Original Article

Transcription factor EB mediates oxidative stress-induced intervertebral disc degeneration via the NF-κB signaling pathway

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Background: It is well known that the intervertebral disc is aggravated by a significant increase in the number of senescent cells, and oxidative stress (OS) is related to the deterioration of this tissue. Transcription factor EB (TFEB) can protect cells from OS. Accordingly, we investigated whether TFEB can prevent OS in human nucleus pulposus (NP) cells.

Methods: First, TFEB expression was investigated in human NP tissue samples with different degrees of degeneration. NP cells were treated with different concentrations of hydrogen peroxide (H_2O_2). The expression of collagen 2, aggrecan, and P65 was detected by quantitative real-time polymerase chain reaction (PCR) and Western blotting. We overexpressed and knocked out the *TFEB* gene to detect the expression of collagen 2, aggrecan, and P65.

Results: We found that the expression of TFEB decreased stepwise as the degree of intervertebral disc degeneration (IDD) increased. When the NP cells were treated with H_2O_2, the expression of TFEB, collagen 2, and aggrecan decreased gradually as H_2O_2 concentration increased. In addition, the expression of collagen2 and aggrecan increased following TFEB overexpression. However, nuclear factor-kappa B (NF-κB) decreased in NP cells after TFEB overexpression. We also found that the previously low cell viability increased and the high level of apoptosis decreased.

Conclusions: This study suggests that OS is associated with the development of IDD. TFEB mediates OS-induced IDD via the NF-κB signaling pathway. The *TFEB* gene can potentially be used as a diagnostic biomarker and therapeutic target.

Keywords: Oxidative stress (OS); transcription factor EB (TFEB); intervertebral disc degeneration (IDD)

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Introduction

Low back pain (LBP) affects approximately 84% of the population worldwide, and is reported to disable as much as 11–12% of the population (1). The cost and health care associated with LBP can become burdensome (2). The burden of LBP is regarded as a difficult public health problem. It is widely accepted that the cause of LBP is intervertebral disc degeneration (IDD). However, its underlying mechanism is not yet completely clear.

Oxidative stress (OS) is the imbalance between the

oxidation system and the antioxidant system in the body, which leads to excessive or reduced free radical production, and ultimately, to damage in the body (3,4). Oxygen can form free radicals in the process of normal metabolism. Generally, these free radicals are designated reactive oxygen species (ROS) (5). ROS produced in the process of OS directly cause the lipid peroxidation of biomembranes, destroy nucleic acids and chromosomes, denature some proteins or enzymes in cells, and ultimately lead to apoptosis (6). An increasing number of studies have shown that OS, as a pathological mechanism, can induce many diseases, such as degenerative disc disease, arteriosclerosis, and diabetes (7). Chen *et al.* found that ginsenoside Rg3 (Rg3) inhibits OS-induced disc degeneration by inhibiting the NF-κB signaling pathway (8).

Transcription factor EB (TFEB) is a member of the leucine zipper family (9). It regulates lysosomal production and autophagy by increasing coordinated lysosomal expression (10). Several studies have shown that TFEB-activated autophagy is a protective process under OS in neurodegeneration (11,12). Further, it has been proven that enhancing autophagy by TFEB promotes the formation of the extracellular matrix (ECM) (13). However, the role of TFEB in IDD remains very unclear.

Previous study focus on the inflammation, unbalance of mechanical force and nutrition deficiency. Only a small number of studies investigate the effect of oxidative stress in IDD and the mechanism of oxidative stress promotes IDD is still unclear. It is reported TFEB protects nucleus pulposus cells against apoptosis and promotes ECM synthesis. In this study, we investigate the role of TFEB in the oxidative stress-induced intervertebral disc degeneration and show TFEB mediates the NF-κB signaling pathway to protect nucleus pulposus cells from oxidative stress damage.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-3756).

Methods

Clinical study population/human specimens

Intervertebral disc tissues were obtained from patients undergoing posterior lumbar decompression and fusion surgery. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Patients or relatives were informed of the study, and patient or guardian consent was obtained preoperatively. This study was approved by the Ethics Committee of Shanghai General Hospital of Nanjing Medical University (ID: 2018KY038). Magnetic resonance imaging (MRI) was performed on patients before the surgery, and the Pfirrmann scale was used to score the degeneration of the intervertebral disc based on the imaging results.

