The therapeutic effect of TBK1 in intervertebral disc degeneration via coordinating selective autophagy and autophagic functions

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Introduction: While its innate immune function has been known, recent works of literature have focused on the role of Tank binding kinase 1 (TBK1) in regulating autophagy and it is unknown whether TBK1 protects against intervertebral disc degeneration (IVDD) through affecting autophagy.

Objectives: Here, we aim to explore whether TBK1 is implicated in the pathogenesis of IVDD, and investigated the potential mechanism.

Methods: Western blotting and immunohistochemistry were used to detect the TBK1 expression in human and rat NP tissue. After TBK1 overexpression in NP cells with lentivirus transfection, autophagic flux, apoptosis and senescence percentage were assessed. Si-RNA, a utophagy inhibitors and protein phosphatase inhibitors were applied to study the mechanism of autophagy regulation. In vivo study, we further evaluated the therapeutic action of lentivirus-TBK1(Lv-TBK1) injection in a rodent IVDD model.

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Introduction

These days, intervertebral disc degeneration (IVDD) is seriously increasing the disability and social burdens in everyday worldwide [1,2]. Nowadays, it has also become a major cause of low back pain, which imposes a huge clinical and social economic burden on the society and family in the world [3]. However, there are various etiological factors including mechanical stress, genetic predisposition, metabolic diseases and age, which can largely led to the initiation and progression of IVDD [4–6]. These factors are associated with a common disease phenotype: loss of mass nucleus pulposus cells (NPCs) [7]. Nevertheless, the exact molecular mechanism of IVDD development is remained unclear and the corresponding specific molecule is not available until now. Three kinds of cells consist intervertebral disc: 1) the gelatinous internal NPCs; 2) the outer annulus fibrosus (AF) cells; 3) the endplate chondrocytes (EP) in the upper and lower endplates [8]. Among them, NPCs are generally regarded as primary cell residents in the disc tissues, disguised the extracellular matrix molecules including collagen II as well as proteoglycan, which enormously regulate the regular disc structures, normal functions and play a crucial role in resisting the compressive pressure. In addition, several pathological factors such as apoptosis senescence and mitochondrial or lysosomal dysfunction have been demonstrated to be critical roles in the pathogenesis and progression of IVDD [9–11].

TANK binding kinase 1 (TBK1) is usually recognized as a serine or threonine protein kinase, which notably mediates the activities of TANK and TRAF2 to activate the nuclear factor-kB (NF-kB) signaling pathway [12]. Previous investigations have clearly demonstrated that TBK1 plays significant roles in cellular innate immune responses against several viral and bacterial pathogens [13,14]. In addition to playing an important role in immune responses facing various exogenous pathogen infections, TBK1 also regulates cell homeostasis through selective autophagy process in the total different pathologic contexts [15]. Interestingly, TBK1 phosphorylates autophagy receptor protein, which binds to ubiquitinated protein aggregates, damaged organelles, and other toxic cellular components [15]. Microtubule-related protein 1 light chain 3 beta (LC3) is known as an ATG8 relative protein component, which can directly bind with autophagy receptors [16]. Additionally, the interactions between receptors and cargos and/or ATG8 family members and receptors were enhanced by the TBK1 phosphorylation of autophagy receptors. Then, the autophagic process is promoted.

Autophagy is a natural, regulated cellular process that plays protective roles through removing damaged proteins and dysfunctional organelles [17]. Low level of autophagy is crucial for maintaining cell homeostasis. Accumulating evidences have shown that autophagy dysfunction causes in several diseases including IVDD and osteoarthritis [18–20]. In addition, autophagy dysfunction is regarded as an important factor inducing IVDD development.

Macro-autophagy (autophagy) is generally recognized as a substantial intracellular degradation process by which damaged organelles, misfolded proteins and other cytoplasmic materials are degraded, which play important roles in cellular protections [21]. Numbers of evidences have demonstrated that all the cytoplasmic materials are degraded in the lysosomal compartments. Canonical autophagy plays a critical role in random protein degradation process. Autophagy also contains selective type, which is related to targeted phagocytosis autophagic cargo by autophagosomes [22]. Support selectivity of autophagy is depended on autophagic adaptor proteins, which includes SQSTM1 (p62), NBR1, NDP52 (COCOZ), optineurin (OPTN), TAX1BP1, as well as NIX, combine with ATG8 through an LC3 interacting region (LIR) motif and cargo via other domains. In addition, a notable investigation clearly showed that SQSTM1 played positive roles in directing depolarized mitochondria to the autophagosome [24]. Emerging evidences demonstrate that SQSTM1 plays significant roles in the mitochondrial aggregation but not in the mitophagy process [25]. Although SQSTM1 has no significant role in the autophagy process of MEFs and HeLa cells, but it remains unclear until now whether SQSTM1 has an effect and specific form of action in other cell types such as NPCs. Autophagic adaptor proteins are activated and then changed to depolarized mitochondria [26–28]. A recent study has shown that TBK1 triggering as a consequence to mitochondrial depolarization facilitates the autophagic adaptor proteins phosphorylation except TAX1BP1. In addition, TBK1 plays key roles in NDP52, OPTN and SQSTM1 recruitments to transduce mitochondrial functions, whereas TAX1BP1 recruiting is actually PINK1 substituted, unfortunately TBK1 is independent [29].

