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Skp2/p27 axis regulates chondrocyte proliferation under high glucose induced endoplasmic reticulum stress

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Abstract. – OBJECTIVE: Diabetes mellitus is closely related to osteoarthritis (OA) and may be an independent risk factor for the development of OA. As one of the main characteristics of diabetes, endoplasmic reticulum (ER) stress resulting from glucose metabolism disorder is one of the main causes of cartilage degeneration. The aim of our study is to illuminate the effect of high glucose to chondrocytes (CHs) and the role of Skp2 in high-glucose induced ER stress in CHs.

PATIENTS AND METHODS: We compared the ER stress status between healthy and diabetic OA cartilage using Western blot and quantitative reverse-transcription polymerase chain reaction (RT-PCR) methods. Different concentration of glucose was used to culture CHs for both 24 h and 72 h. Furthermore, Tunicamycin (TM) and 4-Phenylbutyric acid (4-PBA) were used to mediate ER stress of CHs, and human recombinant Skp2 protein was used to promote Skp2 expression. CH viability was determined by CCK8 assay, and cell proliferation was determined by flow cytometry. Western and RT-PCR were performed to measure related gene expression.

RESULTS: ER stress makers GADD34, GRP78, and MANF were upregulated in diabetic OA cartilage. The long-term high glucose increased GADD34, GRP78, and MANF expression, but decreased collagen II and proliferation of CHs, and Skp2 expression was negative related to the ER stress level. Additionally, Skp2 overexpression partly reversed ER stress-induced collagen II and proliferation suppression by the suppression of p27 expression.

CONCLUSIONS: High glucose raises the ER stress in CHs and overexpression of Skp2 promotes CH proliferation under high glucose treatment.

Key Words:
Skp2, Endoplasmic reticulum stress, Diabetes, Chondrocyte, Proliferation.

Introduction

The pathological manifestations of osteoarthritis (OA) contain cartilage degeneration, subchondral bone sclerosis, joint bone hyperplasia, and osteophyte formation, contracture of the joint capsule, and its surrounding ligaments, usually involving the weight-bearing joint joints such as the knee joint. Eventually, OA leads to joint deformity, disorders, and labor loss, which seriously affects the quality of life of patients[1,2]. However, the etiology of OA is complicated and still not very clear. With in-depth research, Schett et al[3] have found that diabetes is considered to be one of the important risk factors for OA, and inflammation and high glucose environment caused by diabetes contributes to the destruction of articular cartilage. However, some researches[4,5] report that both of them are normal multiple diseases in elderly patients, and there is no correlation between them. Whether the two disease states are only a simple metabolic disease accompanying phenomenon, or there is a close relationship between the pathogenesis, no clear conclusion has been made so far.

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from the disability to produce or use insulin. High levels of glucose increase the incidence of connective tissue and skeletal muscle lesions in diabetic patients[6,7]. Endoplasmic reticulum (ER) stress is a protective response of cells affected by harmful external stimulus, which is mediated by unfolded protein response (UPR). UPR alleviates endoplasmic reticulum stress by inhibiting protein synthesis, inducing endoplasmic reticulum chaperone protein expression to promote protein folding and accelerate the degradation of unfold-

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ed proteins or misfolded proteins. In addition, ER stress-coupled inflammatory response is closely related to the occurrence and development of various diseases, including diabetes and OA[8,9]. Glucose regulatory protein 78 (GRP 78), the ER stress marker of OA cartilage, and Bcl-2 interacting protein-1 are indicated to be much higher than healthy cartilage[10,11]. Yamabe et al[12] found that aggregation of endogenous advanced glycation end products (AGEs) induces chondrocyte apoptosis through ER stress.

ER stress is also detected to inhibit cell proliferation and viability[13-15]. S phase kinase-associated protein-2 (Skp2) is a member of the human F-box protein family required for DNA replication. Skp2 is involved in the adjusting of cell proliferation and transcriptional regulation, functions by promoting cyc-induced S-phase transition and activation of c-Myc target genes[16]. Han et al[17] elucidated that ER stress inhibits cell cycle progression via the Skp2/p27 pathway in melanoma cells. Chen et al[18] found ER stress delays cell proliferation through the regulation of the Cdh1-Skp2-p27 axis. Whereas, the function of Skp2 in the diabetic OA remains unknown. We suggest that ER stress plays a key role in OA caused by high glucose exposure. We cultured chondrocytes (CHs) with high glucose to establish an ER stress model. The damage of CHs by high glucose has been confirmed, and we aim to explore whether ER stress inhibits CH proliferation by inhibiting the expression of skp2. This project will contribute to understand the impact of high glucose status on the OA process and present an idea for early prevention and treatment of diabetic OA.

