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A20 of nucleus pulposus cells plays a self-protection role via the nuclear factor-kappa B pathway in the inflammatory microenvironment

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Aims

Inflammatory response plays a pivotal role in the pathophysiological process of intervertebral disc degeneration (IDD). A20 (also known as tumour necrosis factor alpha-induced protein 3 (TNFAIP3)) is a ubiquitin-editing enzyme that restricts nuclear factor-kappa B (NF- κ B) signalling. A20 prevents the occurrence of multiple inflammatory diseases. However, the role of A20 in the initiation of IDD has not been elucidated. The aim of the study was to investigate the effect of A20 in senescence of TNF- α -induced nucleus pulposus cells (NPCs).

Methods

Immunohistochemical staining was performed to observe the expression of A20 in normal and degenerated human intervertebral discs. The NPCs were dissected from the tail vertebrae of healthy male Sprague-Dawley rats and were cultured in the incubator. In the experiment, TNF- α was used to mimic the inflammatory environment of IDD. The cell viability and senescence were examined to investigate the effect of A20 on TNF- α -treated NPCs. The expression of messenger RNA (mRNA)-encoding proteins related to matrix macromolecules (collagen II, aggrecan) and senescence markers (p53, p16). Additionally, NF- κ B/p65 activity of NPCs was detected within different test compounds.

Results

The expression of A20 was upregulated in degenerate human intervertebral discs. The A20 levels of NPCs in TNF- α inflammatory microenvironments were dramatically higher than those of the control group. TNF- α significantly decreased cell proliferation potency but increased senescence-associated beta-galactosidase (SA- β -Gal) activity, the expression of senescence-associated proteins, the synthesis of extracellular matrix, and G1 cycle arrest. The senescence indicators and NF- κ B/p65 expression of A20 downregulated group treated with TNF- α were significantly upregulated compared to TNF- α -treated normal NPCs.

Conclusion

A20 has a self-protective effect on the senescence of NPCs induced by TNF- α . The downregulation of A20 in NPCs exacerbated the senescence of NPCs induced by TNF- α .

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Keywords: A20, Nuclear factor-kappa B, Tumour necrosis factor alpha, Nucleus pulposus, Senescence

Article focus

- To clarify the role of A20 in intervertebral disc degeneration to guide clinical practice.

Key messages

- A20 is elevated in a degenerative intervertebral disc.
- Low expression of A20 accelerates senescence of nucleus pulposus cells (NPCs).

- Low expression of A20 weakens cell self-protection by regulating nuclear factor-kappa B (NF- κ B).

Strengths and limitations

- These findings indicate that A20 may be a self-protective protein of NPCs.
- More studies need to be conducted to examine the role of A20 in the pathogenesis of disc degeneration.

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Introduction

Intervertebral disc degeneration (IDD) is a global health problem and a main cause of lower back and leg pain.¹ With the advance of the ageing process of the world population, disc herniation caused by IDD tremendously increases socioeconomical burden and reduces people's quality of life.² The occurrence and development of IDD is affected by multiple factors including age, genes, and adiposis. Among these factors, age-related factors are the most significant.^{3,4} The levels of senescent nucleus pulposus cells (NPCs) increase in the degenerated intervertebral disc,^{5–7} and the level of inflammatory factors is also sharply upregulated in the degenerated intervertebral disc. Previous studies demonstrated that inflammatory response and cellular ageing play pivotal roles in the pathophysiology of IDD, osteoarthritis (OA), and cartilage regression.^{8–12} In particular, the proinflammatory cytokine tumour necrosis factor alpha (TNF- α) is closely related to the pathogenesis of IDD.¹³ It has been reported that TNF- α could promote senescence of NPCs by activating the nuclear factor-kappa B (NF- κ B) pathway.^{14–16} We also found that the expression of TNF- α in the nucleus pulposus (NP) tissue was positively correlated with the age of the rat by extracting the NP tissue proteins from rats of different ages (unpublished).⁸ The senescence of NPCs is regulated by many factors, such as inflammation, injury, oxidative stress, A20, and stress. Therefore, to understand the regulation mechanism of the senescence of NPCs is of great significance for the treatment of disc herniation. So, we hypothesized that the NF- κ B pathway can be inhibited to attenuate TNF- α -induced senescence of NPCs, which would point to new strategies for the treatment of IDD.

A20 is a deubiquitinating enzyme with critical anti-inflammatory functions. Numerous researchers have identified that A20 is a susceptibility gene for inflammatory diseases, and that A20 inhibits inflammation by regulating the NF- κ B pathway.^{17–21} Interestingly, when NF- κ B translocates into the nucleus and binds to the κ B binding site in the A20 gene promoter structure, it can promote the expression of the A20 gene, and A20 acts as an ubiquitinating enzyme to modify the upstream molecules of the NF- κ B pathway, leading to a negative feedback loop between A20 and the NF- κ B.²² The expression level of A20 is affected by multiple factors, including the proinflammatory cytokines TNF, interleukin (IL)-1, and Toll-like receptors. It is currently unknown whether A20 could attenuate premature senescence of NPCs. There is no report regarding the biological function of A20 in senescence of TNF- α -induced NPCs. The purpose of this study was to investigate whether A20 could inhibit TNF- α -induced senescence of NPCs, and further reveal its biological mechanism to guide clinical treatment.

Methods

Immunocytochemistry staining. A clinical collection of human intervertebral discs was divided into a relatively normal group (spine fracture, Pfirrmann II; one 35-year-old male) and a degeneration group (Pfirrmann V; five male patients aged 35 to 40 years). Immunohistochemical staining was performed to observe the expression of A20 protein in the intervertebral disc tissue of the two groups. The human specimens were fixed in formaldehyde, decalcified, dehydrated in gradient solutions of ethanol, and embedded in paraffin. Subsequently, the tissues were cut into 5 μ m sections continuously. Next, endogenous peroxidase activity was blocked by 3% hydrogen peroxide for ten minutes, and non-specific binding sites were blocked by 5% bovine serum albumin for 30 minutes at room temperature. The sections were then incubated with antibodies against A20 (1:2000; Cell Signaling Technology, Danvers, Massachusetts, USA) overnight at 4°C. Negative control sections were incubated with non-specific immunoglobulin G (IgG). Next, the sections were washed with phosphate-buffered saline (PBS) three times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour at 37°C. Finally, counterstaining with haematoxylin, the sections were observed under a microscope.

