

The role of ZFP580, a novel zinc finger protein, in TGF-mediated cytoprotection against chemical hypoxia-induced apoptosis in H9c2 cardiac myocytes

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Received December 19, 2015; Accepted December 22, 2016

DOI: 10.3892/mmr.2017.6236

Abstract. Zinc finger protein 580 (ZFP580) is a novel Cys2-His2 zinc-finger transcription factor that has an anti-apoptotic role in myocardial cells. It is involved in the endothelial transforming growth factor- β 1 (TGF- β 1) signal transduction pathway as a partner against decapentaplegic homolog (Smad)2 binding partner. The aim of the present study was to determine the involvement of ZFP580 in TGF- β 1-mediated cytoprotection against chemical hypoxia-induced apoptosis, using H9c2 cardiac myocytes. Hypoxia was chemically induced in H9c2 myocardial cells by exposure to cobalt chloride (CoCl₂). In response to hypoxia, cell viability was decreased, whereas the expression levels of hypoxia inducible factor-1 α and ZFP580 were increased. Pretreatment with TGF- β 1 attenuated CoCl₂-induced cell apoptosis and upregulated ZFP580 protein

expression; however, these effects could be suppressed by SB431542, an inhibitor of TGF- β type I receptor and Smad2/3 phosphorylation. Furthermore, suppression of ZFP580 expression by RNA interference reduced the anti-apoptotic effects of TGF- β 1 and thus increased CoCl₂-induced apoptosis. B-cell lymphoma (Bcl)-2-associated X protein/Bcl-2 ratio, reactive oxygen species generation and caspase-3 activation were also increased following ZFP580 inactivation. In conclusion, these results indicate that ZFP580 is a component of the TGF- β 1/Smad signaling pathway, and is involved in the protective effects of TGF- β 1 against chemical hypoxia-induced cell apoptosis, through inhibition of the mitochondrial apoptotic pathway.

Introduction

Zinc finger protein 580 (ZNF580) is a novel gene that is associated with low-density lipoprotein stimulation in vascular endothelial cells. ZNF580 gene cloning (1) and subsequent bioinformatics analysis revealed that ZNF580 is a Cys2-His2 (C2H2)-type transcription factor (2). C2H2-zinc finger genes constitute the largest class of transcription factors within the human genome; they are typically involved in critical cell functions, such as survival and growth (3). Our previous study indicated that ZNF580 is ubiquitously expressed in human tissues, and serves important roles in maintaining normal cell functions, including migration and proliferation (4,5). ZFP580 is the murine homologue of ZNF580, research regarding cloned ZFP580 (6) revealed that intermittent hypoxia could induce expression, which displayed an anti-apoptotic role during early phase ischemia-reperfusion injury (7). However, the mechanism and signaling pathways underlying the anti-apoptotic effects of ZFP580 have not yet been fully elucidated.

Members of the transforming growth factor- β (TGF- β) family regulate numerous cellular functions, including cell growth, differentiation, adhesion, migration and apoptosis. TGF- β 1 is a multifunctional cytokine that regulates apoptosis

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Abbreviations: TGF- β 1, transforming growth factor- β 1; CoCl₂, cobalt chloride; T β R-I, TGF- β type I receptor

Key words: transforming growth factor- β , cardiomyocyte, hypoxia, zinc finger proteins, apoptosis

in a cell type-specific and context-dependent manner, with proapoptotic or anti-apoptotic actions depending on the target cell type and the pathophysiological milieu (8-10). Despite its proapoptotic role in several cell types, TGF- β 1 demonstrated cardioprotective effects during reperfusion injury or cardiac inflammatory disease in myocytes (11-13), and exerted an anti-apoptotic role in hypoxia-reoxygenation-induced myocardial cell injury (12,14,15).

Mothers against decapentaplegic homolog (Smad) proteins are the primary intracellular mediators of the TGF- β 1 signaling pathway. Smad2 and Smad3 are particularly important in the transcriptional response to TGF- β 1, in various physiological scenarios. Our previous study determined that ZNF580 is involved in the TGF- β 1 signaling pathway as a binding partner of Smad2 (16,17). However, the actual relationship between ZFP580 and Smad proteins in cobalt chloride (CoCl₂)-induced apoptosis has not been fully elucidated. Therefore, the present study aimed to investigate the involvement of ZFP580 in TGF- β 1-mediated cytoprotection during CoCl₂-induced apoptosis, and its association with Smad2 in embryonic rat heart H9c2 cells.

Materials and methods

Materials. SB431542, CoCl₂, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,7-dichlorofluorescein diacetate (DCFH-DA) were provided by Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Recombinant human TGF- β 1 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Anti-ZNF580 (catalog no. ab59015), anti-hypoxia inducible factor (HIF)-1 α (ab463), anti-phosphorylated Smad (p-Smad)-2 (phospho S467; ab53100), anti-p-Smad3 (phospho S425; ab51177), and anti-active caspase-3 (ab2302) primary antibodies were purchased from Abcam (Cambridge, UK). Anti-Smad2 (sc-393312), anti-Smad3 (sc-101154), anti-B-cell lymphoma 2 (Bcl-2; sc-783), anti-Bcl-2-associated X protein (Bax; sc-526), and anti- β -actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit was supplied by Promega Corp. (Madison, WI, USA), and the high-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific Inc., Waltham, MA, USA). The carbon dioxide independent DMEM was purchased from the Medical Gas Company (Tianjin, China).

Cell culture and hypoxia induction. The embryonic rat heart-derived cell line (H9c2) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). H9c2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. The hypoxia model was established using the hypoxia-inducing agent CoCl₂, according to a previous study (18).

