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The effect of transforming growth factor β1 on the crosstalk between autophagy and apoptosis in the annulus fibrosus cells under serum deprivation

Bin-Bin Ni, Bo Li, Yue-Hua Yang, Jiang-Wei Chen, Ke Chen, Sheng-Dan Jiang, Lei-Sheng Jiang *

Department of Orthopedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

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ABSTRACT

Autophagy and apoptosis are important in maintaining the metabolic homeostasis of intervertebral disc cells, and transforming growth factor-β1 (TGF-β1) is able to delay intervertebral disc degeneration. This study determined the effect of TGF-β1 on the crosstalk between autophagy and apoptosis in the disc cells, with the aim to provide molecular mechanism support for the prevention and treatment of disc degeneration. Annulus fibrosus (AF) cells were isolated and cultured under serum starvation. 10 ng/mL TGF-β1 reduced the apoptosis incidence in the cells under serum starvation for 48 h, down-regulated the autophagy incidence in the cells pretreated with 3-methyladenine (3-MA) or Bafilomycin A (Baf A), partly rescued the increased apoptosis incidence in the cells pretreated with 3-MA, while further reduced the decreased apoptosis incidence in the cells pretreated with Baf A. Meanwhile, TGF-β1 down-regulated the expressions of autophagic and apoptotic markers in the cells under starvation, partly down-regulated the expressions of Beclin-1, LC3 II/I and cleaved caspase-3 in the cells pretreated with 3-MA or Baf A, while significantly decreased the expression of Bax/Bcl-2 in the cells pretreated with Baf A. 3-MA blocked the phosphorylation of both AKT and mTOR and partly reduced the inhibitory effect of TGF-β1 on the expression of LC3 II/I and cleaved caspase-3. TGF-β1 enhanced the expression of p-ERK1/2 and down-regulated the expressions of LC3 II/I and cleaved caspase-3. U0126 partly reversed this inhibitory effect of TGF-β1. In conclusion, TGF-β1 protected against apoptosis of AF cells under starvation through down-regulating excessive autophagy. PI3K–AKT–mTOR and MAPK–ERK1/2 were the possible signaling pathways involved in this process.

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1. Introduction

Intervertebral disc degeneration (IVDD) was recognized as the main cause for lower back pain [1–3], and IVDD did exist in 97% of individuals aged more than 50 years old in an autopsy study [4]. IVDD is definitely a consequence of aging. However, the underlying molecular mechanism implicated in the pathogenesis of IVDD has not been fully understood, although increased death of the intervertebral disc cells has been demonstrated to have an essential role in this process [5–10].

Apoptosis and autophagy are two main programmed cell death patterns [11,12]. In addition to their importance as biological phenomena, defective apoptotic process or excessive autophagy has been implicated in an extensive variety of diseases [10,13,14]. It was reported that increased apoptosis or excessive autophagy in the intervertebral disc cells was also involved in the pathogenesis of IVDD [11,15–17]. Meanwhile, apoptosis and autophagy were closely linked and autophagy could either inhibit or delay the occurrence of apoptosis [18–23], or promote apoptosis [24,25]. However, the crosstalk between autophagy and apoptosis in the intervertebral disc cells and its implication in the pathogenesis of IVDD has not been clarified.

Studies have shown that transforming growth factor-β1 (TGF-β1) was a critical factor in cartilage and spine tissue development [26,27]. It increased the proteoglycan synthesis in human intervertebral discs [28], and decreased the intervertebral disc degeneration in rabbits [29]. Meanwhile, the effect of TGF-β1 on the crosstalk between apoptosis and autophagy in various types of cells has also been studied with contradictory results [30–32]. TGF-β1 could protect against mesangial cell apoptosis via induction of autophagy [19], while it could induce autophagy and promote

* Corresponding author. Address: Department of Orthopedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, China. Tel.: +86 13002195209, +86 21 25077970.
E-mail address: jiangleisheng@126.com (L.-S. Jiang).

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apoptosis in renal tubular epithelial cells [33], bovine mammary epithelial cell [34], and human hepatocellular carcinoma cells [32]. However, the role of TGF-β1 on the crosstalk between apoptosis and autophagy in the intervertebral disc cells has never been comprehensively explored.

Based on the background mentioned above, we believe that understanding the molecular mechanisms involving the survival and death of the intervertebral disc cells has important clinical significance in the prevention and treatment of degenerative discogenic diseases. According to our previous study, intervertebral disc cells underwent programmed cell death via different signal transduction pathways in response to multiple stimuli [5]. In this study, we used serum deprivation to induce programmed cell death in the annulus fibrosus (AF) cells as previous described [35–37], observed the interactions between apoptosis and autophagy in the cells, and investigated the effect of TGF-β1 on this crosstalk. Because the PI3K/AKT/mTOR and ERK1/2 signaling pathways were commonly involved in the regulation of autophagy, we hypothesized that TGF-β1 could protect against apoptosis through inhibition of excessive autophagy in the AF cells under serum starvation via the PI3K/AKT/mTOR and ERK1/2 signaling pathways.

2. Materials and methods

2.1. Isolation and culture of AF cells

Twenty-four Male Sprague–Dawley rats aged 2 months old were killed by an overdose intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg). The AF tissue was obtained from L1–L6 intervertebral discs under aseptic condition. Isolation and culture of the primary-passage AF cells were carried out as previously described [35]. AF cells were collected and seeded into appropriate culture plates maintained in a monolayer at a 37 °C, 5% CO_2 environment, and the first-passage AF cells were used throughout the experiments. All experimental procedures were approved by the Animal Care and Use Committee of our university, conformed to the institutional guidelines.

2.2. Reagents and antibodies

Rabbit monoclonal antibodies T-AKT, P-AKT, P-mTOR, T-mTOR, cleaved caspase-3, Beclin-1 and LC3 II/I, Bcl-2, T-ERK1/2 and P-ERK1/2 and rabbit polyclonal antibody Bax were purchased from Cell Signaling Technologies (Beverly, MA, USA). 3-Methyladenine (3-MA), acridine orange and monodansylcadaverine (MDC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The Lyso-Tracker Kit, Lipofectamine-2000 transfection reagent, the Alexafluor 488-labeled and the Alexafluor 594-labeled secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Annexin V-FITC apoptosis detection kit I was got from BD Pharmingen (San Diego, CA, USA). DMEM/Ham's F12 was purchased from Gibco/Invitrogen (Carlsbad, CA, USA). TGF-β1 was purchased from Peprotech (Rocky Hill, NJ, USA). Hoechst 33258 and Bafilomycin A were purchased from Biotime (Shanghai, China). GFP-LC3 plasmid was kindly provided by Dr. Li Wang (Neonatology, Shanghai Jiao Tong University).

