaction. Thereafter, the amplifications were detected by anti-digoxigenin antibodies conjugated to peroxidase. After addition of the peroxidase substrate (3,3',5,5'-tetramethylbenzidine), the amount of TRAP products was determined by measurement of absorbance at 450 nm using the ELISA plate reader.

Western blot analysis. To obtain total protein, HL-1 cells were lysed with ice-cold lysis buffer (Beyotime Biotechnology). Expression of β-catenin and β-actin were evaluated by western blot. Cellular extracts were prepared according to the manufacturer's instruction. Protein samples were quantified and separated with SDS-PAGE. Western blot assay was performed as described previously (26).

Plasmid transfection. LincRNA-p21 siRNA, β-catenin siRNA and adenoviral vectors expressing lincRNA-p21 (Ad-lnc-p21) were designed and synthesized. Scrambled non-targeting siRNA (siRNA-NT) and adenoviral vectors expressing a control scrambled sequence (Ad-Ctrl) were used as negative controls. HL-1 cells were transfected using Lipofectamine® 2000 (Invitrogen) at a final concentration of 100 nM.

Evolution of $\Delta\Psi m$. Cells in a 96-well microtiter plate were grown at 37˚C for 1 day in complete culture medium to reach $1x10^5$ cells per well. The cells were then washed with PBS and loaded at 37˚C for 15 min with 5 μg/ml JC-1. After two wash cycles with PBS, the time-dependent JC-1 fluorescence was recorded using the ELISA plate reader. The fluorescent probe was excited at 490 nm and the emission was alternately read at 530 and 590 nm.

ROS measurement. Levels of intracellular ROS were determined using 2,7-dichlorodihydrofluorescein diacetate (Beyotine Institute of Biotechnology, Nantong, China), following the manufacturer's instructions. The fluorescence intensity of the cells was measured using a fluorescence spectrophotometer, with excitation and emission wavelengths of 488 and 525 nm, respectively.

SOD activity. SOD activity in HL-1 cells was determined using a colorimetric assay kit (Abcam) according to the manufacturer's protocol. Briefly, protein was isolated from HL-1 cells using lysis buffer, and SOD activity was measured in 10 μg of total protein extract. Absorbance was measured at 450 nm.

Lipid peroxidation assays. Lipid peroxidation was monitored using an assay kit (Abcam) to measure the formation of MDA according to the manufacturer's protocol. Briefly, HL-1 cells ($1x10^6$ cells) were homogenized on ice in 300 μl of MDA lysis buffer (with 3 μl of 100x butylated hydroxytoluene), then centrifuged (13,000 x g, 10 min) to remove insoluble material. The supernatant (200 μl) was added to 600 μl of thiobarbituric acid and incubated at 95˚C for 60 min. The samples were cooled to room temperature in an ice bath for 10 min, and the absorbance at 532 nm was measured spectrophotometrically.

Statistical analysis. Data were expressed as means ± standard deviation. Differences among groups were tested with one-way analysis of variance followed by Tukey's post hoc test, and comparisons between two groups were evaluated with Student's t-tests using SPSS package v19.0 (IBM, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