Isolation and culture of nucleus pulposus cells. A group of 15 Sprague–Dawley rats (male, 150 g to 200 g) were euthanized with an overdose of pentobarbital. The tail discs of Sprague–Dawley rats were removed under aseptic conditions. Gel-like NP tissue was isolated and shaken with 0.25% collagenase for four hours at 37°C. The digested tissue was transferred to Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12" (DMEM/F12; Gibco, Wuhan, China) (10% fetal bovine serum (FBS; Gibco, Shanghai, China) and 1% penicillin/streptomycin) and maintained at 37°C, 5% carbon dioxide (CO₂), in an incubator. Fresh complete medium was changed every two days, and cells were harvested by using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) when the cells reached up to 80% confluence. The third-passage NPCs were identified based on the phenotype of NPCs²³ and used for all of our experiments. The study contained four groups: Group 1 was treated with PBS as control; Group 2 was treated with TNF- α (Beyotime, Shanghai, China); Group 3 was treated with TNF- α +A20 (RNA interference (RNAi)); and Group 4 was treated with TNF- α +A20 (RNAi)CON.

Cell viability assay. Cell viability was evaluated using Cell Counting Kit-8 (CCK-8, Beyotime). The NPCs were cultured in 96-well plates at a density of 2,000 cells per well with different test compounds. Then, CCK-8 reagent was added to each well at different timepoints, and cultured in air at 37°C, 5% CO₂, for four hours. Absorbance at 450 nm was detected with Multiskan MK3 (Thermo Scientific,

Table I. Primers of target genes

| Gene | Forward (5'-3') | Reverse (5'-3') |
|----------------|-------------------------|-----------------------|
| p53 | CCCCTGAAGACTGGATAAAGTGT | TCTCCTGACTCAGAGGGGAGC |
| p16 | TCGTGCGGTATTGCGGTAT | TAGTCTCGCGTTGCCAGAAG |
| A20 | GTGGCGAAGCATACAACCTGA | GGTCGTGGTTCGGGTCCG |
| Aggrecan | ATGGCATTGAGGACAGCGAA | GCTCGGTCAAAGTCCAGTGT |
| Collagen II | GCCAGGATGCCCGAAAATTAG | CCAGCCTTCTCGTCAAATCCT |
| β -actin | CCCATCTATGAGGGTTACGC | TTAATGTCACGCACGATTC |

Waltham, Massachusetts, USA) to calculate cell viability.

Senescence-associated beta-galactosidase activity. NPCs were seeded in six-well plates and incubated with different test compounds. Then, different groups of cells were stained with senescence-associated beta-galactosidase (SA- β -Gal) staining kit (Beyotime), and the proportion of SA- β -Gal staining-positive NPCs was observed under a light microscope. In all, three fields of view were randomly selected under the microscope to count SA- β -Gal-positive cells by two independent and uniform observers (XP and RS).

Cell cycle analysis. NPCs were seeded in petri dishes (10 cm diameter, 3×10^4 cells per dish) and grown to 70% to 80% confluence. After serum starvation for eight hours, NPCs were incubated with different test compounds. Then, the NPCs were digested with trypsin (0.25% without EDTA; Gibco, Thermo Fisher) and centrifuged to collect the cell pellets. After fixation with 70% ethanol and staining with propidium iodide dye (50 μ g/ml, Beyotime) for 30 minutes, NPCs were subjected to flow cytometry analysis.

Transfection. Transfection was performed according to the manufacturer's protocol (Genechem, Shanghai, China). The sequences of the specific small interfering RNAs (siRNAs) were as follows: A20, sense (5'-3') ccGGAGTGGACTTCAGTACAA; antisense (5'-3') TTGACTGAAGTCCACTCCGG. Non-targeting siRNA was used as a control. NPCs were transferred to a 6 cm dish (5×10^6 cells) one day before. Lipofectamine 6000 (Beyotime) served as the transfection reagent. In total, 5 μ g of plasmid and 10 μ l of lipofectamine were diluted with 250 μ l of serum-free medium (DMEM/F12). Then they were mixed and added to the cell culture medium and, after six hours of transfection, cells were serum starved for 12 hours and then treated with different test compounds. The efficiency of plasmid transfection increased up to 80%. Then NPCs were harvested for the subsequent experiments.

Real-time polymerase chain reaction. Total cellular RNA was isolated with Trizol reagent (Invitrogen, Thermo Fisher). Purified RNA was reverse transcribed using PrimeScript RT Master Mix (RR036A; Takara Biotechnology, Dalian, China). The primer sequences are presented in Table I. The complementary DNA (cDNA) produced by the reverse transcription was amplified by using ChamQ SYBR qPCR Master Mix (High ROX premixed) (Vazyme Q341-02/03) following the manufacturer's protocol

through real-time PCR (Applied Biosystems, Foster City, California, USA). Transcription level was quantified by real-time polymerase chain reaction (RT-PCR) using the following cycling conditions: 30 seconds at 95°C, then 40 cycles of ten seconds at 95°C and 30 seconds at 60°C. The expression levels of related genes were normalized to housekeeping gene and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Western blotting analysis. The total protein content of NPCs was obtained using a whole protein extraction kit (Nanjing KeyGen Biotech, Nanjing, China), and equal amounts of protein samples were mixed with a sample buffer (Beyotime) and boiled for ten minutes. Protein samples were separated with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies for A20 (1:2,000; Cell Signaling), p53 (1:1,000; Cell Signaling), p16 (1:1,000; Abcam, Cambridge, UK), aggrecan (1:1,000; Abcam), collagen II (1:5,000; Abcam), NF- κ B (1:1,000; Cell Signaling), phosphorylated (p)-NF- κ B (1:2,000; Abcam), and β -actin (1:2,000; Abcam) overnight at 4°C. Afterwards, the membranes were incubated with HP-conjugated secondary antibody for two hours at room temperature. Protein bands were visualized using a SuperSignal West Pico Trial Kit (Thermo Fisher). Bands were detected and assessed through Grayscale analysis.

