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TFEB protects nucleus pulposus cells against apoptosis and senescence via restoring autophagic flux

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SUMMARY

Objective: Excessive apoptosis and senescence of nucleus pulposus (NP) cells are major pathological changes in intervertebral disc degeneration (IVDD) development; previous studies demonstrated pharmacologically or genetically stimulation of autophagy may inhibit apoptosis and senescence in NP cells. Transcription factor EB (TFEB) is a master regulator of autophagic flux via initiating autophagy-related genes and lysosomal biogenesis. This study was performed to confirm whether TFEB was involved in IVDD development and its mechanism.
Methods: TFEB activity was detected in NP tissues in puncture-induced rat IVDD model by immunofluorescence as well as in tert-Butyl hydroperoxide (TBHP), the reactive oxygen species (ROS) donor to induce oxidative stress, treated NP cells by western blot. After TFEB overexpression in NP cells with lentivirus transfection, autophagic flux, apoptosis and senescence percentage were assessed. In *in vivo* study, the lentivirus-normal control (LV-NC) or lentivirus-TFEB (LV-TFEB) were injected into the center space of the NP tissue, after 4 or 8 weeks, Magnetic resonance imaging (MRI), X ray, Hematoxylin-Eosin (HE) and Safranin O staining were used to evaluate IVDD grades.
Results: The nuclear localization of TFEB declined in degenerated rat NP tissue as well as in TBHP treated NP cells. Applying lentivirus to transfect NP cells, TFEB overexpression restored the TBHP-induced autophagic flux blockage and protected NP cells against apoptosis and senescence; these protections of TFEB are diminished by chloroquine-medicated autophagy inhibition. Furthermore, TFEB overexpression ameliorates the puncture-induced IVDD development in rats.
Conclusions: Experimental IVDD inhibited the TFEB activity. TFEB overexpression suppressed TBHP-induced apoptosis and senescence via autophagic flux stimulation in NP cell and alleviates puncture-induced IVDD development *in vivo*.

Introduction

Intervertebral disc degeneration (IVDD) is a frequent musculoskeletal disease and an important cause of low back pain, which affects up to 80% of adults at different stages of their life[1]. Multifarious factors including age, adiposis, gene, sexuality are associated with IVDD initiation and development[2]. However, the exact pathogenesis and effective therapy for IVDD are still under development. Intervertebral disc mainly consists of three cell types: 1)

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the gelatinous internal nucleus pulposus (NP) cells; 2) the outer annulus fibrosus (AF) cells; 3) the endplate chondrocytes (EP) in the upper and lower endplates[3]. NP cells secret extracellular matrix (ECM) molecules including collagen and proteoglycans to maintain the structural stabilization and biomechanical equilibrium of the intervertebral disc[4], dysfunction and hypocellularity of the gelatinous NP cells are the hallmark of IVDD. Pathological factors such as inflammatory cytokines and oxidative stress may induce reactive oxygen species (ROS) production; consequently, ROS overproduction results in immoderate apoptosis and senescence of NP cells[5]. Hence, intervening apoptosis and senescence of NP cells is considered to be an effective therapeutic strategy for IVDD[6,7].

Autophagy is a cellular degradation process, it is a cytoprotective mechanism helps to maintain intracellular homeostasis and improve cellular survival and function[8]. Autophagy is a dynamic process, in which subcellular membranes undergo structural changes, encapsulate cytoplasmic components, form autophagosomes and then fuse with lysosomes to form autolysosomes for degrading the contents[9]. This process is termed autophagic flux, which is also considered as a reliable indicator of autophagic activity. Various studies showed that the autophagic flux was closely related to IVDD development[10–12]. Rapamycin, the classical autophagic activator, was reported to suppress ECM degeneration in rat NP cells exposed to inflammatory factors such as TNF-α and IL-1β[13], showing the protective role of autophagy in IVDD. Wei and his colleagues reported that the expression of LC3-II and Beclin-1, the autophagosomes membrane proteins, decreased in human degenerative NP cells, suggesting autophagosome initiation is damaged[14], however how this process is dysregulated in IVDD is still unknown.

As a member of microphthalmia-associated transcription factor (MITF)/transcriptional factor E (TFE) family, transcription factor EB (TFEB) has been identified as a master regulator of autophagic flux via inducing lysosome biogenesis and promoting autophagosome formation as well as its fusion with lysosome[15–17]. Several *in vitro* and *in vivo* models of neurodegenerative disorders showed that genetic or pharmacological upregulation of TFEB promoted the clearance of accumulated toxic proteins[18–22]. Nevertheless, the role of TFEB in IVDD remains to be verified. In the present study, we found that TFEB activation is damaged in rat IVDD model, whereas TFEB overexpression could suppress the excessive apoptosis and senescence of NP cells through regulation of autophagy lysosome pathway (ALP).

Materials and methods

Ethics statement

The experimental procedures and the animal use and care protocols were according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Animal Care and Use Committee of Wenzhou Medical University.

Rat NP cells culture

30 Sprague–Dawley rats (150–200 g) were euthanized with an overdose of pentobarbital. The spinal columns from L1 to L6 were removed under aseptic conditions and lumbar disks were collected. Gel-like NP tissues was isolated by a dissecting microscope and treated with 0.1% collagenase (Sigma–Aldrich, St Louis, USA) and 2U/ml hyaluronidase (Sigma–Aldrich, St Louis, USA) for 4 h at 37°C. Then the digested tissues were transferred as explants to high glucose Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Logan, UT) and antibiotics (1% penicillin/streptomycin) in the incubator maintaining 5% CO2 at 37°C. NP Cells moved out of the explants after 1 week. The complete medium was changed every 2 days. When up to 80–90% confluence, the cells were harvested by using 0.25% Trypsin– EDTA (Gibco, Invitrogen). The second-passage NP cells were used for all of our experiment. Six independent cell isolations were performed.

Transmission electron microscopy

Rat NP cells were fixed in 2.5% glutaraldehyde overnight, post fixed in 2% osmium tetroxide for 1 h and stained with 2% uranyl acetate for 1 h. After dehydration in a series of acetone, samples were embedded into araldite and cut into semi-thin sections, which were stained with toluidine blue to locate cell position and observed under a transmission electron microscope (Hitachi, Tokyo, Japan). The 30 cells of each section were randomly selected and captured.

TUNEL staining

The level of deoxyribonucleic acid (DNA) damage was detected by transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining. In brief, NP cells were collected and prepared in a 12-well plate. After the treatment, the cells were fixed with 4% paraformaldehyde for 1 h and incubated with 3% H2O2 and 0.1% Triton X-100 for 10 min and washed with phosphate buffer saline (PBS) for three times in every step. Finally, according to the manufacturer's instructions, the cells were stained with *in situ* cell death detection kit (Roche, Basel, Switzerland) for 30 min at 37°C and the nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI). Twenty-five fields of each slide were randomly selected and captured under a fluorescence microscope (Olympus Inc., Tokyo, Japan) to count TUNEL positive cells by two independent and blinded observers.

