

17 β -Estradiol protects against interleukin-1 β -induced apoptosis in rat nucleus pulposus cells via the mTOR/caspase-3 pathway

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Abstract. Intervertebral disc degeneration (IVDD) is the main pathological basis of spinal degenerative diseases, and aberrant apoptosis of nucleus pulposus cells (NPCs) is the main cellular process that causes IVDD. In our previous studies, 17 β -estradiol (E₂) was demonstrated to protect rat NPCs from interleukin-1 β (IL-1 β)-induced apoptosis via the PI3K/Akt signaling pathway. However, the downstream signaling pathway of PI3K/Akt is currently unclear. The present study aimed to explore the signaling pathways that are downstream of the PI3K/Akt pathway, including mTOR, NF- κ B and glycogen synthase kinase-3 β (GSK-3 β). Annexin V/propidium iodide double staining was used to determine the incidence of apoptosis. Cell Counting kit-8 and MTS assays were used to determine the proliferation and viability of NPCs, respectively. Cellular binding was evaluated using a cell-collagen binding assay. Western blotting was used to determine the protein expression levels of mTOR, NF- κ B and GSK-3 β , and their phosphorylation levels, as well as the expression levels of active caspase-3. The results revealed that IL-1 β induced NPC apoptosis and increased the early apoptotic rate of NPCs. However, E₂ reduced the early apoptosis of NPCs induced by IL-1 β . In addition, E₂ suppressed the decrease in cell viability and binding ability caused by IL-1 β cytotoxicity. Western blotting revealed that E₂ also reduced the expression of activated caspase-3, and increased the expression of activated mTOR. As a specific inhibitor of mTOR, rapamycin

effectively attenuated the effects of E₂. These findings indicated that E₂ protected NPCs against apoptosis via activation of the mTOR/caspase-3 pathway.

Introduction

Clinically, intervertebral disc (IVD) degeneration (IVDD) causes lower back pain and disc herniation, which are some of the most common disorders leading to morbidity and deterioration in quality of adult life. A decrease in the number of living cells is one of the initial inducers of IVDD in nucleus pulposus (NP) cells (NPCs) (1,2). Notably, aberrant apoptosis and accelerated aging of NPCs are considered the two major cellular processes associated with IVDD (3,4). As cellular loss due to excessive apoptosis may serve an important role in IVDD (3), suppression of apoptosis has been suggested as a potential strategy to alleviate IVDD (5,6).

17 β -Estradiol (E₂) has been widely studied for its role in suppressing apoptosis, mainly through the involvement of integrins and extracellular matrix (ECM) receptors (7-12). In our previous studies, it was demonstrated that E₂ protects against aberrant apoptosis in rat NPCs by upregulating integrin α 2 β 1 and type II collagen (COL2), and downregulating matrix metalloproteinase (MMP)-3 and MMP-13 (13,14). Previous studies have reported that integrins, including β 1, β 4 and α v β 3, can promote cell proliferation and inhibit cell apoptosis via the PI3K/Akt pathway (15-18). Activation of the PI3K/Akt pathway has been shown to promote cell survival and protect rat NPCs from apoptosis (19-21). However, the pathway downstream of PI3K/Akt that is associated with the anti-apoptosis process remains unclear.

Glycogen synthase kinase-3 β (GSK-3 β), mTOR and NF- κ B are key proteins in the pathways downstream of PI3K/Akt (22-25); these proteins are inhibited by SB216763, rapamycin and SC75741, respectively. The present study hypothesized that these signaling pathways may take part in retarding the progress of IVDD-associated diseases. The present study aimed to explore the three downstream signaling pathways of PI3K/Akt and determine which pathways were involved in the effect of estrogen against apoptosis. Notably, this work is expected to provide a novel target for a therapeutic strategy to prevent and treat IVD degenerative diseases.

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Abbreviations: IVD, intervertebral disc; IVDD, IVD degeneration; ECM, extracellular matrix; NP, nucleus pulposus; NPCs, NP cells; IL-1 β , interleukin-1 β ; E₂, 17 β -estradiol; ER, estrogen receptor; COL2, type II collagen; COL2 α 1, COL2 α 1 chain; S6K, S6 kinase

