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FBW7 inhibits nucleus pulposus cells proliferation by downregulation of cyclin E in the intervertebral disc degeneration

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Abstract. – OBJECTIVE: TF-box and WD repeat domain-containing 7 (FBW7), a component of SCF ubiquitin ligase complex, usually acts as a tumor suppressor because it has an ability in the inhibition of cell proliferation. Nevertheless, the role of FBW7 in intervertebral disc degeneration (IDD) is not quite understood.

MATERIALS AND METHODS: The total protein and RNA were isolated from patients' disc tissues. WB was carried out to analyze the collagen II and FBW7 protein levels of different Pfirrmann grades disc degeneration. Reverse transcription-polymerase chain reaction (RT-PCR) was used to test the collagen II and FBW7 mRNA expression in these disc samples. NP cells were transfected with siRNA-FWB7 to downregulate the FBW7 expression. SiRNA-NC was used as the sham group. Cyclin E, E2F1 and E2F2 were analyzed with WB and RT-PCR.

RESULTS: In this study, different grads of degenerated disc tissues were analyzed, and it was found that FBW7 was overexpressed in much severe degeneration condition, which was also proved by the IL-1β stimulated nucleus pulposus (NP) cell degeneration model *in vitro*. Interestingly, the results showed that FBW7 suppression could reverse the degeneration of NP cells. Furthermore, we found that FBW7 induced NP cell cycle arrest in G1 phase and inhibited cell proliferation by upregulating p27 expression *in vitro*. The overexpression of p27 resulted in the inhibition of cyclin E, which promotes cell proliferation.

CONCLUSIONS: Taken together, our study uncovered that FBW7 played an essential inhibitory role in NP cells proliferation, providing new insights that FBW7 may be a potential strategy for IDD treatments.

Key Words:
Nucleus pulposus cells, Intervertebral disc degeneration, Cell proliferation, FBW7, Cyclin E.

Introduction

Low back pain, a major symptom of intervertebral disc degeneration (IDD), affects millions of people globally each year[1,2], which leads to many economic and social burdens that affect millions of individuals globally[3]. The progression of IDD involves complex multiple factors, such as inflammation, oxidative stress, mechanical injury and senescence[4]. The main clinical manifestations of IDD are disc herniation, vertebral instability, and spinal stenosis. Currently, the pathogenic mechanisms of IDD remain unknown. At present, IDD is mainly treated with surgery for symptomatic interventions, but the outcomes are poor with proneness to recurrence[5]. It is important to elucidate the exact mechanisms of IDD, so as to identify novel treatment for this disabling disorder.

Even though the etiology of IDD is always multifactorial, genetic factors are considered to be the greatest parts of them. In these years, the molecular functions of disc degenerative disease have got much attention in research, which has substantially improved the understanding of the biology in IDD[6,7]. F-box and WD repeat domain-containing 7 (FBW7) is a substrate component of the evolutionary conserved SCF-type E3 ubiquitin ligase. FBW7 targets multiple well-known oncoproteins, including c-Myc[8], cyclin E[9], and the mammalian target of rapamycin (mTOR)[10] for ubiquitination-mediated destruction, thus, it is a recognized tumor suppressor[11,12]. In most cases, FBW7 functions as a trigger to degrade target protein which possesses a potent in the regulation of cell proliferation, cell division, and differentiation. FBW7 complexed with SCF targets cyclin E for degradation and inhibits cell cycle progression, which

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can inhibit the cell proliferation[13]. Cyclin E is a key component of the cell cycle machinery that is frequently deregulated in cancer. During the cell cycle, cyclin E has a very important role in S-phase entry from either G1 or quiescence by the activation of E2F, which is quite meaningful for cell proliferation[14].

The potential association between FBW7 and IDD is still unknown. To investigate the function of FBW7 in IDD, we isolated the nucleus pulposus (NP) cells from surgery samples, established the NP cell degeneration model with IL-1β stimulation, and downregulated the expression of FBW7 gene by siRNA transfection. What we found showed that FBW7 targeted cyclin E and deregulated NP cell cycle arrest, which inhibited the proliferation of NP cells. These findings enable us to comprehend the FBW7 as a pro-degeneration molecule in NP cells during the progress of IDD.

