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TGF-β1 aggravates degenerative nucleus pulposus cells inflammation and fibrosis through the upregulation of angiopoietin-like protein 2 expression

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Abstract. – OBJECTIVE: Inflammation and fibrosis progress of nucleus pulposus (NP) cells participate in the pathologic changes of intervertebral disc degeneration (IDD). ANGPTL2 is well known for its angiogenesis and proinflammatory properties and transforming growth factor β1 (TGF-β1) is also responsible for tissue fibrosis. However, the role of ANGPTL2 in IDD and whether it is related to TGF-β1 remains unclear. This study aims to explore the relation of TGF-β1 and ANGPTL2 in the degenerative process of NP cells.

PATIENTS AND METHODS: We isolated NP cells of NP tissues provided from the spine fracture patients. IL-1β was used to induce the NP cells degeneration. To determine the effect of TGF-β1 and ANGPTL2 on NP cell degeneration, we regulated the cellular TGF-β1 and ANGPTL2 expression by Recombinant human protein stimulation and siRNA transfection. Quantitative real-time polymerase chain reaction (qRT-PCR) or Western blot was employed to investigate the expression of TGF-β1, ANGPTL2, IL-6, TNF-α, collagen I, and collagen III.

RESULTS: TGF-β1 overexpression aggravated the ANGPTL2, IL-6, TNF-α, collagen I, and collagen III expressions of NP cells that caused by IL-1β, which was rejected by ANGPTL2 gene silencing. Besides, the silencing of TGF-β1 weakened the ANGPTL2 expression. ANGPTL2 overexpression promoted the NP cells inflammation and fibrosis via increasing IL-6, TNF-α, collagen I, and collagen III expression, which was sharpened by a consequent increase of TGF-β1 expression.

CONCLUSIONS: This study, for the first time, points that TGF-β1 aggravates degenerative NP cells inflammation and fibrosis via the mediation of ANGPTL2.

Key Words:
Nucleus pulposus cells, TGF-β1, ANGPTL2, Inflammation, Fibrosis.

Introduction

Intervertebral disc degeneration (IDD) is a disease with multiple causes. The etiology of IDD is not clear, and it is generally considered to be related to various factors such as aging, inflammation, biomechanics, and molecular biology[1,2]. IDD mainly occurs in the central nucleus pulposus (NP), referring to the gradual degeneration, necrosis, and apoptosis of nucleus pulposus (NP) cells in the intervertebral disc, degradation of extracellular matrix (ECM), and changes in the proportion of collagen[3,4]. The progress is accompanied by progressive fibrosis, causing the disc tissue to lose its typical structure and function. In recent years, the role of cytokines during the development of IDD has been paid more attention. Among them, transforming growth factor β1 (TGF-β1) plays an essential role in the NP tissue fibrosis[5]. TGF-β is a critical factor in the fibrotic cytokine network, involving multiple organ fibrosis of human tissues. Its overexpression is closely

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related to the occurrence and development of certain proliferative or fibrotic diseases[6].

The vast majority of tissue and organ fibrotic diseases, including NP, which is based on the excessive deposition of type I and some type III collagen, are related to the overexpression of TGF-β[7]. TGF-β has been recognized as the most closely associated with collagen metabolism. Among the three different mammalian isoforms of TGF-β[8] (TGF-β 1 to 3), TGF-β1 accounts for the highest proportion (> 90%) in somatic cell lines with the most energetic activity. Additionally, the process of intervertebral discs fibrosis usually accompanies by angiogenesis[9], which increases the invasion of inflammatory factors, production of matrix-degrading enzymes, and results in an imbalance in the synthesis and catabolism of ECM[10]. TGF-β1 has a stimulating effect on angiogenesis, which can induce endothelial cells to adhere to and promote endothelial cell proliferation. Angiopoietin-like protein 2 (ANGPTL2) is reported to be a potential target of TGF-β1[11], which plays a role in promoting the formation of capillaries[12]. Besides, ANGPTL2 is an essential signaling pathway mediator involved in the occurrence and development of chronic low-grade inflammatory diseases, such as obesity, diabetes mellitus, cardiovascular disease, and tumors, which is different from the inflammation obtained by microbial infection[13]. But whether ANGPTL2 is related to the NP fibrosis and inflammation has not been reported.