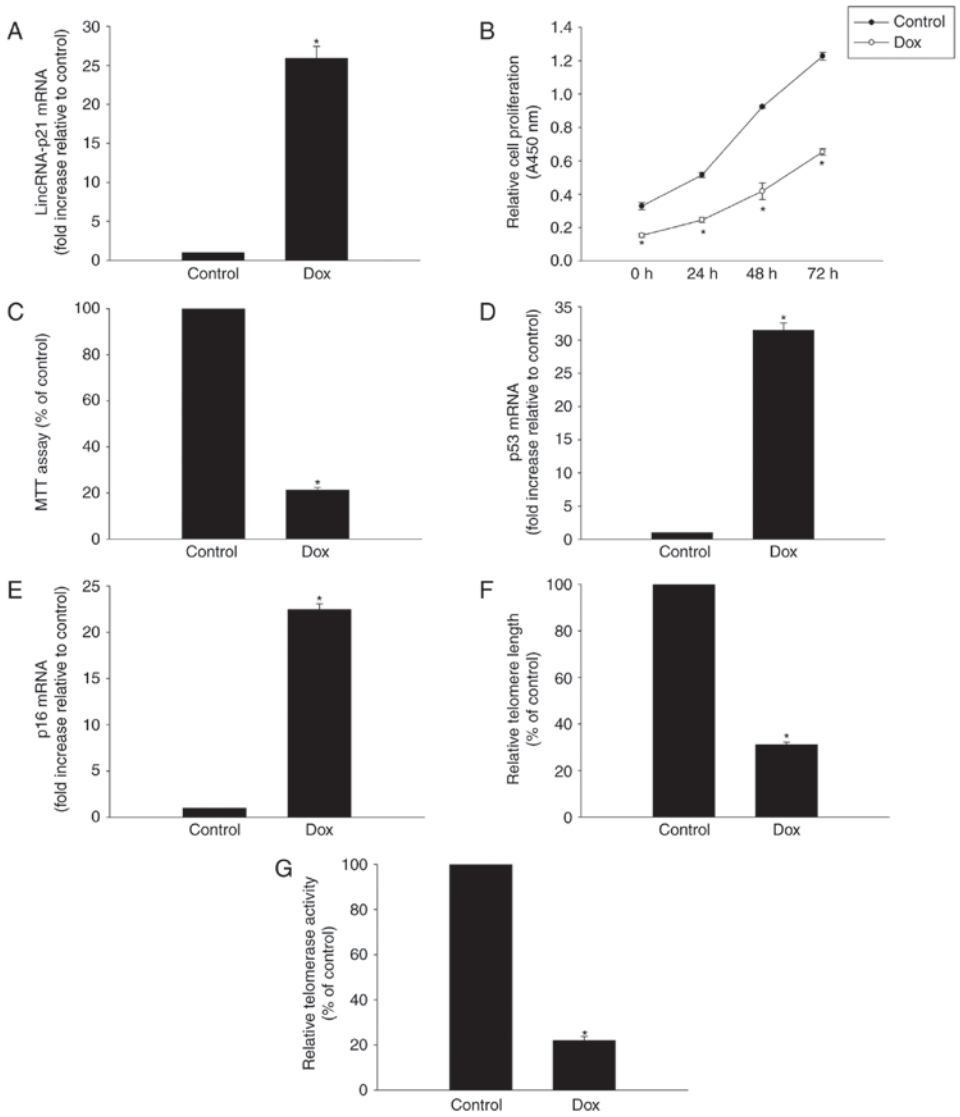

Figure 1. Dox induced cellular senescence and generation of lincRNA-p21. (A) RT-qPCR analysis of lincRNA-p21 mRNA levels in HL-1 cells after treatment with Dox. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control. (B) Proliferation growth curves of HL-1 cells incubated with Dox at concentrations of 5 μM, determined by the WST-1 proliferation assay. (C) Dox was added to culture media and cell viability was analyzed by MTT assay. (D-F) RT-qPCR analysis of p53 and p16 mRNA levels and telomere length in HL-1 cells treated with Dox. (G) Relative telomerase activity. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control.

Results

Dox induced cellular senescence and generation of lincRNA-p21. To determine whether lincRNA-p21 is involved in Dox-induced cardiac senescence, its expression was evaluated in HL-1 murine cardiomyocytes exposed to 5 μM Dox for 24 h. RT-qPCR analysis revealed a significant increase of linRNA-p21 in HL-1 cells following Dox treatment (Fig. 1A).

To further explore the role of Dox in inducing senescence in HL-1 cells, we tested HL-1 cell viability and proliferation and demonstrated that both proliferation and the percentage of viable cells were decreased following Dox treatment (Fig. 1B and C). Furthermore, expression of senescence-related genes p53 and p16 was markedly increased in the Dox treatment group (Fig. 1D, E). Finally, we demonstrated that treating cells with Dox resulted in decreasing telomere length and telomerase activity (Fig. 1F and G).

Modulating lincRNA-p21 attenuated cellular senescence induced by Dox. The role of lincRNA-p21 in cellular senescence induced by Dox was further investigated by knockdown of endogenous lincRNA-p21 by siRNA. LincRNA-p21 expression levels were significantly reduced by transfection with lincRNA-p21 siRNA compared with siRNA-NT (Fig. 2A). Knockdown of lincRNA-p21 was associated with significantly increased proliferation and cellular viability of HL-1 cells (Fig. 2B and C) compared with the HL-1 cells treated with Dox only. In addition, inhibition of lincRNA-p21 in the presence of Dox decreased the expression of senescence-related genes p53 and p16 (Fig. 2D and E), while telomere length and telomerase activity increased (Fig. 2F and G), compared to cells treated with Dox without inhibition of lincRNA-p21. In contrast, siRNA-NT treatment did not attenuate cellular senescence induced by Dox.

