Upregulation of SIRT1 inhibits H_2O_2-induced osteoblast apoptosis via FoxO1/β-catenin pathway

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Abstract. Osteoporosis is a disease that significantly influences life expectancy and quality in humans. Oxidative stress may stimulate bone marrow osteoclast differentiation and inhibit osteoblast (OB) differentiation. OB proliferation and differentiation are affected by the forkhead box O (FoxO)1/β-catenin signaling pathway. The osteogenic differentiation of mesenchymal stem cells (MSCs) may be promoted by silent information regulator type-1 [sirtuin (SIRT)1]. However, the molecular mechanism of SIRT1 regulation of osteogenic differentiation of MSCs remains unclear, and further elucidation is needed. The present study investigated the role of SIRT1 in the FoxO1/β-catenin signaling pathway in oxidative stress and its mechanism in the osteoblastic progenitor cell line (MC3T3-E1). The results demonstrated that OB apoptosis and elevated oxidative stress in cells were simulated by H_2O_2, which was inhibited by moderate SIRT1 overexpression through reducing the oxidative stress. Further studies revealed that FOXO1 and β-catenin pathway activity was downregulated by SIRT1 and eventually resulted in inhibition of target genes, including the proapoptotic gene B cell lymphoma-2 interacting mediator of cell death, DNA repair gene growth arrest and DNA damage inducible protein 45 and the OB differentiation suppressor gene peroxisome proliferator activated receptor (PPAR)-γ. Furthermore, β-catenin and PPAR-γ were inhibited by SIRT1. Overall, the results of the present study suggest that moderate overexpression of SIRT1 (~3-fold of normal level) may directly or indirectly inhibit apoptosis of OBs via the FOXO1 and β-catenin signaling pathway.

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Introduction

Osteoporosis (OP) is a disease characterized by structural deteriorationist, low bone mass, and porous bone associated with higher fracture risk, significantly influencing expectancy and quality of life in humans. Osteoblasts (OB) play pivotal roles in mediation of formation, resorption and remodeling of bones (1). OB and osteoclast (OC) are units of bone remodeling and healthy of bone is dependent on balance and interaction between them (1). The imbalance caused by reduction of OB differentiation and increased differentiation of OC were the major reasons of OP (1). OP was increased year by year with aging population and per-capita life expectancy, especially for postmenopausal womenfolk (2). In the past decades, researches showed that estrogen deficiency and immune and inflammatory responses are the main factors inducing OP (3,4). However, recent investigations indicated that oxidative stress can stimulate bone marrow OC differentiation and inhibit OB differentiation, thereby promoting the development of OP (5,6). Therefore, as a risk factor for the development of OP, oxidative stress has received more attention (6,7). And the focus of pathologic mechanism of OP was gradually shifted from estrogen-centric to aging and oxidative stress (8).

Oxidative stress is a highly reactive molecule in living organisms, such as reactive nitrogen radicals (RNS) and reactive oxygen species (ROS) (9,10). High production of them would lead to oxidative damage to bimolecular, which further induces tissues and organs damaged (10). ROS is closely related with biomedical and pathogenic bases of variety diseases and pathological processes. Oxidative stress levels in the body depend on the balance between ROS and antioxidant defense system *in vivo* (10).

Investigations showed that oxidative stress can inhibit bone marrow stromal cell line (M2-1 OB4) and OB progenitor cell line (MC3T3-E1) differentiation into OB, and inhibit OB mineralization and induce its apoptosis (11). Studies revealed that H_2O_2 in human bone marrow stromal cells (HBMSCs) increased level of ROS through activation of c-Jun N-terminal kinase (JNK) and nuclear factor κB (NF-κB) Pathway, which result in reduction of glutathione, activation of caspases 3, 9 and 8, and finally inducing apoptosis of HBMSCs (12).

ROS is capable of regulating the osteoclastic bone resorption from bone cells (13). Superoxide produced by OC may be directly involved in the degradation of bone tissue, and inhibiting production or utilization of ROS and bone resorption (13). Researches showed that production of ROS and OC differentiation were completely inhibited by blocking nicotinamide adenine dinucleotide phosphate oxidase 1 (Noxl) in OC precursor cells (14). In other words, ROS can stimulate the growth and differentiation of OC, while OC, in turn, increases the production of ROS, and if the body's antioxidant defense mechanisms impaired, it will form a vicious circle, leading to increased bone resorption and OP.

Currently, there are two kinds of drugs in the treatment of OP: The bone resorption inhibitor such as calcium, vitamin D, estrogen (15), the other is bone enhancers, such as fluoride and parathyroid hormone (16). Antioxidant treatment is a new and effective approach to the prevention and treatment of OP (17).

OB is the main functional cell of bone formation in bone metabolic processes, and its proliferation and differentiation were affected by multiple signaling pathways, including ER, BMP-2/Smads, OPG/RANKL, Wnt and forkhead box O (FoxO)1/β-catenin signaling pathways (18,19). FoxO transcription factor family is a community of transcription factors with conservative Fork domain, including four family members: FoxO1 (Fkhr), FoxO3 (Fkhrl 1), FoxO4 (Afx) and FoxO6 (20,21). Investigators reported that among four family members of FoxOs, only the FoxO1 is the transcription factor that promotes OB proliferation and is necessary to maintain redox balance and control bone formation (22). FoxOs activities are regulated by multiple signaling pathways, including insulin, phosphatidylinositol-3-kinase/serine threonine kinase (PI3K/Akt), which mainly involving regulation of phosphorylation/dephosphorylation of specific amino acid sites (23). After phosphorylation of specific amino acid sites, FoxOs were transferred from the nucleus to the cytoplasm, where transcriptional activity of FoxOs and its functions are inhibited (23). Therefore, the nuclear translocation of FoxOs determines the regulation of transcription of target genes.