To explore the potential role of ANGPTL2 and TGF-β1 in the NP fibrosis and inflammation progress, we isolated NP cells from the NP tissue donated by patients with a spine fracture. We evaluated the ANGPTL2 and TGF-β1 expression in the degenerative NP cells caused by IL-1β. NP cells were also cultured with human TGF-β1 and ANGPTL2 protein to test the fibrosis and inflammation-related gene expression. Apart from this, we used siRNA to knockdown TGF-β1 or ANGPTL2 expression in NP cells and evaluated the fibrosis and inflammation level by analyzing collagen I/III, IL-6, and TNF-α, respectively. What we found provides further insight into the mechanism of TGF-β1 and ANGPTL2 in the IDD.

Patients and Methods

Patient NP Tissue

This project was approved by the Ethics Committee of the Xi'an Hospital of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study. We recruited 5 patients (2 males, 3 females; every age: 39 years, from 28 to 45 years) with a burst fracture of the vertebral body undergoing lumbar fixation operations in our hospital. They donated the NP tissue after knowing informed consent. We conserved the tissues in cold Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) and delivered to the laboratory for NP cell isolation as soon as possible.

NP Cells Preparation and Drug Treatment

NP tissue was shaved off from the endplate cartilage and cut into fragments as smaller as possible. The samples were pretreated with 0.2% trypsin-EDTA (ethylenediaminetetraacetic acid) solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h, and then digested with 0.4% collagenase II (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37°C. After filtration, NP cell pellets were re-suspended in cell growth media (DMEM/F12 1:1), containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). We used the first passage NP cells for the following experiment. We used Recombinant human TGF-β1 protein (rh-TGF-β1) (ab50036, Abcam, Cambridge, MA, USA) and Recombinant human ANGPTL2 (rh-ANGPTL2) (9795-AN, R&D Systems, Minneapolis, MN, USA) to culture NP cells.

siRNA Transfection

NP cells were cultured in 6-well plates, and we replaced the fresh medium (serum-free) until the cell density reached 70 %. For the NP cells transfection, Opti-MEM (Sigma-Aldrich, St. Louis, MO, USA) and siRNA targeting ANGPTL2 (Catalog #AM16708S, Thermo Fisher Scientific, Waltham, MA, USA) or TGF-β1 (Catalog #AM16708, Thermo Fisher Scientific, Waltham, MA, USA) were added to Lipo2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). After 30 min incubation, the Lipo2000–siRNA reagent was added to the NP cells Cultured for 24 h after transfection, the medium was changed freshly, and the cells could be used for subsequent experiments.

Western Blot Analysis (WB)

After treatments, the protein of NP cells samples was isolated by the radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime,

Shanghai, China). The purity of the protein was measured by the bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). Protein was separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% milk and following incubated with primary antibodies against ANGPTL2 (Abcam, Cambridge, MA, USA) and TGF-β1 (Abcam, Cambridge, MA, USA) at 4°C overnight. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, the protein was exposed by the enhanced chemiluminescence (ECL) solution (Beyotime, Shanghai, China) using the Odyssey Infrared Imager (Seattle, WA, USA). Densitometric measurement was performed using the ImageJ software (NIH, Bethesda, MD, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After treatments, total mRNA was isolated from NP cells samples with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the introductions. The quality of RNA was measured by Nanodrop and reverse-transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript Kit (TaKaRa, Tokyo, Japan). Then, PCR was performed using an SYBR Green Master according to the introductions (TaKaRa, Tokyo, Japan). Data were analyzed by the normalization of the relative quantitative glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression using the $2^{-\Delta\Delta CT}$ method. Primer sequences for PCR are listed in Table I.

Statistical Analysis

All the data were analyzed by the Statistical Product and Service Solutions (SPSS) 20.0 package (IBM Corporation, Armonk, NY, USA), and the cartograms were generated by the GraphPad 6.0 Software (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. A comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). *p*-values <0.05 were considered statistically significant.

