Transcriptional activation of ENPP2 by FoxO4 protects cardiomyocytes from doxorubicin-induced toxicity

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Abstract. It has been shown that ferroptosis is involved in doxorubicin (DOX)-induced cardiotoxicity and that ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) can protect cardiomyocytes from ferroptosis. Thus, the present study aimed to investigate whether ENPP2 could protect cardiomyocytes from DOX-induced injury by inhibiting ferroptosis. H9c2 cardiomyocytes were exposed to various concentrations (0.625, 1.25, 2.5, 5 or 10 μM) of DOX for different time periods. Cell viability and ENPP2 expression were determined. ENPP2-overexpressing H9c2 cells were treated with DOX and subsequently cell viability, oxidative stress, autophagy and ferroptosis were measured using the corresponding assays (MTT assay, commercial kits and western blot analysis). Dual-luciferase reporter and chromatin immunoprecipitation assays, as well as bioinformatics analysis, were applied to detect the interaction between ENPP2 and FoxO4. Following FoxO4 overexpression in H9c2 cells, the aforementioned cellular processes were assessed. The results indicated that ENPP2 expression was downregulated following treatment of the cells with DOX. DOX also led to the decreased cell viability, reduced autophagy and elevated ferroptosis in H9c2 cells, which were notably reversed by ENPP2 overexpression. In addition, FoxO4 bound to the ENPP2 promoter, resulting in inhibition of its expression. Following FoxO4 overexpression in H9c2 cells, further experiments conducted using commercial kits and western blot analysis revealed that FoxO4 overexpression partially inhibited the effects of ENPP2 overexpression on DOX-induced oxidative stress, autophagy and ferroptosis in H9c2 cells. In conclusion, the data indicated that ENPP2 was transcriptionally regulated by FoxO4 to protect cardiomyocytes from DOX-induced toxicity by inhibiting ferroptosis. Therefore, specific treatment approaches targeting the FoxO4/ENPP2 axis and ferroptosis may provide potential therapies for alleviating DOX-induced cardiotoxicity.

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Introduction

Cardiovascular complications due to cancer therapy cause a significant reduction in treatment efficacy for patients with cancer (1). Doxorubicin (DOX) is a potent anthracycline that has been confirmed to cause long-term cardiovascular side effects and decrease the quality of life of cancer survivors, which eventually reduces the clinical application of DOX (2). Among the diverse mechanisms involved in the cardiotoxicity of DOX, the contribution of ferroptosis has been recently reported by an increasing number of studies (3-5).

Ferroptosis is a novel type of regulated cell death that is different from apoptosis and necrosis. It is an iron- and reactive oxygen species (ROS)-dependent cell death, characterized by the accumulation of ROS and inactivation of the cellular antioxidant glutathione (GSH), leading to redox dysregulation (6). Ferroptosis has been implicated in the pathological processes associated with carcinogenesis, degenerative diseases, stroke and kidney ischemia/reperfusion injury (7). Recently, ferroptosis was shown to exhibit a crucial role in DOX-induced cardiotoxicity. For instance, it has been documented that ferroptosis promoted DOX-induced cardiomyopathy and mortality in mice, whereas the ferroptosis inhibitor ferrostatin-1 and iron chelation ameliorate this process and effectively improve the survival rate of mice (4). In addition, mitochondrial ferroptosis has been shown to be a major cause of DOX-induced cardiotoxicity (2). It has also been reported that overexpression of GSH peroxidase 4 (GPX4) ameliorated cardiac impairment in mice, whereas GPX4 knockdown exacerbated this process (3). Therefore, inhibition of ferroptosis may effectively reduce DOX-induced cardiotoxicity.

Autotaxin (ATX) is a secreted enzyme encoded by the ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) gene, which is important for generating lysophosphatidic acid (LPA). The ATX/LPA pathway serves a major role in embryonic, vascular and neuronal development (8). Disturbances in normal ATX/LPA signaling are associated with the development of multiple diseases, including cardiovascular disease (9). A previous study reported that ENPP2/LPA protected

cardiomyocytes from erastin-induced ferroptosis, indicating the inhibitory effect of ENPP2 on ferroptosis induction in cardiomyocytes (10). The transcription factor FoxO4, which is a member of the FoxO transcription factor family, is predicted to bind to the promoter of ENPP2. The FoxO proteins are involved in a variety of biological processes, including cell proliferation, oxidative stress response, metabolism, immunity and apoptosis (11). It has been suggested that FoxO4 may be involved in aggravating cardiovascular diseases (12). FoxO4 was also reported to regulate DOX-induced toxicity in liver and kidney cells both *in vivo* and *in vitro* (13). In the present study, it was hypothesized that FoxO4 may be a transcriptional regulator of ENPP2 by inhibiting ferroptosis, which in turn can protect cardiomyocyte H9c2 cells against DOX-induced injury.

Materials and methods

Cell culture and treatment. H9c2 rat cardiomyocytes were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were exposed to 0.625, 1.25, 2.5, 5 or 10 μM DOX (Sigma-Aldrich; Merck KGaA) at 37˚C for 12, 24 or 48 h to detect cell viability. Cells were treated with 0.625, 1.25, 2.5, 5 or 10 μM DOX (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C or with 5 μM DOX for 12, 24 and 48 h at 37˚C to determine the mRNA and protein expression of ENPP2. In subsequent experiments, 5 μM DOX was used for 24 h to treat H9c2 cells.

Cell transfection. ENPP2 and FoxO4 were overexpressed in H9c2 cells to produce overexpressing (Oe)-ENPP2 and Oe-FoxO4 cell lines. The rat ENPP2 and FoxO4 cDNA sequences were synthesized by GenScript and inserted into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). An empty pcDNA3.1 vector was used as the negative control (NC). H9c2 cells were seeded into 6-well plates at the density of $4x10^5$ cells/well 10 h prior to transfection, and the vectors (2.5 μg) were transfected into cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, at 37˚C for 48 h. At 48 h post-transfection, the cells were exposed to 5 μM DOX for 24 h at 37˚C and collected for subsequent analysis. Control cells were devoid of treatment.

Cell viability assessment. Cell viability was determined using the MTT assay kit (Beyotime Institute of Biotechnology). Briefly, control or transfected H9c2 cells were seeded into 96-well plates at the density of $2x10^3$ cells/well. Following treatment with DOX for 12, 24 or 48 h, the culture medium was replaced with DMEM without DOX and then added to 10 μl MTT solution at 37˚C for 4 h. The cells were incubated with formazan lysis solution at room temperature for 10 min until the purple crystals were completely dissolved. Finally, the absorbance was measured at 570 nm using a spectrophotometer.

Oxidative stress measurement. To detect cellular total ROS status, the $2x10^3$ cells/well were seeded into 96-well plates and stained with 2',7'-dichlorodihydrofluorescein diacetate (Abcam) at 37˚C for 45 min. Fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm using a multimode plate reader (PerkinElmer, Inc.).