Cell viability assessment under various hypoxic conditions. Cells at a density of 1x10⁴/200 μ l were plated in DMEM supplemented with 10% FBS, in 96-well microtiter plates (Corning Life Sciences, USA) and incubated for 24 h at

37°C. Cells were subsequently incubated in serum-free CO₂-independent DMEM supplemented with various concentrations of CoCl₂ (200, 400, 600, 800 and 1,000 μ M) for 24 h, or with 600 μ M CoCl₂ for various time intervals (0, 4, 8, 12, 16, 20 and 24 h). Normoxic control cells were incubated under the same conditions but in a normal atmosphere. Normoxic cells received normal serum and no CoCl₂ treatment, and the hypoxic cells were then incubated in a hypoxic chamber. MTT was added at a final concentration of 0.5 mg/ml. After a 4-h incubation at 37°C, the reaction was halted by adding 200 μ l dimethyl sulfoxide, and the relative optical density was measured at 490 nm by a microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was calculated according to the following equation: Cell viability (%) = (OD_{treatment} - OD_{blank}) / (OD_{control} - OD_{blank}) x 100. Experiments were performed in triplicate. To analyze TGF- β 1 function under hypoxic conditions, cell viability was measured following pretreatment with 2 ng/ml TGF- β 1 for 30 min, prior to exposure to 600 μ M CoCl₂ for 8, 16 or 24 h. To analyze the role of Smad2/3 in the TGF- β 1-mediated cytoprotection against CoCl₂-induced hypoxia, H9c2 cells were pretreated with SB431542 (20 μ M, diluent with DMSO, at 37°C), a selective inhibitor of T β R1-Smad2/3, prior to stimulation with TGF- β 1, and were subsequently exposed to 600 μ M CoCl₂ for 24 h.

RNA extraction, cDNA synthesis, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from H9c2 cells treated with 600 μ M CoCl₂ at various time intervals (0, 4, 8, 12, 16, 20 or 24 h) was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. First-strand cDNA samples were synthesized using a TransScript First-Strand cDNA Synthesis Supermix kit according to the manufacturer's protocol (Beijing Transgen Biotech, Co., Ltd., Beijing, China). GAPDH RNA levels were quantified in all of the samples as an internal control, and mRNA levels were calculated relative to GAPDH mRNA. qPCR was performed in a 25 μ l volume with SYBR[®] Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each gene analysis was repeated at least 3 times, and all RT-qPCR experiments were performed in triplicate using the ABI 7500 Real-Time PCR platform (Applied Biosystems; Thermo Fisher Scientific, Inc.). The specific primers used were as follows: Forward, 5'-ACATCA TTTCGTCTTTTCTTCTG-3' and reverse, 5'-GGTGCTTTT GTCATTTCTTCCAC-3' for ZFP580. The PCR conditions for ZFP580 were 15 sec at 95°C, 34 sec at 63°C and 45 sec at 72°C for 40 cycles. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, served as an internal control. The fold-change in expression of the gene of interest between the two samples was calculated using the $\Delta\Delta$ Cq method (19).

Western blot analysis. Cells were lysed with radioimmuno-precipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% NP-40, and protease inhibitor cocktail] for 30 min and centrifuged at 12,000 x g for 10 min prior to supernatant collection. The protein concentration was quantified using the bicinchoninic acid assay. Equal amounts (60 μ g) of protein were loaded into each lane and separated by

10% SDS-PAGE, then transferred to polyvinylidene fluoride (PVDF) membranes (Roche Diagnostics GmbH, Mannheim, Germany). After blocking in 5% fat-free milk in Tris-buffered saline-Tween-20 (TBST) for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies (1:2,000) overnight at 4°C. The membranes were washed with TBST and then incubated with horseradish peroxidase-conjugated anti-rabbit (L3012, Signalway Antibody, Nanjing, China) or anti-mouse (L3032, Signalway Antibody) secondary antibodies in TBST (1:5,000) for 1 h at room temperature, and then visualized by a super enhanced chemiluminescence detection reagent (Beyotime Institute of Biotechnology, Haimen, China). The signals were detected using Image Station 4000R (Kodak, Rochester, NY, USA). Quantification of results was performed using ImageJ version 1.44 (National Institutes of Health, Bethesda, MD, USA). Each experiment was repeated at least three times.

Flow cytometric analysis. The aforementioned treated H9c2 cells were collected as previously described (20). Briefly, cell supernatants were incubated with 5 μ l Annexin V-FITC and 10 μ l propidium iodide (PI) for 15 min in the dark. Following incubation, 400 μ l binding buffer was added to each sample and the samples were then filtered through a 50 μ m nylon mesh. Fluorescence was analyzed by flow cytometry Epics Elite flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Cells display phosphatidylserine on their outer cell membranes, which is readily detected by Annexin V during the early stages of apoptosis. The plasma membrane becomes increasingly permeable during the later stages of apoptosis, and PI may move across the cell membrane to bind to cellular DNA. The data were analyzed using FlowJo 7.6 software (FlowJo, LLC, Ashland, OR, USA). Cells in the fourth quadrant indicated the presence of apoptotic cells.

Lentiviral infection. Lentiviral vectors expressing small interfering RNAs (siRNAs) directed against ZFP580 (Lenti-RNAi) or a negative control (Lenti-NC) were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China). H9c2 cells were cultured to 30-40% confluence and were then infected with either the Lenti-RNAi or Lenti-NC for 72 h at 37°C [multiple of infection (MOI)=50]. The infected cells were treated with or without 2 ng/ml TGF- β 1 for 30 min prior to exposure to 600 μ M CoCl₂ for 24 h.

Fluorescence assay of intracellular reactive oxygen species (ROS). Intracellular ROS generation was determined using the ROS-dependent oxidative conversion of cell-permeable DCFH-DA to the fluorescent dichlorofluorescein. H9c2 cells were treated as aforementioned, washed 3 times with PBS, and incubated with a 10 μ M DCFH-DA solution in serum-free medium at 37°C, for 30 min, in the dark. Cells were washed 3 times with PBS, and ROS concentrations were quantified using a Leica TSP SP8 confocal microscope at a wavelength of 485 nm (Leica Microsystems GmbH, Wetzlar, Germany) and a SpectraMax M2 microplate reader at a wavelength of 530 nm (Molecular Devices, LLC, Sunnyvale, CA, USA).