Immunofluorescence of p-NF- κ B/p-p65 and A20. NPCs were seeded into 24-well plates at 8×10^3 cells per well, and the cells with or without siRNA interference were treated using TNF- α , then fixed with 4% paraformaldehyde at room temperature and permeabilized with 0.5% Triton X-100. The treated cells were incubated with rabbit A20 (1:500; Cell Signaling) and rabbit phospho-p65 (1:500; Abcam) at 4°C overnight. Consequently, the cells were stained with the respective secondary antibodies Alexa Fluor 647 goat anti-rabbit IgG (1:1,000; Abcam). Nuclei were counterstained with DAPI and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis. Significant difference was analyzed using SPSS v19.0 software (IBM, Armonk, New York, USA). All quantitative data were expressed as the mean (SD) of three independent experiments in this study.

One-way analysis of variance (ANOVA) was conducted to compare differences among multiple groups. Values of $p < 0.05$ were considered statistically significant.

Results

The expression of A20 in degenerated disc is upregulated.

We found that the protein expression of A20 in the intervertebral disc with a high Pfirrmann grade was significantly higher than that in the low disc. Thus, the result demonstrated that A20 is involved in the progress of IDD through some mechanisms (Figure 1).

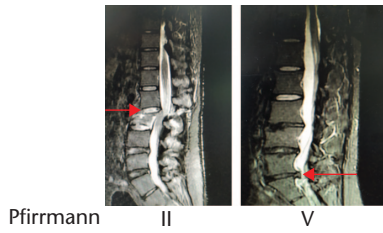


Fig. 1a

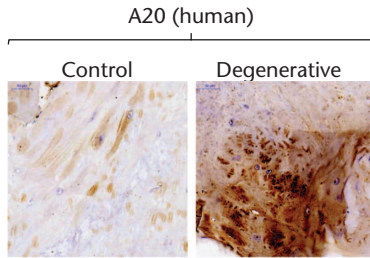
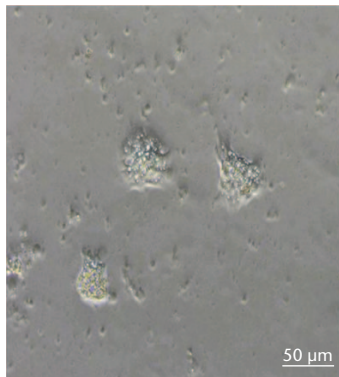


Fig. 1b

The expression of A20 is increased in the degenerated human intervertebral disc. a) Representative MRI images of two different intervertebral discs in patients from the normal group (spine fracture, Pfirsman II, one 35-year-old male; as indicated by red arrow) and degeneration group (Pfirsman V, five male patients aged 35 to 40 years, as indicated by red arrow). b) The expression of A20 from intervertebral discs in patients was analyzed by immunohistochemical staining. Magnification: 127 mm × 35 mm (300 × 300 DPI).

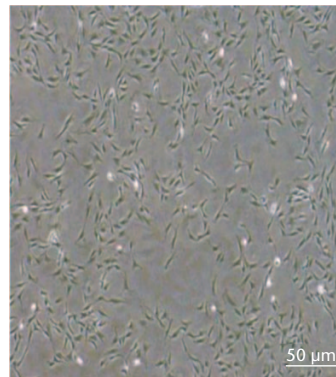
A20 expression is upregulated in senescent NPCs induced by TNF- α . NPCs of Sprague-Dawley rats were extracted and purified to obtain relatively uniform NPCs. To investigate the expression of A20 in NPCs treated with TNF- α , we treated NPCs at different timepoints with different doses of TNF- α (0 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml). We found that the cell viability of TNF- α -treated NPCs for 48 hours and 72 hours was sharply weaker than that of the control group ($p < 0.05$). Therefore, we chose TNF- α to treat NPCs at a timepoint of 48 hours. As expected, the results indicated that the level of A20 expression in TNF- α -treated NPCs was dramatically increased compared to the control group. In addition, there is a certain quantitative relationship between the expression level of A20 in NPCs and the concentration of TNF- α . It has also been confirmed that TNF- α can induce premature senescence of NPCs,¹⁶ so we chose TNF- α (10 ng/ml, 48 hours) to induce senescence of NPCs for the subsequent experiments. (Figure 2)

A20 has a self-protective effect on senescence of TNF- α -induced NPCs. SA- β -Gal staining is one of the momentous criteria for identifying senescent cells. Compared with the control group, the positive rate of SA- β -Gal staining of TNF- α -treated NPCs showed a significant increase. When



NPCs (P0) (X40)

Fig. 2a



NPCs (P3) (X40)