Real-time PCR

Following the manufacturer's instructions, we extracted ribonucleic acid (RNA) from the nucleus pulposus (NP) cells with TRIzol (Invitrogen, USA). Total RNA was measured by an ultra-violet spectrophotometer, and complementary deoxyribonucleic acid (cDNA) was synthesized by real time (RT) Mix (Takara, Japan). Real-time polymerase chain reaction (PCR) was performed using a 2-step real-time system (Takara), and the relative amount of transcripts was calculated by the cycle threshold (CT) method.

Western blotting

After sodium dodecyl sulphate-polyacrylamide gel electrophoresis, the prepared protein samples were transferred to polyvinylidene fluoride membranes (Millipore, USA), and then sealed with a blocking solution containing 5% bull serum albumin (BSA) and kept at room temperature for 2 h. The membranes were incubated with a rabbit anti-human primary antibody (1:1,000) overnight in a refrigerator at 4 ℃. The next day, membranes were washed 3 times for 10 min each time and then incubated with horseradish peroxidase (HRP) labeled anti-rabbit immunoglobulin G (IgG) secondary antibody at room temperature for 2 h. After being washed 3 times, the membranes were visualized by western blotting substrate (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase was used as the control protein.

Annexin V-APC/7-AAD double-stain

Apoptosis was detected by an annexin V-APC/7-AAD double-stain procedure (BD Biosciences, USA). NP cells were digested by 0.25% trypsin and then centrifuged at 1,000 r/min for 5 min. The supernatant was discarded, and the precipitate was washed with phosphate saline buffer twice. The cells were resuspended in a suitable amount of annexin V binding buffer. After annexin V-APC and 7-AAD

were added, the cells were incubated for 15 min at room temperature away from light. The cells were analyzed by flow cytometry.

Cell Counting Kit-8 (CCK-8)

A CCK-8 assay was used to detect NP cell viability. The NP cells were cultured in 96-well plates, and 10 μL CCK-8 reagent (Dojindo, Japan) was added to each well. The cells were then incubated for 2 h at 37 ℃. The absorbance at 450 nm was measured by a microplate reader.

Immunohistochemical staining

Decalcified intervertebral disc sections were boiled in 10 mM of sodium citrate (pH 6.0) for 5 min to retrieve the antigens. Sections were quenched with 3% (volume per volume) hydrogen peroxide for 15 min to reduce endogenous peroxidase activity and blocked with 3% (weight per volume) normal goat serum in tris-buffered saline. Next, the sections were incubated with a rabbit anti-human primary antibody or a goat IgG as a control at 4 ℃ overnight and biotinylated secondary antibodies sequentially, and a peroxidase-labeled streptavidin-biotin staining technique (Invitrogen, USA) was then used. The nuclei were counterstained with hemalum (FARCO Chemical Supplies, Hong Kong). The slides were visualized with a microscope (Zeiss, Germany).

Overexpression and knockouts

Second-generation NP cells were selected for the overexpression experiments. The NP cells were inoculated into 6-well plates, and lentivirus transfection was performed when the fusion rate was 50%. The multiplicity of infection (MOI) was 50, and the NP cells were cultivated for 48 h after lentivirus transfection.

Second-generation NP cells were selected for the knockout experiments. Opti-Minimal Essential Medium (MEM) containing small inhibitory RNA (siRNA) was slowly dropped into tubes containing Lipofectamine® RNAIMAX Reagent (Invitrogen, USA) and incubated at room temperature for 5 min. A 250 μL mixture was added to each well of a 6-well plate and cultured at 37 ℃ for 48 h.

IDD model

This study complied with the ARRIVE guidelines and was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act. Animal experiments were performed under a project license (No.: 2017KY043) granted by institutional board of Shanghai General Hospital of Nanjing Medical University, in compliance with Shanghai General Hospital of Nanjing Medical University institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration. The New Zealand rabbits were all male and were aged 8 years old. The rabbits were randomly divided into a control group, IDD group, IDD + TFEB knockout group, and IDD + TFEB overexpression group. The animals were anesthetized with 2% pentobarbital sodium by a marginal ear intravenous injection. Skin preparation was performed on the right side of the abdomen, on the portion near the 12th rib to the ilium. The boundary of the abdominal oblique muscle and paravertebral muscle was exposed. Blunt dissection was performed along the boundary between the 2 muscles and ultimately to the front of the vertebral body. A 16G needle was used to puncture the annulus fibrosus into the intervertebral disc. After the needle was withdrawn, a jellylike outflow of NP could be seen.

