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SIRT2 inhibits oxidative stress and inflammatory response in diabetic osteoarthritis

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Abstract. – OBJECTIVE: Diabetes mellitus is involved in inflammation, immunity, and metabolism during osteoarthritis (OA). It destroys the normal synthesis and degradation balance of chondrocytes (CHs) and extracellular matrix (ECM). The purpose of this study was to explore the possible way of SIRT2 influencing the progress of diabetic OA.

PATIENTS AND METHODS: Proteins of diabetic OA and normal OA cartilage samples were extracted from patients undergoing knee joint operation. CHs were also isolated from the cartilage exempted from diabetes for cell culture. Glucose was used to treat CHs for imitating the microenvironment of diabetes. The expressions of SIRT2, acetylated H3K9, H3K14, and H3K56 protein were determined by Western blotting. SIRT2, 8-hydroxy-2' deoxyguanosine (8-OH), and MMP-13 expressions were analyzed using immunofluorescence. RT-PCR was performed to measure the mRNA levels of SOD1, SOD2, CAT, MMP-13, ADAMTS-4, and ADAMTS-5. Total ROS level was performed by flow cytometry assay.

RESULTS: SIRT2 expression was reduced, whereas acetylated H3K9, H3K14, and H3K56 were upregulated in diabetic cartilage compared to normal. High glucose suppressed the expression of SIRT2 but accelerated the acetylation of H3K9, H3K14, and H3K56. Besides, high glucose promoted the expression of 8-OH, and inhibited SOD1, SOD2, and CAT mRNA expressions, resulting in the up-regulated ROS level of CHs. In addition, high glucose activated the inflammatory response by upregulation of MMP-13, ADAMTS-4, and ADAMTS-5 expressions. SirReal2 suppressed SIRT2 and resulted in several acetylations of H3, more ROS, less antioxidant enzymes, and stronger inflammatory response caused by high glucose. However, supplied rh-SIRT2 reversed these negative effects of high glucose in CHs.

CONCLUSIONS: SIRT2 expression is reduced along with the diabetic OA process with increased acetylation of H3, oxidative stress, and inflammatory response. Suppression of SIRT2 accelerates the progress of diabetic OA and upregulation of SIRT2 alleviates diabetic OA development by suppressing oxidative stress and inflammatory response that are likely to be related to the deacetylation of H3.

Key Words:
Diabetes, Osteoarthritis, SIRT2, Oxidative stress, Inflammation, Deacetylation.

Introduction

Osteoarthritis (OA) is the main hazard in the elderly[1]. Although the incidence of OA is high, its early diagnosis and prevention are difficult. Clinical diagnosis mainly depends on morphological examination (such as X-ray film), but at this time OA has often developed into the middle and late-stage with significantly destroyed cartilage, the drug is difficult to cure, with large risk of surgical treatment and many postoperative complications[2]. OA is reported as a "metabolic disorder" associated with obesity, diabetes mellitus, dyslipidemia, hypertension, and insulin resistance. These diseases can increase the production of proteolytic enzymes, accelerate the degradation of ECM of cartilage, and cause the destruction of cartilage, which is the main feature of OA[3]. At the same time, a large number of clinical studies have also shown that diabetes may be related to the occurrence and development of bone degenerative diseases[4].

Diabetes is a multi-pathogenic metabolic disease characterized by chronic hyperglycemia accompanied by insulin secretion or defects in the metabolism of sugar, fat, and protein[5]. In China, with the development of society and the aging of the population, the incidence of diabetes is also increasing year by year[6]. Epidemiological investigations have found that diabetes or elevated blood glucose is associated with OA. In addition, experimental studies have shown that OA CHs exposed to high glucose have an impaired ability to down-regulate glucose transporter-1, resulting in an higher level of reactive oxygen species (ROS), which exacerbates the catabolic process and promotes OA progression[7]. With the in-depth study

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of the association between diabetes and OA, the concept of diabetes OA has been proposed.

Sirtuins are NAD+-dependent deacetylase involved in the regulation of a variety of physiological functions. SIRT2 tends to deacetylate tubulin and histone H3 and plays an important role in a variety of physiological processes that maintain metabolic balance[8]. A decrease in SIRT2 levels was observed in visceral adipose tissue of human obese individuals. In addition, reducing SIRT2 expression promotes adipogenesis. In cultured human cell and animal experiments, SIRT2 regulates gluconeogenesis and insulin-mediated systemic glucose uptake[9]. SIRT2 protects cells by mediating ROS, DNA repair, and inflammation, which are important for the incidence of diabetes[10].

In the present study, it was hypothesized that diabetes and high glucose suppressed SIRT2 expression that promoted the process of OA. OA cartilages were collected from the patients with or without OA and isolated chondrocytes (CHs) *in vitro* coupled with high glucose culture to imitate diabetic environment, so as to explore the function of SIRT2 in the deacetylation of diabetic OA to provide a theoretical basis for the future treatment of diabetic OA.

