SIRT4 suppresses the inflammatory response and oxidative stress in osteoarthritis

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Abstract: Sirtuins have been involved in the osteoarthritis (OA) process. However, the functions of SIRT4 in the degeneration of human chondrocytes and OA are not fully understood. This study aimed to explore the role of SIRT4 during OA and mechanisms implicated. We extracted total protein and mRNA of the cartilage from OA patients and isolated the chondrocytes from the cartilage in different degenerated degrees for cell culture. Collagen II and SIRT4 levels of the tissues were analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) and Western blot. Chondrocytes were transferred with SIRT4-siRNA, treated with recombinant human SIRT4 protein for 24 h, respectively. Aggrecan, collagen I, collagen II, MMP-13, IL-6, TNF-α, SOD1, SOD2, and CAT expression, and ROS levels were investigated by Western blot, RT-PCR, immunofluorescence, enzyme-linked immunosorbent assay (ELISA), or flow cytometry. Collagen II decreased significantly in severely degenerated cartilage compared to the mild one, paralleling with SIRT4 expression both in protein and mRNA levels. Chondrocytes in severe OA grade were observed with a decrease in aggrecan, collagen II, SOD1, SOD2, CAT expression, nonetheless, an increase in collagen I, reactive oxygen species (ROS), MMP-13, IL-6, and TNF-α levels. However, SRIT4 protein treatment significantly upregulated aggrecan, collagen II, an antioxidant enzyme, and suppressed ROS and inflammatory response. Further analysis revealed that silencing of SIRT4 expression induced healthy chondrocytes, a decrease in aggrecan, collagen II and antioxidant enzyme expression, and an increase in ROS and inflammatory response, importantly, which can be reversed by SIRT4 protein stimuli. Our results elucidated that SIRT4 was tangled with the development of OA, and SIRT4 overexpression contributes to suppresses the inflammatory response and oxidative stress.

Keywords: SIRT4, inflammation, oxidative stress, osteoarthritis

Introduction

Osteoarthritis (OA) is a chronic disease that usually happens in the joints of the body and its surrounding tissues. It is one of the most common diseases affecting human health [1]. OA often causes clinical reactions such as redness, pain, dysfunction, or joint deformity in the joints of patients and further leads to progressive joint disability, which seriously affects the quality of life of patients [2]. The incidence of OA has no visible regional characteristics. The pathogenesis factors are complex and more common in middle-aged and older adults. Among them, secondary OA is associated with acute and chronic joint damage, inflammatory joint disease, metabolic abnormalities, endocrine disorders, and neurological defects; however, the pathogenesis of primary OA remains unclear [3, 4]. It has been universally accepted that destruction or damage of cartilage is the most important part of OA, and its incidence increases significantly with age, which may be mainly due to a series of reactions caused by aging changes in cartilage matrix, such as decreased anabolic capacity, decreased antioxidative stress ability, and higher secretion of inflammatory factors, promote the occurrence and development of OA [5].

Sirtuin (silent information regulator) belongs to human Sir2 gene, which behaves essential mediated functions in many cellular processes, such as aging, transcription, apoptosis, inflam-
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...mation as well as stress resistance [6, 7]. There are seven members (SIRT1-7) in the SirTuin family that show diversity in cellular localizations and functions depending on eukaryotic core domain sequences [8]. Matsuzaki et al. [9] found SIRT1 disruption in chondrocytes may accelerate the progression of OA. Wang et al. [10] uncovered the upregulation of SIRT3 protected against OA through PINK1/Parkin-dependent mitophagy in primary chondrocytes. Duarte et al. [11] also proved SIRT6 prevented chondrocyte senescence and DNA damage in OA. However, SIRT4 was the last of less well-understood sirtuins, especially for its modulators in OA, which thus brings some obstacles for the application of SIRT4 biological functions or developing SIRT4 modulators.

Our study aimed to investigate SIRT4 functions in the progress of OA and potential mechanisms involved. According to these discoveries, for the first time, we found that SIRT4 prevented the development of OA by suppressing inflammatory response and reactive oxygen species (ROS) levels in chondrocytes. It provides a theoretical basis for SIRT4 to become a therapeutic target for OA.