Patients and Methods

Cartilage Samples Collection

Patients with knee trauma who underwent surgery at Xijing Hospital from March to September 2018 were selected as the control group, all of which had no significant arthritis diagnosis. In the same period, the OA patients accompanying diabetes who underwent joint replacement surgery in our hospital were selected as the experimental group (diabetes group). There were 5 patients in each group, including 8 males and 2 females, aged 42-65 years old. The knee joint tissue obtained during the operation was washed with physiological saline, placed in the sterile culture solution, and stored in an icebox. This protocol was approved by the Ethics Committee of our Hospital, and the informed consent from the patient or relatives was obtained before the operation. This research was conducted in accordance with the Declaration of Helsinki.

Chondrocytes Isolation and Culture

The cartilage of the knee joint without bone tissue was scraped under aseptic conditions. After rinsing with phosphate-buffered saline (PBS), cartilage was cut into small particles by ophthalmic scissors, and digested with 0.25% trypsin at 37°C for 30 min; the digestion was terminated with Dulbecco's Modified Eagle's Medium (DMEM; Millipore, Billerica, MA, USA) containing 10% fetal bovine serum (FBS; Millipore, Billerica, MA, USA), followed by centrifugation and collection of the precipitate; the digested pellet was resuspended with 0.25% type II collagenase and incubated at 37°C for 4 h; following with filtration, the suspension was collected, centrifuged, and the CHs were collected; the pellets were resuspended in DMEM containing 10% FBS and the medium was replaced every other day. We used a cultural medium with different concentrations of glucose (from 10 mM to 40 mM) to treat CHs for 24 h or 72 h, and set 10 mM as control. CHs were pretreated with Tunicamycin (TM, 5 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) to induce ER stress[19] for 6 h, and pretreated with 4-Phenylbutyric acid (4-PBA, 1 mM; Sigma-Aldrich, St. Louis, MO, USA) to clear ER stress[20] for 12 h, or treated with human recombinant Skp2 protein (rh-Skp2, 50 nM, LS-G81526, LifeSpan BioSciences, Seattle, WA, USA) to overexpress Skp2.

Western Blot

The cartilage tissues or collected cells were lysed with the lysis buffer and the protein was quantified to ensure that the sample loading of each well was consistent. After electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% skim milk powder for 4 h, and the primary antibody was added to incubate overnight. After washing, the horseradish peroxidase (HRP)-labeled secondary antibody was added and incubated for another 2 h. Finally, bands on membrane were exposed using enhanced chemiluminescence

(ECL) assay (Thermo Fisher Scientific, Waltham, MA, USA). Primary antibody was purchased from Abcam (Cambridge, MA, USA) as followed: collagen II (ab34712), aggrecan (ab3778), MMP-13 (ab39012), TNF-α (ab1793), SOX-9 (ab185966), TIMP-3 (ab39184), GADD34 (ab236516), GRP78 (ab108615), MANF (ab126321).

Immunofluorescence

CHs grown in 6-well plates were incubated with 4% paraformaldehyde and TritonX-100. After blocking with BSA for 15 min, CHs were incubated with primary antibodies against collagen II (ab34712, Abcam, Cambridge, MA, USA) and PCNA (ab29, Abcam, Cambridge, MA, USA) overnight at 4°C and then with secondary antibodies (IgG Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). The intensity of fluorescence was measured using the ImageJ software (NIH, Bethesda, MD, USA).

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added directly to the splintery cartilage tissues or CHs, followed by repeat blow of the lysate. Following the manufacturer's protocol, mRNA was dissolved in enzyme-free water, quantified by a UV spectrophotometer (Shanghai Drawell Scientific Instrument Co., Ltd., Shanghai, China) to determine RNA quality, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). An optimal PCR reaction system was established, and the corresponding production was amplified. The primers used for RT-PCR were list in Table I (designed by Shanghai Biotech Biotech, Shanghai, China).

Cell Viability Assay

Cell viability was measured with the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Kumamoto, Japan). CHs were seeded at 1×10^4 cells/well in a 96-well plate and were then treated with specific drugs for 24 h and 72 h. After treatments, CHs were incubated with CCK-8 reagent according to the manufacturer's instructions. The intensity of the CCK-8 product was measured at 450 nm by enzyme-linked immunosorbent assay (ELISA).

Flow Cytometry

Cell proliferation was assessed using 5-Ethynyl-2'-deoxyuridine (EdU) Flow Cytometry Assay Kits (Invitrogen, Carlsbad, CA, USA). CHs were harvested and prepared in PBS, labeled with EdU according to the manufacturer's instructions, and then incubated for 30 min at 37°C. Finally, CHs were detected by a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ, USA).

