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Inhibition of p53/p21 by TWIST alleviates TNF-α induced nucleus pulposus cell senescence *in vitro*

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Abstract. – OBJECTIVE: This study aims to reveal the TWIST protein expression in the degenerated nucleus pulposus (NP), its effect on the TNF-α treated NP cells, and to explore its specific mechanism of anti-senescence.

PATIENTS AND METHODS: NP tissues from spine fracture patients without intervertebral disc degeneration (IDD) and the IDD patients were collected to detect the TWIST1/2 protein expression by Western blot (WB). NP cells isolated from the healthy tissue was treated with TNF-α to induce senescence, and the TWIST1/2 protein expression was also analyzed. We transfected NP cells with the plasmid coding TWIST to upregulate its expression, which was also cultured in the TNF-α condition. Besides, the TNF-α pretreated NP cells were further stimulated with the recombinant human TWIST1/2 protein. The collagen II and senescent marker β-galactosidase (β-gal) were determined by immunofluorescence (IF); the MMP-13, TIMP-3, IL-10, IL-1β mRNA expression level was detected by quantitative Real Time PCR; the cell proliferation was analyzed by CCK8 assay; the cell cycle was measured by flow cytometry.

RESULTS: TWIST1/2 protein was decreased both in the degenerated NP tissue, and TNF-α treated NP cells. The overexpression of TWIST1/2 could prevent the p53, p21, β-gal, MMP-13, and IL-1β expression, moreover, it protected the collagen II, TIMP-3, and IL-10 expression in the TNF-α treated NP cells. Additionally, TWIST overexpression also promoted cell proliferation by ensuring the process of the cell cycle. Furthermore, the supplement of TWIST protein was functional to reverse these senescent phenotypes caused by TNF-α partly.

CONCLUSIONS: TWIST alleviates the TNF-αinduced NP cells senescence *via* the inhibition of the p53/p21 pathway.

Key Words:
TWIST, Nucleus pulposus cells, Senescence, p53/p21, Cell cycle.

Introduction

Disc degeneration (IDD) is considered to be the most common cause of chronic low back pain. Recently, the specific pathophysiological mechanism of IDD is not fully understood. Still, it is generally believed that it is a multi-factor induction, multi-factor participation, and a very complex biological process[1]. The functions of various tissues and organs of the human body gradually decrease with age, and the degeneration of the intervertebral disc is earlier than any other musculoskeletal tissue. Boos et al[2] found that definite degeneration can occur in the lumbar intervertebral discs of patients in the 11 to 16 age group. Data of 600 clinical autopsy specimens from Miller et al[3] suggest that 20% of the population had early signs of IDD during adolescence, and degeneration increased with age. The pathological changes of degenerated discs originate from the reduction of the number of intervertebral disc cells and the abnormal function caused by cell aging, which in turn leads to the destruction of matrix components, such as proteoglycan and collagen II. Eventually, biomechanical functions, such as dispersal of spine compression force and maintenance of intervertebral space height are lost. IDD is an aging disease, and its aging starts earlier. Therefore, early intervention and treatment of the aging process of intervertebral discs have great clinical significance[4].

Unlike programmed cell death (apoptosis), cell senescence is a process in which somatic cells grow slowly, reduce vitality, lose the ability to divide and proliferate, and decline in replication under normal or pathological factors. Although senescent cells still maintain a particular metabolic activity, their ability to respond to growth

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factor stimulation weakens, and they lost the ability to repair after damage[5]. Specific changes in cell gene expression patterns can indicate the existence of cellular senescence. Aging-related β-galactosidase (β-gal) is the most commonly used marker of nucleus pulposus (NP) cell aging. Roberts et al[6] found that the number of β-gal positive cells in protruding intervertebral disc tissue increased, and the positive rate of NP cells was higher than that of annulus fibrosus, indicating that the NP cell senescence was more severe than the annulus fibrosus in IDD. Kim et al[7] further reported that with the increase of IDD, intervertebral disc tissue contains a higher proportion of β-gal positive NP cells, indicating the close relationship between cell aging and IDD. Nucleus pulposus cell senescence is a programmed activation process in which normal cells respond to various stimuli. The abnormally activated p53/p21 signaling pathway can arrest the cell cycle at the G0/G1 phase, affecting the normal mitosis of the cells and aggravating the NP cell senescence phenotype. On the contrary, the process of cell senescence can also be reversed by the inactivation of p53[8].

The twist is a highly conserved primary helix-loop-helix transcription factor involved in embryo development, tumorigenesis, and metastasis[9]. In humans, the TWIST gene encodes two isomers: TWIST1 and TWIST2, both of which play an essential role in regulating senescence and the cell cycle[10]. Kwok et al[11] showed that TWIST protein could cross the defense barrier of tumorigenesis to avoid senescence and apoptosis caused by oncogenes. TWIST inactivation can lead to cell growth arrest and senescence. By contrast, TWIST overexpression can inhibit cell senescence in response to genotoxic damage and promote cell proliferation through the accumulation of DNA damage[12]. Several articles have mentioned the TWIST inactivates p53/p21 signaling pathway13-15 to promote cell survival. However, whether TWIST1 and TWIST2 could protect NP cells from the senescent progress is not fully elucidated.