Patients and methods

Patient tissue samples collection and chondrocytes isolation

This project was accepted by the Ethics Committee of the West China Hospital, Sichuan University. Human articular cartilage tissues of the knee joints were donated from six patients who took arthroplasty knee surgery treated for OA (4 males, 2 females; every age: 47 years, from 39 to 73 years). All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki. In operation, degenerated articular cartilage with bone tissues were adequately resected for joint replacement. We divided the cartilage obtained from patients into two groups based on the degree of degeneration: mild group, with smooth, even, shiny and light pink or red cover (Figure 1A). Cartilage samples were cut into small pieces using scalpel, totally washed with sterile phosphate buffered saline (PBS) and then, mixed with collagenase XI (2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and Penicillin/Streptomycin (100 U/mL, Thermo Fisher Scientific, Waltham, MA, USA) in Dulbecco’s modified eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) to digest on a shaking platform overnight at 37°C. The cell solution was centrifuged and diluted in fresh DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) containing Penicillin/Streptomycin.

Cell treatment

The chondrocytes in passage one were transferred with Lentiviruses inducing the silencing of SIRT4. Non-treated and transferred chondrocytes over 70% density were partly treated with recombinant human SIRT4 protein [12] (50 ng/mL, Active Motif, Carlsbad, CA, USA) for another 24 h. The cell was collected at different time-points as mentioned to be applied to different experimental procedures.

Western blot analysis

Briefly, chondrocytes were lysed with cold radio-immunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), including phosphatase inhibitor cocktail (PI, Beyotime, Shanghai, China). Protein quality was measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). The antibodies used in this assay were: SIRT4 (1:3000; Cell Signaling Technology, Danvers, MA, USA), collagen II (1:1000; Abcam, Cambridge, MA, USA), collagen I (1:3000; Abcam, Cambridge, MA, USA), and β-Actin (1:1000; Abcam, Cambridge, MA, USA) as normalized overnight at 4°C. Finally, membranes were then incubated with secondary antibody 37°C for another 1 h. membranes were incubated in chemiluminescent electrochemiluminescence (ECL) substrate (Beyotime, Shanghai, China) and exposed using a developing film.

Immunofluorescence (IF)

Briefly, chondrocytes were first blocked with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100 for 15 min at room temperature, separately. After 30 min blocking with
bovine serum albumin (5%, BSA), chondrocytes were following cultured with primary antibodies against aggregan (1:200, Proteintech, Rosemont, IL, USA) and MMP-13 (1:500, Cell Signaling, Danvers, MA, USA) overnight at 4°C. After washing with PBS, chondrocytes were incubated with Alexa Fluor 488 conjugated secondary antibody (Abcam, Cambridge, MA, USA) at room temperature in the dark for 1.5 h, and finally staining with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was detected with Leica confocal microscope (Leica, Wetzlar, Germany). The staining intensity was determined by the Image-Pro Plus software (Silver Springs, MD, USA).

**Quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis**

Briefly, total RNA of chondrocytes was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Then, 1 g RNA of each group was reverse transcribed using RT Master Mix (TaKaRa Bio, Otsu, Japan). Diluted cDNAs were analyzed by RT-PCR using SYBR Green I master mix (TOYOBO, Tokyo, Japan) on a LightCycler 480. Relative gene expression was achieved by normalization to the amount of GAPDH and calculated according to the method of $2^{-\Delta\Delta C_T}$. The primer sequences applied were shown in Table 1.

**Lentivirus transfection**

The siRNA against SIRT4 was purchased from a biotechnology company (Bio-Primer, China), the sequence targeting described as 5'-TCCTATACAGCTACGCTC-3’. Chondrocytes were transfected by si-SIRT4 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. 95% of the cells were viable 12 h later.

**Enzyme-linked immunosorbent assay (ELISA)**

The levels of TNF-α, MMP-13, and IL-6 in the culture medium were determined using an ELISA.
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Intracellular ROS analysis

ROS levels of the chondrocytes were determined using the flow cytometry by peroxide-sensitive fluorescent probe DCFH-DA (KeyGene BioTECH, Nanjing, China) according to the manufacturer’s instructions. We used excitation wavelength at 488 nm and emission wavelength at 525 and calculated the mean fluorescence by the program Cell Quest (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All generated quantitative data were expressed as mean ± standard deviation. Differences between two groups were analyzed by using the Student’s t-test. A comparison between multiple groups was made using one-way ANOVA test followed by Post Hoc Test (Least Significant Difference). Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) Version 22.0 (IBM Corp, Armonk, NY, USA). P-value <0.05 was considered statistically significant for all tests.