Statistical Analysis

Software Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was used for data processing. Data were expressed at mean ± standard deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered to be significant.

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

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
Aggrecan	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
SOX-9	AGCGAACGCACATCAAGAC	CTGTAGGCGATCTGTTGGGG
TIMP	CTTCTGCAATTCCGACCTCGT	ACGCTGGTATAAGGTGGTCTG
GADD34	ATGATGGCATGTATGGTGAGC	AACCTTGCAGTGTCCTTATCAG
GRP78	CATCACGCCGTCCTATGTCG	CGTCAAAGACCGTGTTCTCG
MANF	TTTACCAGGACCTCAAAGACAGA	TTGCTTCCCGGCAGAACTTTA
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

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

Results

ER Stress is Upregulated in Human Diabetic OA Cartilage

To determine whether the level of ER stress is upregulated in OA patients with diabetes, we isolated total protein and mRNA of healthy cartilage from the patients undergoing joint replacement due to trauma without significant degeneration and OA cartilage from the patients undergoing joint replacement accompanying with diabetes. Western blot and RT-PCR were performed to analyze the degenerated and RE stress associated gene expression. As shown in Figure 1A and 1B, collagen II and aggrecan the main content of extracellular matrix (ECM) that secreted by CHs were

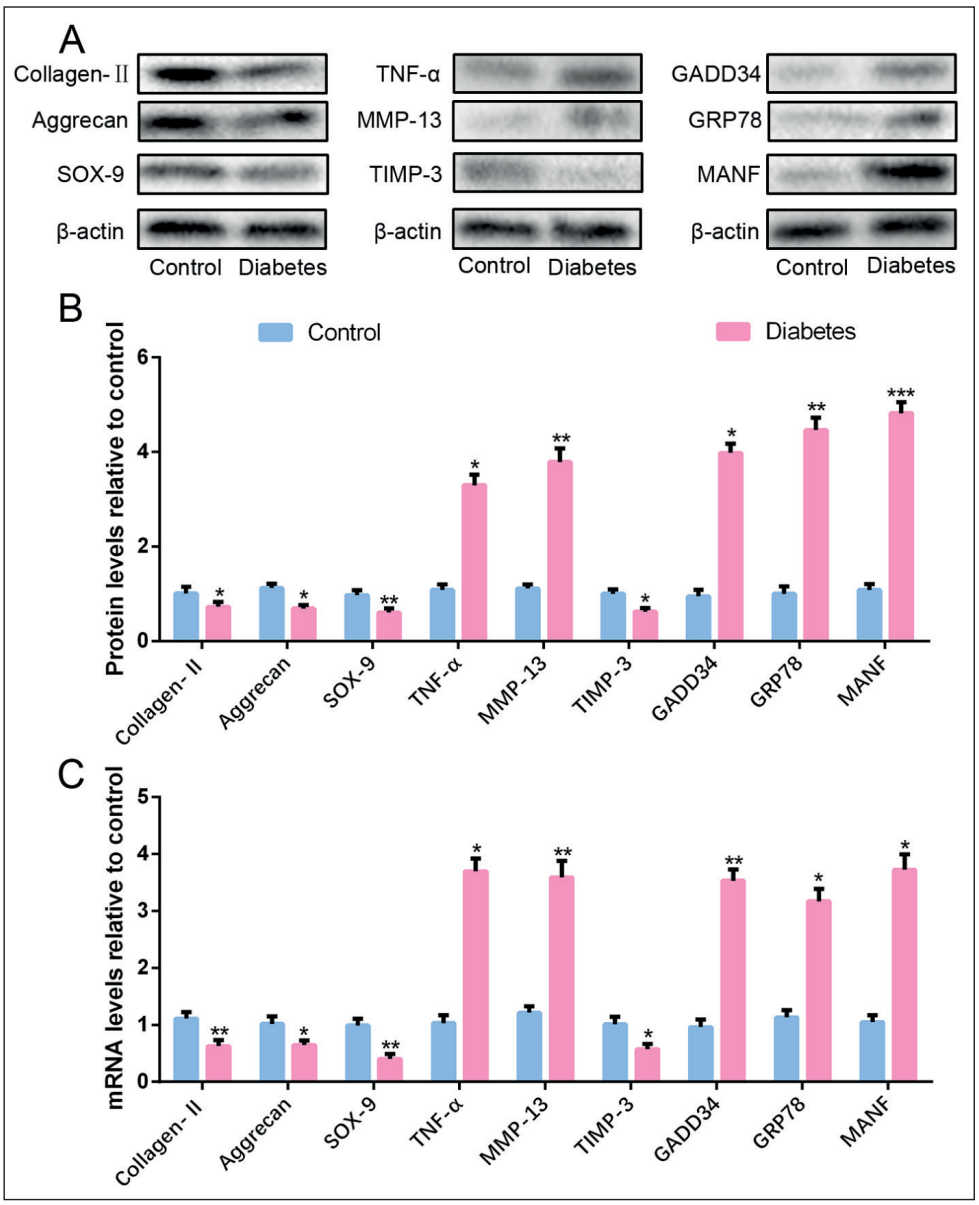

Figure 1. ER stress levels in human diabetic OA cartilage tissues. We collected knee joint cartilage tissues form joint replacement surgery due to trauma with no significant OA (as control) and OA accompanying with diabetes. **A**, **B**, Protein levels of collagen II, aggrecan, MMP-13, TNF-α, SOX-9, TIMP-3, GADD34, GRP78, and MANF were determined by Western blot (**A**) and quantification analysis (**B**). **C**, MRNA levels of collagen II, aggrecan, MMP-13, TNF-α, SOX-9, TIMP-3, GADD34, GRP78, and MANF were determined by RT-PCR. The values are mean ± SD of three independent experiments (n=3; *p<0.05, **p<0.01, ***p<0.001 compared to control).

decreased in diabetic OA cartilage as well as the chondrogenic gene SOX-9, the inflammation-related MMP-13 and TNF-α were increased, anti-catabolic gene TIMP-3 was reduced compared to the control one. In addition, the markers of ER stress containing GADD34, GRP78 and MANF were all significantly upregulated in diabetic OA cartilage. Hopefully, the data of mRNA levels were parallel to the proteins (Figure 1C). These results suggest that diabetes OA cartilage stays in a severe degenerated status with decreased ECM synthesis and increased inflammation and a higher level of ER stress. Though it is not clear whether the ER stress was activated by diabetes, we know they could be potentially related.