Patients and Methods

OA Samples Collection

This investigation was approved by the Ethics Committee of the Affiliated Zhongshan Hospital of Dalian University. 5 OA patients aged 39-65 years old with an average age of 47 years old (4 males and 1 female) based on diabetes and 5 simple OA patients aged 41-67 years old with an average age of 52 years old (3 males and 2 females) without diabetes undergoing operations of total knee replacement in our hospital from July to August 2018 participated in the project. We conserved the tissues in a sterile cell culture medium immediately after being separated from patients for the following protein extraction and CH isolation. All patients provided written informed consent. This investigation was conducted in accordance with the Declaration of Helsinki.

CHs Isolation and Culture

The cartilage was separated from the knee joint and washed with sterile phosphate-buffered saline solution (PBS; Invitrogen, Carlsbad, CA, USA). Then, tissues were totally chipped and digested with 0.2% collagenase XI (Sigma-Aldrich, St. Louis, MO, USA) in medium overnight at 37°C. After that, the CHs pellets were filtered, centrifuged, and re-suspended in cell culture medium [contains DMEM/F12 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA)]. CHs in passage 1 were treated as the following described: 1) CHs were cultured with different concentration of glucose (5, 15, 25, 35 mM; Beyotime, Shanghai, China), 2) CHs were cultured with 5 mM glucose combined with 50 nM SirReal2, and 3) CHs were cultured with 35 mM glucose combined with 100 μM recombinant human SIRT2 protein (rh-SIRT2). CHs were harvested after 24 h incubation for the next steps.

Western Blot (WB) Analysis

CHs were lysed by lysis buffer (Beyotime, Shanghai, China). Total protein was isolated and quantified with the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Equal amounts of protein from CHs were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). 5% nonfat milk was used to block membranes, which were incubated overnight at 4°C with the primary antibodies against: SIRT2 (1:1000; Cell Signaling, Danvers, MA, USA), acetylated H3K9, H3K14, and H3K56 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:1000, Abcam, Cambridge, MA, USA). After washing with Phosphate-Buffered Saline with Tween-20 (PBST), membranes were incubated again with secondary antibody for 1 h at room temperature. Finally, the enhanced chemiluminescence (ECL) substrate (Beyotime, Shanghai, China) was used to expose the band on the membranes.

Immunofluorescence (IF)

CHs were seeded on the coverslips and treated as described above. After fixation in 4% paraformaldehyde, CHs were incubated with 0.2% Triton-X to permeabilize the membrane. The coverslips were next blocked with 5% bovine serum albumin at room temperature, and then incubated with SIRT2 (1:500, Abcam, Cambridge, MA, USA), 8-hydroxy-2' deoxyguanosine (8-OH) (1:800, Abcam, Cambridge, MA, USA), MMP-13 (1:300, Abcam, Cambridge, MA, USA) primary antibodies at 4°C overnight. Subsequently, coverslips were incubated with secondary antibodies

and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) for 1 h in the dark at room temperature. Images were captured with the Nikon ECLIPSE (Nikon, Tokyo, Japan) and measured by Image-Pro Plus software.

Real-Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted from CHs *via* miRNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then reversely transcribed into complementary deoxyribose nucleic acid (cDNA) with a reverse transcription kit (Ambion, Life Technologies, Gaithersburg, MD, USA). PT-PCR analysis of SOD1. SOD2, CAT, matrix metalloproteinases-13 (MMP-13), ADAMTS-4, and ADAMTS-5 were performed using SYBR Green Master (TOYOBO, Osaka, Japan). Gene expression was normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in RT-PCR are shown in Table I.

Flow Cytometry

Total ROS level of CHs was determined using flow cytometry. CHs were harvested (1×10^5/group), washed with cold PBS, centrifuged, and fixed in 4% formaldehyde at room temperature. The cells were incubated with DCFH-DA (10 μm; Keygen, Nanjing, China) for 30 min at room temperature to detect the total ROS level according to the manufacturer's instructions.

Statistical Analysis

All data were expressed as mean ± standard deviation that analyzed by the Statistical Product and Service Solutions (SPSS) software package (Version 20.0; IBM Corp, Armonk, NY, USA). Differences between the two groups were analyzed by using the Student's *t*-test. Comparisons among multiple groups were done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). *p*-value <0.05 (two-tailed) was considered as statistical significant.

Results

SIRT2 and Acetylated H3 Levels in Diabetic OA Cartilage

To explore whether SIRT2 expression in diabetic OA is different from the OA without diabetes, cartilages were collected from these two conditions (Figure 1A) for protein measurements and CHs isolation. SIRT2 is one of the key enzymes for histone acetylation. Therefore, protein levels of SIRT2 and three acetylated H3 members, including H3K9, H3K14, and H3K56 in the cartilage of OA with or without diabetes were examined *via* Western blotting. Diabetes significantly decreased the expression of SIRT2, whereas increased the expressions of acetylated H3K9, H3K14, and H3K56 (Figure 1B and 1C). The results indicate that SIRT2 and acetylated H3 play an unknown role in the progress of diabetes.

