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The crosstalk between IGF-1R and ER-α in the proliferation and anti-inflammation of nucleus pulposus cells

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Abstract. – OBJECTIVE: Insulin-like growth factors-1 receptor (IGF-1R) and estrogen receptor (ER) are reported to co-express and engage in crosstalk involving in the synergistic effect of various aspects. It is unknown whether this crosstalk exists in the nucleus pulposus (NP) cells. We aimed to investigate the interaction between IGF-1R and ER-α in regulating NP cell proliferation and inflammation response under IGF-1 stimulation.

PATIENTS AND METHODS: We analyzed the IGF-1, IGF-1R, and ER-α in different degenerated degree human NP tissues. NP cells were cultured with IGF-1 protein with or without the inhibitor of IGF-1R or ER-α to investigate their effects on the proliferation and inflammation response. In addition, we also upregulated the IFG-1R and ER-α expression by plasmid transfection to investigate the impact on each other. The content of IGF-1, IFG-1R, and ER-α was analyzed by enzyme-linked immunosorbent assay (ELISA). The proliferative cell rate was determined by flow cytometry. Additionally, intracellular collagen-II, p16, PCNA, IL-1β, IL-6, TNF-α, and MMP-13 expression were also detected.

RESULTS: We found IGF-1, IFG-1R, and ER-α content were decreased in higher degenerated NP tissues. IGF-1 protein treatment upregulated the IFG-1R and ER-α expression and promoted NP cell proliferation, collagen-II, and PCNA expression. However, the suppression of IGF-1R (or ER-α) weakened the IGF-1 induced collagen-II expression, proliferation, and anti-inflammation effects on NP cells, decreased ER-α (or IGF-1R) expression, and partly reversed the protective effect of NP cells caused by IGF-1 Similarly, the upregulation of one of IGF-1R and ER-α may increase the other as well.

CONCLUSIONS: There is an interaction between IGF-1R and ER-α acts synergistically to promote the proliferation and suppress inflammation in NP cells.

Key Words:

Nucleus pulposus cells, IGF-1R, ER-α, Proliferation, Anti-inflammation.

Introduction

Intervertebral discs play an extremely important role in spinal bearing and motor function. Intervertebral disc degeneration (IDD) has a higher incidence in adults. During IDD, the nucleus pulposus (NP) is dehydrated, with a weakened ability to synthesize proteoglycans, the changed arrangement of collagen type, and severely damaged cell functions[1]. The mechanism underlying IDD is very complicated. The traditional view is that IDD is related to injuries caused by excessive physical load. At present, many studies have shown that senescence and apoptosis of NP cells contribute to the progress of IDD[2]. Early prevention or reversal of apoptosis and promotion of proliferation of NP cells are issues of interest to many researchers. The study of the relationship between growth factors and disc regeneration will show a good prospect for the treatment of IDD.

Various growth factors can regulate the degeneration of intervertebral discs[3]. Insulin-like growth factors (IGFs) are considered to be one of the essential cytokines associated with degenerative disc disease, closely relating to the promotion in the proliferation of NP cells. IGFs are a class of peptides that participate in several physiological activities, like cell proliferation, growth, and differentiation[4]. The IGFs system includes two polypeptide factors containing IGF-1 and IGF-2. Most of the cellular effects of IGFs are achieved through the IGF-1R. IGF-I is a low-molecular-weight, anabolic, growth-promoting, insulin-like peptide that binds and activates its receptor IGF-1R[5]. It plays a role in maintaining the NP cell phenotype, promoting the synthesis of ECM and cell proliferation, and anti-inflammatory effects[6,7]. Due to the fact that cells can respond to multiple signaling molecules at the same time,

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one of the new research hotspots is to test the response of cells to various receptors, rather than studying a single ligand and receptor response alone. IGF-1R is the critical role-playing inducer in regulating the metabolic function of IGF-1. Additionally, IGF-1R is also reported to play the biological role *via* the crosstalk with estrogen receptor-α (ER-α) in wound repair process[8], breast cancer[9], nervous system[10], and so on. ER-α has shown a protective effect on IDD by mediating inflammation, proliferation, cell cycle, apoptosis, and ECM gene expression[11,12]. However, whether the interaction among IGF-1R and ER exists in the NP cells remains unknown.

