Sirt1 regulates apoptosis and extracellular matrix degradation in resveratrol-treated osteoarthritis chondrocytes via the Wnt/β-catenin signaling pathways

SHUAN LIU, HONGPING YANG, BING HU and MINGYONG ZHANG

Department of Orthopaedics, Tianyou Hospital Affiliated to Wuhan University of Science and Technology, Wuhan, Hubei 430064, P.R. China

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Abstract. Osteoarthritis (OA) has become a major public health problem with the increased aging population. Previous studies have demonstrated that resveratrol (RES) was able to increase the level of sirtuin 1 (Sirt1) in OA chondrocytes. However, further investigations are required to elucidate the precise molecular mechanism of RES and the potential link between Sirt1 and RES. Therefore, the present study used 30 clinical OA chondrocyte to examine chondrocyte viability, apoptosis rate and the mRNA and protein expression levels of Sirt1 and relevant genes implicated in apoptosis, extracellular matrix (ECM) degradation and Wnt/β-catenin signaling pathway. RES and nicotinamide were used as the stimulus and inhibitor, respectively. The results demonstrated that the apoptotic rate reduced as the cell population decreased from 13.83 to 6.55% in response to 10 μM RES. Expression levels of B-cell lymphoma 2 (Bcl-2) associated X protein (Bax), procaspase-3 and -9, matrix metalloproteinase 1 (MMP1), MMP3, MMP13, Wnt3a, Wnt5a, Wnt7a and β-catenin were significantly inhibited ($P<0.01$), whereas the level of Bcl-2 was significantly increased ($P<0.01$) in OA chondrocytes treated with 10 μM RES. These observations suggested that Sirt1 may regulate apoptosis and ECM degradation in RES-treated osteoarthritis chondrocytes via the Wnt/β-catenin signaling pathway.

Introduction

Osteoarthritis (OA) is a progressively degenerative joint disorder affecting ~10% of males and ~18% of females aged >60 years old worldwide (1). Risk factors of OA include obesity, bone mass, joint injury and deformity, trauma and age (2). Although OA influences the adjoining bone, synovial lining and periarticular muscle, the cause may be primarily attributed to a progressive structural and functional compromise of the cartilage (3). Typical symptoms include activity-related or mechanical pain and brief morning stiffness (3). Therapeutic approaches currently available are limited for patients with OA prior to prosthetic joint replacement, which may be required (4).

Sirtuin 1 (Sirt1), the homolog of silence information regulator 2, is a highly conserved and well-characterized nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylase in mammalian cells (5). Increased cellular ionized NAD (NAD^+) as a substrate induces the activation of Sirt1, whereas a high concentration of nicotinamide (NAM) inhibits the activity of Sirt1. Numerous studies have demonstrated that Sirt1 has a central role in the regulation of cell proliferation, apoptosis and inflammation (6-8). Abnormal Sirt1 expression has been considered to be involved in OA pathogenesis. Compared with normal cartilage from cadavers, Sirt1 protein was highly expressed in OA cartilage (9). Sirt1 mRNA expression was also higher in severely degenerated cartilage, when compared with less damaged cartilage (10). Previous studies have demonstrated that Sirt1 induced abnormal sclerostin expression in human osteoarthritis subchondral osteoblasts (11) and regulated the osteoarthritic gene expression changes in human chondrocytes (12).

Resveratrol (RES) is a polyphenolic compound commonly identified in grape skin, berries and peanuts that has multiple functions, including anti-apoptosis, anti-inflammation and anti-oxidation effects (13). It has been indicated that RES may be an activator of Sirt1 in articular chondrocytes (14) and RES has been demonstrated to have a positive effect against OA (15). Although Sirt1 activation induced by RES appears to improve the survival and metabolism of OA chondrocytes (9,10), the precise molecular mechanism of RES and the potential link between Sirt1 and RES remain to be elucidated (16).

The present study aimed to examine the effect of Sirt1 on the Wnt/β-catenin signaling pathway. To do this, the present study detected the expression levels of a number of pivotal genes and corresponding translated proteins implicated in

Correspondence to: Dr Mingyong Zhang, Department of Orthopaedics, Tianyou Hospital Affiliated to Wuhan University of Science and Technology, 9 Tujialing, Dingzi Bridge, Wuhan, Hubei 430064, P.R. China
E-mail: zmytianyouchina@126.com

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apoptosis, extracellular matrix (ECM) degradation and the Wnt/β-catenin signaling pathway in RES-treated osteoarthritis chondrocyte.