High Glucose Reduces SIRT2 Expression and Promotes H3 Acetylation in CHs In Vitro

To explore the effect of diabetes in the SIRT2 expression and H3 acetylation, a different concentration of glucose (5 mM to 35 mM) was used to culture the CHs from the OA cartilage without diabetes, which imitated the microenvironment of diabetic OA *in vitro*. Subsequnetly, the protein level of SIRT2 was detected by IF. As shown in Figure 2A and 2B, the expression of SIRT2 was significantly reduced within the increased concentration of glucose, which meant an excess

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

Gene name	Forward (5'>3')	Reverse (5'>3')
SIRT2	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD1	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
SOD2	TTTTGCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC
CAT	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
ADAMTS-4	ACATGGAGACTTTGTCCCTTTTG	TTGGCTGAGTGGTAGAGTCCC
ADAMTS-5	CTGGACAGCCAGACACTAAAC	CTCGCGGCAAGTCTTCAGAG
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

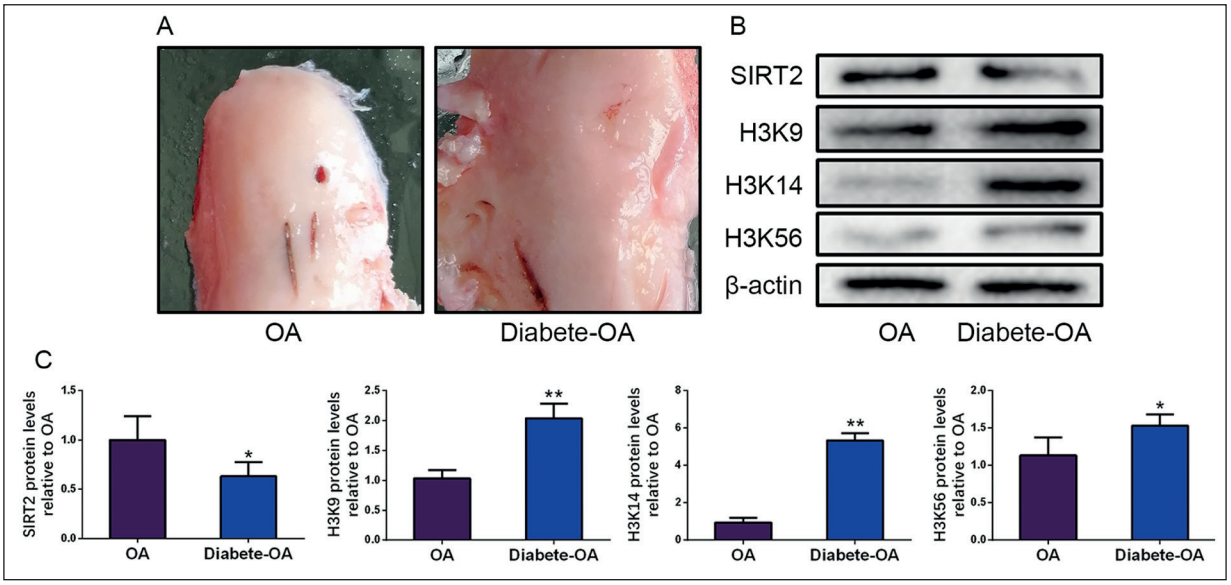

Figure 1. SIRT2 and acetylated H3 levels in OA and diabetic OA cartilage from the patients. Cartilage tissues are divided into two groups. **A,** Protein levels of SIRT2 and acetylated H3K9, H3K14, and H3K56 from the cartilage tissue are determined by Western blotting (**B**) and quantification analysis (**C**). Values are mean ± SD of three independent experiments. *p<0.05, **p<0.01 compared with OA group.

