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TFDP1 overexpression promotes apoptosis of nucleus pulposus cells in intervertebral disc degeneration through regulating ADAM15/MMP9 axis

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Abstract. Intervertebral disc degeneration (IVDD) is a common contributor for low back pain, which is featured by loss of extracellular matrix and nucleus pulposus cells (NPCs). Hence, our current study is undertaken to explore the potential mechanism of NPC apoptosis during IVDD. Transcription factor Dp-1 (TFDP1) expression in degenerative and non-degenerative intervertebral disc tissues was analyzed by bioinformatics. After transfection as needed, viability and apoptosis of NPCs were evaluated by cell counting kit-8 assay and flow cytometry, respectively. Western blot or quantitative real-time reverse transcription polymerase chain reaction was applied to assess expressions of TFDP1, matrix metallopeptidase 9 (MMP9), a disintegrin and metalloproteinase 15 (ADAM15), and apoptosis-associated proteins. TFDP1 expression was upregulated in degenerative intervertebral disc tissues. TFDP1 overexpression repressed viability, promoted apoptosis, increased expressions of Bax, Cleaved caspase 3, MMP9 and ADAM15, and decreased Bcl-2 expression in NPCs, while TFDP1 silencing did conversely. ADAM15 silencing promoted viability, inhibited apoptosis, increased Bcl-2 expression, and decreased Bax, Cleaved caspase 3, and MMP9 expressions in NPCs, which were reversed by TFDP1 overexpression. TFDP1 overexpression promotes apoptosis of NPCs in IVDD through regulating ADAM15/MMP9 axis, highlighting its role as a molecular target for the treatment of low back pain.

Key words: Transcription factor Dp-1 — Nucleus pulposus cells — A disintegrin and metalloproteinase 15 — Matrix metallopeptidase 9 — Apoptosis

Introduction

Low back pain is a global disease that seriously affects people's quality of life and health (Chou 2021), and possesses multifactorial etiologies, mainly including discogenic pain, facet joint pain, and myofascial pain (Urits et al. 2019). Intervertebral disc degeneration (IVDD) is a common contributor for low back pain (Knezevic et al. 2021), featured by loss of extracellular matrix and nucleus pulposus cells (NPCs). More specifically, in IVDD tissues, the increased inflammatory cytokines induce apoptosis of NPCs, which is conducive to IVDD (Risbud and Shapiro 2014; Johnson et al. 2015; Wang et al. 2016). Hence, NPC apoptosis is a vital risk factor for IVDD, suppression of which can effectively alleviate IVDD (Liao et al. 2019; Hu et al. 2021). Howbeit, there are still limited researches on the mechanisms related to NPC apoptosis in IVDD.

Firstly, we analyzed the differential genes in degenerative and non-degenerative intervertebral disc tissues using GSE34095 dataset. Transcription factor Dp-1 (TFDP1) caught our attention due to its abnormal high expression in IVDD. TFDP1, as a member of the TFDP family, is highly

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expressed in various human tissues (Zhang et al. 2021), and participates in the development of non-small cell lung cancer (Zhan et al. 2017), colon cancer (Morimoto et al. 2020), and liver cancer (Drucker et al. 2019). Also, the effect of TFDP1 on cell apoptosis has been highlighted in a prior study (Huang et al. 2021). Nevertheless, the involvement of TFDP1 in NPC apoptosis of IVDD has yet to be elucidated.

A disintegrin and metalloproteinase 15 (ADAM15), belonging to ADAM family, is a membrane-related proteinase widely expressed in breast cancer (Mattern et al. 2019), prostate cancer (Burdelski et al. 2017), and lung cancer (Zhou et al. 2022), functioning as a promoter or suppressor in the progression of cancers. Accordingly, the role of ADAM15 in IVDD is worthy of exploration. It has been evidenced that ADAM15 can stimulate matrix metallopeptidase 9 (MMP9) expression (Dong et al. 2015), and MMP9 can boost the progression of IVDD (Wang et al. 2020). More interestingly, according to the data from hTFtarget database, ADAM15 is predicted to bind with TFDP1. These results support us to delve into the mechanism of TFDP1 on apoptosis of NPCs.

Collectively, we focused on the specific role of TFDP1 in NPC apoptosis during IVDD, in an attempt to reveal whether TFDP1 could regulate NPC apoptosis *via* ADAM15/MMP9 axis.