Silent information regulator type-1 [sirtuin (SIRT)1] is a NAD^+-dependent deacetylase (24). Because of the substrate diversity of SIRT1, it has a wide range of physiological functions, including genome stability, cell survival, apoptosis, inflammation and metabolism (24,25). Among them, SIRT1 and apoptosis are closely related. It has been proved that SIRT1 can not only deacetylate histone and regulate the transcriptional activity of transcription factors in preadipocyte differentiation (26), but also can deacetylate lipids to produce key transcription factors (27). In addition, SIRT1 can promote the osteogenic differentiation of mesenchymal stem cells (MSCs) and inhibit the adipogenic differentiation of MSCs (28). However, the molecular mechanism of SIRT1 regulation of osteogenic differentiation of MSCs remains unclear, and further elucidation is needed. Nuclear localization of FoxO1 is regulated by SIRT1 deacetylase (29).

In summary, the aim of this study is to investigate the inhibition efficiency of overexpressing SIRT1 on apoptosis of OB cell induced by H_2O_2. Our results revealed that upregulated SIRT1 can inhibit apoptosis of OB cell induced by H_2O_2 though FoxO1/β-catenin pathway.

Materials and methods

Cell culture. MC3T3-E1 cells, derived from newborn mice calvaria, were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics, at 37˚C under a humidified atmosphere with 5% CO_2 and 95% air, as previously described. Cells were grown to about 70-80% confluence and used for following experiments.

Cell transfection and experiments. Exponentially growing cells at $1x10^5$ were seeded to 6-well plates and cultured at 37˚C and 5% CO_2 for 24 h. After cells were grown to about 80% subconfluence, medium was discarded and replaced by serum-free DMEM-F12 medium overnight. According to the instructions, Lipofectamine 2000 10 μl was diluted with 250 μl serum-free DMEM-F12 and incubated for 5 min at room temperature. Serum-free DMEM-F12 medium (250 μl) were added into two groups of centrifuge tubes. The blank control plasmid and SIRT1 overexpression plasmid were constructed by Shanghai Jike Gene Chemical Technology Co., Ltd. (Shanghai, China) and 4 mg of Sirtl overexpression plasmid and blank control plasmid were then added into the two groups, respectively. Plasmids were added to Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) diluted solution and incubated at room temperature for 20 min. The DNA-liposome mixtures obtained were added to 6-well plates, respectively. After incubation for 6 h, culture medium was replaced by normal culture medium to continue cultivation. After transfection for 48 h, transfection efficiency was assessed by western blot and RT-PCR assays. Cells used in this study were divided into four groups as follows: Normal cells (untransfected, control), normal cells with H_2O_2 treatment (H_2O_2), negative transfection cells with H_2O_2 treatment (H_2O_2 + NT), and positive transfection cells with H_2O_2 treatment (H_2O_2 + SIRT1). All cells were then cultured in complete DMEM and harvested at 24, 48, and 72 h.

CCK-8 assay. Cell viability was determined by CCK-8 assay as described previously (30). Briefly, after treatment, cells harvested at 24, 48 and 72 h were seeded on 96-well plates at $1x10^4$ and 10 μl of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was added into the cell solution. The absorption of cell solution was measured at 450 nm.

ROS determination by flow cytometer assay. After treatment for 24, 48, and 72 h as described above, cells collected from all groups were washed with PBS and detached using a trypsin solution (Sigma-Aldrich, St. Louis, MO, USA) containing 0.05% trypsin and 0.02% EDTA for 2 min at 37˚C, and then cells were resuspended within 1 ml Hanks' Balanced Salt Solution (HBSS; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Subsequently, cells were incubated in 20 μM dichlorofluorscein-diacetate (DCFH-DA) (Sigma-Aldrich) solution [dissolved in dimethylsulfoxide (DMSO)] at 37˚C for 1 h. After cells were washed with PBS, ROS production in cells was detected using flow cytometry.

Apoptosis determination by flow cytometer assay. After treatments for 24, 48, and 72 h as described above, cells in

all groups were detached using a trypsin solution containing 0.05% trypsin and 0.02% EDTA for 2 min at 37˚C. When cells were observed to be round and float, 10% serum medium was added to stop digestion. Cells were then centrifuged at 1,000 rpm for 4 min. After washed with PBS, cells were placed into diluted binding buffer (500 μl). Then FITC-labeled Annexin V and 5 μl PI (BioDesign, Quakertown, PA, USA) were added, respectively. After incubation at room temperature for 10 min, apoptosis was finally measured using flow cytometer. Annexin V-FITC positive and PI negative represent early apoptotic cells and late apoptotic cells were expressed as Annexin V-FITC positive and PI positive.

Western blot assay. Cultured cells from all groups were homogenized in a 100 μl regular lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Quantifications of total protein extracts were performed by BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins was isolated by 10% sodium dodecyl sulphate-polyacrylamide gel and then transferred to immobilon-P-polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Membranes were then blocked in 5% non-fat dry milk in TBST (20 mM Tris-HCl pH 7.2, 137 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and incubated with appropriate monoclonal antibody [FoxO1, 1:1,000, ab60270; β-catenin, 1:5,000, ab32572; growth arrest and DNA damage inducible protein 45 (Gadd45), 1:100, ab76664; Bim, 1:1,000, ab32158; peroxisome proliferator activated receptor (PPAR)-γ, 1:500, ab45036; GAPDH, 1:1,000, ab8245; all from Abcam, Cambridge, UK] at 4˚C overnight. Membranes were then washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000 dilution, DAKO) for 1 h. Each sample was performed in triplicate and GAPDH expression was used to normalize the sample values. Proteins were then determined by enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Band intensity was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Total RNA (1 μg) was converted to cDNA using the Omniscript™ Reverse Transcriptase kit (Qiagen). Real-time PCR was performed using a SYBR-Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and determined on an ABI PRISM 7700 Sequence Detection system (Applied Biosystem; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The PCR products were confirmed by melting curve analysis and garose gel electrophoresis. Data were normalized to GAPDH and were calculated using delta-delta cycle threshold (CT) method. All primer sequences are listed as follows: SIRT1 forward, 5'GGGTTTCTGTCTCCTGTG GGA3' and reverse, 5'GCTTGAGGGTCTGGGAGGTC3'; Bax forward, 5'TCATGGGCTGGACACTGGAC3' and reverse, 5'CACAGTCCAAGGCAGTGGGA3'; Bcl-2 forward, 5'GCC TGAGAGCAACCCAATGC3' and reverse, 5'CGGAGG GTCAGATGGACCAC3'; FoxO1 forward, 5'GTCTACGCT GCCCAGTCTGT3' and reverse, 5'TGTTTGGCGGTGCAA ACGAA3'; β-catenin forward, 5'GGATACGGCCAGGAT GCCTT3' and reverse, 5'CCAGATCAGGCAGCCCATCA3'; Gadd45a forward, 5'ATTACGGTCGGCGTGTACGA3' and reverse, 5'ACATCCCGGTCGTCGTCTTC3'; Bim forward, 5'GTTTCCCTTGCCTCCTCGGT3' and reverse, 5'CAGCAG GCTGCAATTGTCCA3'; PPAR-γ forward, 5'GCTGAACGT GAAGCCCATCG3' and reverse, 5'GGCGAACAGCTGAGA GGACT3'; and GAPDH forward, 5'AAGGCTGTGGGCAAG GTCAT3' and reverse, 5'CGTCAGATCCACGACGGACA3'.