Key words: estrogen, apoptosis, IVDD, NP, IL-1 β , mTOR

Materials and methods

Reagents and antibodies. The following reagents and antibodies were used in this study: DMEM/F12 (HyClone; GE Healthcare), FBS (HyClone; GE Healthcare), trypsin (Sigma-Aldrich; Merck KGaA), DMSO (Beijing Solarbio Science & Technology Co., Ltd.), Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.), FITC Annexin V Apoptosis Detection kit I (BD Pharmingen; BD Biosciences), IL-1 β (PeproTech, Inc.), E₂ (Sigma-Aldrich; Merck KGaA) and ICI182780 (Sigma-Aldrich; Merck KGaA). Secondary antibodies (cat. no. 7074; Cell Signaling Technology, Inc.), and primary antibodies against mTOR (cat. no. 2972), phosphorylated (p)-mTOR (cat. no. 2971), GSK-3 β (cat. no. 9315), p-GSK-3 β (cat. no. 9322), NF- κ B (cat. no. 4764), p-NF- κ B (cat. no. 3031) and cleaved caspase-3 (cat. no. 9664) (all Cell Signaling Technology, Inc.), and β -actin (Wuhan Sanying Biotechnology). Rapamycin, SC75741 and SB216763 (Selleck Chemicals). A protease inhibitor and phosphorylase inhibitor (Beijing Solarbio Science & Technology Co., Ltd.), PVDF membranes (EMD Millipore) and an enhanced chemiluminescence reagent (Beijing Solarbio Science & Technology Co., Ltd.).

Ethics statement. The animal protocols were approved by the Institutional Animal Care and Use Committee of The Third Hospital of Hebei Medical University.

Cell culture. Briefly, three male Sprague-Dawley rats (weight, 200 g; age, 2 months) were purchased from the Animal Experimental Center of Hebei Medical University, and were raised under standard conditions: Temperature, 25 \pm 1 $^{\circ}$ C; 12-h light/dark cycle; and humidity, 60-70%. All rats were given free access to food and water. Rats were sacrificed by intravenous administration of 150 mg/kg pentobarbital sodium. Lumbar spinal columns were removed en bloc under aseptic conditions, and lumbar IVDs were collected. The gel-like NP was separated from the annulus fibrosus under a dissecting microscope and was sequentially treated with 0.25% type II collagenase (Sigma-Aldrich; Merck KGaA) for 1 h and 0.2% trypsin with EDTA (1 mmol/l) for 5 min. The solution containing partially digested tissue was then transferred to a 50-ml culture flask containing DMEM and 20% FBS and cultured at 37 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂. NPCs adhered to the bottom of the culture flask after 3 days. When confluent (after 1 week), the NPCs were dissociated using a 0.25% trypsin solution with EDTA (1 mmol/l) and further subcultured. First-passage cells maintained in monolayers were used for subsequent experiments. The cells were serum starved for an additional 24 h with phenol red-free medium and underwent cell apoptosis, binding and viability assays. Rat NPCs were divided into seven groups as follows: Control (treated with an equivalent volume of PBS), IL-1 β , IL-1 β + E₂, IL-1 β + E₂ + ICI182780, IL-1 β + E₂ + rapamycin, IL-1 β + E₂ + SC75741 and IL-1 β + E₂ + SB216763.

CCK-8 and MTS assays. Cell proliferation and viability were assessed using a CCK-8 kit and the MTS method using CellTiter 96[®] Aqueous MTS Reagent Solution (Promega Corporation), respectively. Both kits were conducted

according to the manufacturers' protocols. Briefly, 100 μ l cells (2 \times 10³ cells/well) were transferred into 96-well plates following trypsin digestion, and six parallel wells were used for each treatment. The culture plate was incubated at 37 $^{\circ}$ C in an incubator containing 5% CO₂ for 24 h. After attachment, the cells were cultured for 90 min in serum-free, phenol red-free medium, after which, E₂ (1 μ M) (13) was added to the medium. After 5 min, the inhibitors rapamycin, SC75741 and SB216763 were added. In the medium, the final concentrations of rapamycin, SC75741 and SB216763 were 100 nM, 1 and 1 μ M, respectively. After 30 min, IL-1 β (75 ng/ml) was added, and the cells were cultured at 37 $^{\circ}$ C in an incubator containing 5% CO₂ for 1, 2, 4, 8, 12, 24 and 48 h (26). Subsequently, 10 μ l CCK-8 was added to each well, and the cells were cultured for 1 h. The density of living cells was determined by measuring absorbance with a microplate reader (Shimadzu) at 450 nm (CCK-8).

For the MTS assay, 12 h after the cells were processed as for the CCK-8 analysis, 20 μ l MTT solution was added to each well and NPCs were incubated for 4 h at 37 $^{\circ}$ C in an incubator containing 5% CO₂. Subsequently, 150 μ l DMSO was added to each well and the plate was oscillated for 10 min to fully melt the crystals at room temperature. The density of living cells was determined by measuring absorbance with a microplate reader at 492 nm (MTS). Cell proliferation and viability were normalized by calculating the relative value to the control. The concentration of ICI182780 (1 μ M) used was the same as that reported in our previous study (13).