Statistical analysis. All data are presented as the mean \pm standard error. Data were analyzed using SPSS software version

18.0 (SPSS, Inc., Chicago, IL, USA). Statistically significant differences were determined using one-way analysis of variance, followed by the least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CoCl₂ reduces cell viability and increases expression of ZFP580 and HIF-1 α in H9c2 myocardial cells. MTT assay revealed that CoCl₂ treatment decreased cell viability in a concentration- and time- dependent manner (Fig. 1A and B). Cell viability decreased following a 24 h incubation with increasing concentrations of CoCl₂ (400, 600, 800 or 1,000 μ M); 600 μ M CoCl₂ was sufficient to decrease viability by ~50%. Based on these results a concentration of 600 μ M CoCl₂ was selected for a time-course experiment investigating the effects of CoCl₂ on cell viability at different time intervals (0, 4, 8, 12, 16, 20 or 24 h). The results indicated that cell viability was significantly decreased at 8, 12, 16, 20 and 24 h after CoCl₂ treatment compared with the control group ($P < 0.05$; Fig. 1B). Western blot analysis indicated that the protein expression levels of HIF-1 α , a marker of hypoxia, rose during hypoxia and peaked at 12 h; however, HIF-1 α mRNA levels, as determined by RT-qPCR, did not significantly increase during the same time period (Fig. 1C and D); this result is consistent with previously reported research (21,22). CoCl₂ treatment increased ZFP580 expression at mRNA and protein levels; ZFP580 mRNA expression peaked at 12 h, and protein expression peaked at 16 h (Fig. 1C and E). Notably, cell viability steadily decreased in hypoxia-induced cells, whereas the expression of ZFP580 increased and then decreased after 16 h. This may be due to the role of ZFP580 as an anti-injury marker (7), where it is first activated then degraded with increasing hypoxia. These findings indicated that a CoCl₂-induced hypoxia model of H9c2 cells had been successfully established; in addition, it was observed that CoCl₂-induced hypoxia increases ZFP580 expression.

TGF- β 1 attenuates CoCl₂-induced cytotoxicity and upregulates ZFP580 protein expression. To analyze TGF- β 1 function under hypoxic conditions, H9c2 cells were pretreated with 2 ng/ml TGF- β for 30 min prior to exposure to 600 μ M CoCl₂ for 8, 16 or 24 h. MTT assay revealed that pretreatment with TGF- β 1 reduced CoCl₂-induced cytotoxicity and increased cell viability by ~10% at 16 and 24 h, compared with that of the untreated hypoxic group (Fig. 2A; $P < 0.05$). Western blot analysis indicated that ZFP580 protein expression was upregulated by pretreatment with TGF- β 1 and subsequent exposure to CoCl₂ (Fig. 2B and C). ZFP580 expression was upregulated at 8 and 24 h following TGF- β 1 and CoCl₂ treatment ($P < 0.05$); however, there was no difference in ZFP580 expression at the TGF- β 1-treated 16 h time-point, regardless of CoCl₂ treatment. It is possible that ZFP580 protein levels had plateaued at this time point. At 24 h there were clear differences in both cell viability and ZFP580 expression in the TGF- β 1 pretreatment group, with or without CoCl₂ treatment; therefore, at 24 h, 600 μ M CoCl₂ treatment was selected for subsequent experimental conditions. From these findings it was hypothesized