In our study, we first tested the different content of IGF-1, IGF-1R, and ER-α of human NP tissue in different degenerated degrees. To explore the relation between IGF-1R and ER-α, we treated human NP cells with exogenic IGF-1 to activate them expression, combining with the inhibitor of IGF-1R and ER-α, respectively, and compared the change of IGF-1R and ER-α expression. Apart from this, we also transfected NP cells with plasmid targeting IGF-1R or ER-α to understand the effect on the expression to the other. We aimed to clarify the interaction of IGF-1R and ER-α in NP cells, providing a reference for the strategy of IDD treatment.

Patients and Methods

NP Cell Isolation and Culture

To compare the gene expression in human NP tissue of different degenerated degrees. We used the Pfirrmann classification score to value the disc degenerated status, according to magnetic resonance imaging (MRI). We chose fifteen NP tissues (five grade 3, five grade 4, five grade 5) from the patients undergoing lumbar fusion operations resulting from lumbar disc herniation. This retrospective study was registered by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

Disc samples were cut into small fragments and washed with phosphate-buffered saline (PBS) to get rid of excessive blood. The fragments were then digested with 0.2% collagenase II and 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) overnight in the incubator. We got the NP cell pellets after filtration. The NP cells were seeded in 6-well plates with Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium (Gibco, Rockville, MD, USA), containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) plus 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). NP cells were treated with recombinant human IGF-1 protein (500 ng/mL, ab9573, Abcam, Cambridge, MA, USA) for 72 h. In addition, the IGF-1R inhibitor Picropodophyllotoxin (PPP, 5 nM, ab144623, Abcam, Cambridge, MA, USA) or selective ER-α inhibitor MPP dihydrochloride (5 nM, CAS289726-02-9, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 72 h. NP cells were also co-cultured with 500 ng/mL IGF1 with 5 nM MPP or PPP for 72 h.

Hematoxylin and Eosin (HE) Staining

NP specimens were fixed with 4% formaldehyde for 24 h, dehydrated with gradient alcohol, embedded in paraffin, and sliced at 5 μm. Sections were dewaxed and hydrated, hematoxylin staining for 5 min, acetic acid differentiation for 1 min, eosin staining for 1 min, xylene transparent, and neutral gum seals.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IGF-1, IGF-1R, and ER-α in both NP tissue and NP cells were measured using human IGF-1 ELISA Kit (LS-F36954); human IGF-1R ELISA Kit (LS-F7034); human ER-α ELISA Kit (LS-F23792) purchased from LifeSpan BioSciences (Seattle, WA, USA) according to the manufacturer's instructions.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by TRIzol reagent (Solarbio, Shanghai, China). Then, we reverse-transcribed the mRNA into complementary deoxyribose nucleic acid (cDNA) by a reverse transcription kit (Solarbio, Shanghai, China) according to the manufacturer's instructions. The gene expression of IL-1β, p16, collagen-II, proliferating cell nuclear antigen (PCNA), IL-6, TNF-α, and MMP-13 were determined by RT-PCR analysis using SYBR Green Master (Invitrogen, Carlsbad, CA, USA). The method of $2^{-\Delta\Delta Ct}$ was used to calculate the mRNA expression by normalization to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for RT-PCR are listed in Table I.

Immunofluorescence (IF)

NP cell was fixed by 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton

Table I. Primer of the genes for RT-PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
IL-β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
p16	GATCCAGGTGGGTAGAAGGTC	CCCCTGCAAACTTCGTCCT
PCNA	CCTGCTGGGATATTAGCTCCA	CCTGCTGGGATATTAGCTCCA
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

X-100 for 15 minutes, and then blocked with 5% serum for 30 min at room temperature. After that, NP cells were washed and incubated with collagen-II primary antibodies (Cell Signaling, Danvers, MA, USA) at 4°C overnight. Then, cells were incubated with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h in the dark and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for the nucleus.

Flow Cytometry

The proliferative NP cell rate was detected by flow cytometry using 5-Ethynyl-2'-deoxyuridine (EdU) Flow Cytometry Assay Kits (Invitrogen, Carlsbad, CA, USA). NP cells were prepared in phosphate- buffered saline (PBS) and treated with specialized reagent according to the manufacturer's instructions. After incubation for 30 min at 37°C, cell pellets were washing and analyzed by flow cytometry.