LysoTracker red staining

LysoTracker Red (LTR) staining was applied to assess the number and function of lysosomes. In brief, NP cells were collected and prepared in a 12-well plate. After the treatment, the cells were washed by PBS for three times and then stained with 50 nM LTR (Invitrogen, Grand Island, NY) for 30 min at 37°C, and nuclei were stained with Hoechst33258 for 10 min. After the staining, the cells were washed by PBS and then to mounting by PBS. The slides were observed with a fluorescence microscope (Olympus Inc., Tokyo, Japan). Red fluorescence images of at least 25 random microscopic fields were acquired per slide and fluorescence intensity was measured by Image J (Bethesda, MD, USA) with two independent and blinded observers.

SA-β-gal staining

The level of senescence was measured by SA-β-gal staining kit (Beyotime, Shanghai, China) according to the instruction. Aging cells showing high SA-β-gal activity were stained blue. In brief, NP cells were collected and prepared in a 12-well plate. After the treatment, the cells were fixed with 0.2% glutaraldehyde for 15 min at room, washed three times with PBS and then stained with X-gal staining solution at pH 6.0 overnight. For the observation, twenty-five fields of each slide were randomly selected and captured under a microscope (Olympus Inc., Tokyo, Japan) to count SA-β-gal positive cells by two independent and blinded observers.

Rat IVDD model

Adult male Sprague–Dawley rats (200–230 g) were purchased from the Animal Center of the Chinese Academy of Sciences in Shanghai, housed in standard temperature conditions with a 12-h light/dark cycle and regularly fed with food and water. IVDD rats

were performed as described previously[23]. Briefly, rats were injected intraperitoneally with 2% (w/v) pentobarbital (40 mg/kg). The experimental level rat tail disc (Co7/8) was located by digital palpation on the coccygeal vertebrae and confirmed by counting the vertebrae from the sacral region in a trial radiograph. Needles (21G) were used to puncture the whole layer of AF though the tail skin. To make sure the needle won't be punctured too deep, the length of the needle was decided according to the AF and NP dimensions which were measured in the preliminary experiment is about 5 mm. All the needles were rotated 360° and kept in the disc for 1 min. To eliminate the influences of the injected volume, only 3 μL lentivirus-normal control (LV-NC) or lentivirus-TFEB (LV-TFEB) were injected into the center space of the NP tissue by using a microliter syringe with a needle of 27 gauge (10 μl, Gaoge, Shanghai, China). All operators were blinded to animal experimental grouping. Daily monitoring of the rats was carried out to ensure their well-being and all animals were allowed free unrestricted weight bearing and activity and regularly fed with food and water.

X-ray and MRI

The 20 rats experiencing the puncture were randomly divided into two groups: IVDD+LV-NC and IVDD+LV-TFEB and then injected with LV-NC or LV-TFEB separately. The X-ray and Magnetic resonance imaging (MRI) were performed at 0, 4 and 8 weeks after puncture. For X-ray analysis, disc height was measured using the ImageJ software and expressed as the disc height index (DHI) using the method as previously described[24]. Changes in the DHI of the punctured IVDs were expressed as % DHI (%DHI = post-punctured DHI/pre-punctured DHI×100%). Magnetic resonance imagings were performed to evaluate the signal and structural changes in sagittal T2-weighted images using a 3.0 T clinical magnet (Philips Intera Achieva 3.0 MR). T2-weighted sections in the sagittal plane were obtained in the following settings: fast spin echo sequence with time to repetition (TR) of 5,400 ms and time to echo (TE) of 920 ms; 320 (h) 9,256 (v) matrix; field of view of 260; and four excitations. The section thickness was 2 mm with a 0-mm gap. The MRIs were evaluated by another blinded orthopedic researcher using the classification of intervertebral disk degeneration as reported by Pfirrmann *et al.*[25]. (1 point = Grade I, 2 points = Grade II, 3 points = Grade III, 4 points = Grade IV, 5 points = Grade V).

Histopathologic analysis

For the experiment about the TFEB activity change in IVDD development, the 24 rats were randomly divided into four groups: control (4w), IVDD (4w), control (8w) and IVDD (8w). The rats in IVDD group were performed to puncture surgery in tail disc rat (Co7/8). For the experiment to assess the effect of TFEB overexpression in IVDD development, the 40 rats experiencing the puncture were randomly divided into 4 groups: IVDD+LV-NC (4w), IVDD+LV-TFEB (4w), IVDD+LV-NC (8w) and IVDD+LV-TFEB (8w) and then injected with LV-NC or LV-TFEB separately. The rats were sacrificed at 4 weeks and 8 weeks after puncture. And rat tail disc tissue (Co7/8) was isolated, fixed with 4% paraformaldehyde, decalcified with 10% ethylenediaminetetraacetic acid solution and paraffin-embedded for serial sectioning. Sections were stained with Hematoxylin-Eosin (HE) and safranin O-fast green (SO) and then captured under a microscope. The HE staining was used to evaluate morphological change of NP cells. For the SO staining, the cellularity and morphology of NP and AF were examined by another group of experienced histology researchers in a blinded manner using a microscope, and evaluated by using a grading scale, as described previously[23,26]. The histologic score was five for normal disc, 6–11 for moderately degenerated disc and 12–15 for severely degenerated disc. At least three sections from each specimen were used to assess histologic score in the rat.

Statistical analysis

The results were presented as mean ± S.D. Statistical analyses were performed using SPSS statistical software program 20.0 (IBM, Armonk, NY, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison between the two groups. $P < 0.05$ was considered significant.

Results

TFEB activity is declined in rat degenerated NP cells

To illustrate the relationship between TFEB activity and IVDD, we established rat IVDD model and applied immunofluorescence to detect TFEB nuclear expression in NP cells from rat degenerated intervertebral disc. As shown in Fig. 1(A), the gelatine NP cells showed gradually diminished and translated fibrocartilage, accompanied by the disappearance of border between NP and AF at 4 and 8 weeks after puncture. Rats suffering IVDD surgery showed higher histological scores relative to sham surgery especially at 8 weeks, suggesting our rat IVDD model is available [Fig. 1(B–C)]. The immunofluorescence staining results showed the rate of TFEB nuclear positive NP cells was decreased in rat degenerated intervertebral disc, especially at 8 weeks [Fig. 1(A), (D), and (E)]. In addition, we detected TFEB activity in ageing rats. As compared to 8-weeks-old rats, TFEB mRNA level in NP tissue of 24-weeks-old was reduced [Fig. S6]. Thus, we conclude that IVDD leads to abnormal TFEB activity in NP cells.

