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ORIGINAL ARTICLE

TNF-α Regulates ITGβ1 and SYND4 Expression in Nucleus Pulposus Cells: Activation of FAK/PI3K Signaling

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Abstract— Integrins can function synergistically with syndecan-4 (SYND4) and bind to the fibronectin (FN) matrix, resulting in the regulation of tissue regeneration. This study aimed to explore the effects of TNF-α on the formation of FN/ITGβ1/SYND4 complex and the relative mechanism in NP cells. The expression of FN-ITG-SYND4 at the cellular level under TNF-α stimulation was detected by immunofluorescent staining, western blotting, and RT-PCR. ITGβ1 is a crucial component of ITG FN-induced FAK signaling, which was detected using dual mode. And, the involved signaling down stream pathways were also detected. FN is a preferred adhesion substrate for NP cells and that integrin β1 (ITGβ1) and SYND4 work synergistically during ECM engagement in a focal adhesion kinase (FAK)-dependent fashion. The PI3k/Akt pathway is obviously down-regulated, resulting in decreased adherence capacity and increased anoikis. TNF-α induction could weaken FAK activity and downstream levels of phospho-PI3K and Akt, resulting in decreased adherence capacity and increased apoptosis. Thus, TNF-α is essential for the formation of FN/ITGβ1/SYND4 complex in NP cells and further elucidates the inflammatory mechanism of NP cells degeneration.

KEY WORDS: intervertebral disc; degeneration; integrins; fibronectin; adhesion; anoikis.

INTRODUCTION

The molecular mechanism of Intervertebral disc degeneration (IDD) is very complicated and some of the factors responsible include mechanical strain, hypoxia, and acidic environment. Millions of Americans will experience some form of low back pain during their lifetime, resulting in direct and indirect annualized costs amounting to $50 billion. ECM contains two main components: collagens and proteoglycans. Alterations in the composition of ECM may cause changes in the mechanical properties of the disc, participating in the process of IDD. The available evidence indicates that disc degeneration is the result of progressive decrease in nucleus pulposus (NP) proteoglycan content leading to the dehydration of the disc and decrease. However, the complete mechanism is still unknown and needs extensive investigation.

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The IVD is typically composed of NP and annulus fibrosus (AF) cells, which show differences in morphology and ECM production. Both NP and annulus fibrosus (AF) cells synthesize similar extracellular matrix components. Although the exact mechanism is still unclear, inflammatory cell infiltration has been identified as a cue [24]. Disc degeneration is characterized by increased levels of inflammatory cytokines, including IL-1b, TNF-α, IL-2, IL-4, IL-10, IL-12, and IL-17 [8, 15]. Under the stimulatory effect of inflammatory cytokines, protease production significantly increases, resulting in excessive matrix degradation and a pathological condition [3, 4, 9–11]. The inflammatory factors could reduce the expression of anabolic ECM proteins, aggrecan, and collagen II, and concurrently promote the expression of the catabolic enzymes, matrix metalloproteinases (MMPs) 1, 2, 3, 4, 13, and 14, and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 and 5.

ITGs, which are well-known receptors of ECM, play key roles in mediating communication between cells and ECM. Because syndecans can function by clustering with ITGs, they initiate or modulate signaling molecules. Syndecans play important roles in tissue injury and repair. During the process of tissue regeneration, syndecan (SYND) 4 and 2 and ITGα5β1 can function synergistically and bind to the fibronectin (FN) matrix [12, 18–20]. SYND-4 acts as a receptor of FN, and the engagement of SYND4 with FN is required for the activation of FAK in NP cells, inducing ITGβ1-dependent focal adhesion. The binding of FN to SYND4 is mediated by its high-affinity heparin-binding domain [22]. In addition, PI3K/Akt signaling has been identified to contribute to IVD degeneration by inhibiting NP cell apoptosis [14]. The upregulation of ECM was related to the activation of the FAK//PI3K/Akt pathway in degenerative NP cells [26].

We found that the degradation of FN from the degenerated ECM impairs NP cell function by affecting SYND4-ITGβ1 (ITGβ1) signaling in an FAK-dependent and FAK-independent fashion. This study aimed to investigate the effects of pro-inflammatory cytokine TNF-α on the expression of SYND4-ITGβ1 complex and the downstream signaling pathway.

MATERIALS AND METHODS

This study was reviewed and approved by the Ethics Committee of Tongji Medical College; written informed consent was obtained from all patients.