Statistical analysis

SPSS 13.0 software was used for the statistical analysis. The grades of Pfirrmann degeneration of the intervertebral discs were compared using a Pearson chi-square test, and the quantitative data are expressed as the mean ± standard deviation (SD). In addition, a one-way analysis of variance (ANOVA) was used for the intergroup data. For the above two analytical methods, a $P<0.05$ indicated a statistically significant difference.

Results

Expression of TFEB in human NP tissues with different Pfirrmann grades and in NP cells treated with H_2O_2

Based on their Pfirrmann grade, the human NP tissues were divided into two groups: the control group and the IDD group. The immunohistochemistry (IHC) results showed that TFEB was highly expressed in the IDD group (see *Figure 1A*). RT-PCR showed that TFEB was highly expressed in the IDD group and was lowly expressed in the control group (see *Figure 1B*). The relative mRNA expression of TFEB was found to be negatively correlated with the Pfirrmann grades of NP tissues (n=10, r=–0.9144)

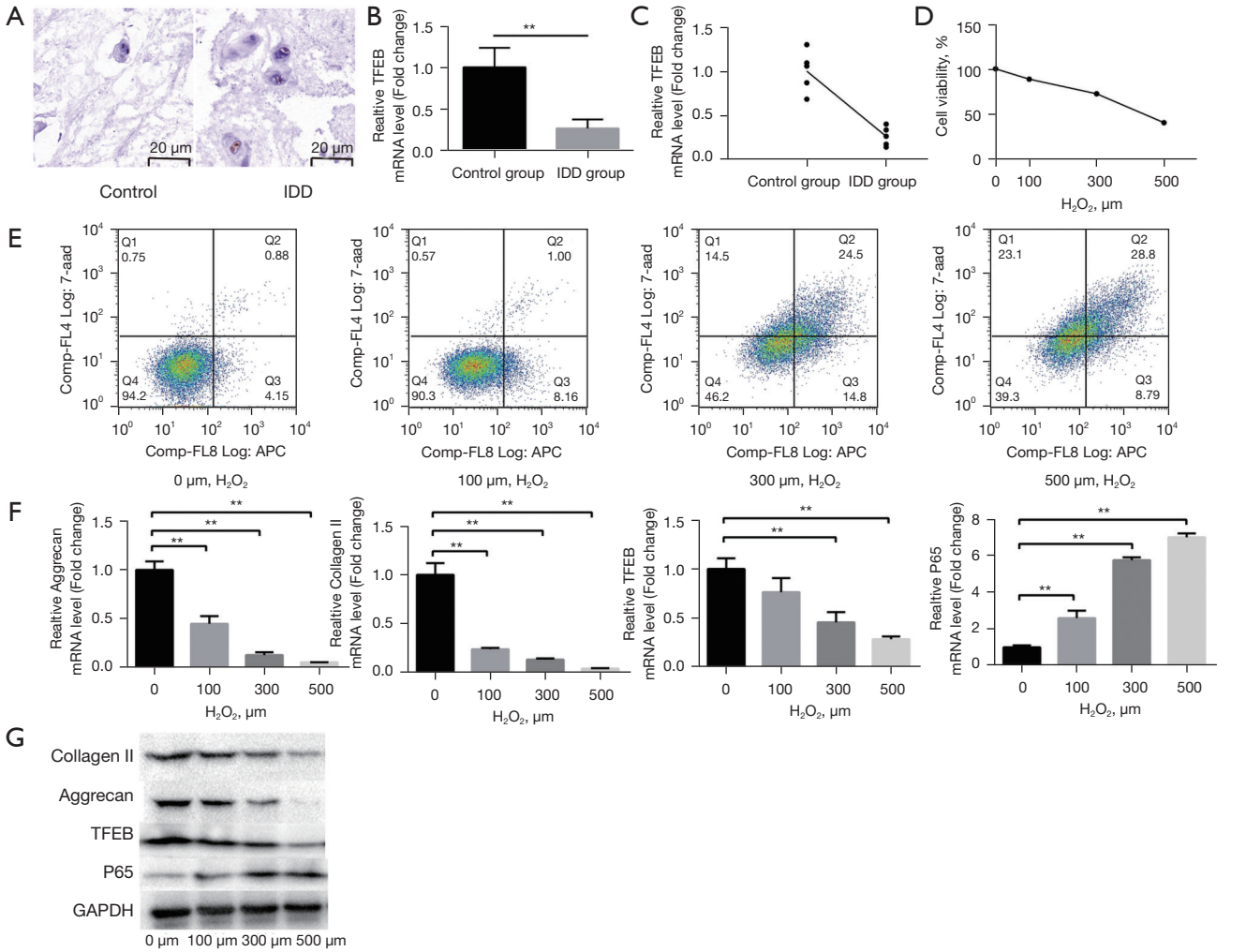

Figure 1 Expression of TFEB in human NP tissues with different Pfirrmann grades; OS inhibited cell viability, apoptosis, and ECM synthesis, and promoted apoptosis in NP cells. (A) IHC results showed the expression of TFEB in human NP tissues. (B) RT-PCR analysis results showed that the mRNA expression level of TFEB decreased with the severity of IDD. (C) The mRNA expression of TFEB was negatively correlated with the Pfirrmann grades of 10 human NP tissues (n=10, r=–0.9144). (D) CCK-8 analysis results showed that the viability of the NP cells decreased gradually as H_2O_2 concentrations increased. (E) Annexin V-APC/7-AAD double-stain results showed the apoptosis of the NP cells. (F) Real-time RT-PCR analysis results showed the mRNA expression levels of aggrecan, collagen 2, and TFEB due to OS induced by H_2O_2. (G) Western blotting showed protein levels of aggrecan, collagen 2, and TFEB due to OS induced by H_2O_2. All data were obtained from 3 independent experiments and are shown as means ± SD. Significant differences between groups are indicated as **P<0.01. TFEB, transcription factor EB; NP, nucleus pulposus; ECM, extracellular matrix; IHC, immunohistochemistry; RT-PCR, real-time polymerase chain reaction; IDD, intervertebral disc degeneration; OS, oxidative stress.

(see *Figure 1C*).

High concentrations of H_2O_2 inhibit viability and promote the apoptosis of NP cells

OS inhibits cell viability, induces apoptosis, and finally promotes IDD (14). To evaluate the effects of OS induced by H_2O_2 in human NP cells, we examined the viability and apoptosis of NP cells that had been treated with several H_2O_2 concentrations using CCK-8 assays and a flow cytometry analysis. The CCK-8 assays showed that the viability of NP cells decreased gradually as concentrations