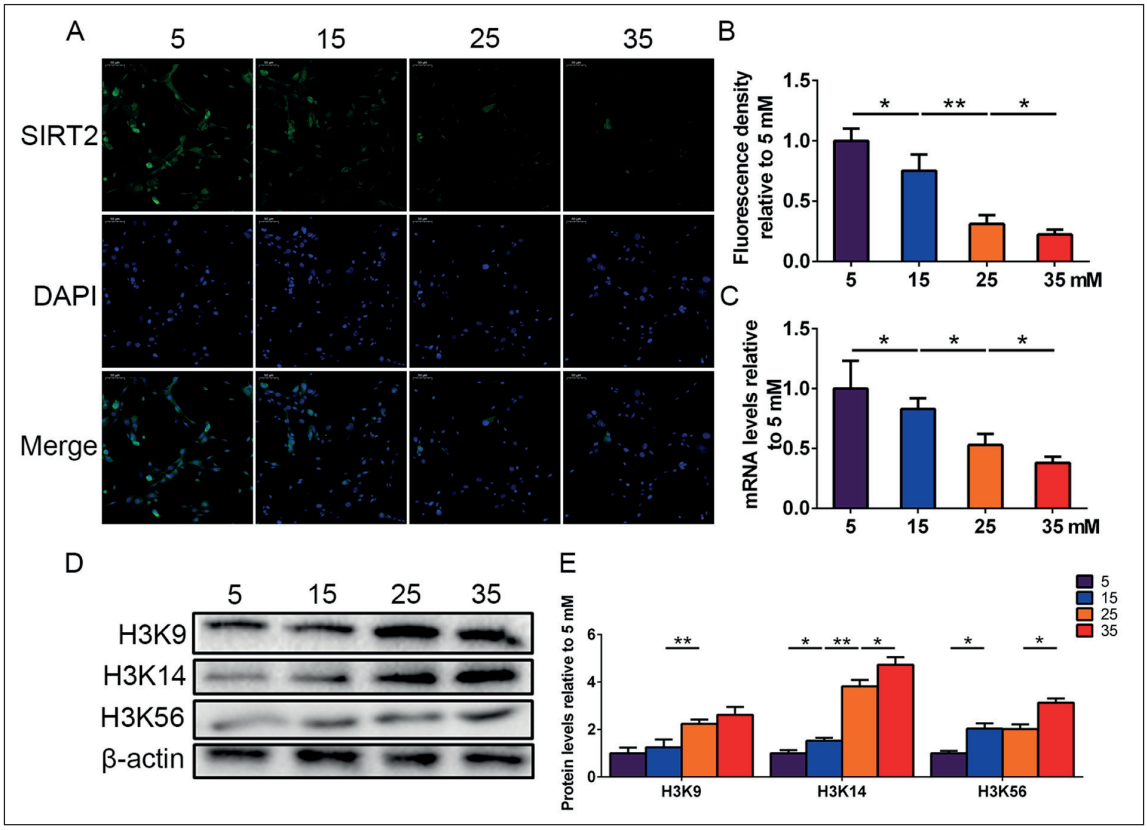

Figure 2. High glucose mediates SIRT2 and acetylated H3 expressions of CHs. CHs are isolated in OA condition and pretreated with glucose in ranged concentration (5, 15, 25, 35 mM). The protein expression level of SIRT2 is determined by immunofluorescence (**A**) and quantification analysis (magnification: 40×) (**B**). The mRNA expression level of SIRT2 is determined by RT-PCR (**C**). Protein expression levels of H3K9, H3K14, and H3K56 are determined by Western blotting (**D**) and quantification analysis (***E***). The values are mean ± SD of three independent experiments. *p<0.05, **p<0.01.

of glucose down-regulated SIRT2 expression in human CHs. RT-PCR was also used to determine the mRNA levels of SIRT2, which was paralleled with the protein level (Figure 2C). In addition to this, a gradual increase in glucose promoted the expression of acetylated H3K9, H3K14, and H3K56 to varying degrees. The results revealed that the decreased SIRT2 affected the histone deacetylation resulting in the accumulation of acetylated H3 (Figure 2D and 2E). These findings support the results in the cartilage samples that glucose regulates SIRT2 and the H3 acetylation in the metabolism of CHs.

High Glucose Actives ROS and Inflammation In CHs Vitro

During oxidative stress, SIRT2 regulates ROS levels, maintains cellular nicotinamide adenine dinucleotide phosphate (NADPH) balance, and coordinates energy production for cell survival[11,12]. SIRT2 regulates inflammatory response by regulating the specificity of NF-κB gene expression by deacetylating transcription factor p65[13]. To explore whether high glucose-mediated oxidative stress and inflammatory response in the CHs, the oxidative stress damage to marker 8-OH and MMP-13 was analyzed through IF. With the ranged concentration of glucose, both of the expressions of 8-OH and MMP-13 were raised along with the increased glucose (Figure 3A and 3B). Flow cytometry was used to determine total ROS level of the CHs, and it was found that higher glucose resulted in higher ROS production in the cells (Figure 3C). The mRNA expressions of several anti-oxidant enzymes and inflammatory factors were determined by RT-PCR as well. The results indicated that SOD1, SOD2, and CAT were markedly inhibited by high glucose. However, MMP13, ADAMTS-4, and ADAMTS-5 were promoted in higher glucose (Figure 3D).