Culture and Stimulation of NP Cells

NP cells were isolated and cultured as previously described [23]. Fibronectin (FN) was dissolved in purified water at the concentration of 1 mg/ml, and then the solution was diluted 100 times to obtain a concentration of 10 μg/ml. Add 1 ml dilute solution to each well of the plate. After sealed, the plate was placed in a refrigerator at 4 °C overnight. Remove coating solution and wash it twice with PBS. Additionally, collagen (COL) was dissolved acetic acid (0.5 M), and the wells were coated with COL as the method for fibronectin. NP cells were cultured in 6- or 96-well plates coated with FN (F2006; Sigma) or COL (C7774; Sigma) until 100% confluency. After being serum-starved for 2 h, NP cells were incubated in the growth medium or in a stimulatory medium containing TNF-α (25 and 50 ng/mL) for 6, 24, 72, and 96 h. Each treatment was conducted and replicated in three different wells.

Cell Adhesion Assay

Cell adhesion is essential for cell proliferation and survival. NP cells from different groups were collected and seeded in 96-well plates coated with MatrigelTM Matrix at a density of 2×10^5 cells/mL (volume of the medium per well is 100 μL) and incubated overnight at standard conditions. After washing with DMEM medium, non-adherent NP cells were discarded. Then, 10 μL of MTT substrate was added to each well, and the plates were incubated for 4 h. After that, the MTT-treated cells were lysed with 150 μL of DMSO, and the absorbance was quantified by measuring OD 568 using a microplate reader.

Immunofluorescence Studies

NP cells were treated and fixed in 4% paraformaldehyde. After blocking with 10% skimmed milk, the slides were permeabilized in 0.1% Triton X in PBS. After washing, the slides were incubated with primary antibody anti-FAK (1:100) and anti-FN (ProteinTech Group, 1:100) overnight at 4 °C. Then, fluorescein-conjugated secondary antibodies were added, and the mixture was incubated for 2 h at 37 °C. Nuclei were visualized by staining with 4,6-diamidino-2-phenylindole (DAPI; Beyotime) for 5 min. For double-immunofluorescence studies of FAK and EDU, after the addition of secondary antibody, the slides were kept in dark and stained according to the instructions provided by the manufacturer of the EDU staining kits (Ribobio, China). Finally, the images were captured and analyzed using a fluorescence microscope (Olympus, Tokyo, Japan).

TUNEL Apoptosis Assay

The cells were fixed in 4% paraformaldehyde (pH 7.4) at room temperature for 25 min, washed thrice in PBS containing 0.1% Triton X-100, and analyzed using *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. Then, the slides were dried with wipes and sealed with fluorescence quenching liquid, and TUNEL-positive cells were calculated under fluorescence microscopy. The percentages of TUNEL-positive cells of total cells in each section were counted and expressed as the TUNEL indices.

Immunoprecipitation and Western Blotting

Co-immunoprecipitation (Co-IP) is a widely used method to identify protein-protein interactions. RIPA lysis buffer was used for protein extraction, and Bio-Rad protein assay (Bio-Rad) was used to measure protein concentration. For Co-IP, 50% Protein A/G Agarose was added to the sample solution to eliminate non-specifically-binding proteins. The solution was centrifuged at 3000 rpm at 4 °C for 5 min to discard Protein A/G Agarose beads. The supernatant was transferred to another tube, and the total protein was quantified by BCA assay. About 2 μg of ITGβ1 antibody (NOVUS) was added to 500 μL of total proteins. The antigen-antibody complex was slowly shaken overnight on a rotating shaker at 4 °C. After centrifuging, the above precipitants were used for Western blot analysis. For Western blotting, equal amounts of the protein extract and Co-IP precipitants were separated using 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat milk in Tris-buffered saline with Tween (TBST), the PVDF membranes were incubated with primary antibodies (Table 1) overnight at 4 °C overnight. The density of protein bands was quantified and analyzed using Image System (Bio-Rad) and normalized according to the controls.

H-E Staining

NP cells were cultured in 6- or 96-well plates coated with FN (F2006; Sigma) or COL (C7774; Sigma). After 6 h, 24 h, and 96 h culture, the plates were counterstained with hematoxylin, and images were captured using a microscope (Olympus).

ELISA Assay

The expression level of FN in supernatant was detected by ELISA method using the stored supernatant from each group at different time points after induction, according to the manufacturer's procedures (USCN, SEA037Hu).

Statistical Analysis

All experiments were conducted independently at least in triplicate, and the data were expressed as the mean ± SD. Comparisons between the groups were performed by Student's *t* test or analysis of variance (ANOVA) using GraphPad IPrism 5 software. A p value of < 0.05* or 0.01** was considered statistically significant.