Results

SIRT4 levels in different degree of human OA tissues

To determine the potential connection between SIRT4 and OA, we collected cartilage tissues of different OA degrees from the patients (Figure 1A). Collagen II is the most important extracellular matrix (ECM) in cartilage secreted by chondrocyte. As shown in Figure 1B-D, protein and mRNA levels of collagen II were gradually reduced in severely degenerated chondrocytes compared to the mild one. What’s more, SIRT4 expression was also obviously decreased in severe condition compared to the mild in both protein and mRNA level, suggesting that SIRT4 was strongly associated with the progression of OA.

SIRT4 protein treatment prevents human chondrocytes from degeneration in vitro

To determine the function of SIRT4 on OA, chondrocytes in the severe group were treated with SIRT4 protein 24 h. As shown in Figure 2A, 2B, chondrocytes in severe OA condition expressed a lower amount of aggrecan compared to the mild, which was upregulated after SIRT4 treatment. Besides, collagen I, the osteogenic marker of a chondrocyte, turned to a higher dose in the progress of OA but suppressed by exterior SIRT4 protein stimuli (Figure 2C, 2D). As expected, the results showed SIRT4 contributed chondrocytes to secrete much more collagen II compared to the non-treated group (Figure 2C, 2D). Taken together, these data suggest that SIRT4 stimulation seems to be a positive effect on the progress of OA, which contributes to the balance of ECM and inhibition of ossification (Figure 2E).

SIRT4 protein treatment suppresses the inflammatory response and ROS of human chondrocytes in vitro

To determine the potential mechanism underlying the SIRT4 in OA. IF against SOD1 was carried out to detect the difference of antioxidant ability among three groups. The intensity of green fluorescent in the cells was defined as SOD1 protein level, indicating SOD1 expression decreased along with the OA progression, whereas SIRT4 could promote SOD1 expression (Figure 3A, 3B). Oxidative stress has been considered a key mechanism underlying cartilage destruction. As shown in Figure 3C, the ROS level in severely degenerated chondrocytes was gradually increased compared to the mild but reversed by SIRT4. In addition to this, mRNA expression of the antioxidant enzymes was also increased with the treatment of SIRT4 (Figure 3D), suggesting that SIRT4 somehow

Table 1. Primer sequences of the genes for RT-PCR

<table>
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<tr>
<th>Gene name</th>
<th>Forward (5’&gt;3’)</th>
<th>Reverse (5’&gt;3’)</th>
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<tr>
<td>aggrecan</td>
<td>GGTGAACCGTGGTGTTGTC</td>
<td>CCGTCCTTCCAGCAGTC</td>
</tr>
<tr>
<td>Collagen II</td>
<td>TGGACGATCAGGGAACCC</td>
<td>GCTGCGGATGCTCTCAATCT</td>
</tr>
<tr>
<td>Collagen I</td>
<td>TGGATTCGATCAGGCTTCAG</td>
<td>GCTGCGGATGCTCTCAATCT</td>
</tr>
<tr>
<td>IL-6</td>
<td>TAGTCCTCTCACCACCAATTCCC</td>
<td>TTGTCCTTACGACCCTTCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTCCTCTCAATCAGGCTTCG</td>
<td>GAGGACCTGGAGATGAGAG</td>
</tr>
<tr>
<td>MMP13</td>
<td>ACTGAGAGGCCTCGAGAATG</td>
<td>GAACCCCGCATCTTGCTT</td>
</tr>
<tr>
<td>SOD1</td>
<td>GGTGAACCGTGGTGTTGTC</td>
<td>CCGTCCTTCCAGCAGTC</td>
</tr>
<tr>
<td>SOD2</td>
<td>CAGACCTGCTTACCAGCTAGTGG</td>
<td>CTCGGTGCCGTTGAGATTG</td>
</tr>
<tr>
<td>CAT</td>
<td>TGGAGCCTGGTGAACCCAGTGG</td>
<td>CTCGGTGCCGTTGAGATTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACAACTTTGATCGTGGAAGG</td>
<td>GCCATCACGCCACAGTTC</td>
</tr>
</tbody>
</table>

RT-PCR, quantitative reverse-transcription polymerase chain reaction.
triggered the antioxidant program in the chondrocytes. We further studied the mechanism of SIRT4 related to inflammation in OA. As shown in Figure 3D and 3E, MMP-13, IL-6, and TNF-α were obviously overexpressed in severe OA chondrocytes both detected in the cells and the culture medium, whereas SIRT4 successfully inhibited the inflammatory response. The data above suggest that ectogenic SIRT4 stimuli can suppress inflammatory response and oxidative stress in human chondrocytes in vitro.

