European Review for Medical and Pharmacological Sciences 2020; 24: 3448-3458

Prx II reduces oxidative stress and cell senescence in chondrocytes by activating the p16-CDK4/6-pRb-E2F signaling pathway

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Abstract. – OBJECTIVE: Osteoarthritis (OA) is a common clinical degenerative disease and has a high incidence in the elderly. The purpose of this study was to explore the anti-oxidative stress and anti-aging effects of Peroxiredoxin II (Prx II) on articular chondrocytes, as well as its molecular mechanism.

MATERIALS AND METHODS: Articular cartilage tissues and culture human articular chondrocytes were selected. By constructing Prx II overexpressing lentivirus, the effects of Prx II on oxidative stress and cell senescence in chondrocytes were studied. Besides, the p16 overexpression lentivirus was constructed to investigate the effect of Prx II on the p16-CDK4/6-pRb-E2F signaling pathway (p16 signaling pathway).

RESULTS: Articular cartilage tissues in patients with OA and IL-1β-induced chondrocytes expressed lower Prx II and had higher p16 signaling pathway activity. The overexpression of Prx II significantly increased the expression of SOD1 and SOD2 and decreased the expression of β-gal and P53/P21, indicating that Prx II can reduce the oxidative stress and senescence level of chondrocytes. Moreover, the overexpression of Prx II increased the expression of p16 signaling pathway-related molecules and the activation of the p16 signaling pathway attenuated the anti-oxidative stress and anti-aging effects of Prx II.

CONCLUSIONS: Prx II can inhibit the p16 signaling pathway in chondrocytes to reduce the level of aging in chondrocytes, thereby reducing the level of oxidative stress in chondrocytes, and ultimately inhibiting the progression of OA.

Key Words:
Peroxiredoxin II, Oxidative stress, Cell senescence, Osteoarthritis, p16.

Introduction

Osteoarthritis (OA) is the most common type of degenerative joint disease in the world and the most common cause of pain and disability in patients, mostly in older people[1]. OA affects various tissues around the joint, such as articular cartilage, subchondral bone, synovium, and ligaments, and it mainly occurs in the articular cartilage. The cartilage has no blood vessels and nerves, and contains only one type of cells, namely chondrocytes. The decomposition of chondrocytes is a sign of OA[2]. Chondrocytes are responsible for the secretion and transformation of extracellular matrices (ECM) and are essential for maintaining their homeostasis. Therefore, the balance between the synthesis and degradation of ECM in OA is an important factor in determining pathological progression[3]. In addition, the occurrence of OA is mainly caused by the aging of chondrocytes, resulting in a decrease in the number of chondrocytes and decomposition and degeneration of cartilage tissue[4].

Peroxiredoxin (Prx) is a type of peroxidase that protects cells from oxidative stress. The Prx family has six members, Prx I-Prx VI, which are widely distributed in mammalian cells[5]. Prx II is widely expressed in mammalian cytoplasmic matrix and cell membrane, has the function of scavenging reactive oxygen species (ROS), and participates in multiple signaling pathways in cells to regulate cell proliferation, senescence, and apoptosis[6]. Under pathological conditions, due to the imbalance of ROS production and clearance, it often causes damage to the tissues and organs by ROS[7]. Embryonic fibroblasts from Prx II knockout mice

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show severe cell senescence characteristics[8], and Prx II knockout mice show significant skin age symptoms, indicating the importance of Prx II in cell senescence. However, the anti-aging effects of Prx II in chondrocytes have not been studied. Therefore, human primary articular chondrocytes were used, and Prx II and p16 overexpressing lentiviruses were constructed to explore the anti-oxidative stress and anti-aging effects of Prx II on chondrocytes *in vitro*.

Materials and Methods

Patient Tissue Samples

In the OA group, the articular cartilage tissue was collected from patients who underwent total knee replacement. X-rays showed a significant stenosis in the patient's joint space, and the patient had significant clinical manifestations of arthritis. The articular cartilage tissue of the control group was taken from patients who underwent amputation. This study was approved by the Ethics Committee of Shanghai Changzheng Hospital. All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cells Culture and Drug Treatment

Human primary chondrocytes were purchased from Shanghai Saibaikang Biotechnology Company (Shanghai, China) and cultured in DMEM/F12 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco, Rockville, MD, USA) and 1% penicillin plus streptomycin (Gibco, Rockville, MD, USA) in an incubator at 37°C and 5% CO_2. Recombinant human IL-1β (Lianke, Hangzhou, China) was used to stimulate degeneration of chondrocytes.

Western Blot Analysis

Total protein was extracted from cartilage or chondrocytes and measured protein concentration using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After blocking the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) with 5% skim milk-PBST, primary antibodies (collagen II, 1:3000, Rabbit, Prx II, 1:3000, Rabbit, p16, 1:2000, Rabbit, E2F1, 1:3000, Rabbit, SOD1, 1:3000, Rabbit, SOD2, 1:3000, Rabbit, β-gal, 1:2000, Rabbit, p53, 1:1000, Rabbit, p21, 1:3000, Rabbit, CDK4, 1:5000, Rabbit, CDK6, 1:1000, Rabbit, β-actin, 1:3000, Rabbit) from Abcam (Cambridge, MA, USA) were used for incubation at 4°C overnight. The next day, after washing the PVDF membrane, incubation was conducted for 2 hours at room temperature using a secondary antibody (Goat anti-rabbit, 1:3000, Abcam, Cambridge, MA, USA). Finally, enhanced chemiluminescence (ECL) method was used to analyze protein expression.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from cartilage tissues or chondrocytes using the TRIzol (Invitrogen, Carlsbad, CA, USA) method. After reverse transcription of mRNA into cDNA, the RNA expression was analyzed by RT-PCR. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous controls. Finally, $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression level. The primer sequences of mRNA are shown in Table I.

