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Research Article

The resistant effect of SIRT1 in oxidative stress-induced senescence of rat nucleus pulposus cell is regulated by Akt-FoxO1 pathway

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Objective: The senescence of nucleus pulposus (NP) cells induced by oxidative stress is one of the important causes of intervertebral disc degeneration (IDD). Herein, we investigated the role and action mechanism of silent information regulator 1 (SIRT1) in oxidative stress-induced senescence of rat NP cell.

Methods: Premature senescence of rat NP cells was induced by sublethal concentration of hydrogen peroxide (H_2O_2) (100 μM). SIRT1 was activated with SRT1720 (5 μM) to explore its effect on NP cells senescence. FoxO1 and Akt were inhibited by AS1842856 (0.2 μM) and MK-2206 (5 μM), respectively, to explore the role of Akt-FoxO1-SIRT1 axis in rat NP cells. Pretreatment with the resveratrol (20 μM), a common antioxidant and indirect activator of SIRT1, was done to investigate its role in senescent rat NP cells.

Results: The mRNA and protein levels of SIRT1 were decreased in H_2O_2-induced senescent rat NP cells, and that specific activation of SIRT1 suppresses senescence. And the Akt-FoxO1 pathway, as the upstream of SIRT1, might be involved in the regulation of H_2O_2-induced senescence of rat NP cells by affecting the expression of SIRT1. In addition, the resveratrol played an anti-senescence role in rat NP cells, which might affect the Akt-FoxO1-SIRT1 axis.

Conclusion: SIRT1 ameliorated oxidative stress-induced senescence of rat NP cell which was regulated by Akt-FoxO1 pathway, and resveratrol exerted anti-senescence effects by affecting this signaling axis.

Introduction

Intervertebral disc degeneration (IDD) is a widely known cause of low back pain. The pathogenesis of IDD is complicated, associated with reactive oxygen species (ROS) and oxidative stress, proinflammatory cytokines storm, reduction of the number of functional cells (programmed cell death, cell necrosis and senescence) and degeneration of extracellular matrix [1,2]. However, our understanding of the pathogenesis of IDD is still limited. Although the rarely number of intervertebral disc cells (mainly nucleus pulposus [NP] cells) are embedded in the IVD, they play a dominating role in maintaining the stability of the microenvironment of discs. The senescent disc cells lost the capability of replication to generate new cells, thus the number of functional cells in discs decreases gradually due to cell death eventually. Furthermore, the senescence-associated secreted phenotype (SASP) of senescent disc cells is characterized by a catabolic and pro-inflammatory phenotype [3–6]. Subsequently, these results led to accelerated development of IDD. The excessive ROS and oxidative stress are tightly

associated with the development of premature senescence, and contribute to the establishment and progression of IDD. It has been reported that ROS are produced in the form of hydrogen peroxide (H_2O_2) in NP cells *in vivo* [7], but the process of H_2O_2-induced premature senescence of NP cells needs further verification.

silent information regulator 1 (SIRT1) is a member of the silent information regulator 2 protein family. It is a highly conserved nicotinamide (NAD^+)-dependent deacetylases and has been found to be associated with age-related diseases, cancer and degenerative disorders [8,9]. SIRT1 has been shown to regulate cellular oxidative stress burden and its toxicity, while cellular redox status can also affect SIRT1 level and activity through multiple manners [10]. Although previous study has shown that oxidative stress led to a reduction of SIRT1 abundance and transcription in lung epithelial cells, endothelial cells and macrophages, the SIRT1 expression was significantly elevated in an early degeneration stage [11,12]. Thus, the expression of SIRT1 in NP cells modulated by oxidative stress is controversial. In addition, SIRT1 is able to regulate the expressions of p53 and p16 by deacetylation in NP and other type cells to relieve the progress of senescence [13–15]. However, the role of SIRT1 in senescent NP cells has not been well studied.

Transcription factor FoxO family members (and FoxO1 in particular) also play important roles in aging, cell metabolism, insulin resistance and oxidative stress resistance [16]. FoxO1, as a transcription factor, binds to a large set of target gene promoters and has been shown to regulate the transcription of genes even in the absence of direct DNA binding [17]. And it has been shown that FoxO1 could bind directly to the SIRT1 promoter through insulin receptor substrate-1 and forkhead (FKHD)-L sites and to positively regulate its transcription [18]. However, whether this regulatory relationship between FoxO1 and SIRT1 exists in senescence NP cells induced by oxidative stress has not been verified. Besides, the best characterized of all FoxO regulatory pathways is the phosphatidylinositol-3-kinase (PI3K)/Akt-mediated suppression of FoxO activity. Functionally, active FoxO1 protein is mainly localized in the cell nucleus, where its transcriptional functions can be executed. Conversely, the activation of PI3K/Akt pathway phosphorylates FoxO1 in the nucleus, creating a docking site for 14-3-3 proteins that translocate FoxO1 to the cytoplasm and inactivate them [19,20]. At physiologic levels, ROS signaling is frequently associated with growth factor–receptor activation and stimulation of cellular metabolism and growth through the PI3K/Akt pathway. And previous study had found that Akt activation induced premature senescence by increasing intracellular ROS through increased oxygen consumption and inhibiting the expression of ROS scavengers downstream of FoxO [21]. Akt, FoxO1 and SIRT1 play important roles in regulating oxidative stress and senescence, but their relationship and function in oxidative stress-induced premature NP cells are poorly characterized.

In the present study, we demonstrated that SIRT1 played a crucial role in oxidative stress-induced senescent rat NP cells, and Akt-FoxO1 pathway was involved in the regulation of SIRT1 expression. Additionally, we found that resveratrol, a known plant-derived polyphenol antioxidant and activator of SIRT1 [22,23], shown an anti-senescence effect via suppressing the activity of Akt and activating the FoxO1-SIRT1 pathway. It is suggested that Akt-FoxO1-SIRT1 axis might be a potential therapeutic approach to relieve chronic disc degeneration.

Materials and methods
Reagents and antibodies

SRT1720 HCL (S1129), a selective activator of SIRT1 was purchased from Selleck (Houston, TX, U.S.A.) [24]. AS1842856 (HY-100596), a potent and cell-permeable Foxo1 inhibitor was purchased from MedChem Express (MCE) (Princeton, NJ, U.S.A.) [25]. MK-2206 dihydrochloride (T1952), a highly specific inhibitor of Akt1/2/3 [26], was purchased from TargetMol (Boston, MA, U.S.A.). Resveratrol (R5010), a phenolic phytoalexin found in grape skin and other plants was purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

The antibodies against Collagen II (ab34712), p53 (ab26), p21 (ab80633), p16 (ab51243) and SIRT1 (ab110304) were purchased from Abcam (Cambridge, MA, U.K.). The antibodies against β-actin (4970S), Phospho-Histone H2A.X (Ser139) (9718S), Phospho-Rb (Ser807/811) (8516S), Akt (4691S), Phospho-Akt (Ser473) (4060S), FoxO1 (2880S) and Phospho-FoxO1 (Ser256) (9461S) were purchased from Cell Signaling Technology (CST, Danvers, MA, U.S.A.).