Several neurodegenerative disease models including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) in vitro and in vivo illustrated that TBK1 genetic or pharmacological up-regulation significantly suppressed apoptosis and removed the accumulated toxic proteins [30,31]. In our current investigation, we clearly pointed that TBK1 regulates autophagic flux via restoring mitochondria and lysosomal functions and promoting autophagosomes formation. In addition, TBK1 overexpression could considerably alleviate excessive NPCs apoptosis and senescence and then ameliorate IVDD progression. Further investigations might be carried out on the potential role of TBK1 in IVDD development in the near future.

Materials and methods

Ethics statement

The experimental procedures and animal uses and care protocols were strictly followed according to the guidance for the animal Care and Use of Laboratory protocols of the National Institutes of Health and approved by the Animal Care and Use Committee of Wenzhou Medical University (wydw2014-0129). All experiments to conduct our study were followed the guidelines of the Helsinki Declaration [32].

Human NPCs

The degenerated NP tissues from IVDD patients (Pfirrmann grades II, n = 3; Pfirrmann grades III, n = 3; Pfirrmann grades V, n = 3) were selectively collected for further investigations accord-
ing to the Pfirrmann grading scale. The patients were no other complications related to IVDD including obesity and diabetes mel-
itus. The sample tissues were cut up into 1 mm³ and properly
washed by phosphate buffered saline (PBS) solution for three times
each time 5 min. Furthermore, the slices were assimilated with 0.25% type II collagenase for 3 h at 37 °C. After cleansing and cen-
trifugation, NPCs were cultured in plate containing in DMEM/F12
with 10% FBS as well as 1% penicillin in 5% CO₂ at 37 °C incubator.

Rat NPCs culture

Gel-like NP tissues was carefully isolated from Sprague-Dawley
rats (15 male, average weight about 100–130 g), according to the
previous briefly methods and properly treated with 0.1% colla-
genase (Sigma Aldrich, St Louis, USA) and 2μ/ml hyaluronidase
(Sigma Aldrich, St Louis, USA) for 4 h. Next, the cells were carefully
plated for 2 weeks in DMEM/F12 medium with 10% FBS also 1% penicillin 37 °C. Then, the culture medium was refreshed by third
times in every week. The cells from the second passage were care-
fully applied in following investigations in our experiments.

TUNEL staining

Transfere-mediated deoxyuridine triphosphate-biotin nick end
labeling (TUNEL) staining was notably conducted to detect the
deoxyribonucleic acid (DNA) damage level. After treatment,
NPCs were carefully stained with cell death detection kit (Roche,
Basel, Switzerland) for 30 min at 37 °C, according to the manufac-
turer's instructions. 4, 6-diamidino-2-phenylindole (DAPI) solution
was carefully performed for the nuclei staining. Three independent
and blinded observers chose twenty fields of each sample and cap-
tured images under a confocal fluorescence microscope (Nikon,
Japan).

Senescence analysis

β-galactosidase (SA-β-gal) staining kit (Beyotime, Shanghai,
China) was performed to measure senescence level, according
to the manufacturer’s protocols.

Western blotting

The NPCs were carefully lysed in ice-cold RIPA buffer solution
with 1 mM PMSF (phenyl methane sulfonyl fluoride, Beyotime). To measure the protein concentration of each sample, BCA protein
assay kit (Beyotime) was used according to the manufacturer's pro-
tocols. Thereafter, the protein samples were carefully separated via
sodium dodecylsulfate-polyacrylamide (SDS) gel electrophoresis
and then carefully transferred to polyvinylidene difluoride mem-
brane (PVDF) (Millipore, USA). Then, the membranes were suffi-
ciently blocked with 5% nonfat dry milk solution for 2 h at room
temperature. After blocking, the membranes were carefully washed by PBS for 3 times each time 7 min. Next, the bands were
stained with 50 nM LTR (Invitrogen, Grand Island, NY) at
8 h. After washed with PBS for 3 times, the cells were stained with
DAPI and images were obtained using a confocal fluorescence
microscope (Nikon, Japan).

RT-PCR

TRizol reagent was used to extract the total RNA tissues, accord-
ing to the manufacturer's instructions. Then, cDNA was synthe-
sized and augmented using the Prime Script-RT reagent kit and
SYBR Premix Ex Taq (Sangon). Afterwards, the target gene expres-
sion levels were measured by using DDct method, as previously
described. Additionally, primers were synthesized by Invitrogen
(GAPDH primers: forward GGCCTTCCGTGTCTTACC and reverse
TGCCGTCACACCCCTC; TBK1 primers: forward GGAGACCTG-
TATGCGTGC and reverse CAATACTCATCACCCGC).