Long-Term High Glucose Activates ER Stress in Human CHs In Vitro

To explore whether diabetes contributes to the degeneration of CHs, we treated CHs with high glucose to imitate the microenvironment of diabetic cartilage. We used 10 mM glucose-DMEM as control and cultured CHs with glucose from 10 mM to 40 mM for 24 h, and 72 h. After 24 h treatment, there was no significant difference of cell viability and proliferation in these subgroups (Figure 2A, 2B), and we obtained a minor upregulated mRNA expression of collagen II and aggrecan in 30 mM and 40 mM group, besides, an increased level of GADD34, GRP78, and MANF mRNA in high glucose treatment (Figure 2C). However, the data from 72 treatments indicated high glucose inhibited CH viability and proliferation (Figure 2D, 2E) along with the collagen II and aggrecan mRNA expression especially in the concentration of 40 mM compared to the controls. We also got a significant upregulation of GADD34, GRP78, and MANF mRNA expression after 72 h treatments (Figure 2F). Although high glucose treatment caused ER stress, glucose as an essential source for CH metabolism and substrate for the synthesis of ECM, it promoted the ability of ECM synthesis in the short term. However, long-term exposure to a high glucose environment was suggested to stable increased stress in the ER, which ultimately reduced the viability of CH so as to affect the ability to ECM synthesis.

Suppression of ER Stress Promotes CH Proliferation and Attenuates High-Glucose Induced Skp2 Downregulation

To determine whether high glucose promotes CH degeneration by the activation of ER stress, we used 4-PBA to suppress ER stress in long-term high glucose (GH, 40 mM) treated CHs. As shown in Figure 3A and 3B, 4-PBA promoted the collagen II and proliferating cell nuclear antigen (PCNA)[21] expression in the high-glucose treated CHs, which proved suppression of ER stress played a positive role in the CH viability and proliferation. The flow cytometry also suggested 4-PBA promoted CH proliferation in the condition of high glucose treatment (Figure 3C). Cell proliferation is regulated by lots of signaling pathways especially the mediation of cell cycle[22,23], among which related to ER stress is Skp2/p27 axis[17,24,25]. We found high glucose significantly suppressed the Skp2 protein expression and promoted p27 expression, however, 4-PBA attenuated the glucose-induced Skp2 inhibition and p27 upregulation (Figure 3D, 3E). Absolutely, the function of 4-PBA in the protection of CHs was performed by the downregulation of GADD34, GRP78, and MANF (Figure 3F). These data indicated that ER stress caused by high glucose suppressed the Skp2 expression resulting in the upregulation of p27, and a reduction of ER stress promoted CH proliferation and mediation of the Skp2/p27 axis.