Silencing of SIRT4 accelerates the progress of human chondrocytes degeneration in vitro

To determine whether the absence of SIRT4 may affect the chondrocytes behavior, we silenced the activation of the SIRT4 gene in healthier chondrocytes compared with the control. SIRT4 protein was also used to compensate for the decrease of SIRT4 resulting from siRNA transfection. As shown in Figure 4A, 4B, silencing of SIRT4 caused chondrocytes a reduction severe OA aggrecan expression compared to the control one, which was significantly changeover after SIRT4 treatment. What’s more, collagen I expression was promoted with the absence of SIRT4, but down-regulated by SIRT treatment as expected (Figure 4C, 4D). The data suggested silencing of SIRT4 partly destroys the anabolism of collagen II compared to the control (Figure 4C, 4D). Taken together, these results indicate that SIRT4 deficiency may play a decisive role in the acceleration of the progress of OA (Figure 4E).
Silencing of SIRT4 promotes inflammatory response and ROS of human chondrocytes in vitro

To determine whether the absence of SIRT4 may help the inflammation and ROS reaction, we used SIRT4-silenced chondrocytes, making a comparison with the control. The result of IF against SOD1 indicated SOD1 expression decreased by the suppression of SIRT4, whereas rescued through the supply of SIRT4 protein (Figure 5A, 5B). As shown in Figure 5C, the ROS level in SIRT4-silenced chondrocytes was gradually increased compared to the control, inter-
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Figure 4. The silencing of SIRT4 accelerates human chondrocytes degeneration. Chondrocytes in mild OA conditions were silenced by siRNA transfected, pretreated with or without SIRT4 protein (50 ng/mL) for 24 h. A, B. The protein expression level of aggrecan was determined by immunofluorescence and quantification analysis. C, D. The protein expression levels of collagen II and collagen I was determined by Western blot and quantification analysis. E. The mRNA expression levels of aggrecan, collagen II, and collagen I was assayed by RT-PCR. The values are mean ± SD of three independent experiments. *P<0.05, **P<0.01.

Discussion

As a common chronic joint disease, OA undoubtedly plagues mainly hips, knees, hands, and feet, causing the health and quality of people’s lives, particularly in the elderly population [13]. However, because of the complex characteristics, the pathogenesis of OA has always been one of the problems that perplex people, which makes it the key to cure or control OA. Sirtuin is a family of NAD+-dependent deacetylases that are detected both in the simplest living organisms and complex organisms such as humans [14]. This family of proteins has been claimed to take part in the progress of stress resistance, inflammation, aging, and even cancer. Sirtuin family contains seven members, of which SIRT1 and SIRT2 are present in the nucleus and cytoplasm, SIRT3, SIRT4, SIRT5 are mitochondrial proteins, and SIRT6 and SIRT7 are nuclear proteins [15]. Previous studies have shown that SIRT1 promotes chronic inflamma-
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by inducing TNF-α overexpression in joint synovial cells, thereby improving the expression of pro-inflammatory cytokines [16]. Overexpression of SIRT6 has a significant inhibitory effect in inflammation in mice arthritis [17]. The role of these two members of the sirtuin family in the pathogenesis of arthritis has raised our interest in other family members. Based on past research, whether other family members affect OA has become the source of this research. Actually, in the present study, it was discovered that the protein and mRNA levels of SIRT4 were significantly decreased in the cartilage tissue of the severe OA patients group

Figure 5. The silencing of SIRT4 promotes inflammatory response and ROS of human chondrocytes. Chondrocytes were treated as mentioned above. A, B. The protein expression level of SOD1 was determined by immunofluorescence and quantification analysis. C. The total ROS levels were assessed by flow cytometry. D. The mRNA expression levels of SOD1, SOD2, CAT, MMP-13, IL-6, and TNF-α, were assayed by RT-PCR. E. The protein expression level of MMP-13, IL-6, and TNF-α in the medium were determined by ELISA. The values are mean ± SD of three independent experiments. *P<0.05, **P<0.01.
than in the mild group. This phenomenon suggested that SIRT4 may play a more critical role in the development of OA.