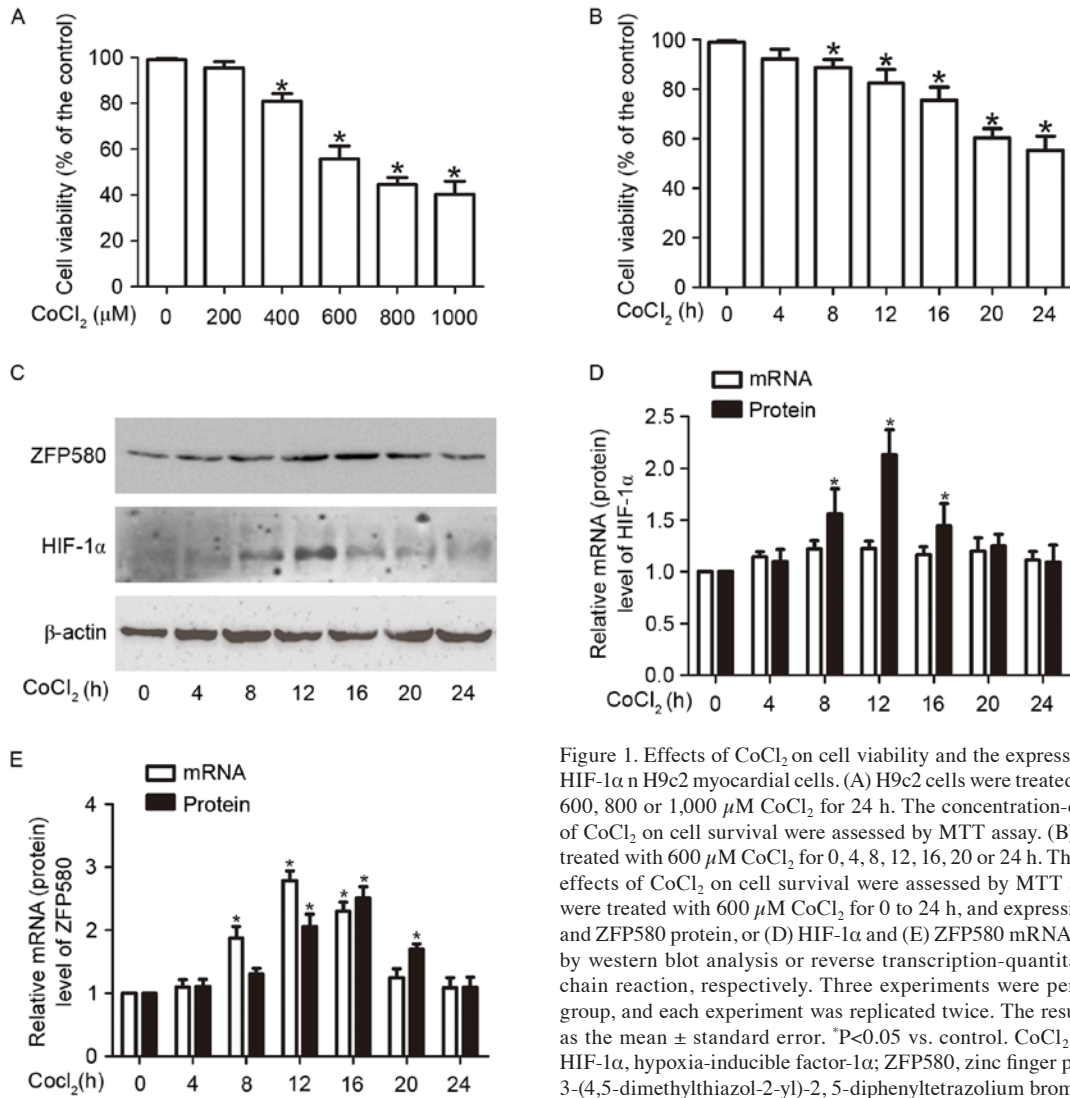


Figure 1. Effects of CoCl₂ on cell viability and the expression of ZFP580 or HIF-1α in H9c2 myocardial cells. (A) H9c2 cells were treated with 0, 200, 400, 600, 800 or 1,000 μM CoCl₂ for 24 h. The concentration-dependent effects of CoCl₂ on cell survival were assessed by MTT assay. (B) H9c2 cells were treated with 600 μM CoCl₂ for 0, 4, 8, 12, 16, 20 or 24 h. The time-dependent effects of CoCl₂ on cell survival were assessed by MTT assay. H9c2 cells were treated with 600 μM CoCl₂ for 0 to 24 h, and expression of (C) HIF-1α and ZFP580 protein, or (D) HIF-1α and (E) ZFP580 mRNA were determined by western blot analysis or reverse transcription-quantitative polymerase chain reaction, respectively. Three experiments were performed for each group, and each experiment was replicated twice. The results are presented as the mean ± standard error. *P<0.05 vs. control. CoCl₂, cobalt chloride; HIF-1α, hypoxia-inducible factor-1α; ZFP580, zinc finger protein 580; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

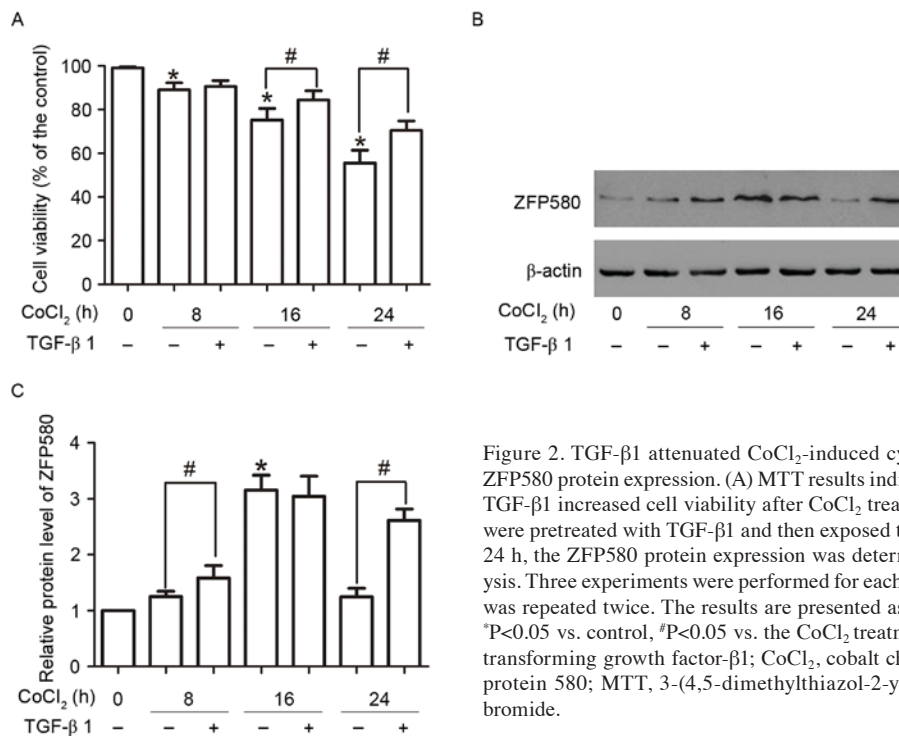


Figure 2. TGF-β1 attenuated CoCl₂-induced cytotoxicity and upregulated ZFP580 protein expression. (A) MTT results indicated that pretreatment with TGF-β1 increased cell viability after CoCl₂ treatment. (B and C) H9c2 cells were pretreated with TGF-β1 and then exposed to 600 μM CoCl₂ for 8, 16 or 24 h, the ZFP580 protein expression was determined by western blot analysis. Three experiments were performed for each group, and each experiment was repeated twice. The results are presented as the mean ± standard error. *P<0.05 vs. control, #P<0.05 vs. the CoCl₂ treatment control group. TGF-β1, transforming growth factor-β1; CoCl₂, cobalt chloride; ZFP580, zinc finger protein 580; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