Materials and Methods

Patient Tissue Samples

17 degenerative disc samples were coll from patients undergoing disc herniation op- tions (mean age: 43; age range: 33-50; 10 ma 7 females). This research project approve by the Ethics Committee of Th Affil- iated Hospital of the Xi'an Jiaotong U versity. The written informed con patients or relatives bef the ions. This study was conducted accordan with the Declaration of Hels st took nucleus pulposus witho he endp We divided the samples into groups acc g to the Pfirrmann sc of dis egeneration: Mild group (grade II/I ere gr up (grade IV/V). We conser the ues in quid nitrogen for the isol mR proteins.

Cells Culture and Drug Treatment

The tissues were washed three times with a phosphate-buffered saline solution (PBS) before isolating the NP cells. The samples were minced into small pieces and digested for 1 hour with 0.25% trypsin solution at 37°C. Next, the samples were incubated in 0.15% type II collagenase at 37°C overnight. The cell solution was filtered with a 100 μm cell st iner and resuspended with Dulbecco's M gle's Medium and Ham's F-12 ium (D M/F12, 1:1, Thermo Fisher, Wa n, MA, A) containing 10% fetal bo e se (FBS lb- co, Rockville, MD, U). Then, t were seeded in six-well cul e plat at 1×10^5 cells per well. Aft tment (or not) with IL-1β (10 ng/ , or 5 mL) iRNA transfection, th s are cul d 5 days for the next ste

erse Transcription-Polymerase ain Reac n (RT-PCR)

e total A was isolated from NP tissues or c RIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's nstructions. After the determination of the concentration, RNA was reverse transcribed with the PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed to quantify FBW7, type II collagen, p27, cyclin E, E2F1, E2F2, β-Galactosidase (β-gal), and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression levels. GAPDH was used for normalization. The primers used for RT-PCR are shown in Table I. RT-PCR was conducted using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ methods.

ces of the genes for RT-PCR.

Ge me	Forward (5′>3′)	Reverse (5′>3′)
FBW7	GGCCAAAATGATTCCCAGCAA	ACTGGAGTTCGTGACACTGTTA
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
p27	AGGAGGAGATAGAAGCGCAGA	GTGCGGACTTGGTACAGGT
Cyclin E	GGTGAGCTTTTTGCCTGGG	TTCTCCGGCAGAAATCTGAGC
E2F1	ACGCTATGAGACCTCACTGAA	TCCTGGGTCAACCCCTCAAG
E2F2	CGTCCCTGAGTTCCCAACC	GCGAAGTGTCATACCGAGTCTT
β-gal	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

Western Blot (WB) Analysis

The tissues and NP cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was isolated using a Nuclear/Cytosol Fractionation Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The concentrations of protein were measured with the enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). After blocking with 5% skim milk for 1 hour at room temperature and overnight with anti-FBW7 (Abcam, Cambridge, MA, USA, 1:1000), anti-p27 (Abcam, Cambridge, MA, USA, 1:1000), anti-cyclin E (Abcam, Cambridge, MA, USA, 1:1000), anti-type II collagen (Millipore, Billerica, MA, USA, 1:1000), anti-E2F1 (Millipore, Billerica, MA, USA, 1:1000), anti-E2F2 (Abcam, Cambridge, MA, USA, 1:1000), anti-β-gal (Abcam, Cambridge, MA, USA, 1:3000), and anti-β-actin (Cell Signaling Technology, Danvers, MA, USA, 1:2000), the membrane was washed for 3 times with Phosphate-Buffered Saline and Tween-20 (PBST), followed by incubation with the secondary antibody (Abcam, Cambridge, MA, USA, 1:3000) for h at room temperature. The protein bands were visualized and detected using the enhanced chemiluminescence (ECL) system. β-actin was used as the controls.

Immunocytofluorescence and Staining

NP cells (1×10^5/group) cultured in each group were washed three times with PBS, fixed with 4% formaldehyde for 15 min at room temperature, and then, blocked with 5% bovine serum albumin (BSA) for 30 min. Subsequently, the cells were incubated with a primary antibody against FBW7 (Abcam, Cambridge, MA, USA, 1:1000) or type II collagen (Abcam, Cambridge, MA, USA, 1:100) overnight at 4°C. Followed by Cy3-conjugated goat anti-rabbit IgG antibody (Abcam, Cambridge, MA, USA, 1:200) incubated for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China, 1:500), and the cells were visualized using a fluorescence microscope (Zeiss, Oberkochen, Germany).

SiRNA Transfection

NP cells were transfected by siRNA-FBW7 or siRNA-NC (Invitrogen, Carlsbad, CA, USA). 95% of the cells were viable 12 h later. FBW7 was downregulated *via* transfection of siRNA-FBW7. The cells were incubated for other 3 days and passaged after transfecting. The transfection efficacies were measured *via* WB and RT-PCR.