RESULTS

Protein Expression of FN-ITG-SYND4 in NP Cells under TNF-α Induction

TNF-α is a kind of pro-inflammatory cytokine that induces NP cell degeneration. To determine the protein expression of FN-ITG-SYND4 at the cellular level during degeneration, NP cells were cultured under stimulation with TNF-α and the expression was detected by immunofluorescent staining, western blotting, and RT-PCR at different time points. As shown in Fig. 1a, immunofluorescent staining of NP cells showed downregulated expression of FN and upregulated expression of ITGβ and SYND4 in the TNF-α-stimulated group, compared to the control group. Cell lysates from different groups were collected and examined by western blotting (Fig. 1b), which also confirmed a significant decrease in FN expression at 6, 12, and 24 h of TNF-α stimulation (Fig. 1c). The results also indicated that the protein expression levels of ITGβ (Fig. 1d) and SYND4 (Fig. 1e) increased at the corresponding time points. FN expression by the cells conditioned in the growth medium was determined by ELISA, which indicated that FN concentration decreased significantly, in a time-dependent manner, in the NP cells after TNF-α induction (Fig. 1f). Thus, TNF-α induction could decrease the gene expression of FN and increase the expression of ITGβ and SYND4 in NP cells.

Lower Adhesive Capacity and Higher Apoptosis in NP Cells in Response to TNF-α

FN is a preferred adhesion substrate for NP cells. NP cells were grown on FN for 6, 24, and 96 h, using TNF-α for induction. As shown in Fig. 2a, TNF-α induction could

Table 1. Antibodies for Western Blot Analysis

Primary antibody	Size	Company	Catalog no.	Dilution
Anti-rabbit GAPDH	37KD	GOOD HERE, Hanzhou	AB-P-R 001	1:1000
Anti-rabbit syndecan 4	24KD	Abcam	ab24511	1:200
Anti-mice integrin β1	88KD	NOVUS	NBP2-36561	1:500
Anti-rabbit FN	263/100/70KD	ProteinTech Group	15613-1-AP	1:500
Anti-rabbit AKT	62KD	ProteinTech Group	10176-2-AP	1:500
Anti-rabbit P-AKT	60KD	Cell signaling	13461	1:1000
Anti-mice FAK	110KD	ProteinTech Group	66258-1-lg	1:2000
Anti-rabbit P-FAK	125KD	Cell signaling	3281	1:1000
Anti-mice PI3K	85KD	ProteinTech Group	60225-1-lg	1:2000
Anti-goat P-PI3K	85KD	Santa Cruz	SC-12929	1:300

significantly reduce the adhesive capacity of NP cells in a time-dependent manner. After 96 h of culture, the number of attached cells was reduced to 42% of the control group. TUNEL assay was used to investigate whether the mechanism led to anchorage-dependent programmed cell death (anoikis) (Fig. 2b). TNF-α induction resulted in NP cell apoptosis, reaching as high as 2.63-folds of the total number of TUNEL+ NP cells at 96 h, compared to the control group (Fig. 2c). Owing to the decreased adherence capacity, NP cells tend to be more prone to anoikis during the degenerative state.

Decreased Formation of the SYND4/ITGβ1 Complex by TNF-α Stimulation

To characterize the relationship between FN and the formation of SYND4/ITGβ1 complex in NP cells, Co-IP