SIRT2 Mediates Histone Acetylation In CHs Vitro

To explore the role of SIRT2 in the glucose-induced CH metabolism, SirReal2 (50 nM), the specific inhibitor of SIRT2, and rh-SIRT2 protein (100 μM) were used to suppress and upregulate SIRT2 levels in the CHs[14,15]. Compared with the 5 mM glucose, the SIRT2 protein level significantly decreased after the SirReal2 treatment. Besides, SIRT2 expression was increased by rh-SIRT2 stimuli in 25 mM glucose (Figure 4A and B). The result of mRNA was also consistent with the IF (Figure 4C). What's more, the acetylated H3K9, H3K14, and H3K56 proteins were upregulated by the inhibition of SIRT2 and promoted by the overexpression of SIRT2 (Figure 4D and 4E). These findings suggest high glucose or SirReal2 can down-regulates SIRT2 expression leading to the H3 acetylation, and overexpressed SIRT2 by exogenous rh-SIRT2 protein supplement can decrease H3 acetylation.

SIRT2 Suppresses ROS and Inflammation In CHs Vitro

To explore the mechanism underlying SIRT2 in the progress of diabetic OA, the oxidative stress and inflammatory response were measured in the glucose-treated CHs with different SIRT2 levels. The expressions of 8-OH and MMP-13 were increased within the suppression of SIRT2 by SirReals compared with 5 mM glucose-treated CHs, however, decreased by the rh-SIRT2 treatment compared with 25 mM glucose-treated CHs (Figure 5A and 5B). Total ROS level was promoted by the blocked SIRT2 and reduced at the condition of rh-SIRT2 treatment (Figure 5C). Finally, the mRNA levels of anti-oxidant related enzymes and inflammatory factors were analyzed by RT-PCR. The data that showed SOD1, SOD2, and CAT were significantly decreased with the suppression of SIRT2, but increased by the upregulation of SIRT2. However, MMP13, ADAMTS-4, and ADAMTS-5 were activated with the suppression of SIRT2 and inhibited by the upregulation of SIRT2 (Figure 5D).

Discussion

The etiology of OA is complex, and the traditional view is that it is mainly caused by mechanical factors and age-related cartilage degeneration. However, incresing evidence[16] shows that metabolic syndrome is closely related to OA. Epidemiological investigations have found that the incidence of metabolic syndrome in patients with OA increased significantly by 2.2-5.3 times. Diabetes has become an important chronic non-communicable disease (NCD) after cardiovascular and cerebrovascular diseases and cancer. As the incidence of diabetes continues to rise, the incidence of complications associated with diabetes also increases. In recent years, it has been observed that patients with type 2 diabetes mellitus complicated with OA (more common in knee arthritis) gradually increase, and the symptoms of these patients are generally more severe than those of patients