LincRNA-p21-β-catenin signaling was involved in Dox-related cellular senescence. LincRNA-p21 has previously been

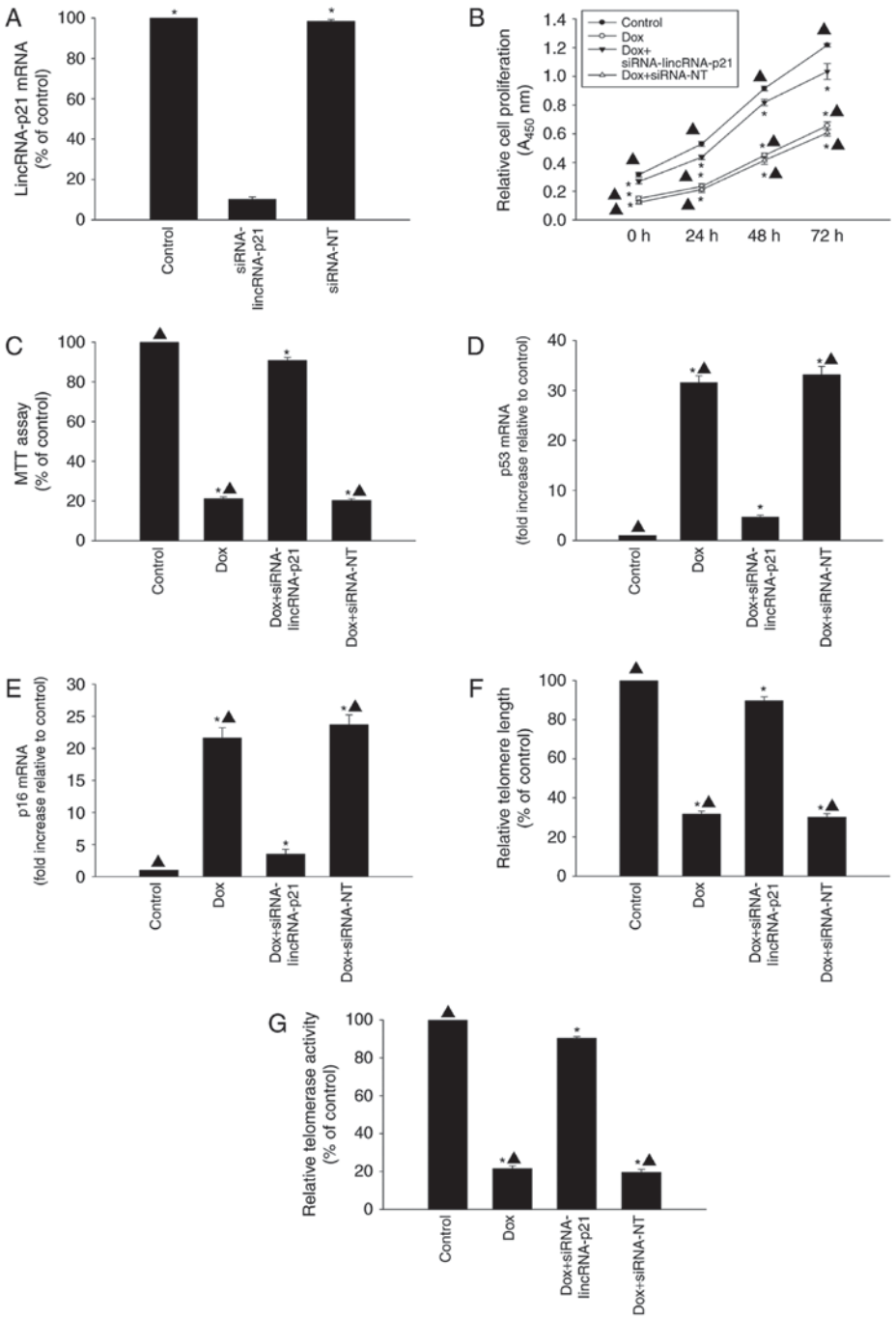

Figure 2. Modulating lincRNA-p21 attenuated cellular senescence induced by Dox. (A) RT-qPCR analysis of lincRNA-p21 mRNA levels in untransfected HL-1 cells and HL-1 cells transfected with lincRNA-p21-specific siRNA or control siRNA-NT; *P<0.05 vs. siRNA-lincRNA-p21. (B-G) HL-1 cells were transfected with siRNA-lincRNA-p21 or siRNA-NT in the presence of Dox. (B) Proliferation of HL-1 cells determined by the WST-1 proliferation assay. (C) Cell viability analyzed by MTT assay. (D-F) RT-qPCR analysis of p53and p16 mRNA level and telomere length in HL-1 cells. (G) Relative telomerase activity. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control, ▲P<0.05 vs. Dox+siRNA-lincRNA-p21.