of H_2O_2 increased (see *Figure 1D*). The flow cytometry analysis demonstrated that the apoptosis of NP cells correspondingly increased (see *Figure 1E*).

High concentrations of H_2O_2 inhibit ECM synthesis

Maintaining ECM homeostasis is essential for keeping NP cells healthy, and an imbalance between anabolism and catabolism of the ECM promotes IDD (15). We cultured NP cells in H_2O_2 at 3 concentrations. The RT-PCR showed that collagen 2 and aggrecan was more decreased in the high H_2O_2 concentration group than the control group and the low H_2O_2 concentration group. However, NF-κB (P65) was highly expressed in the high H_2O_2 concentration group (see *Figure 1F*). The Western blot also revealed the same results (see *Figure 1G*).

Effects of TFEB on cell viability, apoptosis, and ECM synthesis in NP cells

TFEB is a master regulator of cell viability and apoptosis (16,17). In this study, the effects of TFEB on the viability and apoptosis of NP cells under OS was assessed and measured by CCK-8 and flow cytometry. SiRNA TFEB-816 and lentivirus had excellent transfection efficiency (see *Figure 2A-2C*). OS resulted in a decrease in cell viability and an increase in apoptosis (see *Figure 2D,2E*). The knockout of TFEB made the process correspondingly worse, while the overexpression of TFEB produced the opposite results (see *Figure 2D,2E*) by promoting cell viability and inhibiting the apoptosis of NP cells.

TFEB also plays a role in the synthesis of ECM (18). To determine whether TFEB had the same effects on NP cells, we performed PCR and Western blotting. The results showed that OS inhibited collagen 2 and aggrecan expression, and TFEB knockout resulted in a stronger inhibition. Conversely, TFEB overexpression enhanced the synthesis of collagen 2 and aggrecan (see *Figure 2F,2G*). These results suggest that TFEB alleviated H_2O_2-mediated OS in NP cells.

Effects of the TFEB/NF-κB pathway on H_2O_2-mediated OS in NP cells

The NF-κB pathway is known to modulate the OS response (19). Thus, immunofluorescent staining was performed to determine whether the NF-κB pathway was associated with H_2O_2-mediated OS in NP cells. When the NF-κB pathway is activated, P65 translocates into the nucleus (20). Our experiment showed that OS led to P65 nuclear translocation, and TFEB knockout enhanced P65 translocation (see *Figure 3*). Conversely, TFEB overexpression inhibited this process. These results revealed that TFEB inhibited H_2O_2-mediated OS in NP cells by NF-κB pathway activation.