Since the SIRT4 level in severe OA cartilage is much lower than the mild. We wondered whether it was beneficial for the chondrocytes with an exogenous supplement of SIRT4. Collagen II and aggrecan are significant components of the ECM of articular cartilage. Their biosynthesis and catabolism are regulated by chondrocytes. Collagen I turns out generally in the condition that chondrocyte differentiates into ossification. The degeneration of chondrocytes usually accompanies with the disorder of collagen II and aggrecan, and the accumulation of collagen I. In our study, SIRT4 protein stimuli contributed chondrocytes with a changeover to a healthier state according to an increase of collagen II and aggrecan secretion.

Nowadays, molecular changes such as inflammation and oxidative stress cause more and more people’s attention to the mechanism and therapy of OA. Oxidative stress is thought to play a significant role as a stressor in the aging process, resulting from the amount of imbalance between ROS and the antioxidant capacity of the cell. It is well known that SIRT1 activators may prevent cartilage degeneration due to antioxidant defenses [18]. SIRT1 and SIRT6 are reported to inhibit TNF-α-induced inflammation by the ROS pathway [19]. SIRT4 is also elucidated to play a role in the regulation of mitochondrial ROS production and the senescence-associated secretory phenotype [20]. However, whether SIRT4 affects the ROS production remains unknown. We measured ROS level of the degenerated chondrocytes with or without the presence of SIRT4 protein and found that extra SIRT4 supplement resulted in a marked reduced ROS accumulation with an upregulation of antioxidant enzymes, such as SOD1, SOD2, and CAT. Furthermore, SIRT4-silencing of chondrocytes in mild degeneration showed a break of the ROS balance by inhibiting the expression of the antioxidant enzymes, which even could be reversed by SIRT4 treatment. These results suggested that SIRT4 may take part in oxidative stress homeostasis of human chondrocyte.

Another main character of the pathological process of OA is the destruction of the cartilage structure and the dysregulation of the repair process, accompanied by secondary inflammatory changes, which can also involve itself through negative feedback [21]. The maintenance of the typical structure and function of cartilage depends on the metabolic balance of the ECM, which is regulated by a series of cytokines in the body. In the pathogenesis of OA, the ECM is always in a state of catabolism higher than anabolism, resulting in the disintegration and the destruction of cartilage [22]. The reason based on is that OA continues to stimulate the secretion of degrading cytokines, such as MMP-13, IL-6, and TNF-α, from the beginning of the disease, which is far higher than the synthetic cytokines that chondrocytes can produce. With the development of the disease, the normal function of chondrocytes is continuously lost, the artificial cytokines collapse, and the degrading cytokines occupy an absolutely dominant position, leading to the degradation of large pieces of chondrocytes, finally resulting in irreversible diseases [23]. Sirtuins are adequately verified to be associated with the inflammatory response in many cells. Tao et al. [24] recovered SIRT4 inhibits inflammatory responses using human umbilical vein endothelial cells. We mainly analyzed three important that involved in the OA progress, which are MMP-13, IL-6, and TNF-α. Then we investigated they all reduced at a higher present of SIRT4 condition both within the cells and in the dilution of culture medium. In addition to this, we found the silencing SIRT4 of chondrocytes in good condition accelerated the speed to degenerate, which also could be disturbed by SIRT4 stimuli. These evidence support the notion that SIRT4 might mediate inflammatory responses of human chondrocyte via inflammatory cytokines suppression.

Conclusions

In conclusion, our results elucidate the protective function of SIRT4 during the development of OA. This study may partly explain a possible mechanism underlying which is downregulating of SIRT4 accelerate OA procession by disturbing oxidative stress and inflammatory responses, and finally result in ECM destruction and osteogenesis. Besides, the ectogenous SIRT4 supplement contributes a decisive role in the inhibition of oxidative stress and inflammatory responses. This study provides an idea that
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SIRT4 may offer novel opportunities for delaying OA by keeping chondrocyte heathier.

Disclosure of conflict of interest

None.

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