TBHP restrains TFEB nuclear expression in rat NP cells

Oxidative stress is accompanied by the entire pathophysiological process of IVDD[5,27]. We exposed NP cells to Tert-Butyl hydroperoxide (TBHP), the exogenous ROS donors with more steady releasing peculiarity than H_2O_2, to establish IVDD model *in vitro*. From CCK8 assay results we could found that TBHP inhibited NP cells viability [Fig. S1]. TFEB nuclear expression in NP cells was reduced in both time and concentration dependent manner by TBHP stimulation, as indicated by western bolt results [Fig. 1(F–K)]. Furthermore, TFEB cytoplasm expression seems to decline in NP cells exposing to TBHP, but it showed not statistically difference. The results above collect with our *in vivo* experimental results suggest that TFEB activaty is declined in IVDD process.

TFEB overexpression inhibits senescence and apoptosis in TBHP treated rat NP cells

Next, we upregulated TFEB expression in NP cells by transfecting them with lentivirus-TFEB (LV-TFEB) to assess the effects TFEB on IVDD *in vitro*. The lentivirus transfection efficiency was confirmed by western blot [Fig. S2]. We analyzed the SA-β-gal activity and p16INK4a expression in NP cells, which are commonly used indicators of senescence. As shown in Fig. 2(A), (C), (E), and (G), TFEB overexpression significantly prevented TBHP-induced increase of SA-β-gal activity and p16INK4a expression. In addition, we detected the expression of IL-6 and MMP-3, which are classical senescence-associated secretory phenotype factors; the results showed that TFEB overexpression may suppress TBHP-induced upregulation of IL-6 and MMP-3 [Fig. S5]. Cleaved caspase3 expression and DNA damage level were detected to evaluate TFEB-medicated anti-apoptotic effect. From western blot and TUNEL staining results we could see that TBHP administration may stimulate cleaved

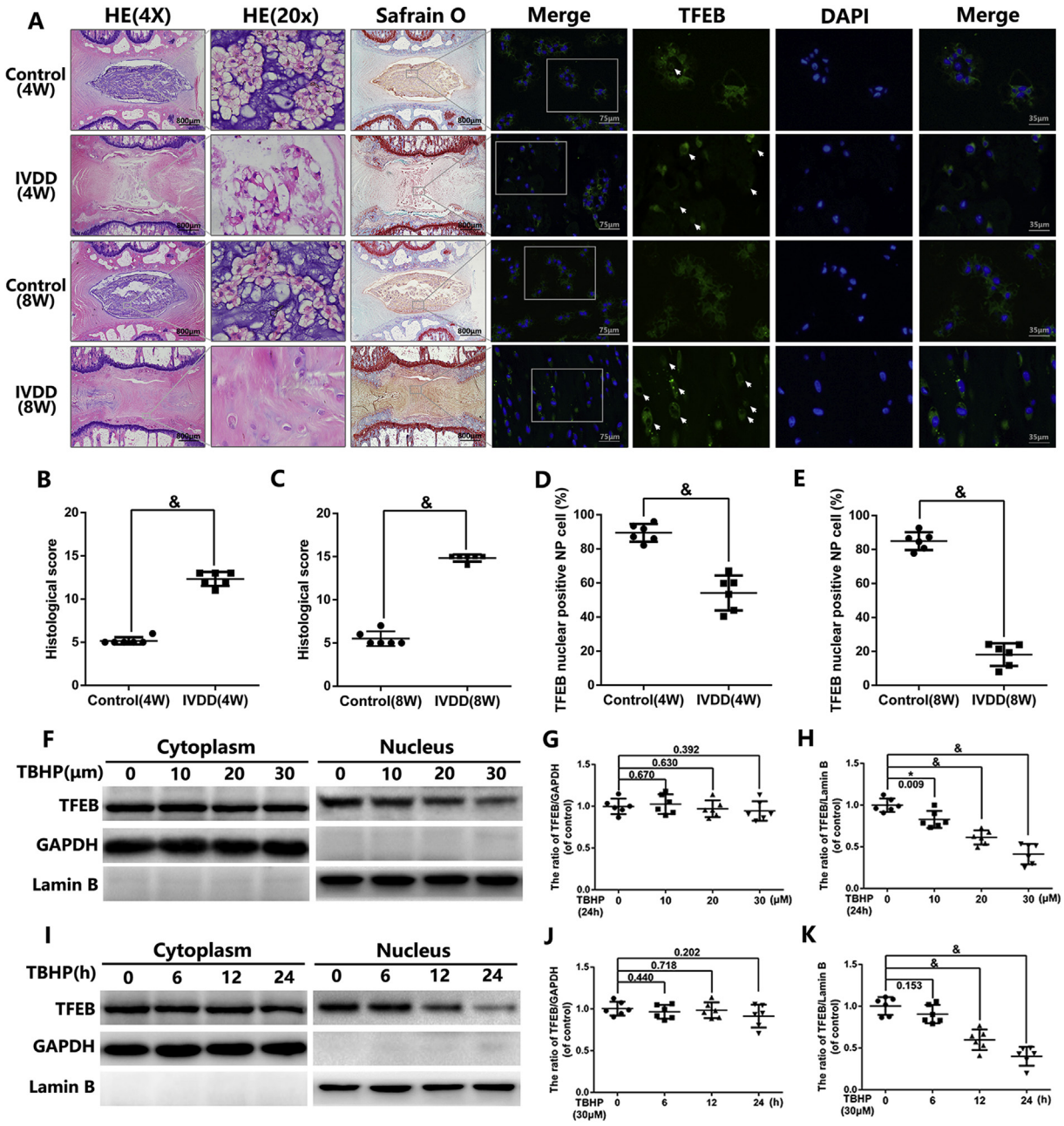

Fig. 1. The nuclear expression of TFEB in NP cells is decreased in rat IVDD model in vivo and in vitro. (A) Representative HE staining, SO staining and immunofluorescence staining of TFEB in rat NP tissue (bar: 800 μm in HE and SO staining, bar: 75 μm or 35 μm in immunofluorescence staining). **(B, C)** The histological grades evaluated at week 4 and week 8 in two groups. **(D, E)** Quantitation of immunofluorescence staining of TFEB. **(F-K)** The protein expression of TFEB in cytoplasm and nuclear in rat NP cells after TBHP treatment. The NP cells were incubated with 0, 10, 20 or 30 μM TBHP for 24 h or 30 μM TBHP for 0, 6, 12 or 24 h. All data were shown as mean ± S.D. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, & $P < 0.0001$.

caspase three expression and DNA damage, which were reversed after TFEB overexpression [Fig. 2(A), (B), (D), and (F)]. Conversely, TFEB down-regulation by siRNA amplified TBHP-induced cleaved caspase3 and p16INK4a expression [Fig. S2]. These results indicate that excessive senescence and apoptosis in NP cells caused by TBHP are relieved by LV-TFEB transfection.