We collected the human NP tissue of different degeneration degrees and analyzed the TWIST1 and TWIST2 protein expression. Combined with the TNF-α induced NP cell degeneration *in vitro*, we confirmed that the NP degeneration is related to the TWIST1/2 level. Furthermore, the senescent cell markers containing p53/p21 protein levels were also tested to reveal the mechanism of how TWIST prevents IDD development.

Patients and Methods

NP Tissue Source

This experiment was supported by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. To explore the relation between TWIST expression and the IDD degree, we collected the NP tissue with or without significant IDD based on the Pfirrmann score[16] form the spine fracture patients and lumbar disc herniation patients. A total of six fracture patients and six IDD patients undergoing surgery in our hospital joined in the study. The NP tissues were grouped into two groups, from which the fractured disc without IDD history was arranged as control, and the herniated disc was bundled as IDD. The fresh tissue was conserved in the medium for NP cells isolation or in liquid nitrogen for protein extraction.

NP Cells Isolation

Half part of the NP tissue from the fracture patients was used for NP cells isolation and culture. Generally, the NP tissue was cut into fragments and incubated with the digest solution overnight at 37°C, which contained 0.1% type II collagenase (Sigma-Aldrich, Darmstadt, Germany). The following day, the digested solution was filtered and centrifuged to get the NP cell pellets. We were resuspended in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) cell culture medium (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). NP cells were seeded into 12-well plates at 1×10^5 /mL and cultured under 37°C and 5% CO_2 incubator.

Cell Treatments and Transient Transfection

After the cell density up to 70%, the primary NP cells were passaged and used for the following treatments. To overexpress the TWIST1 and TWIST2 expression, we transfected the NP cells with the plasmids, including pCMV6-TWIST1, pCMV6-TWIST2, (#SC321467; #SC123104; OriGene, Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To induce NP cells senescence *in vitro*, we cultured the NP cells with TNF-α (Boehringer, Mannheim, Germany) for three days. To test the effect of the exogenic supplement of TWIST1 (TP302920, OriGene Technologies, Rockville, MD, USA) and TWIST2 TP305006, OriGene Technologies, Rockville,

MD, USA) on the senescent NP cells, we cultured the TNF-α pretreated NP cells with the TWIST1 or TWIST2 protein for an extra three days.

Western Blot Analysis

The whole protein of the NP tissue or the NP cells was extracted using the radioimmunoprecipitation assay (RIPA) lysis buffer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, proteins (40 μg) were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, electrophoresed, and transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). As follows, the membrane was blocked with 5% milk and incubated with the primary antibodies: anti-TWIST1 (PA5-49688, Invitrogen, Carlsbad, CA, USA), anti-TWIST2 (PA5-993931:1000, Invitrogen, Carlsbad, CA, USA), anti-p53 (ab1314421, Abcam, Cambridge, UK), anti-p21 (ab109520, Abcam, Cambridge, UK), and anti-GAPDH (ab9485, Abcam, Cambridge, UK) overnight at 4°C. One the next day, the membranes were incubated with a secondary antibody for an extra one hour at room temperature.

Cell Counting Kit 8 (CCK-8) Assay

NP cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM/F12 medium. When the confluence up to 70%, NP cells underwent transfection, treated with TNF-α, or TWIST protein, separately. Cell proliferation was examined with CCK-8 kits (Beyotime, Shanghai, China). The intensity of CCK-8 product was measured at a wavelength of 450 nm using a microplate reader (Labsystems Multiskan, MS, Finland).

Immunofluorescence (IF)

The collagen II and β-gal protein expression were determined using IF staining. NP cells were seeded in the 12-well plates at 1×10^5 /mL. After treatments, NP cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then blocked with 5% bovine serum albumin (BSA) for one hour at room temperature. The cells were incubated with the primary antibodies: anti-collagen II (ab34712, Abcam, Cambridge, UK), anti-β-gal (PA5-102503, Invitrogen, Carlsbad, CA, USA) overnight at 4°C. The following day, NP cells were washed and incubated with Alexa Fluor488/568 conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for one hour in the dark.