Data analysis. Multiple groups are compared by one-way ANOVA followed by post hoc Tukey's comparison test. Data was carried out using Student's t-test for paired data and $P<0.05$ was considered as a statistically significant.

Results

Western blot assay showed high efficiency of cell transfection. To evaluate the transfection effectiveness, we tested the expression levels of both mRNA and protein of SIRT1. After transfection for 48 h, cells were harvested to determine the expression efficiency of SIRT1. As showed in Fig. 1, expression of SIRT1 mRNA and protein were upregulated compared to the control (untransfected) and negative transfection.

Cell viability determination revealed that SIRT1 overexpression protected cells against H_2O_2-induced damage. H_2O_2 is one of main forms of ROS that is relatively more stable *in vivo* and membrane permeable (31). Cell viability assay performed by CCK-8 showed that the cytotoxicity of H_2O_2 increased in a dosage dependent manner. Compared to the control, cell viabilities were significantly reduced in cells with 0.5, 1, 2 mM H_2O_2 treatment for 24 h (Fig. 2A).

To explore the protection effect of SIRT1 overexpression on cells against oxidative damage, we compared the cell viabilities for normal cells, negative transfected cells and positive transfected cells in the present of $H_2O_2$with normal cells without H_2O_2. As showed in Fig. 2B, cell viabilities in H_2O_2 NT and H_2O_2 group were significantly reduced in a time dependent manner, compared to control. While, cell viabilities in H_2O_2 + SIRT1 group were increased from 24 to 72 h and up to no significant difference levels at 48 and 72 h with respect to control group.

High levels of ROS induced by H_2O_2 was reduced in cells with upregulated SIRT1 expression. ROS is capable of regulating the osteoclastic bone resorption from bone cells (13). Superoxide produced by OC may be directly involved in the degradation of bone tissue, and inhibiting production or utilization of ROS and bone resorption (13). Flow cytometer assay revealed that ROS levels in cells were significantly increased after cells treated with 1 mM H_2O_2 for 48 h. As showed in Fig. 3A-E, ROS levels H_2O_2 NT and H_2O_2 group were more 3-fold of control. While in H_2O_2 + SIRT1 group, ROS levels were only half of that in H_2O_2 NT and H_2O_2 group. However, ROS levels in H_2O_2 + SIRT1 group was significant higher than that in control group.

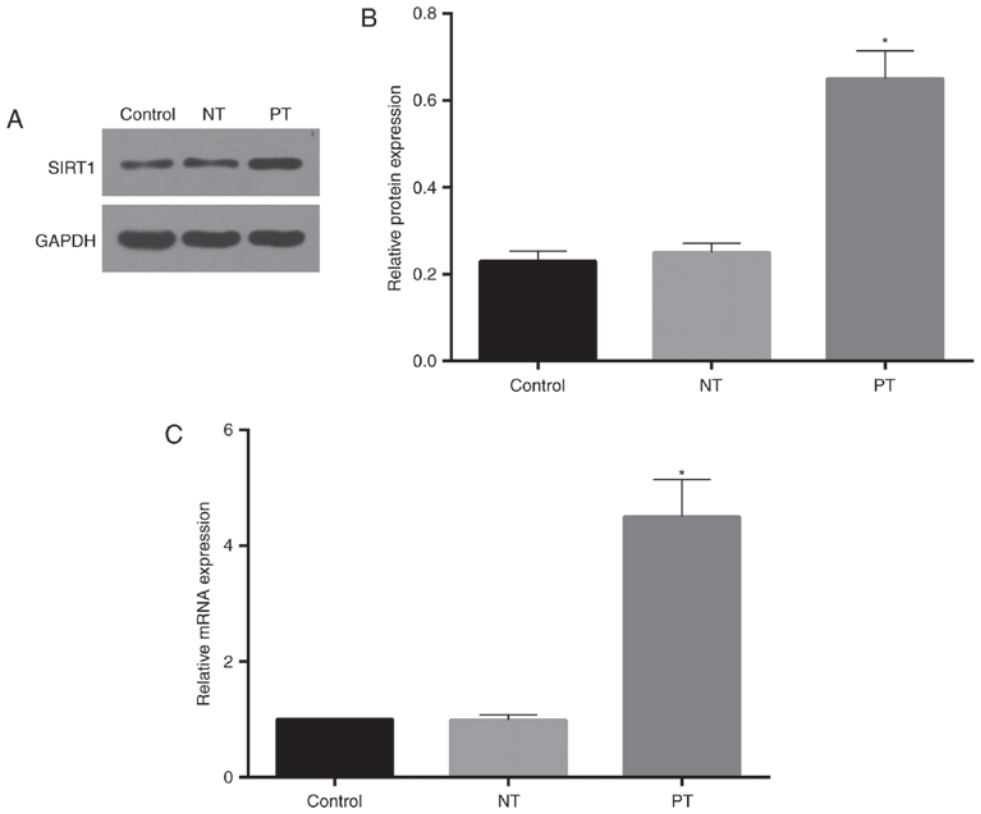

Figure 1. Cell transfection significantly increased the expression level of SIRT1. (A and B) Western blot analysis showed that protein level of SIRT1 was increased after transfection, compared to the control and negative transfection. (C) Real-time RT-PCR analysis showed that SIRT1 mRNA level was significantly increased after transfection with respect to the control and negative transfection. *P<0.05, significantly different from control group. SIRT1, sirtuin 1.