Plasmid Transfection

NP cells were transfected by plasmid (human tagged ORF clone) targeting IGF-1R (NM_000875), ER-α (NM_000125) or Untagged Clone (null, AK098156) purchased from OriGene Technologies (Rockville, MD, USA) using TurboFectin regent (TF81001, OriGene Technologies, Rockville, MD, USA). Transfection efficacies were measured *via* ELISA.

Statistical Analysis

Data analysis was performed by Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) and presented as Mean ± standard deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. A comparison between multiple groups was made using One-way ANOVA test followed by Post-Hoc analysis (Least Significant Difference). p-value < 0.05 was regarded as statistically significant.

Results

IGF-1, IGF-1R, and ER-α Decreased in Degenerated Human NP Tissues

To determine the content of IGF-1, IGF-1R, and ER-α in the different degenerated NP tissues, we used the human NP tissue from Pfirrmann grade 3 to grade 5. From the HE staining, the texture of grade 3 was bright and regular, and the texture of grade 4 or 5 was much chaotic and irregular compared to grade 3. Besides, the size of the NP cells became much bigger and hypertrophic in a higher degenerated condition (Figure 1A). The IGF-1, IGF-1R, and ER-α levels all decreased with the IDD development (Figure 1B). As expected, the inflammatory effector IL-1β was increased in grade 5 compared to grade 4 and grade 3. Additionally, the mRNA level of the senescent marker p16 was raised in a higher degenerated disc. On the contrary, collagen-II was decreased with the disc degeneration (Figure 1C). As other disc cells study reported[7,13], we concluded that IGF-1, IGF-1R, and ER-α significantly decreased along with the disc degenerated.

Suppression of IGF-1R Decreased ER-α Expression in IGF-1 Treated NP Cells In Vitro

As previously described[14,15], IGF-1 stimuli can activate IGF-1R and ER-α expression. We treated NP cells with recombinant human IGF1 protein, which highly promoted the IGF-1 content of NP cells. Besides, we noticed an increased IGF-1R and ER-α expression after IGF-1 stimulation. However, the IGF-1R inhibitor PPP significantly suppressed the IGF-1R expression compared to