TFEB overexpression upregulates autophagic flux in rat NP cells

Although growing evidences identified TFEB as a upstream protein of the ALP regulation, its function in NP cells is still nuclear, we addressed this question here. Firstly, the transmission electron microscopy (TEM) was performed to observe the organelle difference between LV-NC and LV-TFEB treated NP cells. More autophagic vesicles, including autophagosome and autolysosome, were found in LV-TFEB transfected NP cells relative to LV-NC cells [Fig. 3(B)]. Meanwhile, quantitative polymerase chain reaction (qPCR) results showed that overexpression of TFEB in rat NP cells can significantly upregulated the mRNA expression of LC3, Beclin1, p62, CTSD, CTSB and Lamp1, which are TFEB downstream genes regulating autophagosome formation and lysosomal biogenesis [Fig. 3(A)]. During the dynamic process of autophagy, LC3-II level may decrease along with autolysosomes degeneration and autophagosomes generation; therefore, the expression level of LC3-II between bafilomycin

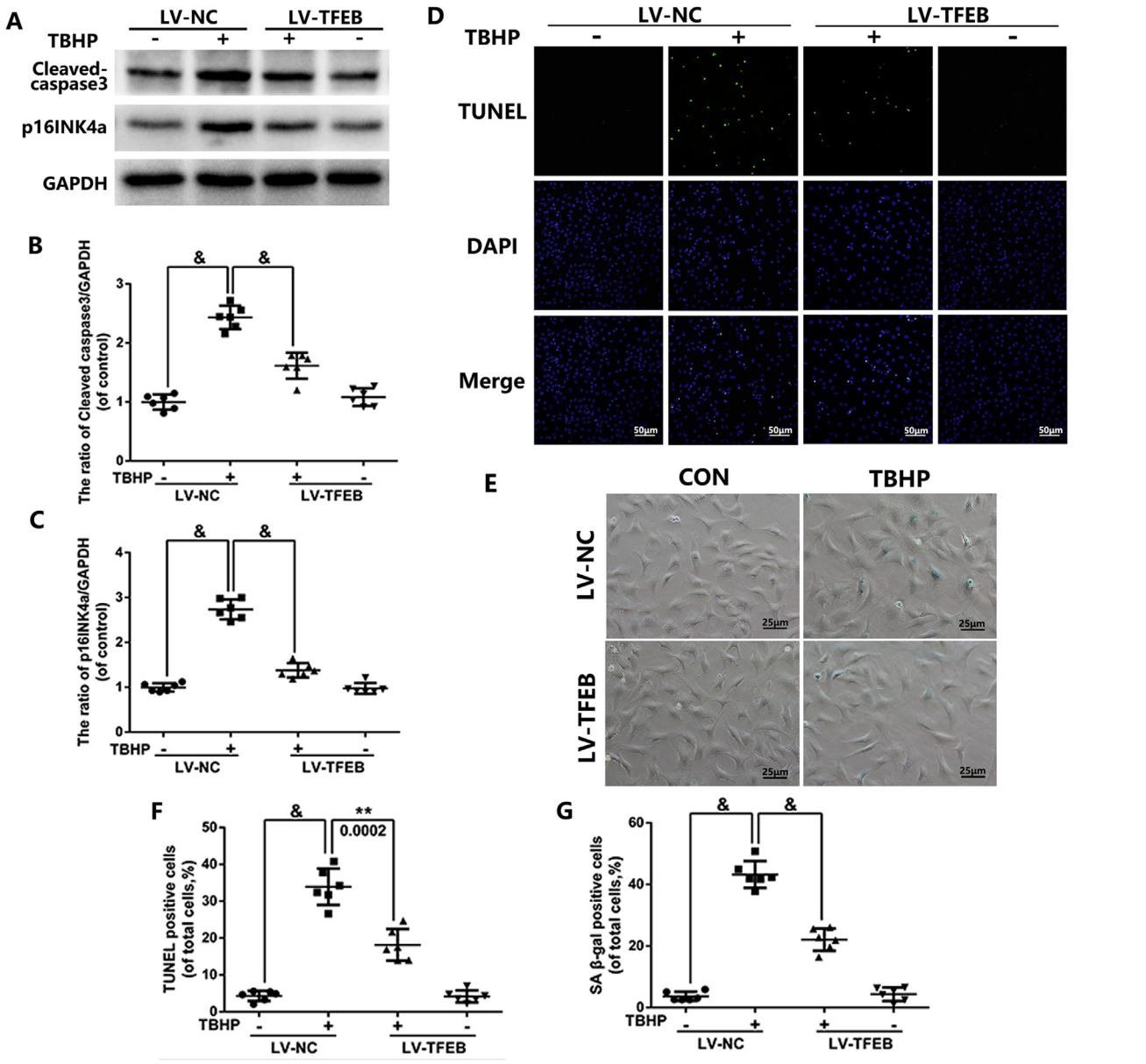

Fig. 2. TFEB overexpression attenuates the TBHP-induced apoptosis and senescence in rat NP cells. The cells were transfected with LV-NC or LV-TFEB before receiving TBHP (30 μM). **(A–C)** The protein expression of Cleaved-caspase3 and p16INK4a in rat NP cells. **(D, F)** TUNEL staining assay was performed in rat NP cells (bar: 50 μm). **(E, G)** SA-β gal staining assay was performed in rat NP cells (bar: 25 μm). All data represent mean ± S.D. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, & $P < 0.0001$.

A1 (a typical lysosomal inhibitor) treated and untreated cells is applied to quantize the autophagic flux activity. Our results in Fig. 3(C) and (D) indicated autophagic flux was increased following TFEB overexpression.

TFEB overexpression alleviates TBHP-induced autophagy and lysosome dysfunction in rat NP cells

Next, we assessed the effects of TFEB overexpression on autophagy and lysosome in NP cells. From western blot results we could see that TBHP stimulation may lead to declined CTSB, LAMP2 level as well as increased p62 and LC3-II level, suggesting autophagic flux blockage occurred under TBHP exposure. Meanwhile, TFEB overexpression reversed TBHP-induced changes of the indexes above [Fig. 4(A–E)]. LC3-II and LAMP1 immunofluorescence double staining showed that autophagy-lysosome fusion was reduced in NP cells exposed to TBHP, which was reversed by LV-TFEB transfection [Fig. 4(H–I)]. LTR is a specific lysosomotropic probe which may exhibit red fluorescence dependenting on lysosomal pH. It showed weakened red fluorescence intensity in NP cells treated by TBHP, indicating TBHP may lead to lysosomal dysfunction, and this phenomenon was restored after TFEB overexpression [Fig. 4(F) and (G)]. The results above suggest that TFEB overexpression alleviates TBHP-induced autophagy and lysosome dysfunction in NP cells.