Skp2 Overexpression Promotes ER Stress-Induced CH Proliferation In Vitro

To determine whether ER stress affects CH proliferation by Skp2 suppression, we treated CHs with TM to cause an ER stress and upregulated Skp2 expression by rh-Skp2 protein stimuli. We used RT-PCR to measure the mRNA levels of GADD34, GRP78, and MANF, and the results showed TM caused a significantly ER stress in CHs, but rh-Skp2 protein made no effects to ER stress (Figure 4A). However, rh-Skp2 treatment increased the expression of Skp2 and inhibited p27 compared to the group treated by TM alone (Figure 4B, 4C). In addition, Skp2 overexpression promoted the proliferation of CHs (Figure 4D) and raised the content of collagen II and PCNA protein (Figure 4E, 4F) in the condition of TM. This result indicated again that ER stress suppressed the Skp2 expression and delayed the proliferation of CHs, but Skp2 overexpression reversed the negative effects of TM to CHs which suggested Skp2 played a vital role in the ER stress-induced CH degeneration. However, we found Skp2 upregulation could not reduce the ER stress level, which meant Skp2 was mediated by ER stress, not vice versa.

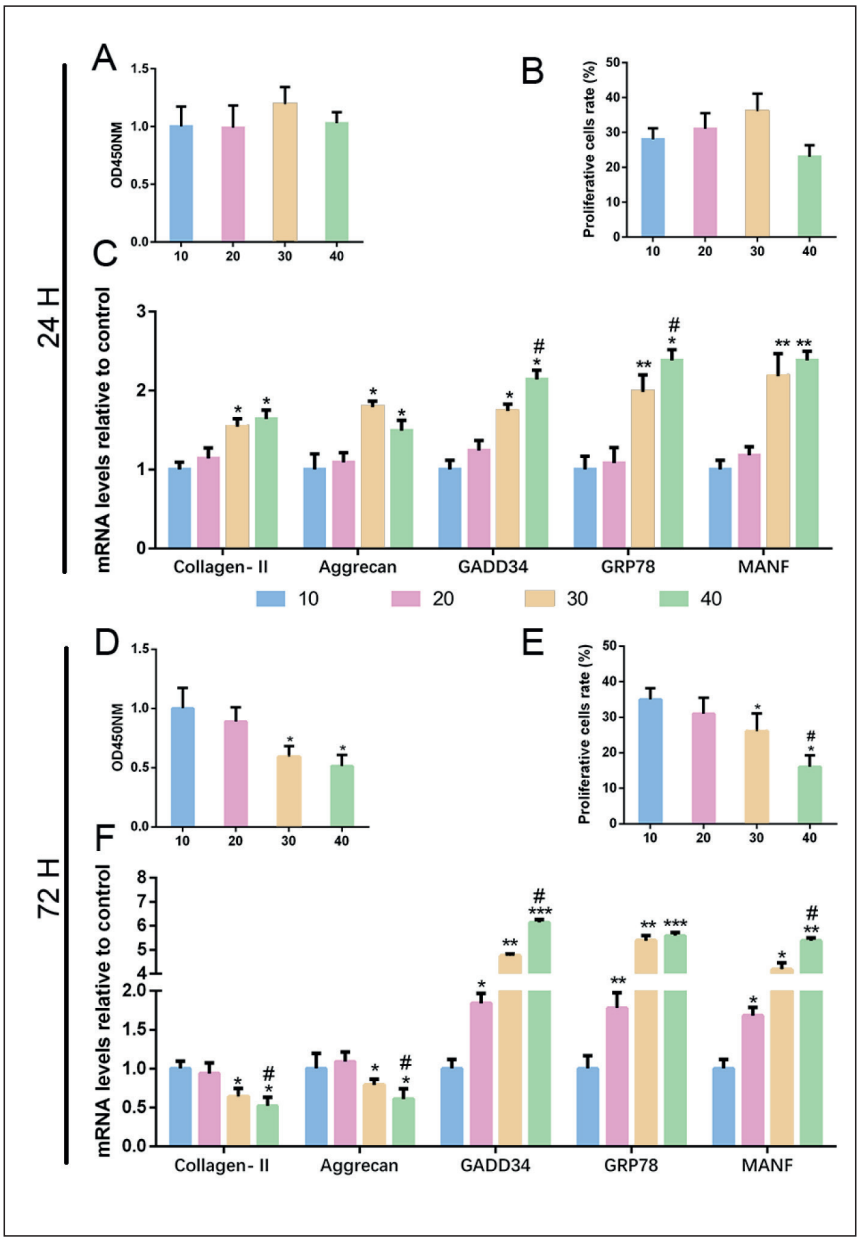

Figure 2. ER stress levels in high-glucose treated CHs in vitro. CHs isolated from cartilage tissues of traumatic joint replacement were cultured with different concentrations of glucose (10 to 40 mM) for 24 h (**A**-**C**) or 72 h (D-F). **A**, **D**, Cell viability was measured by CCK8 assay. **B**, **E**, Proliferative cell rate was determined by flow cytometry. **C**, **F**, MRNA expression levels of collagen II, aggrecan, GADD34, GRP78, and MANF were assayed by RT-PCR. The values are mean ± SD of three independent experiments (n=3; *p<0.05, **p<0.01, ***p<0.001 compared to 10 mM; $^{\#}p$<0.05 compared to 30 mM).