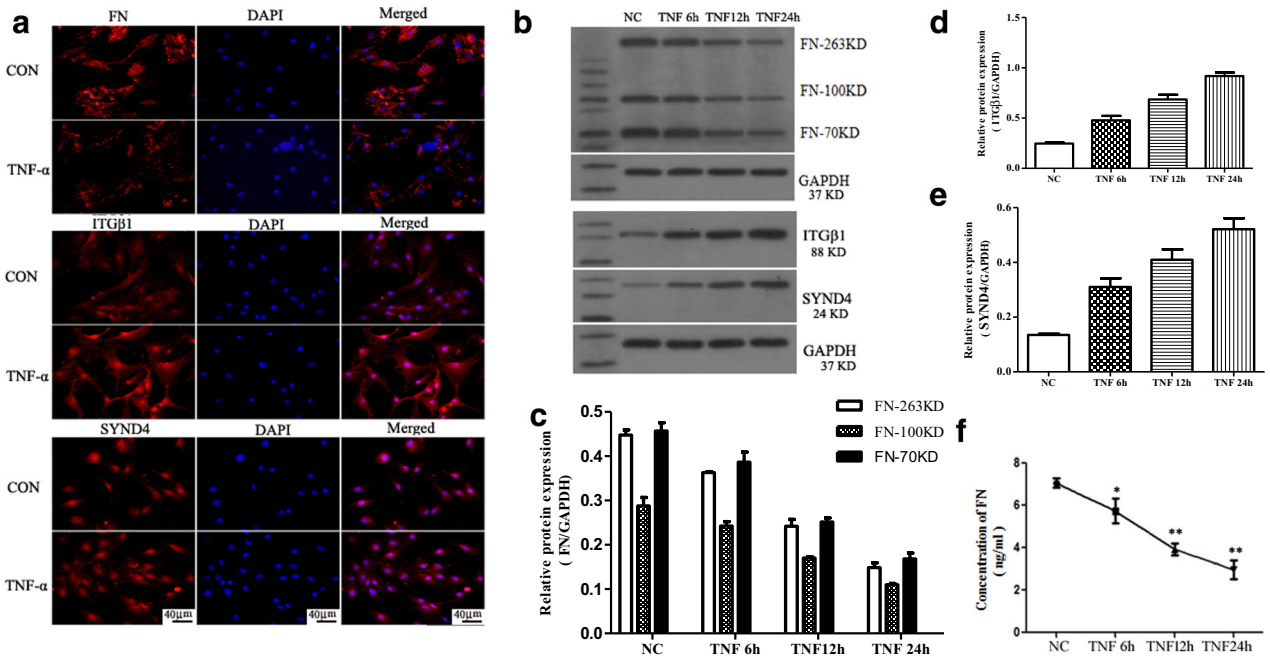

Fig. 1. TNF-α decreased the expression level of FN but increased that of ITG and SYND4 in NP cells. **a** Representative photomicrographs of immunofluorescence staining for FN-ITG-SYND4 expression in NP cells of different groups. **b** Expression of FN-ITG-SYND4 proteins as assessed by western blotting. **c**–**e** Densitometry analysis shows the suppression of FN (**c**), ITG (**d**), and SYND4 (**e**) proteins in NP cells treated with TNF-α at different time points. **f** ELISA of the conditioned cell medium of TNF-α-treated NP cells, showing a decrease in FN expression. Data are presented as the mean ± SD, from at the least three independent experiments. $*p < 0.05$, $**p < 0.01$ (IF: scale bar magnification × 400); $*p < 0.05$, $**p < 0.01$, compared with NC.