2.3. Transmission electronic microscopy for autophagosomes

The AF cells were cultured in DMEM with complete serum deprivation for 24 and 48 h. Then the cells were separately fixed in 0.2% glutaraldehyde in phosphate-buffered saline (PBS, PH = 7.4) for 2 h at room temperature and postfixed in 1% osmium tetroxide in water for 1 h, and stained in 2% uranyl acetate in water for 1 h in the dark. After dehydration in an ascending series of ethanol, the AF cells were embedded in Durcopan ACM for 6 h, cut into 80 nm sections. All these sections were stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Philips CM, Netherlands). AF cells cultured in DMEM containing 10% fetal bovine serum (FBS) were served as control.

2.4. Detection of apoptotic cells by Hoechst 33258 staining

The AF cells in starvation for 12, 24, 36 and 48 h were prepared at a density of 5000 cells per well in 24-well plates. Apoptotic cells were detected by using the Hoechst 33258 (2 μg/mL) staining as previously described [36,38]. Morphologic changes in the apoptotic nuclei were evaluated under a fluorescence microscope with excitation wave length 350 nm, emission filter 460 nm (Olympus Fluoview, Japan). A percentage was calculated by the strong blue staining cells/total cells ratio × 100% in five random fields. AF cells cultured in DMEM containing 10% FBS were served as control.

2.5. Autophagy incidence quantified by flow cytometry

The first-passage AF cells were cultured in 6-well plates at 2×10^5 cells per well with DMEM containing 10% FBS. After reaching 90% confluence, the medium was changed to DMEM containing 1% FBS for 12 h to synchronize the cells and the cells were subjected to serum starvation. Autophagy incidence was measured by quantifying the development of acidic vesicular organelles (AVOs) after AO staining (0.5 μg/mL) for 15 min at 37 °C [39], and immediately determined as percentage of AO positive cells analyzed with a FACS Canto II software (BD, USA).

2.6. Measurement of apoptosis by flow cytometry

The apoptosis incidence of AF cells was detected by using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Pharmingen) as previously described [35]. Apoptotic cells, including those staining positive for Annexin V-FITC and negative for PI and those double positive for Annexin V-FITC and PI, were counted and represented as a percentage of the total cell count.

2.7. Real-time PCR analysis

Total RNA from the AF cells were extracted using RNAiso Plus Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The purity and concentration of isolated RNA were measured spectrophotometrically at OD_{260} and OD_{280}. Complementary DNA was synthesized from 1 μg of total RNA using the PrimeScript RT Master Mix Kit (Takara, Dalian, China). The gene expression of Beclin-1, LC-3, and GAPDH was measured by real-time PCR using the Premix EX Taq Kit (Tli RnaseH Plus, TakaRa) and the ABI Prism 7500 sequence detection system (Applied Biosystem, Foster City, CA, USA). The specific primers were designed and synthesized commercially (Takara, Dalian, China), and the sequences were listed below:

Beclin-1 (bp:154): forward, 5′-GAAACTGGACACGAGCTTCAAGA-3′; reverse, 5′-ACCATCCTGGCGAGTTTCAATA-3′ (Primer set ID: RA056559; GenBank Acc. NM 001034117.1).

LC3 (bp:102): forward, 5′-AGCTCTGAAGGCAACAGCAACA-3′; reverse, 5′-GCTCCATGCAGGTAGCAGGAA-3′ (Primer set ID: RA019913; GenBank Acc. NM 022867.2).

GAPDH (bp:143): forward, 5′-GGCACAGTCAAGGCTGAGAATG-3′; reverse, 5′-ATGGTGGTGAAGACGCCAGTA-3′ (Primer set ID: RA 015380; GenBank Acc. NM 017008.3).

The reaction mixture was amplified at 50 °C for 2 min and denatured at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The optimal concentrations of primers and templates

used in each reaction were established according to the standard curve created before the reaction. The fold change in all sample mRNA expressions was calculated by $2^{-\Delta\Delta C}$.

2.8. Western blot analysis

The AF cells were treated according to the study protocol and harvested at indicated time points. 40 μg total protein from different samples were electrophoresed in different concentrations (6%, 10%, 12%) of sodium dodecyl sulfate polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (Beyotime, Shanghai, China). After washing for three times with 10 mM Tris-buffered saline with 1.0% Tween-20, the polyvinylidene difluoride membranes were incubated with 5% dehydrated skim milk to block nonspecific protein binding and then incubated with rabbit monoclonal antibodies against cleaved caspase-3 (CST, #9964, 1:1000 dilution), Bcl-2 (CST, #2870, 1:2000 dilution), T-AKT (CST, #4691, 1:1000 dilution), Phospho-AKT (CST, #4060, 1:1000 dilution), Beclin-1 (CST, #3495, 1:1000 dilution), LC3 II/I (CST, #3868, 1:500 dilution), T-mTOR (CST, #2983, 1:2000 dilution), Phospho-mTOR (CST, #5536, 1:2000 dilution), T-P44/42 MAPK–ERK1/2 (CST, #4695, 1:1000 dilution), Phospho-P44/42 MAPK–ERK1/2 (CST, #4370, 1:1000 dilution), and rabbit polyclonal antibodies against Bax (CST, #2772, 1:1000 dilution) overnight at 4 °C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibodies (diluted 1:1000–1:2000, Dako, Carpinteria, CA, USA) and visualized by the Super Signal Chemiluminescent Substrate system (Millipore or BeyoECL Plus). Protein band images were quantified by densitometry analysis using the Bio-Rad Image Lab 2.0 software (Bio-Rad Laboratory, Hercules, CA, USA).

2.9. Lysosomal activity detected by monodansylcadaverine and LysoTracker staining

Monodansylcadaverine (MDC) is a marker for autolysosomes. After starvation for 48 h, AF cells with or without 10 ng/mL TGF-β1 were incubated with MDC (0.05 mM) for 10 min at 37 °C. Subsequently, the cells were washed four times with PBS and analyzed by fluorescence microscopy (excitation wavelength 380 nm, emission filter 525 nm) (Olympus Fluoview, Japan). Lysosomal activity was assessed by using the LysoTracker kit (Invitrogen, USA). After cultured at a density of 5000 cells per well, the starved AF cells with or without TGF-β1 were incubated with LysoTracker Red (100 nM) for 1 h at 37 °C and then observed using confocal microscopy (Olympus Fluoview, Japan). The cells cultured under serum condition were served as control groups.