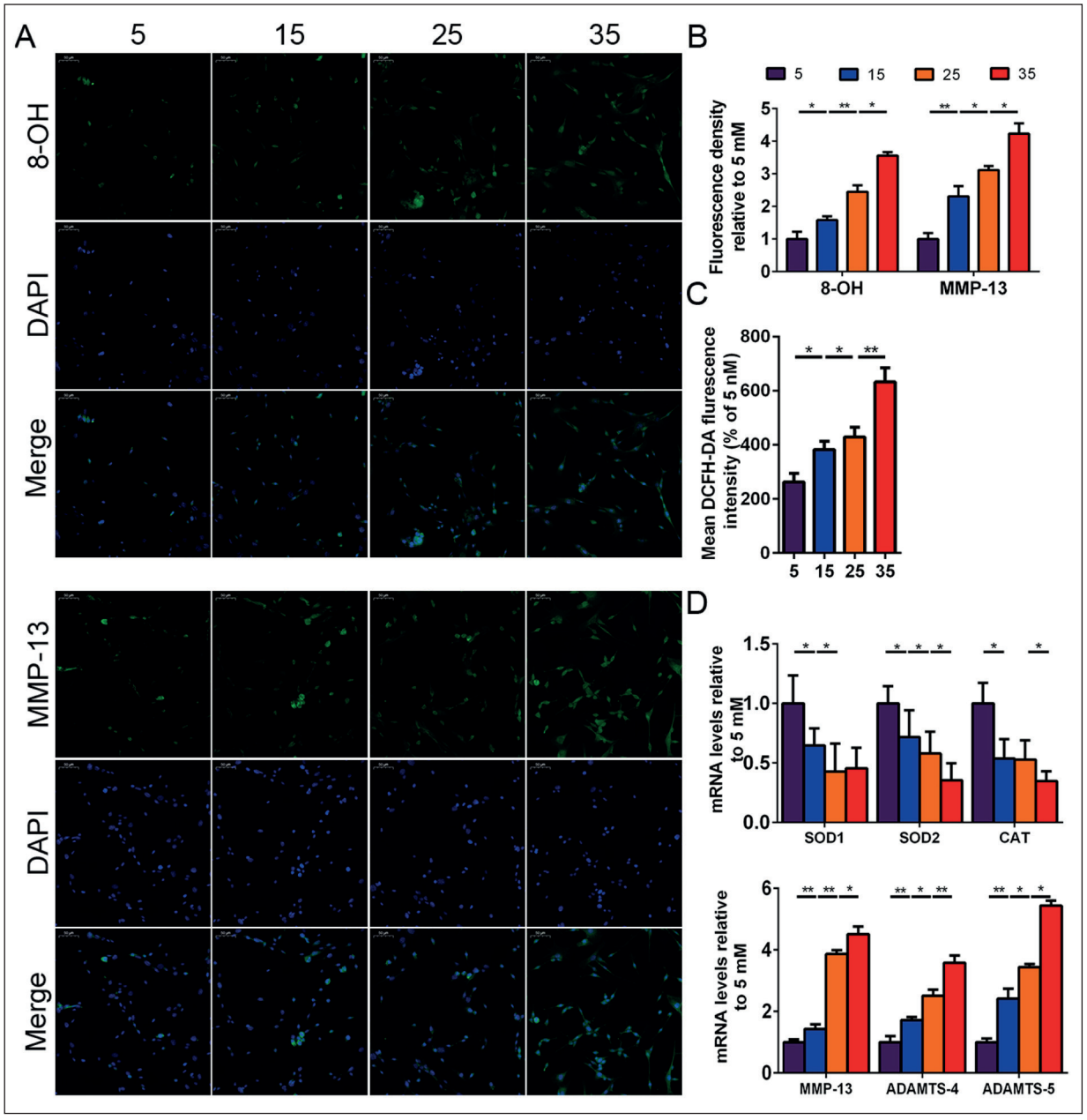

Figure 3. High glucose activates oxidative stress and inflammatory response in CHs. CHs are treated as mentioned above. The protein expression levels of 8-OH and MMP-13 are determined by immunofluorescence (**A**) and quantification analysis (magnification: 40×) (**B**). Total ROS level is determined by flow cytometry (**C**). The mRNA expression levels of SOD1, SOD2, CAT, MMP-13, ADAMTS-4, and ADAMTS-5 are assayed by RT-PCR (**D**). The values are mean ± SD of three independent experiments. *p<0.05, **p<0.01.

with osteoarthritis alone, and X-ray findings are more typical[17]. The risk of arthritis in diabetic patients is more than four times higher than that in non-diabetics, and its incidence increases with the prolonged duration of diabetes and is more common in patients with poor long-term glycemic control. Therefore, it has been suggested that it is also one of the chronic complications of diabetes. The pathological process of diabetes is often accompanied by oxidative damage and excessive inflammatory response[18-20]. ROS injury is closed to diabetic cardiomyopathy, and upregulation of SIRT1 is reported to be capable to attenuate oxidative stress[21]. Yu et al[8] found SIRT2