Isolation of rat NP intervertebral disc cells and cell culture conditions

The present study has been approved by the IACUC of Soochow University. All the Sprague–Dawley (SD) rats were obtained from the Laboratory Animal Center of Soochow University. All experimental procedures were in accordance with the guidelines of animal research of Science and technology department of China, and have been approved by the ethic committee of animal research in Soochow University. For isolation of rat NP cells, 24 SD male rats (8 weeks old) were euthanized by an overdose of pentobarbital sodium. The choice of appropriate age for the rat is based on previous studies [27]. The NP tissues were dissected from the thoracic and lumbar IVDs of rats under the microscope

and cut into 1 mm^3 small pieces. Subsequently, digested with 0.1% type II collagenase for 5 h until most of pieces were disappeared, then centrifuged at 800 rpm for 5 min to take the precipitate and cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Hyclone, LA, U.S.A.) with 10% fetal bovine serum (Gibco, Carlsbad, CA, U.S.A.) and 1% antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) (Gibco, Carlsbad, CA, U.S.A.) at 37°C in a 5% carbon dioxide incubator. The medium was refreshed every 3 days. All the animal experiments were performed in the Laboratory Animal Center of Soochow University.

Hematoxylin–eosin and toluidine blue staining

When the cells converged about 80% on the coverslip, washing the slide with PBS solution. Cells were fixed with 4% paraformaldehyde for 20 min and washed three times with PBS, and staining nuclei with the hematoxylin solution for 8 min, and rinsing in running tap water. Then differentiating with 1% acid alcohol solution for 30 s, and rinsing in running tap water. Bluing in saturated lithium carbonate solution for 30 s, and rinsing in running tap water. After that, counterstaining in eosin solution for 1 min, and rinsing in running tap water.

Toluidine blue staining was also performed when cells were converged about 80%, washing the slide with PBS solution. Cells were fixed with 95% alcohol for 15 s and washed three times with PBS. Then staining with the toluidine blue solution for 5 min, and adding the equal volume distilled water for next 15 min. And rinsing in running tap water, drying by airing, glycerol sealing piece. Hematoxylin–eosin staining and toluidine blue staining were used to observe cellular morphology under an inverted microscope (Leica Microsystems CMS GmbH, DFC450 C, Wetzlar, Germany).

Cell counting kit-8

The effect of different concentrations of H_2O_2 (0–2 mM) on the viability of rat NP cells was investigated using the Cell Counting Kit-8 (CCK-8) assay. Briefly, approximately 2000 cells were seeded in a 96-well plate and grown at 37°C with 5% carbon dioxide supply in a humidified incubator. The adherent NP cells were subjected to H_2O_2 exposure treatment for 2 h after culture for 24 h. Then the H_2O_2 was removed, and cells were rained with PBS for three times, replaced the normal medium and continued to culture for 24 h. The CCK-8 assay reagent (BOSTER Biological Technology, China) was added to each culture media of different groups and then incubated for 2 h in an incubator containing 95% air and 5% carbon dioxide incubator. A microplate reader was used to measure the absorbance at 450 nm. Each assay was performed in triplicate.

Hoechst staining kit

Hoechst 33258 (Beyotime Institute of Biotechnology, Shanghai, China) was used to analyze nuclear morphology under fluorescence microscopy. Briefly, the treated cells were fixed with 4% paraformaldehyde for 10 min at room temperature and rinsed twice with PBS. Subsequently, the cells were incubated with Hoechst 33258 staining for 5 min. After washing twice with PBS, the cells were observed using a fluorescence microscope (Leica Microsystems CMS GmbH, DFC450 C, Wetzlar, Germany) at 350 nm excitation wavelength.

Flow cytometry

The level of ROS in cellular was detected by Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). The rat NP cells in dish were washed three times with PBS before loading the DCFH-DA fluorescence probe. Then cells were treated with a certain concentration of H_2O_2 in the incubator to stimulate ROS production. Removed H_2O_2 and rinsed three times with PBS. Then DCFH-DA fluorescent probes were loaded in the cell incubator for 20 min, and the redundant probes were washed off with serum-free medium. Finally, cells were collected, and FITC was set as the parameter to detect the fluorescence intensity after stimulation at 488 nm excitation wavelength.

The cell cycle was detected by flow cytometry using the Cell Cycle Staining Kit (Multisciences, Hangzhou, China). Following the cell treatments, rat NP cells were collected (1×10^6/group), rinsed twice with PBS, and fixed in 75% ethyl alcohol. Added 1 ml DNA Staining solution and 10 µl permeabilization solution to the tube, vortexed for 5–10 s, and incubated at room temperature for 30 min in the dark. The analysis was carried out on the Flow cytometry (BD Biosciences, San Jose, CA, U.S.A.). The cells in different cycles including G0/G1, G2/M and S phases were counted and represented as a percentage of the total cell count.

The apoptotic rat NP cells were detected using and Annexin V-APC/7-AAD apoptosis kit (Multisciences, Hangzhou, China) by flow cytometry. The cells were collected (1×10^6/group) and washed twice with PBS. The cells were resuspended with 100 µl 1× Binding Buffer, 100 µl Annexin V-APC and 10 µl 7-ADD, respectively. After

mixed gently and vortexed, incubated at room temperature for 15 min in the dark, tubes were added 380 µl pre-cooled $1\times$ Bingding Buffer. Finally, the analysis was carried out on the Flow cytometry. The early apoptotic cells contained Annexin V^+/PI^-, the late apoptotic cells contained Annexin V^+/PI^+, and the normal cells contained Annexin V^-/PI^-. The early and late stage apoptotic cells were counted, and the results were expressed as a percentage of the total cell count.

Real-time PCR analysis

Total RNA was extracted using RNAiso Plus (Takara, Shiga, Japan). Reverse transcription was performed using RevertAid First-Strand cDNA Synthesis Kit (Thermo, Waltham, MA, U.S.A.) according to the manufacturer's specification. Real-time PCR was performed in triplicate in 20 µl reactions with iQ SYBR Premix Ex Taq Perfect Real Time (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), 50 ng of first-strand cDNA and 0.2 mg of each primer (Supplementary Table S1). Samples were cycled once at 95°C for 2 min, and then subjected to 35 cycles of 95, 56 and 72°C for 30 s each. The expression of each gene was defined from the threshold cycle (C_T) and melting temperatures (Tm) were recorded. The relative mRNA content was calculated using the $2^{-\Delta\Delta C_T}$ method with GAPDH as an endogenous control.

Western blot analysis

The cells were washed with PBS before being incubated in cell lysis buffer containing protease inhibitor PMSF (Beyotime, Shanghai, China) on ice for 10 min and centrifuged at 4°C at 8000 $\boldsymbol{g}$ for 5 min. Collecting the supernatant and the concentration of protein was measured with the BCA method. Samples containing 50 µg proteins were electrophoresed by performing SDS/PAGE on 10% gel (Invitrogen, Mississauga, ON, Canada), and then transferred to the PVDF membrane (EMD Millipore, IPFL00010, U.S.A.). The membrane was blocked with 5% non-fat milk for 1 h and incubated overnight with primary antibodies at 4°C. The membrane was then washed with TBST three times and incubated with HRP-conjugated secondary antibody at 37°C for 1 h. After washing the membrane, the blotted proteins were visualized using Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, U.S.A.) and CLINX Imaging System (CLINX Science Instruments, Shanghai, China).

Senescence-associated β-galactosidase staining

Cellular senescence was detected with senescence β-Galactosidase staining kit (CST, Danvers, MA, U.S.A.). Rat NP cells were cultured in six-well plates and treated with different sublethal concentrations of H_2O_2 for 2 h, and then cultured for another 72 h without H_2O_2 in complete culture medium. The treated cells were fixed with fixative solution for 15 min at room temperature and rinsed twice with PBS. Subsequently, add 1 ml of the β-Galactosidase Staining Solution ([a] 930 µl $1\times$ Staining Solution; [b] 10 µl $100\times$ Solution A; [c] 10 µl $100\times$ Solution B; [d] 50 µl 20 mg/ml X-gal stock solution) to well and then incubated at 37°C at least overnight in a dry incubator (without CO_2). The cells were checking under a microscope for the development of blue-stained β-gal-positive cells.