Results

The Effect of TGF-β1 Overexpression on the NP Cells

The process of IDD is complex and diverse, and the role of TGF-β1 is also dual. In the early stage of IDD, TGF-β1 plays a protective role by promoting ECM synthesis. However, in the late phase, TGF-β1 activates the production of large amounts of type I and type III collagen and accelerates the degradation of proteoglycan. To detect the effect of TGF-β1 on the NP cells *in vitro*, we used the rh-TGF-β1 protein to culture healthy NP cells and degenerative NP cells caused by IL-1β[14], separately. For inducing degeneration, NP cells were pretreated with 10 ng/mL IL-1β for 24 h. The NP cells, with or without IL-1β pretreatment, were then cultured with rh-TGF-β1 (10 ng/mL) for an extra two days. NP cells without IL-1β and rh-TGF-β1 treatments were set as control.

We used the WB method to analyze the TGF-β1 and ANGPTL2 protein expression. The result indicated the IL-1β upregulated the TGF-β1 and ANGPTL2 expression. Besides, the rh-TGF-β1 supplement further increased both TGF-β1 and ANGPTL2 protein expression (Figure 1A and 1B). To determine the inflammatory and fibrous levels of NP cells, we measured the mRNA expression of IL-6, TNF-α, collagen I, and collagen III by qRT-PCR. Compared to the control, IL-1β significantly triggered the IL-6, TNF-α, collagen I, and collagen III expressions. Without IL-1β pretreatment. rh-TGF-β1 was only observed to increase the collagen I expression compared to the control. However, in the IL-1β pretreated groups, rh-TGF-β1 aggravated the increase of IL-

Table I. Primer sequences for qRT-PCR.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Collagen I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Collagen III	ATGTTGTGCAGTTTGCCCAC	TCGTCCGGGTCTACCTGATT
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

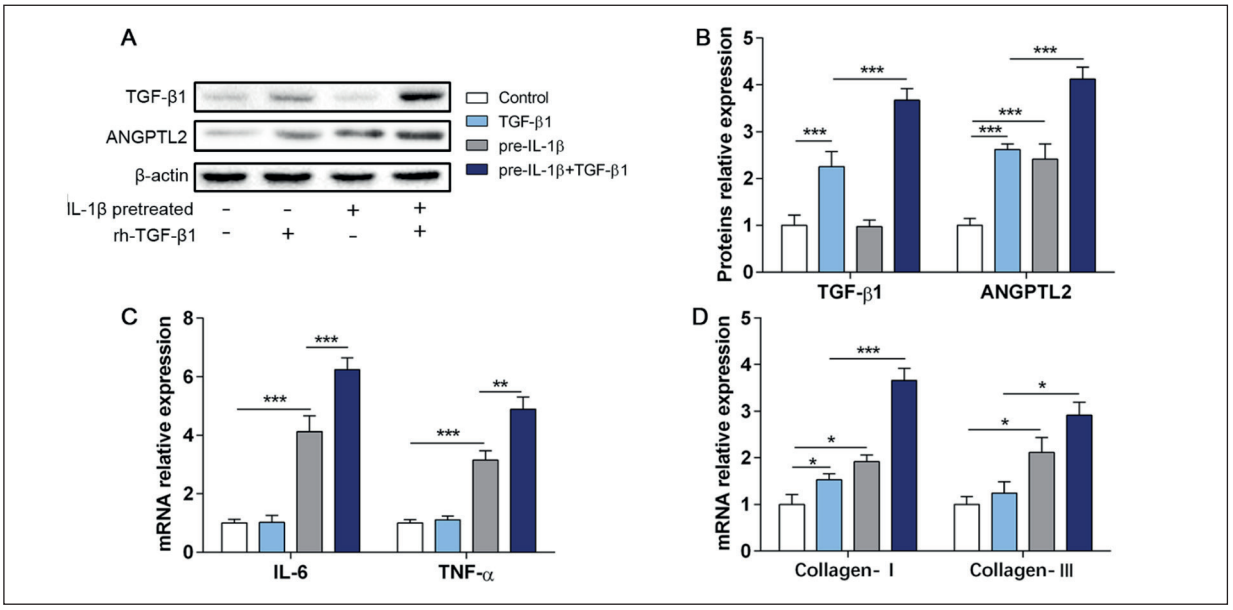

Figure 1. The effect of TGF-β1 overexpression on the NP cells. **A,** WB analysis for the protein expression of TGF-β1 and ANGPTL2 isolated from NP cells, and **B,** quantification of protein bands measured by Image J software. qRT-PCR analysis for the mRNA expression levels of **C,** IL-6, TNF-α, and **D,** collagen I, and collagen III isolated from NP cells normalized to GAPDH expression. Results are expressed as mean ± SD. (*$p < 0.05$, **$p < 0.01$, **$p < 0.001$).