Materials and Methods

Bioinformatics

The dataset GSE34095 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34095) from gene expression omnibus (GEO) was used to analyze TFDP1 expression in degenerative and non-degenerative intervertebral disc tissues.

Cell culture and transfection

Human NPCs (#4800, ScienCell Research Laboratory, San Diego, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (21041025, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS, abs972, Absin, Shanghai, China) at 37°C with 5% CO_2.

For transfection, small interfering RNAs targeting TFDP1 (siTFDP1, forward, 5'-AGAAAAAUGAUUUUUCUGGCU-3'; reverse, 5'-CCAGAAAAAUCAUUUUUCUUC-3') and ADAM15 (siADAM15, forward, 5'-UUCACUAAACCCUACAAGCCA-3'; reverse, 5'-GCUUGUAGGGUUUAGUGAAGA-3'), together with their negative controls (siNCs), were all customized from GenePharma (Shanghai, China). At the same time, overexpression plasmid of TFDP1 was also constructed using pcDNA 3.1 vector (VT1001, YouBio, Changsha, China), with the empty vectors as NCs. With the help of lipofectamine^{TM} 3000 transfection reagent (L3000015, Thermo Fisher Scientific, USA), the abovementioned siRNA and plasmids were transfected into NPCs after the cells reached 90% confluence in a 96-well plate by 48-h incubation at 37°C, and the transfection efficiency was detected by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

QRT-PCR

NPCs were firstly lysed using Total RNA Extraction Kit (19221ES50, Yeasen, Shanghai, China) to isolate the total RNA. The RNA then underwent concentration measurement employing NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and reverse transcription into the first-stand complementary DNA (cDNA) through reverse transcription kit (4366596, Thermo Fisher Scientific, USA). Thereafter, the synthesized cDNA was subject to ProFlex™ PCR system (4484078, Thermo Fisher Scientific, USA) along with the application of TaqPath™ ProAmp master mix (A30865, Thermo Fisher Scientific, USA). The PCR was conducted at the indicated conditions: 95°C for 5 min, and 40 cycles of 95°C for 15 s and 60°C for 90 s. All data were processed by $2^{-\Delta\Delta CT}$ method (Chen et al. 2019).

Primer sequences were listed as follows: TFDP1: forward, 5'-AGCCAACGGAGAACTCAAGG-3'; reverse, 5'-GCAGACCAAGGTGAGGAGTC-3'. ADAM15: forward, 5'-TAGTACTGTCTTGGGGTGTCC-3'; reverse, 5'-GCCTGGGACCAACTCCCTAT-3'. GAPDH (internal reference): forward, 5'-CAGCCTCAAGATCATCAGCA-3'; reverse, 5'-TGTGGTCATGAGTCCTTCCA -3'.

Cell counting kit-8 (CCK-8) assay

The principle of CCK-8 detection of cell viability is based on the reduction of WST-8 in CCK-8 reagent to yellow formazan product by dehydrogenase in cells. The amount of formazan produced is proportional to the number of living cells, therefore cell proliferation can be analyzed by measuring the amount of formazan products. CCK-8 assay kit (CK04, Dojindo, Kumamoto, Japan) was applied to detect the viability of NPCs. In detail, NPCs were added into 96-well plates (5×10^3 cells/well) and cultured for 24/48/72 h, and then incubated with 10 μl CCK-8 assay solution for 4 h at 37°C. The absorbance at 450 nm was determined by Varioskan LUX microplate reader (Thermo Fisher Scientific, USA).

Flow cytometry

Annexin V-fluorescein isothiocyanate (V-FITC) apoptosis detection kit (C1062S, Beyotime, Shanghai, China) was purchased to measure apoptosis of NPCs. Firstly, NPCs

were subject to digestion in 0.25% Trypsin solution (C0201, Beyotime, China), centrifugation, and resuspension as needed. Thereafter, NPC cell suspension (5×10^5 cells) was centrifuged at 1000×*g* for 5 min, and the supernatant was removed. 195 µl Annexin V-FITC binding solution was added to resuspend the cells, followed by the addition of 5 µl Annexin V-FITC and 10 µl propidium iodide. 20-min incubation at 25°C was proceeded in the dark, and then observation and analyses were performed using BD FACSAria™ Fusion flow cytometer (BD Biosicences, San Diego, CA, USA) and BD FACSDiva™ software (BD Biosicences, USA), respectively.