Flow Cytometry

The cell cycle was detected by flow cytometry. Four groups of NP cells were collected and washed three times with PBS, and then fixed 4% formaldehyde for 15 min at room temperature. Subsequently, the cells in different cycles were counted and represented as a percentage of the total cell count by propidium iodide staining (0.2 mg/mL, Kaiji, Nanjing, China) using the CellQuest Diva software.

Cell Viability Assay

NP cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay. Four groups of NP cells were seeded (10^4 cells/well) in 96-well plates and cultured with CCK-8 cell viability and cytotoxicity assay kit (C0009; Beyotime, Shanghai, China), according to the manufacturer's instructions. Absorbance was then measured using a microplate reader (Multiskan MS, Labsystems, Helsinki, MS, Finland) at 570 nm.

Statistical Analysis

All data were normality displayed by the means ± standard deviations. Data compared between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way analysis of variance (ANOVA) test followed by a post-hoc test (Least Significant Difference). p-values <0.05 were considered statistically significant.

Results

Expression of FBW7 in Human NP Tissues with Pfirrmann Grades

The total protein RT-PCR and RNA were isolated from patients' disc tissues. WB was carried out to analyze the collagen II and FBW7 protein level of different Pfirrmann grades disc degeneration. RT-PCR was used to test the collagen II and FBW7 mRNA expression of these disc samples. We chose 4 samples of each patient group randomly, as expected, that the collagen II protein was significantly decreased in the Severe group in both protein and mRNA level (Figure 1A, 1B). Meanwhile, FBW7 gene was markedly upregulated in the Severe group compared to