Flow cytometric evaluation

Cell apoptosis rate was assessed by flow cytometry strictly
according to the manufacturer's protocol. NPCs and cell super-
natant were collected and centrifuged for 3 min at 1200 r.p.m.
The NPCs were gently resuspended in 500 μL of binding buffer.
Next, PI solution and FITC-labelled Annexin V were incubated with
cells for 20 min. The ratio of cell apoptosis was detected using a
CytoFLEX LX Flow Cytometer - Beckman Coulter.

EDU assay

Cultured overnight for 24 h before treatment, each dish was
added with Edu assay solution (Beyotime, Shanghai, China) for
8 h. After washed with PBS for 3 times, the cells were stained with
DAPI and images were obtained using a confocal fluorescence
microscope (Nikon, Japan).

Immunofluorescence

NPCs were properly washed in PBS and carefully fixed with 4%
paraformaldehyde solution. Then, the NPCs were carefully perme-
ated in 0.1% Triton X-100 solution for 3 min. Then, the cell samples
were blocked with 5% bovine serum albumin (BSA) for 30 min at
37 °C. After proper incubation, the cell slides were properly washed
with PBS for 3 times each time 5 min. Then, the cells were incu-
bated with specific primary antibodies (LC3, 1:200; LAMP1, 1:50;
p-TBK1, 1:100; p62, 1:100; TOM20, 1:100) at 4 °C for overnight
incubation. After overnight incubation, the cells were cleansed
with PBS for 3 times each time 5 min. Next, the cell sample slides
were carefully incubated with Alexa Fluor 488 or Alexa Fluor 594
conjugated anti-bodies for 2 h at room temperature and stained with 4', 6-diamidino-2-phenylindole (DAPI) solution staining for 5 min. A confocal fluorescence microscope (Nikon, Japan) was finally used to capture the images.

Lysotracker red staining

To determine the number and function of lysosomes, Lysyo-
tracker Red (LTR) staining was used. Briefly, the NPCs were resus-
pended and prepared in a 6-well plate. Thereafter, the treated NPCs
were stained with 50 nM LTR (Invitrogen, Grand Island, NY) at
37 °C for 30 min. Then, DAPI solution was used for 5 min for nuclei
staining. The cells were properly washed by PBS for 3 times each
time 5 min. Then, the cell sample slides were carefully observed with a confocal fluorescence microscope (Nikon, Japan). Finally,
Image J (Bethesda, MD, USA) was performed to measure the fluo-
rescence intensity with two independent and blinded observers.

Lentivirus transfection

Lenti-TBK1 transfection (GeneChem, Shanghai, China) was
applied for TBK1 overexpression. Then, the cells were cautiously
transfected with Lenti-virus at 40%; >95% confluences of the cells
were viable after 12 h later. After changing the medium, the cells were thoroughly incubated for a further 3 days. Therefore, western blotting was performed to measure the transfections efficacy.

**Si-RNA transfections**

Specific si-RNAs for Parkin and control were purchased from Santa Cruz Biotechnology. Si-RNA against TBK1 (Invitrogen, Carlsbad, CA, USA) was applied for TBK1 knockdown. Then, NPCs were carefully transfected with specific si-RNAs followed by the manufacturer's instructions.

**Rat IVDD model**

Adult--male Sprague-Dawley rats (normal average weight 200–250 g) were acquired from The Laboratory Animal Center of Wenzhou Medical University. Rat IVDD experimental model was constructed, according to the previous methods [33]. In preparation for the tested animal, rats were intraperitoneally injected with 2% w/v pentobarbital (40 mg/kg) dose. Furthermore, the experimental level was determined by counting the vertebrae using trial radiograph. Together with the rat tail disc (C07/8) was located by digital palpation on the coccygeal vertebrae. To puncture the whole AF layer on the tail skin, needles (21G) were cautiously applied. Then, all the needles were scrupulously rotated at 360° and followed in the disc for 30 sec. Then, 5 μl lentivirus-normal control (Lenti-Ctrl) or lentivirus-TBK1 (Lenti-TBK1) was meticulously injected into the center space of the NP tissues by a micro-letter syringe with a needle of 27 gauges (10 ml, Gaoge, Shanghai, China). Finally, all rats were haphazardly divided into three groups: Sham, IVDD + Lv-Ctrl, IVDD + Lv-TBK1. All operators were indiscriminate to experimental animal grouping.

**Histopathological analysis**

To measure the degree of IVDD, all rats were sacrificed at 8 weeks after surgery. After tissue collection, tissue sections (5 μm) were carefully cut. In addition, the disc tissue sample sides were carefully stained by Safranin O fast green (SO), haematoxylin and eosin (H.E) and Alcian Blue (A.B), according to the manufacturer’s protocols. For SO staining, deparaffinized sections were stained with SO solution (Sigma-Aldrich) and subsequently counterstained with 0.2% fast-green solution (Sigma-Aldrich). For HE staining, deparaffinized sections were stained with Haematoxylin (Thermo Scientific, UK) for 7 min and counterstained in Eosin (Thermo Scientific, UK) for 1 min. A.B staining was performed using Alcian Blue solution (0.1% A. B 8GX in 0.1 N HCl) for 30 min at room temperature. H. E staining was performed to evaluate the morphological changes of NPCs. The SO staining and A. B staining were performed to detect the cellularity and morphology of NP tissues examined by other three histology analysts in a blinded manner by a light microscope and carefully evaluated by using a grading scale, as described previously [34]. Therefore, the histopathological scores were 5 for the normal disc, 6–11 for the moderate degenerated disc and 12–15 for the severe degenerated disc. In the end, images were scrupulously captured using a light microscope (Nikon, Japan).