Western blot

NPCs were subject to lysis in RIPA lysis buffer (SL1010, Coolaber, Beijing, China) to obtain total protein, and the protein concentration was assessed in a bicinchoninic acid (BCA) protein assay kit (23227, Thermo Fisher Scientific, USA). The protein was separated on 6% and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (SK60112, Coolaber, China), transferred to polyvinylidene fluoride membrane (YA1701, Solarbio, Beijing, China) and blocked in 5% nonfat-dried milk blocking buffer (SL1330, Coolaber, China) for 1 h at room temperature.

The primary antibodies (Abcam, Cambridge, UK) against ADAM15 (ab137387, 93 kDa, 1:1000), BCL2 associated X protein (Bax, ab32503, 21 kDa, 1:1000), B-cell lymphoma-2 (Bcl-2, ab182858, 26 kDa, 1:2000), Cleaved caspase 3 (ab32042, 17 kDa, 1:500), MMP9 (ab76003, 92 kDa, 1:1000), and housekeeping control GAPDH (ab181602, 36 kDa, 1:10000) were used to incubate the membrane at 4°C overnight. Then the membrane was rinsed in Tris Buffered Saline with Tween® 20 buffer (TBST, #9997, Cell Signaling Technology, Danvers, MA, USA), and experienced 1-h incubation in horseradish peroxidase-conjugated Mouse anti-Rabbit IgG (ab99697, 1:1000, Abcam, UK) secondary antibody at room temperature.

The membrane was rinsed by TBST about three times, and developed in the chromogenic liquid (34577, Thermo Fisher Scientific, USA), followed by the detection in Amersham Imager 680 (GE Healthcare, Chicago, IL, USA) and quantification in ImageJ 5.0 (Bio-Rad, Hercules, CA, USA).

Co-immunoprecipitation (Co-IP) assay

Co-IP is a classical method for studying protein interactions based on the specific interaction between antibodies and antigens. The basic principle is to lyse cells under non-denatured conditions in order to preserve many protein-protein interacting complexes in living cells. If X is immunoprecipitated with antibodies to protein X, then the protein Z bound to X in the body can also be precipitated. Briefly, the cells were collected and lysed in IP lysis buffer (87787; Pierce IP Lysis Buffer, Thermo Fisher Scientific, USA). The cell lysates were incubated with antibody against ADAM15 (LS-C124907, LSBio, Seattle, USA) and Protein A/G magnetic beads (88803, Pierce, Thermo Fisher Scientific, USA) at 4°C overnight. The immunoprecipitated pellets were collected by centrifugation (12000 rpm, 5 min), and then pellets were washed 3 times with the cold IP lysis buffer (87787; Pierce IP Lysis Buffer, Thermo Fisher Scientific, USA), boiled in 2X SDS loading buffer (P0015B, Beyotime, China), and run on 10% SDS-PAGE for Western blot analysis.

Statistical analyses

The experiments were performed in triplicate. All data were expressed as mean ± standard deviation (SD) and analyzed in GraphPad Prism 8 (GraphPad, Inc., La Jolla, CA, USA). Data among multiple groups were compared with one-way analysis of variance. Statistical analysis was considered significant by $p < 0.05$.

Results

TFDP1 overexpression repressed viability and promoted apoptosis of NPCs, while TFDP1 silencing did conversely

In this study, we analyzed the differential genes in degenerative and non-degenerative intervertebral disc tissues through bioinformatics. TFDP1 expression was upregulated in degenerative intervertebral disc tissues relative to the non-degenerative intervertebral disc tissues (Fig. 1A). Then siTFDP1 and TFDP1 overexpression plasmids were transfected into NPCs, respectively. TFDP1 expression was downregulated or upregulated in NPCs, which confirmed the successful transfection ($p < 0.001$) (Fig. 1B). Next, we probed into the specific effects of TFDP1 on viability and apoptosis of NPCs following the corresponding transfection. TFDP1 overexpression led to suppressed viability (at 24/48/72 h) and promoted apoptosis in NPCs ($p < 0.05$), whereas TFDP1 silencing caused the opposite trend ($p < 0.05$) (Fig. 1C–E). All findings hinted that TFDP1 may promote the apoptosis of NPCs.