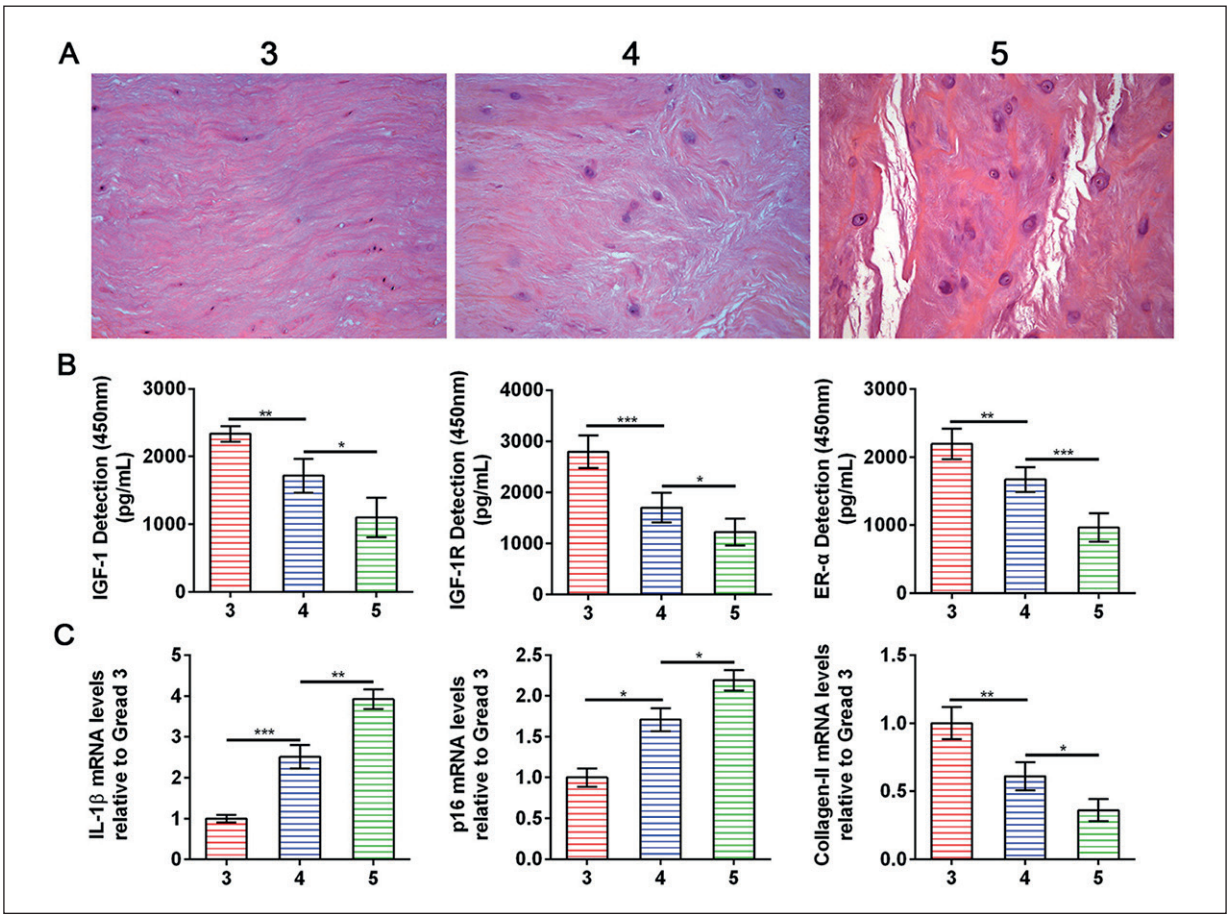

Figure 1. IGF-1, IGF-1R, and ER-α expression in different degenerated degree disc tissue. **A,** Representative HE staining of NP tissue from Pfirrmann grade 3 to 5 (magnification: 400×). **B,** The content of IGF-1, IGF-1R, and ER-α was determined by ELISA. **C,** The mRNA expression levels of IL-1β, p16, and collagen-II were assayed by RT-PCR. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

the control, even if under the presence of IGF-1 treatment. Hopefully, we found the decreased IGF-1R also led to the reduction of ER-α level, though not as drastic as the IGF-1R (Figure 2A). Furthermore, the collagen-II expression was increased, resulting from the IGF-1 treatment and was decreased by PPP. After adding PPP, the data suggested decreased IGF-1R weakened the upregulated collagen-II caused by IGF-1 (Figure 2B, C). As a growth promotor, IGF-1 increased the proliferative NP cell rate compared to the control. The decreased IGF-1R expression affected proliferative NP cells with or without IGF-1 treatment (Figure 2D). Apart from this, the mRNA expression of PCNA was suppressed by the inhibition of IGF-1R. On the contrary, the mRNA levels of p16, IL-1β, IL-6, TNF-α, and MMP-13 were increased because of the decreased IGF-1R expression (Figure 2E). The data suggested that IGF-1R played a vital role in the IGF-1 induced proliferation, and the anti-inflammation response of NP cells, and the content of IGF-1R affected the ER-α expression.

Suppression of ER-α Decreased IGF-1R Expression in IGF-1 Treated NP Cells In Vitro

As mentioned above, IGF-1R suppression led to a reduction of ER-α. We further wondered whether the suppression of ER-α would affect IGF-1R expression. In addition to PPP, MPP, the inhibitor of ER-α, was applied to the NP culture and with or without the presence of IGF-1. MPP significantly suppressed the ER-α level even under the condition of IGF-1 treatment. As PPP, MPP did not affect the expression of IFG-1. However, it downregulated the IGF-1R expression compared to the control. Under the activation of IGF-1, the