Immunofluorescence staining

A total of 1×10^6 cells were seeded into the coverslip in six-well plate. Cells were fixed with 4% paraformaldehyde for 20 min and washed three times with PBST, and permeabilized in 0.2% Triton X-100 for 20 min. After that, the cells were incubated overnight with primary antibodies at 4°C and then incubated with secondary antibodies for 1 h. Finally, using the antifade mount medium with DAPI (Invitrogen, Mississauga, ON, Canada) staining nuclei and sealing piece. Pictures were sequentially photographed with fluorescent microscope.

Statistical analysis

Experiments were conducted at least three times. Similar results were obtained in all assays using cells deriving from different donors. SPSS 25 statistical software program (SPSS Inc., IL, U.S.A.) was used for statistical analyses. Statistical differences were measured with Student's t-test for comparison between two groups or an analysis of variance (ANOVA) followed by the Turkey's t-test for comparison of multiple groups. Values presented are the means $\pm$ standard deviations. Differences were considered statistically significant when $P<0.05$.

Results
Identification of rat NP cells

Currently, NP cells lack specific identification methods, but because of their chondrocyte-like characteristics, these features are widely used as basic identification methods of NP cells [28]. The hematoxylin–eosin staining results
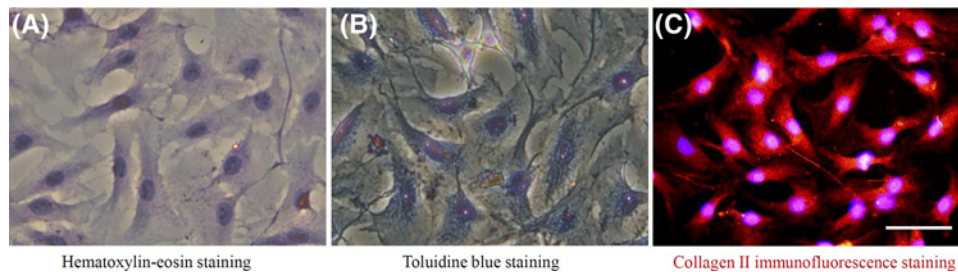
Figure 1. Different staining assessment of rat NP cells

(**A**) Hematoxylin–eosin staining: rat NP cells were spindle shaped and polygonal, and the nucleus was round or oval. (**B**) Toluidine blue staining: rat NP cells stained strongly positive with toluidine blue. (**C**) Immunofluorescence staining: the nucleus was blue-fluorescent by DAPI, while the red fluorescence showed high expression of collagen II by rat NP cells. Scale bars 50 μm.

showed that the rat NP cells were of polygonal, short spindle, or other irregular shapes (Figure 1A). After staining with toluidine blue, the accumulated proteoglycans were stained indigo blue (Figure 1B). The nucleus was in the center of the cell or leaned to one side. In addition, the immunofluorescence staining showed the extensive expression of fluorescence of type II collagen (exhibited in red fluorescence), most of which was located in the cytoplasm. The fluorescence intensity was stronger when closer to the nucleus (Figure 1C). The nucleus exhibited blue fluorescence.

Sublethal concentration of H_2O_2-induced senescence in rat NP cells

H_2O_2 has been the most commonly used inducer of stress-induced premature senescence (SIPS), partially because it is classically thought of as a natural inducer of oxidative stress [29,30]. In order to identify the concentration of H_2O_2 that could cause premature senescence of rat NP cells, we evaluated the difference in cell viability and apoptosis after treatment with different concentrations of H_2O_2. The initial dose range of H_2O_2 (0–2 mM) according to previous study [31]. First, DCFH-DA fluorescence probe was used to detect ROS content in rat NP cells. FITC fluorescence value schematic diagrams of different groups (negative group, control group and H_2O_2 group) were fused in Figure 2A, and we found that ROS could be significantly produced in cells when the concentration of H_2O_2 was only 25 μM compared with the control group (Figure 2B). This suggested H_2O_2 acted in the rat NP cells in the form of ROS. Then we further explored the effects of different concentrations of H_2O_2 on rat NP cells. The low concentration of H_2O_2 (25 μM) promoted cell growth, while cell viability was slightly affected until its concentration upgraded to 50 μM. And when the concentration of H_2O_2 reached 200 μM, the cell viability decreased by about half compared with the control group (50.6 ± 1.9%) (Figure 2C). A certain degree of oxidative stress induced cell senescence, but excessive oxidative stress could also cause cell apoptosis or death; hence, Hoechst staining test showed that when the concentration of H_2O_2 was ≥200 μM, dense and hyperchromatic apoptotic cells appeared (Figure 2D). Subsequently, the same results were obtained by detecting apoptotic cells via flow cytometry. When the concentration of H_2O_2 was ≤150 μM, the early- and late-stage apoptotic of rat NP cells were not statistically significant compared with the control group, and the apoptosis rate was significantly higher than that of the control group ($P<0.001$) while the concentration ≥200 μM (Figure 2E,F). Consequently, based on these results, we chose a concentration gradient of H_2O_2 from 50 μM to 150 μM for the following experiments (50, 75, 100, 125 and 150 μM) and chose 100 μM as a typical concentration for inducing premature senescent cells.

The formation of phosphorylated H2A.X foci is a marker of DNA damage caused by oxidative stress [32], so we investigated the extent of H_2O_2 induced DNA damage in NP cells. Indeed, after exposed to the concentration gradient of H_2O_2, the result showed that the phosphorylation of H2A.X on Ser139 was gradually increased (Figure 3A). Subsequently, in order to induce premature senescence of rat NP cells, we adopted three consecutive sublethal concentrations of H_2O_2 for a long-term treatment. Then we found that the expression of p53, p21, p16 and hypo-phosphorylated form of p-Rb was increased follow the increasing concentration of H_2O_2, revealing that two central senescence pathways (p53-p21-pRb and p16-pRb pathway) were activated (Figure 3B,C), and leading to a cell cycle arrest increased at G0/G1 phase compared with the control group (Figure 3D,E).