The increase in ROS levels was also examined using a commercially available thiobarbituric acid reactive substances (TBARS) assay kit (cat. no. 700870; Cayman Chemical Company) according to the manufacturer's instructions. GSH peroxidase (GSH-Px) activity was measured using a GSH-PX assay kit (cat. no. A005-1-2; Nanjing Jiancheng Bioengineering Institute) in accordance with the manufacturer's instructions.

Measurement of Fe^{2+} activity. Cellular Fe^{2+} activity was measured using the Iron Assay kit (Abcam) according to the manufacturer's instructions. Briefly, control or transfected H9c2 cells were seeded into 96-well plates at the density of $1x10^3$ cells/well. Following treatment with 5 μM DOX for 24 h at 37˚C, 5 μl assay buffer was added to each sample and incubated at 37˚C for 30 min. Following addition of 100 μl Iron Probe to each well and incubation at 37˚C for 60 min in the dark, the optical density (593 nm) was measured using a colorimetric microplate reader.

Dual-luciferase reporter assay. The JASPAR database (http://jaspar.genereg.net/) was searched and FoxO4 was identified as a potential target that could bind to the promoter of ENPP2. To confirm the interaction between FoxO4 and the ENPP2 promoter, a dual-luciferase reporter assay was performed. Wild-type or mutant ENPP2 E1 and E2 sequences were amplified and cloned downstream of the luciferase reporter gene in pMIR-REPORT luciferase vectors (Thermo Fisher Scientific, Inc.). Subsequently, they were co-transfected with pcDNA3.1-FoxO4 (Oe-FoxO4; 2.5 μg) or Oe-NC (2.5 μg) using Lipofectamine 2000 reagent. At 48 h post-transfection, luciferase activity was measured with a dual-luciferase reporter system (Promega Corporation). *Renilla* luciferase activity was used for normalization.

Chromatin immunoprecipitation (ChIP) assay. H9c2 cells were crosslinked with 1% formaldehyde for 10 min at 37˚C and a ChIP assay was performed with a high-sensitivity kit (Abcam). Cells were washed with cold PBS and suspended in 1 ml PBS containing 5 μl protease inhibitors. Cells were centrifuged at 4˚C at 716 x g for 5 min. A total of 300 μl SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl pH 8.0) was then used to lyse the cells, which were subsequently sonicated at 150 Hz and sheared with four sets of 10 sec pulses on wet ice using a high intensity ultrasonic processor. An equal amount of chromatin (100 μl) was immunoprecipitated at 4˚C overnight. The antibodies used in this assay included anti-FoxO4 (cat. no. ab128908; 1:50; Abcam) and IgG (as the NC; cat. no. ab172730; 1:50; Abcam). Immunoprecipitated products were collected after incubation with magnetic beads coupled with anti-IgG or anti-FoxO4. The beads were washed using a magnetic separation rack and the bound chromatin was eluted in ChIP Elution Buffer with Proteinase K mixer. The primer sequences used for ENPP2 detection were as follows: Forward 5'-TTCCAATGTACCCCGCCTTC-3' and reverse

5'-AGCTGCCTTCCACATACTGTT-3'. The recovered DNA fragments were evaluated via RT-qPCR. The relative level of ENPP2 was normalized according to the average level of the IgG group.

Lipid ROS measurement. Lipid ROS generation was measured by adding C11-BODIPY (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 1.5 μM for 20 min at 37˚C before cell harvesting, according to the manufacturer's instructions. Lipid ROS-positive cells were finally assessed using a FACSCanto II flow cytometer (BD Biosciences). The lipid ROS can be differentiated by adding C11-BODIPY, which is a lipid soluble, ratio type fluorescent probe used to indicate lipid peroxidation and antioxidant properties in model membrane systems and in living cells (14).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from H9c2 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The sample concentration was quantified via spectrophotometry. Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. qPCR was performed using a SYBR® Premix Ex Taq kit (Takara Bio, Inc.) in accordance with the manufacturer's instructions. The thermocycling conditions were as follows: Initial denaturation for 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 30 sec; and a final extension of 10 min at 72˚C. The fold difference in gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (15) following normalization to the expression levels of GAPDH mRNA. The primer sequences were as follows: ENPP2 forward, 5'-TTCCAATGT ACCCCGCCTTC-3' and reverse, 5'-AGCTGCCTTCCACAT ACTGTT-3'; FoxO4 forward, 5'-AGCGACTGACACTTGCCC AGAT-3' and reverse, 5'- AGGGTTCAGCATCCACCAAGA G-3'; and GAPDH forward, 5'-CGTGCCGCCTGGAGAA-3' and reverse, 5'-CCCTCAGATGCCTGCTTCAC-3'.

Western blot analysis. Total proteins were isolated from H9c2 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology) and proteins (40 μg/lane) were separated via 12% SDS-PAGE and subsequently transferred to a PVDF membrane. The membranes were blocked with 10% non-fat dry milk at room temperature for 2 h and incubated with primary antibodies at 4˚C overnight. Following the primary antibody incubation, the membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies. The immunoreactive bands were visualized using DAB (Wuhan Boster Biological Technology, Ltd.). GAPDH was used as a loading control, which was in accordance with the previous studies showing that GAPDH expression could be used as a loading control in for DOX investigation (16-18). Densitometric analysis was performed using ImageJ software (version 1.52r; National Institutes of Health). Anti-LC3I/II (cat. no. 4108S; 1:1,000), anti-Beclin1 (cat. no. 3495T; 1:1,000), anti-autophagy related 5 (ATG5; cat. no. 12994T; 1:1,000), anti-p62 (cat. no. 23214S; 1:1,000) and anti-GAPDH (cat. no. 5174T; 1:1,000) antibodies were purchased from Cell Signaling Technology, Inc. Anti-FoxO4 (cat. no. ab128908; 1:1,000), anti-ENPP2 (cat. no. ab140915; 1:1,000), anti-solute carrier family 7 member 11 (SLC7A11; cat. no. ab175186; 1:1,000), anti-GPX4 (cat. no. ab125066; 1:1,000), anti-ferroportin 1 (FPN1; cat. no. ab239511; 1:1,000), anti-NADPH oxidase 4 (NOX4; cat. no. ab133303; 1:1,000), HRP-conjugated anti-mouse (cat. no. ab6728; 1:5,000) and HRP-conjugated anti-rabbit IgG (cat. no. ab6721; 1:5,000) antibodies were provided by Abcam.