Immunocytofluorescence (IF) Staining

The treated cells were fixed with 4% paraformaldehyde for 30 minutes. After washing the cells with phosphate-buffered saline (PBS), the cells were soaked with 0.5% Triton-PBS for 15 minutes. Then, 10% of goat serum was used to block non-specific antigens in cells, and a primary antibody dilution (Prx II, 1:500, Rabbit, Abcam, Cambridge, MA, USA, and β-gal, 1:500, Rabbit, Abcam, Cambridge, MA, USA) was used for incubation at 4°C overnight. The next day, after washing the cells with PBS, cells were incubated with the secondary antibody dilution (Goat anti-rabbit, 1:500, Abcam, Cambridge, MA, USA) for 1 hour. Then, 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) was used to stain the nucleus. Finally, the staining results were observed by fluorescence microscopy.

Cell Viability Assay

The cells were cultured and treated using a 96-well plate. The Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Kumamoto, Japan) was used to detect cell viability. After that, 10 μl of CCK-8 reagent was added into each well. After the cells were incubated in the incubator for 2 hours, the cells were taken, and the absorbance of each well was measured using a microplate reader.

Intracellular ROS Levels

Intracellular ROS level was detected by flow cytometry. The cells were intubated with DCFH-

DA (10 μM Keygen, Nanjing, China) for 20 min in 37°C followed immediately by flow cytometry analysis in a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) to detect the total ROS level.

Enzyme Linked Immunosorbent Assay (ELISA)

The well-grown log phase chondrocytes were transferred to 96-well plates and adjusted to a cell concentration of $1' 10^5$/ml. After different treatment of the cells, the cell supernatant was extracted, and relevant molecular expression levels were tested according to the ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA) instructions.

Lentiviral Transfection

Chondrocytes were transfected with Lentivirus-Prx II, Lenti-p16 or Lentivirus-NC. Prx II was overexpressed by Lentivirus-Prx II and p16 was overexpressed by Lenti-p16. The cells were incubated for other 3 days and passaged. Finally, transfection efficacies were measured *via* Western blot and RT-PCR.

Statistical Analysis

GraphPad (La Jolla, CA, USA) and Statistical Product and Service Solution PSS 20 software (SPSS; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Each set of experiments was repeated 3 times. For measurement data, we use the mean ± standard deviation for analysis. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Changes in the Expression of Prx II and the Activity of the p16-CDK4/6-pRb-E2F Signaling Pathway (p16 signaling pathway) in the Articular Cartilage of Patients with OA

In order to detect the expression of Prx II and the activity of p16 signaling pathway in articular cartilage tissue of patients with OA, articular cartilage tissues were taken from patients with OA and normal subjects, and the expressions of Prx II and p16 signaling pathway-related molecules were detected. Western blot results (Figure 1A-1E) showed that the expression of collagen II in articular cartilage of patients with OA was significantly reduced, indicating that the articular cartilage was significantly damaged. Besides, the ex-

Table I. RT-PCR primer sequences.

Name	sense/anti-sense	Sequence (5'-3')
Collagen II	sense	GGGAATGTCCTCTGCGATGAC
	anti-sense	GAAGGGGATCTCGGGGTTG
Prx II	sense	GTTCACATAGAAGCCTAGCCCAC
	anti-sense	GTGTGCTCAGGGCAGGAYCACY
p16	sense	GGTAGCATGCAACCGGTAACGG
	anti-sense	GCTTAGCCCTTAGGCATGCATG
E2F1	sense	AATGCCATGGCAATGCGTACGC
	anti-sense	TGTGCAGTGCAAACGCGTGAC
SOD1	sense	GGTGAACCAGTTGTGTTGTC
	anti-sense	CCGTCCTTTCCAGCAGTC
SOD2	sense	CAGACCTGCCTTACGACTATGG
	anti-sense	CTCGGTGGCGTTGAGATTGTT
Bmi-1	sense	GCATGCAGTAGCCGTACGACG
	anti-sense	TGCATGCAGCAATGCGTGAGTC
p53	sense	GGTTCCTGCCCCAGGATGTTG
	anti-sense	GGAACATCTCGAAGCGCTCA
p21	sense	AGTATGCCGTCGTCTGTTCG
	anti-sense	GACTGCAAGACAGCGACAAG
CDK4	sense	GGCTTAGCACGTAGCATCGACG
	anti-sense	TTGACGTACGATCGAGCTCGACG
CDK6	sense	ATGCTAGCACGTACGATCGAGC
	anti-sense	GTGGCATGCTGCTAGCTACGTA
GAPDH	sense	ACAACTTTGGTATCGTGGAAGG
	anti-sense	GCCATCACGCCACAGTTTC