Cellular binding assay. Rat NPCs were treated as aforementioned and their ability to bind COL2 α 1 chain (COL2 α 1) was assessed. Briefly, 24-well plates were coated overnight at 4 $^{\circ}$ C with COL2 α 1 (20 μ g/ml; Sigma-Aldrich; Merck KGaA). Nonspecific binding sites were blocked by incubating the coated plates with 10 mg/ml bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) for 60 min, followed by two washes with ice-cold PBS. A total of 3 \times 10⁴ cells were placed in each well and allowed to adhere at 37 $^{\circ}$ C for 60 min. After adhesion, the cells were stained with 0.5% toluidine blue for 60 min at 37 $^{\circ}$ C, fixed with 4% paraformaldehyde for 15 min and solubilized in 1% SDS for 60 min at room temperature. Extracted dye was quantified by measuring the absorbance value at 590 nm using a plate reader (Dynatech MR5000).

Fluorescence-activated cell sorting (FACS) analysis. Cell apoptosis was detected by FACS using the FITC Annexin V Apoptosis Detection kit I according to the manufacturer's protocol. Rat NPCs were washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1 \times 10⁶ cells/ml. Subsequently, 1 \times 10⁵ cells were transferred to a 5-ml culture tube, and 5 μ l FITC-Annexin V and 5 μ l PI were added to the tube. The cells were vortexed and incubated for 15 min at room temperature in the dark. Finally, 400 μ l 1X binding buffer was added to each tube, and the cells were analyzed by Cell Quest Pro 6.0 software (BD Biosciences) after 1 h.

Western blotting. The protein expression levels of p-mTOR, mTOR, p-GSK-3 β , GSK-3 β , p-NF- κ B, NF- κ B and cleaved caspase-3 were determined by western blotting, with β -actin

