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Protective effect of estrogen receptors (ERα/β) against the intervertebral disc degeneration involves activating CCN5 *via* the promoter

M.-X. SONG[1], X.-X. MA[1], C. WANG[1], Y. WANG[1], C. SUN[1], D.-R. XU[1], K. ZHU[1], G.-H. LI[1], H. ZHAO[2], C. ZHANG[1]

[1]Department of Orthopedics, Affiliated Hospital of Qingdao University, Qingdao, China
[2]Department of Pathology, Affiliated Hospital of Qingdao University, Qingdao, China

Mengxiong Song and Xuexiao Ma contributed equally to this work

Abstract. – OBJECTIVE: Due to the decrease of estrogen and estrogen receptor (ER) in postmenopausal women, they have a higher risk of intervertebral disc degeneration (IDD) than men. This study aims to explore how ERα and ERβ interact with CCN5 and protect IDD.

PATIENTS AND METHODS: We used Chromatin immunoprecipitation (ChIP) and Luciferase reporter assay to determine whether the ERα/β protein binds to CCN5 promoter and activates its expression. We used TNF-α to induce nucleus pulposus (NP) cell degeneration to simulate the IDD process. The change of the expression of ERα/β and CCN5 was measured in the degenerated NP cells. To understand the function of ERα/β in the NP cells degeneration, we upregulated the ERα/β gene expression by vector transfection or 17β-estradiol (E2) stimulation. Besides, we also used the CCN5 gene-silenced NP cells by siRNA transfection as a comparison to determine the role of CCN5. We tested the cell proliferation and principal components of the extracellular matrix (ECM) to value the degree of NP cell degeneration.

RESULTS: ERα and ERβ protein can bind to the same promoter regions of CCN5 and activate its expression, respectively. TNF-α degraded NP cells with a reduction of cell proliferation, collagen II, ACAN, ERα, ERβ, and CCN5 expression, and increased collagen I/III, and MMP-13 expression. Upregulated ERα or ERβ resulted in the maintains of CCN5 and alleviated the NP cell degeneration. Besides, 17β-E2 supplement increased the ERα, ERβ, and CCN5 expression, as well as stable NP cells phenotype. However, it was partly abolished by the silencing of CCN5.

CONCLUSIONS: Upregulation of ERα and ERβ protects the NP cell degeneration during IDD through the activation of CCN5 by binding to its promoter.

Key Words:
Nucleus pulposus cells, Intervertebral disc degeneration, Estrogen receptors, CCN5.

Introduction

Intervertebral disc degeneration (IDD) is the leading cause of chronic low back pain, especially in elderly patients[1]. The pathology of IDD involves the foundation of various spine diseases, including disc herniation, spinal stenosis, lumbar spondylolisthesis, and spinal segment instability. Its etiology results from multiple factors containing molecular biological factors, genetic factors, nutritional factors, biomechanical factors, of which there is still no consensus to determine the critical factor leading to IDD[2-4]. Clinical studies[5] have shown that postmenopausal women have a higher incidence of lower back pain than men. Endogenous estrogen-deficient is thought to lead to several health problems in women's life. Whether IDD is gender preference has not been fully explained, but it is presumed that estrogen plays a role in the pathogenesis of spinal diseases[6].

Similar to cartilage, the intervertebral disc is a sex-hormone-sensitive tissue. Several authors[7-10] have sought to identify the estrogen responsible for IDD. Jin et al[7] reported estrogen replacement therapy alleviates IDD *via* modulating oxidative stress and autophagy in menopause rats. Estrogen also plays an anti-apoptotic effect on IVD cell apoptosis through the suppression of the inflammatory cytokines, matrix metalloproteinases (MMPs), and promotes the nucleus pulposus (NP) anabolism[8]. Estrogen works by binding to estrogen receptors (ERs), and the presence of ERs raises the cellular sensitivity to estrogen[9]. Song et al[10] found both ERα and ERβ are significantly reduced along with the aggravation of IDD, of which in the NP of males is much higher than that of females, indicating that the degree of NP tissue degeneration is closely related to the level of ERs. ER is a member

Corresponding Author: Chi Zhang, MD; Email: zhch1091962268@163.com

of the nuclear receptor superfamily containing two subtypes of ERα and ERβ, which can mediate estrogen to achieve its function[11]. ERα is expressed as the classic estrogen target, and ERβ is a newly discovered in recent years, which exists in the cells that were not previously considered as estrogen targets, such as vascular cells, osteoblasts, and chondrocytes[12]. ER protein can act as a ligand-induced transcription factor to activate the transcription of the target gene. However, there are few studies on the relationship between ERs and IDD.