TFEB alleviates IDD in a rabbit degeneration model in vivo

The annular puncture model is a classical method for studying IDD (21). In this study, a retroperitoneal approach was adopted to build the model (see *Figure 4A*). MRI, hematoxylin and eosin (HE) staining, and safranin O-fast green (SO-FG) staining were performed to assess the degeneration of NP cells. In both the puncture model group that received TFEB knockout treatment and the group that did not receive TFEB treatment, the MRI images showed hypointense signals in the intervertebral discs and a decrease in the height of the intervertebral space (see *Figure 4B*). The results of the HE staining and SOFG staining showed that the expression of ECM was reduced and that the annulus fibrosus became disorganized (see *Figure 4B*). Conversely, in the puncture model that received TFEB overexpression treatment, the MRI images showed a hyperintense signal, and the height of the intervertebral space remained unchanged (see *Figure 4B*). The results of the HE staining and SOFG staining also showed that expression of ECM was adequate, and the annulus fibrosus was maintained (see *Figure 4B*).

Discussion

IDD can place burdens on people's lives or even lead to disability. Thus, solutions to cure IDD need to be found urgently. However, the etiology and molecular mechanism of IDD remain unclear. Recent studies have focused on OS. Notably, Patil *et al.* found that OS-induced senescence significantly aggravates IDD (4). Zhuang *et al.* reported that TFEB alleviates cell death under OS (11). Interestingly, we found that TFEB functioned to effectively inhibit the process of IDD. In this study, we detected the expression of TFEB in patients with different degrees of IDD and found that the IDD group had a higher expression of TFEB than the control group. These results implied that TFEB level may be negatively correlated with the degree of IDD and that an increase in TFEB may alleviate the degree of IDD.