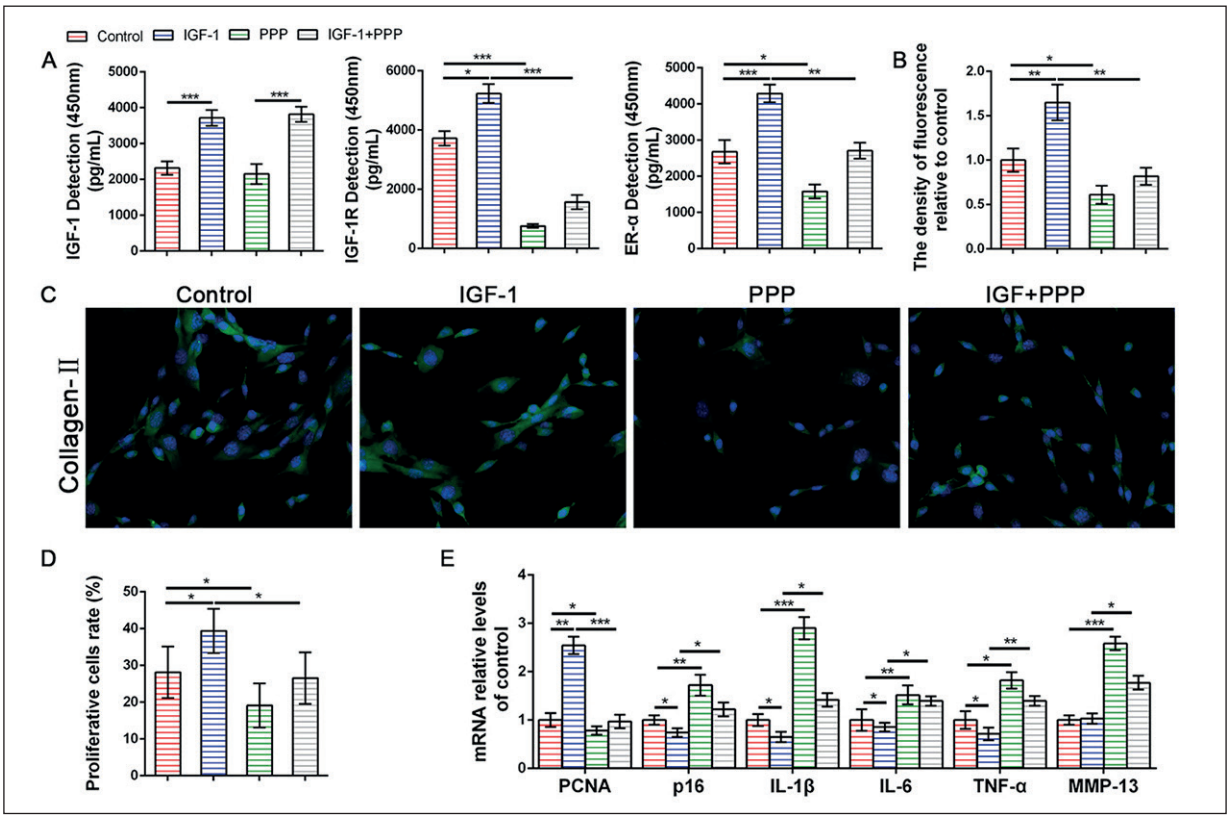

Figure 2. Suppression of IGF-1R led to a decreased ER-α expression under IGF-1 stimulation. NP cells were treated with 500 ng/mL IGF-1 or 5 nM PPP for 72 h. Besides, NP cells were also co-cultured with 500 ng/mL IGF-1 and 5 nM PPP for 72 h. **A,** The content of IGF-1, IGF-1R, and ER-α was determined by ELISA. **B, C,** IF staining of collagen-II (magnification: 400×), and quantification analysis. **D,** Proliferative cells rate was determined by flow cytometry. **E,** The mRNA expression levels of PCNA, p16, IL-1β, IL-6, TNF-α, and MMP-13 were assayed by RT-PCR. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

content of IGF-1R was also suppressed, causing the MPP treatment (Figure 3A). The expression of collagen-II was suppressed due to the decreased ER-α level, which suggested a weak synthetic status compared to the control (Figure 3B and 3C). ER-*α* is an important regulator of proliferation in various cell types, especially in cancer[16,17]. In our study, the decreased ER-α significantly reduced the population of proliferated NP cells. Though the IGF-1 stimulation promoted cell proliferation, it could be partly rejected by the ER-α inhibition (Figure 3D). The mRNA level of PCNA was also reduced caused by the MPP treatment, but the tendency of p16 expression was opposite to ER-α (Figure 3E). Additionally, ER-α also shows good anti-inflammatory ability in many aspects[18,19]. We found the suppression of ER-α triggered the IL-1β, IL-6, TNF-α, and MMP-13 expression in mRNA level, especially affected IL-1β and MMP-13 expression and weakened the anti-inflammatory effect of IGF-1 (Figure 3E). These results indicated that the suppression of ER-α affected IGF-1R expression and played a negative role in the proliferation and anti-inflammatory process of NP cells metabolism.