6, TNF-α, collagen I, and collagen III compared to the non-pretreated group (Figure 1C and 1D). Therefore, the TGF-β1 overexpression played a negative role in the degenerative NP cells by the further acceleration of inflammation and fibrosis, which might be related to the secondary upregulation of ANGPTL2.

TGF-Ð1 Silencing Aggravates IL-1β Induced Fibrosis of NP Cells

TGF-β1 aggravated the degenerative NP cell degeneration. We further explored the effect of TGF-β1 silencing on the ANGPTL2 expression and NP cell degeneration. We treated the healthy NP cells and the TGF-β1 silenced NP cells with 10 ng/ml IL-1β for 21 h. The cells without silencing and IL-1β treatment were set as control. As shown in Figure 2A and 2B, compared to the control, TGF-β1 silencing decreased the ANGPTL2 protein expression. Meanwhile, the deficiency of TGF-β1 also alleviated the upregulation of ANGPTL2 caused by IL-1β. However, the silencing of TGF-β1 did not weaken the IL-1β induced a high level of inflammation (Figure 2C). In contrast with the normal NP cells, TGF-β1-silenced NP cells surprisingly expressed more collagen I and collagen III mRNA under the treatment of IL-1β (Figure 2D). Collectively, TGF-β1 silencing decreased the ANGPTL2 expression; under the normal condition, blocking of TGF-β1 did not affect NP cells inflammatory and fibrous level; however, TGF-β1 silencing aggravated the NP cells fibrosis with the appearance of IL-1β.

ANGPTL2 Silencing Alleviates TGF-β1 Caused Fibrosis and Inflammation of NP Cells

To determine the effect of ANGPTL2 in the NP cell degeneration, we silenced the ANGPTL2 gene by siRNA transfection and treated with IL-1*β* and rh-TGF-*β1.* NP cells were grouped into a normal and si-ANGPTL2 group, and each of them was pretreated with 10 ng/mL IL-1β for 24 h and then cultured with or without 10 ng/ml rh-TGF-β1 protein for two days. The cells without any treatments were set as control. We collected the protein of each group and tested the TGF-β1 and ANGPTL2 levels shown in Figure 3A and qualified as Figure 3B. As a result of Figure 1A and 2A, IL-1β did not affect the expression of TGF-β1 protein, but it increased the ANGPTL2 expression that further aggravated by rh-TGF-β1 supplement. The silencing of ANGPTL2 significantly suppressed the ANGPTL2 expression but not affect the cellular TGF-β1 protein expression. Compared to the non-silenced NP cells, ANGPTL2 gene blocking suppressed the IL-1β related IL-6 and TNF-α upregulation. Besides,