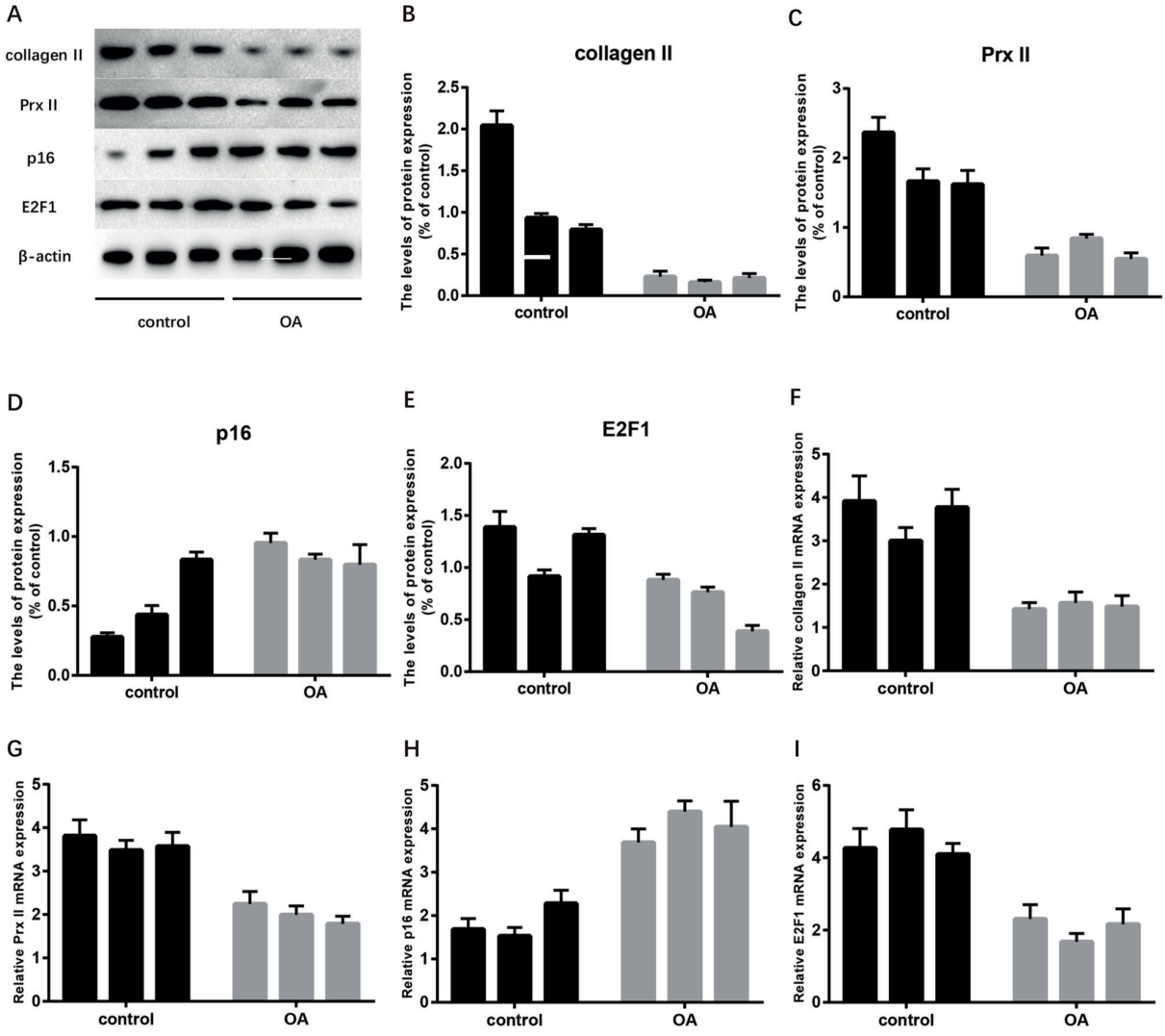

Figure 1. The expression of Prx II is decreased and the activity of the p16 signaling pathway is increased in the articular cartilage of patients with OA. Expressions of collagen II, Prx II, p16, and E2F1 in control and OA groups are determined by Western blot (**A-E**) and RT-PCR (**F-I**).

pressions of Prx II and E2F2 in articular cartilage of patients with OA were significantly decreased and the expression of p16 was increased, indicating that the expression of Prx II is decreased in the articular cartilage tissue of patients with OA, and the activity of p16 signaling pathway is increased. The results of RT-PCR were similar to those of Western blot (Figure 1F-1I).

Prx II Expression was Decreased and p16 Signaling Pathway Activity was Increased in IL-1β-Induced Chondrocytes

IL-1β (10 ng/ml, 50 ng/ml) was used to induce human chondrocytes to make a cell-level OA model. Western blot results (Figure 2A, 2B) showed that with the increase of IL-1β concentration, the expressions of collagen II, Prx II, and E2F1 were decreased and the expression of p16 was increased. The results of RT-PCR (Figure 2C-2F) were similar to those of Western blot. The results of immunofluorescence (Figure 2G) showed that IL-1β induced chondrocytes to cause a decrease of Prx II expression in a dose-dependent manner.

Overexpression of Prx II Reduced Oxidative Stress Levels in Chondrocytes

Lenti-Prx II was constructed to study the effect of Prx II on the anti-oxidative stress of chondrocytes. Western blot results (Figure 3A, 3B)

showed that cells overexpressing Prx II expressed more SOD1 and SOD2, which reduced the effect of IL-1β on the oxidative stress level of chondrocytes. Flow cytometry results (Figure 3C) also indicated that overexpression of Prx II reduced ROS levels in chondrocytes. ELISA results (Figure 3D, 3E) were similar to Western blot. In addition, RT-PCR results (Figure 3F-3I) showed that the overexpression of Prx II increased the expressions of anti-oxidative stress-related molecules SOD1, SOD2, GPX1, and GPX3 in chondrocytes.