The Interaction of IGF-1R and ER-α in the NP Cells In Vitro

The suppression of IGF-1R had an inhibiting effect on ER-α; similarly, decreased ER-α also led to a reduction of IGF-1R expression. We further explored whether the upregulation of one would raise another expression. As shown in Figure 4A, the content of IGF-1 did not change after the transfection of IGF-1R or ER-α plasmid. However, we noticed the crosstalk between IGF-1R and ER-α expression, which was the upregulated IGF-1R, surprisingly made an overexpression of ER-α and vice versa. Both the upregulation of IGF-1R and ER-α could promote the synthesis of collagen-II and proliferative cell rates compared to the control (Figure 4B, 4C, 4D). Finally, RT-

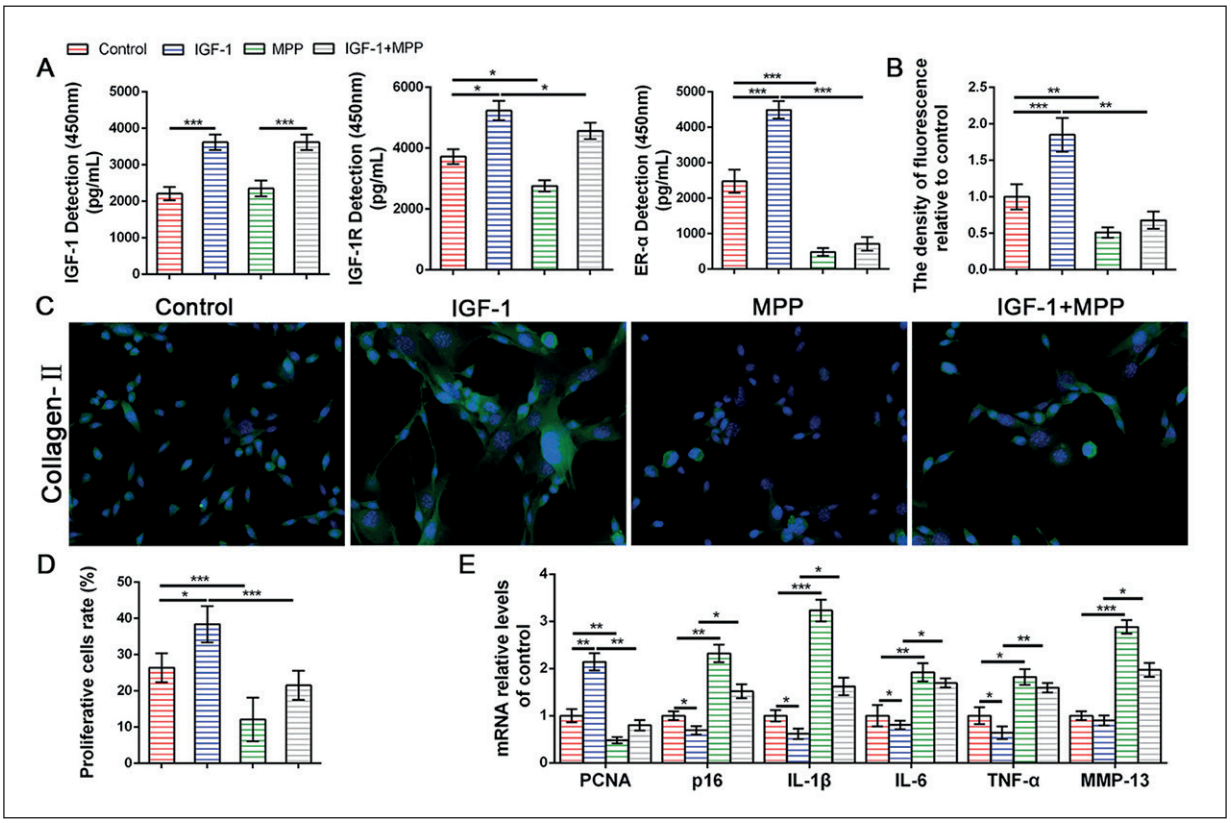

Figure 3. Suppression of ER-αled to a decreased IGF-1R expression under IGF-1 stimulation. NP cells were treated with 500 ng/mL IGF-1 or 5 nM MPP for 72 h. Besides, NP cells were also co-cultured with 500 ng/mL IGF-1 and 5 nM MPP for 72 h. **A,** The content of IGF-1, IGF-1R, and ER-α was determined by ELISA. **B, C,** IF staining of collagen-II (magnification: 400×), and quantification analysis. **D,** Proliferative cells rate was determined by flow cytometry. **E,** The mRNA expression levels of PCNA, p16, IL-1β, IL-6, TNF-α, and MMP-13 were assayed by RT-PCR. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