Although losing the replicative capability, senescent cells aberrantly secretes pro-inflammatory cytokines via autocrine and paracrine, which is defined as SASP [4,33]. We found that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 were highly expressed in rat NP cells after long-term H_2O_2 induction (Figure 3F). Then, a classical senescence-associated β-galactosidase (SA-β-Gal) staining was applied to detect senescent cells. We observed that senescent cells exposed to long-term H_2O_2 had more enlarged and flattened cell morphology and
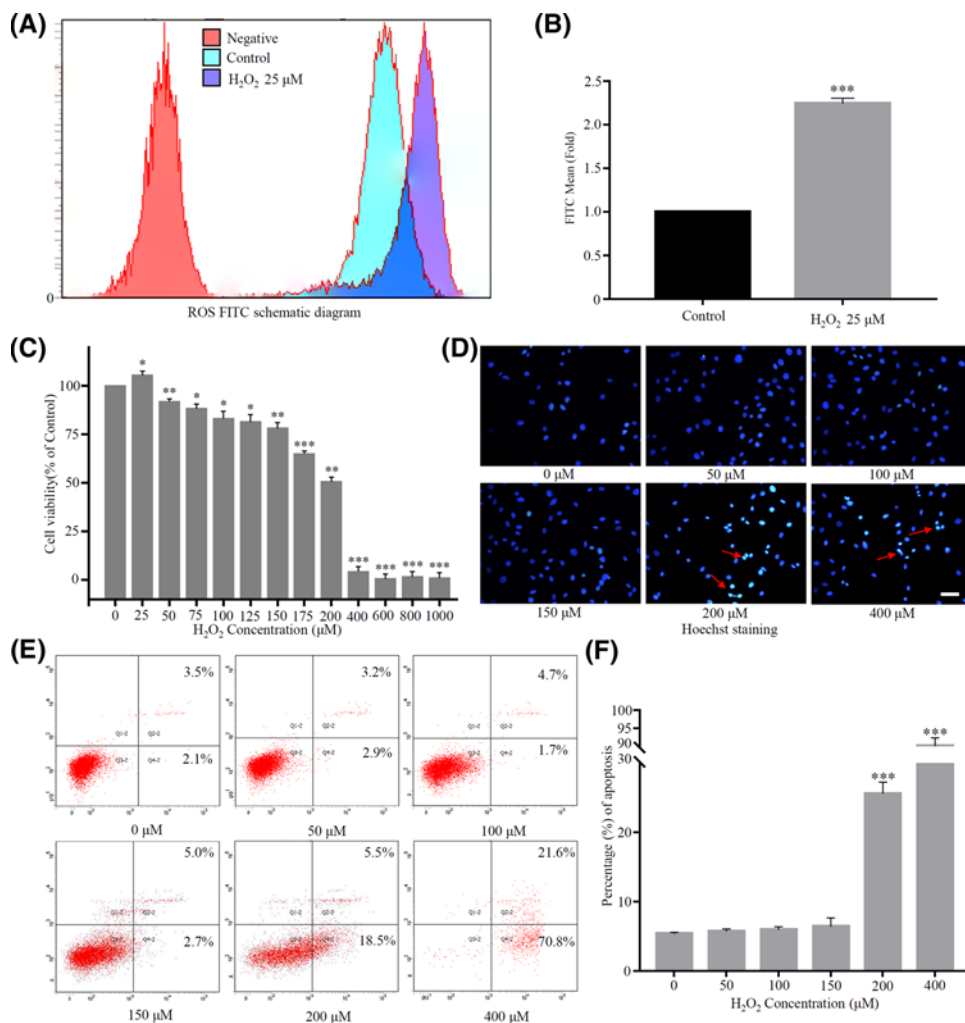
Figure 2. Effect of H_2O_2 on the viability, proliferation and apoptosis of rat NP cells

(**A**) and (**B**) Flow cytometry for detection of intracellular ROS content. Red, blue-green and purple represented the negative, control and H_2O_2 treatment groups, respectively ($^{***}P<0.001$ vs control group) (**C**) Effect of different concentration gradient H_2O_2 on viability and proliferation of rat NP cells detected by Cell Counting Kit (CCK8). ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group). (**D–F**) Hoechst and flow cytometry to detect apoptosis of rat NP cells to determine sublethal H_2O_2 concentration. Scale bars 100 μm. ($^{***}P<0.001$ vs control group).

blue-stained β-gal-positive cells than the control group (Figure 3G). Combined with the above results, we confirmed that long-term exposure to sublethal concentration of H_2O_2 could induce premature senescence of rat NP cells, and the number of senescent cells was positively correlated with the concentration of H_2O_2.

Oxidative stress suppressed SIRT1 expression in senescent rat NP cells

SIRT1 is a redox-sensitive protein, in addition to its role in regulating cellular oxidative stress burden, SIRT1 per se is also regulated by oxidative stress [34]. Therefore, we first investigated the expression changes of SIRT1 in H_2O_2-induced rat senescent NP cells. Real-time PCR analysis revealed the suppress expression of SIRT1 in senescent NP cells after H_2O_2 exposure (Figure 4A). Parallel, the protein expression of SIRT1 was gradually down-regulated with the increasing concentration of H_2O_2 as well (Figure 4B,C).

It is worth mentioning that, in addition to some post-translational modifications such as phosphorylation, SUMOy-lation, S-nitrosylation, S-glutathionylation and carbonylation [11,35,36], nucleocytoplasmic shuttling of SIRT1 is an-other form that affects its activity [10]. Some previous studies have shown that oxidative stress caused by H_2O_2 resulted in cytoplasmic localization of SIRT1 [11,37]. We have found that SIRT1 was indeed a protein mainly localized in the
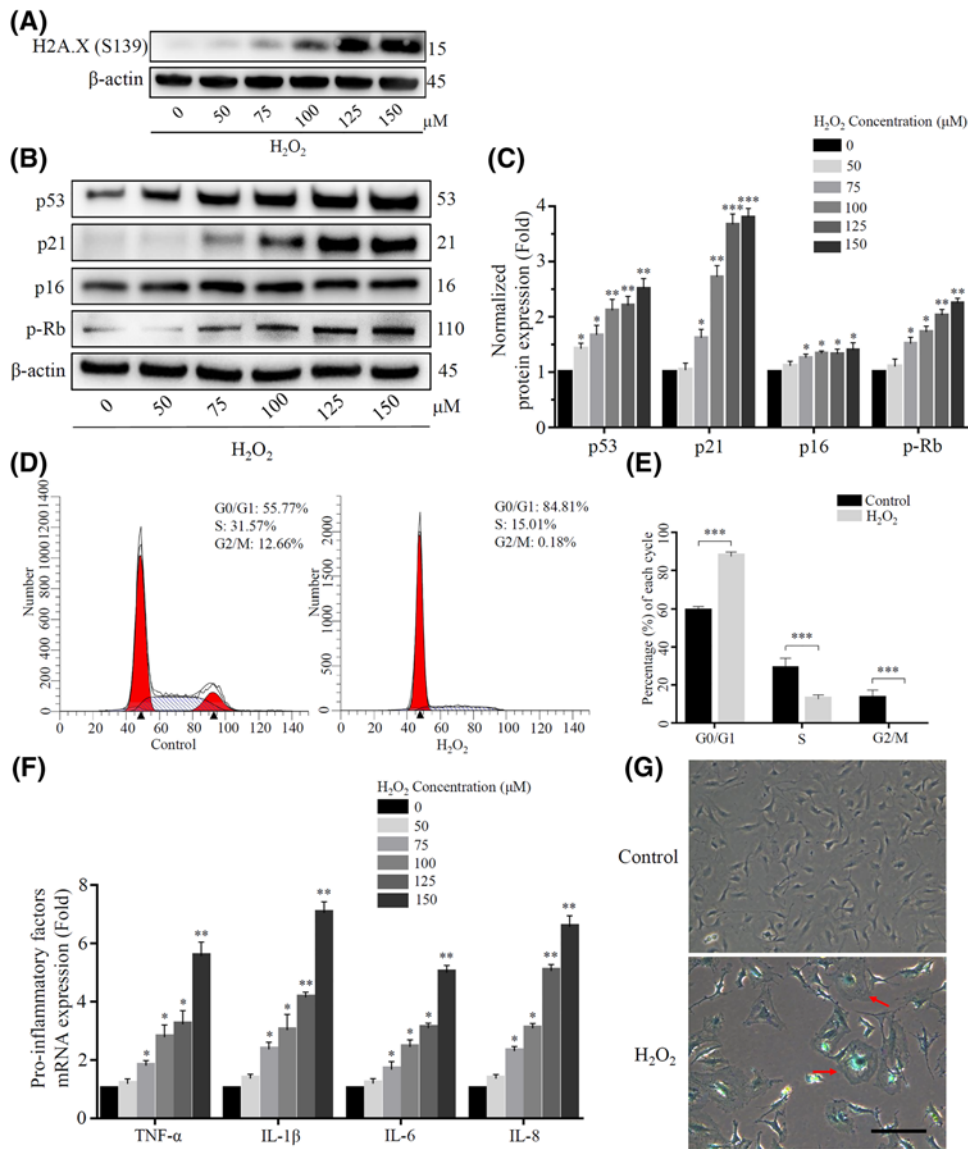
Figure 3. Sublethal concentration of H_2O_2 induced senescence in rat NP cells