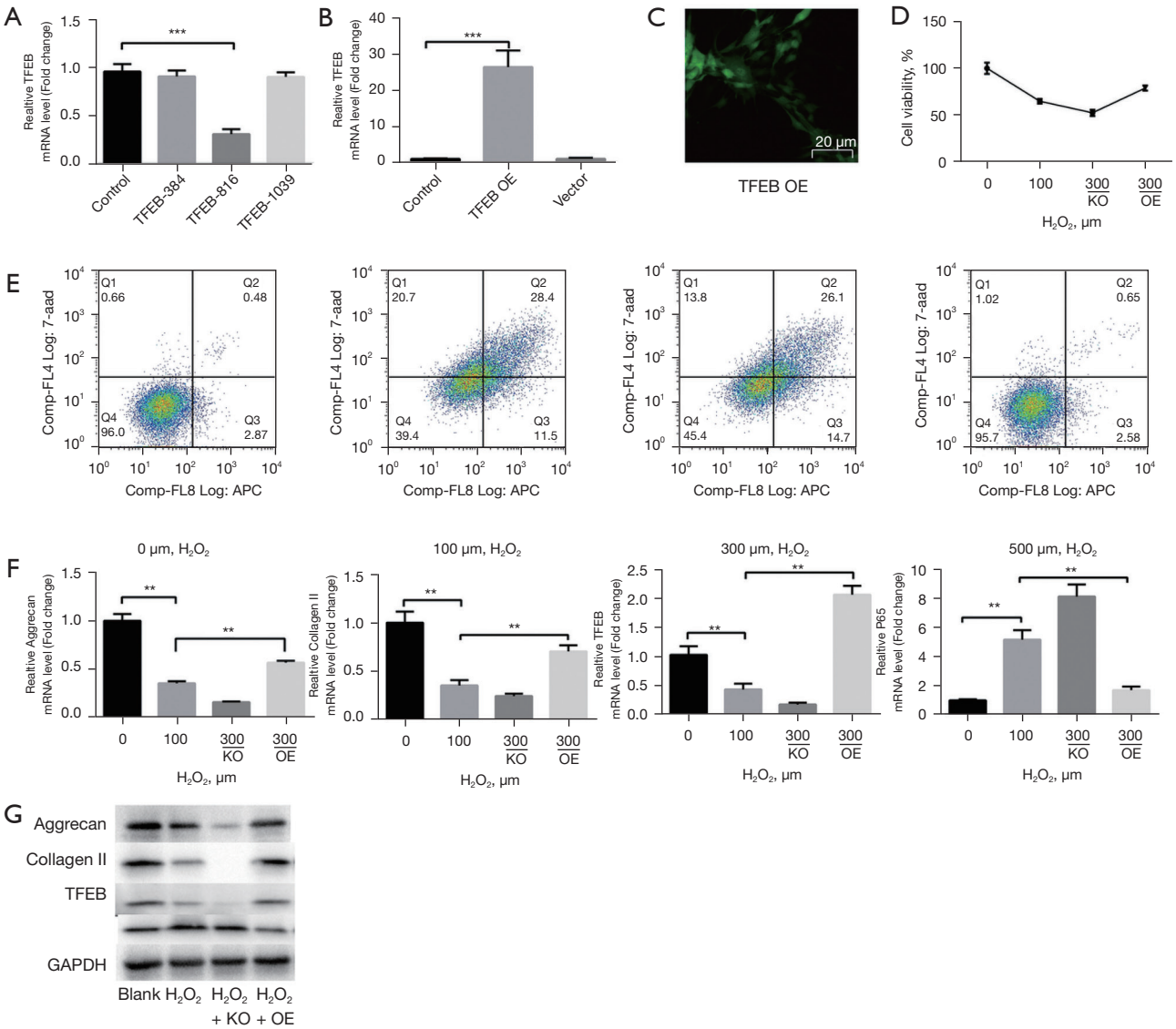

Figure 2 Effects of TFEB on cell viability, apoptosis, and ECM synthesis in NP cells. (A) RT-PCR results validated the TFEB siRNA transfection efficiency. (B) RT-PCR was used to assess lentiviral transfection efficiency in NP cells. (C) Fluorescence of the transfected NP cells 72 h after transfection. (D) CCK-8 was used to assess the viability of the NP cells. (E) Annexin V-APC/7-AAD double-stain results showed the apoptosis of the NP cells. (F) RT-PCR results showed the mRNA expression levels of aggrecan, collagen 2, and TFEB in the TFEB knockout and TFEB overexpression cells. (G) Western blotting showed the protein levels of aggrecan, collagen 2, and TFEB in the TFEB knockout and TFEB overexpression cells. All data were obtained from 3 independent experiments and are shown as the means ± SD. Significant differences between the groups are indicated as **P<0.01 and ***P<0.001. OE, TFEB overexpression; KO, TFEB knockout; TFEB, transcription factor EB; ECM, extracellular matrix; NP, nucleus pulposus; RT-PCR, real-time polymerase chain reaction; CCK-8, Cell Counting Kit-8.