PCR was also performed, and the result indicated IGF-1R and ER-α promoted PCNA expression but suppressed p16, IL-1β, IL-6, TNF-α, and MMP-13 expression (Figure 4E). Therefore, IGF-1R and ER-α were involved in the regulation of proliferation and inflammation response in NP cells, and increased expression of one of them will also cause an increase of the other.

Discussion

During IDD, it is normal to detect the increased inflammatory reaction, degraded number and function of NP cells, and the changed type and arrangement of collagen in the ECM[20]. There is no doubt that the promotion of the proliferation and regeneration of NP cells can prevent or reverse the early disc degeneration. Several growth factors have been verified to modulate the proliferation and matrix synthesis of intervertebral disc cells, and different growth factors can act on different disc areas or cells[21]. Therefore, many researchers focus their interest on the study of the growth factors, such as IGF-1[22], Neurotrophins[23], and transforming growth factor-β (TGF-β) super family[24] in the tissue engineering of IDD. IGF-1 is a well-known agent for intervertebral disc (IVD), promoting both cell proliferation and ECM biosynthesis. Furthermore, the exogenous IGF-1 protein stimulation has been applied for the IDD treatment in many therapeutic strategies. Current studies have shown that IGF-1 promoting cell proliferation and growth is mainly regulated by IGF-1R, and the inhibited IGF-1 and IGF-1R level are strictly related to IDD[7]. At the beginning of our research, we verified that IDD significantly decreased the IGF-1 and its receptor expression.

Estrogen is a steroid hormone and has the characteristics of wide distribution and diverse