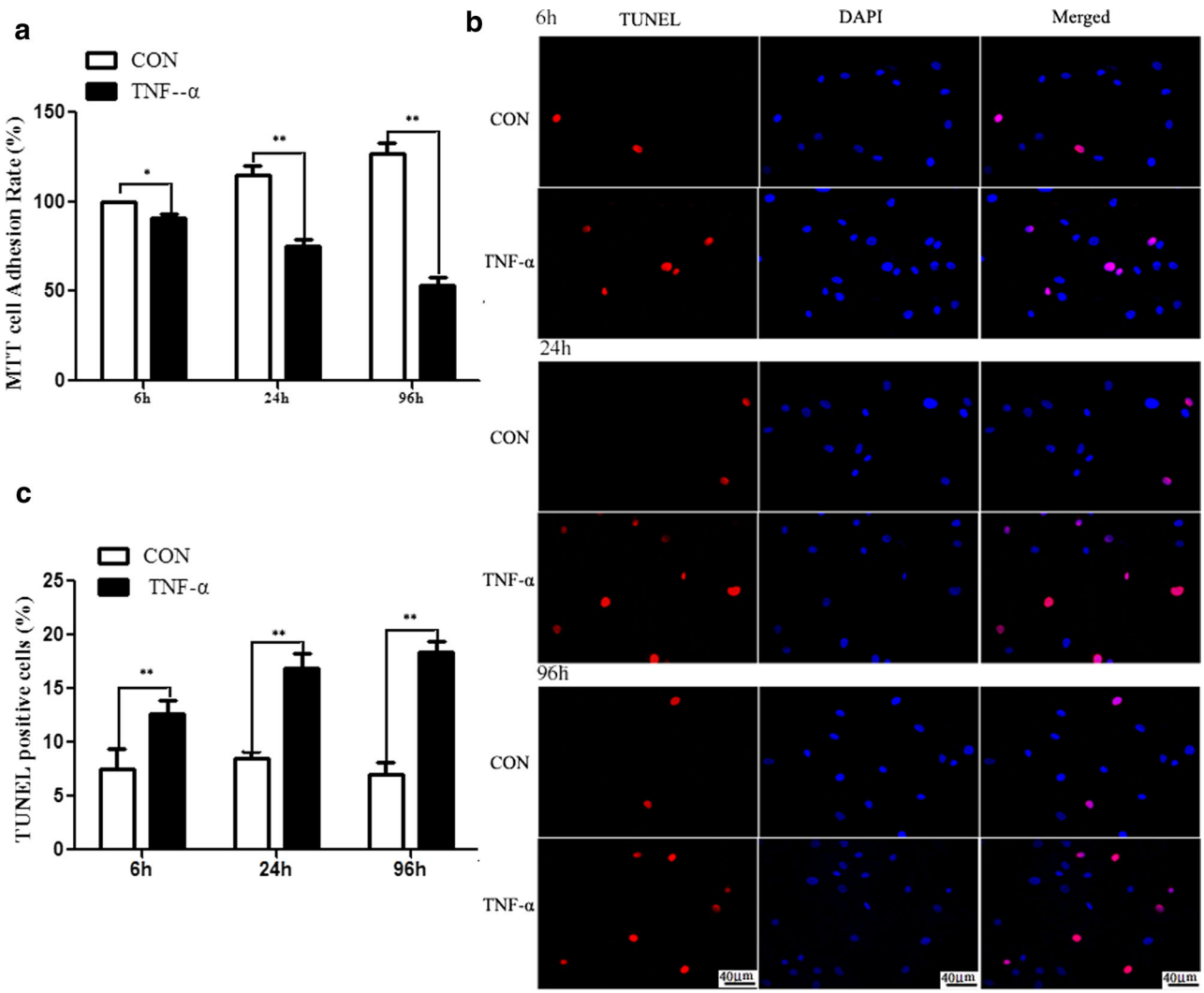

Fig. 2. TNF-α decreased the adhesive capacity but increased apoptotic activity in NP cells. **a** Adhesive capacity of NP cells was measured as the percentages of adherent cells (absorbance OD570) of total cells in each well. **b** TUNEL assay was used to identify apoptotic cells (red) in TNF-α-treated NP cells. **c** Statistical analysis of the results of apoptosis in different groups at different time points. Data are presented as the mean ± SD, from at the least three independent experiments. $*p < 0.05$, $**p < 0.01$ (IF: scale bar magnification × 400); $*p < 0.05$, $**p < 0.01$, compared with NC.

protein was studied. The expression of FN decreased in the presence of TNF-α in a dose-dependent manner, whereas the expression of ITGβ1 and SYND4 increased in the presence of both FN and COL (Fig. 3a). When the NP cells were plated with FN under the stimulation of TNF-α, the amount of FN co-immunoprecipitated with ITGβ1 was remarkably higher than that when the cells were grown in the presence of COL (Fig. 3b–d). The amount of SYND4 co-immunoprecipitating with ITGβ1 reduced with the decrease in FN expression, but it was higher than that observed in the cells plated with COL. Our findings indicate that the formation of SYND4/ITGβ1 complex was enhanced by FN but decreased by inflammatory TNF-α stimulation.

Decreased Adhesion Capacity of NP Cells in the Presence of TNF-α

To investigate the effects of TNF-α on NP cell function and adhesion capacity, the cells were cultured on either FN or COL. After 72 h of culture, NP cells of control group showed higher cell viability over TNF-α treatment group, with a similar preference for FN and COL (Fig. 4a). When comparing the growth of NP cells in the presence of FN