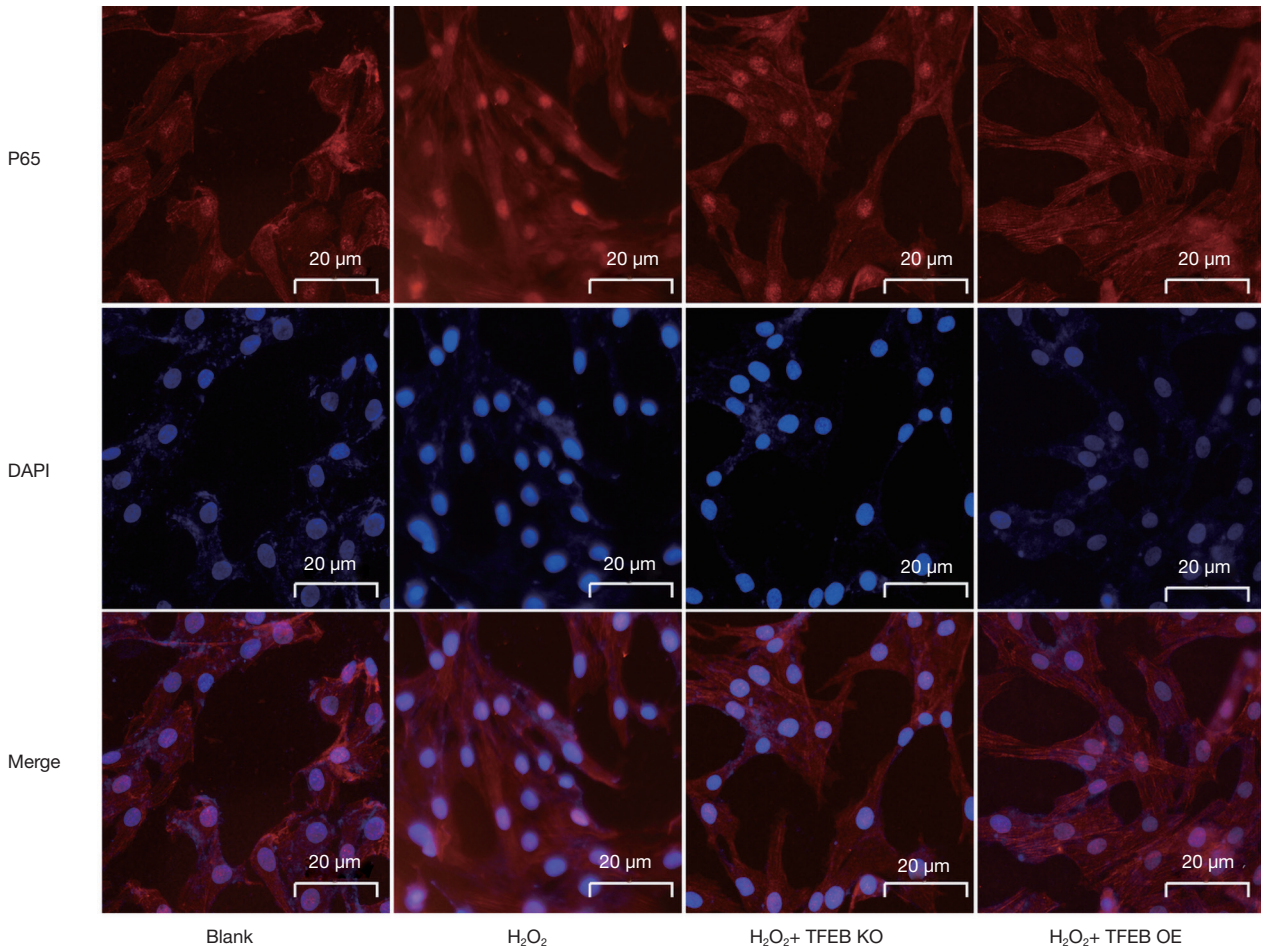

Figure 3 The NF-κB signaling pathway was active under OS. Immunofluorescence showed that P65 was expressed in the cytoplasm in the blank group and the TFEB overexpression group, while P65 was expressed in the nucleus under H_2O_2-induced OS and in the TFEB group. All data were obtained from 3 independent experiments. OE, TFEB overexpression; KO, TFEB knockout; OS, oxidative stress; TFEB, transcription factor EB.

Some studies have found that H_2O_2-induced OS leads to cellular senescence and inhibits proliferation (6,22). In the present study, this effect was also proven in NP cells under H_2O_2-induced OS. Notably, we also discovered that the expression of collagen 2, aggrecan, and TFEB decreased gradually as H_2O_2 concentration increased. ECM exhaustion is one of the mechanisms of disc degeneration. The low expression of aggrecan and collagen 2 results in ECM exhaustion, which in turn aggravates IDD. Thus, it can be concluded that OS suppresses the proliferation and ECM expression of NP cells and ultimately promotes IDD.

A recent study revealed that TFEB alleviates neuronal death under OS (11). In our study, we also found that TFEB confers OS resistance. TFEB overexpression reversed the trend of ECM exhaustion and cell apoptosis. Research has shown that the NF-κB pathway is involved in OS induced by cell injury (23). In this study, we also found the expression of P65 in NP cells following H_2O_2 treatment was high. Interestingly, TFEB overexpression inhibited P65 expression and suppressed P65 nuclear translocation. These results revealed that the TFEB/NF-κB pathway modulated IDD caused by OS. Thus, TFEB plays an important role in the occurrence and development of IDD, and represents a new therapeutic target in the prevention and treatment of IDD.