Materials and methods

Cartilage sample. Cartilage samples were harvested from 30 individual patients with OA (69.0±14 years; 14 males and 16 females) between March 2015 and March 2016. Patients were admitted to the Department of Orthopaedics, Tianyou Hospital (Wuhan, China) and samples were collected during surgery. An OA diagnosis in these patients was determined according to the criteria of the American College of Rheumatology (17). The present study was performed following approval from the Experimental Animal Ethics Committee of Tianyou Hospital Affiliated to Wuhan University of Science and Technology and informed consent was provided by all participants.

Preparation of chondrocytes. OA cartilage tissue was washed with sterile phosphate-buffered saline (PBS; pH 7.2) and sliced into 1-mm^3 sections with ophthalmic scissors. OA chondrocytes were acquired via incubation at 37˚C in 0.25% trypsin for 30 min and 0.2% type II collagenase (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 2 h. Chondrocytes were filtered, centrifuged at 37˚C at 1,500 x g for 10 min and resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. Chondrocytes began to converge following 4-5 h incubation. Second generation chondrocytes were used in the present experiment.

Groups. Log phase chondrocytes were randomly divided into three groups: Control group without any stimulus, the RES group administered the ultimate concentration of 10 μM RES, and the NAM group administered the ultimate concentration of 20 μM NAM. Chondrocytes were cultivated for 48 h separately prior to subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following three washes with PBS for 90 sec, total RNA was extracted from OA chondrocytes using a RNAprep pure kit (DP430; Tiangen Biotech, Co., Ltd., Beijing, China) according to the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). Isolated RNA was transcribed into cDNA using a PrimeScript One Step miRNA cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. qPCR (2X SYBR Green qPCR kit; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) was subsequently performed with a reaction mixture of 20 μl containing: 10 μl 2X SYBR Fast qPCR Mix, 0.8 μl PCR forward and reverse primers, 0.4 μl 50X ROX Reference Dye II and 2 μl cDNA template. The PCR reaction was performed as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 13 sec. The reaction was performed using an Applied Biosystems® 7500 Fast Dx Real-Time PCR instrument (Thermo Fisher Scientific, Inc.). Expression of Sirt1 was normalized to GAPDH, as a reference control. Primer sequences were as follows: Sirt1 forward, 5'-TGGACTCCACGACGTACT-3' and reverse, 5'-TCTCCTGGGAGGCATAGACC-3' (total length, 122 bp); and GAPDH forward, 5'-AGCCACATCGCTCAG ACA-3' and reverse, 5'-TCTCCTGGGAGGCATAGACC-3' (total length, 314 bp). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Relative target gene expression was calculated as a fold change of $2^{-\Delta\Delta Cq}$ value, in which $\Delta Ct = Ct_{tarfet\ gene} - Ct_{endogenous\ control}$ (18). The experiments were performed in triplicate on three independent occasions.

MTT assay. The three groups of OA chondrocytes were seeded at a density of $5x10^3$ cells/ml into 96-well plates. Following 24, 48 and 72 h incubations at 37˚C, 20 μl 5 mg/ml cells were combined with 10 μl sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Beyotime Institute of Biotechnology, Haimen, China) for 4 h at 37˚C. The solution containing MTT was removed and cells were mixed with 150 μl dimethyl sulfoxide for 10 min. The plate was read spectrophotometrically at a wavelength of 560 nm by a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to detect cell viability.

Apoptosis detection. Apoptosis of OA chondrocytes was detected using fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) kit (cat. no. C1063; BestBio, Co., Shanghai, China), according to the manufacturer's instructions. Briefly, the chondrocytes were collected following digestion in EDTA-free 0.25% trypsin for 1 h at 37˚C. Following centrifugation at room temperature (1,000 x g; 10 min), 8 ml Annexin-FITC and 10 ml PI were added to the cell suspension and incubated for 15 min at room temperature. The rate of apoptosis was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and inbuilt software.