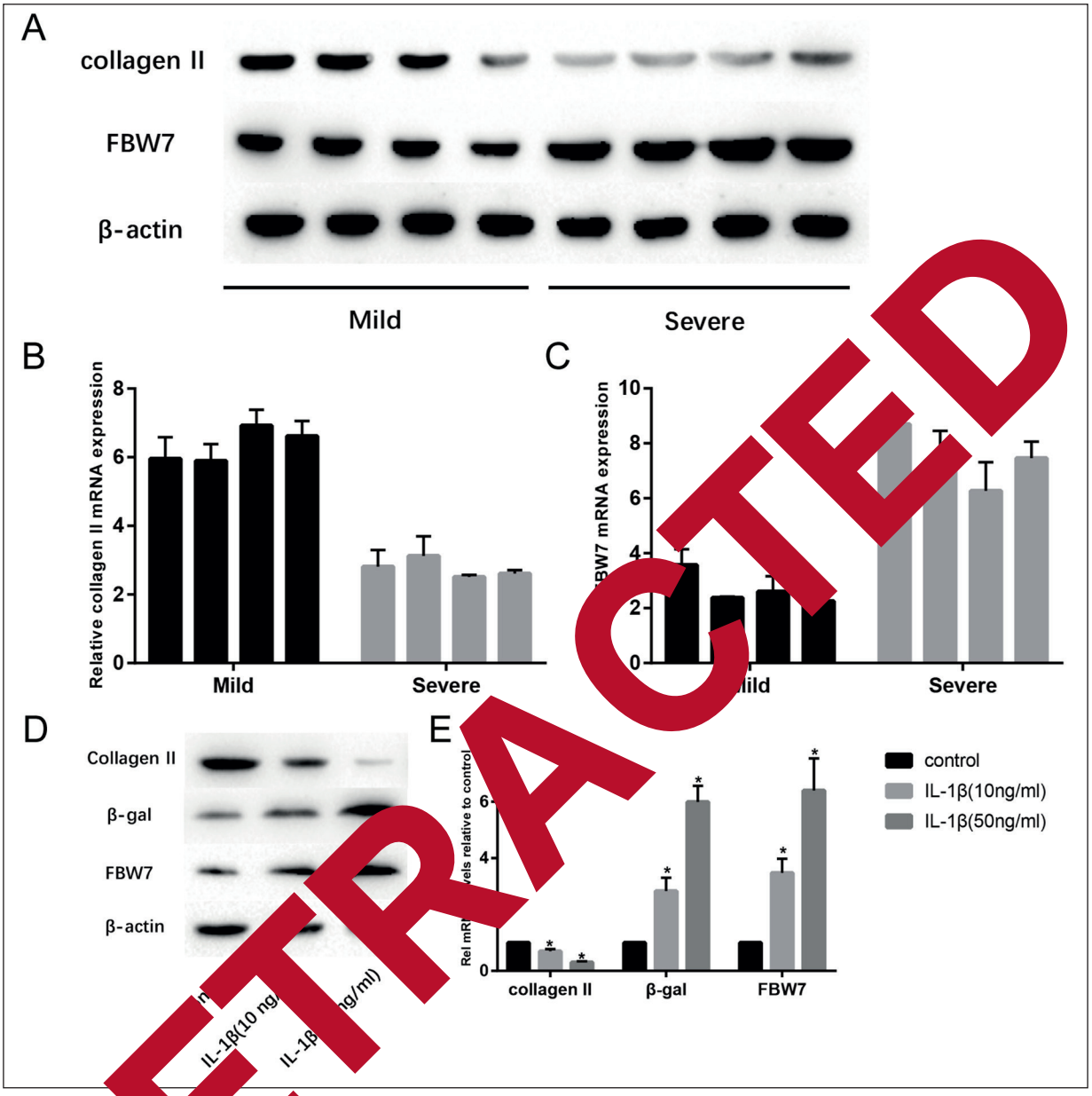

Figure 1. Expre[illegible] of FBW7 [illegible] human NP tissues with Pfirrmann grades and in senescent NP cells *in vitro*. **A, B, C,** Collagen II an[illegible] hu[illegible]P [illegible]es were detected by Western blot (**A**) and RT-PCR (**B, C,**): as the degree of intervertebral disc de[illegible]eratio[illegible] grava[illegible] expression of collagen II decreased, while the expression of FBW7 increased. **D,** collagen II, [illegible], and [illegible]7 in sen[illegible]cent NP cells *in vitro* were detected by Western blot. **E,** collagen II, β-gal, and FBW7 in senescent [illegible]lls [illegible]etected by RT-PCR. ("*" means there is a statistical difference with the control group).

the Mild group in both protein and mRNA level (Figure 1A, 1C). The primer sequences used for RT-PCR are listed in Table I. Collagen II is mainly secreted by the NP cells, and the degeneration of NP cells will less affect the collagen II expression[15]. The results showed that the FBW7 level decreased with the disc got into much severe degeneration.

FBW7 Level Overexpressed in Senescent NP Cells In Vitro

Different concentration of IL-1β (10 ng/ml and 50 ng/ml) was applied into the establishment of NP cell degeneration model according to the previous method[16]. We explored the collagen II and Sirt2 expression with WB and RT-PCR. The results showed that IL-1β significantly decreased the

collagen II expression, especially NP cells treated with 50 ng/ml (Figure 1D). β-gal is the maker of cell senescence. NP cells expressed much β-gal with a higher IL-1β stimulation. Meanwhile, the FWB7 expression increased when the cells were treated with IL-1β and much significant with a higher IL-1β concentration compared with the control (Figure 1D). The RT-PCR showed the same results as those of WB (Figure 1E). In summary, NP cells degenerated under the stimulation of IL-1β, and the FBW7 expression level was upregulated at the same time.

Cyclin E Level and Cell Proliferation Decreased in IL-1β-Induced Senescent NP Cells

We analyzed the Cyclin E expression with WB and RT-PCR. We found that Cyclin E expression decreased in senescent NP cells, especially in the 50 ng/ml dose of IL-1β (Figure 2A, 2B). The CCK8 assay showed that compared with the control group, the cells had less viability, especially in 50 ng/ml group (Figure 2C).

FBW7 Low-Expression Alleviated IL-1β-Induced Human NP Cell Senescence

We transfected NP cells with si[illegible]7 to downregulate the FBW7 expr[illegible]on. SiR[illegible]NC was used as the sham grou[illegible]e verifie[illegible]he function of the siRNA t[illegible]sfecti[illegible]hroug[illegible]/B analysis (Figure 3A)[illegible] treated [illegible]with 50 ng/ml IL-1β t[illegible]stabli[illegible]he de[illegible]eration model. FBW7 l[illegible]w[illegible]re[illegible]n gr[illegible]p exhibited less FBW7, n[illegible]and [illegible] acti[illegible] than IL-1β-induced gr[illegible]d the co[illegible] expression was