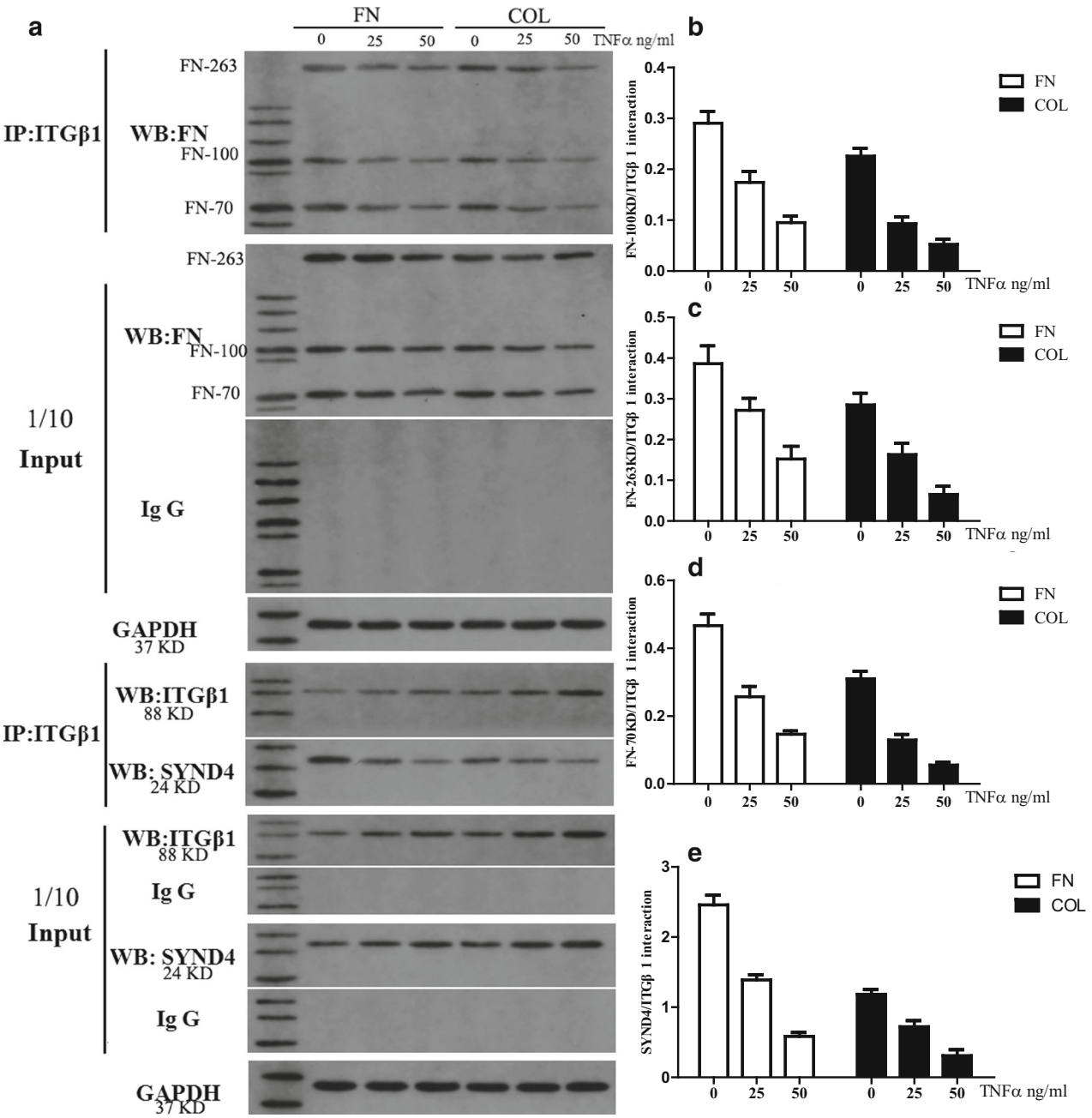

Fig. 3. TNF-α decreased the formation of SYND4/ ITGβ1 complex in NP cells plated with FN. **a** Co-IP-based detection of the FN-induced formation of the SYND4/ITGβ1 complex in NP cells. **b**–**d** Statistical analysis of the results of co-immunoprecipitation of FN with ITGβ1. **e** Statistical analysis of the results of co-immunoprecipitation of SYND4 with ITGβ1. FN (coating) is an essential ECM protein for maintaining the function and survival of NP cells, while COL (coating) was chosen as a control. Data are presented as the mean ± SD, from at the least three independent experiments. $*p < 0.05$, $**p < 0.01$.

and COL, we observed a higher proportion of non-treated cells than TNF-α-treated cells on both FN- and COL-coated surfaces (Fig. 4b). Cell-ECM adhesion is necessary for cell survival, proliferation, and differentiation. Cell adhesion assay for non-treated and TNF-α treated NP cells indicated that the adhesion