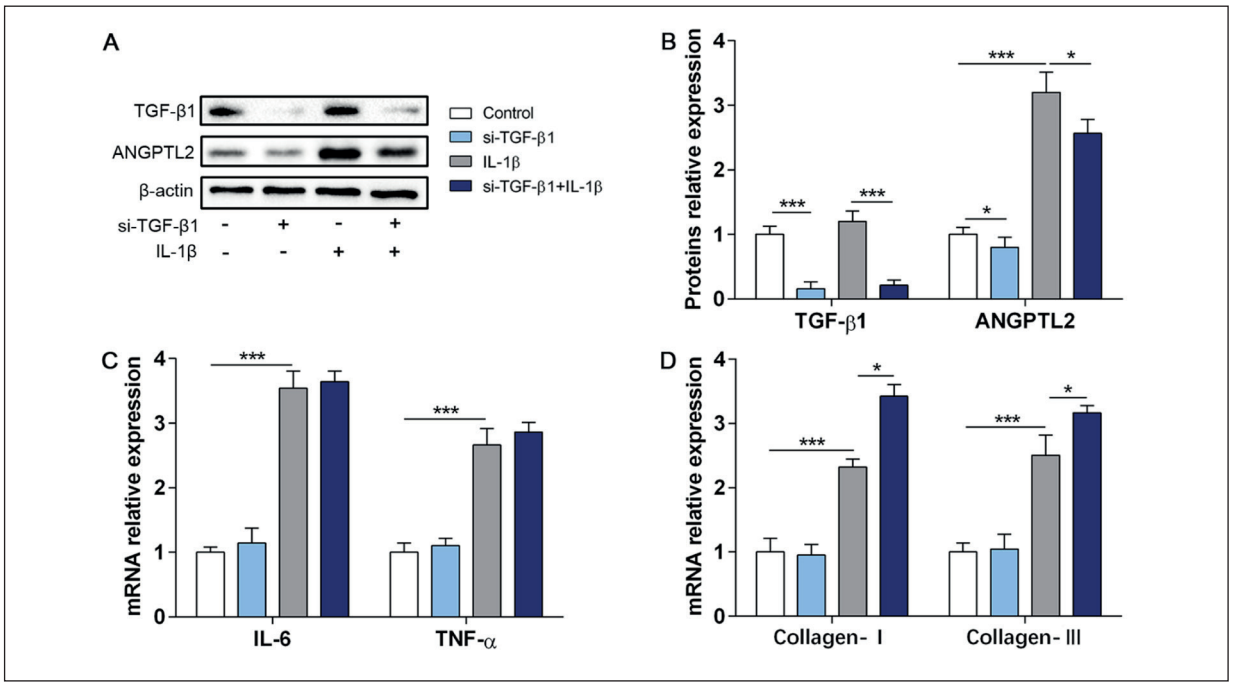

Figure 2. TGF-β1 silencing aggravates IL-1β induced fibrosis of NP cells. **A,** WB analysis for the protein expression of TGF-β1 and ANGPTL2 isolated from NP cells, and **B,** quantification of protein bands measured by Image J software. qRT-PCR analysis for the mRNA expression levels of **C,** IL-6, TNF-α, and **D,** collagen I, and collagen III isolated from NP cells normalized to GAPDH expression. Results are expressed as mean ± SD. (*$p < 0.05$, **$p < 0.001$).