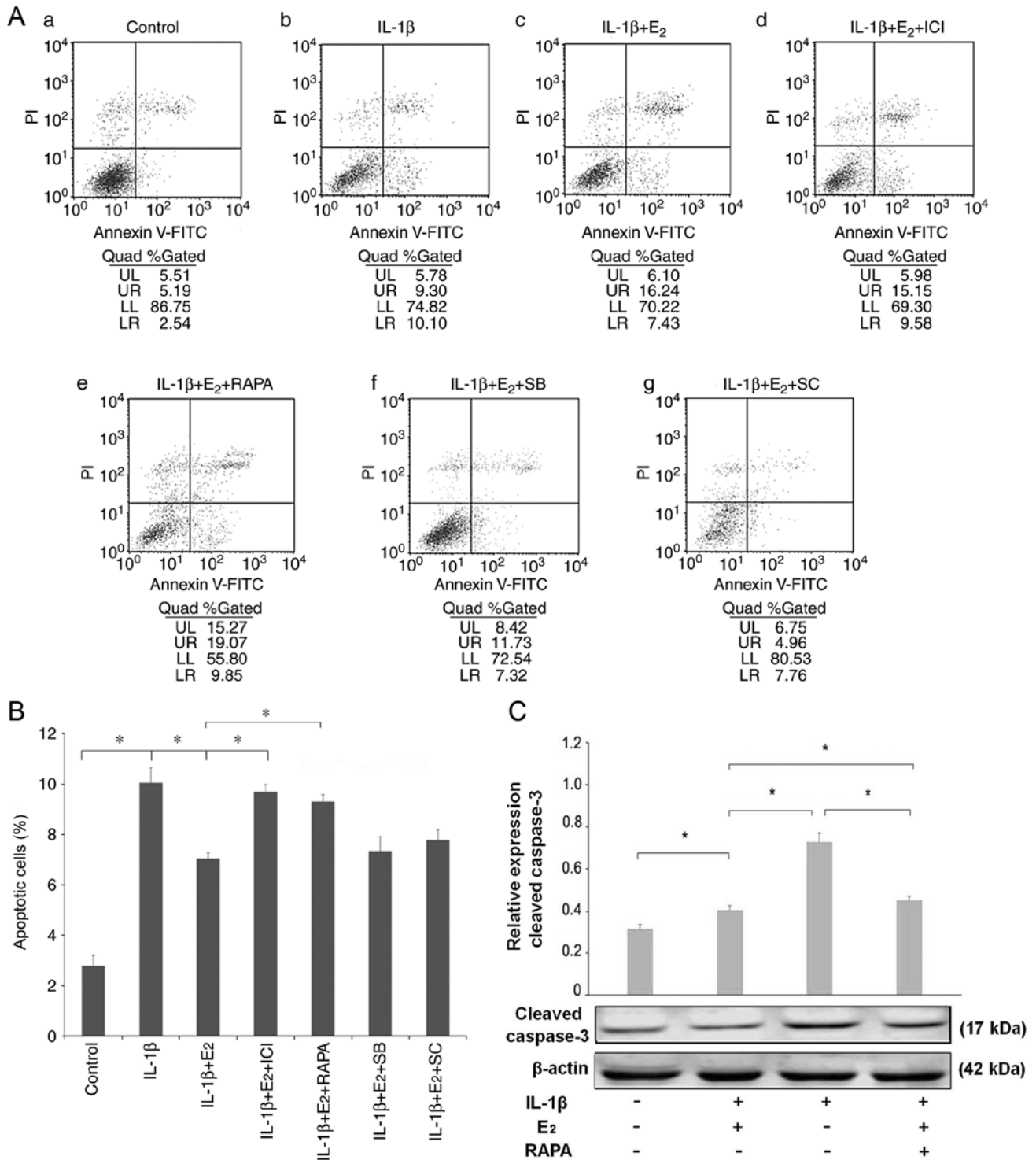


Figure 1. Fluorescence-activated cell sorting analysis of apoptosis. (A) Apoptotic nucleus pulposus cells were detected using an Annexin V-FITC/PI kit. Apoptotic cells, which were stained positive for Annexin V-FITC and negative for PI, were counted. (a) Control, (b) IL-1 β , (c) IL-1 β + E₂, (d) IL-1 β + E₂ + ICI, (e) IL-1 β + E₂ + RAPA, (f) IL-1 β + E₂ + SB, (g) IL-1 β + E₂ + SC. (B) Data are presented as a percentage of the total cell count. Data analysis was determined by one-way analysis of variance accompanied by pairwise comparison using the SNK-q test. (C) Western blot analysis of cleaved caspase-3. Data are presented as the means \pm standard deviation, n=6. *P<0.05. E₂, 17 β -estradiol; ICI, ICI182780; IL-1 β , interleukin-1 β ; PI, propidium iodide; RAPA, rapamycin; SB, SB216763; SC, SC75741.

used as the internal reference protein. Rat NPCs were washed with ice-cold PBS and harvested in 100 μ l RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 1% protease inhibitor and 0.5% phosphorylase inhibitor (Beijing Solarbio Science & Technology Co., Ltd.). The bicinchoninic acid method was used to determine protein concentration. The lysates were centrifuged at 4°C for 15 min at 16,000 x g

and equal amounts of protein (20 μ g) were electrophoresed. Specifically, for the detection of mTOR and p-mTOR, proteins were separated by 10% SDS-PAGE, whereas for GSK-3 β , p-GSK-3 β , NF- κ B and cleaved caspase-3 detection, proteins were separated by 12% SDS-PAGE. Proteins were transferred by electroblotting to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in TBS [50 mmol/l Tris