Statistical analysis. All experiments were repeated three times and the results are presented as the mean ± SD. Data were analyzed using GraphPad Prism 8 software (GraphPad Software, Inc.). Comparisons between two groups were conducted using an unpaired Student's t-test, while comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ENPP2 expression is downregulated following DOX treatment. Different concentrations (0.625, 1.25, 2.5, 5 and 10 μM) of DOX were used to treat H9c2 cells for 12, 24 and 48 h, respectively. Cell viability was significantly reduced following DOX treatment in a time- and dose-dependent manner (Fig. 1A). In addition, the protein expression level of ENPP2 were significantly decreased compared with those of the control cells, following treatment of the cells with 2.5, 5 and 10 μM DOX for 24 h and treatment of the cells with 5 μM DOX for 24 and 48 h. In addition, the mRNA expression levels of ENPP2 were downregulated in cells following DOX treatment in a dose- and time-dependent manner (Fig. 1B-E). Moreover, 5 μM DOX treatment was used for 24 h to treat H9c2 cells in subsequent experiments.

Overexpression of ENPP2 enhances autophagy and inhibits ferroptosis induced by DOX in H9c2 cardiomyocytes. Overexpression of ENPP2 was achieved in H9c2 cells and the results verified the effective increase of ENPP2 protein and mRNA expression levels (Fig. 2A and B). Subsequently, control or ENPP2-Oe cells were exposed to DOX in order to examine the changes noted in cell viability, oxidative stress and autophagy. The reduction in cell viability caused by DOX induction was effectively rescued by ENPP2 overexpression (Fig. 2C). As shown in Fig. 2D, DOX induction significantly elevated in the level of total ROS compared with the control group, which was notably restored by ENPP2 overexpression. Moreover, DOX treatment resulted in a significant increase in TBARS content and a decrease in GSH-Px expression, which were both partially recovered by ENPP2 overexpression (Fig. 2E and F).

The expression levels of the proteins involved in autophagy, including LC3II/I, Beclin 1, ATG5 and p62, were also assessed. The protein expression levels of LC3II/I, Beclin 1 and ATG5 were significantly increased, but p62 expression was decreased following DOX treatment, indicating the induction of autophagy by DOX in H9c2 cells (Fig. 2G). Concomitantly, ENPP2 overexpression enhanced the changes noted in these proteins following induction of autophagy by DOX.

To evaluate the induction of ferroptosis in DOX-treated H9c2 cells, Fe^{2+} activity, lipid ROS production and the

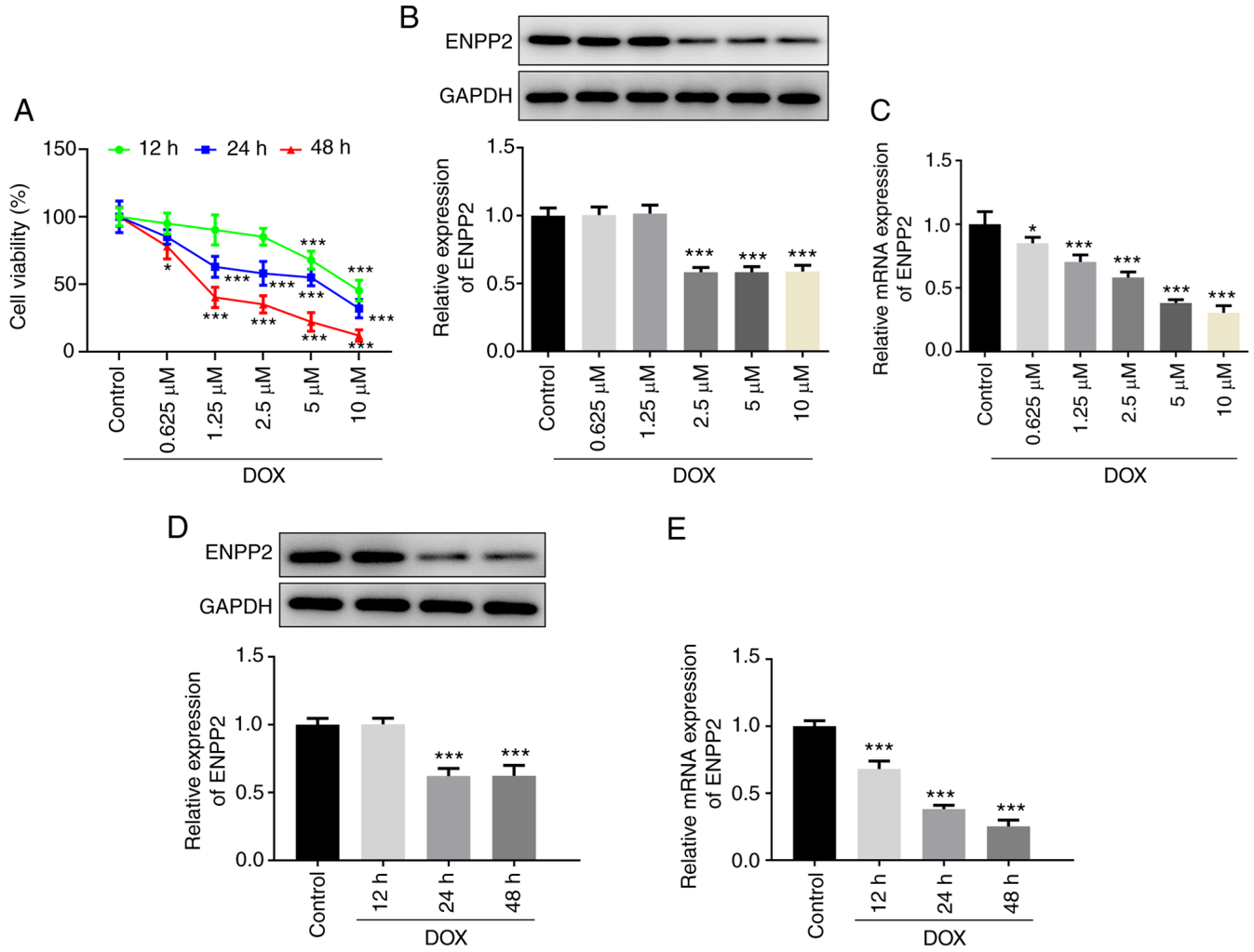

Figure 1. Effects of DOX on ENPP2 expression in H9c2 cardiomyocytes. (A) H9c2 cells were exposed to different concentrations of DOX for 12, 24 and 48 h, and cell viability was measured using the MTT assay. H9c2 cells were exposed to different concentrations of DOX for 24 h and subsequently the ENPP2 (B) protein and (C) mRNA expressions levels were detected. H9c2 cells were exposed to 5 μM DOX for 12, 24 and 48 h, and the ENPP2 (D) protein and (E) mRNA expression levels were detected. *P<0.05 and ***P<0.001 vs. control. DOX, doxorubicin; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2.

expression levels of ferroptosis-associated proteins, including SLC7A11, GPX4, FPN1 and NOX4, were determined. DOX treatment resulted in a significant increase in cellular Fe^{2+} activity and lipid ROS production (Fig. 3A and B), while the cells that overexpressed ENPP2 exhibited decreased Fe^{2+} activity and lipid ROS production compared with the cells transfected with NC vectors (Fig. 3A and B). Furthermore, the decreased expression levels of SLC7A11, GPX4 and FPN1 and increased expression level of NOX4 caused by DOX were significantly reversed by the overexpression of ENPP2 (Fig. 3C). These data suggested the inhibitory effects of ENPP2 on DOX-induced ferroptosis in H9c2 cells.