2.10. Immunofluorescence analysis for autophagy related proteins

AF cells were seeded onto a 24-well plate at a density of 5000/per well with sterile glass cover slips as previous described [35]. After serum deprivation for 48 h with or without 10 ng/mL TGF-β1, the AF cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. After washed three times with PBS, the AF cells were permeabilized with 0.25% Triton-X 100 in PBS for 15 min and washed three times again in PBS, and then blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. The AF cells were then incubated with primary antibodies Beclin-1 (CST, #3495, 1:50 dilution) and LC3 II/I (CST, #3868, 1:30 dilution) respectively in 5% BSA overnight at 4 °C. Cells cultured under serum condition with isotype antibody were served as control groups. Subsequently, the AF cells were washed in PBS for three times and incubated with Alexa Fluor-488 or Alexa Fluor-594 conjugated anti-rabbit secondary antibody (dilution 1:100) for 30 min at room temperature in dark. The cells were then washed three times with PBS and incubated with DAPI for 5 min. Finally, the cells were examined by a fluorescence microscopy (Olympus BX-FLA, Japan).

2.11. GFP-LC3 plasmid transfection and autophagy assay

AF cells were incubated at a density of 2×10^5 on 12-well plates and cultured up to 60% confluence. GFP-LC3 transfection was carried out with Lipofectamine 2000 according to the manufacturer's recommendation. 2 mg/mL GFP-LC3 plasmid DNA in each dish was used. After incubation in Opti-MEM medium for 6 h, the cells were incubated in complete culture medium again for 24 h. Then the transfected cells were treated with serum deprivation for 48 h with or without 10 ng/mL TGF-β1. Finally, the cells were fixed with 4% paraformaldehyde, and then washed twice in cold PBS. Autophagy was evaluated by analyzing the formation of fluorescent puncta of autophagosomes in GFP-LC3 transfected cells under the fluorescent microscope.

2.12. Statistical analysis

Data were presented as means ± SD (standard deviation) for at least three independent experiments. Statistical analyses were performed by using the SPSS 13 statistical software program (SPSS Inc., IL, USA). Multiple comparison of data among the groups were determined by the one-way ANOVA followed by the least significant difference test (Fisher test) and the significance was evaluated by the unpaired Student's test for comparisons between two means. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. The evidence of autophagy and apoptosis in the AF cells under serum deprivation and their interactions

At first, the evidence of autophagy and apoptosis in the AF cells under serum deprivation were investigated. Autophagosomes, characterized as double-membrane vacuolar structures containing different cytoplasmic fragment of damaged organelles or aggregate-prone proteins, could be detected under transmission electronic microscopy in the AF cells after starvation for 24 h, and could be more easily found in the cells after starvation for 48 h (Fig. 1A). Hoechst 33258 staining demonstrated that apoptotic cells began to be detectable after starvation for 24 h, and the number of apoptotic cells increased further after starvation for 48 h (Fig. 1B).

Then the autophagy incidence of the AF cells under serum deprivation for various time periods (12, 24, 36, 48 and 60 h) was measured by quantifying the development of acidic vesicular organelles (AVOs) after AO staining. Flow cytometric analysis revealed that the autophagy incidence in AF cells maintained in a relatively low level (3.6%) when they were under DMEM cultivation with serum *in vitro*. However, the autophagy incidence began to increase in the cells after 12-h serum deprivation, further increased steadily during 24–36 h, reached a peak value (29.9%) at 48 h, and then declined to 19.1% at 60 h (Fig. 1C). Furthermore, the protein and mRNA expressions of the biomarkers of autophagy and apoptosis were evaluated. Real-time PCR assay revealed that the mRNA expressions of Beclin-1 and LC3 in the AF cells under serum deprivation began to increase at 12 h, reached their highest levels at 48 h, and then decreased at 60 h (Fig. 1D). Western blot analysis revealed similar results that the protein expressions of Beclin-1 and LC3 II/I gradually increased when the cells were cultured under serum deprivation for 12–48 h and then decreased at 60 h (Fig. 1E). However, the protein expression of cleaved caspase-3 remained at a low level in the cells under serum deprivation