In order to further verify the promotion of TFDP1 on NPCs apoptosis, apoptosis-related protein expressions were also detected. It was obvious that Bax and Cleaved caspase 3 expressions were enhanced while Bcl-2 expression was suppressed following the overexpression of TFDP1 (Fig. 2A–D, $p < 0.001$). Oppositely, TFDP1 silencing decreased Bax and Cleaved caspase 3 levels, and increased Bcl-2 level in NPCs (Fig. 2A–D, $p < 0.001$). Accordingly, we have preliminarily elucidated the promotion of TFDP1 on NPCs apoptosis.

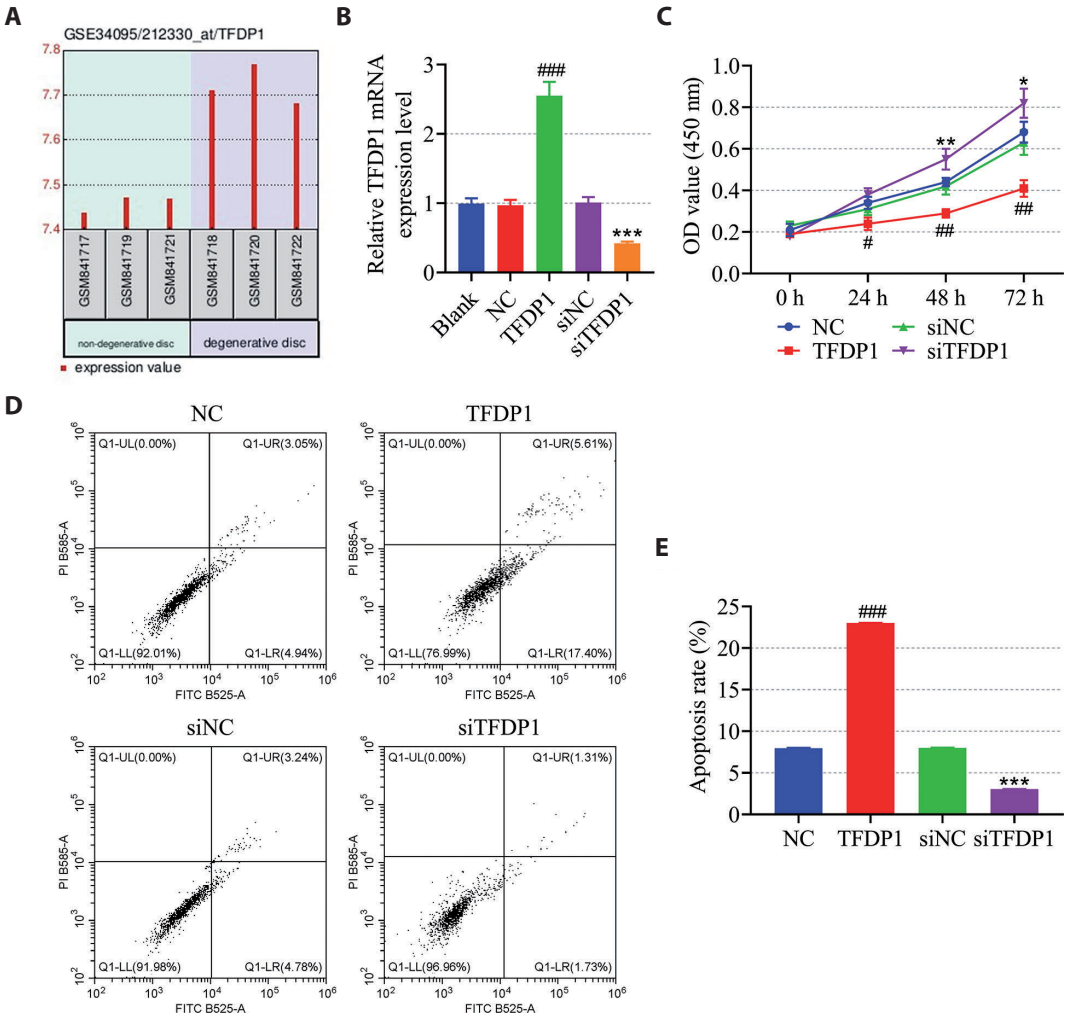

Figure 1. TFDP1 overexpression repressed viability and promoted apoptosis of NPCs, yet TFDP1 silencing did conversely. **A.** TFDP1 expression in degenerative and non-degenerative intervertebral disc tissues was analyzed by bioinformatics. **B.** siTFDP1 and TFDP1 overexpression plasmids were transfected into NPCs, which was verified by qRT-PCR. GAPDH was used as the internal control. After transfection of siTFDP1 or TFDP1 overexpression plasmids into NPCs, the cell viability was tested by cell counting kit-8 assay (**C**) and apoptosis was determined by flow cytometry (**D, E**). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* NC; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* siNC. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; NPCs, nucleus pulposus cells; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; siTFDP1, small interfering RNA targeting TFDP1; TFDP1, transcription factor Dp-1.