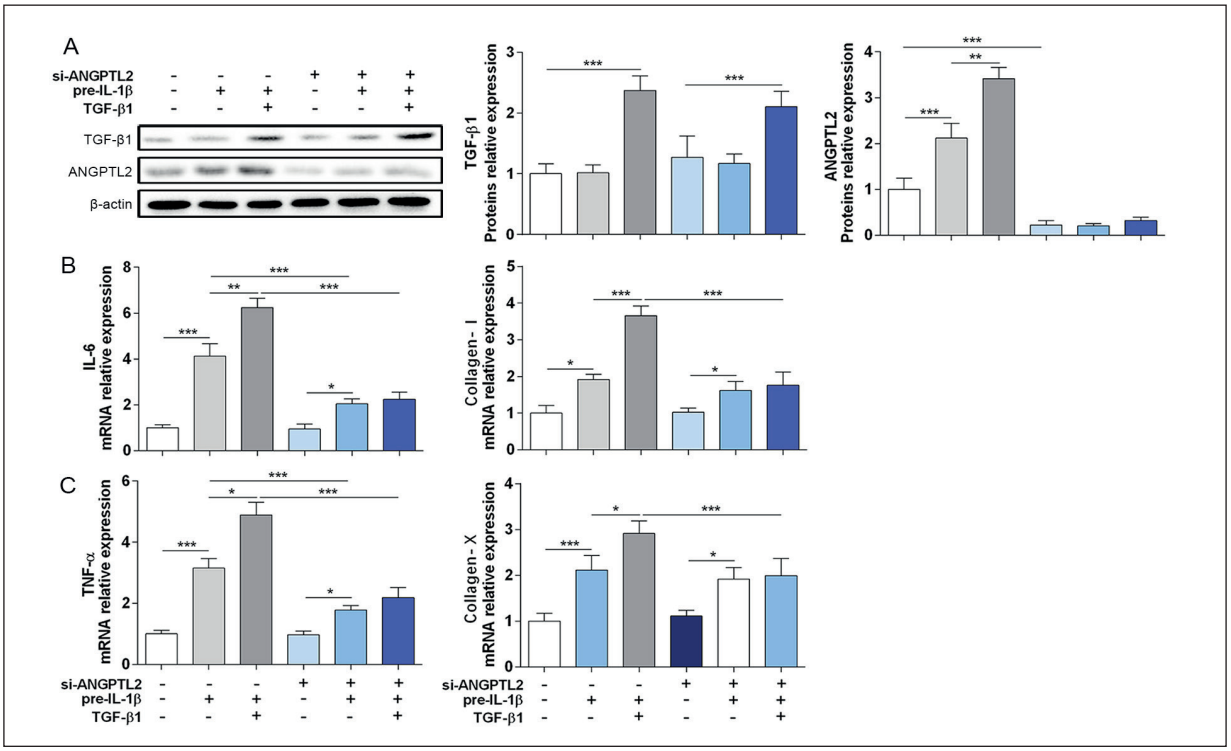

Figure 3. ANGPTL2 silencing alleviates TGF-β1 caused fibrosis and inflammation of NP cells. **A,** WB analysis for the protein expression of TGF-β1 and ANGPTL2 isolated from NP cells, and quantification of protein bands measured by Image J software. qRT-PCR analysis for the mRNA expression levels of **B,** IL-6, TNF-α, and **C,** collagen I, and collagen III isolated from NP cells normalized to GAPDH expression. Results are expressed as mean ± SD. (*$p < 0.05$, **$p < 0.01$, **$p < 0.001$).

rh-TGF-β1 protein treatment continuously increased the IL-6 and TNF-α mRNA expression after IL-1β pretreatment, which was rejected by the silencing of ANGPTL2 (Figure 3B). For the fibrous process, ANGPTL2 silencing was inefficient to inhibit the collagen I and collagen III upregulation caused by IL-1β (Figure 3C). However, in the ANGPTL2 silenced group, rh-TGF-β1 did not aggravate the collagen I and collagen III expressions after IL-1β pretreatment (Figure 3C). Therefore, these findings suggested the TGF-β1 sharpened the NP cells inflammation and fibrosis *via* the mediation of ANGPTL2.

TGF-β1 Aggravates ANGPTL2 Triggered Fibrosis and Inflammation of NP Cells

To confirm the relation between TGF-β1 and ANGPTL2 during the NP cell degeneration, we used rh-ANGPTL2 (5 μg/mL) protein to treated NP cells with or without the presence of rh-TGF-β1 (10 ng/mL) for two days. As shown in Figure 4A and 4B, the cellular TGF-β1 protein level was not significantly changed with the presence of rh-ANGPTL2. On the contrary, exogenic rh-TGF-β1 protein stimuli promoted the cellular ANGPTL2 protein expression. Furthermore, rh-ANGPTL2 significantly triggered the IL-6 and TNF-α mRNA expression, which was aggravated by the rh-TGF-β1 stimuli (Figure 4C). Compared to the control, ANGPTL2 overexpression also contributed to the production of collagen I and collagen III, which was amplificated by the presence of rh-ANGPTL2 and rh-TGF-β1 (Figure 4D). Therefore, ANGPTL2 is a negative factor that promotes NP cells inflammation and fibrosis, which might be a downstream target of TGF-β1.

Discussion

The causes of IDD have not yet been fully elucidated, but its fundamental pathological changes have been roughly apparent, which main contain the gradual reduction of healthy cells, inflammation infiltration, the loss of proteoglycan, and the replacement of collagen proportion in ECM, secondary disc fibrosis, and endplate calcification[15]. In normal discs, type I collagen is mainly distributed in the outer layer of the annulus fibrosus, playing a crucial role in maintaining the tension of the annulus fibrosus; In contrast, type III collagen has very little content, accounting for only 3% of the total collagen, mainly distributed in the type I and II collagen transition area[16,17]. With the approach of IDD, types I and III collagen also appear and