demonstrated to reduce β-catenin protein levels in CSCs (17). In the present study, β-catenin protein expression levels were decreased in the Dox-treated group when compared with the control group; however, after silencing lincRNA-p21, β-catenin protein expression levels increased (Fig. 3A and B). To further investigate the mechanism underlying the effect of lincRNA-p21on Dox-related cellular senescence, we used siRNA to silence β-catenin. β-catenin mRNA expression levels were significantly reduced in cells transfected with siRNA-β-catenin compared to transfection with siRNA-NT control (Fig. 3C). Knockdown of lincRNA-p21 reversed the decrease in proliferation and viability and the increase in expression of senescence-related genes p53 and p16 in HL-1 cells induced by Dox (Fig. 3D-G); it also increased telomere length and telomerase activity (Fig. 3H and I). However, these effects were abolished by silencing β-catenin.

LincRNA-p21 participated in cellular senescence by inducing oxidative stress. To determine whether lincRNA-p21 induced cellular senescence by increasing oxidative stress in the

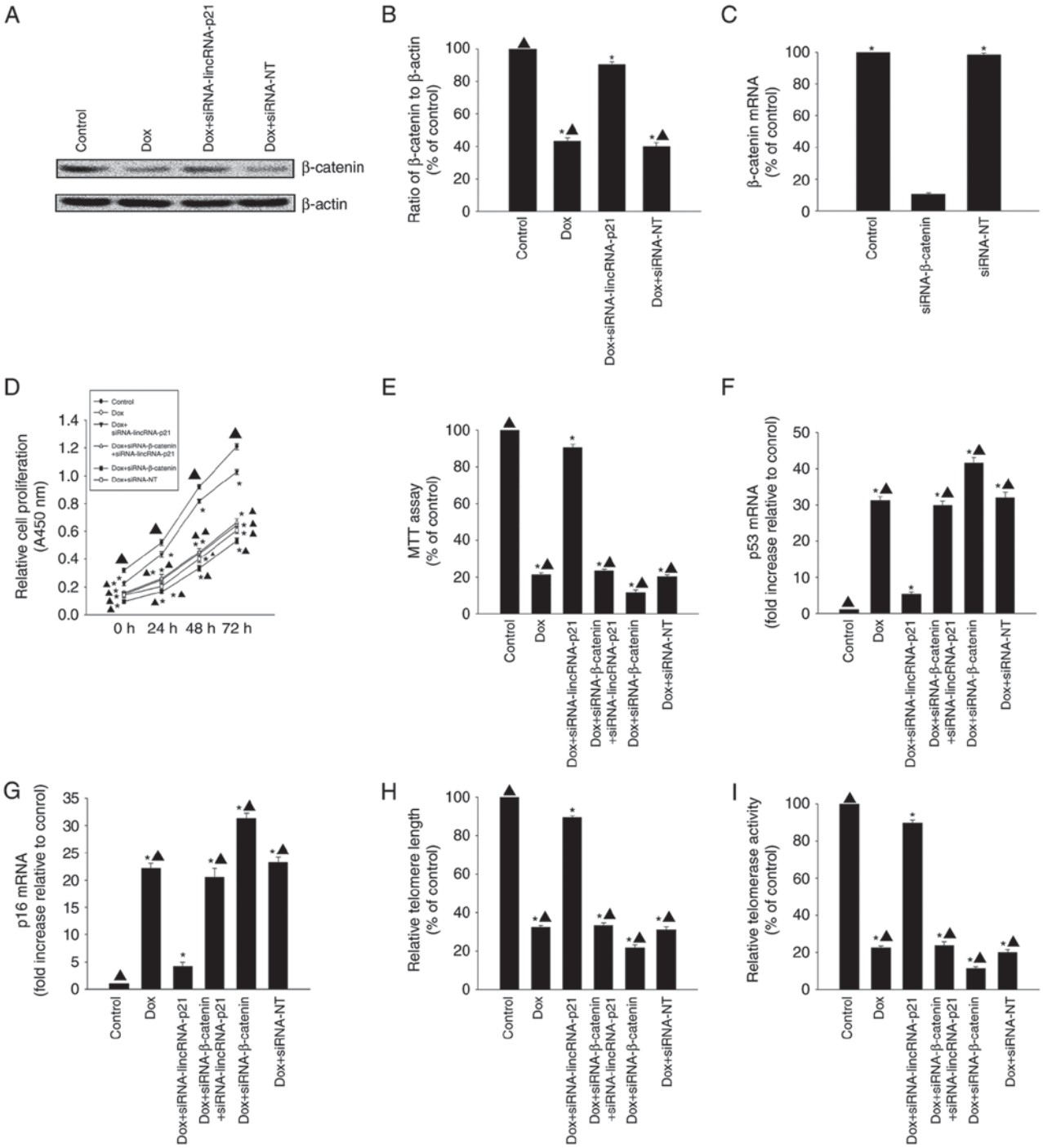

Figure 3. The lincRNA-p21-β-catenin signaling pathway was involved in Dox-related cellular senescence. (A) Image and (B) quantification of western blotting of β-catenin protein expression levels in untransfected HL-1 cells and HL-1 cells transfected with lincRNA-p21-specific siRNA or siRNA-NT in the presence of Dox. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control, ▲P<0.05 vs. Dox+siRNA-lincRNA-p21. (C) β-catenin mRNA levels in HL-1 cells transfected with siRNA-β-cateninor siRNA-NT; *P<0.05 vs. siRNA-β-catenin. (D-I) HL-1 cells were treated with Dox, or transfected with siRNA-lincRNA-p21, siRNA-lincRNA-p21+siRNA-β-catenin, siRNA-β-catenin or siRNA-NT in the presence of Dox. (D) Proliferation determined by the WST-1 proliferation assay. (E) Cell viability analyzed with the MTT assay. (F-H) RT-qPCR analysis of p53 and p16 mRNA levels and telomere length in HL-1 cells. (I) Relative telomerase activity. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control, ▲P<0.05 vs. Dox+siRNA-lincRNA-p21.

presence of Dox, we examined mitochondrial transmembrane potential, generation of ROS, activation of SOD and lipid peroxidation by MDA assay. Dox significantly decreased mitochondrial transmembrane potential (Fig. 4A) and activation of SOD (Fig. 4C) while increasing generation of ROS (Fig. 4B) and MDA activation (Fig. 4D). However, after silencing lincRNA-p21, mitochondrial transmembrane potential and the activation of SOD were increased, but generation of ROS and MDA activation were decreased. siRNA against β-catenin was a potent blocker of the inhibitory effect of siRNA-lincRNA-p21 on oxidative stress, resulting in increased generation of ROS and MDA activation while