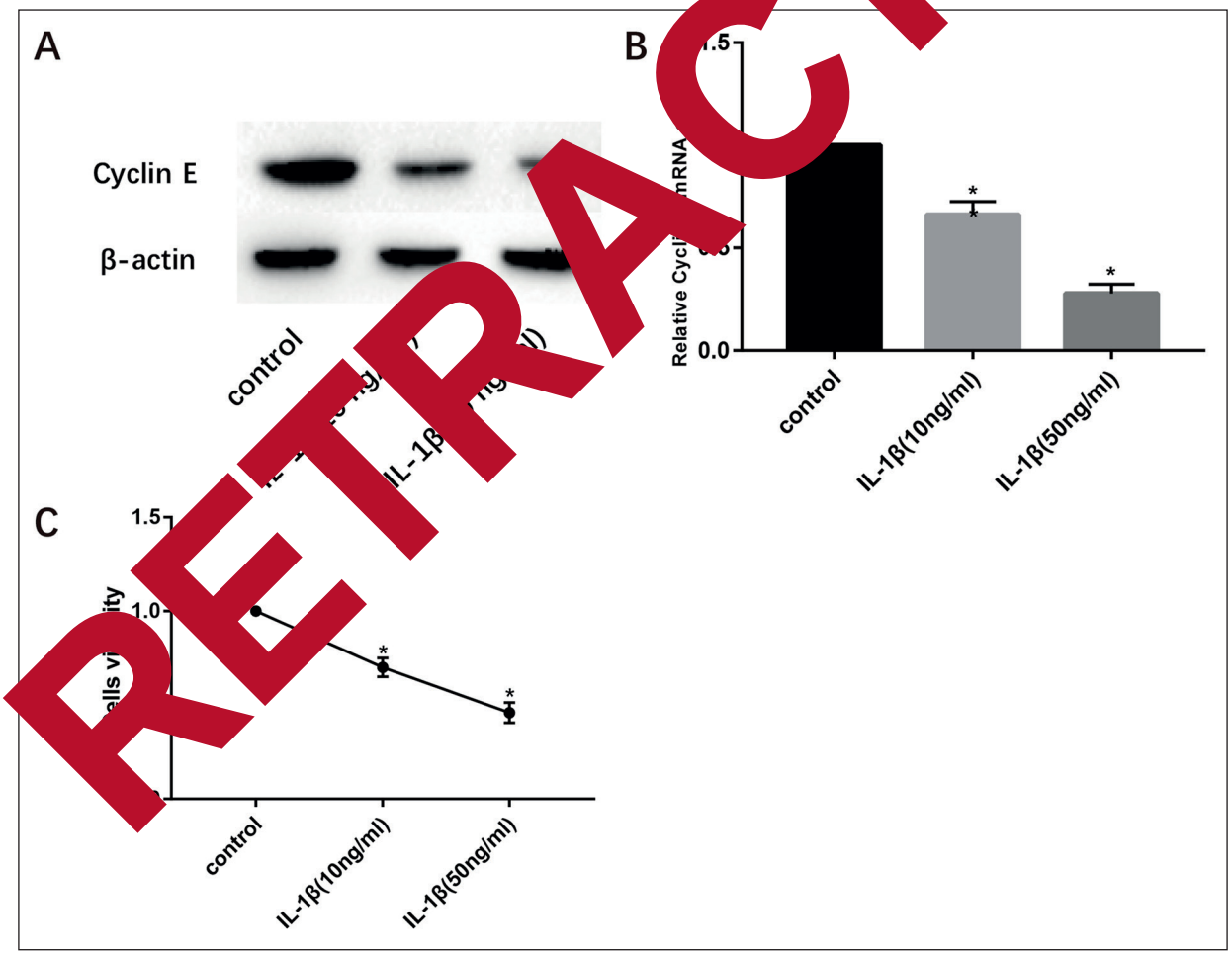

Figure 2. Cyclin E level and cell proliferation decreased in IL-1β-induced senescent NP cells. **A,** Results of the expression of cyclin E in three groups were shown by Western blot. **B,** Results of the expression of cyclin E in three groups were shown by RT-PCR. **C,** Cell proliferation was detected by the CCK-8 assay ("*" means there is a statistical difference with the control group).