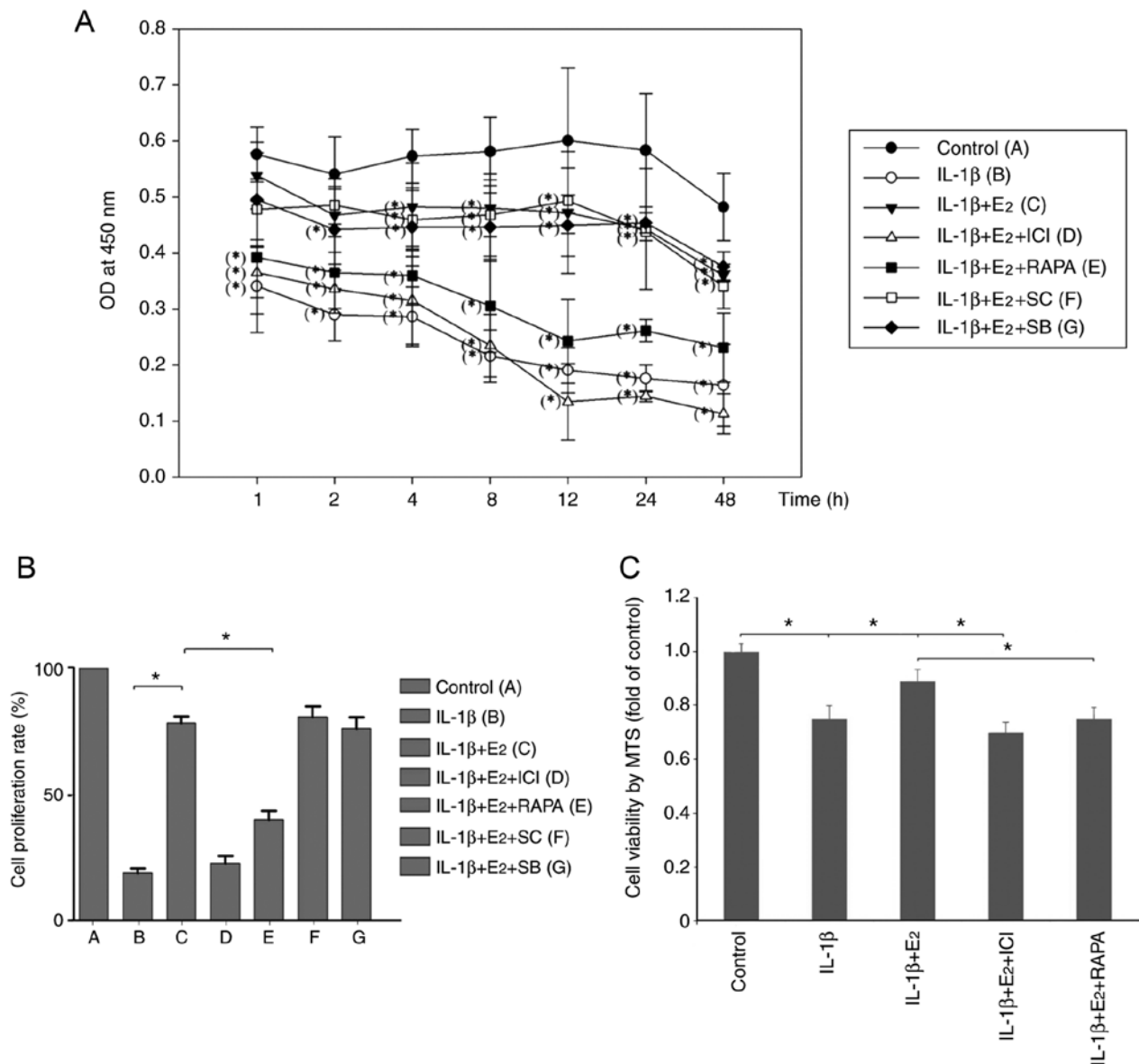


Figure 2. (A and B) CCK-8 and (C) MTS assays of cell proliferation and viability. (A) Optical density was measured after incubating the nucleus pulposus cells with different treatments for 1, 2, 4, 8, 12, 24 and 48 h. * $P < 0.05$ vs. the control at each point. (B) Cell proliferation and (C) cell viability at 12 h was normalized relative to the control. Data are presented as the means \pm standard deviation, $n = 6$. * $P < 0.05$. E₂, 17 β -estradiol; ICI, ICI182780; IL-1 β , interleukin-1 β ; RAPA, rapamycin; SB, SB216763; SC, SC75741.

(pH 7.6) and 150 mmol/l NaCl (0.1%)] and incubated overnight at 4°C in 5% non-fat dry milk in PBS-0.1% Tween (PBST) with the corresponding primary antibodies (1:1,000). After three washes in PBST for 30 min, the membranes were incubated with a secondary antibody (1:5,000) at room temperature for 2 h. Immunolabeling was detected using an enhanced chemiluminescence reagent. The relative intensity of each blot was assessed and analyzed using the AlphaImager 2200 software package (ProteinSimple), and the levels of target proteins were determined as (p-protein/control)/(total protein/control).

Statistical analysis. Statistical analyses were performed using SPSS for Windows, version 18.0 (SPSS, Inc.). All data are presented as the means \pm standard deviation of independent experiments ($n = 6$). If the data satisfied the criteria for normality and homogeneity of variance, statistical analysis among multiple groups was performed using one-way analysis

of variance, which was accompanied by pairwise comparison using the SNK-q test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Apoptosis assay. Rat NPCs were divided into seven groups as follows: Control (treated with an equivalent volume of PBS), IL-1 β , IL-1 β + E₂, IL-1 β + E₂ + ICI182780, IL-1 β + E₂ + rapamycin, IL-1 β + E₂ + SC75741 and IL-1 β + E₂ + SB216763. FACS analysis was then used to determine the number of apoptotic cells. As shown in Fig. 1A and B, the percentage of apoptotic cells increased following treatment with IL-1 β alone (10.10%). Cells pretreated with E₂ (IL-1 β + E₂ group) exhibited a reduced rate of apoptosis (7.43%). To further elucidate the potential contribution of mTOR, the inhibitor rapamycin was used. When cells were incubated with E₂, IL-1 β and

rapamycin, the protective effects of E_2 were reduced (9.85%); a similar result was revealed for cells pretreated with ICI182780, an estrogen receptor (ER) antagonist. Statistical analysis demonstrated that pretreatment with rapamycin promoted apoptosis induced by IL-1 β , which was confirmed by western blot analysis of cleaved caspase-3 (Fig. 1C).