TFDP1 overexpression promoted expressions of MMP9 and ADAM15 in NPCs, while TFDP1 silencing did conversely

As previously reported, ADAM15 can stimulate MMP9 expression (Dong et al. 2015). Based on the data from hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget#!/), we found that ADAM15 could bind with TFDP1. Hence, we wondered whether TFDP1 could affect apoptosis of NPCs *via* regulating ADAM15 expression. The expressions of ADAM15 and MMP9 were evaluated in NPCs following the intervention of TFDP1 overexpression or silencing. In accordance with the results, ADAM15 and MMP9 expressions were enhanced following the overexpression of TFDP1 (Fig. 2E–H, $p < 0.001$), but suppressed due to TFDP1 silencing in NPCs (Fig. 2E–H, $p < 0.01$). In addition, Co-IP assay results showed that ADAM15 and TFDP1 formed a complex and interacted (Fig. 3A).

TFDP1 overexpression attenuated the effects of ADAM15 silencing on viability, apoptosis, and MMP9 expression in NPCs

To further fathom out the role of ADAM15 in TFDP1-overexpressing NPCs, siADAM15 was successfully transfected into NPCs, as verified by the downregulation of ADAM15 (Fig. 3B, $p < 0.001$). Also, by means of CCK-8 assay (Fig.

3C) and flow cytometry (Fig. 3D,E), we proved viability of NPCs was increased while apoptosis was decreased in response to the deficiency of ADAM15 ($p < 0.05$), which was reversed by overexpression of TFDP1 ($p < 0.01$). ADAM15 silencing also counteracted the effects of overexpression of TFDP1 on abovementioned factors (Fig. 3C–E, $p < 0.05$). After the silencing of ADAM15, Bax, Cleaved caspase 3, and MMP9 expressions were downregulated whereas Bcl-2 level was upregulated in NPCs (Fig. 3F,G, $p < 0.001$), which was neutralized by TFDP1 overexpression (Fig. 3F,G, $p < 0.01$). Similarly, silencing of ADAM15 also reversed the effects of TFDP1 overexpression on these protein expressions in NPCs (Fig. 3F,G, $p < 0.01$).

Discussion

Like we have mentioned above, IVDD, as a nonnegligible factor that contributes to the occurrence of low back pain, leads to distinct global disability (Mohd Isa et al. 2022b). Yet little attention has been paid to IVDD-associated mechanism; therefore, this study focused on this mechanism and attempted to identify the cause of IVDD in the progression of low back pain. Herein, we found that TFDP1 overexpression promoted apoptosis of NPCs in IVDD through regulating ADAM15/MMP9 axis, denoting that TFDP1 may act as a molecular target for the treatment of low back pain.