The extracellular matrix (ECM) changes significantly in the distribution, composition, and function during IDD, which is mainly related to the decrease of the number and function of NP cells. The ECM in the NP and cartilage endplate contains mostly type II collagen and proteoglycan (ACAN); the annulus fibrosus mainly contains type I collagen[13]. The total amount of collagen in the degenerated disc is not apparent, but the type is remodeled to be fibrotic with the overexpression of type I and III collagen, resulting in the disorder of the internal environment and accelerating the degeneration of the intervertebral disc[14]. CCN5 (also known as WISP-2) is a member of the CCN (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) family and exerts an anti-fibrotic effect by regulating TGF-β in various tissues[15]. In addition, CCN5 is also characterized as an estrogen-inducible gene in ER-positive cell lines[16]. While much is known about how ERs and CCN5 regulate cell metabolism, little is known about whether ERs interacts with CCN5 and participates in IDD. Therefore, in the present study, we explored the potential effect of ERs and CCN5 regarding the ECM metabolism in the IDD using the human NP cells, *in vitro*.

Patients and Methods

Patient NP Samples

A total of six patients (every age: 39 years, from 28 to 45 years) with spine fracture were enrolled in our study. All the patients underwent internal spinal fixation at one spine center and were diagnosed without apparent IDD diagnosis by two specialized spine surgeons. We obtained informed consent from the patients before the operation. We took the fractured NP tissue during the operation and stored it in the cold growth medium immediately after removing it from the patients. This project was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University and under the guidance of the Declaration of Helsinki.

NP Cells Isolation and Culture

The NP tissue was cut with a scalpel and enzymatically digested with 0.25% trypsin and 0.25% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 6 h. The supernatant was filtered, and cell pellets were re-suspended in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) in the incubator (37°C, 95% humidity, and 5% CO_2). We used the passage 2 NP cells for the experiment and changed the culture medium every 3 days. To induce NP cell degradation, we used recombinant human TNF-α protein (10 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) in the culture medium. In addition, we used the 17β-estradiol (E2) (10 nM, E8875; Sigma-Aldrich, St. Louis, MO, USA) to increase the cellular 17β-E2 level.

Western Blot Analysis (WB)

The protein expression of ERα, ERβ, and CCN5 was determined by WB. Briefly, the total protein was extracted with the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and qualified with the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Following, the same protein was added to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% milk, the membranes were incubated with desired primary antibodies: ERα (PA1-311, Thermo Fisher, Waltham, MA, USA, diluted 1:1000), ERβ (ab3576, Abcam, Cambridge, MA, USA, diluted 1:1000), CCN5 (ab38317, Abcam, Cambridge, MA, USA, diluted 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab181602, Abcam, Cambridge, MA, USA, diluted 1:2000) at 4°C overnight. Membranes were then incubated with the corresponding secondary antibodies at room temperature for 1 h. Band intensities were measured using Image J software (NIH, Bethesda, MD, USA). Finally, the protein level was normalized to GAPDH.

Real-time Polymerase Chain Reaction (PCR) Analysis

The ECM gene expression of collagen I, collagen II, collagen III, ACAN, and MMP-13 was analyzed by real-time PCR. Briefly, total RNA of each NP cells treatment was extracted with TRIzol kit (Invitrogen, Carlsbad, CA, USA), and we used 1 μg RNA as a template to synthesize

Table I. Information of the putative binding sites for ERs to CCN5 promoter.

Predicted sequence	ER	Start	End	Score	Primer sequence for ChIP
AGCCCATGTCTTTCTGTCCC	ERα	1336	1355	11.5513	Sense:5'-GTTTGGACAACTACTGGTGGAG-3'; Anti-sense:3'-TCTTCCGAGGTGAGTATGGTC-5'
CATGGAAGTCAACTTGACTT	ERα	151	170	9.77487	Sense:5'-GTGCAGTAACTGTTGGGGTTT-3'; Anti-sense:3'-AGGTGGCAGGTCGTAAGTAT-5
GGGTCACACCCACCT	ERβ	1949	1963	12.4024	Sense:5'-AACAGGCCGCCCAGGGAAGT-3'; Anti-sense:3'-GGTTAGACGGACGGTCTTTGG-5'
AAGTCAACTTGACTT	ERβ	156	170	10.7644	Sense:5'-GTGCAGTAACTGTTGGGGTTT-3'; Anti-sense:3'-AGGTGGCAGGTCGTAAGTAT-5'

DNA with a reverse transcription kit (Roche, Basel, Switzerland). Then, a reaction system containing complementary deoxyribose nucleic acid (cDNA), SYBR Green Mix (TOYOBO, Osaka, Japan), and primers (Table I) was subjected to real-time PCR machine. The relative gene level was achieved by normalization to the GAPDH, according to the method of $2^{-\Delta\Delta Ct}$.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed to verify the ERα/β protein binding to the primer regions of CCN5 by using the ChIP Assay kit (cat. 17-295, Millipore, Billerica, MA, USA) according to the manufacturer's instruction. NP cells were sonicated to extract the chromatin. Then, 1 μg of anti-ERα (PA1-311, Thermo Fisher, Waltham, MA, USA), ERβ (ab3576, Abcam, Cambridge, MA, USA), or normal goat IgG was used to immunoprecipitated the chromatin. After the DNA was purified from the immunoprecipitation or the original chromatin, real-time PCR was performed to amplify the DNA fragment with the SYBR Green Mix (TOYOBO, Osaka, Japan) and primers (Table II), which was finally imaged by agarose gels electrophoresis.