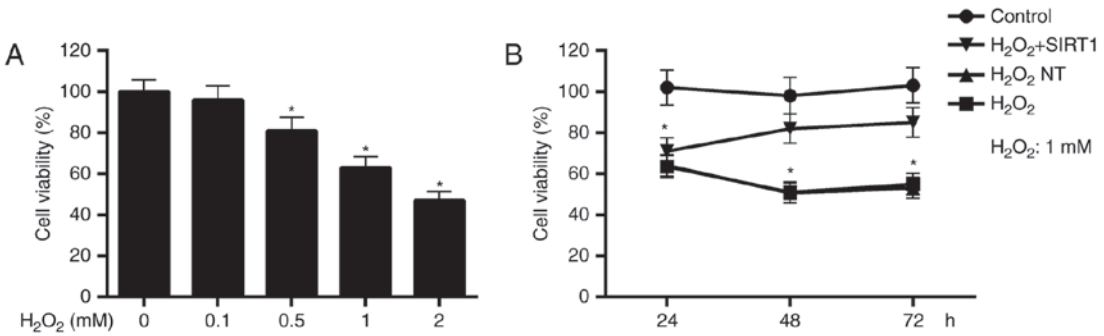

Figure 2. SIRT1 overexpression can elevate cell viability in cells with 1 mM H_2O_2 treatment. (A) H_2O_2 has dosage dependent cytotoxicity on MC3T3-E1 cells; (B) cell viabilities in H_2O_2 NT and H_2O_2 group, and H_2O_2 + SIRT1 group were decrease, and increased from 24 to 72 h, respectively. Cell viabilities were significant lower at 24 h for all group, and 48 and 72 h for H_2O_2 NT and H_2O_2 group, compared to control. *P<0.05, significantly different from control group. SIRT1, sirtuin 1.

SIRT1 overexpression reduced the apoptosis rates induced by H_2O_2. To further assess cytotoxicity of H_2O_2 on MC3T3-E1, we tested the apoptosis rates induced by H_2O_2 under different SIRT1 expression levels. Apoptosis rates were determined in cells in the present of H_2O_2 (Fig. 4). The results showed that after treatment with 1 mM H_2O_2 for 48 h, apoptosis rates in H_2O_2 NT and H_2O_2 group were increased to approximately 32%, which is significantly higher than control. However, apoptosis rate in H_2O_2 + SIRT1 group was <20%, which is one half of that in H_2O_2 NT and H_2O_2 group and more than 2-fold of that in control.

Overexpression of SIRT1 reduced the apoptosis induced by H_2O_2. Based on pro-apoptosis effect induced by H_2O_2, we further explored the evidences on apoptosis-related genes. Bax and Bcl-2 are closely associated with apoptosis, and changes in their levels and ratios are key indicators of apoptosis and surviving. We hereby investigated the effect of SIRT1 overexpression on cells apoptosis induced by H_2O_2. After cells from all groups incubated in 1 mM H_2O_2 for 48 h, the expression levels of Bax and Bcl-2 were significantly changed. As showed in Fig. 5A-C, Bax expression was upregulated in both translation and transcription levels, compared to control, while Bcl-2 expression levels was downregulated in both protein and mRNA. However, expression levels of Bax and Bcl-2 protein and mRNA were reduced and increased in cell from H_2O_2 + SIRT1 group compared to cells from H_2O_2 NT and