**Immunohistochemical staining**

All discs tissue samples were neatly embedded in paraffin and 5 μm sections were carefully cut. Thereafter, the tissue sections were sufficiently deparaffinized and rehydrated, and blocked by 3% (v/v) H₂O₂ solution for 10 min. After washing the sample tissue slides by PBS solution, the sample slides were neatly incubated with 5% BSA solution for 30 min at 37 °C. Then, the slides again properly washed by PBS solution for 3 times. After that, the sample slides were carefully kept with specific primary antibodies (anti-LC-III, anti-cleaved caspase-3) at °C for overnight incubation. After the incubation, the sample slides were cautiously incubated with specific HRP-secondary antibodies. Finally, images were attentively captured by a light microscope (Nikon, Japan).

**Co-immunoprecipitation (co-ip)**

A commercial kit was attentively used for p-TBK1 co-ip construction, according to the manufacturer’s protocols (Pierce Crosslink IP kit). After 24 h treatment, NPCs were kept on the cold ice and properly cleansed with PBS solution for 3 times each time 5 min. However, the total protein was lysated as mentioned earlier. To assess the p-TBK1-p62 binding, protein lysates were thoroughly immunoprecipitated withDynabeadscontentp62specificantibody4°C forovernightincubation. Then, the immunocomplexes were cautiously collected, according to the manufacturer’s protocols. After that, the specific secondary antibodies were incubated. Finally, western blotting was performed to detect the immunolabeling.

**X-ray image acquisition**

X-ray images were attentively captured on all experiment animals, after 8 weeks of surgery, all rats were anaesthetized by intraperitoneally 2% pentobarbital (40 mg/kg) injection. For capturing the X-ray images, rats were tightly fixed in a prone position. In addition, the disc height index (DHI) was tightly measured by using a previously described methods [35].

**Data analysis**

All substantial data are considered as the means ± standard deviations (SDs). Our results were compared through one-way analysis of variance and Tukey’s post hoc test in GraphPad Prism (USA, version. 6). Whereas p values < 0.05 were generally considered as significant value.

**Results**

**TBK1 expression level reduces in degenerated NP tissue and senescent NP cells**

To investigate the correlation between IVDD and TBK1, we carefully collected the intervertebral disc tissues of various degenerative grades to perform western blotting. Our results showed that the expression level of TBK1 was vastly declined with the degree of disc degenerations in human (Fig. 1A–C). IVDD is strictly age-dependent disease; senescence is known to anticipate in IVDD progression. Thus, we explored whether TBK1 could significantly change in normal aging process. The total protein of NP tissues with low degenerative grade was assessed TBK1 level at passages 2, 10 and 15 and p16INK4a (p16) level, a kind specific indicator of senescence. The expression level of TBK1 was markedly down-regulated and p16 expression level was also markedly increased (Fig. 1D–F). Consequently, TBK1 level was considerably lower in NPCs in old rats than young rats, according to the immunohistochemistry staining results (Fig. 1G, H). We also showed in Fig. S1 to demonstrate the TBK1 expression level changed in the rat IVDD tissues. From the immunohistochemistry staining results, the TBK1 expression of IVDD NP tissues was significantly reduced compared to the healthy rat NP tissues. Taken together, the TBK1 expression level was tightly associated with IVDD.
TBK1 overexpression considerably attenuated the replicative senescence of NPCs and TNF-α induced cell damage

In order to explore the relationship between TBK1 and IVDD in vitro, we transfected the rat NPCs with lentivirus-TBK1 to up-regulate the TBK1 expression level (Fig. 2A). Afterwards, western blotting clearly suggested the TBK1 protein expression level precisely was increased, indicating that there are no off-target effects. We markedly found that cells transfected with lentivirus-TBK1 (Lv-TBK1) remarkably exhibited less p16INK4a (p16) activity than cell transfected with lentivirus-Control (Lv-Ctrl) at passage 15, showing that TBK1 exerted an enormous function on NPCs senescence (Fig. 2B, C). In addition to normal cellular senescence, we examined the potential role of TBK1 in pathologic stimulation, for example TNF-α, an inflammatory mediator, plays an important role in the pathogenesis and progression of several degenerative diseases. The p16 and cleaved caspase-3 (CC3) expression levels were assessed to evaluate the TBK1-mediated anti-apoptotic and anti-senescence. Therefore, the substantial results in our study showed that TNF-α significantly increased in the NPCs and the expressions level of p16 and CC3, suggesting that TNF-α induced apoptosis and premature senescence, which were inhibited by Lv-TBK1 (Fig. 2D–F). From the TUNEL staining and SA-β-gal staining, we could observe that TNF-α stimulation markedly increased the positive points of TUNEL and SA-β-gal. Conversely, administration of Lv-TBK1 significantly reversed the TNF-1x induced excessive senescence and apoptosis (Fig. 2G–I).