Chloroquine (CQ) reverses TFEB-induced protective effects in NP cells under oxidative stress

Unlike traditional autophagic activators, TFEB overexpression not only promotes autophagosome formation, but also increases biogenesis and function of lysosome so as to accelerate autophagosome degradation. Hence, we chose CQ, a lysosomal cavity

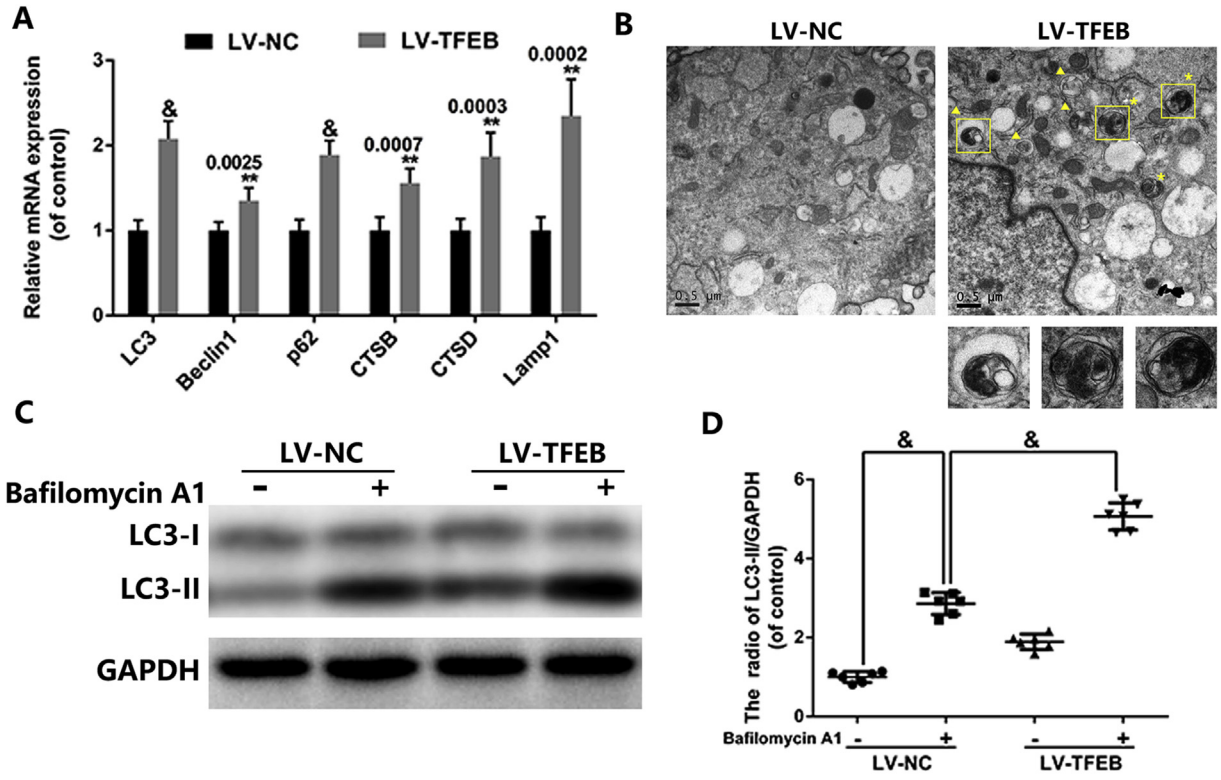

Fig. 3. TFEB regulates autophagic flux in rat NP cells. The cells were transfected with LV-NC or LV-TFEB before receiving bafilomycin A1 (100 nM). **(A)** The mRNA expression of LC3, Beclin1, p62, CTSB, CTSD and Lamp1 in rat NP cells. **(B)** TEM images of autophagic vesicles in rat NP cells (bar: 0.5 μm). (Asterisk: autophagosome with double membrane structure; Triangle: autophagolysosome with single membrane structure). **(C, D)** The protein expression of LC3 in rat NP cells. All data represent mean ± S.D. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, & $P < 0.0001$.

alkalizer which may blocks downstream autophagic flux, rather than 3-Methyladenine (3-MA), to intervene autophagic flux. As shown in Fig. 5, the protection of TFEB overexpression against TBHP-induced apoptosis and senescence are either markedly attenuated or completely abolished by CQ, as indicated by the results from cleaved caspase3 and p16INK4a protein levels, and the percentage of TUNEL and SA-β-gal positive cells. The results above demonstrate that TFEB-induced anti-apoptotic and anti-senescence capability are through autophagic flux regulation.

TFEB overexpression ameliorates IVDD in vivo

To explore the therapeutic effects of TFEB in rat IVDD model, we injected lentivirus into the rat intervertebral disc to overexpress TFEB after IVDD surgery and executed X-ray, MRI, HE and SO staining to estimate imageology and histomorphology change. The TFEB mRNA level in IVDD+LV-TFEB group was found to be increased in NP, AF and EP tissue at 14 days after lentivirus injection (Fig. S4). By X-ray analysis we found that TFEB overexpression delayed the loss of disc height induced by puncture surgery (IVDD surgery) [Fig. 6(A) and (B)]. From MRI results, it could be found that T2-weight signal intensity of the intervertebral disc in damaged section was gradually weakened at 4 and 8 weeks after puncture, and the degeneration was confirmed by Pfirrmann grade scoring. Interestingly, TFEB overexpression delayed MRI signal intensity weakening and showed higher Pfirrmann grade scores [Fig. 6(C) and (D)]. From HE and Safranin O staining results in Fig. 6(E-G) we could found that the gelatin NP cells gradually reduced and were replaced by the fibrochondrocytes in LV-NC group, the structure of AF exhibited a tear or serpentine pattern followed by inward bulging and even became disorganized at 8 weeks after puncture, and the cartilaginous endplate was corroded and collapsed. Nevertheless, LV-TFEB injection obviously delayed these histopathological changes, as shown by the existence of more NP cells, less unordered AF and intact cartilaginous endplate at 4- and 8-week time points. Histological scores from Safranin O staining also demonstrated that the protection of LV-TFEB in IVDD development [Fig. 6(H)]. These results demonstrate that TFEB overexpression may ameliorate IVDD in rats *in vivo*.