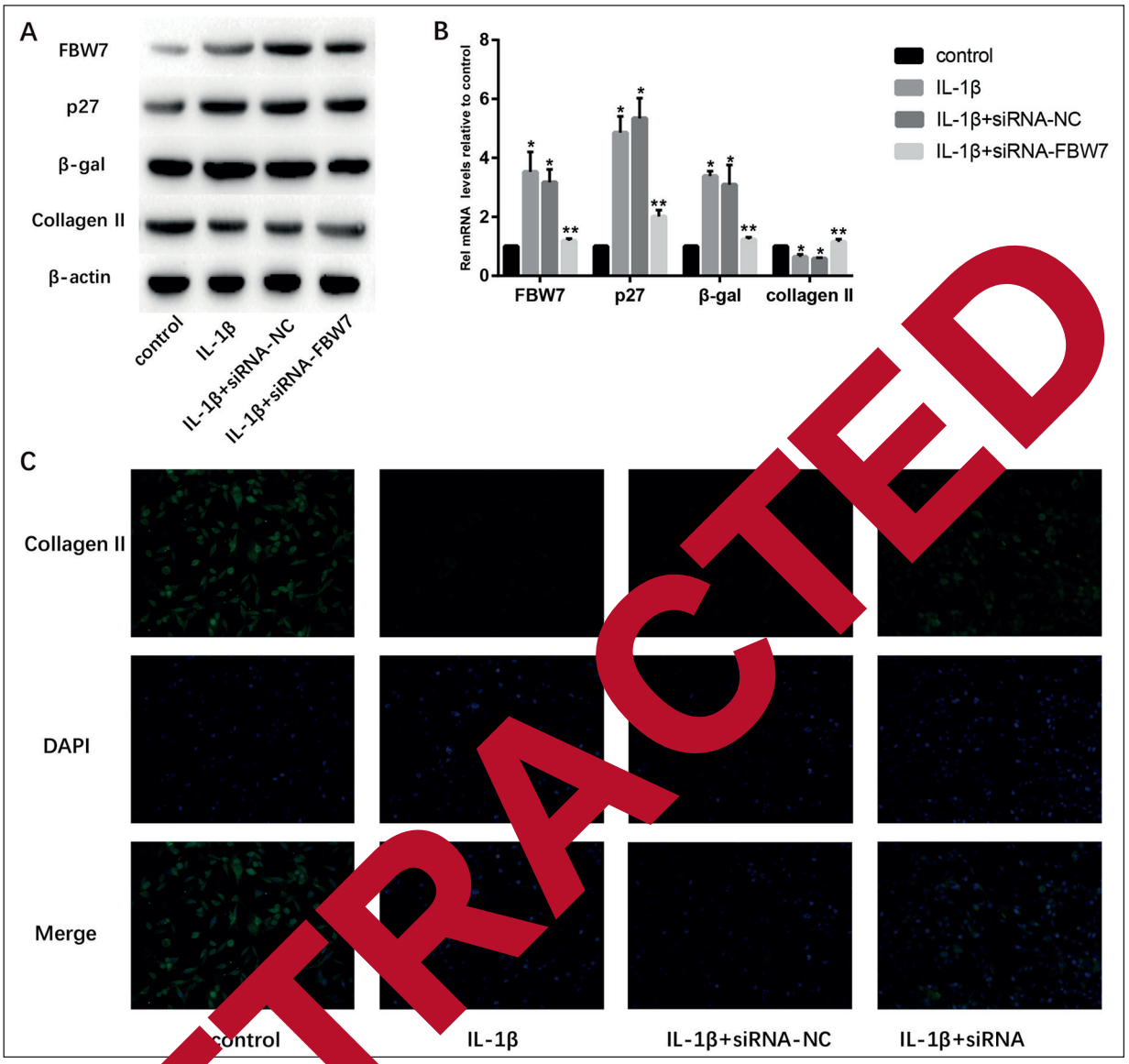

Figure 3. FBW7 low-expression alleviated IL-1β-induced human NP cell senescence. **A,** Results of the expression of FBW7, p27, β-gal, and collagen II of four groups were shown by Western blot. **B,** The results of the expression of FBW7, p27, β-gal, and collagen II of four groups were shown by RT-PCR. **C,** Results of the expression of FBW7, p27, β-gal, and collagen II in four groups were detected by IF (magnification: 40×). ("*" means there is a statistical difference with the control group and "**" means there is a statistical difference with the IL-1β group).

significantly upregulated in IF staining, WB, and RT-PCR analysis (Figure 3A-3C). These results revealed that FBW7 low-expression could reverse the senescence of NP cells caused by IL-1β.

FBW7 Low-Expression Promoted Cell Cyclin Expression and NP Cells Proliferation

Cyclin E, E2F1, and E2F2 were analyzed with WB and RT-PCR. We found that the three-cell cyclin-related genes expression decreased in the senescent NP cells but upregulated at the condition of FBW7 low-expression (Figure 4A, 4B). CCK8 assay showed that the FBW7 low expression promoted NP cells proliferation compared with the degenerated group (Figure 4C). Flow cytometry result showed that compared with the control group more cells stayed in the G0-G1 phase and fewer cells stayed in the S phase with the IL-1β stimulation. Downregulated FBW7 could promote more cells to go through G1 to S phase (Figure 4D).