Fig. 2b

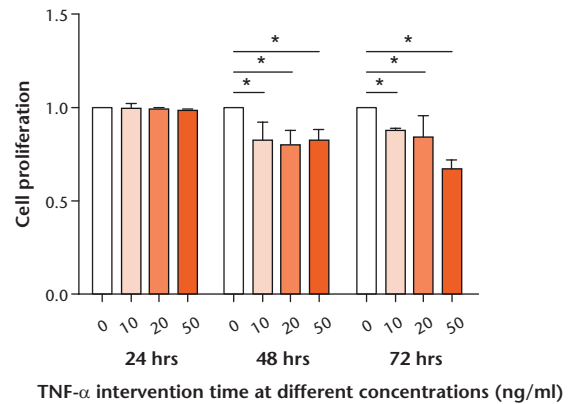


Fig. 2c

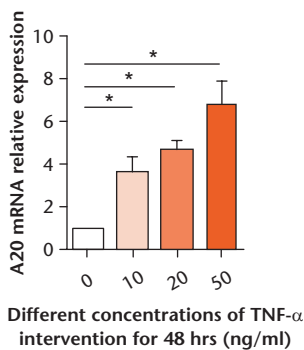


Fig. 2d

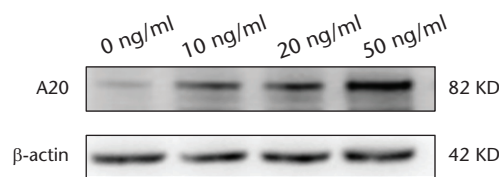


Fig. 2e

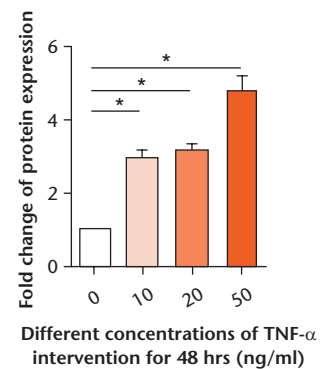


Fig. 2f

Acquisition of rat nucleus pulposus cells (NPCs) and identification of A20 expression in tumour necrosis factor alpha (TNF- α)-induced NPCs. a) Primary NPCs. b) P3 NPCs. c) The cell viability of NPCs induced by TNF- α was evaluated. d) The messenger RNA (mRNA) expression of A20 in NPCs was evaluated by real-time polymerase chain reaction (PCR). e) The expression of A20 from TNF- α -induced NPCs was analyzed by western blot. f) Quantification of immunoblots of A20. Magnification: 127 mm × 71 mm (300 × 300 DPI). Data are expressed as means (SDs; $n = 3$). * $p < 0.05$.

the NPCs after silencing the *A20* gene were treated with $TNF-\alpha$, the positive rate of SA- β -Gal staining of NPCs had a significant superior increase. With regard to these findings, we observed that the decrement of *A20* expression in NPCs increased the senescence staining ratio of $TNF-\alpha$ -treated NPCs. (Figure 3)

It is generally believed that p53 and p16 are two classical markers of cellular senescence,²⁴ and studying their

protein expression is important for clarifying cellular senescence. In the study, it was found that $TNF-\alpha$ could upregulate the expression of p53 and p16 in NPCs at the gene and protein levels compared to the control group. As expected, when $TNF-\alpha$ interfered with *A20* gene-silenced NPCs, this led to a significant increase of the expression of intracellular senescence-associated proteins (Figure 3).