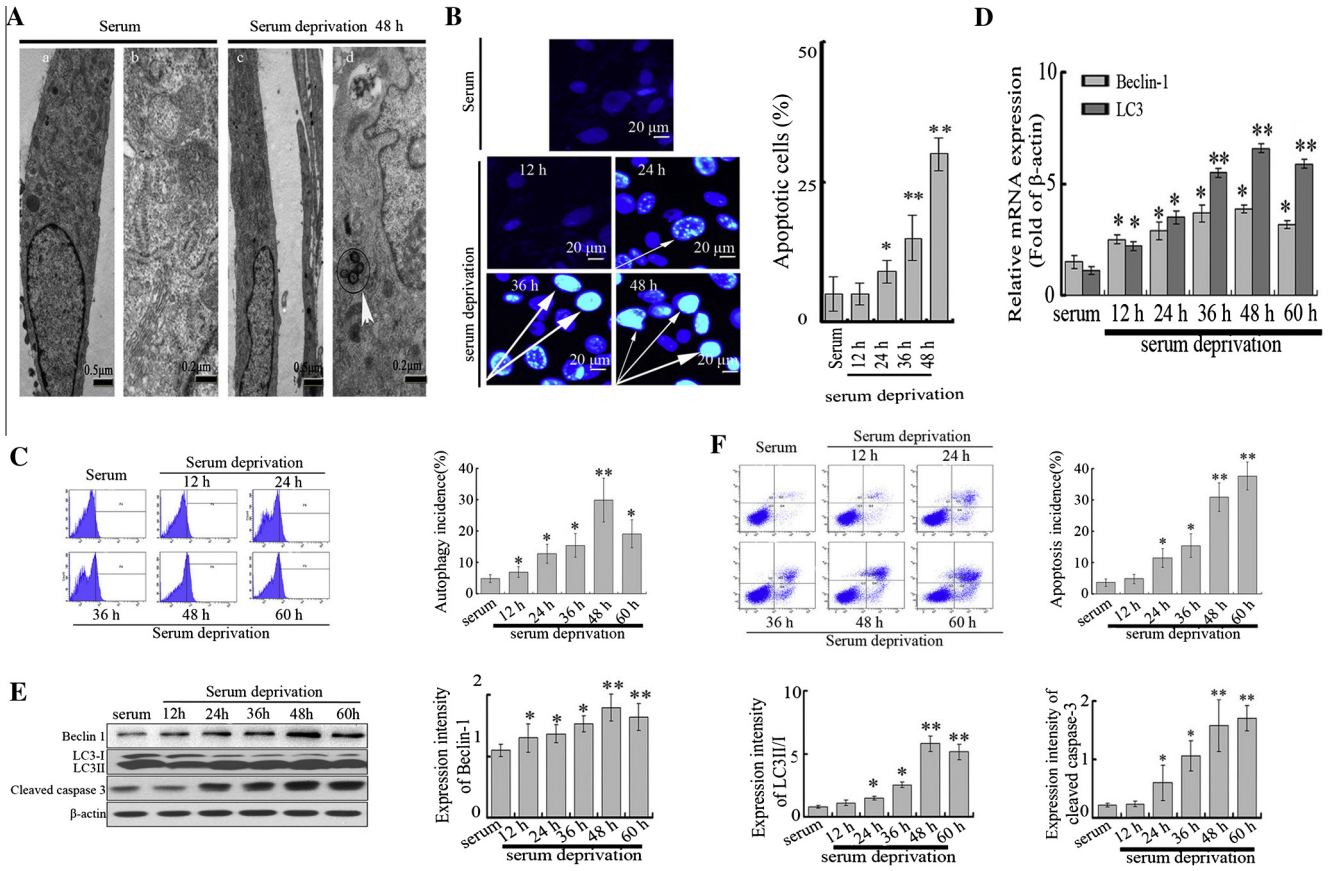

Fig. 1. Evidence of autophagy and apoptosis in the AF cells under serum deprivation. AF cells were cultured under serum deprivation for 12, 24, 36, 48 and 60 h. (A) Transmission electronic microscopy (TEM) for autophagosomes. Much more autophagosomes were detected in the AF cells after 48-h starvation. (B) Hoechst 33258 staining for apoptosis. The number of apoptotic cells significantly increased with the time of serum deprivation. (C) Flow cytometric measurement for autophagy incidence. The autophagy incidence increased gradually after 12 h, and reached the highest level (29.9%) at 48 h after serum starvation. (D) Real-time PCR analysis. The mRNA expressions of Beclin-1 and LC3 increased steadily after starvation for 12–48 h, and then decreased gradually. (E) Western blot evaluation. The protein expressions of Beclin-1 and LC3 II/I gradually increased in the cells under serum deprivation for 12–48 h and then gradually decreased. The protein expression of cleaved casepase-3 began to increase gradually in the cells under serum starvation for 24 h. (F) Flow cytometric measurement for apoptosis incidence. The apoptosis incidence began to increase gradually in the cells after 24 h of serum deprivation. For quantitative evaluations, the data were presented as the mean ± SD. AF cells cultured in serum were served as control. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

for 12 h. Only after the cells were serum-starved for 24 h did cleaved caspase-3 begin to increase steadily in the cells (Fig. 1E). Meanwhile, flow cytometric analysis demonstrated that the level of apoptosis incidence in AF cells under serum starvation gradually increased accordingly (Fig. 1F).

These results suggested that serum deprivation could lead to both autophagy and apoptosis in AF cells, and autophagy occurred earlier than apoptosis.

Finally, the interactions between autophagy and apoptosis in the AF cells under serum deprivation were investigated. According to our results that the AF cells under serum deprivation had the most significant autophagy at 48 h (Fig. 1C–E), we choose this time point in our next studies. The AF cells were pretreated with 5 mM 3-MA (an actually autophagy stimulator in this study [40]), or 100 nM Baf A (a widely used autophagy inhibitor), or vehicle (0.1% dimethylsulfoxide) for 2 h and cultured in serum-free DMEM for 48 h. Flow cytometric measurements demonstrated that treatment with 3-MA definitely increased the autophagy incidence (Fig. 2A), while at the same time increased the apoptosis incidence (Fig. 2B). Western blot analysis also revealed that 3-MA increased the autophagic protein expressions of Beclin-1 and LC3 II/I (Fig. 2C), while at the same time increased the apoptotic protein expressions of cleaved caspase-3 and Bax/Bcl-2 (Fig. 2D). These results suggested that excessive autophagy led to apoptosis in the AF cells under serum starvation.

Interestingly, inhibition of autophagy by Baf A led to unexpected increases of the autophagy incidence (Fig. 2A) and the expression of LC3 II/I (Fig. 2C), but had no effect on the protein expression of Beclin-1 (Fig. 2C). At the same time Baf A did reduce the apoptosis incidence (Fig. 2B), and the protein expression of cleaved caspase-3, whereas actually increased the expression of Bax/Bcl-2 (Fig. 2D).

3.2. The effect of TGF-β1 on autophagy and apoptosis in the AF cells under serum deprivation

To investigate the effect of TGF-β1 on autophagy and apoptosis in the AF cells under serum deprivation, cells were cultured in serum-free medium with various concentrations of TGF-β1 (5, 10 and 20 ng/mL) for 48 h. The concentration range of TGF-β1 was decided according to previous studies which demonstrated that 2–20 ng/mL TGF-β1 was efficient in various cell types [19,28,41].

Flow cytometric analysis demonstrated that TGF-β1 did not alter either the autophagy incidence (Fig. 2A) or the apoptosis incidence (Fig. 2B) of AF cells under DMEM cultivation with 10% FBS. However, the highest autophagy incidence in the AF cells under serum starvation for 48 h decreased most significantly when 10 ng/mL of TGF-β1 was added into the culture medium (Fig. 3A). At the same time, 10 ng/mL of TGF-β1 also most significantly reduced the