Discussion

As a basic helix-loop-helix leucine-zipper (bHLH-Zip) transcriptional factors, TFEB activity and subcellular localization are dependent on TFEB phosphorylation level[15,28]. Normally, TFEB is mainly phosphorylated by mechanistic target of rapamycin complex 1 (mTORC1) at multiple critical serine residues and bund with chaperone 14-3-3 and thereby sequestered at the cytoplasm[29–31]. Once mTORC1 activity is suppressed, the phosphatase calcineurin induces the TFEB dephosphorylation[32]. Then dephosphorylated TFEB transfers to the nucleus and directly binds to the promoter regions of autophagy genes and the palindromic E box (i.e., CACGTG), which called the coordinated lysosomal expression and regulation (CLEAR) network, to regulate autophagy and lysosome biogenesis[15,17]. Nishida and his colleagues found that mTORC1 signaling is aberrantly activated in human degenerated disc NP tissues, which may explain our results about TFEB inactivation[12]. Meanwhile, suppressing mTORC1 activity significantly ameliorated IL-1β-induced apoptosis and senescence of NP cells[12,13]. In addition, cartilage-specific tuberous sclerosis complex 1 (Tsc1, mTORC1 upstream inhibitor) knockout (TSC1CKO) mice showed spontaneous osteoarthritis (OA) with aberrant chondrocyte proliferation and hypertrophic differentiation[33]. These researches

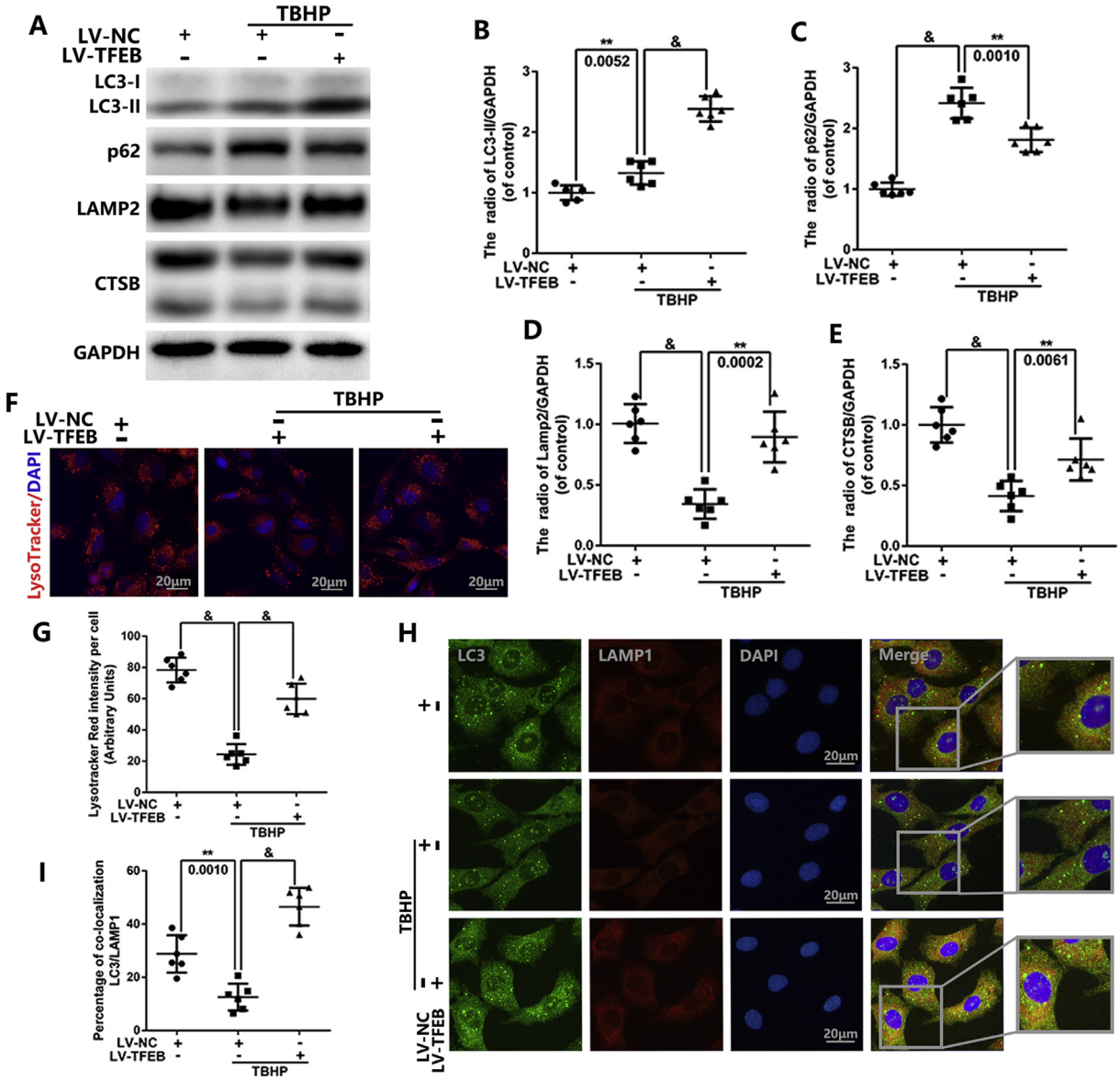

Fig. 4. TFEB overexpression rescues the blockage of autophagic flux induced by TBHP in rat NP cells. The cells were transfected with LV-NC or LV-TFEB before receiving TBHP (30 μM). **(A–E)** The protein expression of LC3, p62, LAMP2 and CTSB in rat NP cells. **(F, G)** The Lysotracker staining in rat NP cells (bar: 20 μm). **(H)** Immunofluorescence double-labeled staining for co-localization of LC3 with LAMP1 in rat NP cells (Green: LC3, red: LAMP1, bar: 20 μm). **(I)** The quantitation of the percentage of co-location of LC3/LAMP1 was detected by image J. All data represent mean ± S.D. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, & $P < 0.0001$.

indicated that the declined activity of TFEB might be a consequence of aberrant mTORC1 activity in IVDD, which of course requires further experimental validation.

In current study, we used TBHP as ROS donor to stimulate apoptosis and senescence in NP cells in *in vitro* study. And we found that TBHP-induced DNA damage as well as increased cleaved caspase3, p16INK4a expression and SA-β-gal activation are reduced in TFEB overexpression cells, suggesting that TFEB overexpression suppresses apoptosis and senescence in condition of oxidative stress. Apoptosis and senescence of NP cells are considered as major characteristics in IVDD process, which could be activated by many factors, oxidative stress in particular[27]. Oxidative stress may induce organelle, e.g., mitochondria and endoplasmic reticulum impairment as well as proteins misfolding, and these impaired organelles and misfolding proteins may be sensed by proteins in pathways of apoptosis and senescence to induce down-stream effects[34,35]. While TFEB-mediated autophage could enhance the clearance of damaged organelles and misfolding proteins, so as to eliminate the inducer of apoptosis and senescence pathways, as a consequence apoptosis and senescence were suppressed.