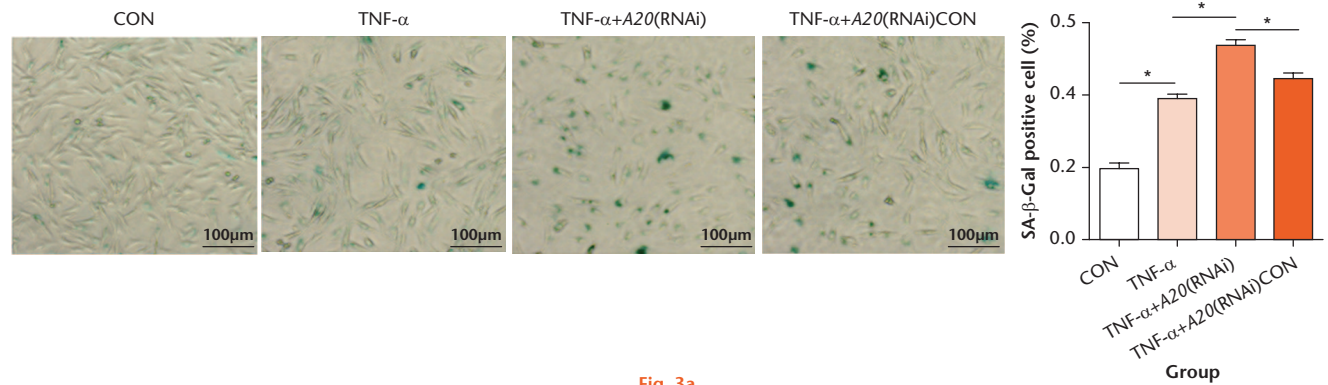


Fig. 3a

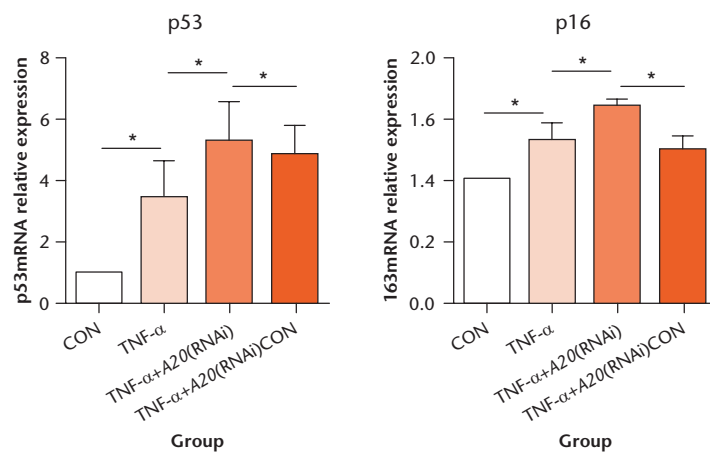


Fig. 3b

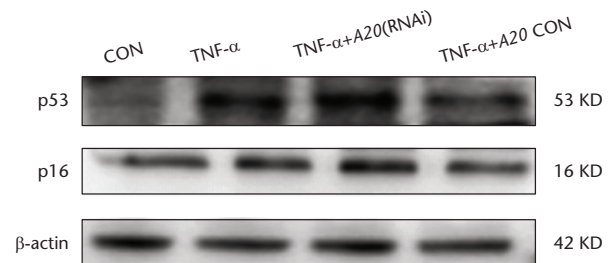


Fig. 3c

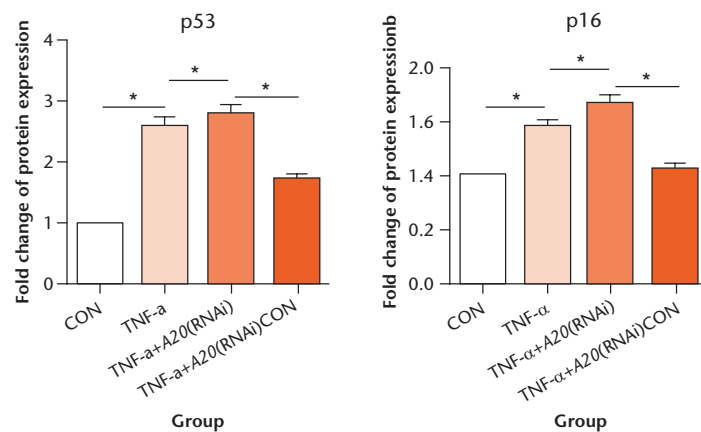
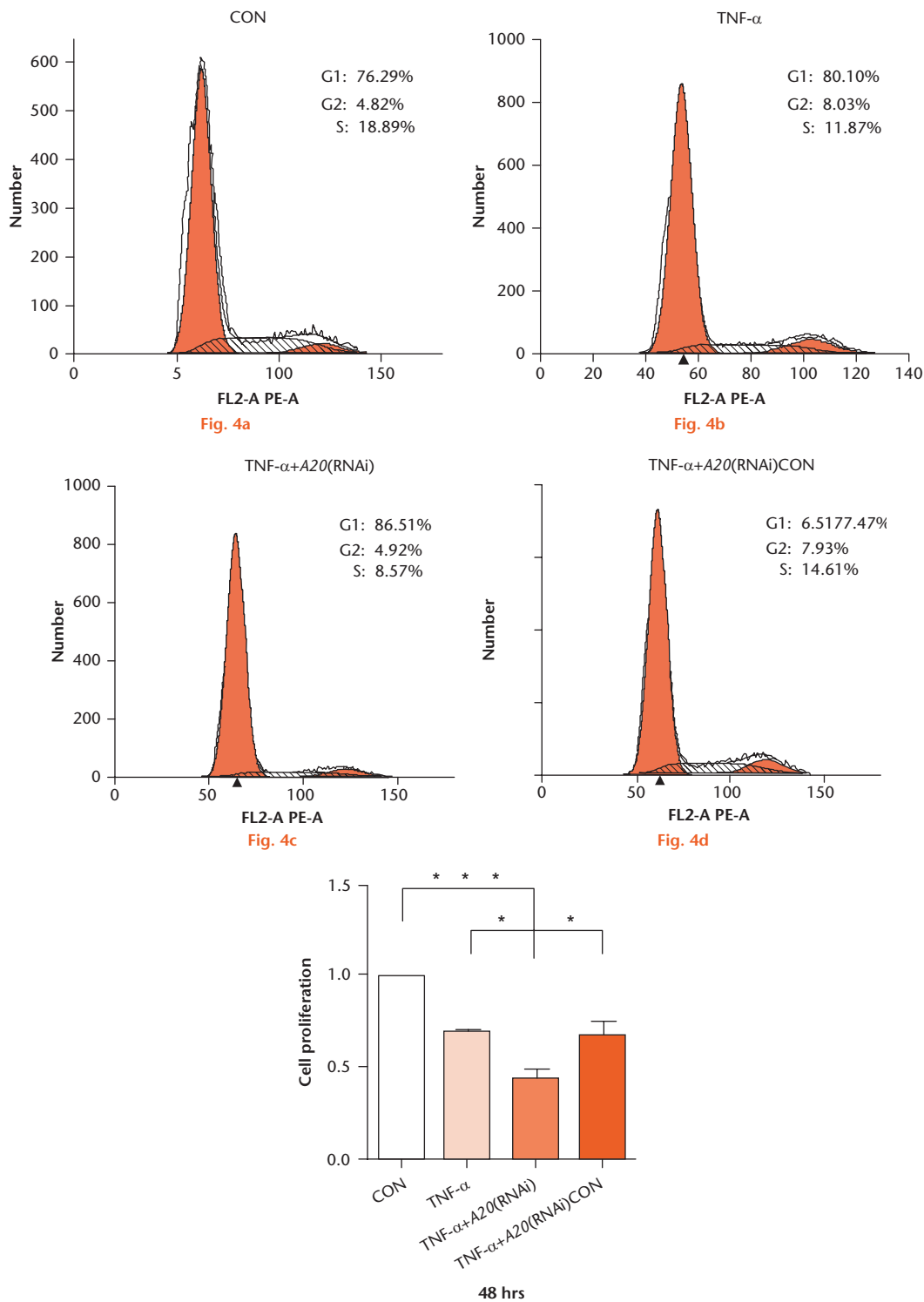


Fig. 3d

Downregulation of *A20* expression increased senescence of tumour necrosis factor alpha ($TNF-\alpha$)-treated nucleus pulposus cells (NPCs). a) The positive ratio of senescence-associated beta-galactosidase (SA- β -Gal) staining is increased in $TNF-\alpha$ -treated NPCs with downregulated *A20* expression. b) The messenger RNA (mRNA) expression of senescence-associated markers (p53, p16) in different test compounds was evaluated by real-time polymerase chain reaction (RT-PCR). c) The expression of p53 and p16 from different test compounds was analyzed by western blot. d) Quantification of p53 and p16 immunoblots. Magnification: 190 mm \times 62 mm (300 \times 300 DPI). Data are expressed as means (SDs; n = 3). *p < 0.05. CON, control treated with phosphate-buffered saline; RNAi, RNA interference.

Attenuation of cell viability is one of the hallmarks of cellular senescence. Cell viability in each group was analyzed via CCK-8 assays. As shown in Figure 4, the cell

viability of TNF- α -treated NPCs was significantly decreased compared to the control group. When TNF- α interferes with NPCs after silencing the A20 gene, the proliferation



Downregulating A20 expression increased the percentage of G1 phase tumour necrosis factor alpha (TNF- α)-treated nucleus pulposus cells (NPCs) and decreased cell viability. a) to d) Cell cycles were analyzed by flow cytometry in each group. The percentage of NPCs in the G1, G2, and S phases in each group is shown in the respective images. e) The cell proliferation of different test compounds was determined for 48 hours using a Cell Counting Kit 8 (CCK-8) assay. Data are expressed as means (SDs; n = 3). *p < 0.05. CON, control treated with phosphate-buffered saline; RNAi, RNA interference.