Inhibition of TBK1 intensified senescence and apoptosis of NPCs induced by TNF-α

Despite results of overexpression of TBK1 induced anti-apoptosis and anti-senescence effects, we also detect its lost-of-function through administration of Si-TBK1. We pretreated NPCs with different Si-TBK1 and followed with TNF-α, and then performed western blot. The result showed that Si-TBK1 significantly decreased TBK1 level (Fig. S2A, B). Treatment with si-TBK1 further exacerbated senescence and apoptosis induced by TNF-α stimulation (Fig. S2C, D). We also assessed apoptosis via flow cytometry of annexin V/PI. TBK1 knockdown in NPCs treated with TNF-α significantly increased cell apoptosis compared to NPCs treated with TNF-α alone. Inversely, TBK1 overexpression markedly reversed higher cell apoptosis induced by TNF-α (Fig. S3A, B). In addition, Si-RNA against TBK1 further attenuated TNF-α induced EDU fluorescence intensity decay. In contrast to this, the result suggested that, TBK1 overexpression rescued impaired proliferative capacity (Fig. S3C, D). Thus, we concluded that TBK1 played a critical role in NPCs senescence and apoptosis.
TBK1 overexpression increases autophagic flux in NPCs and inhibition of autophagy attenuated the protective effects of TBK1 under TNF-α stimulation

Several evidences clearly indicate that targeting autophagic flux is a potential therapeutic strategy for IVDD treatment [36–38]. In our current study, we distinctly demonstrated that TNF-α treatment could cause autophagic flux damage [39]. Regardless, the potential role of TBK1 in the regulation of autophagic flux still remains unclear. Nevertheless, Bafilomycin A1 is called as a typical lysosomal inhibitor was implemented to measure the autophagic flux activity. During normal autophagy process, autolysosomes degeneration and autophagosomes generation lead to reduce the LC3-II/LC3-I ratio. In our study, the LC3-II/LC3-I ratio in the Bafilomycin A1 + Lv-TBK1 group was highest in other three groups and the p62 expression of Lv-TBK1 group was significantly lower than other Bafilomycin A1 group and Bafilomycin A1 + Lv-TBK1 group. Thus, these important findings clearly indicated that TBK1 overexpression significantly increased autophagic flux (Fig. S4). Nonetheless, LTR is known as a specific lysosome tropic probe can considerably exhibit red fluorescence dependent on lysosomal pH. Therefore, our results clearly illustrate that the weakened red fluorescence intensity in NPCs dealt by TNF-α, demonstrating that TNF-α can tremendously lead to lysosomal dysfunctions, as well this event was enormously restored after TBK1 overexpression (Fig. 3A). Autophagy-lysosome fusion was promoted by Lv-TBK1 transfection, demonstrated by LC3-II as well as LAMP1 immunofluorescence double staining (Fig. 3B). To determine the positive role of the overexpression TBK1-induced autophagy in NPCs, we selected CQ, is known as a lysosomal cavity alkalizer can

Fig. 2. TBK1 overexpression attenuated replicative senescence of NPCs and TNF-1α induced cell damage. (A) The protein expression of TBK1 in NPCs treated with Lv-Ctrl and Lv-TBK1. (B, C) The protein expression and quantification of TBK1 in passage 15th NPCs treated with Lv-Ctrl and Lv-TBK1. (D–F) The protein expression of Cleaved-caspase-3 and p16INK4a in NPCs transfected with Lv-Ctrl and Lv-TBK1 before TNF-α (10 ng/ml) treatment. (G, H) SA-β-gal staining assay was performed in rat NP cells as treated above (n = 6). (G, I) TUNEL staining assay was performed in rat NP cells as treated above. All experiments were performed as means ± SD of 3 times in duplicates. *P < 0.05, **P < 0.01, ***P > 0.05.
dramatically inhibit the downstream autophagic flux. In Fig. 3C and D, TBK1 overexpression against TNF-induced apoptosis and senescence was hugely inhibited by CQ treatment, illustrating the cleaved caspase-3, and p16INK4a protein expression levels were immensely inhibited. In addition, TUNEL staining results also showed inhibition of autophagy can substantially reversed the anti-apoptosis effect caused by TBK1 overexpression (Fig. S5). Therefore, these substantial findings surely demonstrate that the anti-apoptosis and anti-aging ability induced by TBK1 is regulated by autophagic flux.