Western blot analysis. Protein expression levels of Sirt1, B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), procaspase-3, -9, matrix metalloproteinase 1(MMP1), MMP3, MMP13, Wnt3a, Wnt5a, Wnt7a and GAPDH were determined by western blot analysis. Briefly, the proteins were extracted from the cells in each group using an EpiQuik Total Histone Extraction kit (Epigentek, Farmingdale, NY, USA) according to manufacturer instructions and quantified using a BCA Protein Quantification kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Subsequently, ~50 mg total protein extracts were separated by 12% SDS-PAGE prior to being transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked using 5% milk solution for 2 h at room temperature and washed three times with Tris-buffered saline with Tween-20 (TBST; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The membrane was respectively incubated with the primary rabbit monoclonal antibodies of Sirt1 (cat. no. ab110304; Abcam, Cambridge, MA, USA), Bax, Bcl-2, procaspase-3, -9 (cat. nos. 1063-1, 1071-1, 1061-1 and 1084-1; Epitomics; Abcam), MMP1, MMP3, MMP13, Wnt3a, Wnt5a, Wnt7a, β-catenin and GAPDH (cat. nos. EP1247Y, ab53015, ab39012, ab28472, ab72583, ab100792, ab32572 and ab8245; Abcam) at 4˚C overnight at a dilution of 1:100. This incubation was followed by a further three TBST washes for 5 min. The membrane was subsequently incubated at room temperature

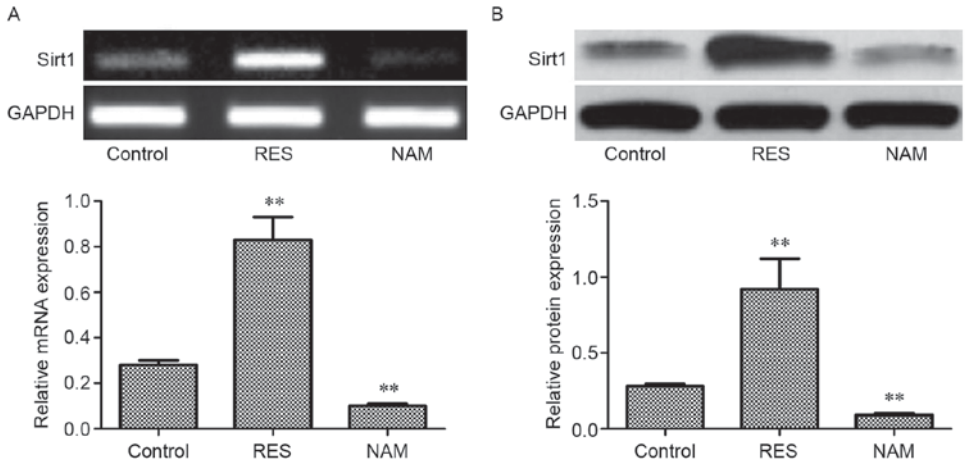

Figure 1. Sirt1 protein expression levels, evaluated via western blotting and densitometry, and mRNA expression levels, evaluated via reverse transcription-quantitative polymerase chain reaction analysis. (A) mRNA expression levels of Sirt1 and GAPDH presented as an electrophoretogram and quantitative analysis. (B) Western blot analysis of Sirt1 and GAPDH protein expression treated with 10 μM RES and 20 μM NAM individually. **P<0.01 vs. the control. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Sirt1, sirtuin 1; RES, resveratrol; NAM, nicotinamide.

with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (cat. no. A0208; Beyotime Institute of Biotechnology) at a dilution of 1:500 for 90 min and washed with TBST. Strip gray levels were quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.).

Statistical analysis. Experiments were performed in triplicate on three independent occasions. Data were presented as the mean ± standard deviation and analyzed using a two-tailed Student's t-test. Statistical analyses were conducted using SPSS version 17 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Sirt1 mRNA and protein in OA chondrocytes. Compared with the control, the expression levels of Sirt1 mRNA and protein in the RES group were significantly increased (P<0.01; Fig. 1A and B, respectively), but decreased significantly in the NAM group (P<0.01).

Cell viability and apoptosis in OA chondrocytes. Compared with the control, the cell viabilities were significantly improved at 24, 48 and 72 h in the RES group (P<0.01; Fig. 2), and significantly suppressed in the NAM group despite the slow growth of chondrocytes (P<0.01). The rate of cell apoptosis was reduced from 13.84 to 6.55% in response to 10 μM RES, but increased from 13.84 to 47.33% in response to 20 μM NAM (P<0.01; Fig. 3).