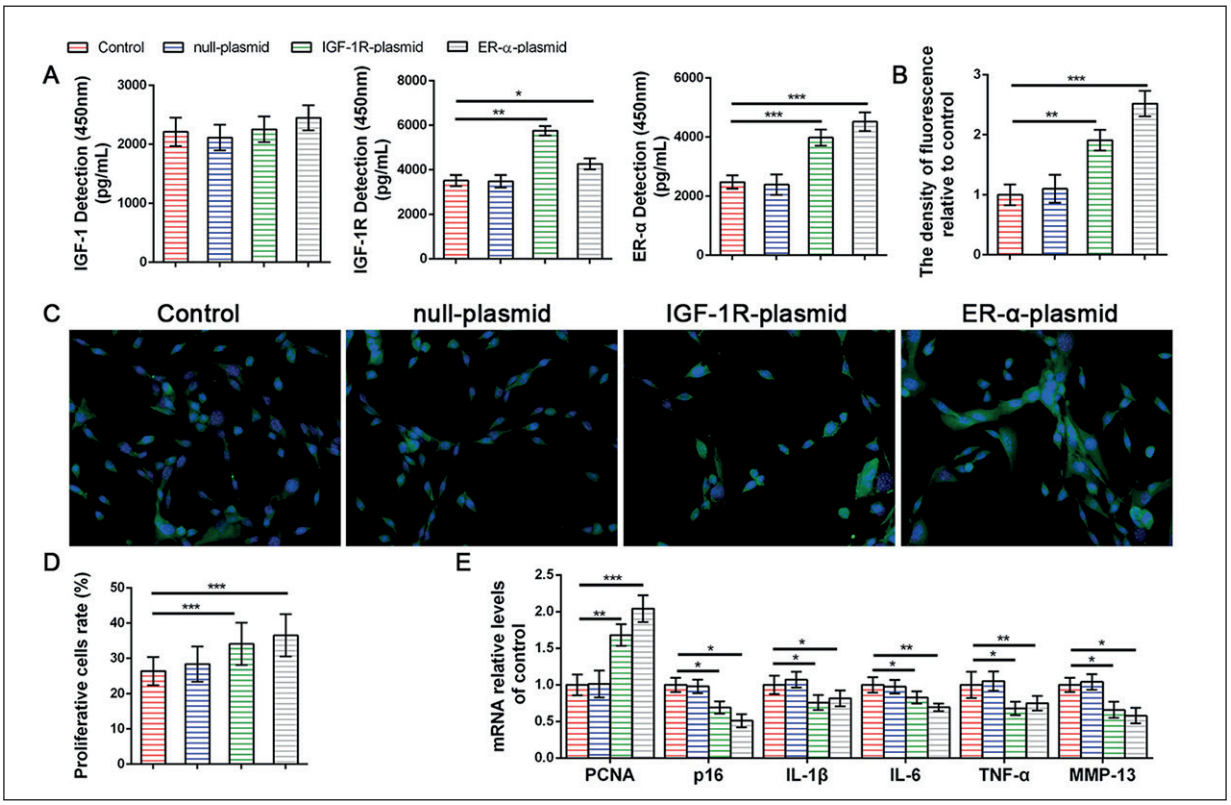

Figure 4. Upregulation of IGF-1R led to ER-α overexpression, and the upregulation of ER-α led to IGF-1R overexpression. NP cells were transfected with null plasmid or IFG-1R-plasmid or ER-α-plasmid. **A,** The content of IGF-1, IGF-1R, and ER-α was determined by ELISA. **B, C,** IF staining of collagen-II (magnification: 400×), and quantification analysis. **D,** Proliferative cells rate was determined by flow cytometry. **E,** The mRNA expression levels of PCNA, p16, IL-1β, IL-6, TNF-α, and MMP-13 were assayed by RT-PCR. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

regulatory functions in target tissues, which are mainly mediated by two classical nuclear receptors ER-α and ER-β. Estrogen regulates the transcription process through ER, including nuclear transcription, combining special effector elements and target genes[25]. In our study, we found IGF-1 promoted the NP cell proliferation in both IGF-1R and ER-α dependent manners and the involvement of ER-α in the process of IDD is an indirect effect through IGF-1R. Therefore, we suspected there might exist a crosstalk underlying IGF-1R and ER-α in NP cells as reported previously. IGF-1 affects the binding of IGF-1R and ER-α, and ER-α can participate in the IGF-1-based IGF-1 signaling pathway as well. Zhou et al[26] elucidated that exogenous IGF-1 and its receptor IGF-1R promote prostatic smooth muscle cell proliferation based on the existence of ER-**α.** They mentioned ER-α causes the upregulation of IGF-1R, otherwise, IGF-1 is indispensable to mediate ER-α. Additionally, Topalli and Etgen[27] found IGF-1 induces IGF-1R and ER-α expression and the receptor interaction between IGF-1R and ER-α is essential in neurite formation and extension of PC12 cells. Apart from this, the suppression of the communication between IGF-1R and ER-α has been verified to prevent the cancer process. The prevention of the crosstalk between IGF-1R and ER-α availably rejected the proliferation of BG-1 ovarian cancer cell[28]. Therefore, the collaboration between them is vitally important for cell proliferation. The positive dose correlation between IGF-1R and ER-α also exists in the NP cells.

IGF-1R and ER-α are vital mediators of IGFs and estrogen. IGF-1 and estrogen both have the anti-inflammation function[29,30]; beyond that, the interaction of them is also noticed in the modulation of an inflammatory response in many diseases such as neurodegeneration[31], Alzheimer's disease[32], and breast cancer[33]. However, whether the interaction between IGF-1R and ER-α is involved in the inflammation response of NP cells remaining unknown. We found the upregulation

of IGF-1R or ER-α independently made a significant reduction of inflammatory factors, as well as a correlated trigger to the other. There may be a context-dependent mode in the anti-inflammatory function between IGF-1R and ER-α in the IDD, which needs to be further verified.

In our researches, the introduction of ER-α in NP cells can increase the level of IGF-1R with or without stimulation compared with the negative control, accompanying with increased cell proliferation and reduced inflammation. Due to the crosstalk between ER-α and IGF-1R, NP cells also showed higher cell growth levels and anti-inflammatory capabilities after transfection with IGF-1R. It is reported that the possible mechanism underlying is that the Ras-MAPK cascade activated ER-α, leading to the quickly binding of ER-α to IGF-1R and inducing the ERK1/2 phosphorylation.

Conclusions

For the first time we recovered the IGF-1 signaling can cross-link with the ER-α pathway and resulting in a feedback amplification effect in the NP cell's physiological activity, which provides a novel insight of the IDD treatments related with the synergy between ER-α and IGF-1R.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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