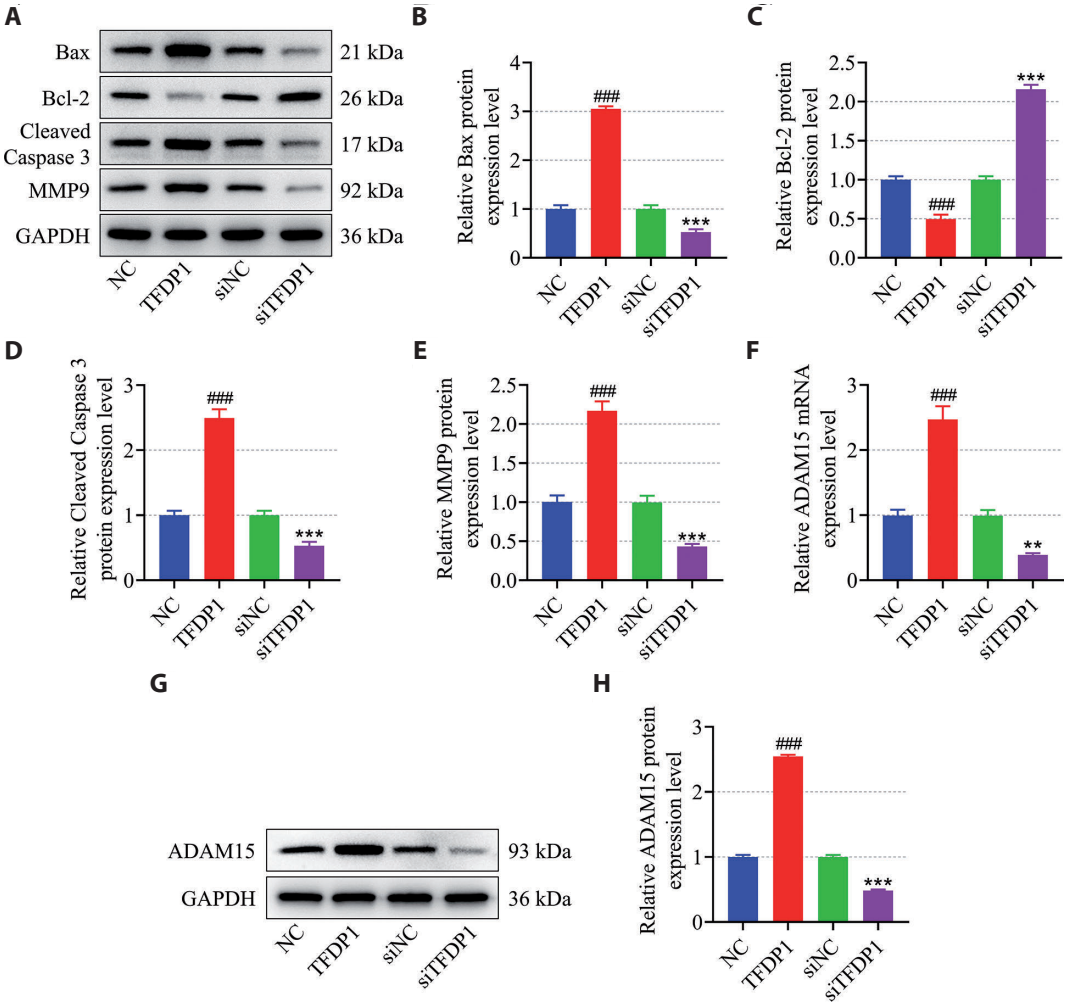

Figure 2. TFDP1 overexpression promoted apoptosis and upregulated MMP9 and ADAM15 expressions in NPCs, yet TFDP1 silencing did conversely. **A.** After transfection of siTFDP1 and TFDP1 overexpression plasmids into NPCs, expressions of Bax, Bcl-2, Cleaved caspase 3 and MMP9 was detected by Western blot. The protein expressions levels of Bax (**B**), Bcl-2 (**C**), Cleaved caspase 3 (**D**) and MMP9 (**E**) was quantified. **F.** The mRNA expression level of ADAM15 in the NPCs were evaluated by qRT-PCR. **G, H.** The protein expression level of ADAM15 in the NPCs were evaluated by Western blot. GAPDH was used as a loading control. $^{###}$ $p < 0.001$ *vs.* NC; ** $p < 0.01$, *** $p < 0.001$ *vs.* siNC. ADAM15, a disintegrin and metalloproteinase 15; Bax, BCL2 associated X protein; Bcl-2, BCL2 B-cell lymphoma-2; MMP9, matrix metallopeptidase 9. For more abbreviations, see Figure 1.