Overexpression of Prx II Reduced the Senescence Level of Chondrocytes

To determine the effect of Prx II on chondrocyte senescence, the expression of senescence-associated molecules in chondrocytes was examined. Western blot analysis (Figure 4A, 4B) showed that IL-1β-induced chondrocytes expressed a large amount of β-gal, p53, and p21, while Prx II could attenuate the effect of IL-1β and decrease the expression of β-gal, p53, and p21. RT-PCR results (Figure 4C-4E) showed that Prx II increased

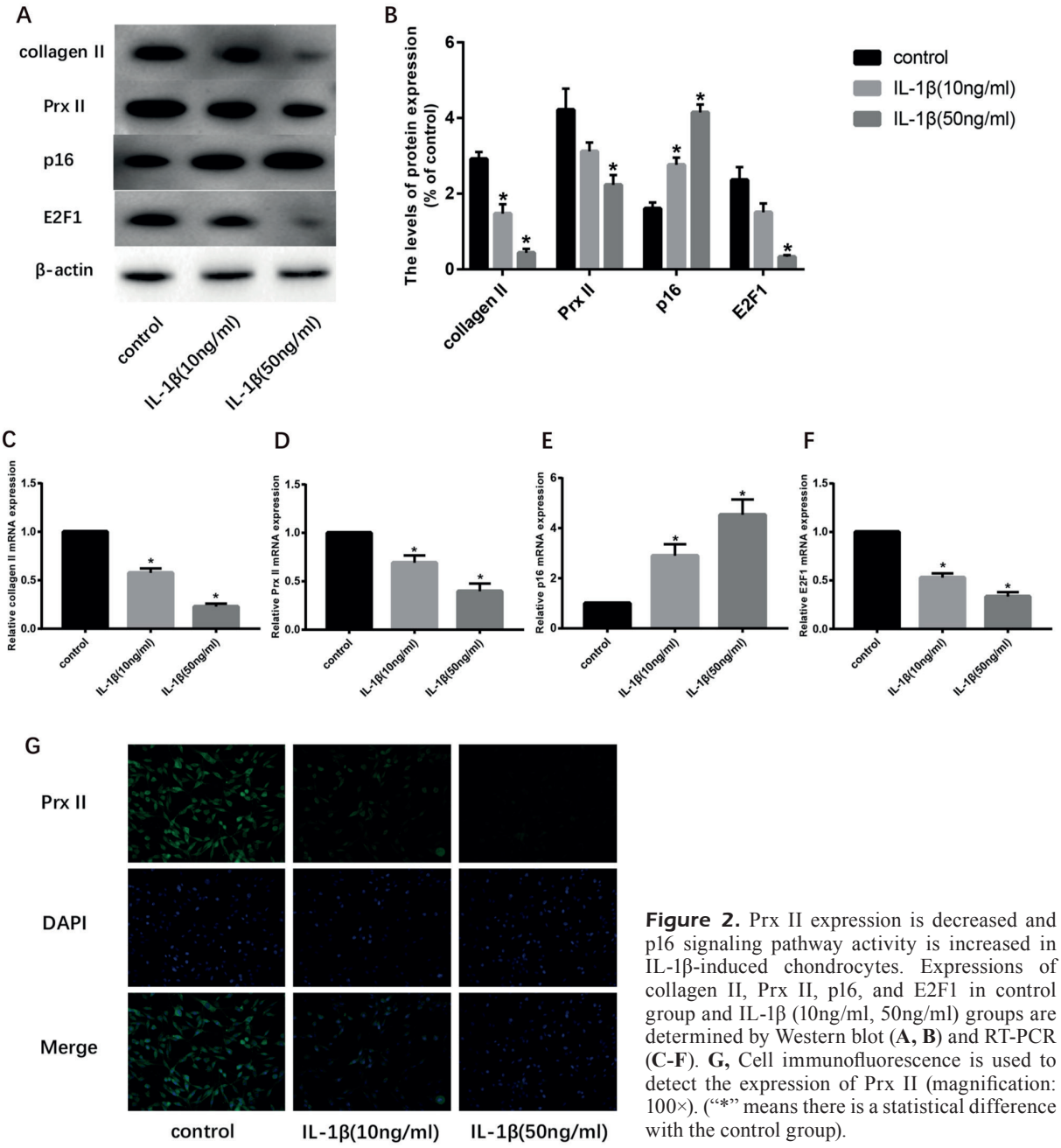

Figure 2. Prx II expression is decreased and p16 signaling pathway activity is increased in IL-1β-induced chondrocytes. Expressions of collagen II, Prx II, p16, and E2F1 in control group and IL-1β (10ng/ml, 50ng/ml) groups are determined by Western blot (**A, B**) and RT-PCR (**C-F**). **G,** Cell immunofluorescence is used to detect the expression of Prx II (magnification: 100×). ("*" means there is a statistical difference with the control group).