Protein expression of critical genes implicated in apoptosis, ECM degradation and the Wnt/β-catenin signaling pathway. To assess the effect of Sirt1 on apoptosis, ECM degradation and Wnt/β-catenin signaling in RES-treated OA chondrocytes, the protein expression levels of Bax, Bcl-2, procaspase-3, -9, MMP1, MMP3, MMP13, Wnt3a, Wnt5a, Wnt7a and β-catenin were determined. It was observed that the expression levels of Bax, procaspase-3 and -9, MMP1, MMP3, MMP13, Wnt3a, Wnt5a, Wnt7a, and β-catenin were significantly

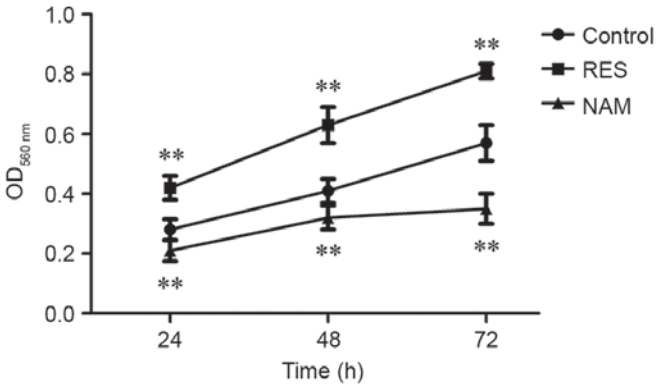

Figure 2. OD (560 nm) curve of cell viability at 24, 48 and 72 h following treatment with 10 μM RES and 20 μM NAM individually. **P<0.01 vs. the control. OD, optical density; RES, resveratrol; NAM, nicotinamide.

inhibited following treatment with RES (P<0.01; Figs. 4-6). However, Bcl-2 expression levels were significantly increased in RES-treated OA chondrocytes (P<0.01; Fig. 4). These variation tendencies were the opposite in NAM-treated OA chondrocytes; when the expression was significantly increased in the RES group, it was significantly decreased in the NAM group (P<0.01; Figs. 4-6).

Discussion

RES is a natural phytoalexin and has been demonstrated to be an effective stimulator of Sirt1 expression in response to OA chondrocyte metabolism, apoptosis and proliferation (15,16,19). Consistent with previous studies, the present study observed that the levels of Sirt1 mRNA and protein were significantly increased in OA chondrocytes treated with RES. Sirt1 has been confirmed *in vitro* and *in vivo* to be essential in the prevention of apoptosis in human chondrocytes, which is associated with cartilage degeneration in OA, via the mitochondrial-related pathway (20,21). Following exposure to RES, the present findings demonstrated that increased Sirt1 expression had a lower apoptotic rate compared with the control,

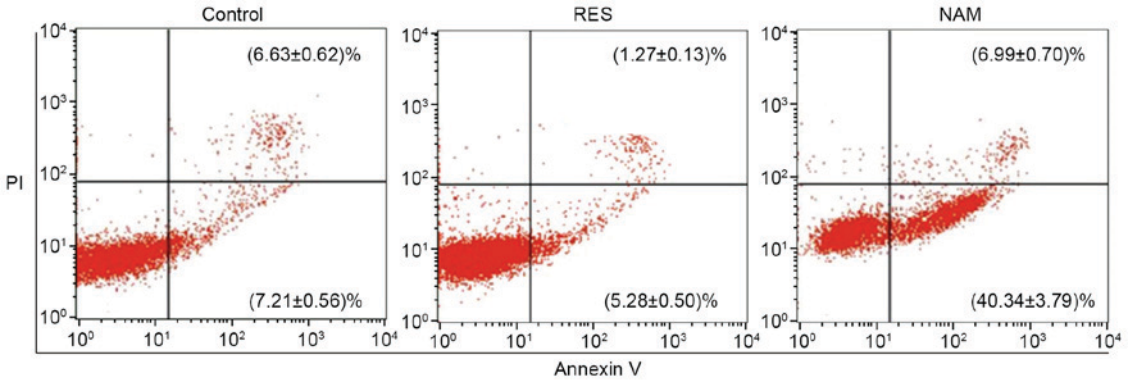

Figure 3. Apoptotic rates detected by Annexin V-PI staining when 10 μM RES and 20 μM NAM were administered. PI, propidium iodide; RES, resveratrol; NAM, nicotinamide.