Cell viability and proliferation. CCK-8 and MTS assays were used to measure cell proliferation and viability, respectively. As shown in Fig. 2A, compared with the control group at each point, the group treated with IL-1 β alone had a lower optical density (OD) value at 1-48 h ($P<0.05$), and the OD value in the IL-1 β + E_2 group was significantly decreased at 4-48 h ($P<0.05$). In the rapamycin group, the OD value was significantly reduced at 1-48 h compared with the control group ($P<0.05$). At 12 h, the proliferation rate of NPCs in the IL-1 β + E_2 group was significantly greater than in the IL-1 β and IL-1 β + E_2 + RAPA groups ($P<0.05$; Fig. 2B). Furthermore, the viability of cells in the IL-1 β + E_2 group was significantly increased compared with in the IL-1 β + E_2 + RAPA group ($P<0.05$; Fig. 2C).

Cellular binding. As shown in Fig. 3, IL-1 β resulted in a decrease of ~30% in cell binding ability compared with in the control group ($P<0.05$). The cytotoxic effects of IL-1 β were partly abolished by the addition of E_2 ; however, this was reversed by the use of ICI182780 or RAPA ($P<0.05$).

Western blot analysis. Rat NPCs were treated as aforementioned. As shown in Fig. 4, IL-1 β significantly decreased the expression ratio of p-mTOR/mTOR, which was reversed by E_2 ($P<0.05$). However, treatment with the ER antagonist (ICI182780) or the mTOR signal pathway inhibitor (rapamycin) decreased the expression ratio of p-mTOR/mTOR compared with in the IL-1 β + E_2 group ($P<0.05$; Fig. 4A). As shown in Fig. 4B, E_2 reversed IL-1 β -induced expression of p-GSK3 β /GSK3 β ($P<0.05$); however, ICI182780 did not reverse the effects of E_2 on p-GSK3 β /GSK3 β . Furthermore, no significant difference in p-NF- κ B/NF- κ B was observed between the control and IL-1 β groups ($P>0.05$; Fig. 4C).

Discussion

In a previous study, E_2 was reported to protect IVD cells against aberrant apoptosis caused by IL-1 β cytotoxicity, which was attributed to upregulation of integrin α 2 β 1 (13). Upon ligand binding, integrins act not only as structural links between the ECM and the actin cytoskeleton, but also as sites of signal transduction from the ECM to intracellular signaling pathways. PI3K and Akt constitute an important signaling pathway of integrin, which has been reported to serve a central role in the effect of estrogen against IL-1 β -induced apoptosis of NPCs (19-21). However, the downstream signaling pathway remains unclear. The results of the present study confirmed that E_2 inhibited IL-1 β -induced apoptosis of NPCs via mTOR, the downstream signaling pathway of PI3K/Akt.

In our preliminary experiments, the NP of female rats was significantly smaller than those of male rats; therefore, separation of the NP from female rat annulus fibrosus was difficult, and a large number of annulus fibrosus cells were able to

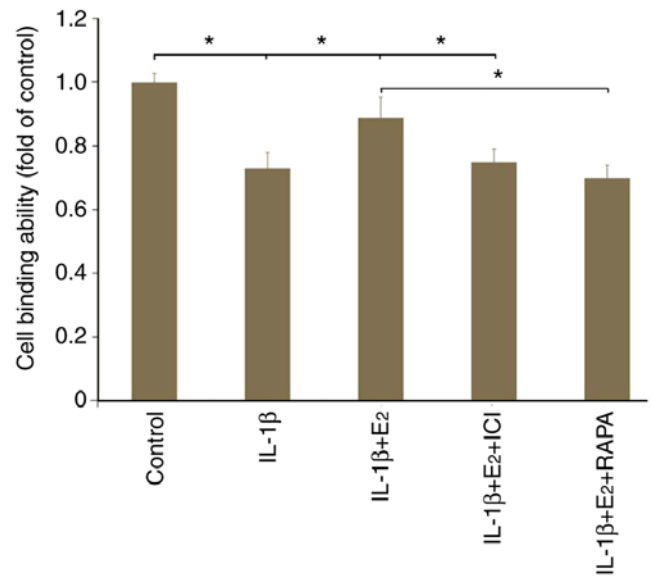


Figure 3. Ability of nucleus pulposus cells to bind to type II collagen. Data are presented as the means \pm standard deviation, $n=6$. * $P<0.05$. E_2 , 17 β -estradiol; ICI, ICI182780; IL-1 β , interleukin-1 β ; RAPA, rapamycin.

mix into NPCs during the subsequent cell culture process. Therefore, male rats were used for tissue isolation and cell culture in the present study, in accordance with our previous report (14). In the present study, a CCK-8 assay confirmed that the proliferation of NPCs was suppressed by the mTOR antagonist rapamycin compared with that in the IL-1 β + E_2 group. Cell binding assay, western blotting and FACS analysis confirmed that E_2 antagonized IL-1 β -induced NPC apoptosis, which in accordance with a previous study (19).