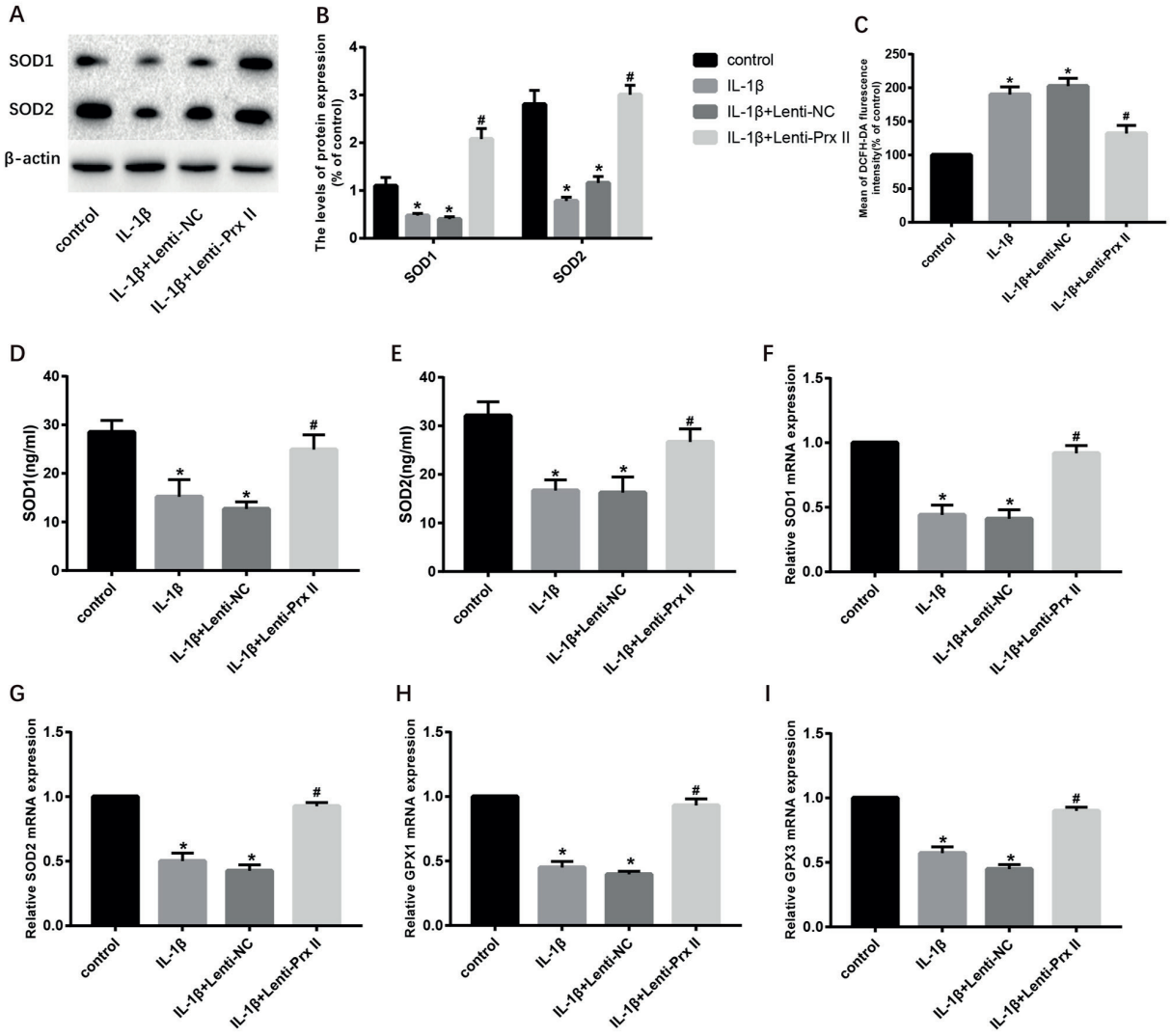

Figure 3. Overexpression of Prx II reduces oxidative stress levels in chondrocytes. Expressions of SOD1 and SOD2 in four groups are determined by Western blot (**A-B**). **C,** ROS level in four groups is detected by flow cytometry. ELISA (**D-E**) and RT-PCR (**F-G**) is used to detect the expressions of SOD1 and SOD2. Expressions of GPX1 (**H**) and GPX3 (**I**) are determined by RT-PCR. ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β+Lenti-NC group).

the expression of anti-aging molecule BMI-1 and decreased the expression of p53 and p21. Cellular immunofluorescence (Figure 4F) also showed that Prx II reduced the expression of β-gal induced by IL-1β. These results indicate that Prx II effectively reduces the senescence level of chondrocytes.

Overexpression of Prx II Inhibited the Expression of the p16 Signaling Pathway Related Molecules

The p16 signaling pathway is a common pathway in cellular senescence. To study the mechanism of anti-aging effects of Prx II in chondrocytes, the expression levels of molecules involved in the p16 signaling pathway were examined. Western blot results (Figure 5A, 5B) showed that IL-1β-induced chondrocyte expressed more p16 while CDK4, CDK6, and E2F1 decreased, and overexpression of Prx II attenuated the effect of IL-1β. The results of RT-PCR were similar to those of Western blot (Figure 5C-5F).

Activation of the P16 Signaling Pathway Attenuated the Anti-Oxidative Stress and Anti-Aging Effect of Prx II

To further validate the anti-oxidative stress and anti-aging effects of Prx II through the activation of the p16 signaling pathway. A double transfect-