p62 phosphorylated by TBK1 at S403 is involved in autophagic process

Effective removal of harmed proteins and protein aggregates plays a critical role in cell homeostasis. However, p62 is characteristically considered as a scaffold protein, can substantially regulate various kinds of selective macro-autophagy. Phosphorylated p62 not only enhances autophagic degradation [40]. We detected all phosphorylated p62 antibodies including Ser403, Ser349 and Thr269/Ser272, possibly related to autophagic degradation, which were reported before. Only p62 Ser 403 and Ser 349 were changed in the TNF-α concentration manner (Fig. 4A, B). If autophagic degradation of p62 was caused by phosphorylation, inhibiting autophagy can remarkably lead to the phosphorylated p62 accumulation. Furthermore, western blotting results clearly showed that p62 Ser 403 protein expression levels were markedly changed with administration of proteasome inhibitor MG132, whereas p62 Ser 349 did not, suggesting that S403 phosphorylation may promote the autophagosome in this condition (Fig. 4C). Meanwhile, to ensure that changes in phosphorylation levels are not due to changes in the basal content of p62, we constructed NPCs with an ATG5 knockdown (ATG5-KD) by using RNA interference techniques. Okadaic acid (OA) is known as a protein phosphatase 2 (PP2) inhibitor and Bafilomycin A1 (BafA1), the autophagosome-lysosome fusion inhibitor was used to detect the S403 level to confirm our hypothesis. Both OA and BafA1 can upregulate S403, while total p62 protein expression did not significantly change (Fig. 4D). To verify whether the S403 phosphorylation in the p62 is dependent on TBK1, we applied lentivirus to up-regulate the TBK1 expression level and detected p62 at S403. Furthermore, western blotting results illustrated that TNF-α significantly increased the S403 and p-TBK1 expression levels at S172, is called as an important form of TBK1 activation, without altering the total TBK1 and p62 protein expression levels. Lv-TBK1 seriously impacted the p62 and Ser 403 expression levels and further promoted the TBK1 phosphorylation, suggesting that there was potential relationship between p-TBK1 and p62 Ser 403 (Fig. 4E–G). Moreover, immunofluorescence double staining and co-immunoprecipitation findings demonstrated P-TBK1 directly contact with p62 (Fig. 4H–J). Therefore, these results substantially demonstrate that TBK1 may promote autophagic process by directly phosphorylation of p62 at S403.

Mitochondrial damage hugely induces the p-TBK1 activation at mitochondria Parkin-dependent manner

Previous investigations notably suggested that TBK1 activation at damaged mitochondria enhanced mitophagy dependent Parkin activity [41]. In addition, we also proved the positive role of Parkin-mediated mitophagy in IVDD progression [39,42]. We hypothesized that whether TBK1 overexpression induced enhanced autophagy in IVDD. For fluorescence kits, we found that...
TNF-\(\alpha\) markedly impair mito-tracker fluorescence intensity and increased mito-SOX level. This phenomenon was restored after TBK1 overexpression (Fig. 5A–C). TNF-\(\alpha\) stimulation also recruited p-TBK1 at mitochondria, which was proved by P-TBK1 and TOM20 immunofluorescence double staining. TBK1 overexpression enhanced the p-TBK1 level and p-TBK1 locating in the mitochondria. After using si-RNA against Parkin, TNF-\(\alpha\) and Lv-TBK1-induced TBK1 phosphorylation was inhibited. The co-localization of p-TBKI and mitochondria was also weakened (Fig. 5D, F). For autophagy situation, increased LC3-II/LC3-I ratio induced by Lv-TBK1 was also inhibited by si-Parkin. Certainly, Lv-TBK1 did not influence the Parkin expression, thus Parkin is the upstream molecule of TBK1 (Fig. 5I, J). Furthermore, si-Parkin also inhibited the LV-TBK1-induced positive function through western blotting, TUNEL staining and SA-\(\beta\)-gal staining (Fig. 5E, G, H). Therefore, these substantial results strongly suggest that Parkin considerably alleviates the TNF-\(\alpha\) induced apoptosis, senescence and mitochondrial dysfunctions in NPCs via enhancing mitophagy, which through phosphorylating TBK1.