Quantitative Real Time-Polymerase Chain Reaction (RT-PCR) Analysis

The mRNA expression of MMP-13 was detected. TIMP-3, IL-1β, and IL-10 were determined by RT-PCR. Briefly, the whole RNA of NP cells after treatment was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative mRNA expression was performed using SYBR Green Master (TOYOBO, Osaka, Japan) and normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) according to the method of $2^{-\Delta\Delta Ct}$. The primers used for RT-PCR were obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/) as follows: MMP-13 Forward (5'-ACTGAGAGGCTCCGAGAAATG-3'), Reverse (5'-GAACCCCGCATCTTGGCTT-3'); TIMP-3 Forward (5'-CATGTGCAGTACATCCATACGG-3'), Reverse (5'-CATCATAGACGCGACCTGTCA-3'); IL-1β Forward (5'-ATGATGGCTTATTACAGTGGCAA-3'), Reverse (5'-GTCGGAGATTCGTAGCTGGA-3'); IL-10 Forward (5'-GACTTTAAGGGTTACCTGGGTTG-3'), Reverse (5'-TCACATGCGCCTTGATGTCTG-3'): GAPDH Forward (5'-ACAACTTTGGTATCGTGGAAGG-3'), Reverse (5'-GCCATCACGCCACAGTTTC-3').

Flow Cytometry

The cell cycle of NP cells was determined using cell cycle Propidium staining (PI staining, Keygen BioTECH, Nanjing, China). After treatments, NP cells were harvested and prepared as a single-cell resuspension solution in phosphate-buffered saline (PBS) at 4°C, and then, the reagent was added to the cells, incubated at 37°C for 30 min. Then, cells were centrifuged and washed with cold PBS. Finally, the cell cycle was analyzed using flow cytometry with a FACS-Calibur flow cytometer according to the manufacturer's instructions.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Statistical Product and Service Solutions (SPSS) Version 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The differences between the two groups were analyzed by using the Student's *t*-test. A

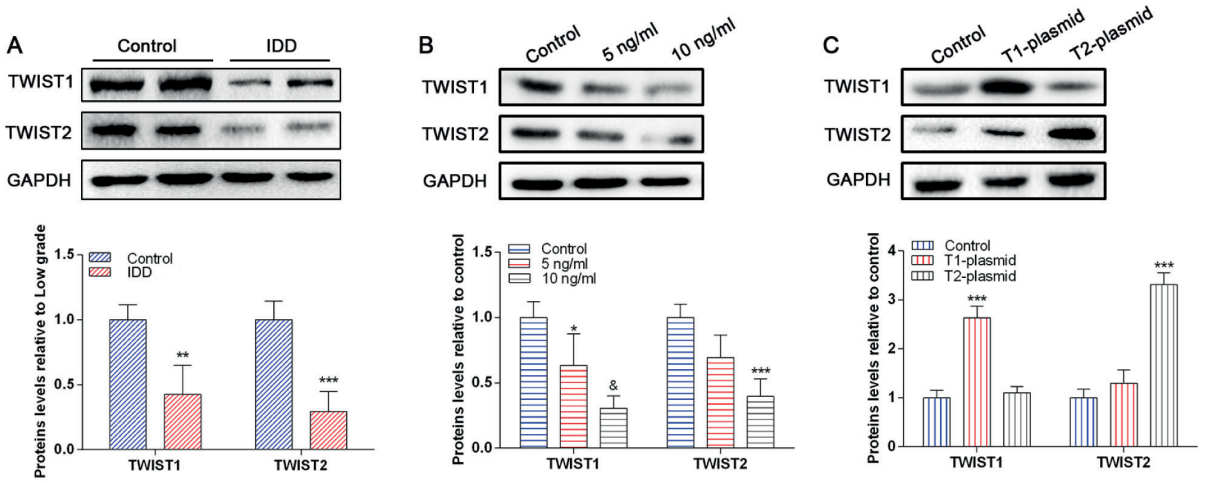

Figure 1. TWIST expresses in the degenerated NP tissue and cells. **A,** TWIST1 and TWIST2 protein level in the NP tissue from the fracture patients (Control) and IDD patients. **B,** TWIST1 and TWIST2 protein level in the non-treated NP cells (Control) and TNF-α (5 ng/mL or 10 ng/mL) NP cells. **C,** TWIST1 and TWIST2 protein level in the non-transfected NP cells (Control), TWIST1 (T1)-plasmid or TWIST2 (T2)-plasmid transfected NP cells. The values are mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001 compared to the Control, &p<0.05 compared to the 5 ng/mL group).

comparison between multiple groups was made using a one-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). p<0.05 represented statistical significance.