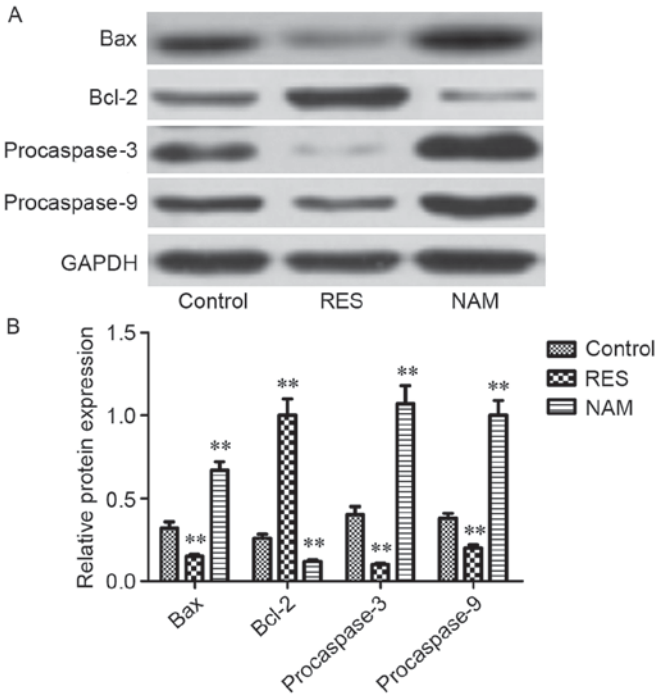

Figure 4. Western blot analysis and semi-quantification of protein expression. (A) Western blot analysis and (B) semi-quantification of Bax, Bcl-2, procaspase-3 and -9 proteins in 10 μM RES-treated and 20 μM NAM-treated OA chondrocytes. **P<0.01 vs. the GAPDH control. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein; RES, resveratrol; NAM, nicotinamide; OA, osteoarthritis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

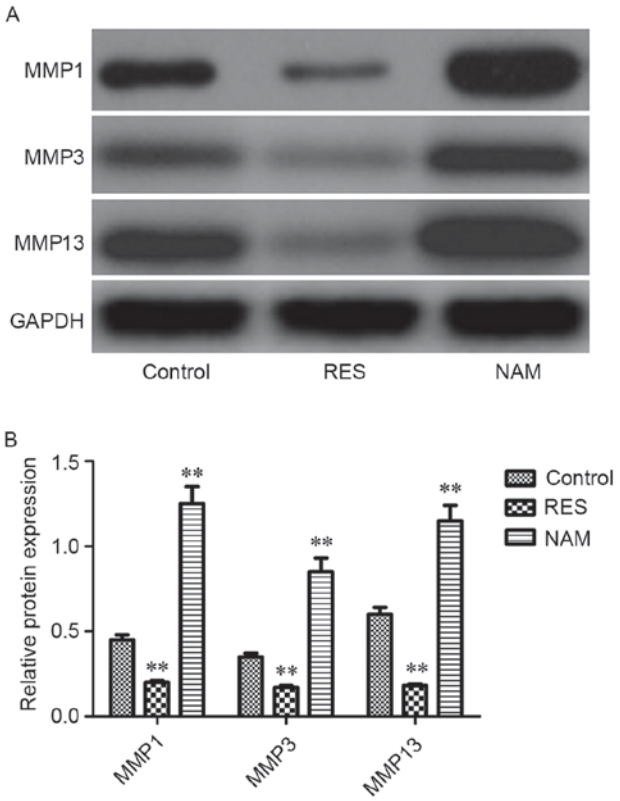

Figure 5. Western blot analysis and semi-quantification of protein expression. (A) Western blot anlysis and (B) semi-quantification of MMP1, MMP3 and MMP13 proteins in 10 μM RES-treated and 20 μM NAM-treated OA chondrocytes. **P<0.01 vs. the GAPDH control. MMP, matrix metalloproteinase; RES, resveratrol; NAM, nicotinamide; OA, osteoarthritis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

indicating that Sirt1 may promote OA chondrocyte survival. Further experiments evaluated the association of Sirt1 with the mitochondrial-related apoptotic pathway. As in mitochondria, the balance between proapoptotic members (such as Bax) and antiapoptotic members (such as Bcl-2) in the Bcl family determines cellular apoptosis and survival. Therefore, the effect of Sirt1 on the protein expression levels of Bax, Bcl-2, procaspase-3 and -9 was surveyed. In line with previous findings, Sirt1 inhibited the expression of Bax, procaspase-3 and procaspase-9 and increased Bcl-2 expression (20), indicating that Sirt1 induced the apoptosis of OA chondrocytes treated with RES through the mitochondria pathway.