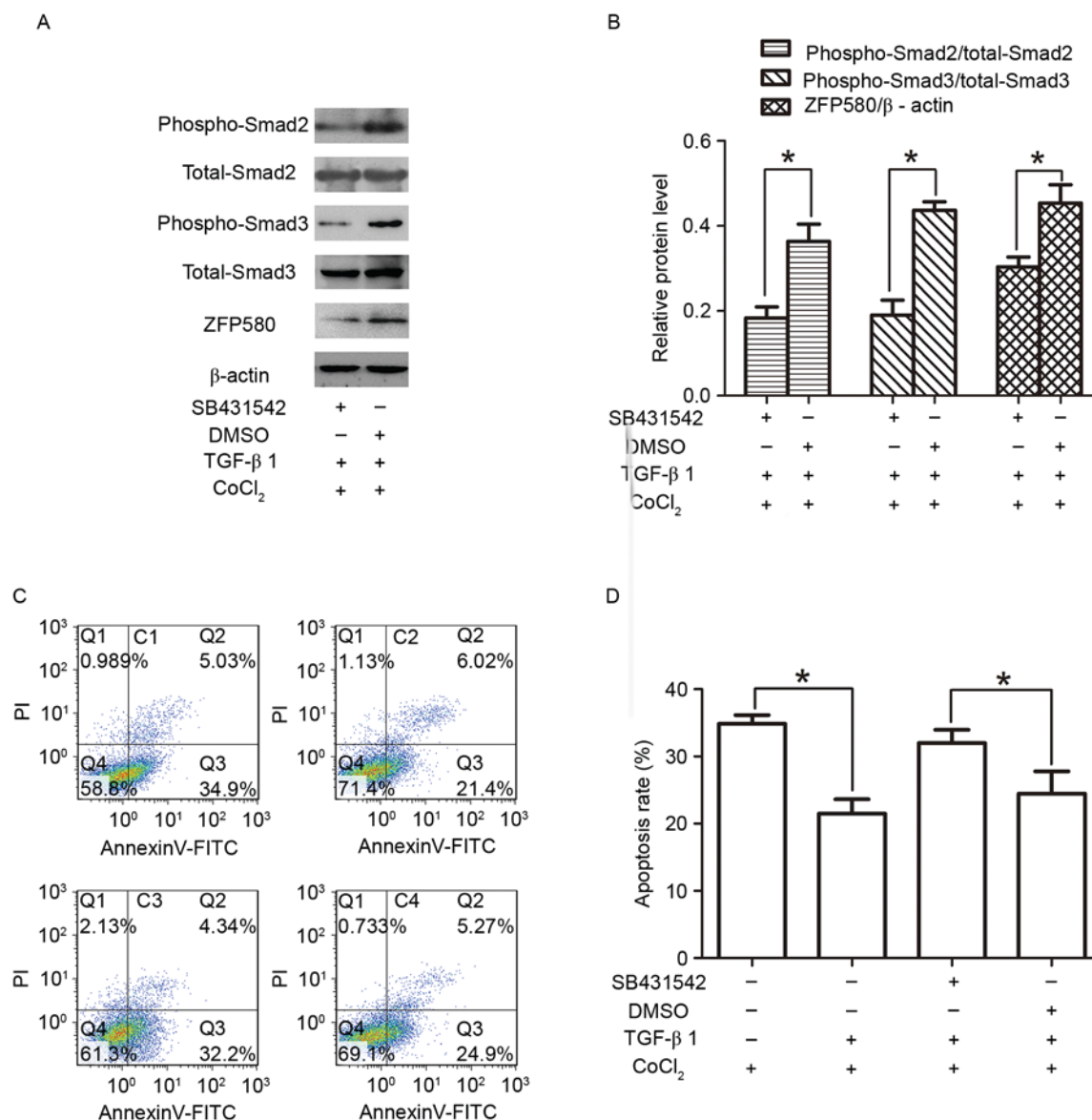


Figure 3. Inhibition of T β R1-Smad2/3 activation attenuated ZFP580 protein expression and the protective effects of TGF- β 1 against CoCl₂-induced apoptosis. H9c2 cells were pretreated with SB431542, a selective inhibitor of T β R1-Smad2/3, prior to stimulation with TGF- β 1, and were subsequently exposed to 600 μ M CoCl₂ for 24 h. (A) Protein expression and (B) histogram analysis detected Smad2/3 activation and ZFP580 expression in H9c2 cells with or without SB431542 pretreatment. (C) Cells (1×10^4) were subjected to AnnexinV-FITC/PI staining and analyzed by flow cytometry. C1, Control group; C2, TGF- β 1 induction group; C3, SB431542 pretreatment and TGF- β 1 induction group; C4, DMSO pretreatment and TGF- β 1 induction group, all exposed to CoCl₂. (D) Representative histograms for the cell apoptosis rates in H9c2 cells. Three experiments were performed for each group, and each experiment was repeated twice. The results are presented as the mean \pm standard error. *P<0.05. TGF- β 1, transforming growth factor- β 1; T β R1, TGF- β type I receptor; Smad, mothers against decapentaplegic homolog; ZFP580, zinc finger protein 580; CoCl₂, cobalt chloride; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

that ZFP580 is involved in the TGF- β 1-mediated defensive mechanisms against CoCl₂-induced injury.

Inhibition of Smad2/3 activation attenuates ZFP580 protein expression and TGF- β 1-mediated cytoprotection against CoCl₂-induced apoptosis. Smad2 and Smad3 are important cofactors in TGF- β 1 signal transduction. To analyze the role of Smad2/3 in the TGF- β 1-mediated cytoprotection against CoCl₂-induced hypoxia, H9c2 cells were first pretreated with SB431542, a well-known selective inhibitor of TGF- β type I receptor (T β R-I) and Smad2/3 activation. Following SB431542 treatment, cells were stimulated with TGF- β 1, and subsequently exposed to CoCl₂. Western

blot analysis indicated that SB431542 partially blocked the TGF- β 1-induced upregulation of ZFP580 expression (Fig. 3A and B). Flow cytometric analysis demonstrated that pretreatment with TGF- β 1 significantly decreased the proportion of apoptotic cells after CoCl₂ treatment (20.64 ± 2.14 vs. $35.68 \pm 1.30\%$; P<0.05); whereas inhibition of Smad2/3 activation appeared to reduce these protective effects by increasing the amount of apoptosis by $\sim 8\%$ (from 23.46 ± 3.33 to $31.97 \pm 2.00\%$; P<0.05; Fig. 3C and D). These findings demonstrated that ZFP580, as a downstream target of the TGF- β 1/Smad2/3 signaling pathway, may be associated with the protective role mediated by TGF- β 1 against CoCl₂-induced cell apoptosis.