TBK1 overexpression considerably ameliorates IVDD development in vivo

To explore the potential therapeutic effects of TBK1 in rats IVDD model, we carefully injected lentivirus into the rat intervertebral disc and performed X-ray, H. E staining, SO staining and immuno-
hithochemical staining to measure the changes of tissue morphology and immunology. The TBK1 mRNA expression of each group was detected. In IVDD + LV-TBK1 group, TBK1 mRNA expression level was significantly higher than other two groups. During puncture injury process, the TBK1 level of IVDD + Lv-Ctrl was slightly lower than in the control group (Fig. 6A). From X-ray images, we carefully found that TBK1 overexpression can delay IVDD surgery induced loss of disc height (Fig. 6B, C). Moreover,
Fig. 6. TBK1 overexpression ameliorates IVDD development in vivo. (A) The TBK1 mRNA of each group. (B, C) The X-ray of a rat-tail disc at 8 weeks after disc puncture surgery. (D) Representative HE, SO and Alcian Blue staining of NP tissues from each groups at 8 weeks post-surgery. (E, G) Immunohistochemical examination and quantification of Cleaved-caspase3 and LC3-II of each group. (F) The histological grades evaluated at 8 week in each group. All experiments were performed as means ± SD of 3 times in duplicates. (n = 10). *P < 0.05, **P < 0.01.
the histomorphological alteration in the IVDD rat model was measured by H, E, A, B and SO staining. H. E staining findings clearly suggested that NPCs structures were clearly disappeared and the fibrous ring was significantly irregular in IVDD + LV-ctrl, while in IVDD + LV-TBK1 group NP also regular fibrous ring were more suitable retained. In addition, the A. B and SO staining showed that NP loss was substantially alleviated by administration of Lv-TBK1 compared to the IVDD + LV-Ctrl group (Fig. 6D). The gelatin NP tissues were almost disappeared and replaced by fibrochondrocytes in IVDD + LV-Ctrl. AB staining demonstrated that the blue color of NP in IVDD + LV-TBK1 group was much stronger than IVDD + LV-Ctrl group. SO staining demonstrated that the NP tissues of IVDD + LV-TBK1 group displayed more red areas than IVDD + LV-Ctrl group. In addition, histopathological scores from SO staining also illustrated that the LV-TBK1 protection plays substantial roles in IVDD progression (Fig. 6F). Furthermore, immunohistochemistry staining and its related quantization markedly indicated that LV-TBK1 increased the LC3-II expression levels in NPCs, while decreased the cleaved caspase-3 positive points (Fig. 6E, G). We also performed TUNEL staining to evaluate further anti-apoptosis of TBK1 in vivo. Increased positive points of TUNEL were significantly reversed after administration of Lv-TBK1 (Fig. 5). In summary, these important findings surely suggested that TBK1 overexpression significantly ameliorated the disc degeneration in puncture-induced rat model.

Discussion

Senescence and apoptosis largely contribute to the pathological alterations of IVDD progression [43]. After several prolonged replication, senescence can be a central factor for aged disc progression. During chronic prolonged replication, we carefully found that TBK1 expression level was dramatically decreased in the NPCs. Moreover, at passage 15 of human NPCs, the p16 protein expression level was markedly up-regulated and TBK1 expression level was substantially decreased compared to the second passage of NPCs. Nevertheless, overexpression of TBK1 can retard natural aging process. Senescent process can be accelerated by several pathological stimulations or factors including inflammation and oxidative stress. In our current investigation, we generally applied TNF-α as a donor to induce the oxidative stress and inflammatory responses, which may trigger apoptosis, senescence and organelles such mitochondria and lysosome deterioration, misfolding proteins and damaged organelles proteins [44,45]. As the same as the human NPCs results, lentivirus-mediated TBK1 overexpression significantly inhibited the TNF-α stimulated apoptosis and senescence in rat NPCs. TBK1 plays central roles in autophagy, mitophagy process and chiefly phosphorylation of autophagy adaptors [46]. Consequently, we hypothesized that the potential anti-apoptotic and anti-senescent effects of TBK1 significantly revealed the autophagy activation. Moreover, we markedly found that CQ, a lysosomal cavity alkalizer and the final-period in autophagic flux, spearhead the autophagy activation. Moreover, we markedly found that CQ, a lysosomal cavity alkalizer and the final-period in autophagic flux, spearhead the autophagy activation. Furthermore, we showed that TBK1 overexpression may not only restore lysosome function but also autophagosomal and lysosomal fusion, which renovates the TNF-α-induced frustration of autophagic flux. Therefore, these remarkable findings were not reported in the previous investigations and further studies should be carried out on the autophagic mechanism of NPCs for the novel therapies of IVDD in the near future.

Previous investigations clearly documented that TBK1 plays crucial roles in suppressing tumor activities, involvement in cells cycle control and inflammatory responses. In addition, further studies revealed that the kinase activity of TBK1 is tightly regulated. During selective autophagy especially mitophagy, TBK1 phosphorylates several autophagy-receptor proteins including OPTN, NDP52 as well as p62. A recent investigation also demonstrates that the tight relationship between TBK1 and neurodegenerative disease, which suggests a possible role for TBK1 in other degenerative diseases including osteoarthritis and IVDD. Afterwards, we carefully collected intervertebral disc tissues and detected the TBK1 expression level. The results clearly showed that TBK1 expression level decreased with increase of the degeneration grade, also indicating that TBK1 may play a crucial role in the course of the pathogenesis and progression of IVDD.