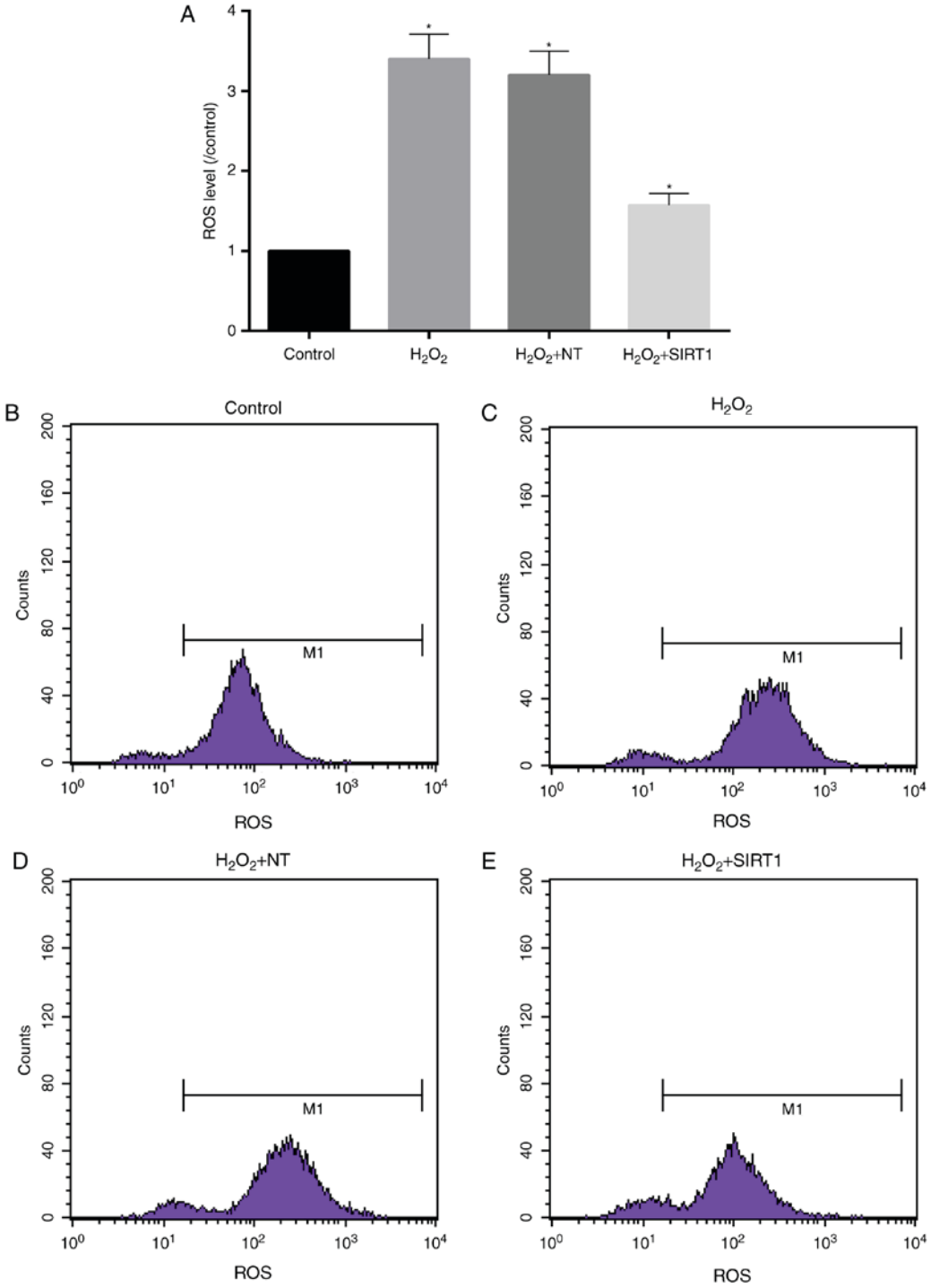

Figure 3. SIRT1 overexpression decreased ROS levels in cells with H_2O_2 treatment. (A-E) Flow cytometer assay showed that after H_2O_2 treatment at 1 mM for 48 h, relative ROS levels in H_2O_2 NT and H_2O_2 group, and H_2O_2 + SIRT1 group were increased >3- and 1.5-fold of the control, respectively. *P<0.05, significantly different from control group. SIRT1, sirtuin 1; ROS, reactive oxygen species.

H_2O_2 group, respectively. The ratios of Bax and Bcl-2 were also significantly changed due to the changes of them. As showed in Fig. 5D, compared to control, the ratios of Bax and Bcl-2 for both protein and mRNA were increased in cells from H_2O_2 NT and H_2O_2 group after cells with 1 mM H_2O_2 treatment for 48 h. While in cells with SIRT1 overexpression (H_2O_2 + SIRT1 group), these ratios were decreased, compared to H_2O_2 NT and H_2O_2 group. Comparing protein expression with mRNA expression, the more significant changes in mRNA ratios of Bax and Bcl-2 were observed (Fig. 5D).

SIRT1 overexpression simulated downregulation of FoxO1/β-catenin pathway and its downstream genes. Our previous results showed that SIRT1 overexpression reduced apoptosis and ROS levels. Here, to clarify the molecular mechanism of these processes, we determined the expression of the FoxO1/β-catenin pathway and its downstream genes, which are associated with proliferation and differentiation of OB. As showed in Fig. 6A and B, protein expression levels of FoxO1 and β-catenin and their downstream genes (Gadd45, PPAR-γ, Bim) were increased in H_2O_2 NT and H_2O_2 group,