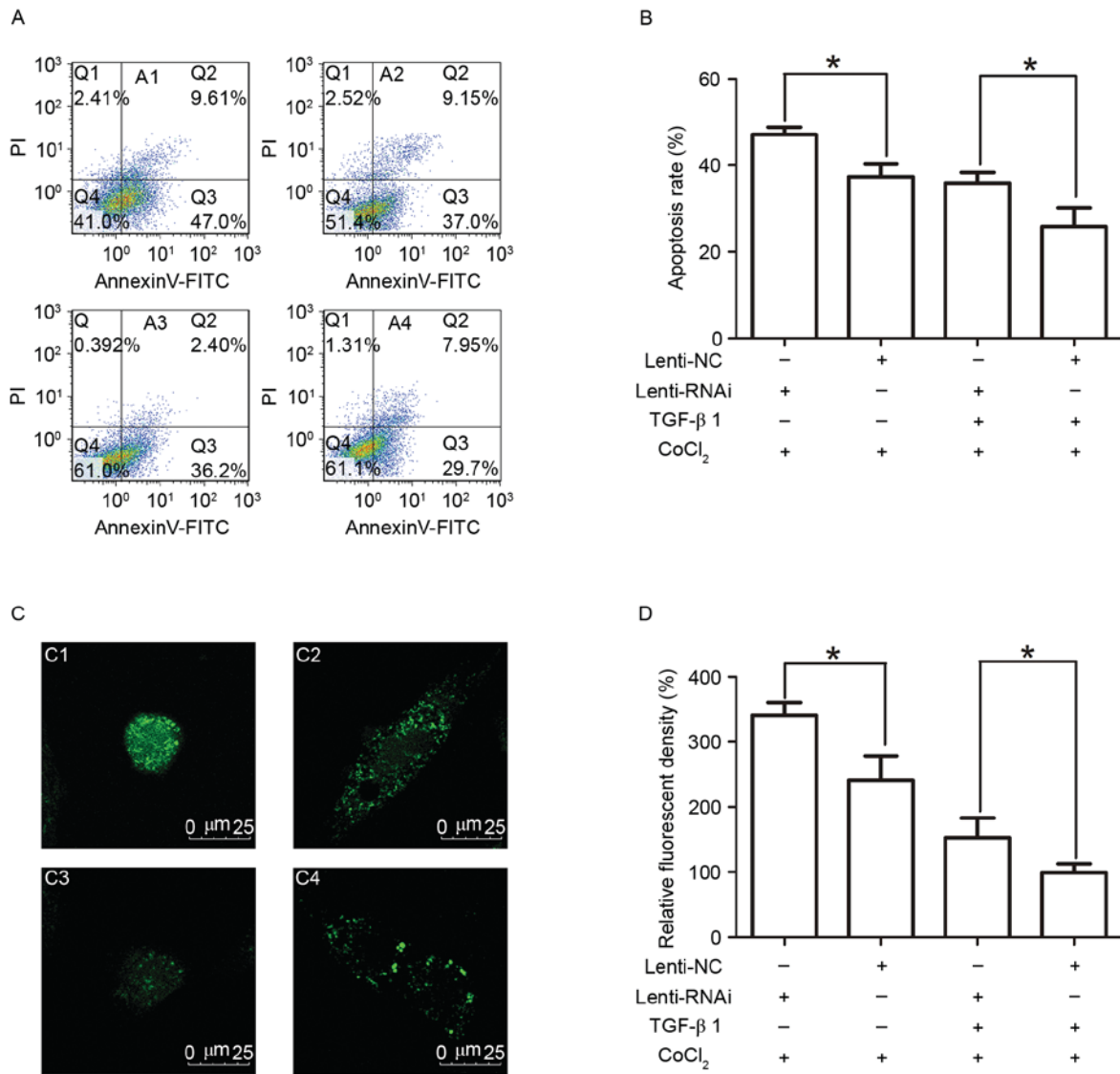


Figure 4. ZFP580 serves an important role in the protective effects of TGF-β1 against CoCl₂-induced apoptosis and ROS generation. H9c2 cells were transfected with Lenti-RNAi or Lenti-NC lentiviral vectors for 72 h, followed by treatment with or without TGF-β1 and subsequent exposure to 600 μM CoCl₂ for 24 h. (A and B) Cells (1x10⁴) were subjected to AnnexinV-FITC/PI staining and analyzed by flow cytometry. (C) DCFH-DA (10 mM) fluorescence probe images were captured using a confocal microscope (Magnification, x200; bars, 25 μm). (D) Intracellular ROS generation was measured by a microplate reader in each of the indicated treatment groups. A1 and C1, Lenti-RNAi with CoCl₂ exposure; A2 and C2, Lenti-NC with CoCl₂ exposure; A3 and C3, Lenti-RNAi with TGF-β1 pretreatment followed by CoCl₂ exposure; A4 and C4, Lenti-NC with TGF-β1 pretreatment followed by CoCl₂ exposure. Three experiments were performed for each group, and each experiment was repeated twice. The results are presented as the mean ± standard error. *P<0.05. ZFP580, zinc finger protein 580; TGF-β1, transforming growth factor-β1; CoCl₂, cobalt chloride; ROS, reactive oxygen species; Lenti-RNAi, small interfering RNA lentiviral vectors directed against ZFP580; Lenti-NC, negative control lentiviral vectors; FITC, fluorescein isothiocyanate; PI, propidium iodide; DCFH-DA, 2,7-dichlorofluorescein diacetate.

ZFP580 serves an important role in the protective effects of TGF-β1 against CoCl₂-induced apoptosis and ROS generation. To evaluate the potential role of ZFP580 in the TGF-β1/Smad2/3-mediated protection against CoCl₂-induced apoptosis, it was hypothesized that TGF-β1 pretreatment of CoCl₂-induced hypoxic cells may stimulate the expression of ZFP580. H9c2 cells were transfected with lentiviral vectors expressing siRNAs directed against ZFP580 (Lenti-RNAi) or a negative control (Lenti-NC) and were subsequently pretreated with or without TGF-β1, prior to exposure to CoCl₂. Flow cytometric analysis indicated that suppression of ZFP580 significantly increased the proportion of apoptotic cells compared with the number of apoptotic cells in the Lenti-NC group (35.90±2.92 vs. 46.13±1.68%; P<0.05).

Furthermore, the anti-apoptotic role of TGF-β1 was reduced by Lenti-RNAi transfection (Fig. 4A and B). Analysis of ROS generation revealed that CoCl₂ significantly induced ROS production in Lenti-RNAi cells compared with in Lenti-NC cells (P<0.05; Fig. 4C-1, C-2, and D). TGF-β1 significantly decreased CoCl₂-induced ROS generation, an effect that was reduced by Lenti-RNAi transfection (Fig. 4C-3, C-4, and D). These results indicated that ZFP580 serves an important role in the TGF-β1-mediated cytoprotective effects against CoCl₂-induced apoptosis and ROS generation.