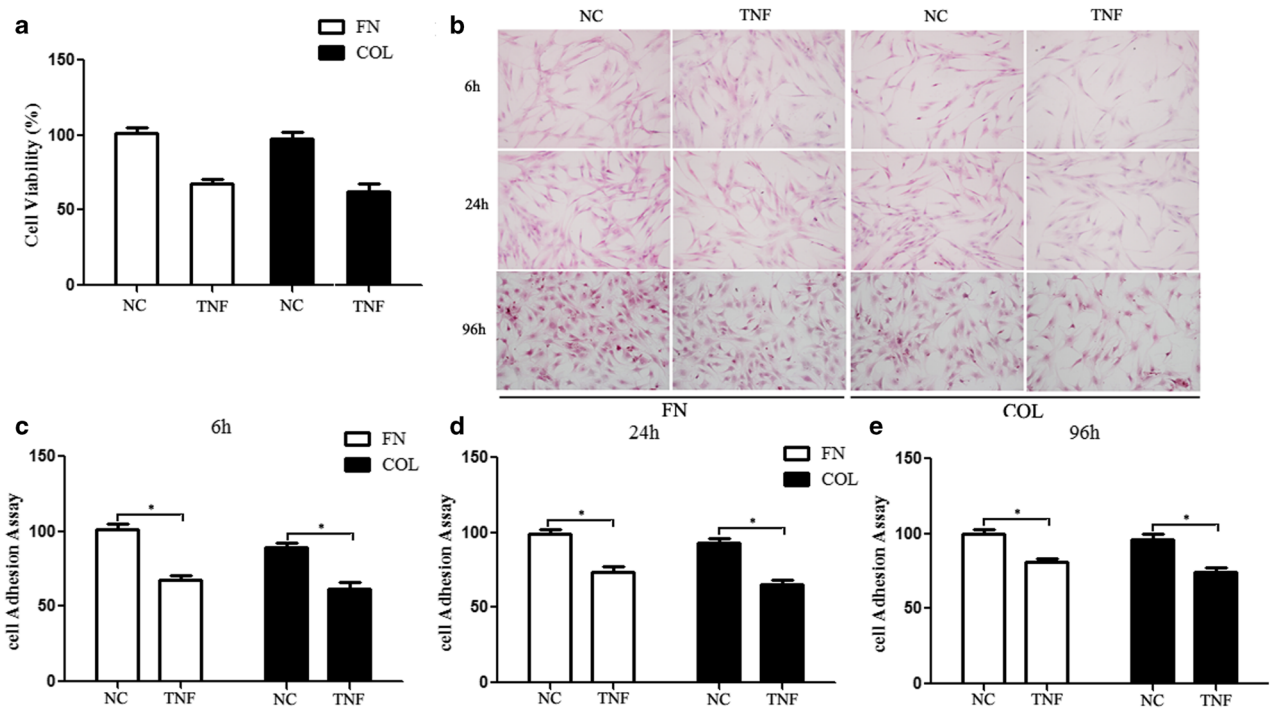

Fig. 4. TNF-α decreased adhesion capacity of NP cells on both FN- and COL-coated surfaces. **a** Cell viability was detected in the NP cells in the presence/absence of TNF-α by the MTT method. **b** Representative images of the growth of NP cells in different groups. **c** Cell adhesion rate of different groups was detected at 6 h **c**, 24 h **d**, and 96 h **e**. FN (coating) is an essential ECM protein for maintaining the function and survival of NP cells, while COL (coating) was chosen as a control. Data are presented as the mean ± SD, from at the least three independent experiments. $*p < 0.05$, $**p < 0.01$. (IF: scale bar magnification × 200).

rate was much higher on FN than on COL at 6 h (Fig. 4c), 24 h (Fig. 4d), and 96 h (Fig. 4e).

FN Rescues the Degenerative NP Cells *via* FAK-Mediated Signaling

We further investigated the potential mechanisms involved in IVD cell adhesion and apoptosis. The protein molecules were detected by western blotting. As shown in Fig. 5a, phosphorylation of FAK was decreased in NP cells under TNF-α stimulation. Next, we investigated the downstream mediators, including PI3K and Akt. Decreased activation of FAK (Fig. 5b) correlated with decreased levels of phospho-PI3K (Fig. 5c) and Akt (Fig. 5d). In addition, immunofluorescence staining showed that FAK levels in NP cells were affected by TNF-α treatment (Fig. 5e). These results suggested that TNF-α treatment might weaken the adhesion capacity and ability to resist anoikis of NP cells *via* the FAK/PI3K/Akt axis (Fig. 5f), and further elucidate the inflammatory mechanism of NP cell degeneration.

DISCUSSION

During IVD degeneration, the matrix homeostasis gets disturbed, resulting in excessive ECM degradation and a pathological condition. NP cells are chondrocyte-like in their morphology and produce ECM components. Alterations in the composition of ECM may be involved in IVD degeneration. A great deal of work has been undertaken on the pathogenesis of IVD degeneration, including progressive decrease in proteoglycan, collagen II, and matrix-anabolic enzymes and increase in collagen type 1, pro-inflammatory cytokines, and matrix-degrading enzymes [1, 6, 9–11]. Ongoing studies are rapidly advancing the scientific understanding of the disorders of ECM [4]. Therefore, the treatment of conditions involving disc degeneration should focus on the homeostasis of the ECM in the disc. In the study, we found decreased level of FN and increased level of ITG-SYND4 during IVD degeneration.

Inflammatory factors can reduce the expression of the anabolic ECM proteins, namely, aggrecan and collagen II, and concurrently promote the expression of catabolic enzymes [9–11, 21]. TNF-α and IL-1β have been found to