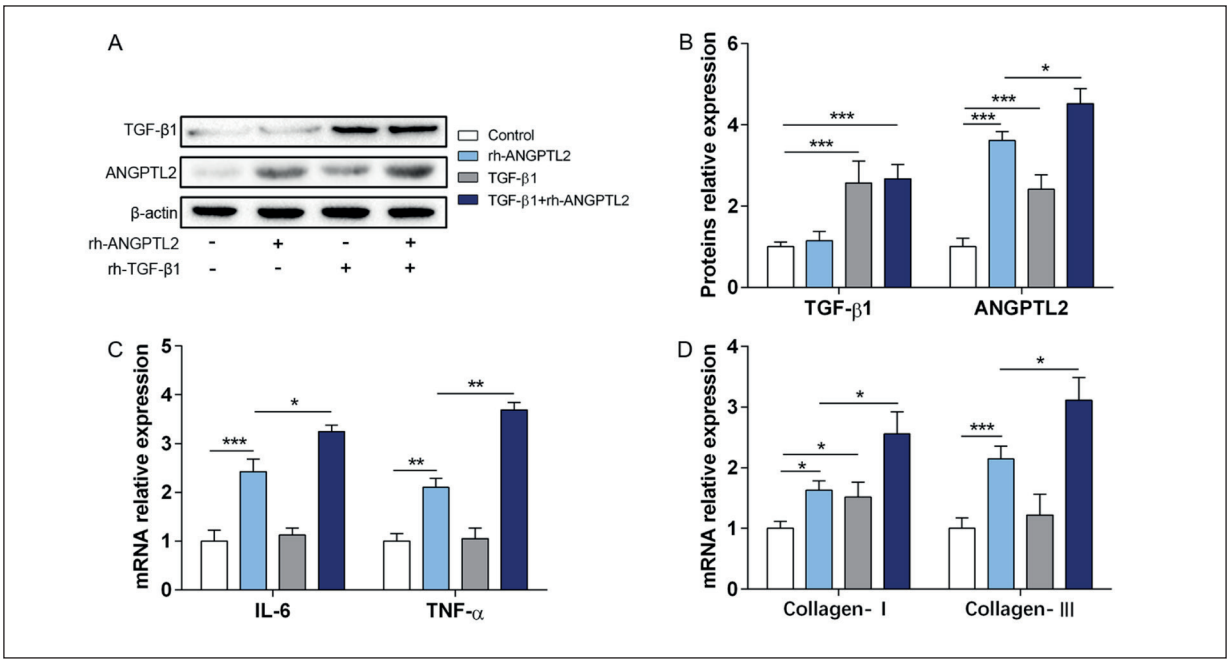

Figure 4. TGF-β1 aggravates ANGPTL2 triggered fibrosis and inflammation of NP cells. **A,** WB analysis for the protein expression of TGF-β1 and ANGPTL2 isolated from NP cells, and **B,** quantification of protein bands measured by Image J software. qRT-PCR analysis for the mRNA expression levels of **C,** IL-6, TNF-α, and **D,** collagen I, and collagen III isolated from NP cells normalized to GAPDH expression. Results are expressed as mean ± SD. (*$p < 0.05$, **$p < 0.01$, **$p < 0.001$).

accumulate in the NP. The role of TGF-β1 in different periods of IDD is varying[18,19]. During the early degeneration process, TGF-β1 promotes the normal ECM synthesis of NP cells, including the proteoglycans secretion and type II collagen production, and it also improves the activity of NP cells and inhibits apoptosis[20]. Therefore, when TGF-β1 was silenced in the normal NP cells, we found the IL-1β was much easier to cause NP cell degeneration. However, with the further development of IDD, the NP cells change into dedifferentiation and fibrosis. Under the stimulation of TGF-β1, the synthesis of type I and type III collagen increases rapidly, resulting in the remolding of collagen proportion and tissue fibrosis[21]. In our study, the normal NP cells expressed a low level of TGF-β1, and TGF-β1 overexpression in the normal NP cells did not significantly trigger the inflammation and fibrosis. In contrast, TGF-β1 stimulation markedly active the inflammatory response and fibrous procedure in the degenerative NP cells. Meanwhile, not only the IL-1β but also TGF-β1 promoted the ANGPTL2 expression, which potentially participates in the TGF-β1 related NP cell degeneration.