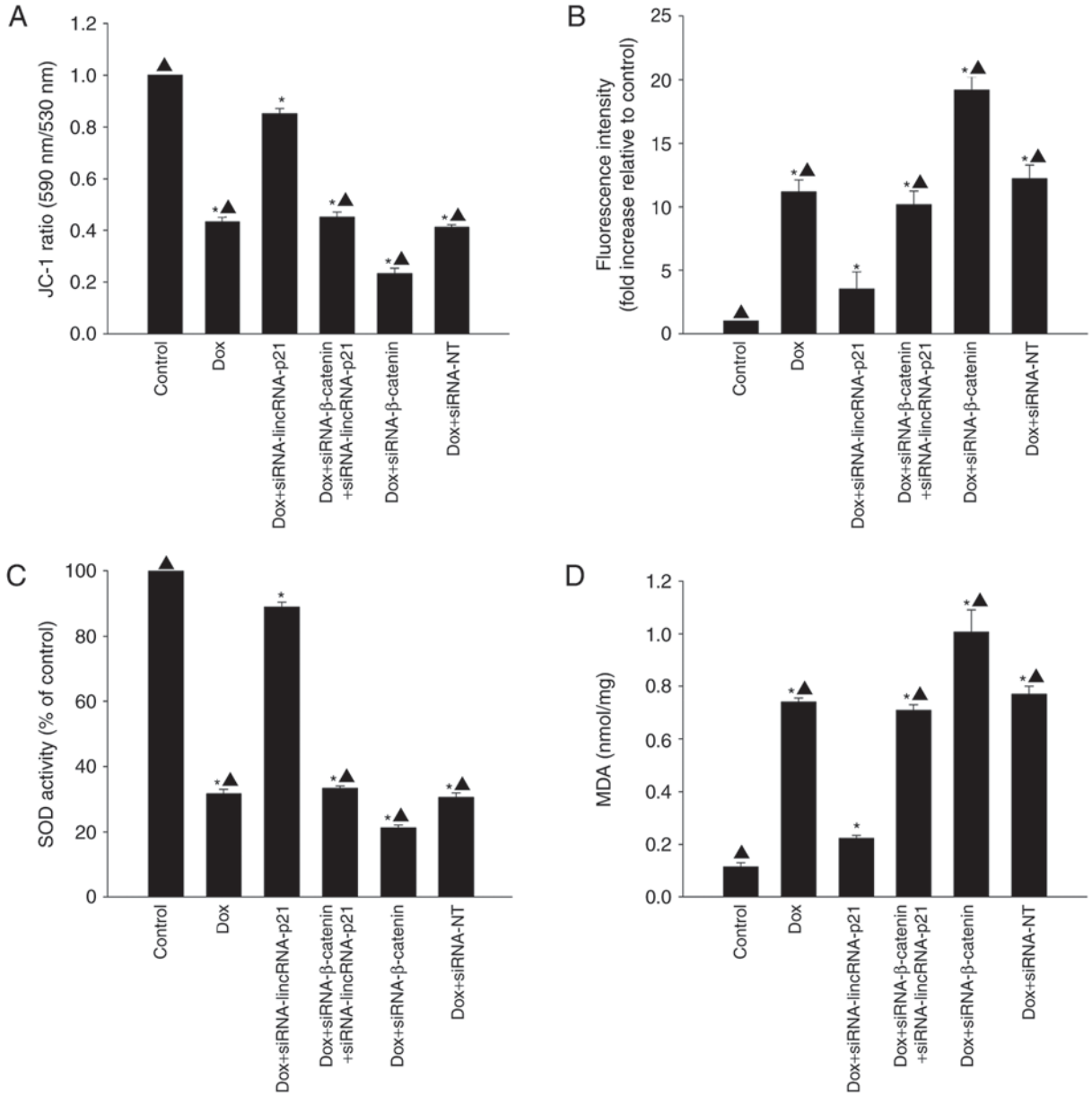

Figure 4. LincRNA-p21 participated in cellular senescence by inducing oxidative stress. HL-1 cells were treated with Dox or transfected with siRNA-lincRNA-p21, siRNA-lincRNA-p21+siRNA-β-catenin, siRNA-β-catenin or siRNA-NT in the presence of Dox. (A) Mitochondrial membrane potential measured using JC-1 stain. (B) Intracellular ROS production analyzed by fluorescence spectrophotometry. (C) SOD activity evaluated by colorimetric assay. (D) Lipid peroxidation evaluated by MDA formation. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control, ▲P<0.05 vs. Dox+siRNA-lincRNA-p21.

decreasing mitochondrial transmembrane potential and activation of SOD (Fig. 4).

Antioxidant treatment suppressed cellular senescence induced by Dox. To confirm that the effects of exogenous Dox on cellular senescence were specifically due to lincRNA-p21-induced oxidative stress, we investigated the effects of the antioxidant agent NAC on cellular senescence in Dox-treated HL-1 cells. NAC treatment apparently decreased the generation of ROS in Dox-treated HL-1 cells (Fig. 5A). We then overexpressed lincRNA-p21 using Ad-lnc-p21 transfection (Fig. 5B). We found that NAC reversed the decrease in proliferation and viability and the increased expression of senescence-related genes p53 and p16 in HL-1 cells induced by Dox (Fig. 5C-F), and also increased telomere length and telomerase activity (Fig. 5G and H); however, these effects were abolished by lincRNA-p21 overexpression.

Discussion

Dox is among the most widely used chemotherapeutic agents and has been shown to be effective in a wide range of tumors (27). However, the clinical effectiveness of Dox is hampered by the development of cardiotoxicity that negatively affects patients' outcomes and severely limits the oncologic therapeutic opportunities (28). Numerous studies have probed the molecular mechanisms of Dox-related cardiomyopathy. As a result, a number of molecular elements have been implicated in the pathogenesis of Dox cardiotoxicity, including DNA and mitochondrial damage and accumulation