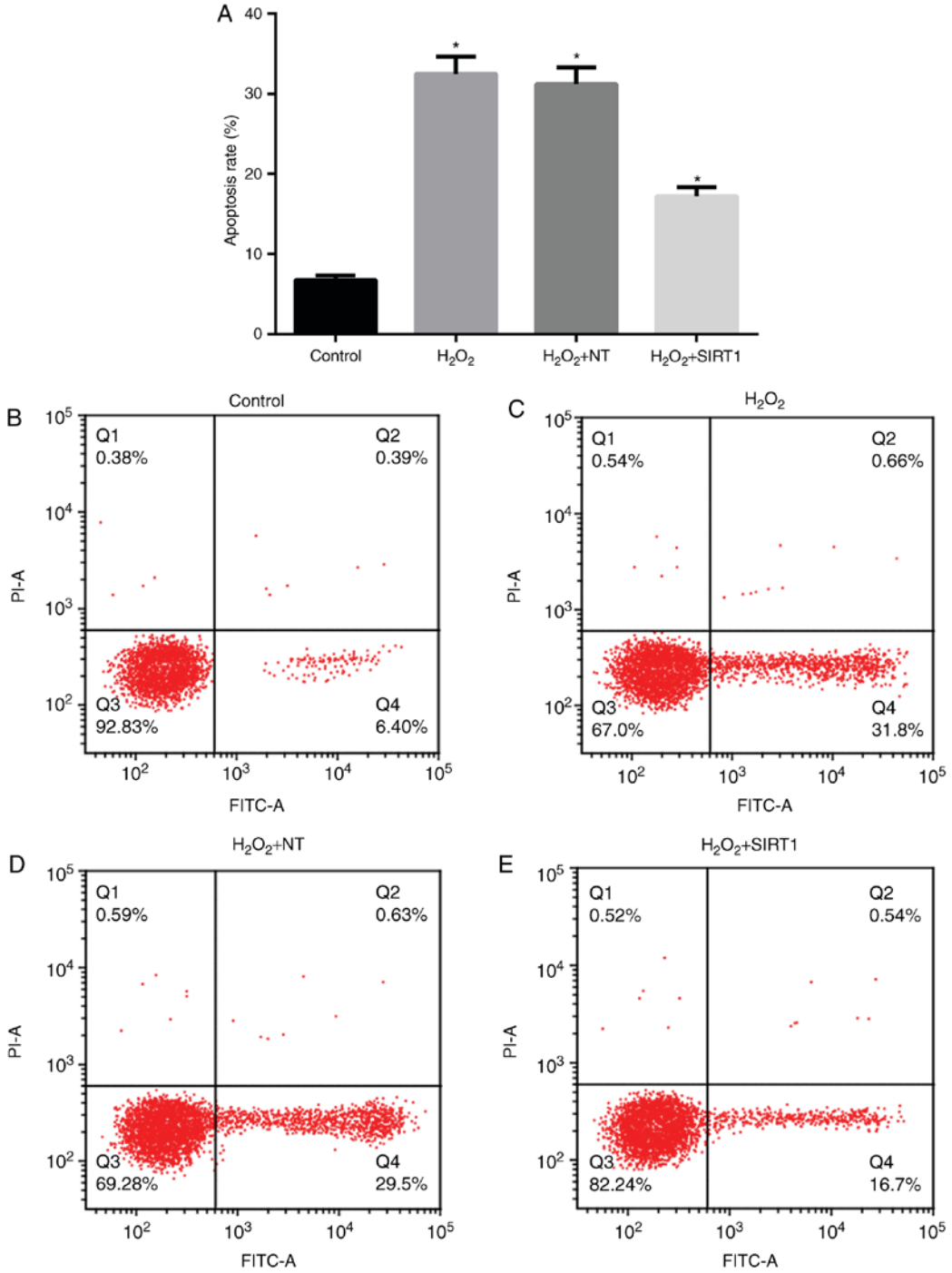

Figure 4. SIRT1 overexpression decreased the apoptosis rates induced by H_2O_2. Apoptosis rates were examined after cells treated with 1 mM H_2O_2 for 48 h using flow cytometer assay. (A-E) The results revealed that apoptosis rates were significantly increased in H_2O_2 NT, H_2O_2 and H_2O_2 + SIRT1 group. Apoptosis rates were reduced in H_2O_2 + SIRT1 group, compared to H_2O_2 NT and H_2O_2 group. *P<0.05, significantly different from control group. SIRT1, sirtuin 1.

compared to control. However, a reduction of expression for all these was observed in H_2O_2 + SIRT1 group compared to H_2O_2 NT and H_2O_2 group.

Fig. 6C shows the relative expression levels of mRNA for FoxO1 and β-catenin and their downstream genes. Expression levels of FoxO1, Gadd45 and Bim mRNA inH_2O_2 NT and H_2O_2 group were apparently increased and approximately 16-fold of that of control. Compared to the control, expression levels of β-catenin and PPAR-γ in H_2O_2 NT and H_2O_2 group were also increased and nearly 4-fold of control. There were no significant differences in expression levels of FoxO1 mRNA in groups from H_2O_2 + SIRT1, H_2O_2 NT and H_2O_2. Expression levels of β-catenin, Gadd45, PPAR-γ and Bim mRNA in H_2O_2 + SIRT1 group were decreased compared to H_2O_2 NT and H_2O_2 group.

Discussion

Metabolism of the skeletal system is a homeostasis process of forming new bone by OBs and absorption of old bone by OCs (32). The process of OB differentiation and proliferation are regulated by multiple signaling pathways and a variety

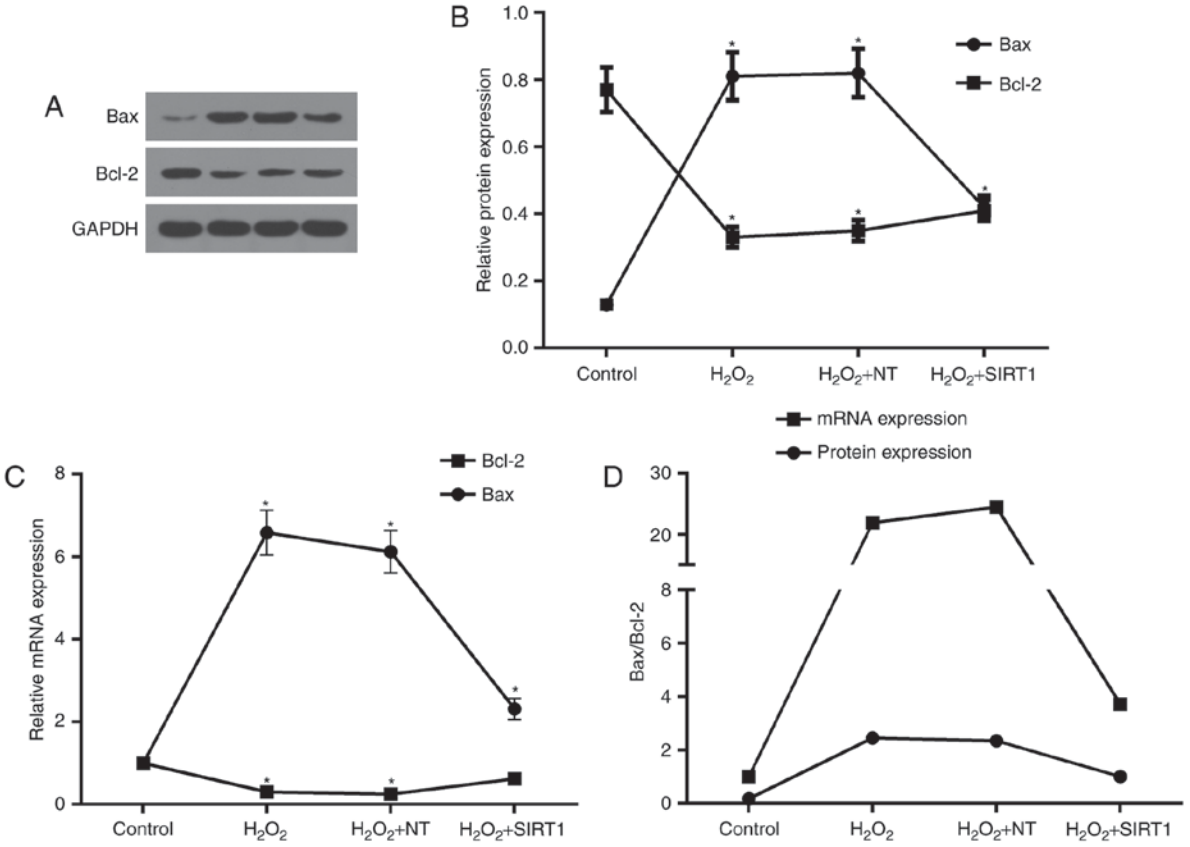

Figure 5. Changes in expression levels of Bax and Bcl-2 in cells confirmed that apoptosis induced by H_2O_2 can be reduced by upregulated expression of SIRT1. (A and B) Western blot analysis showed that in the present of H_2O_2, relative expression levels of Bax and Bcl-2 protein in H_2O_2 NT and H_2O_2 group were increased and reduced compared to control, respectively. However, which were decreased and increased respectively with upregulation of SIRT1 expression, compared to H_2O_2 NT and H_2O_2 group. (C) Real-time RT-PCR revealed that mRNA expression in all groups was similar to protein expression. (D) The protein and mRNA ratios of Bax and Bcl-2 were increased in H_2O_2 NT and H_2O_2 group, and decreased in H_2O_2 + SIRT1 group. *P<0.05, significantly different from control group. SIRT1, sirtuin 1.