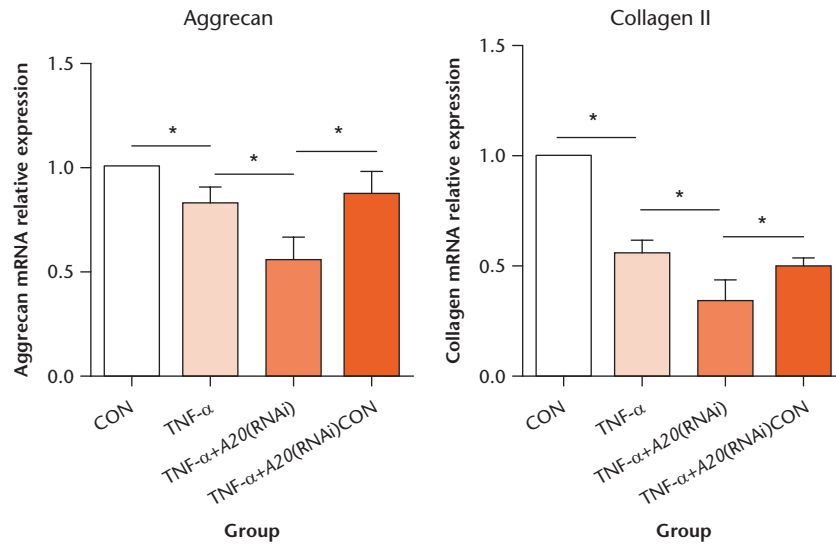


Fig. 5a

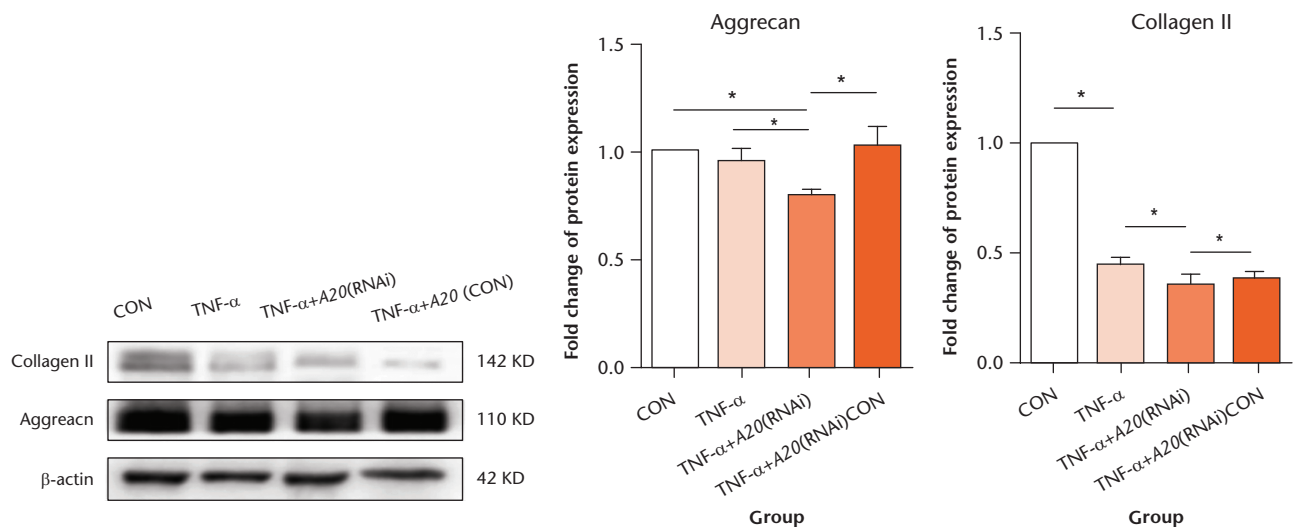


Fig. 5b

Fig. 5c

Decreased A20 expression further downregulated the synthesis of matrix macromolecules in tumour necrosis factor alpha (TNF- α)-treated nucleus pulposus cells (NPCs). a) The messenger RNA (mRNA) expression of aggrecan and collagen II in different test compounds was evaluated by real-time polymerase chain reaction (PCR). b) The expression of aggrecan and collagen II from different test compounds was analyzed by western blot. c) Quantification of immunoblots of aggrecan and collagen II. Data are expressed as means (SDs; $n = 3$). * $p < 0.05$. CON, control treated with phosphate-buffered saline; RNAi, RNA interference.

activity of NPCs will be further attenuated. This result demonstrated that A20 has an effect of resisting the decrease in the cell viability of TNF- α -induced NPCs (Figure 4).

Cell cycle detection is another critical indicator to evaluate cell senescence. G1 cell cycle arrest is an important characteristic of senescent cells. To fully investigate the function of A20 on TNF- α -treated NPC senescence, the cell cycle of each group was analyzed in this study. As shown in Figure 4, TNF- α -treated NPCs were easier to arrest in the G1 phase than those in the control group. Moreover, the NPCs of the A20 gene silencing group were more likely to be arrested in the G1 phase than those of the TNF- α intervention group.

Finally, the degradation of cellular matrix is considered to be another important phenotype of cellular senescence. Previous studies have shown that the anabolism of senescent cells is markedly less than that of normal cells.^{25,26} So we also investigated matrix synthesis of NPCs in each group by evaluating the protein deposition of matrix macromolecules. The matrix molecules' synthesis of TNF- α -treated NPCs was reduced, and TNF- α could further reduce the synthesis of matrix molecules of A20 gene-silenced NPCs. Collectively, these results indicated that A20 had a protective effect on inhibiting the degradation of the extracellular matrix of NPCs induced by TNF- α . (Figure 5)

Decreased A20 expression significantly increased NF- κ B activity in TNF- α -treated NPCs. Activation of NF- κ B has been reported to promote the expression of molecules that regulate various cellular metabolic processes, including immune response, proliferation, inflammation, and senescence.^{10,27,28} As previously studied, TNF- α could cause senescence of NPCs via the NF- κ B signalling pathway.¹⁶ To investigate the mechanism of A20 in senescence of NPCs induced by TNF- α , we examined the activation of the NF- κ B pathway in NPCs of each test group and with different concentrations of TNF- α . As shown in Figure 6, the NF- κ B pathway of NPCs was activated by TNF- α , and TNF- α could further activate the NF- κ B pathway of A20 gene-silenced NPCs.