Discussion

In this study, we identified FBW7 as an important mediator in the pathogenesis of intervertebral disc diseases. First of all, two different degrees of disc degeneration were divided using Pfirrmann scores. The results showed that the tissues in the severe group expressed less collagen II gene which meant that they degenerated more seriously than the mild group. In this condition, we analyzed the FBW7 level in each group and found that the severe group had a higher FBW7 expression compared with the mild group. FBW7 is a ubiquitin ligase of SCF (a complex of SKP1, CUL1, and F-box protein)-type with functions in cell growth, differentiation, and division[11]. The substrates of FBW7 contain Notch, MYC, JUN, and cyclin E are mostly involved in human cancers[9,17]. Besides, FBW7 is also a tumor suppressor according to its disability of cell proliferation *via* cell cycle regulation[18,19]. From these previous studies, we speculated that FBW7 might have a role in the progress of the IDD *via* regulating the NP cells proliferation.

NP cells isolated from the disc tissue with mild degeneration were treated with different concentration of IL-1β, so as to establish cell degeneration model which makes the cell more senescent[20]. We analyzed the colla[illegible]-gal expression of each group. A[illegible]xpected, [illegible]her concentration resulted in les[illegible]llagen II [illegible]el and higher β-gal[21] level, [illegible]ich m[illegible] highe[illegible]on-centration caused seve[illegible]ell degene[illegible]ean-while, the severely [illegible]gener[illegible]d cells [illegible]pressed FBW7 highly a[illegible]ng [illegible]th[illegible]w Cyclin E. Cyclin E-Cdk2 is a[illegible] volut[illegible]ly c[illegible]erved kinase that phosph[illegible]tes retin[illegible]a (Rb) proteins involved [illegible] t[illegible]G1 progr[illegible]ion[22]. Rb bounds