Type II collagen is one of the primary ECM macromolecules in cartilage. MMPs are a family of 23 enzymes with a specific function to hydrolyze the triple helical structure in type II collagen, maintaining the balance between synthesis and degradation in normal cartilage ECM. It is generally hypothesized that the abnormal expression of various MMP members may lead to proteolysis and pathological cartilage breakdown in OA (22,23). The primary function of three MMPs (MMP1, MMP3 and MMP13) has been demonstrated to serve a role in chondrocyte-mediated cartilage matrix degeneration in OA (24), among which MMP13 has a central role in the regulation of OA chondrocytes (22,25). In RES-treated OA chondrocytes, the expression of MMP1, MMP3 and MMP13

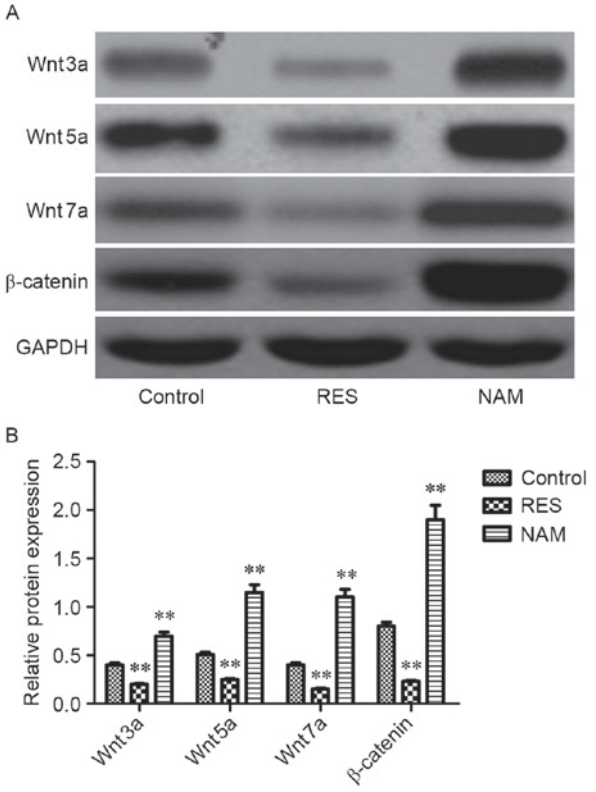

Figure 6. Western blot analysis and semi-quantification of protein expression. (A) Western blot analysis and (B) semi-quantification of Wnt3a, Wnt5a, Wnt7a and β-catenin proteins in 10 μM RES-treated and 20 μM NAM-treated OA chondrocytes. **P<0.01 vs. the GAPDH control. RES, resveratrol; NAM, nicotinamide; OA, osteoarthritis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

decreased along with the increase of Sirt1, and MMP13 exhibited a more evident decrease of expression than MMP1 and 3. Notably, increased Sirt1 induced by RES may inhibit MMP13 and suppress type II collagen degradation (26,27). These observations suggest Sirt1-mediated OA delay may benefit from the suppressions of a number of critical MMP members, including MMP1, MMP3 and MMP13, in RES-treated chondrocytes.

Wnt signaling is a conserved pathway that is associated with the response to cell differentiation and fate determination during embryogenesis and the late stages of development (28). Activation of Wnt/β-catenin, the canonical pathway, has been suggested to be involved in OA aggravation (29). Primary effectors implicated in OA development include three Wnt proteins (Wnt3a, Wnt5a and Wnt7a) (30-32). As described, MMP13, a target protein downstream of the Wnt/β-catenin signaling pathway (2,33), exhibited reduced expression caused by the elevated Sirt1. Notably, the present study demonstrated that an increase in the expression of Sirt1 may effectively suppress the expression of Wnt3a, Wnt5a, Wnt7a and β-catenin in RES-treated OA chondrocytes, indicating that Sirt may reduce OA progression through the Wnt/β-catenin signaling pathway.

In conclusion, the present study used RES-treated chondrocytes to monitor a number of critical factors in the progression of OA, and demonstrated that RES may increase Sirt1, leading to a decrease in the expression levels of Bax, procaspase-3 and -9, MMP1, MMP3, MMP13, Wnt3a, Wnt5a, Wnt7a and β-catenin and an increase in the level of Bcl-2. These results demonstrate that Sirt1 may regulate the apoptosis and ECM degradation in RES-treated osteoarthritis chondrocytes via the Wnt/β-catenin signaling pathway.

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