However, p62/SQSTM1 phosphorylation is generally considered as a multifunctional protein, plays substantial roles in protein degradation through proteasomal regulation and autophagy process. Importantly, p62 is an ubiquitin binding autophagy receptor, which can bond ubiquitin to autophagy through sheltering both an ubiquitin binding domain and an LC-III interaction region. Thus, SQSTM1 remarkably mediates the poly-ubiquitinated proteins sequestration into the autophagosomes. Accumulating studies have documented SQSTM1 phosphorylation plays a significant role in the poly ubiquitinated proteins degradation in various diseases progression including Huntington’s disease [47,48]. Furthermore, prior evidence indicates that SQSTM1 phosphorylation at S403 hugely promotes the affinity between SQSTM1 and polyubiquitin chain, leading to the augmentation of autophagic removal of polyubiquitinated proteins. Consequently, TBK1-induced phosphorylation process causes bacterial or viral immune responses [49,50]. Very few studies on IVDD have been conducted until now. Due to the limited experimental conditions, we examined p62 phosphorylation associated with selective autophagy under TNF-α stimulation without performing tandem mass spectrometry (LC-MS/ MS). Fortunately, protease inhibitors, autophagy inhibitors and phosphorylation inhibitors, selected phosphorylation did not exhibit an obvious role in autophagic degradation, except S403. To demonstrate the substantial relationship between TBK1 and p62, we performed immunofluorescence double staining and immunoprecipitation. Thus, our results clearly indicated that p-TBK1 directly contact with p62. The reason why we did not directly prove the direct relationship between TBK1 and p62 is that the total amount of TBK1 did not change under the stimulation of TNF-α, while the expression level of p-TBK1 and p62 S403 was tremendously increased, indicating that TBK1 did not directly influence the p62 protein expression. Meanwhile, the Lv-TBK1 considerably increased the expression of p-TBK1 and S403 phosphorylation of p62 level.

Next, we explored the TBK1 activation in IVDD progression. Multiple studies have documented that TBK1 plays a significant role in the regulation of mitophagy in several cell types [51,52]. Mitophagy is actually regarded as a special form of selective autophagy, which hugely removes the damaged mitochondria [53]. Mitochondrial membrane depolarization largely initiates mitophagy process, which leads to the recruitment of specific adaptors such as PARK2-Parkin. Currently, mitophagy is triggered with PARK2 accumulation on the mitochondrial membrane under mitochondrial depolarization, then phosphorylation of ubiquitin promoting translocation of the E3 ubiquitin ligase Parkin (PARK2) [54,55]. Then, Parkin recruits specific autophagic cargo-receptors including p62 and NDP52, propelling sequestration into the autophagosomes [56]. In addition, several investigations have definitely demonstrated that Parkin on the mitochondrial outer membrane autop-
hagy cargo receptors in complexes with TBK1 protein kinase [57] and PINK1 and Parkin-mediated TBK1 activation at the mitochondria during mitophagy leads to inhibit mitosis due to the TBK1 sequestration from its physiological role at centrosomes [41], which means some potential relationships may contact Parkin and TBK1. In our current study, the si-Parkin and Lv-TBK1 administration markedly phosphorylate the TBK1 in an indirect way under TNF-α stimulation. In addition, si-Parkin notably inhibits the Lv-TBK1-induced anti-apoptosis and anti-senescence activities in vitro experiments. TBK1 overexpression in vivo with adenovirus injection could not significantly show any negative changes on the rat models in terms of disc status, lifespan and mobility, clearly suggesting the surveillance of Lv-TBK1 transfections can be potential therapeutic option for IVDD in the near future.

There are several limitations in our investigation. Firstly, the potential mechanism of TBK1 restores the mitochondrial and lysosomal functions were not substantially confirmed. In addition, the actual reason for the decreased of TBK expression level during senescent process in vitro and vivo is still unclear. Whether there are transcriptional changes and abnormal post-transcriptional degradation should also be further investigated in more details in the near future. Other signaling pathways can also be related with TBK1 to autophagy process is remained unclear until now. Therefore, further investigations should be carried out in briefly to explore the possible signaling pathways are involved between TBK1 and autophagy in the near future.

In summary, we successfully demonstrated that TBK1 plays critical roles in the pathogenesis and progression of IVDD. Furthermore, our remarkable findings demonstrate a strong genetic interaction in rat NPCs under TNF-1α stimulation, leading us to conclude that TBK1 significantly restores the blocked autophagy flux and influences the cellular selective autophagy through phosphorylation at serine 403 (S403) of p62 in Parkin pathway manner. In addition, overexpression of TBK1 can considerably alleviate the abnormal lysosomes and mitochondria dysfunctions. The comprehensive promotion of autophagy caused by TBK1 can remarkably alleviate the senescence and apoptosis induced by TNF-1α administration.

Conclusions

Our results represent an important first step in exploring the alternative treatments for NPCs loss through using the lentivirus-TBK1 and these substantial findings obviously conclude that exploring the actual mechanisms and pathogenesis of IVDD can provide potential therapeutic targets in the near future.

Compliance with ethics requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors’ contributions

S.L.H. and X.Y.W. designed the project. L.C., L.B.N. and Y.F.S performed the experiments and analyzed the results. W.Y.G and X.L.Z. revised the manuscript. S.L.H., Y.L., H.M.J., X.Y.W. drafted the manuscript and A.A.M. edited languages as well as grammatical concerns of the full manuscript. Finally, all authors successfully revised the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.08.011.

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