Discussion

ER is the largest organelle in the cell, and its function is mainly the folding of membrane proteins and secreted proteins, protein glycosylation modification, and protein secretion. Various physical and chemical factors, such as ultraviolet light, hypoxia, nutrient deficiency, virus, oxidative stress, can cause a stress reaction in the ER, leading to activated unfolded protein reaction and change in the function and survival state of cells[26]. Mild and transient stimulation of ER stress causes cells to maintain normal function and survival, while excessive ER stress ultimately leads to cell death. ER stress is associated with the development of many chronic diseases, which contains

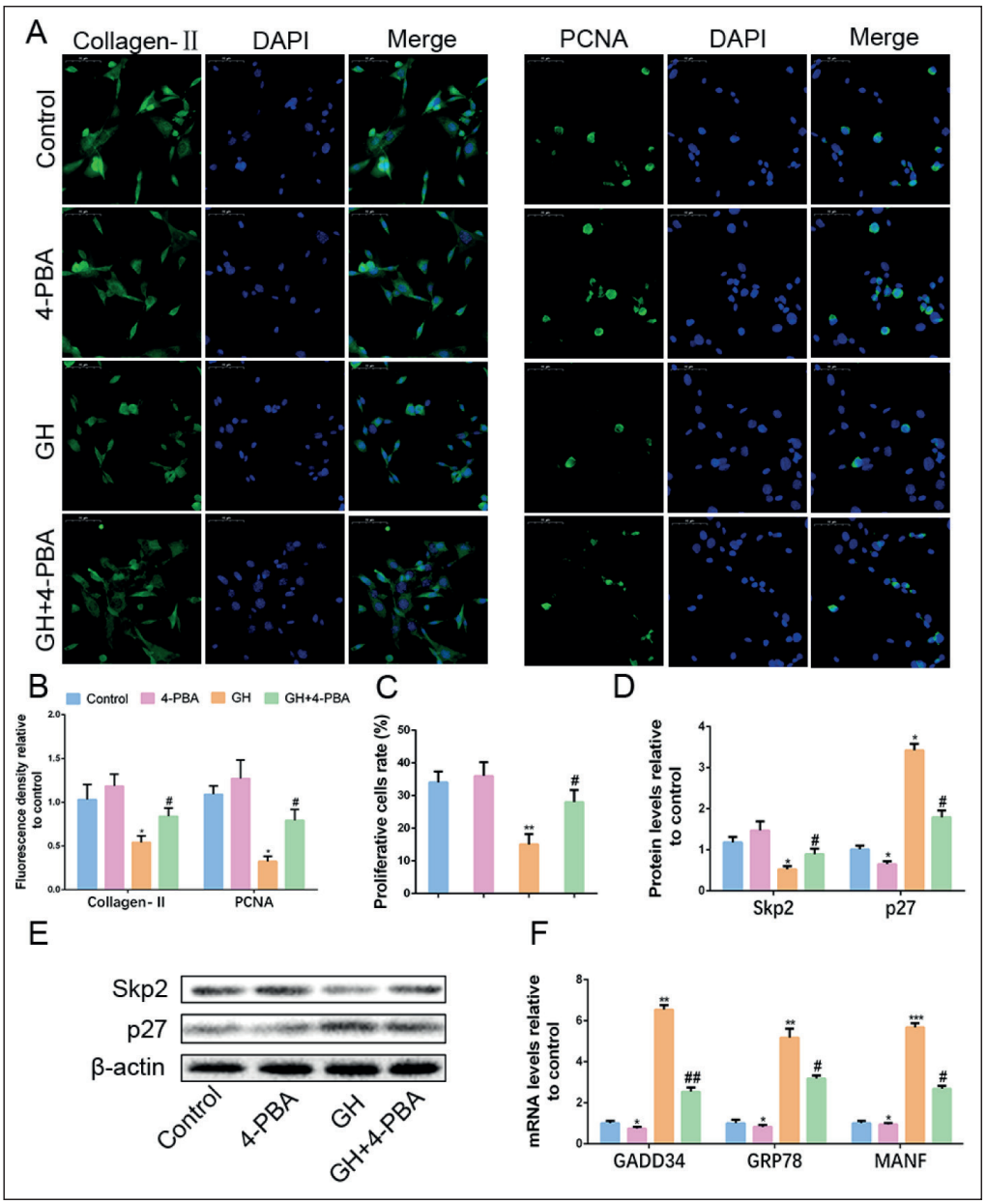

Figure 3. ER stress suppression promotes CHs proliferation and Skp2 expression. CHs were pretreated with 40 mM glucose (GH) for 72 h to induce ER stress. Then, CHs were cultured with or without 1mM 4-PBA for another 24 h. **A**, **B**, The protein expression level of collagen II and PCNA were determined by immunofluorescence (**A**) (magnification: 400×) and quantification analysis (**B**). **C**, The ratio of proliferative cells was analyzed by flow cytometry. **D**, **E**, The protein expression level of Skp2 and p27 were determined by Western blot and quantification analysis (**D**, **E**). **F**, The mRNA levels of GADD34, GRP78, and MANF were measured by RT-PCR. The values are mean ± SD of three independent experiments (n=3; *p<0.05, **p<0.01 compared to control; $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared to GH treatment).

the occurrence and development of OA[27]. There are three makers appearing with ER stress, which are Binding immunoglobulin protein (Grp78), mesencephalic astrocyte-derived neurotrophic factor (MANF), and growth arrest and DNA damage-inducible protein (GADD34). Mice with decreased expression of show inhibition of arthritis, suggesting that the ER molecular chaperone Grp78 plays an important role in the pathogenesis of arthritis[28]. MANF gene is widely distributed in various tissues and cells, mainly located in the cytoplasm and upregulated by the ER stress[29]. GADD34 participates in the ER stress-induced cell death and is increased following stressful

growth arrest conditions[30]. In our research, these ER stress markers were significantly increased in diabetic cartilage compared with the normal one, indicating a potential relationship between diabetes-induced ER stress and cartilage healthy.

Articular cartilage is a special type of tissue with no blood vessels, nerves and lymph nodes, whose main function is to disperse mechanical stress. CHs are mainly responsible for the synthesis of collagen II and proteoglycans in the ECM. Glucose is not only the main energy source of CHs but also the main component of synthetic proteoglycans, the precursor of glycosaminoglycans[31]. Therefore, glucose plays an