of factors, such as hyperglycemia or oxidative stress (16,32). Recent years studies showed that FoxO1 is an important regulatory factor and plays an important role in the skeletal system (22,23). In addition, in response to hyperglycemia or oxygen stress, FOXO1 are acetylated and localized to the promyelocytic leukemia (PML) nuclear bodies, thereby blocking the ubiquitination of FOXO1 and its transcriptional activity (22,33). Subsequently, the acetylated FOXO1 protein is deacetylated in the PML nuclear bodies mediated by SIRT1, inducing FOXO1-dependent transcriptional processes and rapid degradation of FOXO1 (22).

Therefore, in present study, we explored the role of SIRT1 and FOXO1 in the inhibition of apoptosis in OB induced by H_2O_2 (34,35). Our results showed that cell viabilities were inhibited after treatments with H_2O_2 and increased in cells with SIRT1 overexpression. In addition, a reduction of increased ROS level induced by H_2O_2 treatment was observed in cells with SIRT1 overexpression. Together with these results, we suggested that SIRT1 overexpression can increase cell viability by reducing the oxidative stress. Similar researches showed that SIRT1 expression is strongly associated with oxidative stress in mouse myocardium (36). Moderate overexpression of SIRT1 can induce the expression of important antioxidant enzymes, such as catalase, preventing oxidative stress (37). Studies showed that specific overexpression of SIRT1 in the heart tissues delays senescence and prevents oxidative stress; moderate overexpression of SIRT1 (about 3-8-fold) can be effective in preventing drug-induced cardiac stress and apoptosis (37,38). In contrast, oxidative stress and apoptosis were increased in cells with high levels of SIRT1 expression (approximately 12-fold), eventually leading to myocardial infarction and death (38). In this study, both oxidative stress and apoptosis were reduced in cells with moderate SIRT1 expression (approximately 3-fold of control). The ratio of Bax and Bcl-2 expression level was decreased in cells with overexpression of SIRT1, which confirmed the observation of decreasing of apoptosis.

To clarify the role FOXO1 and its downstream genes in the reduction of apoptosis of OB induced by moderate overexpression of SIRT1, we investigated the expression of downstream genes mediated by FOXO1, such as β-catenin, Gadd45, PPAR-γ and Bim. Our results revealed that the expression level of these genes was all increased in cells with H_2O_2 treatment, while it was decreased in cells with additional treatment of moderate overexpression of SIRT1. These results suggested that downregulated expression of β-catenin, Gadd45, PPAR-γ and Bim was induced by SIRT1 via reducing the level of FOXO1 protein. Investigations showed that the interaction of β-catenin and FOXO1 can prevent oxidative damage in cancer cell lines by regulating and controlling the expression of specific target genes (22). It has also been found that under oxidative stress, the competitive binding of