It has long been recognized that there is a continuous increase in cell death during human IVDD. Notably, abnormal apoptosis is considered the main cellular process associated with IVDD (4,27). The Akt signaling pathway is known to have a central role in cell survival, and a role for Akt in the regulation of growth, survival and inhibition of apoptosis of NPCs is well established (28-30). mTOR is downstream of PI3K/Akt and acts as a major sensor of cell growth. This serine/threonine protein kinase is involved in protein translation, proliferation and the anti-apoptosis response (31,32). It was therefore hypothesized that mTOR may participate in PI3K/Akt-induced inhibition of NPC apoptosis. In present study, treatment with the mTOR inhibitor, rapamycin, effectively decreased cellular adhesion and p-mTOR/mTOR expression, and increased IL-1 β -induced NPC apoptosis, thus indicating that mTOR is a downstream protein of the PI3K signaling pathway that may regulate cell apoptosis and adhesion.

In the present study, E_2 pretreatment resulted in an increase in the phosphorylation ratio of mTOR, whereas without this pretreatment, IL-1 β inhibited activation of mTOR. A previous study (33) suggested that estrogen can serve an anti-apoptotic role by uniting with the ER. ICI182780 is an ER antagonist that inhibits the binding of ER to target estrogen response elements and abolishes transcriptional activity in cells. Rapamycin is an inhibitor of mTOR. Both ICI182780 and rapamycin significantly downregulated the p-mTOR/mTOR expression ratio compared with in the IL-1 β + E_2 group, which suggested that