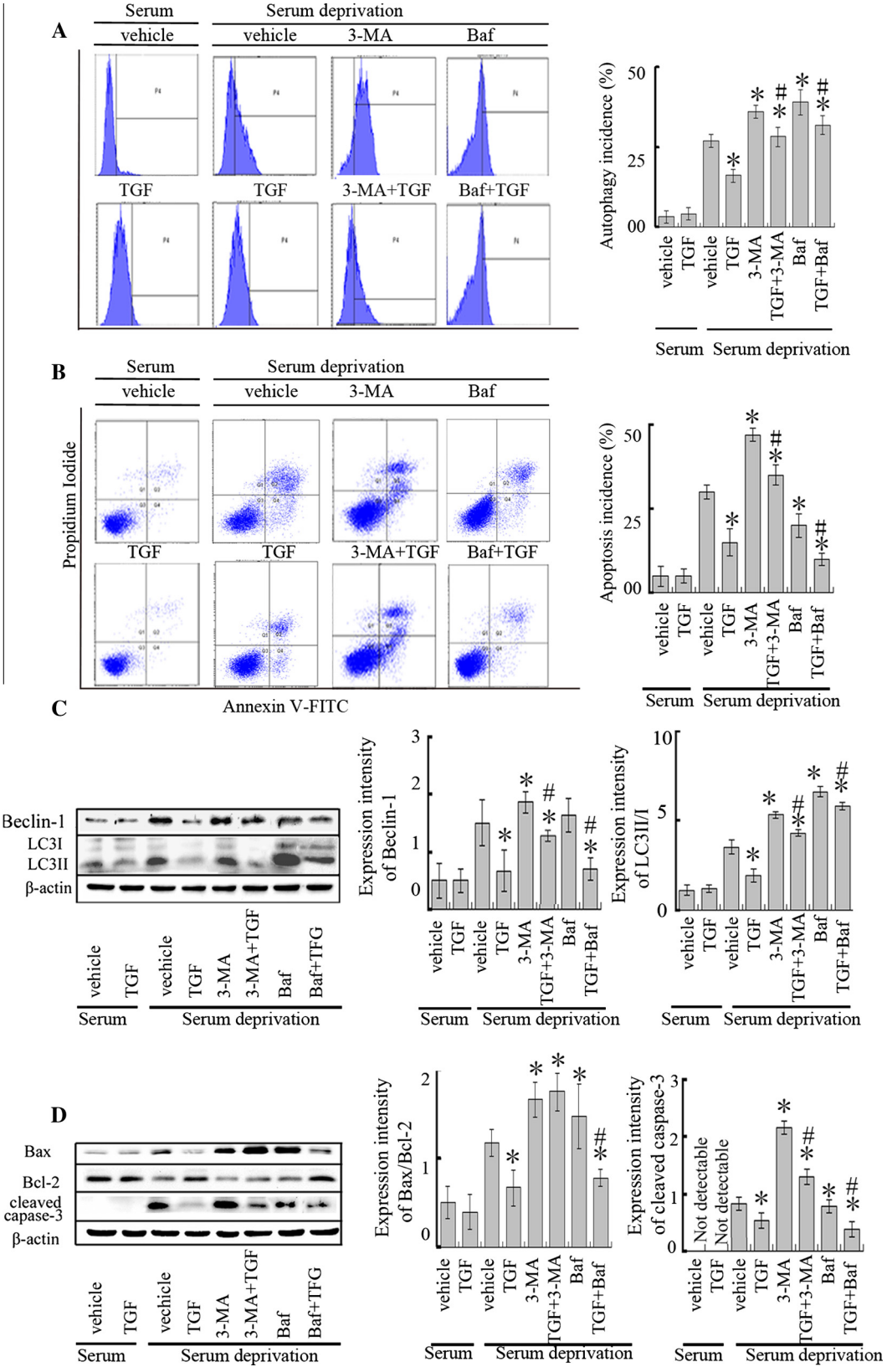

Fig. 2. The interactions between autophagy and apoptosis in the AF cells under serum deprivation. AF cells were cultured under serum deprivation for 48 h. (A) Flow cytometric measurements for the autophagy incidence. Both 3-MA and Baf A increased the autophagy incidence. TGF-β1 partly reversed the increased autophagy incidence in the AF cells pretreated with 3-MA or Baf A. (B) Flow cytometric measurements for the apoptosis incidence. 3-MA increased, while Baf A decreased the apoptosis incidence. TGF-β1 significantly reduced the apoptosis incidence and partly reversed the increased apoptosis incidence in the AF cells pretreated with 3-MA, while further reduced the decreased apoptosis incidence in the AF cells pretreated with Baf A. Western blot evaluation for the protein expressions of autophagic markers (C) and apoptotic markers (D). 3-MA increased the expressions of Beclin-1, LC3 II/I, cleaved caspase-3 and Bax/Bcl-2, while Baf A decreased the expression of cleaved caspase-3, had no effect on Beclin-1, and actually increased the expressions of LC3II/I and Bax/Bcl-2. TGF-β1 significantly decreased the expressions of Beclin-1, LC3 II/I and cleaved caspase-3 in the AF cells pretreated with 3-MA or Baf A. Data were presented as the mean ± SD. AF cells cultured with vehicle under serum deprivation for 48 h were served as control. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus cells pretreated with the corresponding autophagy modulator.

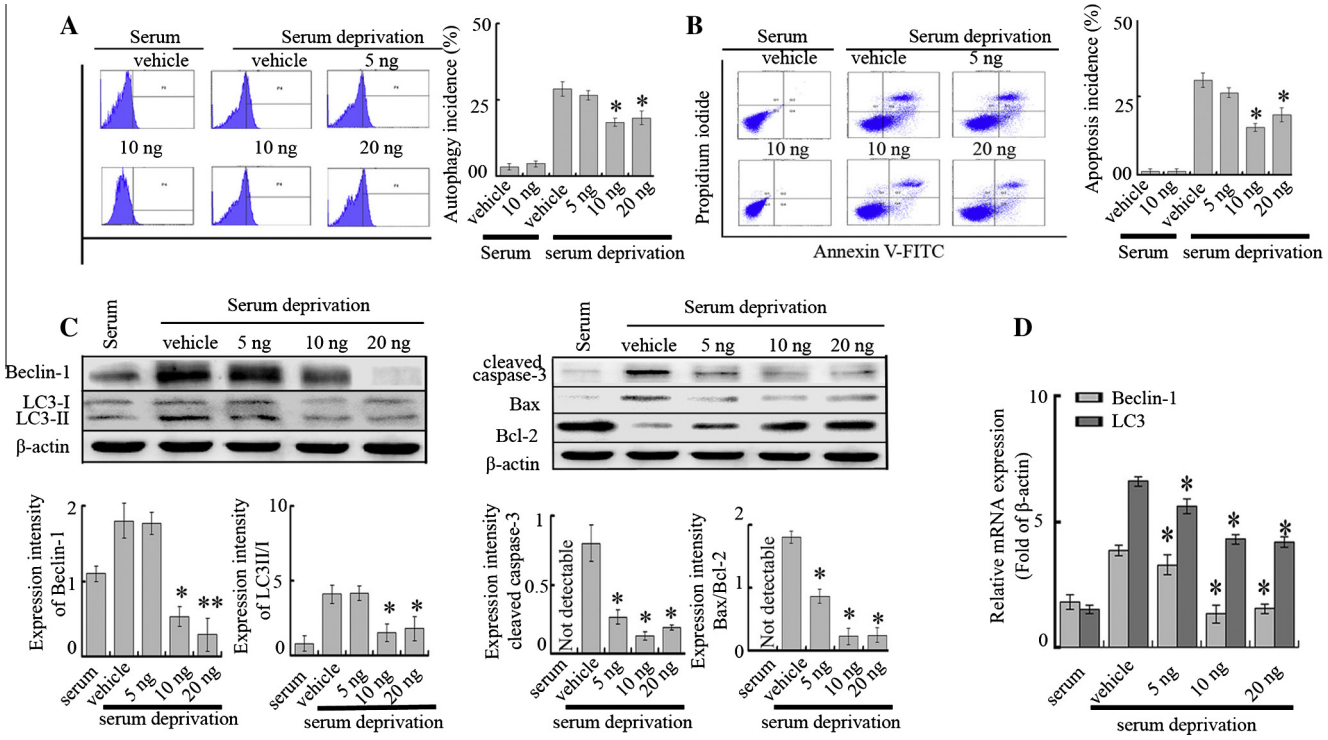

Fig. 3. The effect of TGF-β1 on autophagy and apoptosis in AF cells under serum starvation. AF cells were cultured under serum deprivation with various concentrations of TGF-β1 (5, 10, 20 ng/mL) for 48 h. Flow cytometric analysis for the autophagy incidence (A) and apoptosis incidence (B). 10 ng/mL TGF-β1 most significantly reduced the increased autophagy and apoptosis incidences. (C) Western blot analysis. 10 ng/mL TGF-β1 significantly reduced the increased expressions of Beclin-1, LC3 II/I, cleaved caspase-3 and Bax/Bcl-2. (D) Real-time PCR analysis for Beclin-1 and LC3. 10 ng/mL TGF-β1 most significantly reduced the increased mRNA expressions of Beclin-1 and LC3. Data were presented as the mean ± SD. AF cells cultured under serum deprivation with vehicle for 48 h were served as control. $^{*}P < 0.05$ versus control.

apoptosis incidence in the AF cells under serum starvation for 48 h (Fig. 3B).