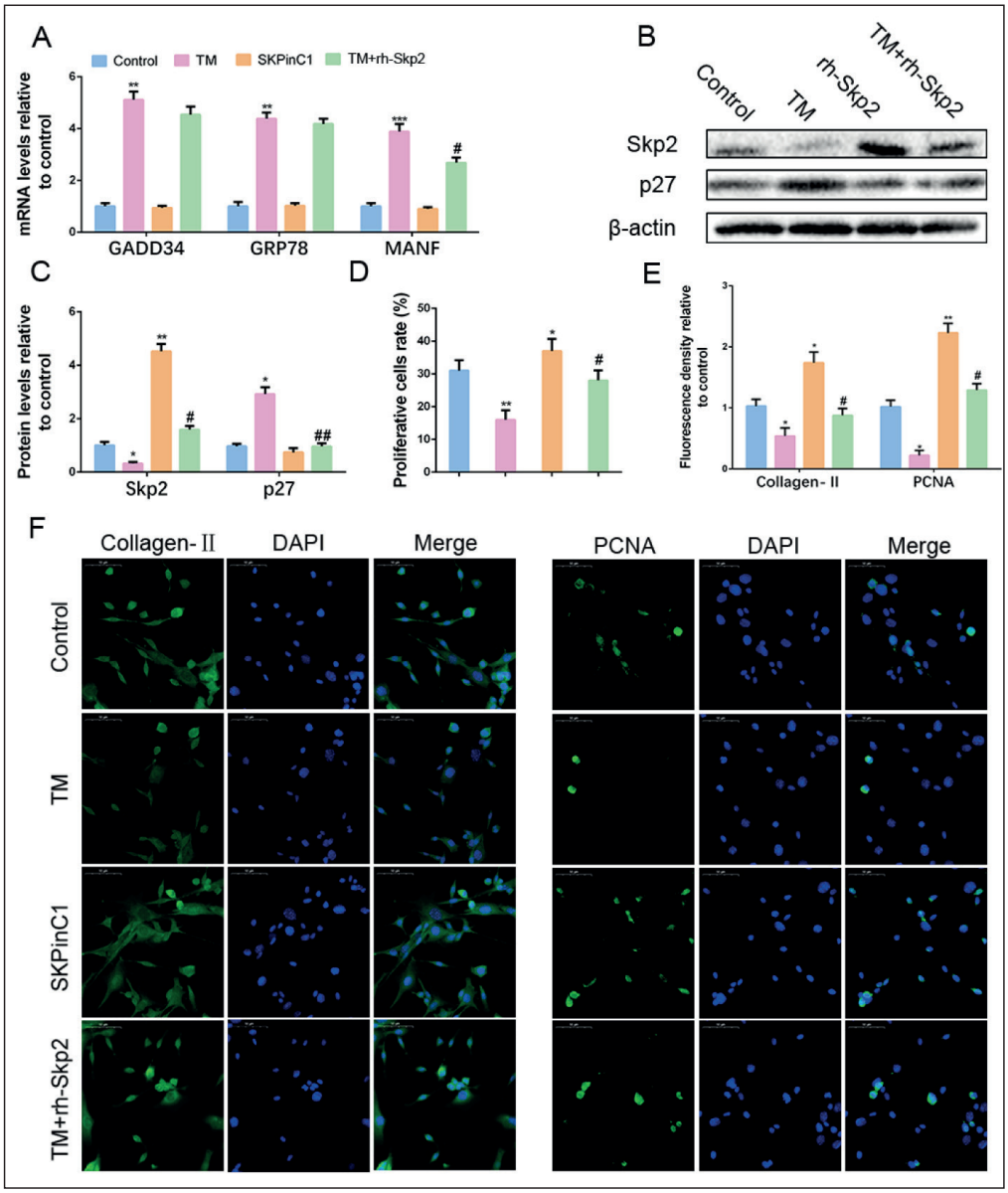

Figure 4. Skp2 overexpression promotes ER stress-induced CHs proliferation. CHs were pretreated with 5 μg/ml of TM for 6 h to induce ER stress. Then, CHs were cultured with or without 50 nM rh-Skp2 for another 24 h. **A**, The mRNA levels of GADD34, GRP78, and MANF were measured by RT-PCR. **B**, **C**, Protein expression level of Skp2 and p27 were determined by Western blot (**B**) and quantification analysis (**C**). **D**, Ratio of proliferative cells was analyzed by flow cytometry. **E**, **F**, Protein expression level of collagen II and PCNA were determined by immunofluorescence (magnification: 400×) and quantification analysis (**E**, **F**). The values are mean ± SD of three independent experiments (n=3; *p<0.05, **p<0.01, ***p<0.001 compared to control; $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared to TM treatment).

important regulatory role in the physiological function of CHs to synthesize ECM. From our experiment, short-term high-glucose treatment promoted the expression of the collagen II and aggrecan expression, promoted mild ER stress, but did not significantly affect the cell viability and proliferation level. However, in the long-term high-glycemic CHs, the secretion of the ECM was decreased, and the ER stress was significantly increased, resulting in the slow proliferative level. Therefore, short-term high-glucose can promote the synthesis of ECM and protect cartilage, but short-term high-glucose contributes to the degeneration of CHs.

To understand the role of ER stress in the high-glucose induced CH degeneration, we used 4-PBA to suppress ER stress. The data suggested a really positive effect of 4-PBA to CHs compared to be treated with high glucose alone, with a promotion in collagen, aggrecan and Skp2 