(**A**) DNA damage caused by H_2O_2 was reflected by the expression of Phospho-Histone H2A.X (Ser139). (**B**) and (**C**) The expression of some senescence relative proteins (p53, p21, p16 and p-Rb) were detected using Western blot after exposure to a long-term H_2O_2. β-actin was used as an internal control. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group) (**D**) and (**E**) The cell cycle was detected by flow cytometry, and the proportion of cells in G0/G1 phase of H_2O_2 treatment group was higher than that of the control group. ($^{***}P<0.001$ vs control group) (**F**) Some pro-inflammatory factors (TNF-α, IL-1β, IL-6 and IL-8) increased at the transcriptional level with the raising of H_2O_2 concentration. ($^*P<0.05$, $^{**}P<0.01$ vs control group) (**G**) The H_2O_2 treatment group showed more abnormal and β-gal-positive cells than the control group. Scale bars 100 μm.

nucleus through immunofluorescence assay. However, interestingly, the ratio of SIRT1 expression in the nucleus to total SIRT1 expression in the H_2O_2 treatment group ($78.3 \pm 2.8\%$) was not significantly different from that in the control group ($79.6 \pm 2.1\%$) ($P>0.05$). It indicated that SIRT1 was not subjected to significant nucleocytoplasmic shuttling under sublethal concentrations of H_2O_2-induced oxidative stress in rat NP cells in our experiments (Figure 4D,E).
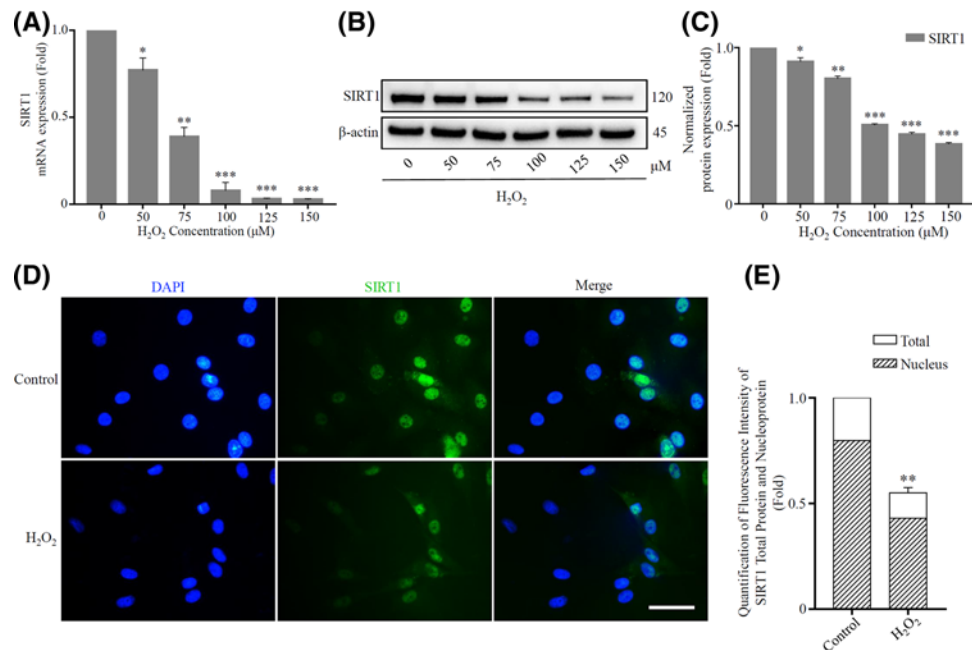
Figure 4. H$_2$O$_2$ inhibited the activity of SIRT1 mainly via affecting its expression

The expression of SIRT1 mRNA (**A**) and protein (**B**,**C**) were detected using real-time PCR analysis and Western blot after exposure to a long-term H$_2$O$_2$. β-actin was used as an internal control. (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control group). (**D**) Immunofluorescence staining was used to detect the expression and localization of SIRT1 in rat NP cells. The SIRT1 showed green fluorescence, and the nucleus showed blue fluorescence stained by DAPI. Scale bars 50 μm. (**E**) Quantification of fluorescence intensity of SIRT1 total protein and nucleoprotein (*$P<0.05$, **$P<0.01$ vs control group).

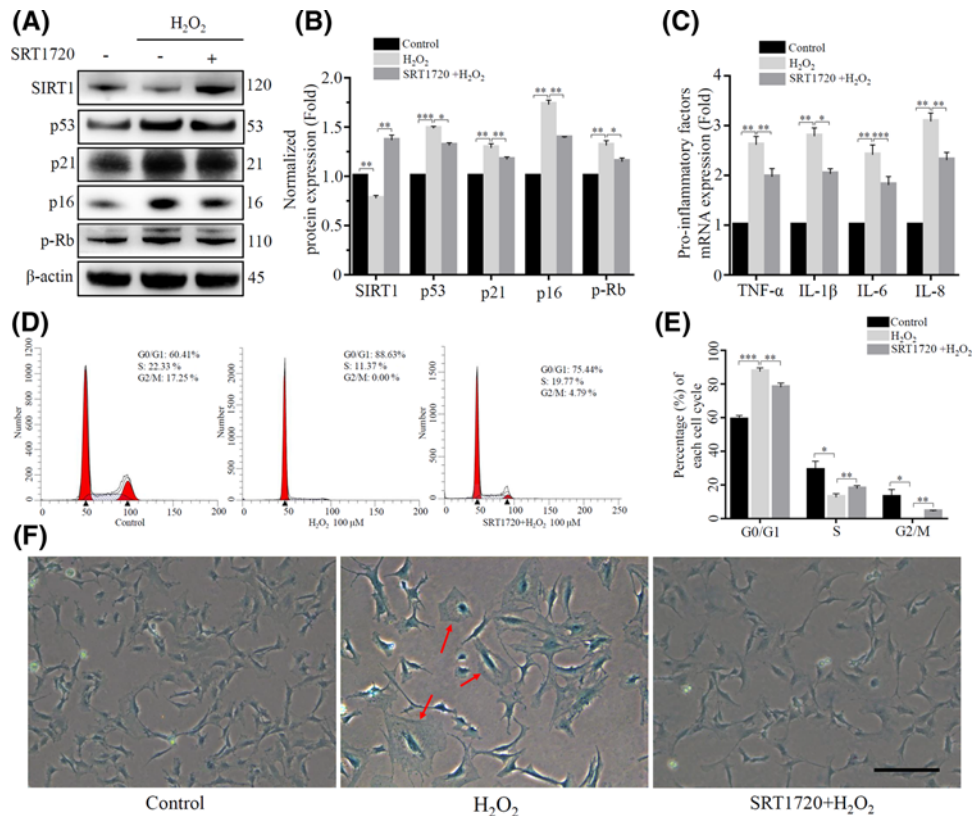## Activation of SIRT1 attenuated oxidative stress-induced senescence in rat NP cells

SRT1720 is a selective activator of SIRT1 [24], we pretreated rat NP cells with 5 μM SRT1720 and then exposed to 100 μM H$_2$O$_2$ for a long term to induce senescence. The expression of SIRT1 indeed increased after treatment of SRT1720 (Figure 5A). And we found that SRT1720 pretreated group had a lower degree of senescence than the H$_2$O$_2$ treatment group, which was reflected in the down-regulation of senescence-related protein (p53, p21, p16 and p-Rb), decreasing expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8), reduction of the G0/G1 phase arrest, and less of hypertrophic and β-gal-positive cells were appeared (Figure 5A–F).