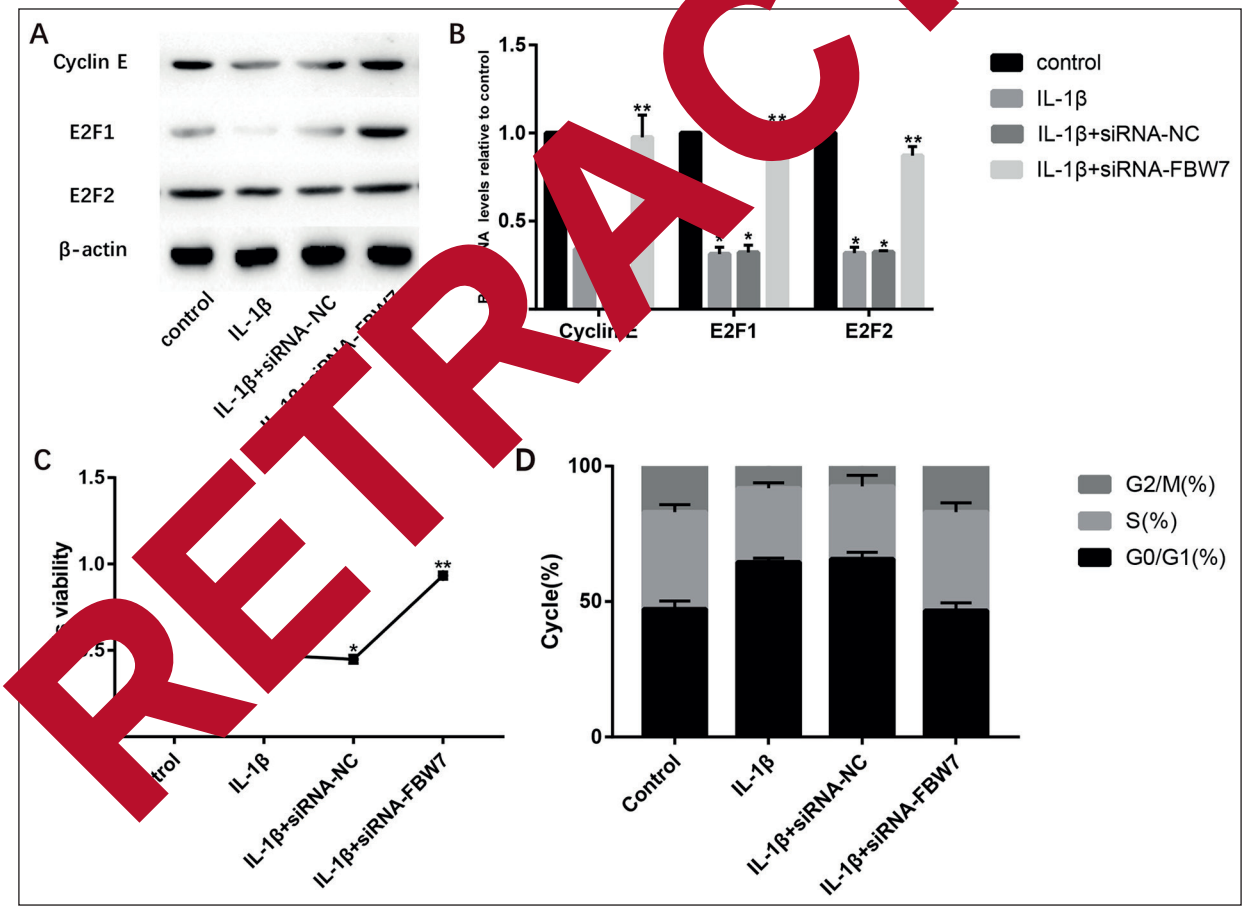

Figure 4. FBW7 low expression promoted cell cyclin expression and NP cells proliferation. **A,** Results of the expression of cyclin E, E2F1 and E2F2 in three groups were shown by Western blot. **B,** Results of the expression of cyclin E, E2F1 and E2F2 in three groups were shown by RT-PCR. **C,** Results of cell proliferation level in four groups were shown by CCK-8 assay. **D,** Results of cell cycle in four groups were shown by Flow Cytometry. ("*" means there is a statistical difference with the control group and "**" means there is a statistical difference with the IL-1β group).

with the transcription factor E2Fs transactivation domain and affects E2Fs lose the ability to activate transcription[23]. When Rb phosphorylated, unbound E2Fs activated the transcription of many genes which are required for G1/S, and the inhibition of E2Fs transcriptional activity induces cell cycle arrest[24]. The results showed that the degenerated NP cells expressed lower Cyclin E but higher FBW7. What we followed with interest was whether the FBW7 took part in the downregulation of Cyclin E, affected the NP cells cycle, and acted as a negative regulator in IDD.

Then, the FBW7 gene was silenced in the degenerated NP cells *via* siRNA transfection and verified the transfection efficiency in both protein and RNA levels.

From the expression of collagen II and β-gal, we found that the degenerated NP cells showed severe senescent phenotype and high concentration of FBW7. CCK8 assay showed that the IL-1β treated group had fewer cell activities than the control group. When the FBW7 gene was silenced, the influence of IL-1β of the NP cells also decreased. The result of the colony formation assay revealed that the absence of FBW7 played a positive role in the prolifer of NP cells. It was reported that the FBW7 diated the Cyclin E expression to regulate G cell cycle transition. We provided th followi evidence to support this conclu ly, th downregulation of FBW7 in ted th xpression of p27 gene. Secondly, t ow of FBW7 induced Cycli E ov ression, as well as the E2F1 and indicate at FBW7 could active E2Fs Cy E. Third Cyclin E promoted G1 cell cy transition in an FBW7 depen way.

In the c nt stu it was first detected that FBW7 was ow arget of IDD. Furthermore, our re s sug ed tha W7 inhibited Cyclin E n, w ther inhibited the NP cycle ough E s. One of the early studies ked tion of FBW7 in regulating the cel in CCA cell lines QBC-939 and found that E biquitin ligase FBW7α inhibited cholangiocar oma cell proliferation by downregulating c-Myc and Cyclin E[13]. FBW7 is frequently mutated in human cancers, however, our study suggested that FBW7 mediated the cycle and inhibited the NP cells proliferation, uncovering the role of FBW7 in IDD. What we found provided a mechanism through which FBW7 might play a negative role in the progress of IDD. In summary, our study not only identified a novel target in IDD but also provided a mechanism for explaining the role of FBW7 in inhibiting NP cells proliferation *via* regulating the expression of Cyclin E. However, the conclusion also needs to be verified *in vivo* in the future study.

Conclusions

Taken together, our study that FBW7 played an essential in tory role NP cells proliferation, providing insights at FBW7 may be a potenti rateg IDD at-ments.

Conflict of rest

The Autho ec at they ha conflict of interests.

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