FoxO4 binds to the promoter of ENNP2 and regulates ENPP2 expression in H9c2 cardiomyocytes. FoxO4 was predicted to bind to the E1 and E2 sequences of the ENPP2 promoter (Fig. 4A). As displayed in Fig. 4B, the protein and mRNA expression levels of FoxO4 were significantly upregulated after transfection with FoxO4 plasmid. Additionally, FoxO4 overexpression significantly reduced ENPP2 mRNA expression, suggesting that FoxO4 may downregulate ENPP2 expression by binding to the ENPP2 promoter (Fig. 4C). Then, the expression level of ENPP2 in DOX-induced H9c2 cells with FoxO4 overexpression was assessed using RT-qPCR. It was found that gain-function of FoxO4 significantly downregulated the expression level of ENPP2 compared with the DOX + Oe-NC group (Fig. 4D). Subsequently, the interaction between FoxO4 and ENPP2 was validated using dual-luciferase reporter (Fig. 4E) and ChIP (Fig. 4F) assays. These results suggested that FoxO4 could bind to the promoter of ENNP2 and regulate ENPP2 expression in H9c2 cardiomyocytes.

Overexpression of FoxO4 partially reverses the effects of ENPP2 on DOX-induced autophagy and ferroptosis in H9c2 cardiomyocytes. Finally, FoxO4 and ENPP2 were co-overexpressed in H9c2 cells, which had been treated with DOX. Cell viability was reduced in cells that co-overexpressed FoxO4 and ENPP2 compared with cells that only overexpressed ENPP2 (Fig. 5A). This indicated that FoxO4 overexpression inhibited the protective effect of ENPP2 on cell viability, which was impaired by DOX (Fig. 5A). Moreover, the effects

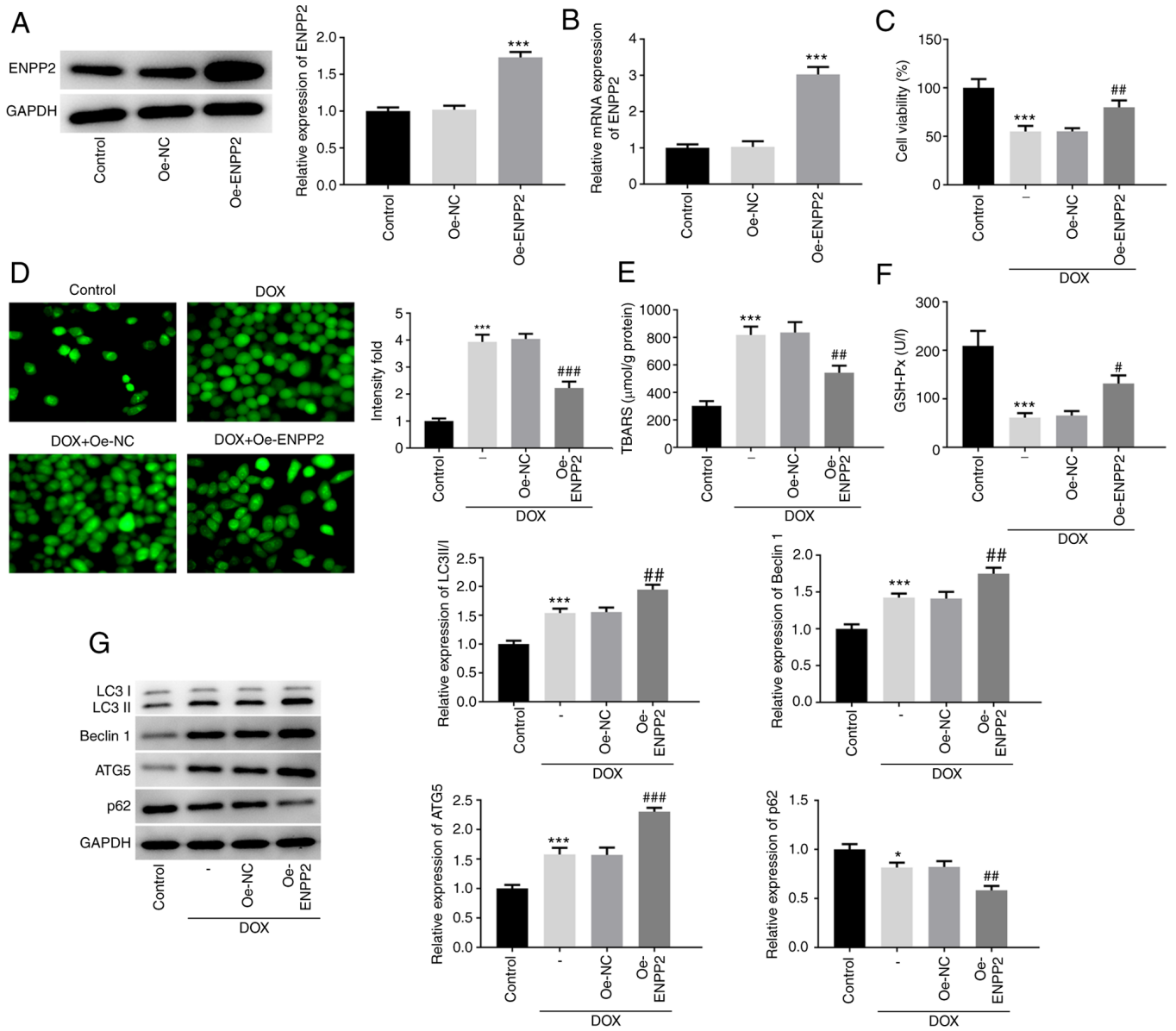

Figure 2. Effects of ENPP2 overexpression on DOX-induced autophagy in H9c2 cardiomyocytes. H9c2 cells were transfected with ENPP2 vector in order to obtain ENPP2-Oe cells. The transfection efficiency was detected via (A) western blotting and (B) reverse transcription-quantitative PCR. ***P<0.001 vs. Oe-NC. (C) ENPP2-Oe H9c2 cells were exposed to 5 μM DOX for 24 h and cell viability was measured. (D) The total reactive oxygen species production was assessed using a 2',7'-dichlorodihydrofluorescein diacetate kit (magnification, x200). ENPP2-Oe H9c2 cells were exposed to 5 μM DOX for 24 h, and subsequently (E) TBARS concentration levels and (F) GSH-Px expression levels were measured. (G) ENPP2-Oe H9c2 cells were exposed to 5 μM DOX for 24 h and the expression levels of the proteins involved in autophagy were evaluated. *P<0.05 and ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. Oe-NC. DOX, doxorubicin; NC, negative control; TBARS, thiobarbituric acid reactive substances; GSH-Px, glutathione peroxidase; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; Oe, overexpressing; ATG5, autophagy related 5.