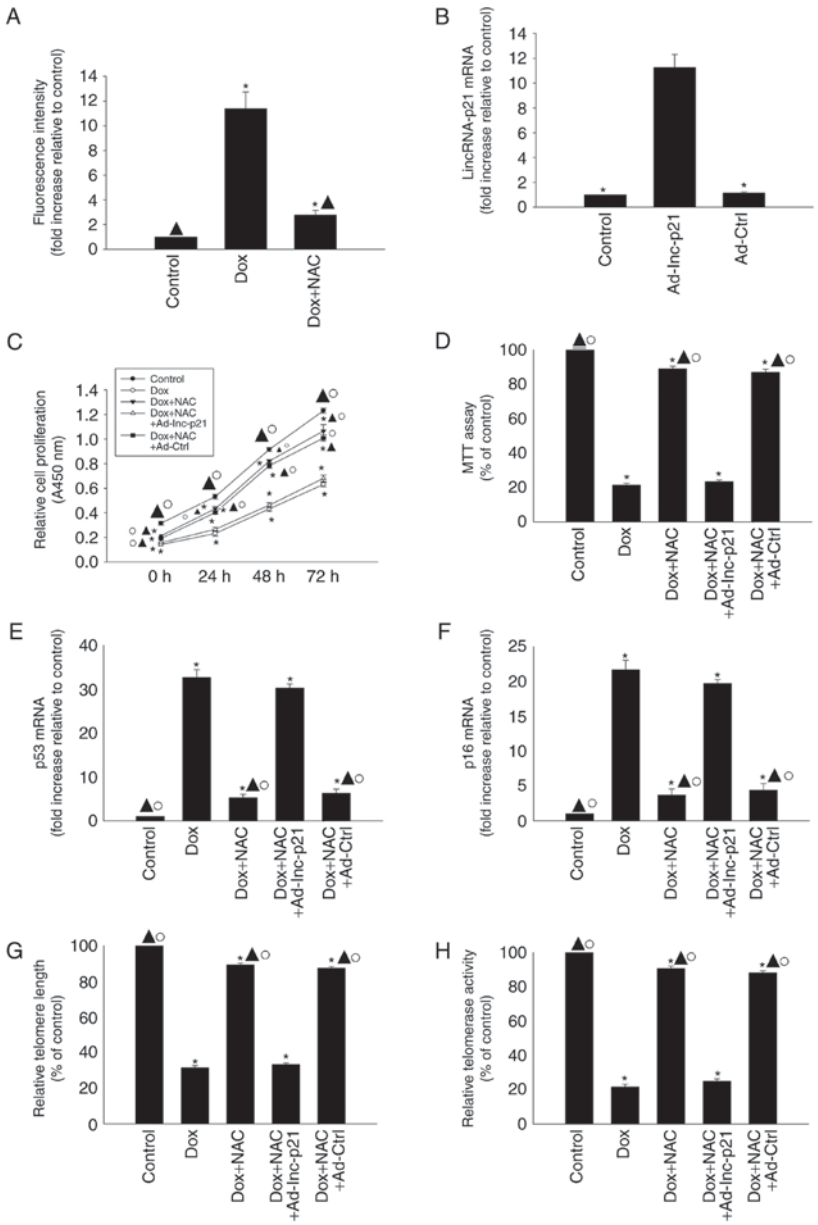

Figure 5. Antioxidant treatment suppressed cellular senescence induced by Dox. (A) Intracellular ROS production analyzed with fluorescence spectrophotometry. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control, ▲P<0.05 vs. Dox. (B) RT-qPCR analysis of lincRNA-p21 mRNA levels in untransfected HL-1 cells and HL-1 cells transfected with adenoviral vectors expressing lincRNA-p21 or a control scrambled sequence; *P<0.05 vs. Ad-lnc-p21. (C-H) HL-1 cells were treated with Dox, Dox+NAC, or were transfected with Ad-lnc-p21 or Ad-Ctrl and treated with NAC in the presence of Dox. (C) Proliferation determined by the WST-1 proliferation assay. (D) Cell viability analyzed by MTT assay. (E-G) RT-qPCR analysis of p53 and p16 mRNA levels and telomere length in HL-1 cells. (H) Relative telomerase activity was measured. Data represent means ± standard deviation from three independent experiments; *P<0.05 vs. Control, ▲P<0.05 vs. Dox, ◦P<0.05 vs. Dox+NAC+Ad-lnc-p21.

of ROS (29,30). These molecular effects indicated that cardiac cellular senescence played a substantial role in Dox-induced cardiomyopathy (31). As previous studies showed that treatment with Dox extensively generated reactive oxidative stress leading to cardiac senescence, these findings confirmed that a number of senescence- and stress-associated proteins and genes were involved in Dox-induced cardiomyopathy (5,32). In our study, we found that treatment with Dox induced apparent cellular senescence, showing that increased expression of senescence related genes p53 and p16 was accompanied by impaired cellular proliferation and viability. Cellular senescence induced by Dox is defined as the arrest of cell cycle progression which can be caused by telomere shortening (5), in agreement with our results finding that treatment of HL-1cells with Dox induced shortening of telomere length and decreased telomerase activity.

Epigenetic modifications can also be mediated by lncRNAs, which play major roles in regulation of gene transcription, chromatin structure and mRNA stability during cell development and diseases (33). They are also involved in the regulation of different cellular functions such as genome maintenance, post-transcriptional modifications, structural maintenance of cellular processes and translational control (34,35). Several lncRNAs have recently been suggested to be involved in the regulation of senescence, and recent research has revealed that lncRNA HOTAIR overexpression reduced adipogenic