Our study found that TFEB nuclear expression was reduced when NP cells were treated with TBHP; while some researchers reported that ROS could induce the TFEB nuclear translocation in other cell models, which seems to be contrary to our results[36–38]. These contradictory results may be caused by the variation in degree of ROS. The studies which showed ROS may induce TFEB nuclear translocation apply a different way to induce ROS than we do. They use a much higher concentration of TBHP (100 μM) for a duration of 4 h in their *in vitro* studies, and this kind of acute stimulation may cause a self-protective responding to transient oxidative stress, which may drive TFEB to translocate to the nucleus; while we use a relative mild concentration of TBHP (30 μM) for total 24 h duration, which might be more able to simulate the pathological conditions *in vivo*.

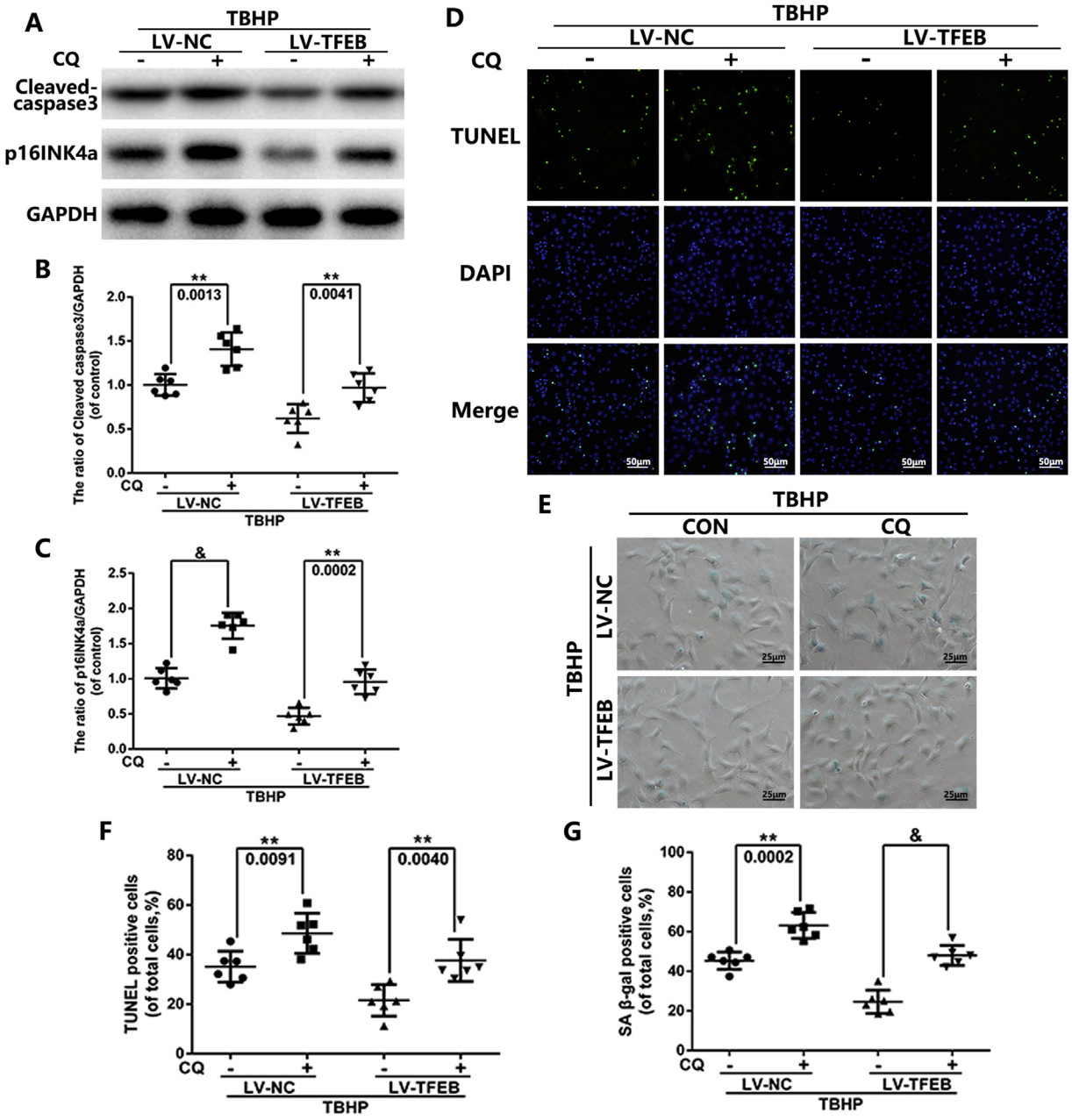

Fig. 5. Chloroquine counteracts the protective effects of TFEB in TBHP-exposed rat NP cells. The cells were transfected with LV-NC or LV-TFEB and then pretreated with 50 μM chloroquine for 6 h before TBHP (30 μM) addition. **(A–C)** The protein expression of cleaved-caspase3 and p16INK4a in rat NP cells. **(D, F)** TUNEL staining assay was performed in rat NP cells as treated above (bar: 50 μm). **(E, G)** SA-β gal staining assay was performed in rat NP cells as treated above (bar: 25 μm). All data represent mean ± S.D. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, & $P < 0.0001$.

Autophagic flux has been widely demonstrated as a potential therapeutic target for IVDD, as it may help to combat apoptosis and senescence. By the TEM results from Hu's study, autophagosomes were reduced in NP cells of patients with IVDD relative to the patients with lumbar vertebral fracture[14]. To our knowledge, the expression alteration of p62 (a protein representing the degradation level of autophagic vacuoles) and lysosome activity in the process of IVDD is still unknown. Our group latterly reported that high accumulation of p62 appears in human OA cartilage and destabilized medial meniscus (DMM) mouse OA model, suggesting that autophagic degradation is blocked in OA chondrocytes[39]. Moreover, Kim *et al.* observed accumulation of lysosomes and decline of lysosomal activity in human OA chondrocytes relative to normal chondrocytes, also they showed that destroying lysosome results in enhanced chondrocyte apoptosis and inhibition of autophagy[40]. These results lead to further hypothesis that targeting autophagosome initiation and lysosomal fitness might restore autophagy flux, which will in turn protects NP cells against cellular damage.