Oxidative stress repressed FoxO1-SIRT1 pathway via activating the Akt

According to previous reports, FoxO1, as a major transcription factor of SIRT1, also plays an important role in oxidative stress resistance [18,38]. Therefore, we wanted to investigate whether the depressive expression of SIRT1 in response to oxidative stress was regulated by FoxO1 in rat NP cells. In our established *in vitro* rat NP cells senescence model, it was found that reduction expression of FoxO1 with the aggregation of ROS (Figure 6A). FoxO1 is regulated by post-transcriptional modifications such as phosphorylation, acetylation and ubiquitination, and these modifications affect FoxO1 subcellular localization, activity as a transcriptional regulator and stability [19,39]. Subsequently, we found that PI3K/Akt pathway was activated during this process, and activated phosphorylated Akt (Ser473) exerted post-translational modification and promoted FoxO1 phosphorylation on Ser256 loci (Figure 6A,B). Previous studies have shown that phosphorylated FoxO1 is localized and inactivated in the cytoplasm [19,20]. Therefore, immunofluorescence assay was used to verify whether nucleocytoplasmic shuttling occurred in FoxO1, and we found that oxidative stress did strongly inhibited the nuclear localization of FoxO1 in rat NP cells (Figure 6C).

In addition, we further explored the relationship between Akt, FoxO1 and SIRT1. We treated rat NP cells with FoxO1 inhibitor AS1842856 (0.2 μM), Akt inhibitor MK-2206 (5 μM) or AS1842856 in combination with MK-2206, respectively. First, MK-2206 inhibited the expression of p-Akt, decreased the phosphorylation of FoxO1 on Ser256 site and further increased the expression of total FoxO1 protein (Figure 7A,B). Besides, as an inhibitor of FoxO1, AS1842856 only reduced the activity of FoxO1 by binding with it, without affecting its transcription [25]. Then in

Figure 5. SRT1720 activated SIRT1 to rescue oxidative stress-induced rat NP cells senescence

The rat NP cells were divided into control, H_2O_2 treatment group and SRT1720 pretreatment group. The expression of SIRT1 and some senescence relative proteins (p53, p21, p16 and p-Rb) were detected using Western blot showed in (**A**) and (**B**). β-actin was used as an internal control. (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control group) (**C**) Pro-inflammatory factors (TNF-α, IL-1β, IL-6 and IL-8) were detected by RT-PCR. (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control group) (**D**) and (**E**) The cell cycle was detected by flow cytometry. (*$P<0.05$, **$p<0.01$, ***$P<0.001$ vs control group) (**F**) β-galactosidase staining was used to detect senescent rat NP cells. Scale bars 100 μm.

our experiment, after AS1842856 treatment, there was no significant difference in the protein expression of p-FoxO1 and FoxO1 compared with the control group, but the expression of p-Akt was decreased compared with the control group (Figure 7A,B). Finally, simply inhibiting p-Akt activity by MK-2206 promoted the expression of SIRT1, while inhibiting FoxO1 activity by AS1842856 suppressed the expression of SIRT1. But if both p-Akt and FoxO1 were inhibited at the same time, the expression of SIRT1 was suppressed, which is different from the result of the simply MK-2206 treatment group (Figure 7A,B).