Results

TWIST Expresses Differently in the Degenerated NP Tissue and Cells

To compare the expressed level of the TWIST1 and TWIST2 protein in the healthy NP tissue and degenerated tissue, we grouped the samples from the patients undergoing spine trauma with the damaged NP tissue as control; meanwhile, the samples from the disc herniation patients were grouped as the IDD group. The degenerative condition of each NP tissue was valued using the Pfirrmann classified method. As shown in Figure 1A, the TWIST1 and TWIST2 protein expression was significantly higher in the control group compared to the IDD group, suggesting that TWIST expression decreased with the progress of IDD. Apart from this, we used TNF-α to induce NP cells senescence *in vitro* as previous description[17] and verified the different distribution of TWIST1 and TWIST2. We cultured NP cells with 5 ng/mL and 10 ng/ml TNF-α for three days, and the cells without any treatment were grouped as control. Compared to the control, the concentration of 5 ng/mL only affected the TWIST1 protein expression statistically; however, the 10 ng/mL suppressed both TWIST1 and TWIST2 expression after treatment (Figure 1B). Therefore, as the degree of senescence of NP cells increased, the protein expression of TWIST1 and TWIST2 also significantly decreased. To explore whether the overexpression of TWIST1 or TWIST2 could delay the senescent progress of NP cells, we upregulated the TWIST1 and TWIST2 gene expression through the pCMV6 vector coding plasmid transfection. The efficiency of transfection was shown in Figure 1C, the TWIST1 and TWIST2 protein expression were markedly increased compared to the control.

TWIST Overexpression Delays NP Cells Senescence In Vitro

To determine the function of TWIST1/2 in the NP cells senescence *in vitro*, we used TNF-α (10 ng/mL) to treat the NP cells without or with TWIST gene overexpression and analyzed the several senescent markers. The result of the WB showed the TWIST1 and TWIST2 protein were suppressed after the stimulation of TNF-α as mentioned in Figure 1B. However, the TWIST1 (T1)-plasmid transfected cells contained a higher level of TWIST1 protein after TNF-α treatment compared to the TNF-α group, and the TWIST2 (T2)-plasmid transfected cells contained more TWIST2 protein as well. Besides, the p53 and p21

protein were upregulated resulting from TNF-α stimulation compared to the control. Of note, in the TWIST overexpression group, the p53 and p21 protein were effectively suppressed compared to the TNF-α group (Figure 2A, 2B). In addition, the collagen II and β-gal protein expression were determined by IF staining. As expected, the collagen II expression was suppressed due to TNF-α treatment, but the plasmid-transfected group maintained the most of collagen II compared to the TNF-α group. On the contrary, T1 or T2-plasmid transfection alleviated the β-gal expression, which was highly increased in the TNF-α group (Figure 2C, 2D). Therefore, a high level of TWIST expression would prevent the NP cell senescence from the

TWIST Overexpression Inhibits SASP and Promotes Proliferation Of NP Cells In Vitro

We further determined the senescence-associated secretory phenotype (SASP) by analyzing the matrix metalloproteinases-13 (MMP-13), TIMP metallopeptidase inhibitor-3 (TIMP-3), and the inflammation associated IL-1β and IL-10 mRNA expression. As shown in Figure 3A, the MMP-13 and IL-1β levels were increased after the TNF-α treatment compared to the control. However, TWIST1/2 overexpressed NP cells were partly efficient in inhibiting the MMP-13 and IL-1β upregulation. Meanwhile, T1-transfection maintained the TIMP-3 mRNA expression, and the IL-10 level was also protected by the T1 or T2-plasmid transfection compared to the TNF-α group. The cell proliferation was determined by CCK-8 assay, and the result indicated the TWIST1/2 overexpressed NP presented a higher proliferative ability than the non-transfected cells under the treatment of TNF-α (Figure 3B). Additionally, TNF-α arrested more cells in the G0/G1 phase and fewer cells in the S phase, which delayed the process of the cell cycle compared to the control. However, the TWIST overexpression protected the normal cell cycle progression by ensuring more cells going through the G1 to S phase (Figure 3C).