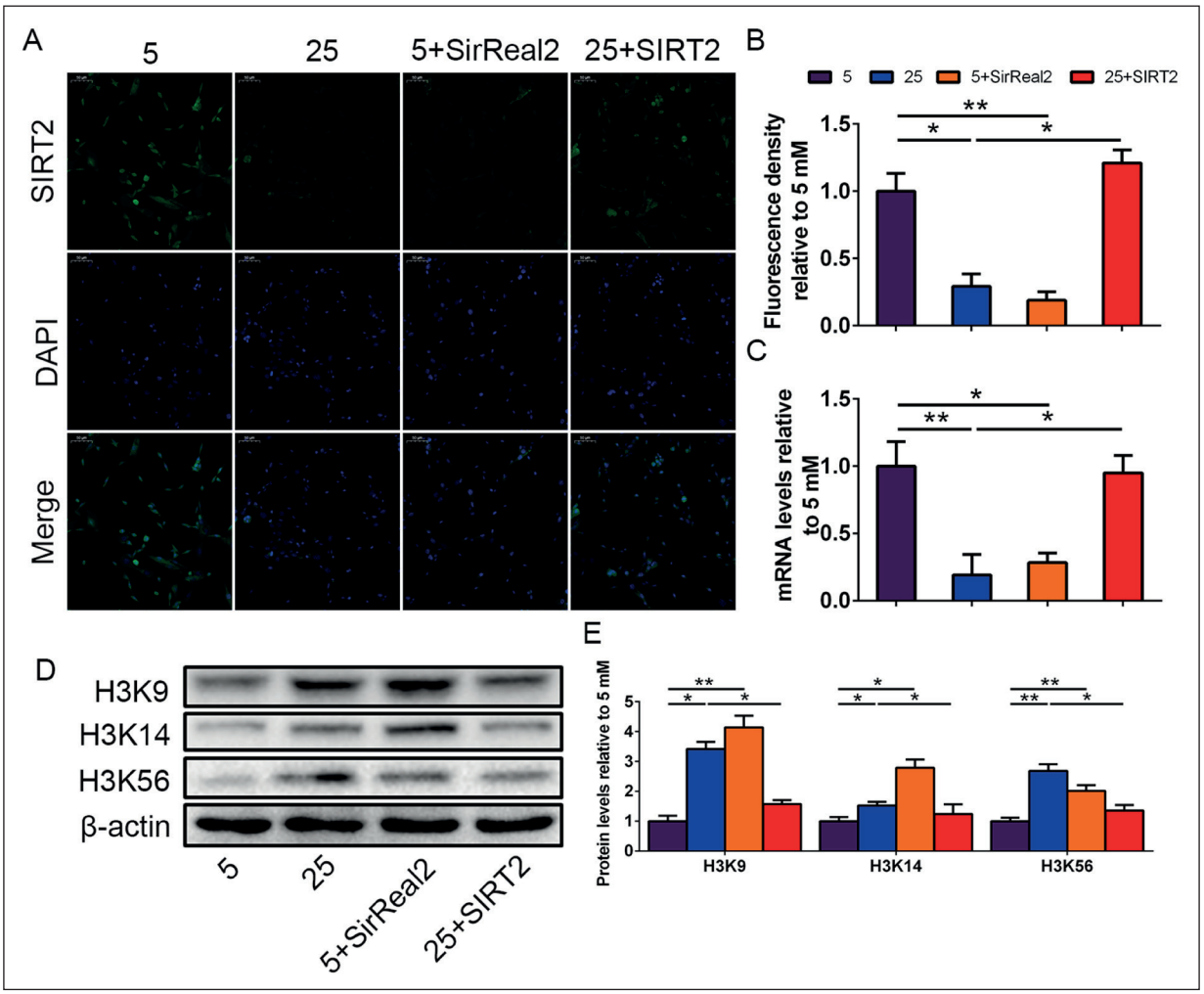

Figure 4. SIRT2 mediates high glucose-induced H3 acetylation. CHs are treated with 5 mM glucose combined with 50 nM SirReal2, and cultured with 35 mM glucose combined with 100 μM rh-SIRT2. The protein expression level of SIRT2 is determined by immunofluorescence (**A**) and quantification analysis (magnification: 40×) (**B**). The mRNA expression level of SIRT2 is determined by RT-PCR (**C**). The protein expression levels of H3K9, H3K14, and H3K56 are determined by Western blotting (**D**) and quantification analysis (**E**). The values are mean ± SD of three independent experiments. *p<0.05, **p<0.01.

and SIRT6 were suppressed in diabetes-induced neural tube defects and upregulated SIRT2 and SIRT6 contributed to histone deacetylation and ROS reduction. Schartner et al[22] revealed that high glucose inhibited SIRT2 in adult sensory neurons. However, whether diabetes affects the SIRT2 level in OA cartilage and how SIRT2 interactives with diabetic OA remains unknown.

In this study, OA cartilage accompanying diabetes was compared with the normal OA and found SIRT2 significantly decreased combining with the upregulation of acetylated H3K9, H3K14, and H3K56. SIRT2 is NAD+ dependent deacetylase and its primary substrate is H3[23,24]. Increased acetylation of histone has been elucidated to DNA breaks and ROS production in OA[25]. Therefore, it was hypothesized that SIRT2 and the downstream deacetylate substrates were involved in the process of diabetic OA. Diabetes is characterized as a metabolic disorder with high blood glucose levels over a long period of time[26]. In this study, the processed high concentration of glucose was used to treat human CHs, and it was found that SIRT2 expression significantly decreased along with the increased glucose. The inhibition of SIRT2, however, promoted the acetylation of H3, including H3K9, H3K14, and H3K56, so high glucose is respon-