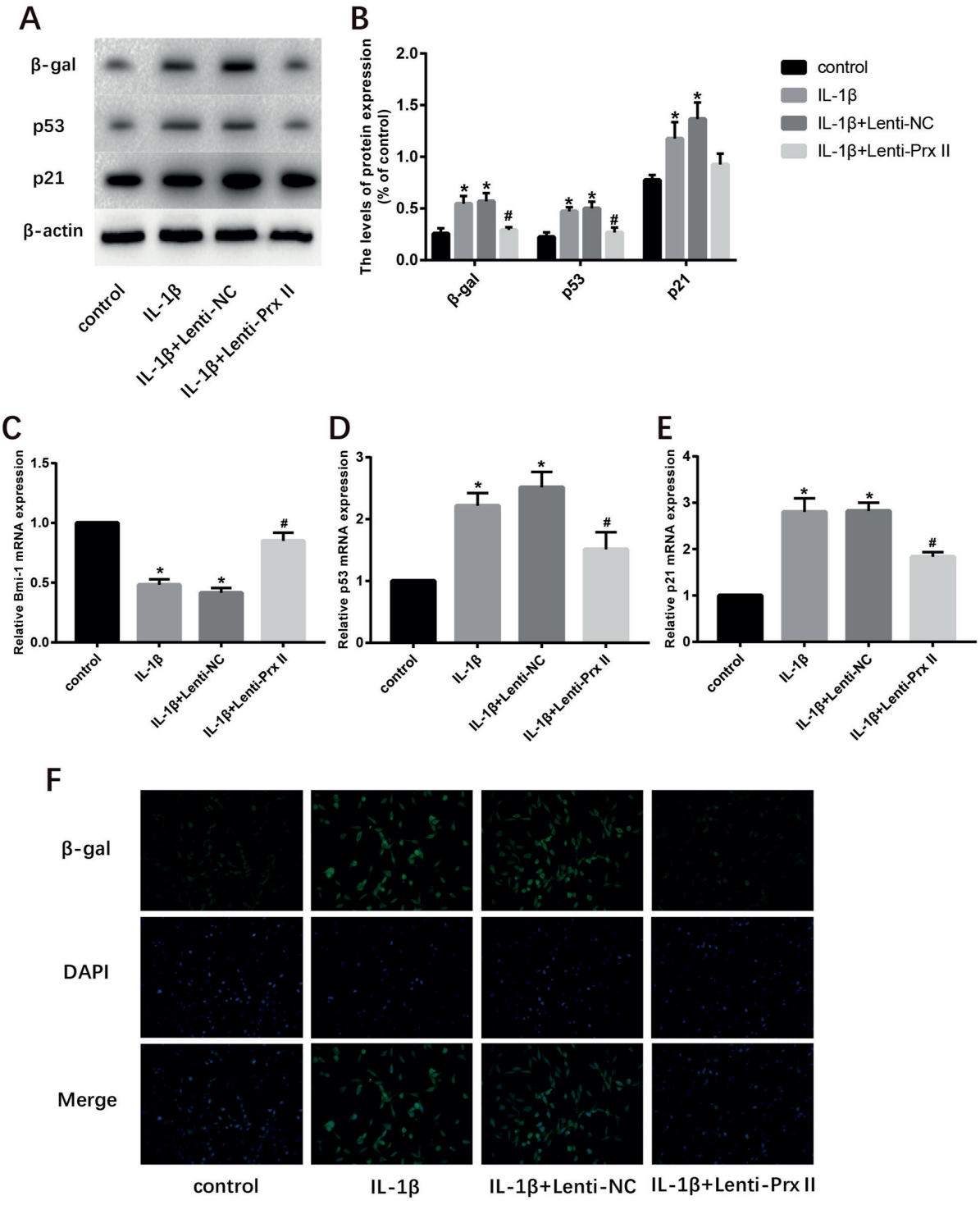

Figure 4. Overexpression of Prx II reduces the senescence level of chondrocytes. Expressions of β-gal, p53 and p21 in four groups are determined by Western blot (**A-B**). RT-PCR is used to detect the expressions of BMI-1 (**C**), p53 (**D**) and p21 (**E**). Cell immunofluorescence is used to detect the expression of β-gal (magnification: 100×) (**F**). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β+Lenti-NC group).