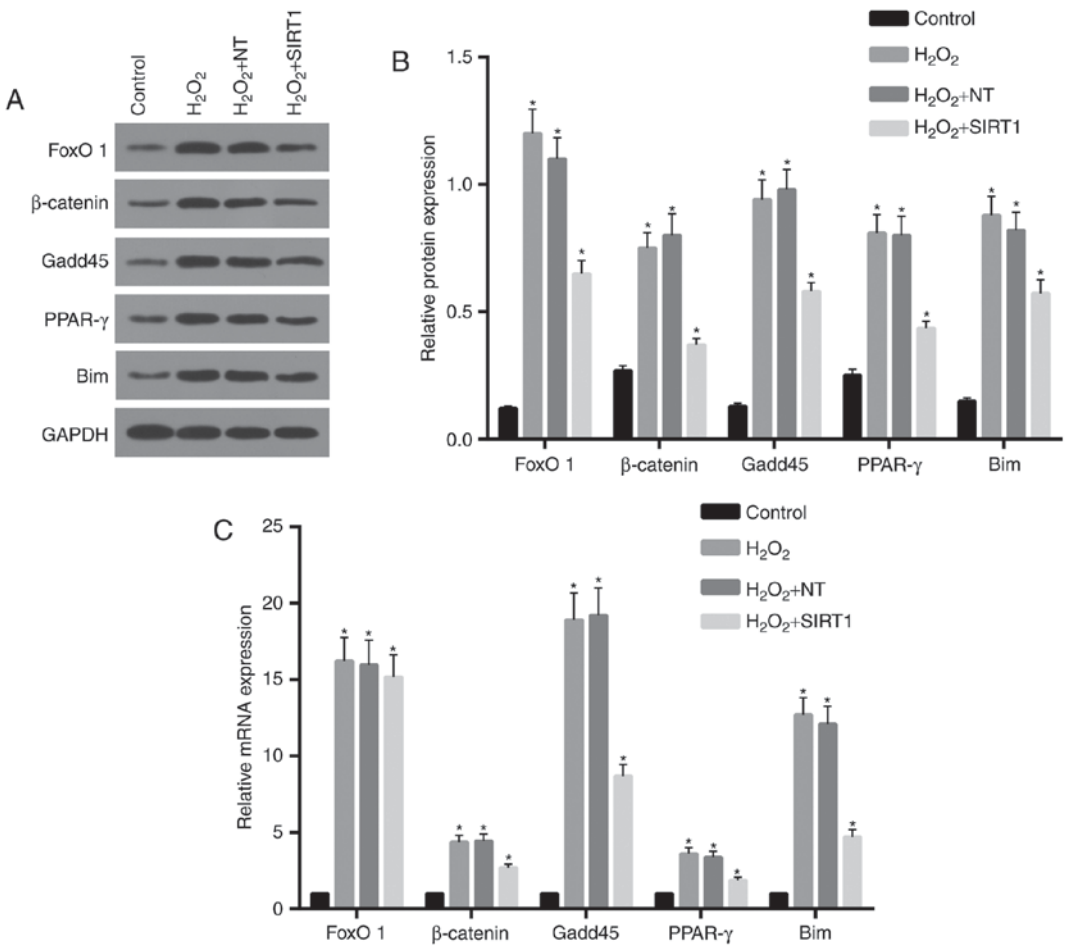

Figure 6. Expression of FoxO1/β-catenin pathway and its downstream genes were downregulated by SIRT1 overexpression. (A and B) Western blot assay was performed and the results showed that protein levels of FoxO1, β-catenin, Gadd45, PPAR-γ and Bim in H_2O_2 NT and H_2O_2 group were significantly increased compared to control. Protein levels for these genes in H_2O_2 + SIRT1 group were reduced compared to H_2O_2 NT and H_2O_2 group. (C) Real-time RT-PCR assay showed that mRNA levels of FoxO1, β-catenin, Gadd45, PPAR-γ and Bim in H_2O_2 NT and H_2O_2 group were significantly increased compared to control. Except for FoxO1, expression levels of β-catenin, Gadd45, PPAR-γ and Bim mRNA in H_2O_2 + SIRT1 group were lower than that of H_2O_2 NT and H_2O_2 group. $^*P<0.05$, significantly different from control group. FoxO1, forkhead box O1; SIRT1, sirtuin 1; Gadd45, growth arrest and DNA damage inducible protein 45; PPAR-γ, peroxisome proliferator activated receptor-γ.

FoxOs to β-catenin in OBs reduces the binding of the latter to the T-cell specific transcription factor (TCF). It's well known that SIRT1 can not only deacetylate histone and regulate the transcriptional activity of transcription factors in preadipocyte differentiation (26). Extensive studies had shown that SIRT1 can directly deacetylate FOXO family transcription factors such as FoxO1, FOXO3a and FOXO4 (22,23,33). In addition, studies had shown that subcellular localization of β-catenin is also affected by SIRT1 via deacetylating it (39,40).

It had been found that PPAR-γ is mainly expressed in adipocytes and its precursor cells, which mediate the differentiation and proliferation of adipocytes (41). It is also expressed in OBs and plays an important role in maintaining bone metabolism balance (42). OBs and bone marrow adipocytes are differentiated by the same precursor, MSCs, which can be transformed into each other under certain conditions (18,22,32). Differentiation of MSCs into OB requires OB-specific transcription factors, namely nuclear protein-binding factor al (cbfa1), which activates OB-specific gene expression such as osteocalcin gene, type I collagen fiber gene, and osteopontin gene (43). PPAR-γ inhibits the expression of cbfal and reduces the expression of the above genes, thereby inhibiting the differentiation of MSCs into OB. On the contrary, it promotes the differentiation of MSCs into adipocytes (41). Transcription of PPAR-γ was simulated by FoxO1 in cells with H_2O_2 treatment and inhibited in cells with overexpression of SIRT1. These results may be explained in two ways. On one hand, SIRT1 overexpression induced ubiquitination of FOXO1 and inhibited β-catenin and FOXO1 pathway way, eventually leading downregulation of Gadd45, PPAR-γ and Bim. On the other hand, investigation showed that the transcriptional activation of PPAR-γ was also directly inhibited by SIRT1 through recruiting the nuclear receptor co-repressors (NCoR and SMRT) to the promoter region of PPAR-γ (41,44).

Gadd45 is a cell cycle-related protein that plays an important role in cell cycle regulation, apoptosis and repair of DNA damage. Increased expression of Gadd45 is necessary for cell cycle arrest and repair of DNA damage (45). The abnormality or dysfunction of Gadd45 expression may lead to abnormal DNA repair and repair pathways mediated by p53, which cannot normally respond to DNA damage, inhibit abnormal cell proliferation and control the regulation of cell cycle, resulting in damage accumulation and malignant transformation and even formation of tumor (45,46). Our studies showed that the expression level of Gadd45 was increased in

cells treated with H_2O_2, where Gadd45 may protect cells from oxidative damage by inducing cell cycle arrest and repair of DNA damage. However, our results revealed that an increased apoptosis was observed in cells treated with H_2O_2. Although Gadd45 is essential for maintaining genomic stability, studies had shown that apoptosis of epithelial cells is inhibited by Gadd45 knockdown by UV irradiation, suggesting that Gadd45 can promote apoptosis (45). The regulation of Gadd45 protein on apoptosis is still controversial, which may be related to cell types and the environmental stimuli inducing apoptosis (46).

The Bcl-2 family is an important regulator of the apoptotic pathway, including the anti-apoptotic Bcl-2 subfamily and pro-apoptotic Bax and BH3 subfamily (also known as BH3-only protein) (47,48). Bim (Bcl-2 interacting mediator of cell death) is one of the BH3-only proteins, which was considered to be a molecule that promotes apoptosis in recent years (49). Previous studies had shown that Bim is one of the downstream target genes of FoxOs (49,50). Here, our results showed that Bim expression was significantly increased in cells treated with H_2O_2 and inhibited in cells with SIRT1 overexpression, suggesting that SIRT1 inhibits Bim expression by mediating FoxOs. Bim can interact with Bcl-2/Bax and then activate Bax-induced mitochondrial pathway (47,49). It had been found that Bim is a very important factor in the process of oxidative stress-induced apoptosis, both *in vitro* and *in vivo* in oxidative stress models (50). Therefore, we suggested that apoptosis induced by H_2O_2 was mediated by SIRT1/FoxOs/Bim pathway.

In conclusion, moderate SIRT1 overexpression can increase cell viability by reducing the oxidative stress, which was involved the expression of specific target genes of β-catenin and FOXO1 pathway, such as Gadd45, PPAR-γ and Bim. FOXO1 and β-catenin pathway was inhibited by SIRT1. In addition, PPAR-γ, an inhibitor of differentiation of MSCs into OB, was also directly inhibited by SIRT1, suggesting that SIRT1 probably promote differentiation of MSCs into OB. However, there is a major limitation that we did not further investigate the deacetylation effect on FoxO1 and β-catenin, which were proved in others studies.

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