differentiation of MSCs, inducing senescence-associated changes (36). In addition, lncRNAs also take part in the process of cardiac senescence, as related research has confirmed that the mitochondrial lncRNA ASncmtRNA-2 is induced in aging and replicative senescence in vascular cells (37). As an important regulator of the cellular senescence process, lincRNA-p21 exerts its effect in multiple ways. A previous study suggested that overexpression of lincRNA-p21 increased p21 expression at both mRNA and protein levels and impeded cell-cycle progression, and thus was involved in cellular senescence (14). LincRNA-p21 has been identified as a regulator of p53 expression, and reciprocally p53 can regulate the expression of lincRNA-p21, which plays a major role in pro-senescence networks (12). LincRNA-p21 is necessary for the recruitment of hnRNPK to the p53 response element and for increasing the binding efficiency of p53 in the p21 promoter region, promoting cellular senescence (38). In the present study, we have characterized the expression profile of lincRNA-p21 in HL-1 cells treated with Dox and found that its expression was closely related to HL-1 cellular senescence induced by Dox. Treatment with Dox induced increased expression of lincRNA-p21 accompanied by decreased cellular proliferation, viability, telomere length and telomerase activity, while increasing expression of senescence-related genes p53 and p16. Furthermore, this senescent condition was reversed by silencing lincRNA-p21, further confirming the pro-senescent effect of lincRNA-p21.

Given the impact of cellular senescence in Dox-associated cardiomyopathy processes, there is much interest in understanding how to modulate senescence for therapeutic purposes. The Wnt/β-catenin pathway is closely related to cardiac senescence, as previous research has revealed a marked decrease in β-catenin in mouse hearts 8 weeks before the mice developed cardiomyopathy at 21 weeks of age after infection with the coxsakie virus (39). Also, sustained inhibition of the Wnt/β-catenin pathway was reported to be involved in Dox-induced cardiomyopathy processes (18). In our study, as indicated by the results of western blots, expression of β-catenin was apparently decreased during Dox treatment. As a target of lincRNA-p21, β-catenin protein has been shown to be directly downregulated by lincRNA-p21 in various cell types, and vector-delivered lincRNA-p21 preferentially blocked the activation of Wnt/β-catenin signaling in CSCs (17). The viability, self-renewal and tumorigenicity of CSCs in this study were compromised by lincRNA-p21 overexpression. It has also been reported that lincRNA-p21 inhibited the Wnt/β-catenin pathway so that inhibition of lincRNA-p21 caused decreased proliferation of hepatic stellate cells (40). In agreement with these previous findings, our research confirmed that treatment with Dox inhibited Wnt/β-catenin signaling, which was reversed by silencing lincRNA-p21. In contrast, inactivating the Wnt/β-catenin pathway using siRNA blocked the anti-senescent effect of silencing lincRNA-p21, as indicated by decreased cellular proliferation and viability, reduced telomere length and telomerase activity and increased expression of p53 and p16.

Oxidative stress has been shown to be a central mediator of cellular senescence (41). Cellular senescence is accompanied by ROS generation, increased oxidant enzyme activity and diminished antioxidant enzyme activity (42). Dox-induced oxidative stress triggered cardiotoxicity leading to cardiomyopathy and heart failure (20). Several theories, including mitochondrial dysfunction, increased ROS production and contractile failure have been proposed as plausible underlying mechanisms for Dox-induced cardiomyopathy (43,44). Regulatory lncRNAs have been identified as key modulators of senescence, oxidative stress-induced apoptosis and cell cycle arrest and have great effects during the cellular senescence process (12,45). LincRNA-p21 was associated with cellular DNA damage and endoplasmic reticulum stress under oxidative stress, thus inducing growth regression of HepG2 cells and apoptosis of hepatocellular carcinoma cells (23). Herein, we found that treatment with Dox induced oxidative stress, decreased mitochondrial transmembrane potential and activation of SOD, while increasing generation of ROS and stimulating MDA activation. These oxidative effects were relieved by silencing lincRNA-p21. To further confirm the alleviation of oxidative stress by lincRNA-p21 in Dox-induced cardiac senescence, we used the antioxidant NAC to alleviate ROS generation and found that it could ameliorate cellular senescence induced by Dox. In addition, the ameliorative effects were abolished by overexpression of lincRNA-p21, confirming that oxidative stress relieved by lincRNA-p21 played a substantial role in Dox-induced cardiac senescence.

In conclusion, our study indicated that lincRNA-p21 is involved in cardiac cellular senescence. Enhancing lincRNA-p21 expression may relieve cardiac senescence induced by Dox by regulating oxidative stress via the Wnt/β-catenin signaling pathway. We demonstrated that attenuation of cardiac senescence may have important therapeutic implications for Dox-induced cardiomyopathy. Targeting lincRNA-p21 expression in cardiomyocytes may be a useful strategy in treatment of Dox-induced cardiomyopathy.

Acknowledgements

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