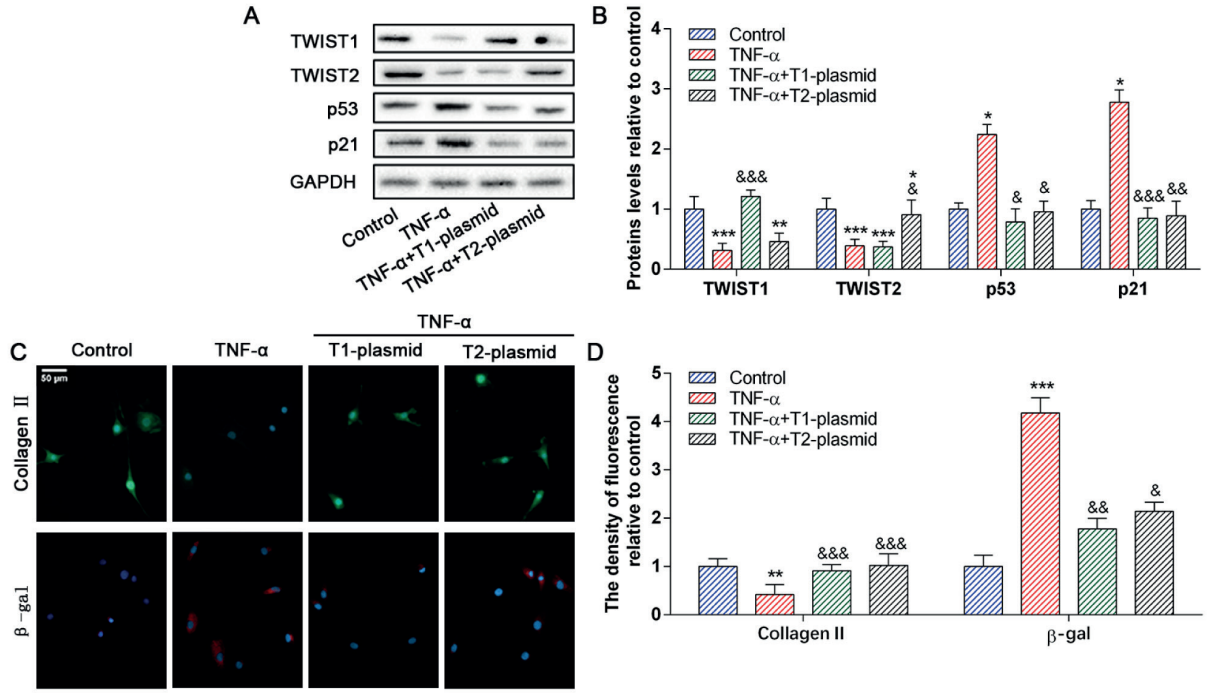

Figure 2. TWIST overexpression delays NP cell senescence. **A, B,** Relative protein expression of the non-treated NP cells (Control), TNF-α (10 ng/mL) treated NP cells, TWIST-1 or TWIST-2 overexpressed NP cells after TNF-α (10 ng/mL) treatment. **C,** The protein expression level of collagen II and β-gal were determined by IF (magnification: 400×) and (**D**) its quantification analysis. The values are mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001 compared to the Control; &p<0.05, &&p<0.01, &&&p<0.01 compared to the TNF-α group).