Resveratrol exerted anti-senescence effects by affecting Akt-FoxO1-SIRT1 axis

Resveratrol is commonly known as an antioxidant which has been shown to be a scavenger of a number of free radicals [22]. It has also been reported that resveratrol exerts some of the gene-regulating effects mediated by the histone/protein deacetylase SIRT1 [23]. However, recent studies have shown that resveratrol is not a direct activator of SIRT1 enzyme activity [40]. To investigate the function of resveratrol to senescent rat NP cells induced by oxidative stress, we pretreated rat NP cells with resveratrol and then exposed to 100 μM concentration of H_2O_2 for a long term to induce senescence. We chose 20 μM as the optimum dose selection of resveratrol which was referred to another study [41]. We found that resveratrol reduced the activation of p-Akt induced by oxidative stress, leading to decreased phosphorylation of FoxO1 and increased the expression of total FoxO1 protein, leading to increased expression of SIRT1 (Figure 8A). Moreover, it was found by immunofluorescence that resveratrol could alleviate the cytoplasmic localization of FoxO1 caused by oxidative stress and enable FoxO1 to accumulate in the nucleus to retain its activity (Figure 8B,C). Subsequently, some senescence-related indicators were conducted, compared with
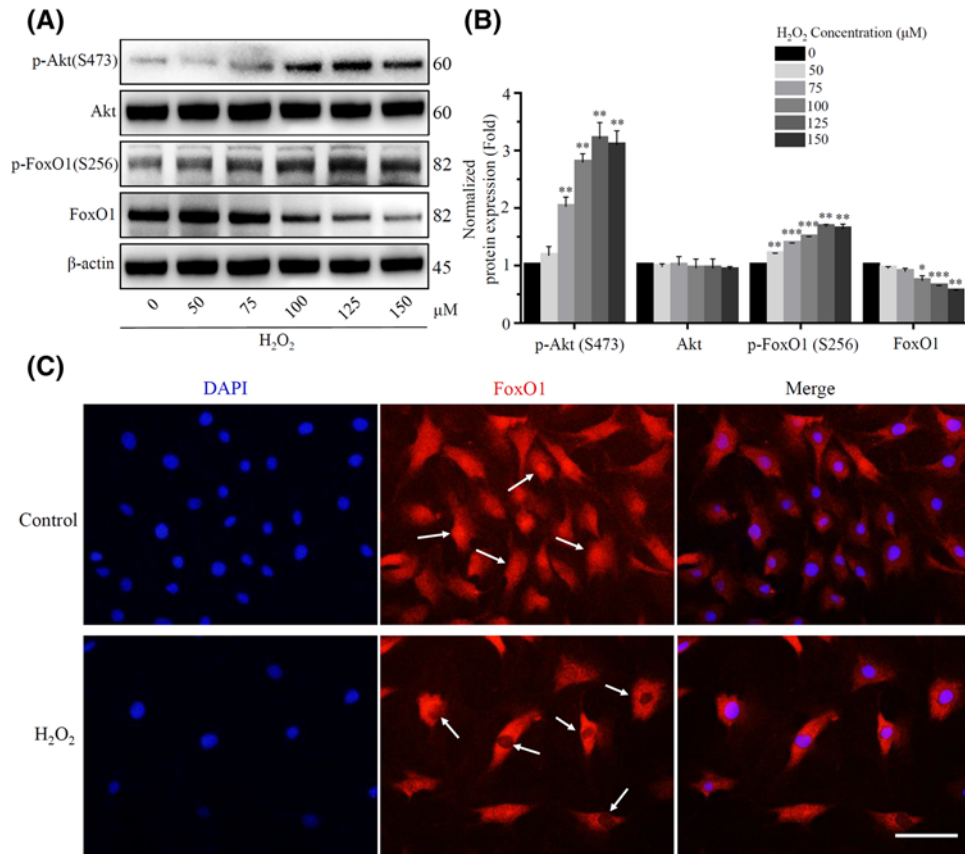
Figure 6. H$_2$O$_2$ increased FoxO1 phosphorylation by activating the PI3K/Akt pathway
(**A**) and (**B**) The proteins expression of Akt, FoxO1 and their phosphorylation were detected using Western blot after exposure to a long-term H$_2$O$_2$. β-actin was used as an internal control. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group) (**C**) Immunofluorescence staining was used to detect the expression and localization of FoxO1 in rat NP cells. The FoxO1 showed red fluorescence, and the nucleus showed blue fluorescence stained by DAPI. Scale bars 50 μm.
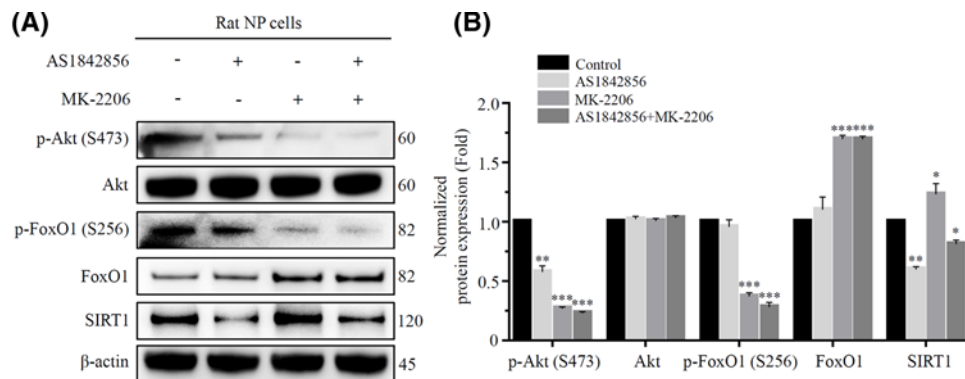
Figure 7. A cascade regulatory relationship among Akt, FoxO1 and SIRT1
(**A**) and (**B**) The rat NP cells were divided into control, AS1842856 group, MK-2206 group and AS1842856 combined with MK-2206 group. The expression of p-Akt (S473), Akt, p-FoxO1 (S256), FoxO1 and SIRT1 were detected using Western blot. β-actin was used as an internal control. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group).
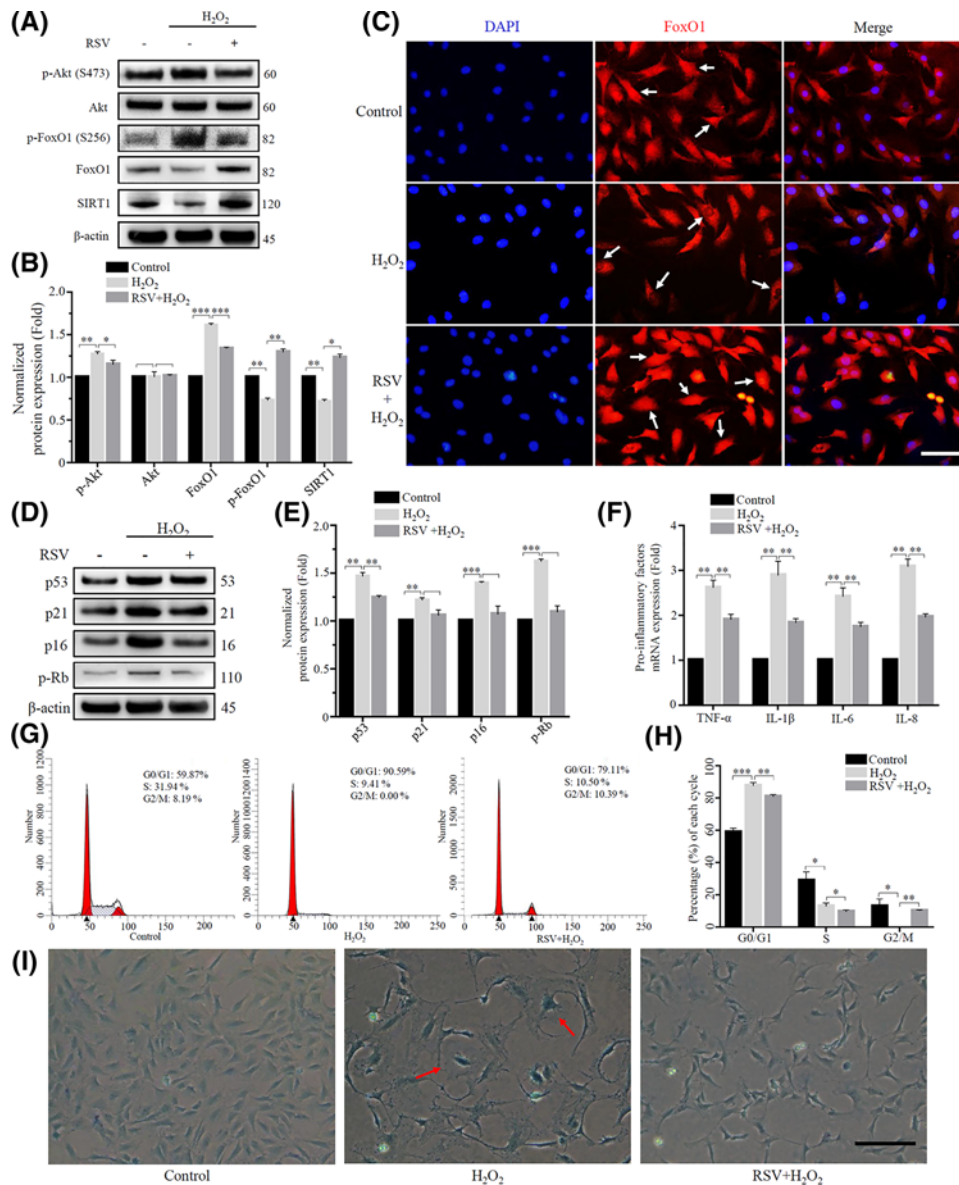
Figure 8. Resveratrol exerted anti-senescence effects by modulating the Akt-FoxO1-SIRT1 axis

The rat NP cells were divided into control, H_2O_2 treatment group and resveratrol treatment group. (**A**) and (**B**) The expression of p-Akt (S473), Akt, p-FoxO1 (S256), FoxO1 and SIRT1 were detected using Western blot. β-actin was used as an internal control. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group) (**C**) Immunofluorescence staining was used to detect the expression and localization of FoxO1 in rat NP cells. The FoxO1 showed red fluorescence, and the nucleus showed blue fluorescence stained by DAPI. Scale bars 50 μm. (**D**) and (**E**) Some senescence relative proteins (p53, p21, p16 and p-Rb) were detected using Western blot. β-actin was used as an internal control. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group) (**F**) Pro-inflammatory factors (TNF-α, IL-1β, IL-6 and IL-8) were detected by RT-PCR. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group) (**G**) and (**H**) The cell cycle was detected by flow cytometry. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group) (**I**) β-galactosidase staining was used to detect senescent rat NP cells. Scale bars 100 μm.

H_2O_2-treated group, the resveratrol-pretreated group showed a down-regulation of senescence-related protein (p53, p21, p16 and p-Rb), decrease expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8), reduction of the G0/G1 phase arrest and less of hypertrophic and β-gal-positive cells were appeared (Figure 8D–I). And these results were similar to those obtained in the previous SRT1720 treatment group.

Discussion

We showed here that sublethal concentration of H_2O_2-induced oxidative stress caused DNA damage and induced premature senescence in rat NP cells. SIRT1, as a common longevity gene, was down-regulated by virtue of oxidative stress instead of a significant nuclear translocation as shown in other studies. Moreover, activation of SIRT1 by SRT1720 inhibited oxidative stress-induced senescence, suggesting that SIRT1 plays a protective role in the process of senescence. Subsequently, we found that PI3K/Akt pathway was activated under low levels of oxidative stress. Activated p-Akt promoted phosphorylation of FoxO1 on Ser256, inhibited the activaion of FoxO1 by reducing nuclear localization of it, and led to a down-regulation of SIRT1 expression. Further, we also found that resveratrol exerted anti-senescence effect in rat NP cells through a similar mechanism.