Meanwhile, Western blot analysis showed that 10 ng/mL of TGF-β1 significantly inhibited both the autophagic protein expressions of Beclin-1, LC3 II/I and the apoptotic protein expressions of cleaved caspase-3 and Bax/Bcl-2 in the AF cells under starvation for 48 h (Fig. 3C). Real-time PCR demonstrated similar results that 10 ng/mL of TGF-β1 significantly inhibited the mRNA expressions of Beclin-1 and LC3 in the AF cells under starvation for 48 h (Fig. 3D). In addition, immunofluorescence assay verified that 10 ng/mL TGF-β1 significantly reduced the increased staining intensities for lysosomes, autolysosomes and autophagy-related proteins in the AF cells under serum deprivation for 48 h. Autophagy assay in GFP-LC3 transfected cells also demonstrated that serum deprivation increased the number of the GFP-LC3 punctate green fluorescence in the cytoplasm, which could be reduced significantly by 10 ng/mL TGF-β1 (Fig. 4).

3.3. TGF-β1 protected against apoptosis through inhibition of excessive autophagy in AF cells under serum deprivation

To investigate the effect of TGF-β1 on the crosstalk between autophagy and apoptosis in the AF cells under serum starvation, AF cells were pre-treated with 3-MA (5 mM) or Baf A (100 nM) for 2 h, and cultured in serum-free DMEM with 10 ng/mL TGF-β1 for 48 h. Flow cytometric analysis revealed that TGF-β1 partly reversed the increased autophagy incidence (Fig. 2A) and apoptosis incidence (Fig. 2B) in the AF cells pre-treated with 3-MA. Western blot analysis revealed that TGF-β1 down-regulated the increased protein expressions of Beclin-1 and LC3 II/I (Fig. 2C), and cleaved caspase-3 (Fig. 2D) in the AF cells pre-treated with 3-MA. Meanwhile, flow cytometric analysis revealed that TGF-β1 reversed the elevated autophagy incidence (Fig. 2A) and further decreased the apoptosis incidence (Fig. 2B) when the cells were pre-treated with Baf A. Western blot analysis also found that when the AF cells were pre-treated with the autophagy inhibitor Baf A, the expressions of Beclin-1 and LC3-II/I (Fig. 2C), cleaved caspase-3 and Bax/Bcl-2 (Fig. 2D) were all down-regulated by TGF-β1.

These results suggested that TGF-β1 could protect against apoptosis through inhibition of excessive autophagy in the AF cells under serum starvation.

3.4. TGF-β1 protected AF cells against apoptosis under serum deprivation through PI3K/AKT/mTOR and ERK1/2 signaling pathways

To explore whether the PI3K/AKT/mTOR and ERK1/2 signaling pathways were involved in the effect of TGF-β1 on the crosstalk between autophagy and apoptosis, AF cells were pretreated with 3-MA (an inhibitor of PI3K and p-AKT [42]), or with U0126 (an inhibitor of ERK1/2), and cultured in serum-free DMEM with 10 ng/mL TGF-β1 for 48 h. Western blot analysis showed that the expressions of p-AKT and p-mTOR were almost completely blocked by 3-MA, no matter whether TGF-β1 was administrated or not. The expressions of LC3 II/I and cleaved caspase-3 in the cells with both TGF-β1 and 3-MA were lower than those with 3-MA alone, although not as low as those with TGF-β1 alone (Fig. 5A). Meanwhile, TGF-β1 significantly enhanced the expression of phosphorylated-ERK1/2. However, when U0126 was applied, the decreased expressions of LC3 II/I and cleaved caspase-3 by TGF-β1 was partly up-regulated (Fig. 5B). These data indicated that the inhibitory effect of TGF-β1 on excessive autophagy could be weakened by either 3-MA or U0126. Thus, both the PI3K/AKT/mTOR and ERK1/2 signaling pathways were involved in the actions of TGF-β1 on the crosstalk between autophagy and apoptosis in the AF cells under serum deprivation.