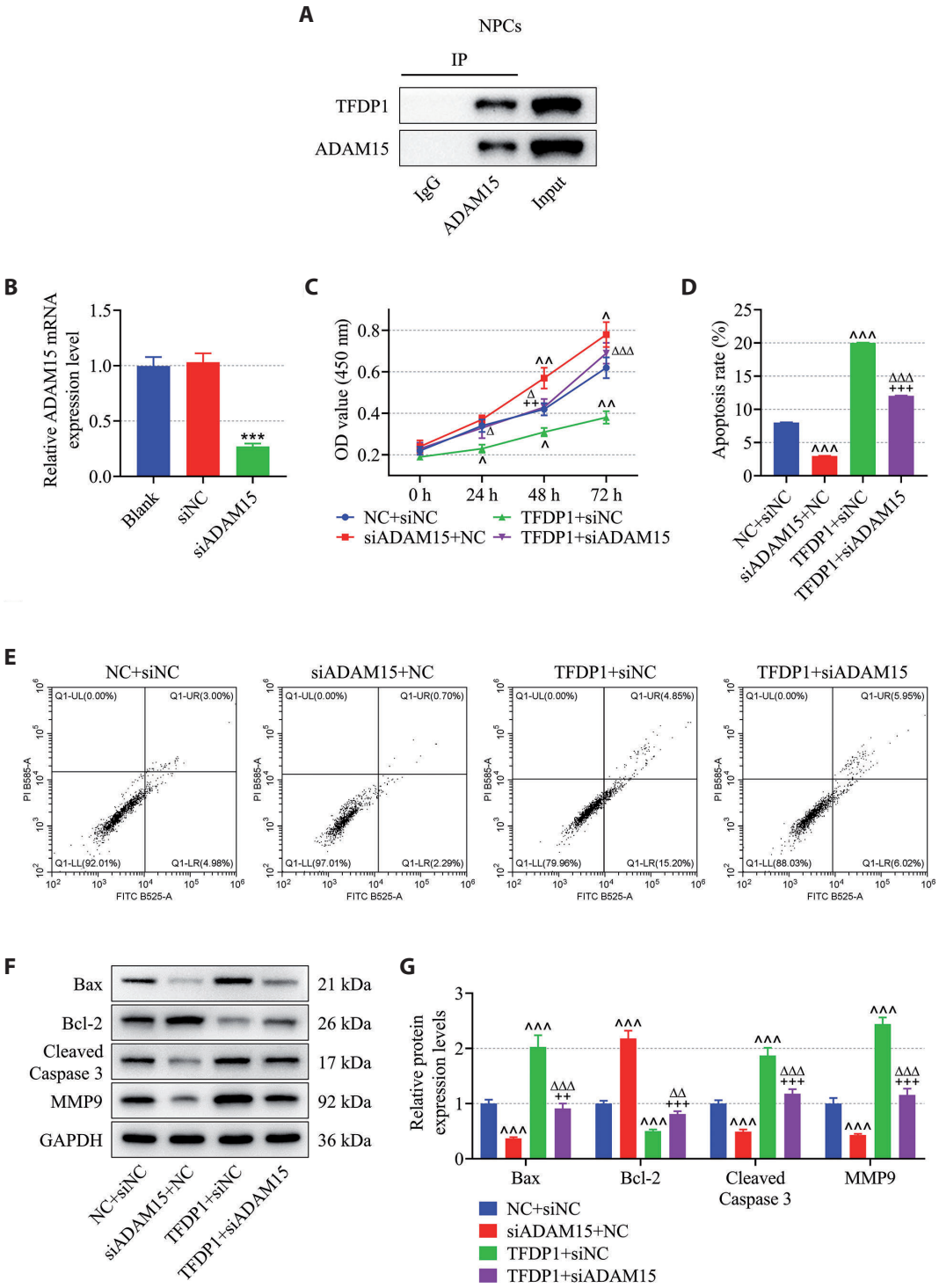

Figure 3. TFDP1 overexpression reversed the effects of ADAM15 silencing on viability, apoptosis, and MMP9 expression in NPCs. **A.** Co-immunoprecipitation analysis of ADAM15 and TFDP1 in NPCs. **B.** SiADAM15 was successfully transfected into NPCs, which was verified by qRT-PCR. GAPDH was used as a loading control. After transfection of TFDP1 overexpression and/or siADAM15, the cell viability was tested by cell counting kit-8 assay (**C**), apoptosis was determined by flow cytometry (**D, E**), and expressions of Bax, Bcl-2, Cleaved caspase 3, and MMP9 in the cells were evaluated by Western blot, along with GAPDH as a loading control (**F, G**). *** $p < 0.001$ *vs.* siNC; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ *vs.* NC+siNC; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ *vs.* TFDP1+siNC; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ *vs.* siADAM15+NC. For abbreviations, see Figure 2.

Intervertebral disc is mainly composed of annulus fibrosus, nucleus pulposus, and cartilage endplate, which plays an important role in maintaining the flexibility and stability of the spine (Mohd Isa et al. 2022a). IVDD is related to various factors such as genetic factors, age, smoking and trauma (Oichi et al. 2020). Much evidence has manifested the association between IVDD and the reduction of NPCs. In other word, the apoptosis of NPCs can accelerate the progression of IVDD (Liao et al. 2019). Herein, we revealed that TFDP1 overexpression promoted apoptosis of NPCs in IVDD through regulating ADAM15/MMP9 axis, indicating that TFDP1 could be a promising target towards apoptosis of NPCs for regulating the progression of low back pain.