NP cells, the foremost functional cell in the intervertebral disc, have been demonstrated to be not anaerobic and to have oxygen-utilizing metabolic processes *in vivo*, accompanied by the ROS being the main by-product [42]. Oxidative stress mediated by excessive ROS accelerates the degeneration of the intervertebral disc through various signaling pathways, including the nuclear factor-κB (NF-κB) pathway, the mitogen-activated protein kinases (MAPKs) pathway, and the lipid pathways (phospholipases, protein kinase C (PKC), and the PI3K/Akt pathway) [43]. Cell senescence is defined as the irreversible proliferation arrest. In addition to cell replicative senescence due to telomerase length shorten, DNA damage aroused by oxidative stress lead to premature senescence [44]. In the present study, we replicated the process of premature senescence of rat NP cells induced by oxidative stress with sublethal concentration of H_2O_2. When cells were exposed to this sublethal oxidative stress for a long term, they would become senescent cells that lose their proliferative ability but still have the function of living cells, which manifest as a large cell deformity, cell cycle arrest in G0/G1 phase, the senescence-related pathway (p53-p21-pRb and p16-pRb pathways) activation and positive for SA-β-Gal. These results provided direct evidence that oxidative stress plays a crucial role in senescence of NP cells.

SIRT1 plays key roles in lifespan extension. Previous studies have investigated the roles of SIRT1 in cell senescence and suggested there is a reciprocal relationship between oxidative stress and SIRT1. SIRT1 has been shown to regulate cellular oxidative stress burden and its toxicity in mammalian cells by deacetylating stress response mediators [45,46]. In addition, oxidative stress affects the activity of SIRT1 by regulating gene expression at transcriptional level, post-translational modification and nucleocytoplasmic shuttling [10]. In our results, we found that SIRT1 decreased at both mRNA and protein levels in response to sublethal H_2O_2-induced oxidative stress. Previous studies found that SIRT1 is mainly localized in the nucleus, and H_2O_2 treatment resulted in cytoplasmic localization of SIRT1 in human bronchial epithelial cells and other cell types [11,37]. Similar to this, SIRT1 was also mainly expressed in nucleus of rat NP cells, but unexpectedly, no significant cytoplasmic translocation of SIRT1 was observed on effect of H_2O_2 in this research. Therefore, it remains to be known whether H_2O_2 has a cell type- or species-specific effect on the nucleocytoplasmic shuttling of SIRT1. But we could assure in rat NP cells, oxidative stress mainly depressed the expression of SIRT1 to affect its activity, rather than nuclear translocation.

Oxidative stress causes cellular senescence, and SIRT1 protects against cellular senescence via regulating FoxO, p53, p21 and p16 as well as molecules involved in DNA damage and repair [13,14,47]. Here, after selectively activating SIRT1 with SRT1720, the rat NP cells showed a significantly resistance to oxidative stress induced premature senescence, and similar results could be obtained after resveratrol treatment. Although the treatment of SRT170 would not completely rescue senescent rat NP cells, we cannot deny the function of SIRT1 in alleviating senescence.

Cell growth is an increase in cell mass (size or volume). In proliferating cells, growth is balanced by divisions. When the cell cycle is blocked, cell growth cannot be compensated by cell division, and cells cannot and do not increase in size indefinitely, then growth turns into senescence. It is assumed that senescence differs from quiescence in that growth stimulation was required while the cell cycle was arrested. Theoretical considerations indicate that mitogenic stimulation might intensify cellular senescence [48]. In agreement, while inhibiting the cell cycle, senescence-inducing agents (radiation, DNA damage) do not inhibit growth-promoting pathways (e.g., Ras/Akt/TOR) and often activate them [49,50]. This view was also confirmed in our research. Sublethal H_2O_2 promoted senescence of rat NP cells in a concentration-dependent manner, accompanied by gradual activation of PI3K/Akt pathway, while activation of p21 and p16 leads to cycle arrest by inhibiting cyclin-dependent kinases (CDK) activity.

In addition, Akt not only stimulates cell growth and leads to senescence, but also affects FoxO1 activity through phosphorylation [51]. Phosphorylated Akt increased FoxO1 phosphorylation in rat senescent NP cells induced by H_2O_2 at sublethal concentration, which resulted in increasing cytoplasm localization of FoxO1. Further, we found that FoxO1 suppression could inhibit the expression of SIRT1, whereas the Akt inhibition could increase SIRT1 expression by reducing its inhibition of FoxO1. These results indirectly demonstrated that there might be a cascade regulatory relationship among Akt, FoxO1 and SIRT1. FoxO1 acted as a transcription factor regulating the expression of SIRT1,

while Akt, as an upstream pathway of FoxO1, regulated the function of FoxO1-SIRT1 pathway. In addition, Wang and co-workers found that SIRT1 participated in growth factor-mediated Akt phosphorylation at residue Ser473, the phosphorylation of Akt at residue Ser473 was impaired in the liver of SIRT1^{LKO} mice [52]. This might explain the decreased expression of p-Akt after application of AS1842856. However, specific regulatory mechanisms need to be further demonstrated in future studies.

Furthermore, we found that resveratrol could retard the senescence of rat NP cells by affecting the Akt-FoxO1-SIRT1 axis. Initially, it was generally believed that resveratrol was a direct activator of SIRT1, while more and more controversy has surrounded it [53]. Recent evidence *in vitro* indicated that resveratrol exerted indirect effects on SIRT1 activation [40,54]. Previous study had found that resveratrol augmented FoxO1-mediated SIRT1 transcription and induced SIRT1 expression [18]. In our experiments, we found that p-Akt was inhibited and both FoxO1 and SIRT1 protein were increased after treatment of resveratrol compared with the H_2O_2-treatment group, and ultimately showed the function of anti-senescence in rat NP cells. However, it was a pity that we have not been able to demonstrate what the direct-action factor of resveratrol is, and it is not clear whether resveratrol directly inhibits p-Akt leading to increased expression of SIRT1, or that SIRT1 inhibits phosphorylation of Akt in the form of negative feedback. So, the role and mechanism of resveratrol *in vivo* also require intensive study in future.

In conclusion, the present study shows the important role of SIRT1 in the resistance of senescent rat NP cells induced by low levels of oxidative stress, which is regulated by Akt-FoxO1 pathway, and finds that resveratrol is able to activate SIRT1 to alleviate the occurrence of senescence. It is suggested that Akt-FoxO1-SIRT1 axis might be a potential therapeutic target to defer the progression of IDD. Therefore, further studies need to be conducted in degenerative NP tissues and animal models.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

J.H. designed experiments, performed research, analyzed data and wrote the manuscript. A.Z. performed research, analyzed data and administrated project. Z.S. designed experiments, revised and wrote the manuscript. S.G. performed research and contributed unpublished reagents. Y.C. performed research and assembled the figure. Z.L. performed research and analyzed data. X.X. analyzed data and assembled the figure. J.Z. revised the manuscript and contributed unpublished reagents. J.L. designed experiments, analyzed data, revised manuscript and approved the final version. LC designed experiments, analyzed data, revised manuscript and approved the final version.

Abbreviations

Akt , PKB, protein kinase B; CCK-8, cell counting Kit-8; CT, threshold cycle; DCFH-DA , 2',7'-Dichlorofluorescin diacetate; FITC , Fluorescein isothiocyanate; FoxO1 , Forkhead box O1; GADPH , glyceraldehyde-3-phosphate dehydrogenase; HRP , Horseradish Peroxidase; H_2O_2, hydrogen peroxide; IDD, intervertebral disc degeneration; IL , interleukin; NP, nucleus pulposus; PI3K, phosphatidylinositol-3-kinase; PMSF , Phenylmethanesulfonyl fluoride; p-Rb , phosphorylated retinoblastoma protein; Ras , rat sarcoma; ROS, reactive oxygen species; RT-PCR , Reverse Transcription-Polymerase Chain Reaction; SASP, senescence-associated secreted phenotype; SA-β-Gal , senescence-associated β-galactosidase; SD , Sprague–Dawley; SIRT1, silent information regulator 1; TNF-α , Tumor Necrosis Factor-α; TOR , Target of Rapamycin.

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