of ENPP2 overexpression on the total ROS levels, TBARS concentration and GSH-Px expression were partially reversed by FoxO4 overexpression (Fig. 5B-E). Similar results were observed in Fig. 5F, as the co-transfection with FoxO4 and ENPP2 plasmids significantly decreased the levels of LC3II/I, Beclin 1 and ATG5 and increased the expression of p62 compared with those in the DOX + Oe-ENPP2 group. In addition, the inhibitory effects of ENPP2 on DOX-induced ferroptosis were significantly reduced by FoxO4 overexpression, as demonstrated by increased Fe^{2+} and lipid ROS activity levels, decreased SLC7A11, GPX4 and FPN1 expression, and increased NOX4 expression, which were observed following FoxO4 overexpression (Fig. 6A-C).

Discussion

DOX is a potent anticancer drug clinically applied for the treatment of various cancer types, such as breast, colon and bladder cancer (19-21). However, its cardiotoxicity is a major challenge, thereby limiting its clinical use. Therefore, further understanding of the molecular mechanisms involved in DOX function can alleviate cardiotoxicity and improve the clinical efficacy of this chemotherapeutic drug. Currently, no effective method exists to relieve the cardiac dysfunction caused by DOX. Thus, the development of effective treatments is a major focus for improving the side effects of DOX. The results of the present study found that ENPP2 mRNA

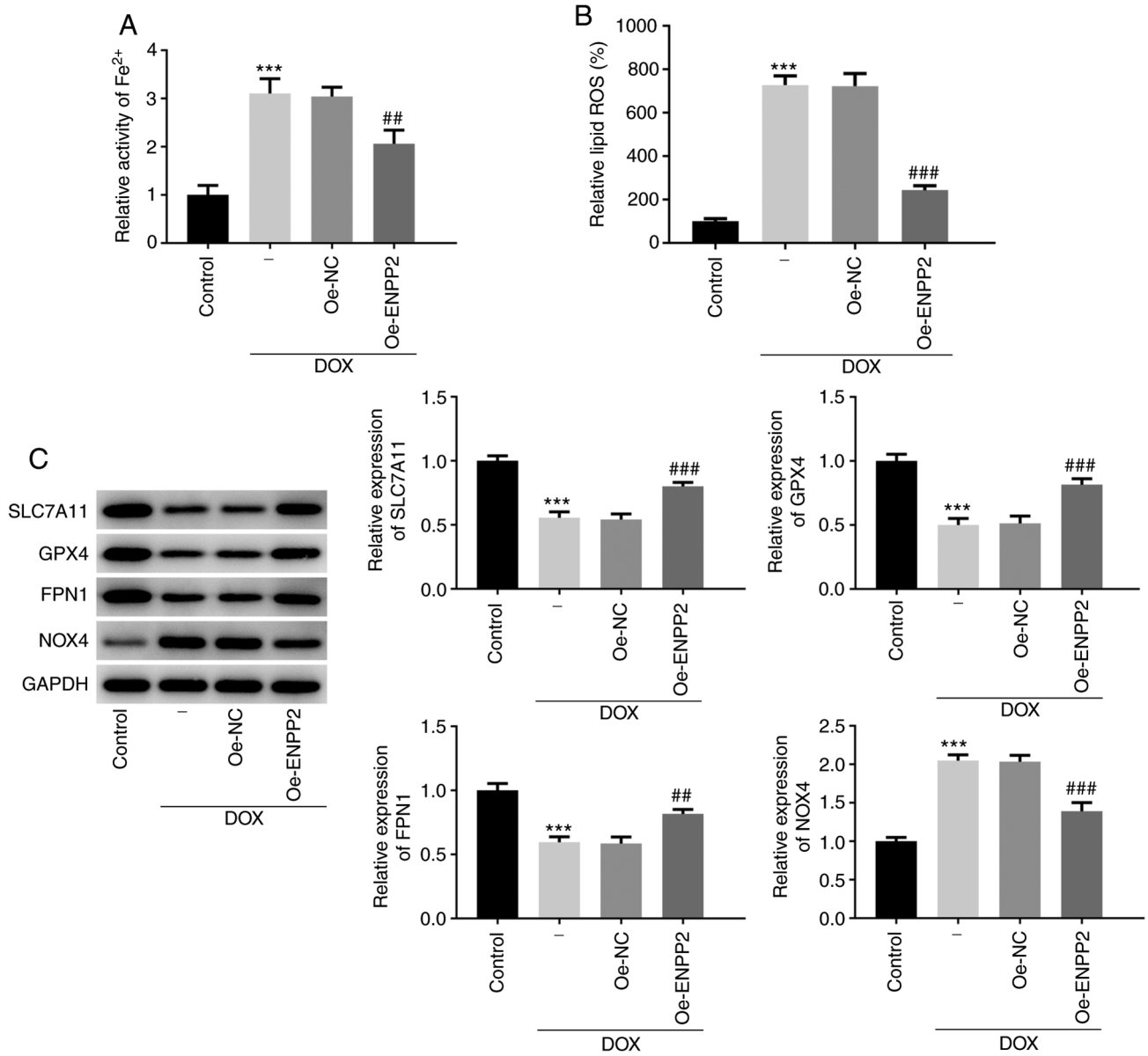

Figure 3. Effects of ENPP2 overexpression on DOX-induced ferroptosis in H9c2 cardiomyocytes. (A) H9c2 cells that overexpressed ENPP2 were exposed to 5 μM DOX for 24 h and cellular Fe^{2+} activity was measured using an Iron Assay kit. (B) The lipid ROS levels were assessed. (C) The expression levels of the proteins involved in ferroptosis were measured using western blot analysis. ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. Oe-NC. DOX, doxorubicin; ROS, reactive oxygen species; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; NC, negative control; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; Oe, overexpressing; SLC7A11, solute carrier family 7 member 11; GPX4, GSH peroxidase 4; FPN1, ferroportin 1; NOX4, NADPH oxidase 4.

and protein expression levels were downregulated following DOX treatment. Gene expression can be divided into two levels: Transcription and translation, that is, the mRNA level and the protein level. The time and site of transcription and translation of gene expression are spatiotemporal. After transcription, there will be post-transcriptional processing, degradation of transcriptional products, translation, post-translation processing and modification. Therefore, transcription levels and translation levels are not completely consistent, which may explain the different degrees of decline between the mRNA and protein expression levels of ENPP2 (22). In the present study, FoxO4 was found to bind to the ENNP2 promoter to improve DOX-induced cardiotoxicity via the induction of ferroptosis.