ZFP580 is involved in the anti-apoptotic effects of TGF-β1 through inhibition of the mitochondrial apoptotic pathway. The role of ZFP580 in the TGF-β1/Smad2/3 signaling

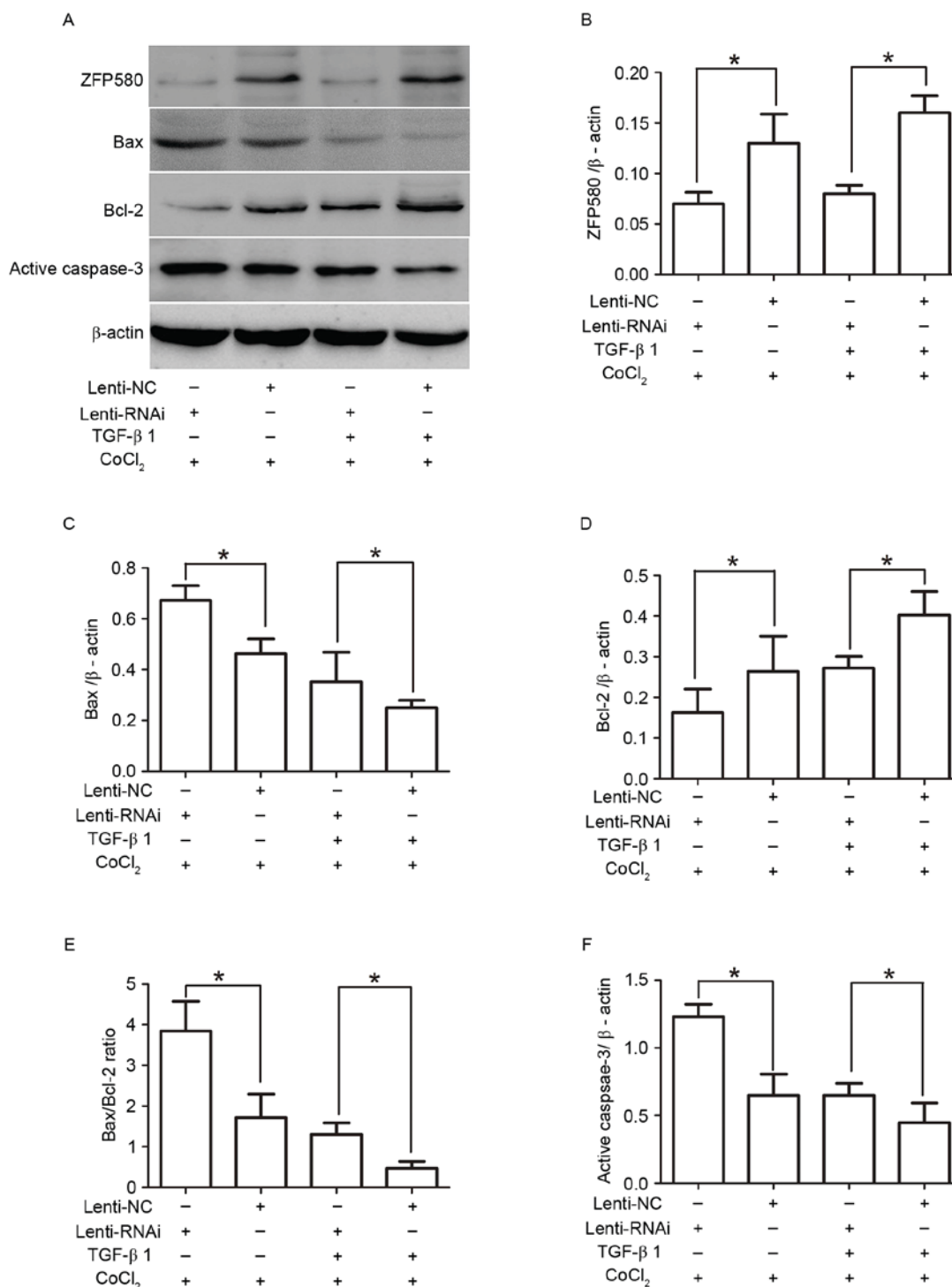


Figure 5. ZFP580 is involved in the anti-apoptotic effects of TGF-β1 through inhibition of the mitochondrial apoptosis pathway. H9c2 cells were transfected with Lenti-RNAi or Lenti-NC lentiviral vectors for 72 h, followed by treatment with or without TGF-β1 and subsequent exposure to 600 μM CoCl₂ for 24 h. (A) Western blot analysis, and histogram analysis of (B) ZFP580, (C) Bax, (D) Bcl-2, (E) Bax/Bcl-2 ratio, and (F) active caspase-3 in each of the indicated treatment groups. Three experiments were performed for each group, and each experiment was replicated twice. The results are presented as the mean ± standard error. *P<0.05. ZFP580, zinc finger protein 580; TGF-β1, transforming growth factor-β1; Lenti-RNAi, small interfering RNA lentiviral vectors directed against ZFP580; Lenti-NC, negative control lentiviral vectors; CoCl₂, cobalt chloride; ROS, reactive oxygen species; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

pathway during CoCl₂-induced apoptosis was investigated. Western blotting indicated that TGF-β1 significantly increased the expression of the anti-apoptotic protein Bcl-2, and decreased the expression of the proapoptotic protein Bax, in Lenti-NC groups compared with Lenti-RNAi groups (Fig. 5). Furthermore, the Bax/Bcl-2 ratio and levels of active caspase-3 were decreased (P<0.05). However,

suppression of ZFP580 reduced the anti-apoptotic effects of TGF-β1, increased the Bax/Bcl-2 ratio, and promoted caspase-3 activation (P<0.05). These results demonstrated that ZFP580 is involved in the protective effects of TGF-β1 against CoCl₂-induced H9c2 cell apoptosis, and may serve an important anti-apoptotic role through inhibition of the mitochondrial apoptotic pathways.

Discussion

ZFP580 is a novel zinc finger protein with three C-terminal C2H2 zinc finger domains and an N-terminal proline-rich domain. The zinc finger is the most abundant protein motif in mammalian cells, and it is involved in the expression and regulation of numerous eukaryotic genes. C2H2 zinc finger proteins in particular often have a crucial role in physiological cellular functions, such as growth and differentiation (3). Previous research has indicated that ZFP580 may be upregulated by hypoxic preconditioning, and may serve a critical anti-apoptotic role in myocardial ischemia and reperfusion injury (7). However, little is known regarding the regulation of ZFP580 expression and function under hypoxic conditions. In the present study, a CoCl_2 -induced hypoxia model was established in H9c2 myocardial cells. The results demonstrated that CoCl_2 treatment decreased cell viability and increased the expression of HIF-1 α protein, but not HIF-1 α mRNA. These findings are consistent with those of previous studies (21,22). Conversely, ZFP580 mRNA and protein expression were upregulated by CoCl_2 treatment. The observed ZFP580 mRNA expression trend was similar to that of the HIF-1 α protein, which is a protein marker of hypoxia. Therefore, it was hypothesized that ZFP580 may serve a role in hypoxia, and its expression may be regulated under hypoxic conditions.