TFDP1 has received our attention due to its high expression in IVDD, and it has been proved to participate in the development of many diseases (Zhan et al. 2017; Drucker et al. 2019; Morimoto et al. 2020). Yet its role in IVDD still awaits to be defined, and there is a dearth of investigation pertinent to the effect of TFDP1 on cell apoptosis. Interestingly, TFDP1 can activate the transcription of E2F1 that is responsible for the cell fate (Pellicelli et al. 2016; Dzreyan et al. 2022). In different cellular environments, E2F1 can stimulate cell apoptosis (Rotgers et al. 2015). More importantly, E2F1-mediated apoptosis has been regarded as a novel target for the treatment of various cancers (Wu and Yu 2009). E2F1 actively promotes gastric cancer cell viability while suppressing apoptosis (Chen et al. 2022). Accordingly, we wondered if TFDP1 may regulate apoptosis of NPCs in IVDD. Following the transfection of siTFDP1 and TFDP1 overexpression plasmids, we firstly confirmed that TFDP1 led to an acceleration of apoptosis in NPCs, which suggested that TFDP1 promoted the progression of IVDD. For further confirmation, we detected three proteins involved in the progression of apoptosis. Heaps of studies have verified that apoptosis is a process strictly controlled by multiple genes, including Bcl-2 family and Caspase family (Gao et al. 2021). Among them, Bax and Bcl-2 belong to the Bcl-2 protein family. Bcl-2 is identified as a factor that promotes cell survival and prevents the release of cytochrome C from mitochondria to the cytoplasm, thereby suppressing the cell apoptosis (Klimentova et al. 2021; Tian et al. 2022). Bax is responsible for the promotion of apoptosis, and overexpression of Bax can antagonize the protective effect of Bcl-2 and lead to cell death (Xiao et al. 2019). Caspase 3 is also an irreplaceable protein involved in apoptosis, which can cleave protein substrates, thereby leading to cell death (Kashaw et al. 2019). In the present study, we detected the increase of Bax and Cleaved caspase 3 levels and decrease of Bcl-2 level following overexpression of TFDP1 in the cells. These data altogether supported the conclusion that TFDP1 could stimulate apoptosis of NPCs.

While seeking the potential mechanism of TFDP1 in NPCs, ADAM15 and MMP9 were recognized as the candidate targets. According to the data from hTFtarget database, ADAM15 can bind with TFDP1 and E2F1, implying that TFDP1 may regulate ADAM15 expression to affect the apoptosis of NPCs. Although little was known about the specific role of ADAM15 in NPCs of IVDD, ample investigations have already underscored its effect on various diseases. For instance, ADAM15 is perceived as a promising biomarker for hepatocellular carcinoma therapy (Xu et al. 2021). ADAM15 can stimulate MMP9 expression to boost the invasion of LC cells (Dong et al. 2015). Intriguingly, MMP9 has been corroborated to stimulate IVDD (Wang et al. 2020). Upregulation of MMP9 has been determined in human degenerative NP tissues (Xu et al. 2016). Herein, ADAM15 and MMP9 expressions were assessed in NPCs under the influence of TFDP1 overexpression or deletion. In accordance with prior findings, their expressions were promoted by TFDP1 overexpression in NPCs, hinting their potential participation in the regulation of NPCs. With the help of siADAM15, we found that deletion of ADAM15 played an inhibitory role in apoptosis of NPCs, which was reversed by overexpression of TFDP1. Additionally, ADAM15 can induce MMP9 expression. Our results, for the first time, demonstrated that TFDP1 overexpression facilitated apoptosis of NPCs in IVDD through regulating ADAM15/MMP9 axis.

However, there are some limitations in this study. We only investigated the role of TFDP1 in IVDD through *in vitro* cell experiments, which needs to be further analyzed through *in vivo* animal experiments. In addition, the downstream genes or pathways regulated by TFDP1 and ADAM15 in IVDD still need exploration.

In conclusion, this study has cast light on another mechanism associated with apoptosis of NPCs, namely TFDP1 enhances the apoptosis of NPCs in IVDD by regulating the ADAM15/MMP9 axis. The results, we hope, can contribute to the deep understanding of IVDD, offering an insight for the therapy of low back pain.

Conflict of interest. The authors report there are no competing interests to declare.

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