Oxidative stress and dysregulation of autophagy have been mainly reported to participate in the cardiotoxicity of DOX (23,24). In the present study, the results demonstrated that DOX treatment impaired cell viability, increased TBARS concentration levels and reduced GSH-Px and p62 expression in H9c2 cardiomyocytes. Concomitantly, DOX increased LC3II/I, Beclin 1 and ATG5 expression levels. The results of the present study confirmed that DOX induced oxidative stress and autophagy in cardiomyocytes. In addition, the relative Fe^{2+} activity and lipid ROS production were enhanced, whereas the expression levels of SLC7A11, GPX4 and FPN1 were decreased following treatment of the cells with DOX. In contrast to these findings, NOX4 expression was increased following incubation of the cells with DOX. Thus, ROS accumulation

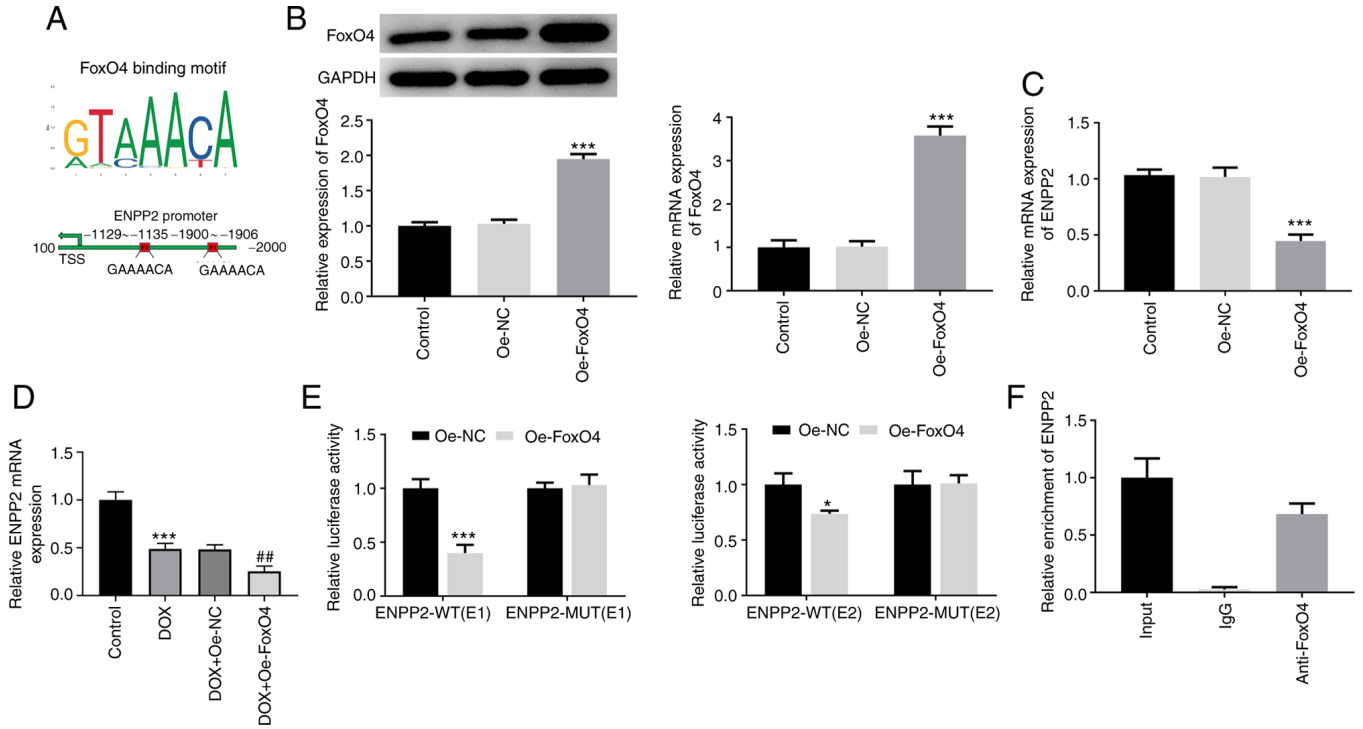

Figure 4. Association between ENPP2 and FoxO4 in H9c2 cardiomyocytes. (A) The binding sequences of FoxO4 and the ENPP2 promoter. (B) FoxO4-Oe H9c2 cells were established and the transfection efficiency was assessed via western blot analysis and RT-qPCR. ***P<0.001 vs. Oe-NC. (C) mRNA expression levels of ENPP2 in H9c2 cells with or without FoxO4 overexpression were measured. ***P<0.001 vs. Oe-NC. (D) The expression level of ENPP2 in DOX-induced H9c2 cells with FoxO4 overexpression was assessed using RT-qPCR. ***P<0.001 vs. control; ##P<0.01 vs. DOX + Oe-NC. The interaction between FoxO4 and the ENPP2 promoter was confirmed by (E) dual-luciferase reporter and the (F) chromatin immunoprecipitation assays. *P<0.05 and ***P<0.001 vs. Oe-NC. NC, negative control; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; Oe, overexpressing; DOX, doxorubicin; RT-qPCR, reverse transcription-quantitative PCR.

in DOX-treated cardiomyocytes was accompanied with deregulation of their antioxidant defense system. GSH serves a major role in the cellular antioxidant defense (25). Depletion of GSH leads to accumulation of lipid ROS levels, protein or membrane damage and subsequent ferroptotic cell death (26). Cellular iron is required in normal life processes, such as DNA synthesis, metastasis, cell cycle progression, angiogenesis and/or mitochondrial iron metabolism. Ferritin is the iron storage protein of the cells. Fe^{2+} promotes lipid peroxide accumulation via the Fenton reaction and lipid oxidation (27). GPX4 can prevent the toxicity of lipid peroxides by its enzyme activity. It can also maintain the homeostasis of membrane lipid bilayers. Furthermore, GSH is the co-factor of GPX4 and aids the catalysis of peroxides into alcohols. Downregulation of GSH expression directly inactivates GPX4 and leads to subsequent induction of ferroptosis (28). Therefore, the present results, when taken together, demonstrated that DOX could induce ferroptosis.

Ferroptosis was recently reported as a novel mechanism of iron-dependent regulated cell death (27). It has been proposed that ferroptosis serves a significant role in DOX-induced cardiotoxicity, and ferroptosis inhibition protects against this process (4,5). Moreover, ENPP2 has been shown to inhibit ferroptosis in cardiomyocytes (10). In the present study, DOX treatment caused the downregulation of ENPP2 expression. It was hypothesized that ENPP2 may protect cardiomyocytes from DOX-induced cardiotoxicity by inhibiting ferroptosis. Therefore, this enzyme was overexpressed in H9c2 cells, which were subsequently treated with DOX. Numerous regulators, such as GPX4, SLC7A11, FPN1 and NOX4, have been identified to be involved in regulation of cell ferroptosis (29-31). It has been reported that ENPP2 overexpression enhances the expression levels of the ferroptosis-associated gene GPX4 in H9c2 cells (10). The current results indicated that ENPP2 overexpression partially recovered TBARS, GSH-Px and ROS levels, which in turn enhanced autophagy and inhibited ferroptosis. Autophagy exerts a dual function in the regulation and progression of cardiac dysfunction. The induction of autophagy has been shown to attenuate DOX-induced cardiotoxicity. In addition, autophagy has been reported to regulate ferroptosis (32-34). In the present study, ENPP2 inhibited ferroptosis by blocking the oxidative stress response, which in turn reduced ROS production and enhanced the induction of autophagy. These processes then promoted the elimination of long-lived proteins and damaged organelles.