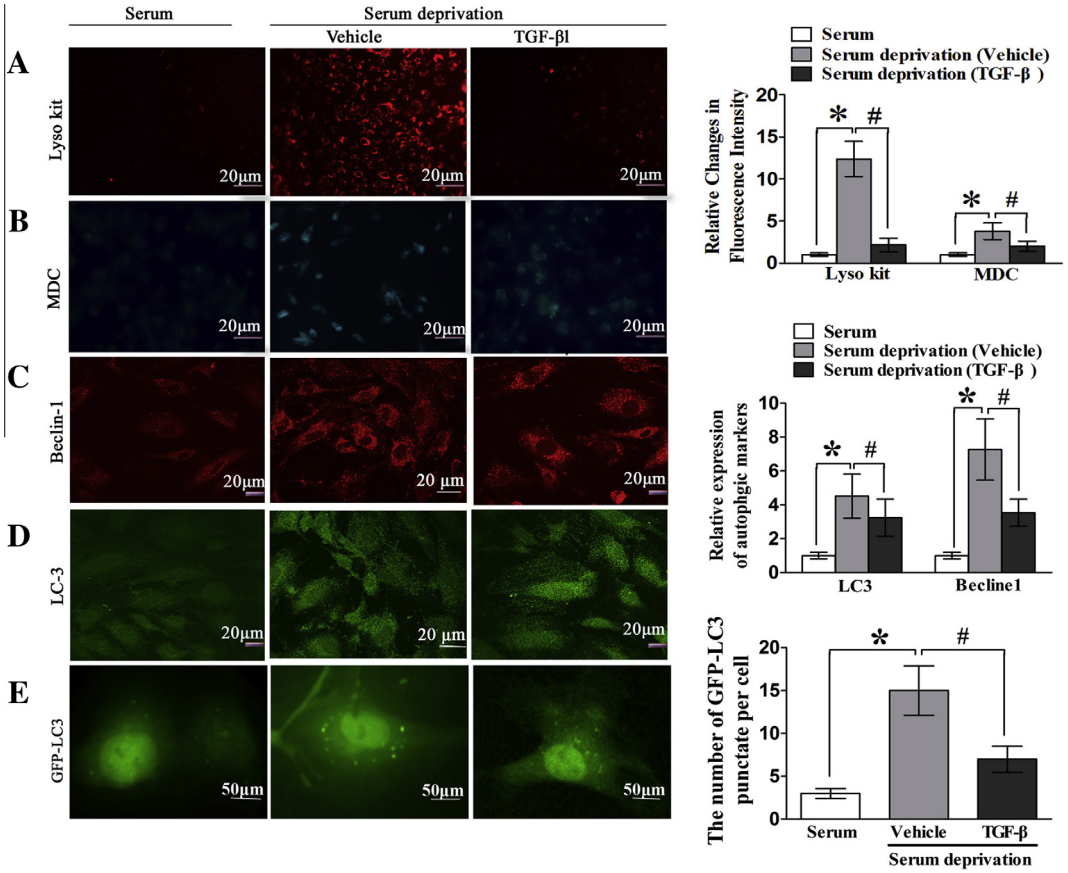

Fig. 4. Immunofluorescence assay for lysosomes, autolysosomes activity and autophagy-related proteins. AF cells were cultured under serum deprivation with vehicle or 10 ng/mL TGF-β1 for 48 h. (A) Lyso-Tracker staining and (B) MDC staining. The AF cells had much higher staining intensity for autolysosomes and lysosomes under serum deprivation than under serum cultivation. TGF-β1 significantly reduced the increased staining intensity. (C and D) Immunofluorescence analysis for the expressions of Beclin1 and LC3. These two proteins were highly expressed and exhibited a punctuated distribution in the cytoplasm of AF cells following 48 h of serum starvation as compared with AF cells cultured in serum condition. TGF-β1 significantly reduced their expressions. (E) Fluorescent microscopy for the formation and distribution of GFP-LC3 punctate. GFP-LC3 plasmid transfected AF cells were incubated with vehicle or 10 ng/mL TGF-β1 under serum deprivation for 48 h. The transfected AF cells showed a higher level of punctate green fluorescence under serum deprivation than under serum condition. TGF-β1 significantly reduced the number of GFP-LC3 punctate in the cells. $^{*}P < 0.05$, $^{\#}P < 0.05$.

4. Discussion

As in our previous study [35], the existence of autophagy and apoptosis was confirmed in the AF cells under serum starvation by the present study. Autophagy increased significantly in the cells as early as 12 h after serum starvation and reached its highest level at 48 h, while gradual increase of apoptosis happened only after 24 h. When autophagy was moderately increased, apoptosis of the cells maintained at a low level. When autophagy was stimulated to an extremely high level, apoptosis of the cells increased. Thus, early and moderate autophagy was beneficial to the survival of the AF cells, while late and excessive autophagy led to death of the AF cells. This inter-relationship between autophagy and apoptosis in the AF cells was in accordance with most studies in other cell types [43,44].

Autophagy and apoptosis are closely linked. Numerous studies verified the existence of complicated crosstalk between apoptosis and autophagy in different cell types under different cultivation conditions [12,45,46]. Autophagy and apoptosis are two main programmed cell death patterns, and both of them had an essential role in the process of IVDD [24,47]. Our findings about the interactions between autophagy and apoptosis in the AF cells would be helpful for better understanding the molecular mechanisms implicated in the pathogenesis of IVDD.

Studies reported that TGF-β1 was involved in the regulation of autophagy and had pro-apoptosis [30,32,33,48] or anti-apoptosis [19] effect in various cell types. But, to our knowledge, there is no relevant research focusing on the effect of TGF-β1 on the crosstalk between autophagy and apoptosis in the intervertebral disc cells. Our present study demonstrated that 10 ng/mL TGF-β1 significantly decreased the enhanced autophagy and apoptosis caused by 48-h serum deprivation in the AF cells. However, when the excessive autophagy flux was further exacerbated, the role of TGF-β1 to down-regulate apoptosis was impaired. When the excessive autophagy flux was suppressed, the role of TGF-β1 to down-regulate apoptosis was promoted. Thus, the above data suggested that TGF-β1 protected against apoptosis through inhibition of excessive autophagy in the AF cells under serum starvation. Previous researches found that the expression of TGF-β1 was significantly up-regulated in the early stage of disc degeneration [49], increased with the pathological grades of disc degeneration [50] and could delay intervertebral disc degeneration via regulating the extracellular matrix production [51]. Thus, our findings about the effect of TGF-β1 on the crosstalk between apoptosis and autophagy in the intervertebral disc cells should be helpful to better understand the molecular mechanism for the involvement of TGF-β1 in the process of disc degeneration.

3-MA and Baf A were autophagy regulators commonly used in numerous studies for different kinds of cells. Consistent with previous reports [19,52], Baf A actually increased the autophagy incidence and elevated the expression of LC3 II/I in our study, although