Hypoxia regulates the expression of numerous genes and the secretion of multiple cytokines. Various growth factors, including hepatocyte growth factor, basic fibroblast growth factor and TGF- β 1, have important roles in tissues exposed to hypoxia or ischemia (9). The present study focused on TGF- β 1 due to previous research indicating that this growth factor exerts cardioprotective effects and mediates important anti-apoptotic effects *in vitro* and *in vivo* (9,10,23). Our previous study demonstrated that the human gene ZNF580 was involved in the TGF- β 1 signaling pathway and interacted with the TGF- β signal molecule Smad2 (16,17). Therefore, it was hypothesized that ZFP580 may be involved in the cardioprotective effects of TGF- β 1. In the present study, MTT assay revealed that TGF- β 1 reduced chemical hypoxia-induced cytotoxicity and increased cell viability. Furthermore, it was discovered that TGF- β 1 upregulates ZFP580 expression in H9c2 cells with or without chemical hypoxia, however expression was highest under hypoxic conditions. These findings support the hypothesis that ZFP580 may be a downstream target of the TGF- β 1 signaling pathway and may mediate the protective effects of TGF- β 1.

Following TGF- β 1 binding to its cognate receptors, downstream intracellular signaling involves activation of the canonical pathway involving Smad 2/3, or the non-canonical signaling pathways, including extracellular signal-related kinase (ERK)1/2 and phosphoinositide-3-kinase-Akt (11,24). Previous research has reported that activation of the TGF- β 1-dependent Smad2/3 pathway is correlated with the cardioprotective effects of TGF- β 1 against ischemia/reperfusion-induced apoptosis (25). The present study indicated that TGF- β 1 could significantly decrease CoCl_2 -induced apoptosis, however these effects were reduced following pretreatment with SB431542, an inhibitor of T β R-I and Smad2/3 phosphorylation. These findings indicated that

TGF- β 1 was able to limit chemical hypoxia-induced H9c2 cell injury via activation of Smad2/3. Furthermore, it was demonstrated that inhibition of TGF β R-I-Smad2/3 activation partially blocked the TGF- β 1-induced upregulation of ZFP580 expression. These results supported the hypothesis that ZFP580 is involved in the protective effect of TGF- β 1 against CoCl_2 -induced apoptosis, as a downstream target of the TGF- β 1/Smad2/3 signaling pathway. ZFP580 has a similar structure to that of the Spl-like/krüppel-like factor (KLF) transcription factor superfamily. Li *et al* reported that TGF- β 1 could increase the phosphorylation of KLF4 via Smad signaling pathways in vascular smooth muscle cells (26). Hence, it was further hypothesized that ZFP580 may be regulated by TGF- β 1 through canonical Smad signaling pathways. Our previous study demonstrated that ZFP580 has an anti-apoptotic function as a downstream target of the ERK pathway in myocardial ischemia and reperfusion injury (7). However, whether other non-canonical signaling pathways have critical roles in the regulation of ZFP580 by TGF- β 1 is worth exploring further.

Hypoxia/ischemia may induce a series of pathological changes, including apoptosis and necrosis. Three major pathways can regulate apoptosis: Extracellular Fas protein; mitochondria; and endoplasmic reticulum. CoCl_2 was previously reported to promote hypoxic/ischemic responses by increasing ROS generation, dissipating the mitochondrial membrane potential, activating caspase-3, decreasing cell viability and inducing apoptosis (20,27,28). A study in rat pheochromocytoma PC12 cells indicated that CoCl_2 -induced apoptosis may be associated with the mitochondrial-mediated apoptosis pathway (29). This mitochondrial pathway is predominantly mediated by members of the Bcl-2 family, and is triggered as a result of cell injury induced by DNA damage or cell distress. This leads to mitochondrial disruption and the release of apoptotic mediators, such as apoptosis inducing factor and cytochrome *c* into the cytoplasm, thereby inducing activation of the caspase cascade (30). Furthermore, numerous reports have demonstrated that Bax, Bcl-2 and caspase-3 act as downstream molecules of TGF- β 1 signaling in some cases of apoptosis (8,31,32). Therefore, the present study investigated the protective mechanisms mediated by ZFP580 and TGF- β 1 against CoCl_2 -induced apoptosis in the mitochondrial-mediated apoptotic pathway. The results demonstrated that TGF- β 1 upregulated the anti-apoptotic protein Bcl-2, downregulated the proapoptotic protein Bax, suppressed ROS generation and reduced the activation of caspase-3, which is a final executioner protein in the apoptotic cascade. Conversely, suppression of ZFP580 expression by RNA interference enhanced CoCl_2 -induced cell apoptosis and reduced the anti-apoptotic role of TGF- β 1. These findings suggested that ZFP580 may be a component of the anti-apoptotic process mediated by the TGF- β 1/Smad signaling pathway.

In conclusion, the present study provided experimental evidence that ZFP580 may function as a novel cytoprotective regulator under hypoxic conditions. It was demonstrated that ZFP580 serves an essential role in mediating the cardioprotective effect of TGF- β 1 against chemical hypoxia-induced cell apoptosis by inhibiting the mitochondrial apoptotic pathway.

Acknowledgements

The authors would like to thank colleagues in the Department of Physiology and Pathophysiology, Logistics University of Chinese People's Armed Police Force for their excellent technical assistance and encouragement. They would also like to thank Dr Xin Zhou and Dr WenJie Ji (Tianjin Key Laboratory of Cardiovascular Remodeling and Target Organ Injury, Pingjin Hospital Heart Center, Tianjin, China) for their help in the flow cytometry study. This study was supported by funds from the National Natural Science Foundation of China (grant no. 81173588); the Tianjin Natural Science Foundation (grant no. 12JCYBJC15900); and the Tianjin Key Laboratory for the Prevention and Control of Occupational and Environmental Hazard (grant no. TJC1409).

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