The JASPAR database was searched and FoxO4 was identified as a potential target that could bind to the promoter of ENPP2. Additional analysis validated the direct interaction between FoxO4 and ENPP2. It was found that FoxO4 overexpression reduced ENPP2 expression. FoxO4 regulates negatively gene transcription via post-transcriptional suppression of coding mRNAs (35). The results of the present study indicated that FoxO4 may negatively control ENPP2 transcription by binding to the ENPP2 promoter. Notably, FoxO4 has been extensively investigated with regards to its effects on promoting myocardial injury, and this transcription factor

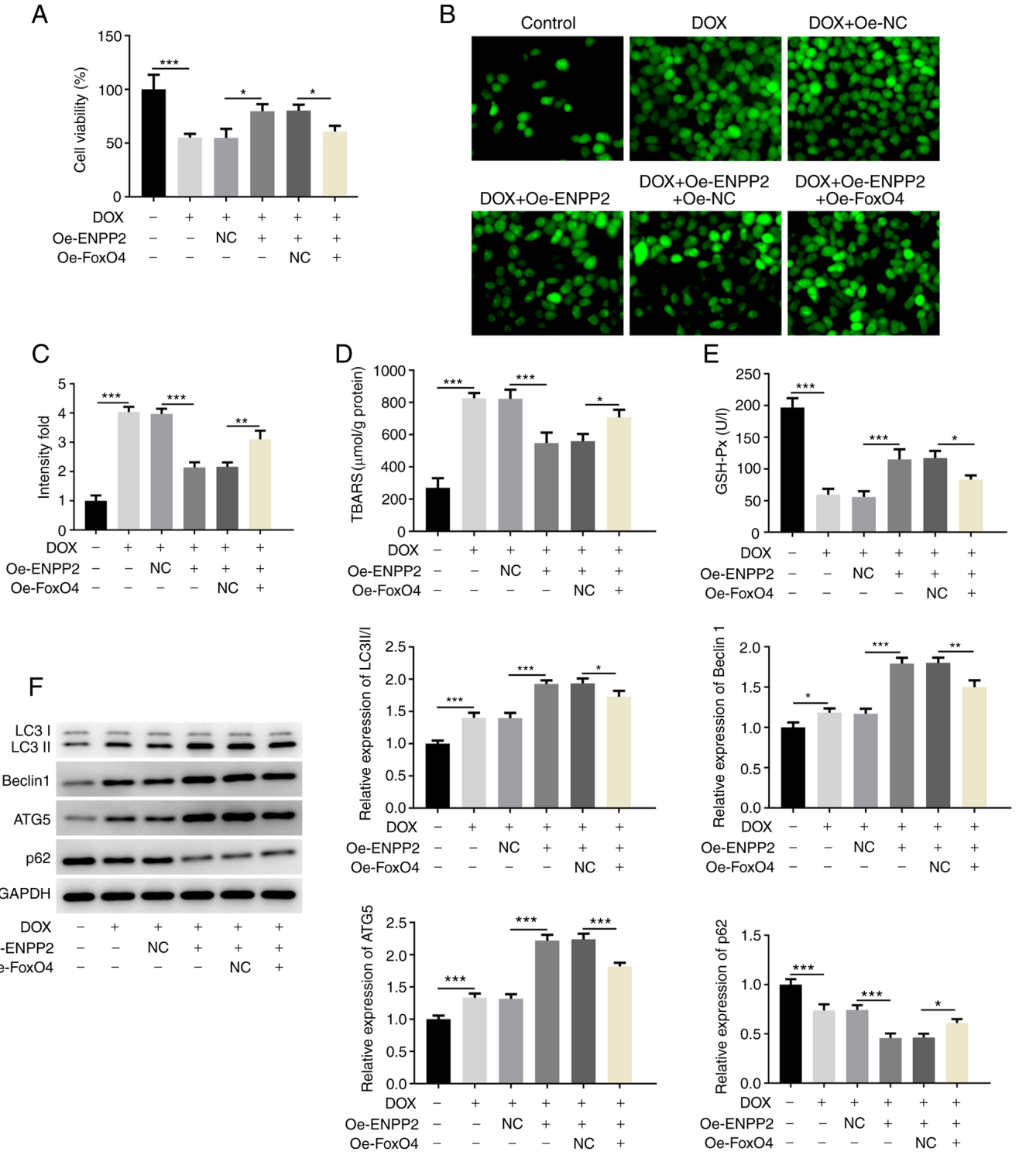

Figure 5. FoxO4 overexpression inhibits the effects of ENPP2 overexpression on DOX-induced autophagy in H9c2 cardiomyocytes. H9c2 cells that co-overexpressed FoxO4 and ENPP2 were exposed to 5 μM DOX for 24 h. Subsequently, (A) cell viability was measured with a MTT assay. (B) Total reactive oxygen species production was assessed using the DCFDA kit (magnification, x200), (C) and the results were quantified. (D) TBARS concentration levels and (E) GSH-Px expression levels were detected using their corresponding kits. (F) The expression levels of the proteins involved in autophagy were evaluated via western blot analysis. *P<0.05, **P<0.01 and ***P<0.001. DOX, doxorubicin; TBARS, thiobarbituric acid reactive substances; GSH-Px, glutathione peroxidase; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; Oe, overexpressing; NC, negative control; ATG5, autophagy related 5.

is involved in myocardial ischemia/reperfusion, myocardial infarction and DOX-induced cardiotoxicity (12,13,36). Subsequently, the overexpression of FoxO4 in ENPP2-Oe H9c2 cells was assessed with regards to the effects of ENPP2 on DOX-induced cardiotoxicity. FoxO4 overexpression inhibited all the effects caused by ENPP2 overexpression on DOX-induced cardiotoxicity, which supported the initial hypothesis.