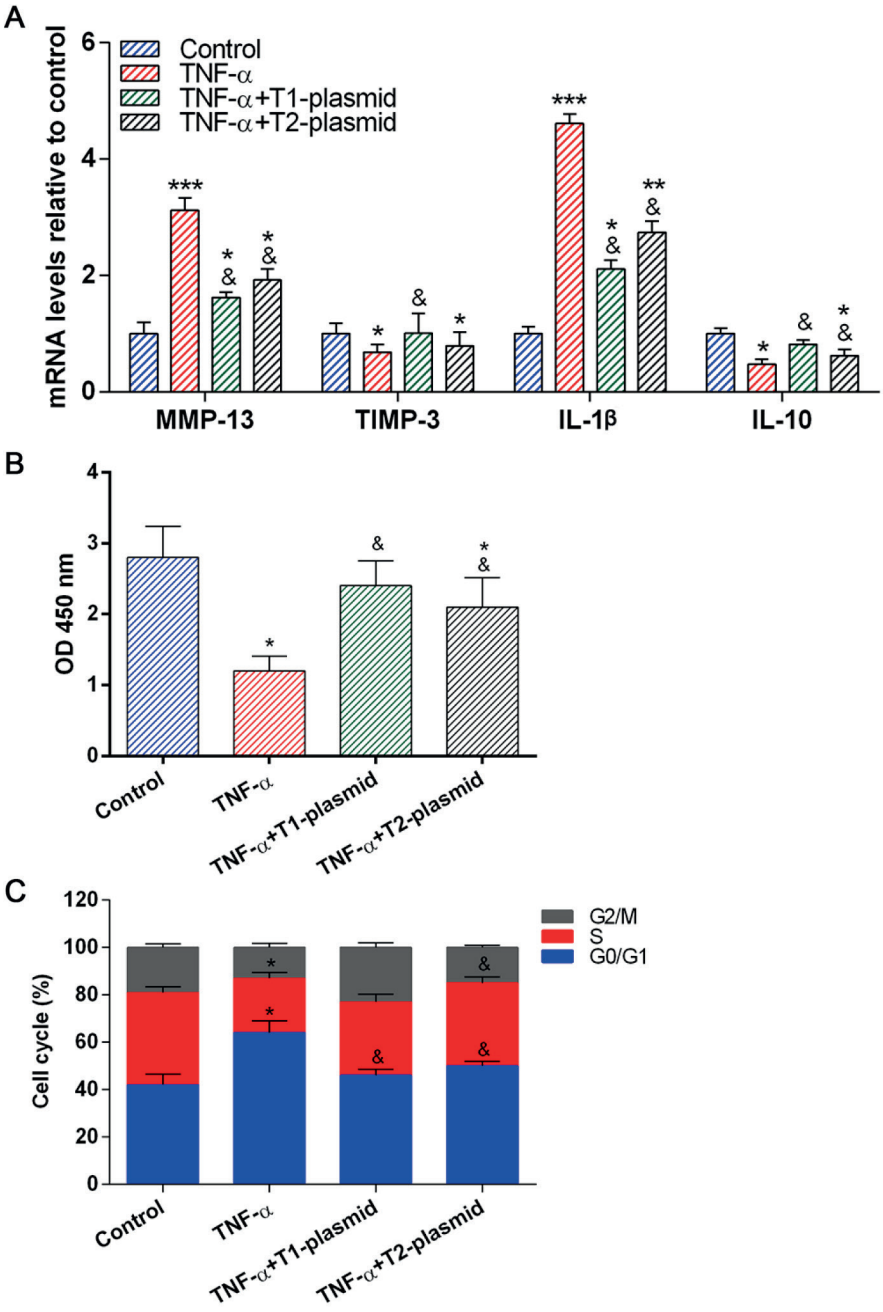

Figure 3. TWIST overexpression inhibits SASP and promotes the proliferation of NP cells. **A,** The relative mRNA expression level of the non-treated NP cells (Control), TNF-α (10 ng/mL) treated NP cells, TWIST-1 or TWIST-2 overexpressed NP cells after TNF-α (10 ng/mL) treatment. **B,** The proliferation of each group was determined by CCK8 assay. **C,** The cell cycle was tested by flow cytometry. (*p<0.05, **p<0.01, ***p<0.001 compared to the Control; &p<0.05 compared to the TNF-α group).

TWIST Supplement Alleviates NP Cells Senescence In Vitro

The above findings suggested that the TWIST1/2 gene overexpressed NP cells had an excellent ability to anti-senescent *via* the suppression of SASP and the protection of proliferation. We wondered whether the supplement of exogenic TWIST protein would reverse the senescent phenotype of NP cells. Therefore, we pretreated NP cells with 10 ng/mL TNF-α for three days to induce cell senescence. After that, the NP cells were cultured with TWIST1 (50 ng/mL) or TWIST2 (50 ng/mL) protein medium for an extra three days. As shown in Figure 4A and 4B, the collagen II expression