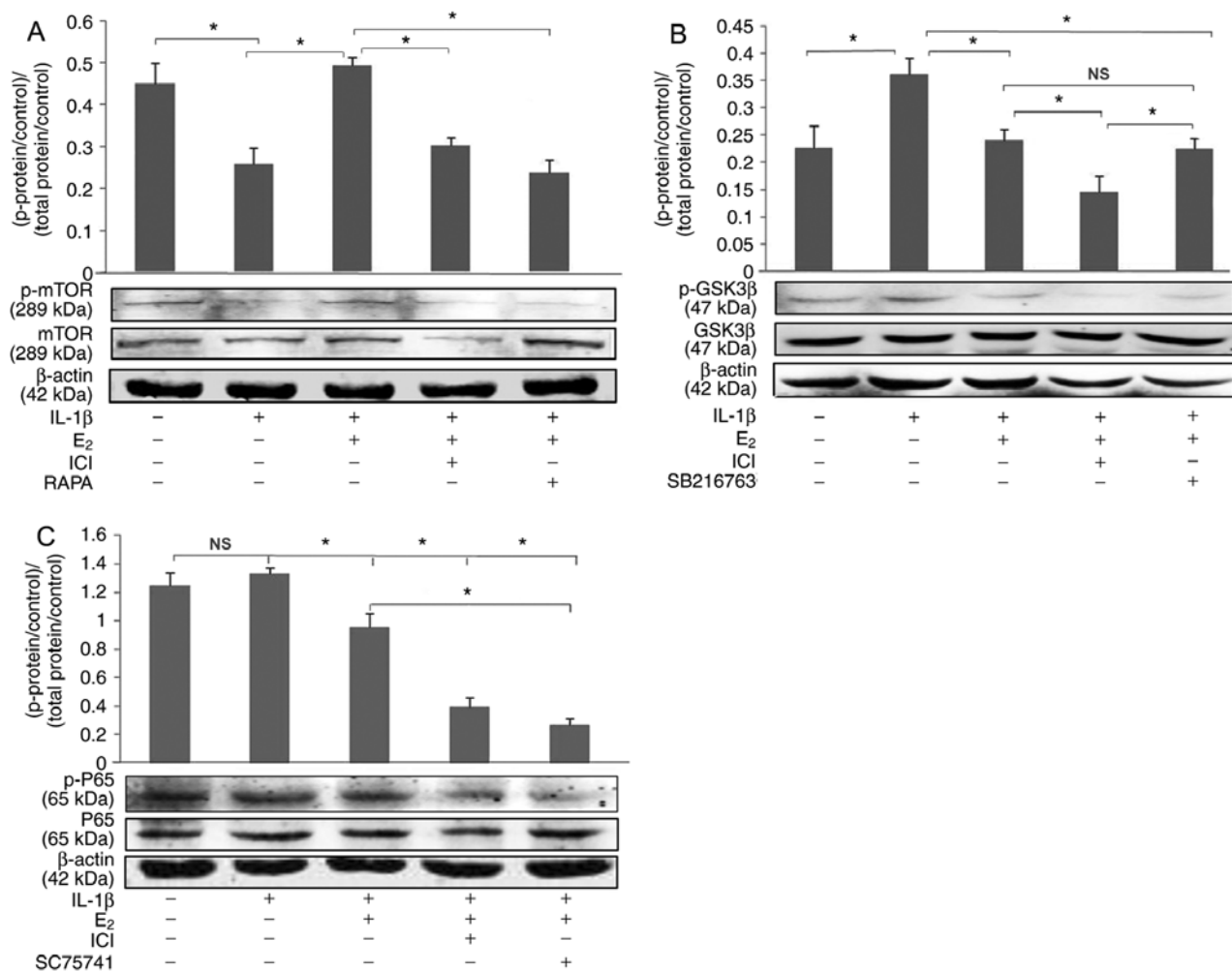


Figure 4. Western blot analysis of (A) mTOR, (B) GSK-3 β and (C) p65, and their phosphorylation levels in nucleus pulposus cells. * $P < 0.05$. NS, no statistical; E₂, 17 β -estradiol; GSK-3 β glycogen synthase kinase-3 β ; ICI, ICI182780; IL-1 β , interleukin-1 β ; p-, phosphorylated; RAPA, rapamycin.

activation of the mTOR pathway in NPCs may have a role in E₂-induced suppression of IL-1 β cytotoxicity. It was hypothesized that activation of the Akt/mTOR pathway may be involved in the protection against apoptosis in rat NPCs. This hypothesis is consistent with the previously described effects in other cell types (24,34,35).

Previous reports have revealed that the mTOR pathway serves an important role in osteoclast and microglia cell apoptosis (36-38). However, to the best of our knowledge, no studies have reported whether mTOR participates in the effects of E₂ against IL-1 β -induced apoptosis of NPCs. mTOR controls apoptotic cell death through eukaryotic translation initiation factor 4E-binding protein 1 and p70S6K. Activation of p70S6K by mTOR blocks apoptosis through pathways that can increase expression of the anti-apoptotic proteins Bcl-2/Bcl-xL and inactivate the pro-apoptotic protein BAD (39). Bcl-2 blocks the release of cytochrome *c* through the mitochondrial outer membrane and inhibits cytochrome *c*-mediated caspase activation (40). Cleaved caspase-3 is associated with the initiation of apoptosis via the mitochondrial (intrinsic) pathway. In this study, activated caspase-3 was suppressed by E₂, which was reversed by rapamycin, as measured by western blotting, thus indicating that mTOR may participate in E₂-induced inhibition of NPC apoptosis.

GSK-3 β and NF- κ B are also key proteins in the pathways downstream of PI3K/Akt. In the present study, FACS results exhibited no significant differences in the apoptotic ratio of NPCs in the IL-1 β + E₂ group treated with or without GSK-3 β and NF- κ B inhibitors. The GSK-3 β signaling pathway serves an important role in inducing cellular apoptosis via mediating mitochondrial functions, which can activate caspase-8 and caspase-2, induce the cleavage of Bid and release cytochrome *c*, thus leading to apoptosis and mitochondrial dysfunction (41,42). The expression ratio of p-GSK-3 β /GSK-3 β was significantly increased in the IL-1 β group, as determined by western blot analysis. Therefore, this study concluded that the GSK-3 β signaling pathway may be involved in the apoptosis of NPCs induced by IL-1 β . Furthermore, NF- κ B regulates the expression of >150 genes involved in inflammation, cell proliferation, differentiation and survival. In the current study, the NF- κ B inhibitor, SC75741, did not decrease NPC apoptosis compared with in the IL-1 β + E₂ group, indicating that NF- κ B did not serve a role in E₂ and IL-1 β -regulated NPC apoptosis.

In conclusion, the present study suggested that the PI3K/Akt/mTOR/caspase-3 signaling pathway may be involved in protection against IL-1 β -induced NPC apoptosis. These findings may provide a novel therapeutic target for the prevention and treatment of IVD degenerative diseases.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

WD conceived and designed the experiments. HG conducted the experiments. FZ and SL acquired the data and provided reagents. SY analyzed the data. HG wrote the manuscript. DY and LM contributed to interpretation of the data and critical revision of the manuscript. HW contributed to interpretation of the data and revision of the manuscript, particularly regarding the FACS section. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocols were approved by the Institutional Animal Care and Use Committee of The Third Hospital of Hebei Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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