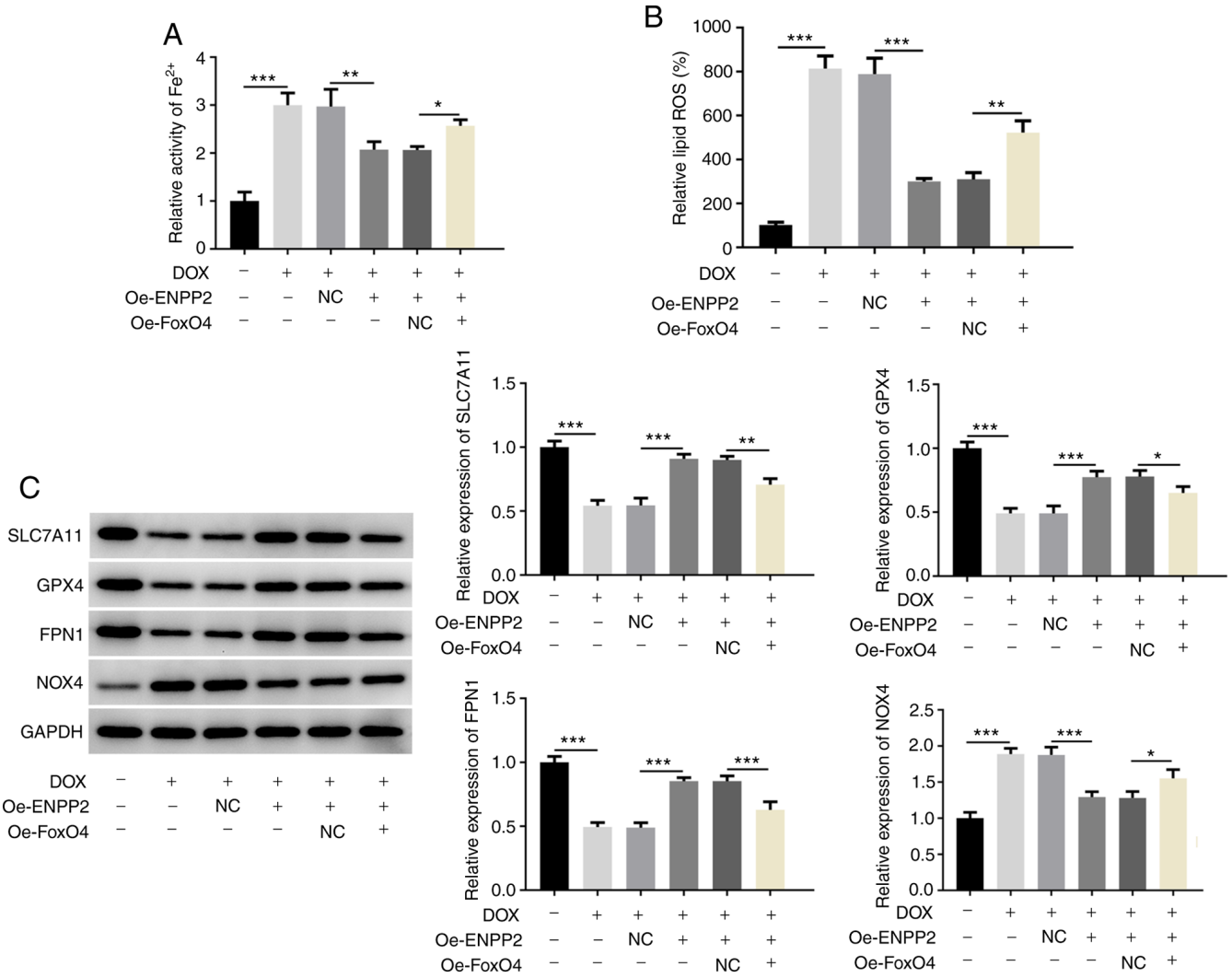

Figure 6. FoxO4 overexpression inhibits the effects of ENPP2 overexpression on DOX-induced ferroptosis in H9c2 cardiomyocytes. (A) H9c2 cells co-Oe FoxO4 and ENPP2 were exposed to DOX (5 μM) for 24 h and cellular Fe^{2+} activity was measured using the Iron Assay kit. (B) The lipid ROS level was assessed. (C) The expression levels of the proteins involved in ferroptosis were measured using western blot analysis. *P<0.05, **P<0.01 and ***P<0.001. DOX, doxorubicin; ROS, reactive oxygen species; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; Oe, overexpressing; NC, negative control; SLC7A11, solute carrier family 7 member 11; GPX4, GSH peroxidase 4; FPN1, ferroportin 1; NOX4, NADPH oxidase 4.

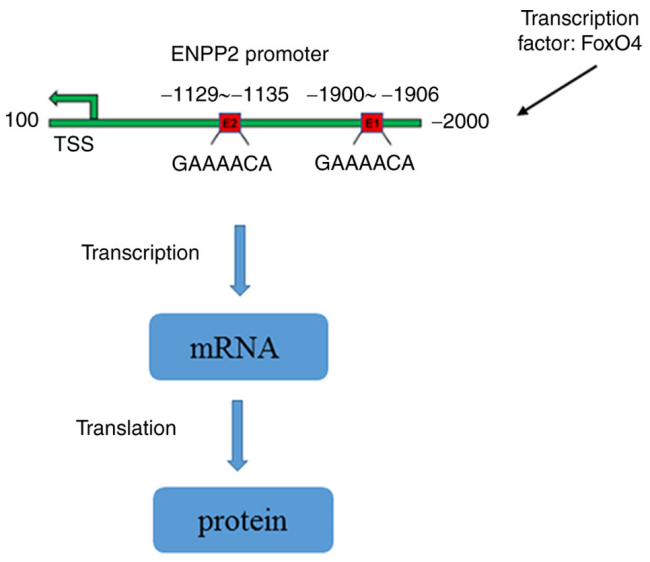

Figure 7. Transcription factor FoxO4 suppresses ENNP2 expression by binding to its promoter. ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2.

However, there are some limitations to the present study that should be addressed, Firstly, *in vivo* experiments were not performed and the effects of FoxO4 overexpression on H9c2 cells in the presence of DOX treatment were not investigated in the present study. Additionally, the absence of clinical data is another limitation of this study. Therefore, further experiments should be performed in future studies to support the present conclusions.

In conclusion, to the best of our knowledge, the current study was the first to investigate the role of ENNP2 in DOX-induced cardiomyocyte injury and its potential regulatory mechanisms. The results of this study demonstrated that FoxO4 could bind to the ENNP2 promoter to affect the post-transcriptional suppression of encoding mRNA and therefore negatively control the transcription of ENNP2 (Fig. 7), thereby regulating DOX-induced cardiotoxicity via the induction of ferroptosis. Specific therapies that target FoxO4/ENPP2 to reduce and increase FoxO4 and ENPP2 expressions, respectively, may provide potential approaches for ameliorating DOX-induced cardiotoxicity.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH, YY and JL contributed to study conception or design. LH and YY performed the experiments and contributed to the acquisition of data. JC and PZ contributed to data analysis or interpretation. JL, LH and YY drafted the work and revised it critically for important intellectual content. All authors read and approved the final manuscript. LH and JL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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