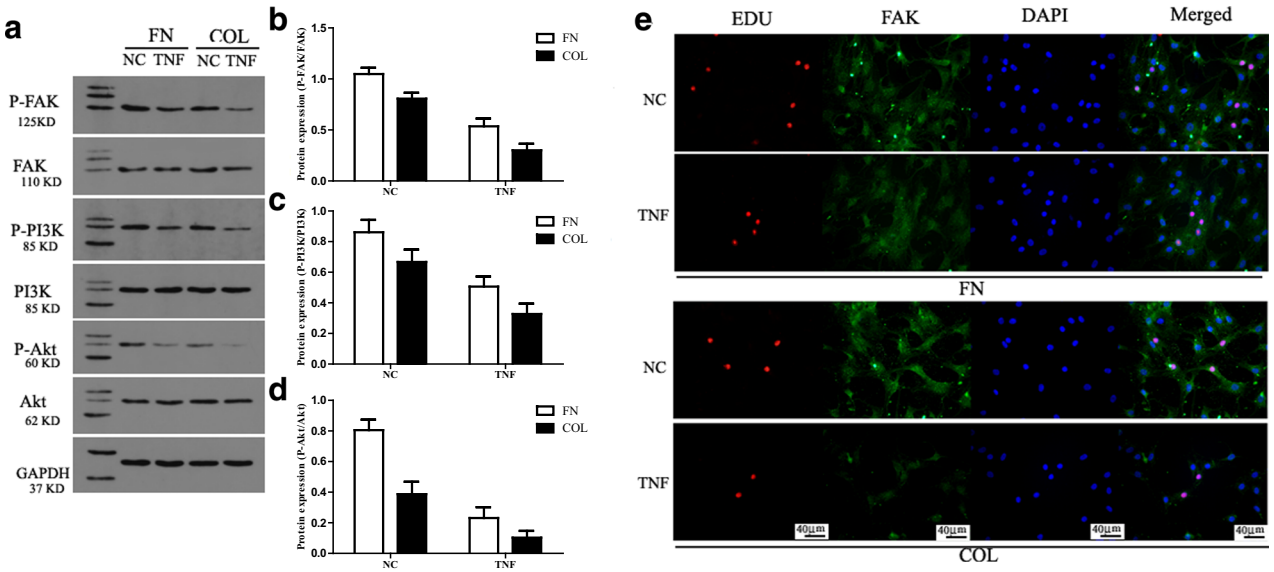

Fig. 5. TNF-α decreased phosphorylation of FAK, PI3K, and Akt in NP cells. **a** The levels of phosphorylated and unphosphorylated FAK, PI3K, and Akt proteins were determined by western blotting. **b**–**d** Statistical analysis of the ratio of p-FAK/FAK (**b**), p-PI3K/PI3K (**c**), and p-AKT/AKT (**d**) in different groups. **e** Immunofluorescence staining for the detection of FAK expression. FN (coating) is an essential ECM protein for maintaining the function and survival of NP cells, while COL (coating) was chosen as a control. Data are presented as the mean ± SD, from at the least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (IF: scale bar magnification × 400).

induce the expression of SYND4 in various cellular systems, besides IVD cells [7, 13, 25]. Moreover, CCN2 has been found to inhibit SYND4 expression induced by IL-1β stimulus [17]. During the process of IVD degeneration, the increase in inflammatory cytokines, namely, TNF-α and IL-1β, enhances the expression of catabolic enzymes within NP cells *via* the MAPK and NF-κB signaling pathways, resulting in an imbalance among the catabolic and anabolic enzymes, which leads to the alteration and loss of function of the IVD [2]. In the study, TNF-α was used to stimulate NP cell degeneration, which could decrease the expression of FN and increase the expression of ITGβ and SYND4 in NP cells.

As emphasized here, SYND4 has dual roles in NP cells during degeneration: facilitating the interactions between FN and ITGβ and promoting ECM degradation. SYND4 exerts its effects *via* the interaction of ITGβ1 and extracellular FN, subsequently leading to the formation of focal adhesion. Apoptosis occurs in anchorage-dependent cells when they detach from surrounding ECM after immunoprecipitation of FAK with anti-FAK antibody; in contrast, apoptosis is inhibited by the constitutive activation of FAK [5]. Thus, preventing loss of adhesion to the matrix and restoring adhesion can indirectly activate FAK activity and inhibit anoikis. SYND4 is also involved in the degradation of aggrecan and collagen during IVD degeneration. SYND4 exerts its effects by proteolytic cleavage and subsequent shedding of the extracellular domain driven by inflammation [7, 16]. Furthermore, SYND4 has been found to promote ADAMTS-5-mediated aggrecan degradation, resulting in IVD degeneration [19, 20]. During IVD degeneration, on one hand, the degradation of FN inhibits the FAK/PI3K/Akt pathway, resulting in decreased adherence capacity and increased anoikis. On the other hand, increased expression of SYND4 accelerates the degradation of ECM, forming a vicious circle. As indicated by our results, FAK signaling activity was partially restored in the degenerative NP cells exposed to FN, with subsequent increase in the adherence capacity as well as decreased anoikis.

Taken together, we found that the formation of ITGβ1/SYND4 complex is essential for maintaining the function and survival of NP cells. Pro-inflammatory cytokine TNF-α could inhibit the formation of ITGβ1/SYND4 complex and the activation of FAK/PI3K signaling, eventually lead to decreased cell adhesion and increased cell apoptosis. Further, we sought to elucidate the inflammatory mechanism of intervertebral disc degeneration.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

This study was reviewed and approved by the Ethics Committee of Tongji Medical College; written informed consent was obtained from all patients.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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