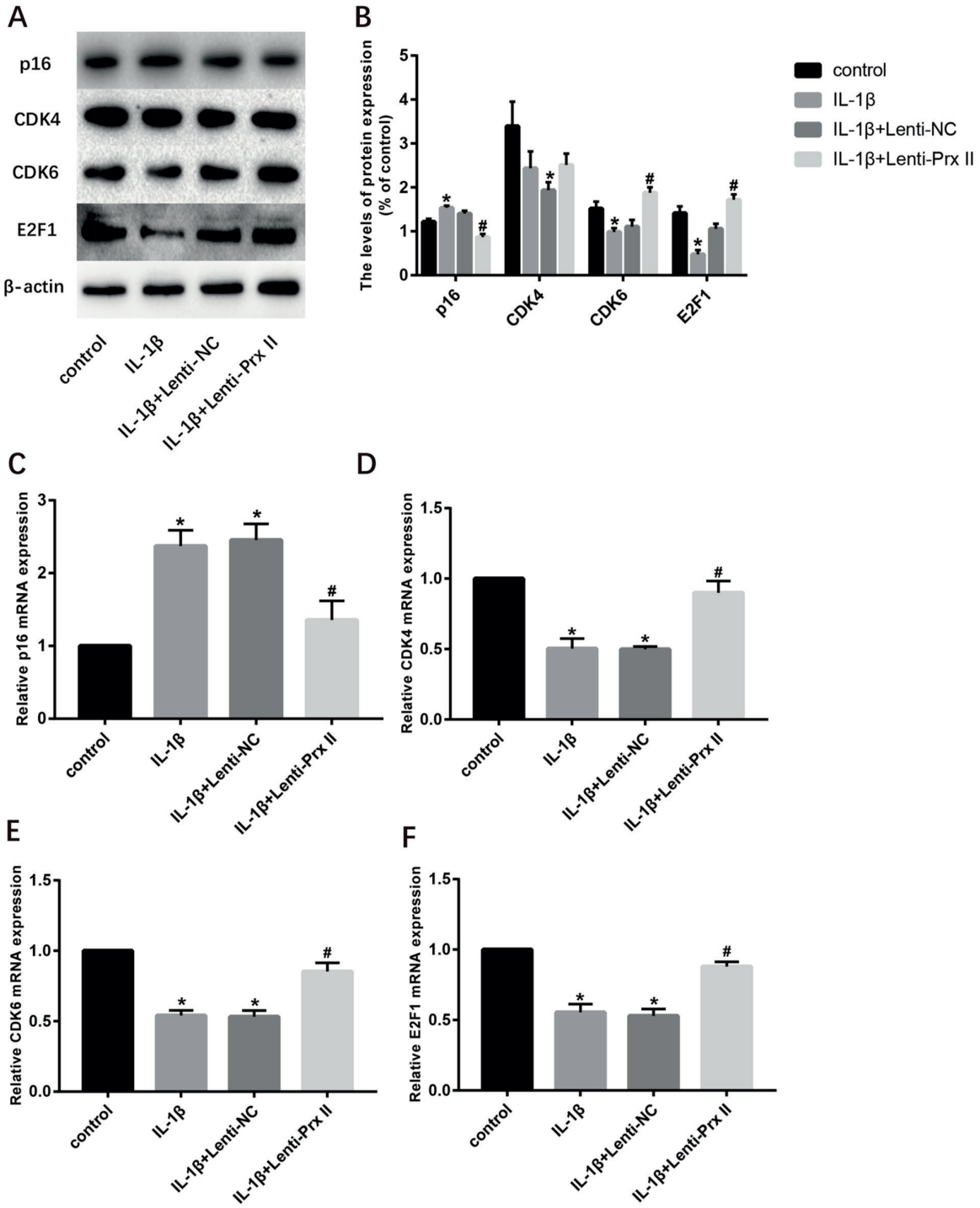

Figure 5. Overexpression of Prx II inhibits the expression of the p16 signaling pathway related molecules. Expressions of p16, CDK4, CDK6 and E2F1 in four groups are determined by Western blot (**A-B**) and RT-PCR (**C-F**). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the IL-1β+Lenti-NC group).

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ed cell model of Prx II and p16 was constructed. The p16 signaling pathway was activated by overexpression of p16. Western blot results (Figure 6A, 6B) showed that overexpression of p16 effectively decreased the expressions of SOD1 and SOD2 and increased the expressions of β-gal, p53, and p21, thereby reversing the anti-oxidative stress and anti-aging effects of Prx II on chondrocytes. The results of RT-PCR (Figure 6C-6F) were similar to those of Western blot.

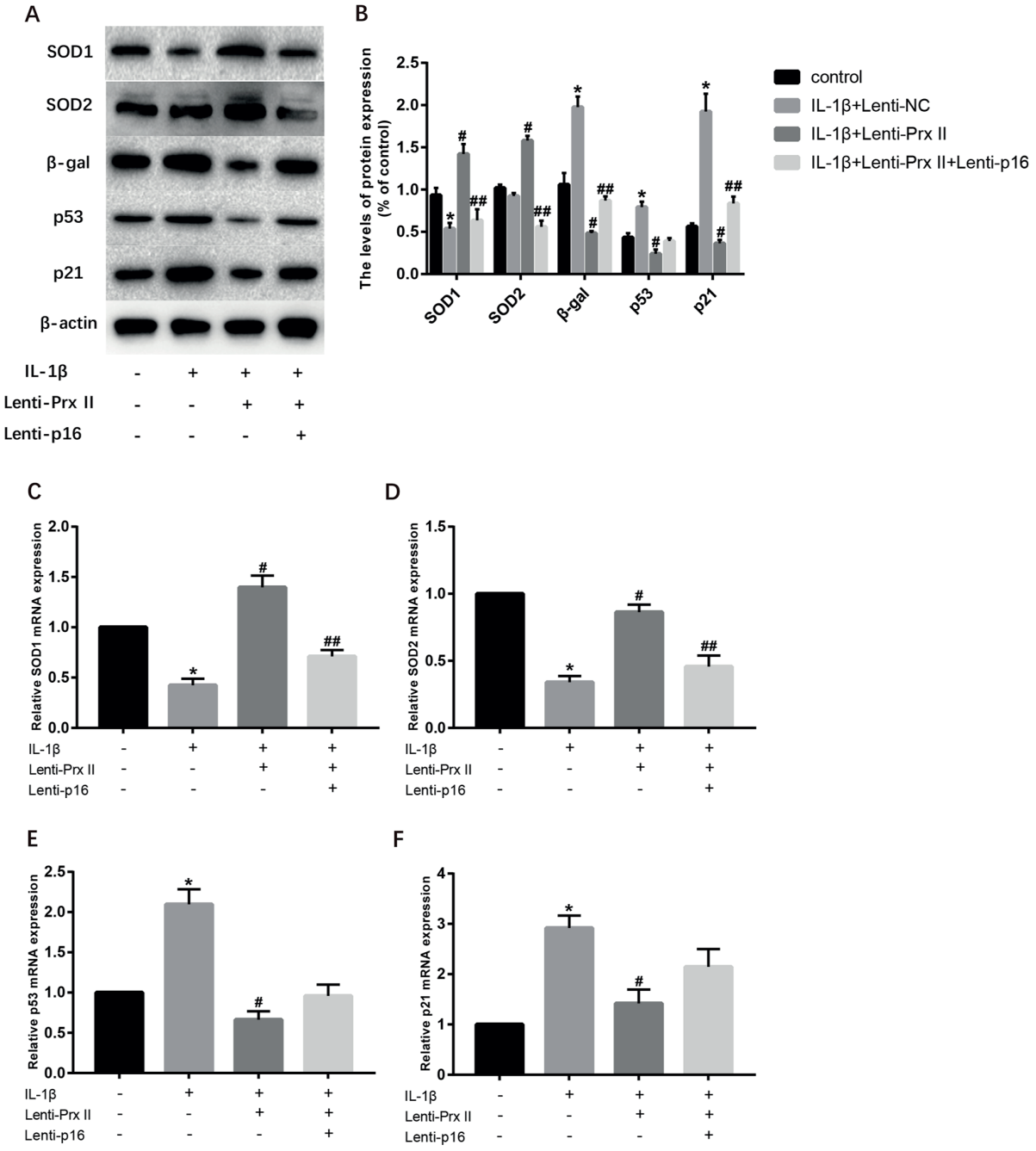

Figure 6. Activation of the P16 signaling pathway attenuates the anti-oxidative stress and anti-aging effect of Prx II. Expressions of SOD1, SOD2, β-gal, p53 and p21 in four groups are determined by Western blot (**A, B**) and RT-PCR (**C-F**). ("*" means there is a statistical difference with the control group, "#" means there is a statistical difference with the IL-1β+Lenti-NC group and "##" means there is a statistical difference with the IL-1β+Lenti-Prx II group).