TFEB has been discovered as the controller of autophagosomes and lysosome biogenesis. We showed here that TFEB overexpression may not only promote autophagosome formation, but also restore lysosome function, and the fusion of autophagosome and lysosome, which ultimately restores the TBHP-induced disruption of autophagic flux. Besides autophagic flux regulation, TFEB also participates in various biological pathways[43,44]. To

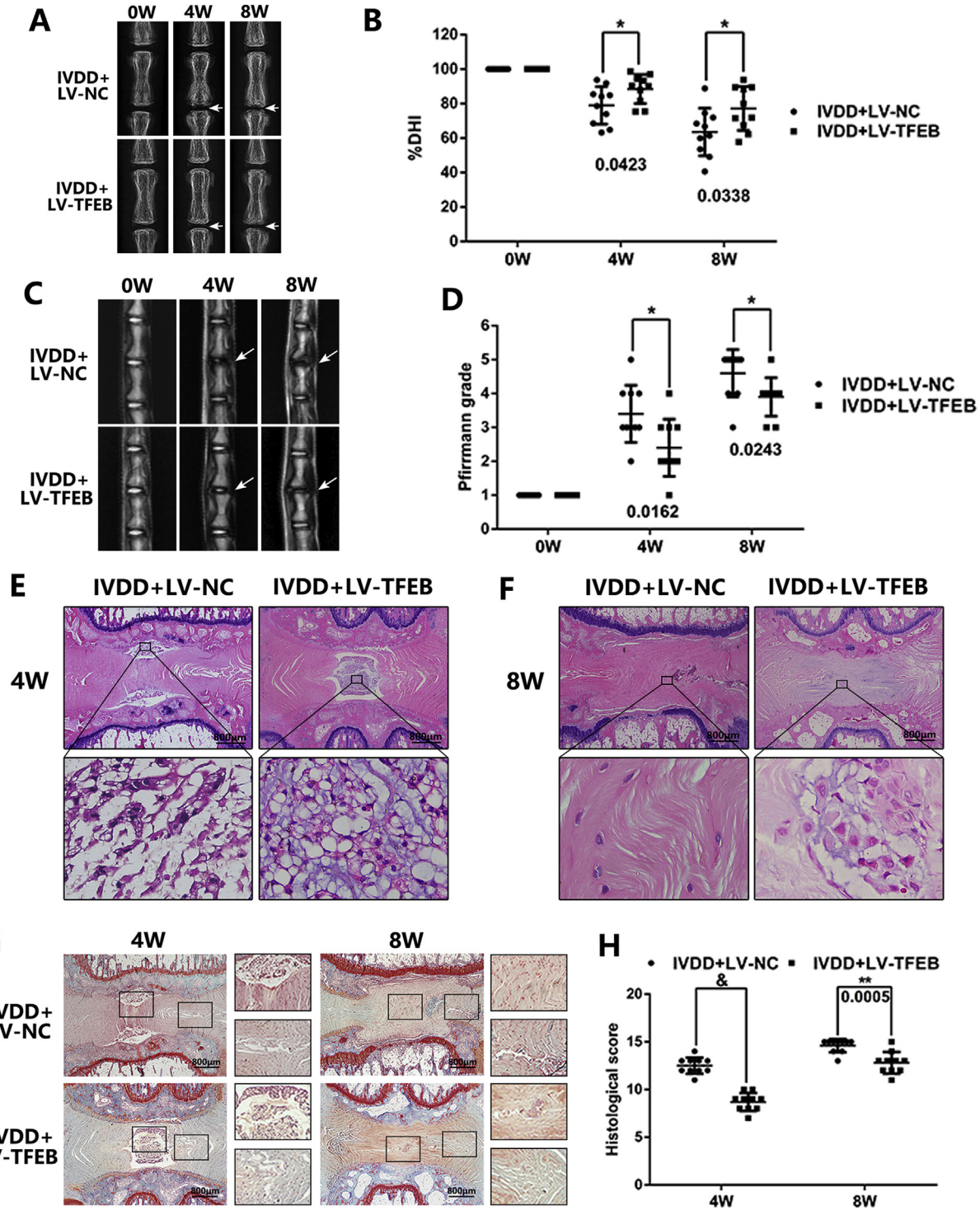

Fig. 6. TFEB overexpression ameliorates IVDD development *in vivo*. **(A)** The X-ray of a rat-tail disc at 0, 4 and 8 weeks after disc puncture surgery (white arrows). **(B)** The DHI of a rat-tail disc at 0, 4 and 8 weeks after disc puncture surgery. **(C)** T2 weighted MRI of a rat-tail disc at 0, 4 and 8 weeks after disc puncture surgery (white arrows). **(D)** The respective Pfirrmann grade scores at 0, 4 and 8 weeks after disc puncture surgery. **(E–F)** Representative HE staining of NP tissues from two groups at 4 and 8 weeks post-surgery (bar: 800 μm). **(G)** Representative S-O staining of NP tissues and AF tissues from two groups at 4 and 8 weeks post-surgery (bar: 800 μm). **(H)** The histological grades evaluated at week 4 and week 8 in two groups. All data represent mean ± S.D. ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, & $P < 0.0001$.

further confirm whether autophagic flux plays an indispensable role in protective effects of TFEB against apoptosis and senescence in TBHP-treated NP cells, CQ, which is a lysosomal cavity alkalizer and commonly used as the classical inhibitor of the downstream in autophagic flux, was used in our study. The results of western bolt, TUNEL and SA-β-gal staining demonstrated that CQ significantly attenuates the protection of TFEB in the cultured NP cells under TBHP-induced oxidative stress, suggesting TFEB exerts its protective effects mainly through autophagy flux regulation.

Pretreating NP cells with wortmannin (autophagy inhibitor) or rapamycin (autophagy activator) in serum deprivation condition may both lead to upregulated apoptosis incidence, suggesting

autophagy functions as double-edge sword in NP cells[41,42]; therefore over-activation of autophagic flux induced side effects, such as apoptosis, should be noted in studies that apply genetic methods to restore autophagy flux. In our study, TFEB overexpression promoted the autophagic flux, however it did not affect the apoptosis and senescence level in NP cells under physiological condition (shown in Figs. 2 and 3), suggesting that LV-TFEB transfection in NP cells is not sufficient to cause unwanted side effects, which also indicates that LV-TFEB transfection is a safe strategy for IVDD therapy.

In conclusion, we demonstrated the activation of TFEB was decreased in puncture-induced rat IVDD model. TFEB overexpression fortified autophagic flux and lysosome function, which may further protect NP cells against apoptosis and senescence under TBHP treatment. In addition, our *in vivo* experiments confirmed that TFEB overexpression may ameliorate IVDD in rats. These results indicate that TFEB can be considered as a potential target for the treatment of IVDD.

Author contributions

GZ wrote the paper, performed the experiments and generated data; GZ, ZYP and YZ performed the experiments and generated data; QT, FHZ and YFZ analyzed data; YSW, YLZ and DHC contributed reagents and materials tools; JXC, XYW and WYG conceived and designed the experiments; HZX, NFT and XLZ designed the experiments and helped write the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary data

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