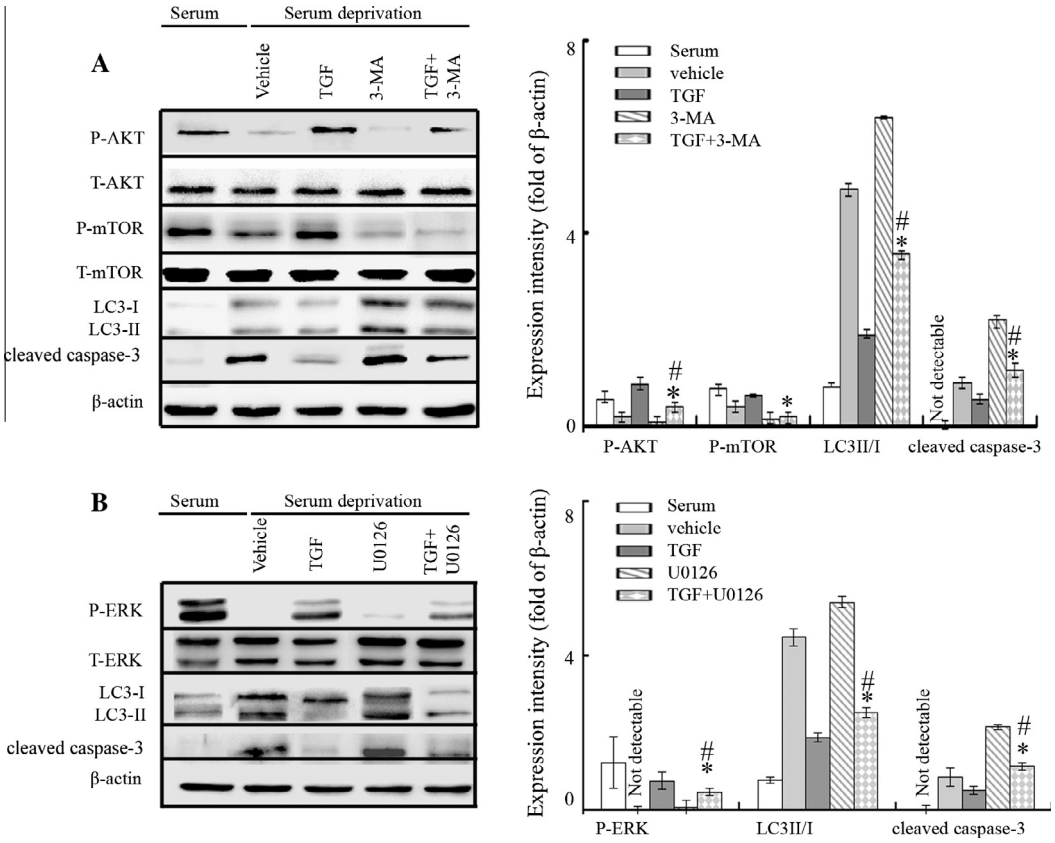

Fig. 5. TGF-β1 protected AF cells against apoptosis through inhibiting autophagy via PI3K–AKT–mTOR and ERK1/2 signaling pathways. (A) Western blot for the expression of T-AKT, p-AKT, T-mTOR, p-mTOR, and LC3 II/I and cleaved caspase-3. Cells were cultured in complete serum-free DMEM with 10 ng/mL TGF-β1, or with 5 mM 3-MA, or with both of these two for 48 h. 3-MA could evidently abrogate the TGF-β1 induced phosphorylation of both AKT and mTOR and partly reduced the inhibitory effect of TGF-β1 on the expression of LC3 II/I and cleaved caspase-3. (B) Western blot for the expressions of T-ERK, p-ERK, LC3 II/I and cleaved caspase-3. AF cells were cultured in complete serum-free DMEM with 10 ng/mL TGF-β1, or with 10 μM U0126, or with both of these two for 48 h. TGF-β1 could significantly enhance the expression of p-ERK1/2 and simultaneously inhibited the expressions of LC3 II/I and cleaved caspase-3. U0126 partly reversed this inhibitory effect of TGF-β1. AF cells pretreated with vehicles and then cultured under serum deprivation for 48 h were served as control. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus cells pretreated with the corresponding modulator.

it is an autophagy inhibitor. The proper explanation was that Baf A inhibited the fusion of autophagosomes/lysosomes and decreased the autophagic degradation, thus blocking the downstream step in the autophagy flux. Thus, increased accumulation of autophagosomes led to increased autophagy incidence measured by flow cytometry for AO staining and elevated expression of LC3 II/I detected by Western blot. Meanwhile, 3-MA was often used as an autophagy inhibitor by inhibiting the class III PI3K [42] and suppressing autophagy [53]. However, we found that 3-MA actually promoted autophagy in AF cells under serum deprivation *in vitro*. Increased protein expressions of Beclin-1 and LC3 II/I and increased autophagy incidence were found in the AF cells pre-treated with
3-MA. Although the accurate mechanism of 3-MA on promoting autophagy was not clear, a similar finding was reported that 3-MA promoted autophagy because of its persistent inhibition on class I PI3K, but transient suppression on class III PI3K [40].

Its well known that apoptosis could be induced by variant upstream stimuli [54] and caspase-3 was a major protease executing apoptosis [55]. Cleaved caspase-3 could be activated by cytochrome C released from mitochondria, which was promoted by Bax or inhibited by Bcl-2 [56–58]. Our results revealed that both Baf A and 3-MA increased the expression of Bax/Bcl-2 in the AF cells under serum deprivation. One possible reason was that autophagy was closely connected with the mitochondrial function [59–61], and inhibition of autophagy with Baf A led to the unexpected high expression of Bax/Bcl-2. We also found that TGF-β1 could down regulate the high expression of Bax/Bcl-2 in the AF cells pretreated with Baf A, but not in the cells with 3-MA. We speculated that TGF-β1 could re-establish the mitochondrial function when the last step of autophagy was inhibited by Baf A, but failed to re-establish the mitochondrial function when the autophagy was promoted by 3-MA. Furthermore, Baf A resulted in a decrease of cleaved caspase-3, although it did not reduce Bax/Bcl-2 expression in our study, suggesting that Baf A reduced apoptosis via nonmitochondrial apoptotic pathway.

These findings proved our hypothesis that TGF-β1 protected AF cells from apoptosis through down-regulating autophagy. The mitochondrial pathway might be involved in this process.

Mammalian target of rapamycin (mTOR) was the key upstream regulator of autophagy-related proteins [62]. Abundance of nutrients, including growth factors, glucose, and amino acids activate mTOR and suppress autophagy, whereas nutrient deprivation could inhibit mTOR and promote autophagy [21,63]. PI3K/AKT lay upstream of mTOR, and activated AKT inhibited autophagy through activating mTOR [64–66]. It has been reported that TGF-β1 could activate AKT in various cell types [19,67,68]. In our present study, we found that TGF-β1 significantly up-regulated the phosphorylation of AKT and mTOR and down-regulated autophagy in the AF cells under serum deprivation. When the signaling pathway PI3K/AKT/mTOR was blocked by 3-MA, TGF-β1 lost part of its down-regulating function on autophagy and apoptosis. These findings proved our hypothesis that the PI3K/AKT/mTOR signaling pathway was involved in the effect of TGF-β1 on the crosstalk between autophagy and apoptosis in the AF cells under serum deprivation.

MAPK–ERK is one of the mitochondrial signaling pathway and it has been reported that the MAPK–ERK signaling pathway could regulate the expression of autophagy and lysosomal genes [69] and TGF-β1 could activate the phosphorylation of ERK [70,71]. As expected, our study further demonstrated that TGF-β1 could significantly enhance the expression of phosphorylated-ERK1/2 and simultaneously inhibited the expression of LC3 II/I and cleaved caspase-3. When the ERK signaling pathway was blocked with U0126, TGF-β1 lost part of its down-regulating function on autophagy. These results confirmed that the MAPK–ERK1/2 signaling pathway was involved in the effect of TGF-β1 on the crosstalk between autophagy and apoptosis in the AF cells under serum deprivation, which further confirmed that the impact of TGF-β1 was through mitochondrial signaling pathway.

We realized that there were several limitations in our present study. Activation or inhibition of autophagy was through the use of chemical modulators in this study. The efficiency of these modulators was not the best. Gene silencing or overexpression technique would be better. The crosstalk between autophagy and apoptosis of the intervertebral disc cells under variant stress conditions and its significance in the pathogenesis of IVDD need to be further investigated.

5. Conclusions

The current study demonstrated that TGF-β1 could protect against the apoptosis of AF cells under serum deprivation through down-regulating excessive autophagy. PI3K–AKT–mTOR and MAPK–ERK1/2 were the possible signaling pathways involved in this process. These findings should be helpful for us to better understand the molecular mechanisms involved in the action of TGF-β1 for AF cells and should be of clinical significance for the prevention and treatment of IVDD.

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