Discussion

During the development of OA, chondrocytes have been shown to undergo many phenotypic changes, which alter their response to external stimuli and promote abnormal remodeling of ECM. These changes are affected by aging[9]. In the early stages of OA, chondrocytes re-proliferate to form clusters of cells, which is a phenomenon that is more common in older patients. Chondrocyte clusters are thought to be an attempt to repair cartilage damage in tissues, but experimental evidence suggests that they have no effect on repair[10]. With age, articular chondrocytes reduce the synthesis of proteoglycan (PG) and change their composition, thereby changing the biomechanical properties of ECM. In addition to affecting PG synthesis, age also affects the ability of chondrocytes to properly fold proteins. The production and aggregation of amyloid, misfolded proteins or peptides from their soluble precursors can be detected in the cartilage of OA patients[11]. Amyloid can promote abnormal gene expression, cell death, and organ dysfunction. The most well-characterized amyloid in articular cartilage is transthyretin, whose expression increases with age and development of OA, promotes chondrocyte death and promotes the secretion of pro-inflammatory factors IL-6 and iNOS, which can further promote the progression of cartilage destruction[12].

The aging of articular chondrocytes in OA has clear characteristics, including the expression of senescence-associated β-galactosidase, irreversible growth arrest, and shortening of telomere length[13]. David et al[14] compared osteoarthritic cartilage taken from normal cartilage and joint replacement in elderly patients with hip fracture and found that β-galactosidase was only present in cases of OA. Besides, aging of osteoarthritic chondrocytes causes a shortening of telomere length and an increase in DNA damage[15]. Palazzo et al[16] showed that there is chondrocyte senescence in the cases of OA, and the positive rate of senescent chondrocytes increases as the severity of cartilage degeneration increases. At the same time, the change of telomere length in senescent chondrocytes further proves the aging and degeneration of chondrocytes. Due to the progressive reduction of aging cartilage tissue, the changes in physical and chemical properties eventually lead to the occurrence of OA. In addition, cell senescence plays a major role in damaging the ability of the articular cartilage to regenerate. Rosenberg et al[17] observed that injection of senescent chondrocytes into joints is sufficient to promote cartilage damage similar to OA in mice. Jeon et al[18] revealed that removal of senescent cells reduces articular cartilage damage and pain in post-traumatic mouse models. Guzik and Touyz[19] indicated that the decline in autophagy in senescent cells leads to restricted cell apoptosis. It also leads to protein misfolding, aggregation, and mitochondrial dysfunction, increasing ROS production and promoting oxidative stress. Oxidative stress can induce the production of immunoinflammatory complexes, leading to inflammation[20]. Therefore, improving the aging state of articular chondrocytes is essential for the treatment of OA.

As a peroxidase widely present in cells of the body, Prx II has the effect of promptly removing low concentrations of ROS in the body[21]. Olthoff et al[22] disclosed that Prx II knockout mice show significant aging symptoms, indicating an important role of Prx II in cell senescence. Therefore, we have studied whether Prx II plays an important role in the aging of articular chondrocytes. Our data showed that overexpression of Prx II significantly increased the expression of collagen II in chondrocytes and reduced the degradation of ECM. In addition, overexpression of Prx II increased the expression of SOD1, SOD2, and BMI-1, and reduced the expression of β-gal and p53/p21, suggesting that Prx II can reduce oxidative stress and cell senescence in chondrocytes.

LaPak and Burd[23] demonstrated that the N terminal of p16 protein has a homologous structure with the cell cycle protein cyclinD, and it can compete with cyc1inD to bind CDK4/6, affecting the phosphorylation of Rb protein and the subsequent activation of transcription factor E2F, and preventing the cell cycle from passing the G1/S phase. p16 protein has also been found to promote the degradation of phosphorylated pRb protein and inhibit the cell cycle. By inhibiting the activity of CDK4/6, p16 protein maintains the phosphorylated pRb protein at a low phosphorylation level, thereby reducing the activity of E2F and keeping the cell in a growth inhibiting state.

The expression of p16 signaling pathway-related molecules in cells overexpressed by Prx II was significantly changed, such as the increase of p16 and the decrease of E2F1, suggesting that Prx II could inhibit the p16 signaling pathway. In addition, the overexpression of p16 gene could attenuate the effect of Prx II on chondrocytes. Therefore, the curative effect of Prx II on OA may be achieved by inhibiting the p16 signaling pathway.

This research also has some limitations, such as the lack of validation *in vivo* experiments. In the next study, Prx II knockout mice will be constructed, and the effect of Prx II on OA *in vivo* will be verified by making a mouse OA model.

To our knowledge, this is the first report to uncover the antioxidant and anti-senescence effect of Prx II on OA *via* the p16 signaling pathway. These data suggest that Prx II can be a useful target to slow down the progression of OA.

Conclusions

Shortly, Prx II alleviates OA by reducing oxidative stress and aging levels in chondrocytes. The role of Prx II may be achieved by activating the p16 signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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