production, reduction of p27, contribution to cell proliferation, and an obvious inhibition of ER stress. Skp2 is an important molecule that mediates the ubiquitination and degradation of p27 protein[32]. Many extracellular anti-proliferative signals induce p27 protein expression, preventing cells from entering the S phase from the G1 phase, thereby inhibiting cell proliferation. Skp2/p27 pathway is implicated as a critical mediator of ER stress-induced growth arrest[17]. Our study also proved Skp2/p27 took part in the ER stress-induced CH degeneration and growth arrest. Besides, we applied TM to induce ER stress in CHs, and observed decreased cell proliferation, ECM secretion, and skp2, but activated p27, which is similar to high glucose-induced pathological changes. In addition, rh-Skp2 was used to treat TM-induced CHs, although the EM stress status did not significantly improve, the level of proliferation, ECM synthesis, and Skp2 were increased, along with significant p27 inhibition. Skp2/p27 mag is a downstream target of the ER stress signaling pathway mediating the proliferation of cell metabolism.

In summary, ER stress induced by high glucose can affect the viability of CHs and inhibit cell proliferation through the Skp2/p27 axis. Either suppressing ER stress or activation of Skp2 contributes to the inhibition of p27 expression resulting in an advanced ECM production and proliferation of CHs. In short, ER stress is potentially related to the development of diabetic OA, and Skp2 may become a novel target for the therapeutic strategy of OA in the future.

Conclusions

High glucose raises the ER stress in CHs and overexpression of Skp2 promotes CH proliferation under high glucose treatment.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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