Conclusions

To conclude, we found that the expression of TFEB

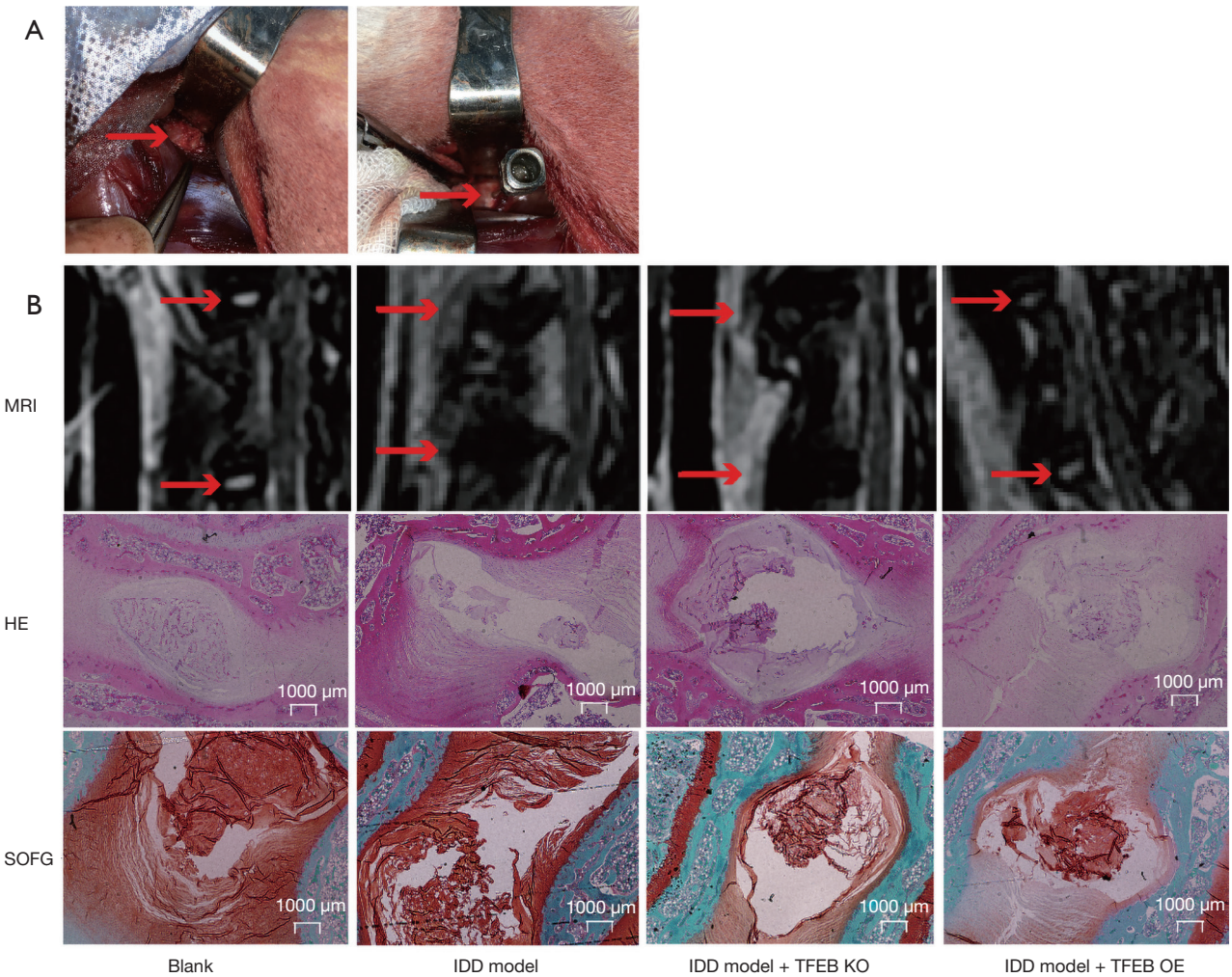

Figure 4 TFEB alleviated IDD in a rabbit degeneration model *in vivo*. (A) An annular puncture IDD model was generated using a retroperitoneal approach. Red arrows stand for the intervertebral disc. (B) MRI showed the degeneration of the intervertebral disc 8 weeks after the annular puncture. Red arrows stand for the intervertebral disc. HE staining showed the NP tissue structure and integrity. SOFG staining showed degenerative and fibrotic changes in the intervertebral discs. All data were obtained from 3 independent experiments. OE, TFEB overexpression; KO, TFEB knock out; TFEB, transcription factor EB; IDD, intervertebral disc degeneration; MRI, magnetic resonance imaging.

decreases in human NP cells and that a TFEB deficiency leads to IDD under OS. TFEB overexpression also aggravates IDD. In summary, our results suggest that excessive OS aggravates IDD through the TFEB/NF-κB pathway.

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Footnote

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uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-3756). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Patients or relatives were informed of the study, and patient or guardian consent was obtained preoperatively. This study was approved by the Ethics Committee of Shanghai General Hospital of Nanjing Medical University (ID: 2018KY038). Animal experiments were performed under a project license (No.: 2017KY043) granted by institutional board of Shanghai General Hospital of Nanjing Medical University, in compliance with Shanghai General Hospital of Nanjing Medical University institutional guidelines for the care and use of animals.

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