Discussion

The increase in senescent cells is a classical pathophysiological change in disc herniation caused by IDD.^{29,30} There is no effective treatment to delay and reverse the

progression of IDD and surgical treatment is also not routinely considered.³¹ Previous studies have demonstrated that inflammation is associated with cellular senescence and degenerated intervertebral discs, and confirmed that TNF- α could induce premature senescence of NPCs via the NF- κ B signalling pathway.^{16,32-34} The NF- κ B pathway could be regulated by a variety of factors and participate in various physiological metabolic activities of the organism.³⁵ Therefore, we hypothesized that inflammatory responses could be attenuated by inhibiting NF- κ B pathway activation to delay or reverse senescence of NPCs. It has been reported that A20 is a cytoplasmic protein that restricts the NF- κ B signalling pathway and regulates the inflammatory response.^{17,19-21} Exhilaratingly, we found that the protein expression of A20 is increased in human degenerative discs. Thus, we investigated the anti-inflammatory effect of A20 on TNF- α -stimulated NPCs and explored the potential mechanism.

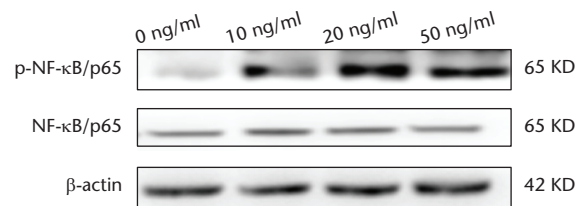


Fig. 6a

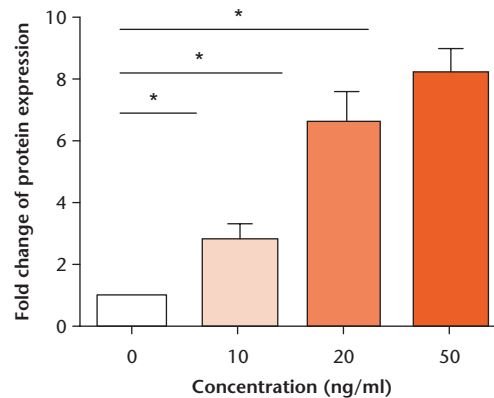


Fig. 6b

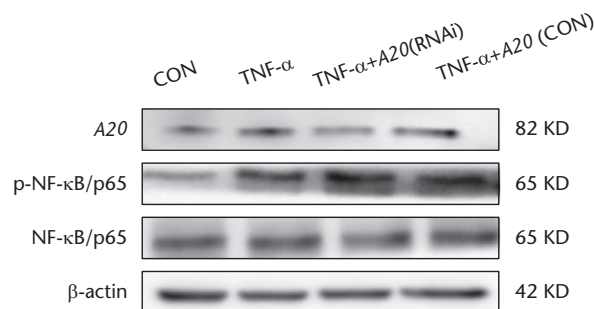


Fig. 6c

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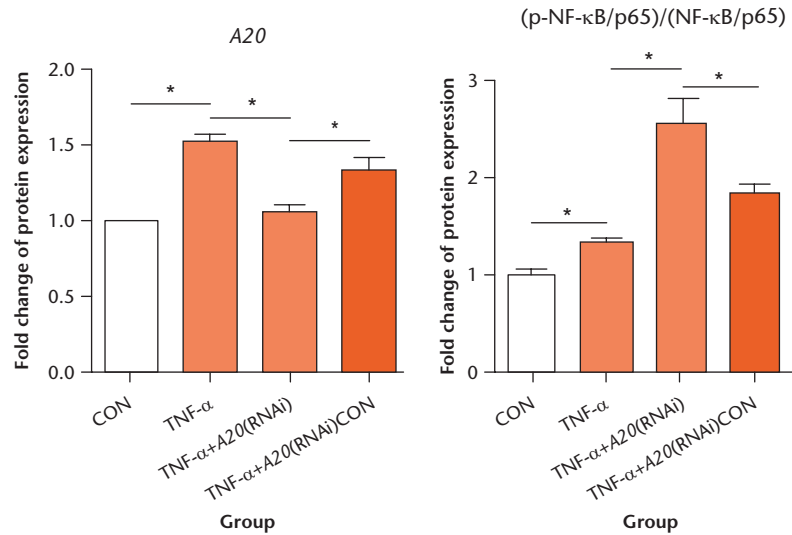