ANGPTL2 belongs to the family of angiopoietin-like proteins (ANGPTLs), a new kind of angiogenesis-related protein factors discovered in recent years pertaining to secreted glycoproteins[22]. The role of ANGPTL2 in regulating the occurrence, reconstruction, and functional maintenance of blood vessels has been agreed in many studies[23]. In recent years, the function of ANGPTL2 in proinflammation, even fibrosis, has gradually attracted attention. Yang et al[24] reported ANGPTL2 deficiency alleviates paraquat-induced lung injury via suppressing inflammation, oxidative stress, and fibrosis through NF-κB signaling. Besides, circulating the ANGPTL2 level presents an inflammatory marker for the diagnosis of diabetes, cardiovascular diseases, chronic kidney disease, and various types of cancers[25]. However, whether ANGPTL2 plays a role in the IDD remains unknown. In our study, IL-1β induced the NP cell degeneration, accompanied by an increase of ANGPTL2 protein expression. Besides, ANGPTL2 also highly expressed in the TGF-β1 caused secondary inflammation and fibrosis after IL-1β pretreatment, and the rh-ANGPTL2 supplement was shown to accelerate NP cell degeneration. Therefore, we hold the opinion that ANGPTL2 is a proinflammatory effector and plays a role in the fibrosis of NP cell degeneration. We confirmed this hypothesis by silencing the ANGPTL2 expression, which alleviated the IL-1β and TGF-β1 caused NP cells inflammation and fibrosis.

Following, to further explore the relation between ANGPTL2 and TGF-β1 in the progress of NP cells degeneration, we silenced TGF-β1 and ANGPTL2 gene by siRNA transfection, respectively. The data indicated TGF-β1 blocking weakened the ANGPTL2 expression, which was more evident under the stimulation of IL-1β. However, the deficiency of ANGPTL2 made no difference to the expression of TGF-β1, suggesting ANGPTL2 might be a downstream target of TGF-β1[11]. Additionally, TGF-β1 is observed to regulate ANGPTL2 expression by the mediation of SMAD3 protein, which binds to the SMAD Binding Element (SBE) region located on the ANGPTL2 promoter[26]. As the TGF-β1/SMAD3 signaling is verified to be significantly activated during IDD[27,28], it is possible that the ANGPTL2 upregulation in the degenerative NP cells also depends on the activated SMAD3 relating to TGF-β1. However, some other studies reported ANGPTL2 could also activate TGF-β1 expression. Therefore, the crosslink between ANGPTL2 and TGF-β1 in NP cells needs depth discovery[29].

During the phase of the development, maturation, and degeneration of the spine, the intervertebral discs undergo vascularization, devascularization, and revascularization[30]. The healthy intervertebral disc is the most abundant avascular tissue in the body, and its nutrition comes from the penetration of endplates cartilage and annulus fibrosus. When degeneration, endplates calcification hinders the supply of nutrients to the disc, resulting in a series of pathological compensatory reactions, including angiogenesis[31]. TGF-β1 is an important growth factor regulating angiogenesis. When hypoxia or injury comes, TGF-β1 upregulates vascular endothelial growth factor (VEGF) expression and promotes a large number of angiogenesis[32]. In fibrous tissue proliferative diseases, such as scleroderma, rheumatoid arthritis, and pathological scars, TGF-β1 and neovascularization also accompany with accumulation[33]. This evidence suggests that the abnormal secretion of TGF-β1 is closely related to the process of angiogenesis and fibrosis[34]. Since ANGPTL2, like VEGF, shows pro-angiogenic activity in various ways. We hypothesized that TGF-β1 upregulated the ANGPTL2 expression via an angiogenesis-associated signaling[35, 36], which needs further verification.

Conclusions

The novelty of this research is to elucidate the proinflammatory and fibrogenic effects of ANGPTL2 in the progression of NP cell degeneration. Additionally, TGF-β1 aggravates the degenerative NP cell›s fibrosis and inflammation, potentially relating to the upregulation of ANGPTL2. Therefore, ANGPTL2 is a promising therapeutic target in IDD. Since the different degenerative states of NP cells respond to TGF-β1 differently, and how IL-1β causes cell degeneration by inflammation may not be the same as a natural way. For the next research, we plan to use the naturally degenerative NP from patients with IDD and continue to explore the relation between ANGPTL2 and SMAD3 in the IDD related disease.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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