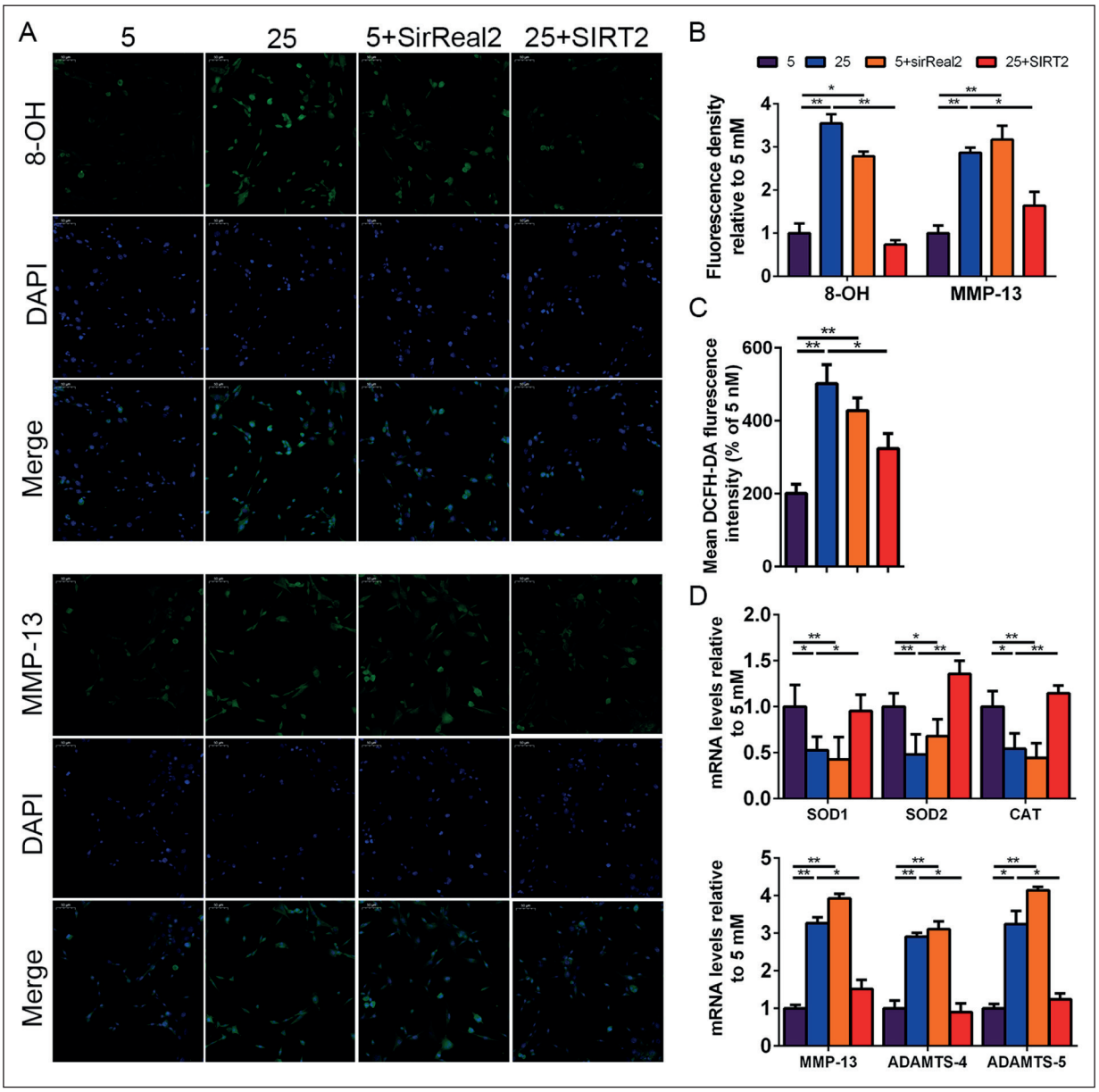

Figure 5. SIRT2 mediates high glucose-induced oxidative stress and inflammatory response. CHs are treated as mentioned above. The protein expression levels of 8-OH and MMP-13 are determined by immunofluorescence (magnification: 40×) (**A**) and quantification analysis (**B**). Total ROS level is determined by flow cytometry (**C**). The mRNA expression levels of SOD1, SOD2, CAT, MMP-13, ADAMTS-4, and ADAMTS-5 are assayed by RT-PCR (**D**). The values are mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$.

sible for the down-regulation of SIRT2 and up-regulation of acetylated H3. The cartilage matrix mainly contains three components: type II collagen, proteoglycans, and glycoproteins. In addition, cartilage contains a number of different proteases and hyaluronidases to decompose these components, such as MMPs, an ADAMTS. Tissue inhibitors of metalloproteinases (TIMPs) antagonize these enzymes. The biosynthesis and degradation of these macromolecules are in a delicate equilibrium and change with age and environmental stimuli. Subsequently, the oxidative stress and inflammatory progress were analyzed under the stimuli of high glucose, and it was discovered that the high glucose aggravated the ROS level, DNA damage, and inflammatory response and suppressed the antioxidant enzyme expression, which also explained the pathologi-

cal process of diabetic OA indirectly. In addition to the regulation of SIRT2 expression by glucose, SIRT2-specific inhibitors and rh-SIRT2 proteins were utilized to regulate SIRT2 levels of CHs. The results proved once again that SIRT2 level turned an opposite trend to the deacetylation of H3. Finally, results revealed the fact that blocking the SIRT2 expression could active the oxidative stress and inflammatory progress, meanwhile, exogenous SIRT2 supplementation inversely tended to inhibit the oxidative stress and inflammatory progress.

Conclusions

In summary, this study indicated that SIRT2 expression was down-regulated in diabetic OA and high glucose *in vitro* resulting in enhanced H3 acetylation. SIRT2 inhibition accelerated high glucose-induced H3 acetylation, oxidative stress activation, and excessive inflammatory response, whereas SIRT2 activation ameliorated high glucose-function. It is the first time that we elucidated SIRT2 involved in the antioxidant and anti-inflammatory function in the diabetic OA progress, which may be associated with the deacetylation of H3 substrates, providing a novel insight in treating diabetes-induced OA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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