Fig. 6d

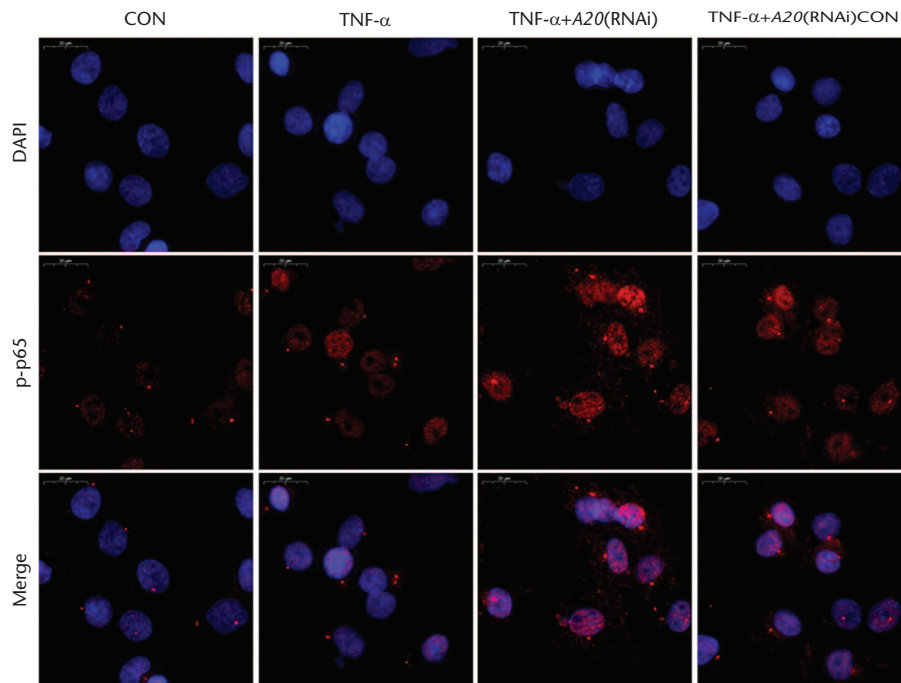


Fig. 6e

The expression of A20 and nuclear factor-kappa B (NF-κB) pathway proteins in nucleus pulposus cells (NPCs) of each group. a) The protein expression of p-NF-κB/p65 and NF-κB/p65 in tumour necrosis factor alpha (TNF-α)-treated NPCs was evaluated by western blots. b) Quantification of immunoblots of (p-NF-κB/p65)/(NF-κB/p65). c) The protein expression of A20, p-NF-κB/p65, and NF-κB/p65 was evaluated by western blots. d) Quantification of immunoblots of A20 and (p-NF-κB/p65)/(NF-κB/p65). e) The nuclei translocation of p65 was detected by the immunofluorescence combined with DAPI staining for nuclei (original magnification × 400). Data are expressed as means (SDs; n = 3). *p < 0.05. CON, control treated with phosphate-buffered saline; RNAi, RNA interference.

In general, senescent cells display decreased cell proliferation ability, increased β-Gal activity, and G1 cell cycle arrest.^{30,36} In this research, CCK-8 assay, SA-β-Gal staining, cell cycle analysis, and synthesis of extracellular matrix and senescence proteins were used to estimate senescence of NPCs induced by TNF-α. The results showed that A20 could resist the damage of NPCs by TNF-α, such as

A20 increased cell proliferation ability, decreased SA-β-Gal activity, and inhibited G1 cell cycle arrest in TNF-α-treated NPCs. Since TNF-α is a critical inflammatory factor in the degenerated discs,^{14,37} these results indicated that A20 could inhibit the senescence of NPCs in the TNF-α inflammatory microenvironment. In line with the previous researches of A20 on other cells, A20 is a deubiquitinating

enzyme with critical anti-inflammatory functions^{18,38} and A20-deficient mice are more susceptible to inflammation and autoimmune diseases.^{39–41} Additionally, A20 could inhibit necroptosis of macrophage through its ZnF7 ubiquitin-binding domain.⁴² All of these studies indicate that A20 should perform a protective role in the pathophysiology of IDD. The catabolic enzymes of senescent NPCs may increase, which would affect normal extracellular matrix production within the tissues.²⁵ Decreased A20 protein expression further aggravates the degradation of extracellular matrix in the TNF- α microenvironment. Metabolic changes in extracellular matrix molecules of NPCs can significantly affect the shape and biomechanics of the intervertebral disc⁴³ and lead to disc herniation. We conclude that A20 could inhibit the premature senescence of NPCs induced by TNF- α .

Cellular senescence is associated with two mechanisms: the p53-p21-pRB pathway and the p16-pRB pathway.²⁴ Previous studies have investigated these two senescent pathways in the intervertebral disc and demonstrated that upregulation of p53 or p16 expression occurs in senescent NPCs.^{6,16,44} In our research, the expression of p53 and p16 was further upregulated in A20 gene-silenced NPCs induced by TNF- α . Furthermore, we also analyzed the changes in the NF- κ B pathway of NPCs in each group to reveal the possible mechanism of the protective effect of A20 against senescence of TNF- α -treated NPCs. The results indicated that NF- κ B activity in TNF- α -treated NPCs was increased compared with that of the control NPCs, and the activation of the NF- κ B pathway

was further increased after attenuating the expression of A20 in TNF- α -treated NPCs. Combined with these results, it is speculated that the protective effect of A20 on the senescence of TNF- α -treated NPCs is related to the NF- κ B pathway. (Figure 7)

Taken together, the senescence phenotype of NPCs was more pronounced when TNF- α stimulated NPCs with suppressed A20 expression. Based upon these findings, we hold the view that the inhibition of NF- κ B by A20 should be an important protective mechanism of NPC senescence.

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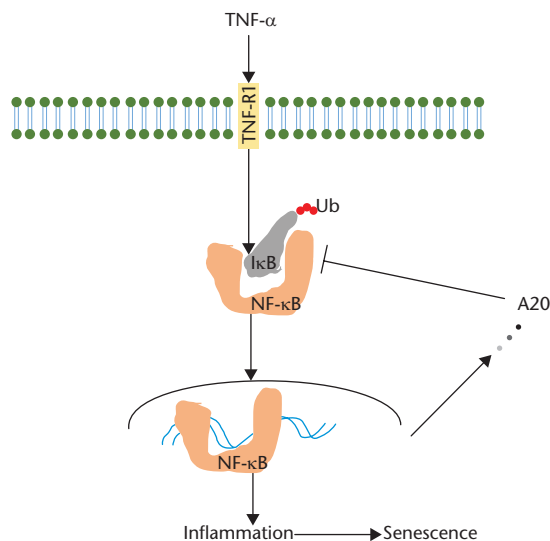


Fig. 7

Mechanism of A20 attenuates senescence of tumour necrosis factor alpha (TNF- α)-induced nucleus pulposus cells (NPCs). TNF- α can stimulate the activation of the nuclear factor-kappa B (NF- κ B) signalling pathway and increase the protein expression of A20. A20 can also negatively regulate the NF- κ B signalling pathway. I κ B, inhibitor kappa B; TNF-R1, tumour necrosis factor receptor 1; Ub, ubiquitin.

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- R. Shi: Evaluated the feasibility of and made corrections to the analysis of the experimental results.
- Z.-Y. Xie: Designed the experiment.
- F. Wang: Provided technical guidance during the experiment.
- K. Wang: Designed the experiment.
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Conflict of interest statement

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Ethical review statement

- The study was conducted in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health.

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