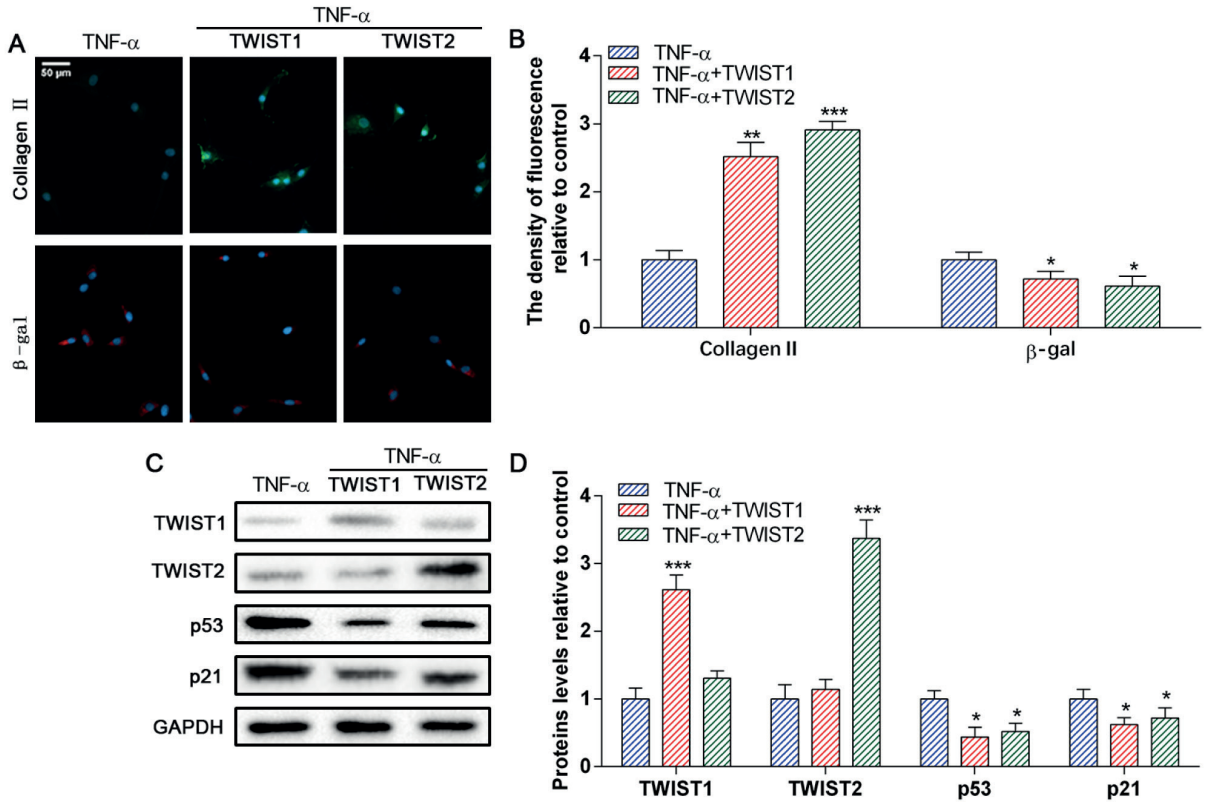

Figure 4. TWIST supplement alleviates NP cells senescence. **A,** The protein expression level of collagen II and β-gal in the TNF-α (10 ng/mL) treated NP cells, or the TWIST-1/-2 protein cultured NP cells after TNF-α (10 ng/mL) pretreatment (magnification: 400×). **B,** IF quantification analysis. **C, D,** Relative protein expression of each group. The values are mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001 compared to the TNF-α group).

was increased after the supplement of TWIST1 or TWIST2 protein. At the same time, the β-gal expression was decreased, suggesting the senescent degree was alleviated after the stimulation of TWIST protein. Following, the WB results indicated the exogenic protein culture increased the TWIST1 and TWIST2 expression compared to the TNF-α group. Besides, the p53 and p21 levels were inhibited due to the supplement of TWIST protein (Figure 4C, 4D).

TWIST Supplement Inhibits Inflammation and Promotes Proliferation of Senescent NP Cells In Vitro

Finally, the mRNA expression of the SASP related gene expression and cell proliferation was also analyzed. MMP-13 and IL-1*β* expression were significantly decreased by the TWIST1 or TWIST2 protein treatment compared to the TNF-α group. Besides, the IL-10 level was also increased resulting from TWIST1 protein stimuli compared to the TNF-α group (Figure 5A). Furthermore, the CCK-8 assay suggested the TWIST protein treatment significantly improved the cell proliferation compared to the TNF-α group (Figure 5B). Both TWIST1 and TWIST2 could promote the cell cycle going through the G1 to S phase, which increased the rate of cells in the S phase (Figure 5C).

Discussion

The NP cell senescence is one of the pathological factors of IDD. Unlike apoptosis, cell senescence mainly manifests in the decline of proliferation capacity and the synthesis ability of extracellular matrix (ECM)[18]. Abnormal activation of the p53 signaling pathway inhibits the normal progression of the cell cycle. It causes mitosis to arrest at the G0/G1 phase, which is the main reason for the cell's loss of proliferation ability[19]. Decreased synthesis capacity of collagen II, increased ECM degradation proteases, and reduced expression of matrix proteinase inhibitors accelerate the breakdown of ECM. TWIST

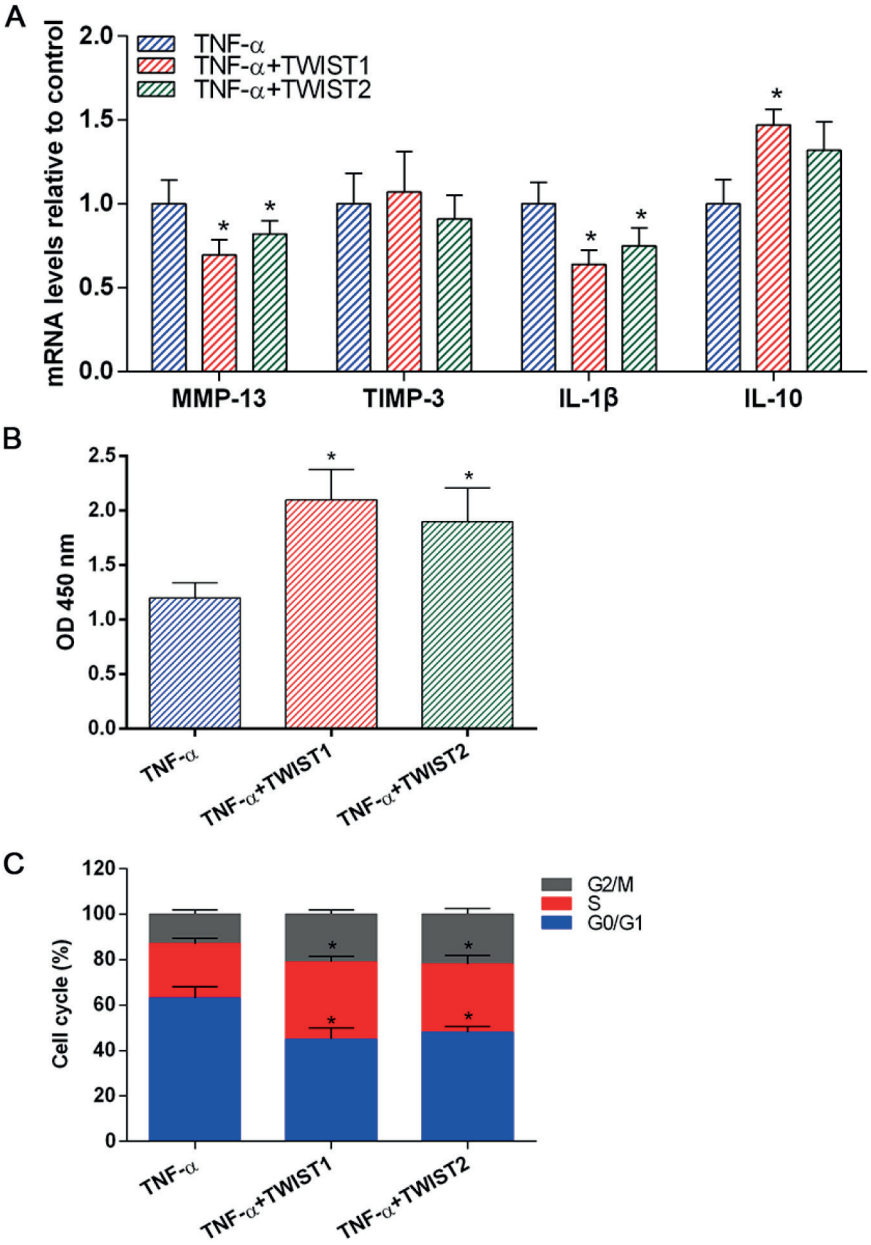

Figure 5. TWIST supplement inhibits inflammation and promotes the proliferation of senescent NP cells. **A,** The relative mRNA expression level of the TNF-α (10 ng/mL) treated NP cells, or the TWIST-1/2 protein cultured NP cells after TNF-α (10 ng/mL) pretreatment. **B,** The proliferation of each group was determined by CCK8 assay. **C,** The cell cycle was determined by flow cytometry. (*p<0.05 compared to the Control; &p<0.05 compared to the TNF-α group)

overexpression promotes cell proliferation and migration[20]. TWIST gene deficiency could lead to significant apoptosis, cell cycle arrest at the G0/G1 phase, and proliferation inhibition[21]. The relationship between TWIST and IDD is still unclear.

At the beginning of our study, we found the TWIST1 and TWIST2 proteins were highly expressed in the healthy NP tissues but significantly decreased in the degenerated tissue. Besides, in the *in vitro* experiment, TNF-α stimulation treatment also suppressed the TWIST1 and TWIST2 expression, suggesting the TWIST could be related to the progress of NP cell degeneration and IDD. *In vitro* cell culture, the NP cell senescent model was constructed by TNF-α. The aging phenotype is characterized by an increase in the ac-

tivity of the aging-related β-gal expression, which is one of the most commonly used and convenient methods for detecting aging biology[22]. After overexpressing the TWIST1 or TWIST2 gene, the collagen II production and proliferation ability of NP cells was significantly protected, the β-gal expression, matrix degradation-related genes expression, and inflammation were also suppressed compared to the TNF-α treated non-transfection cells. These findings indicated the TWIST gene does have an anti-senescence effect *in vitro*.

However, it is still unclear how this effect occurs and by what means. In view of the high correlation between the TWIST gene and the p53 pathway, we further analyzed the p53 and p21 genes. The p53/p21 pathway and p16[23] pathway are the main signal pathways for cell senescence. Abnormally activated p53 causes cyclin-dependent kinase inhibitor (CDKI) p21 transcriptional activation, thereby inhibiting the formation of cyclin/CDK complex, which in turn inhibits the phosphorylation of RB and ultimately leads to cell aging and growth inhibition[24]. From the previous studies, TWIST plays a role in the suppression of p53[14,25] activity as well as the p21[26,27] to inhibit cellular senescence. Similarly, our data proved that the TWIST overexpression could inhibit the p53 and p21 accumulation, which contributed to the natural process of the cell cycle. As one part of the anti-senescence effect, TWIST also showed an anti-inflammatory manner by inhibiting IL-1β[28] and protecting IL-10[29] expression.

Upregulating the expression of TWIST through gene editing can alleviate the senescence of NP cells. However, clinically targeted degeneration of NP cells through drug intervention is more feasible than gene editing. Therefore, we wondered whether the supplement of TWIST1 or TWIST2 protein would reverse the phenotype of senescent NP cells. The results indicated that the exogenic delivery of TWIST protein increased the collagen II synthesis, TIMP-3 expression, and protected the cell proliferation *via* regulating cell cycle, which was also related to the suppression of the p53/p21 expression.

Conclusions

In summary, the novelty of our study is to recover the effect of TWIST in IDD NP cell senescence and IDD. Overexpression of TWIST1/2 by gene editing could prevent the process of TNF-α induced NP cell senescence by suppressing p53/p21 signaling. Besides, the supplement of TWIST1/2 protein is also useful to partly reverse the senescent phenotype of NP cells, which could be related to the inhibition of p53/p21 expression. Therefore, TWIST is a promising therapeutic target to protect the NP cell viability and makes benefits to the intervention of IDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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