Dual-Luciferase Assays

Dual-Luciferase assays were used to verify the ERα/β protein activates the CCN5 promoter expression. To increase the ERα/β expression, we transfected the NP cells with ERα (CAT: SC125287, OriGene Technologies, Rockville, MD, USA) or ERβ (CAT: RC218519, OriGene Technologies, Rockville, MD, USA) gene encoding vector. Besides, the WT or mutated promoter regions (-1 to -260 bp previous to the transcription start site, TSS) of CCN5 encoded pGL6-TA plasmid was also used to upregulated the predicted promoter sequence. The empty vector and pGL6-TA were used as the negative control, and pRL-Renilla plasmid was used as an internal reference of the Luciferase activities. All the vectors and plasmids were designed and synthesized by the VIPOTION (Guangzhou, China). NP cells were seeded in 24-well plates and transfected with vector or plasmid using Lipofectamine 2000 (Beyotime, Shanghai, China) according to the manufacturer's instruction. After 24 h transfection, firefly and Renilla Luciferase activities were finally measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

ERs Plasmid or CCN5 siRNA Transfection

We upregulated the ERα or ERβ gene expression of NP cells by the transfection of vector used in the Luciferase assays and suppressed the CCN5 expression by the siRNA transfection. The mixture of Opti-MEM (Sigma-Aldrich, St. Louis, MO, USA) and vector coding ERα or ERβ (OriGene Technologies, Rockville, MD, USA) or si-CCN5 (#289450, Thermo Fisher Scientific, Waltham, MA, USA) were diluted in Lipofectamine 2000 reagent (Beyotime,

Table II. Primer sequences for real time-PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
Collagen III	ATGTTGTGCAGTTTGCCCAC	TCGTCCGGGTCTACCTGATT
ACAN	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Shanghai, China), and the NP cells were incubated with the mixture according to the manufacturer's instruction.

Cell Viability Assay

NP cell viability was determined with the Cell Counting Kit-8 (CCK-8) assay. Briefly, NP cells were seeded in 48 well plates (5000 cells/well) and treated with indicated methods, respectively. Then, the fresh medium containing CCK-8 working solution (Beyotime, Shanghai, China) was added to the cells for 2 h incubation, the intensity of CCK8 at 450 nm wavelength presents the cell proliferation potency using an automatic microplate reader (Labsystems Multiskan, Helsinki, Finland).

Enzyme-Linked Immunosorbent Assay (ELISA) for 17β-E2

To compare the cellular 17β-E2 level between primary NP cells, TNF-α treated NP cells, and exogenic 17β-E2 supplied NP cells, we used the 17β-E2 ELISA Kit (ab108667, Abcam, Cambridge, MA, USA) following the manufacturer's instruction.

Statistical Analysis

Statistical analysis was performed with the Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA), and data were expressed as mean ± standard deviation (SD). Differences between the two groups were analyzed using the Student's *t*-test. A comparison between multiple groups was done using a one-way ANOVA test followed by Post Hoc Test (Least Significant Difference). p-value <0.05 was considered statistically significant.

Results

ERs and CCN5 Decrease in the TNF-α Treated NP Cells

To understand the changes of ERα, ERβ, and CCN5 expression in NP during IDD, we used TNF-α to induce the degeneration of NP cells and tested the cell proliferation and the main components of ECM that synthesized by NP cells. The growth medium with different concentrations of TNF-α (5 or 15 ng/mL) was used to culture NP cells for three days, and the nontreated group was divided as control. We used real-time PCR to determine the ECM-related gene expression. In contrast with the control, we found that the 5 ng/mL TNF-α treatment upregulated the collagen-I and MMP-13 expression and suppressed the ACAN gene expression. Additionally, a higher dose of TNF-α further affected the collagen-I, ACAN, and MMP-13 expression, and disordered the collagen-II, and collagen-III expression, as well (Figure 1A). Besides, 15 ng/mL TNF-α also significantly damaged the cell proliferation compared to the control and 5 ng/ml group (Figure 1B). From the WB results, it is easy to observe the decrease of the protein level of ER***α,*** ERβ, and CCN5 within the presence of TNF-α, which is more significant in the 15 ng/mL group (Figure 1C). Therefore, in our study, TNF-α is an efficient way to disrupt the original ECM generation of NP cells, accompanied by a reduction of ERα, ER β, and CCN5.

ERα and ERβ Protein Bind to the Same Promoter Regions of CCN5

CCN5 is regarded as an estrogen-inducible gene that widely expressed in ER-positive cell lines[16]. Hence, we wondered there might be a direct interaction between ERs and CCN5 in the NP cells. Therefore, we detected the potential transcriptional effect of both ERα and ERβ on the DNA promoter regions of CCN5. We collected the 2000bp upstream of the transcription initiation region of the CCN5 gene from the Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) and matched the ERα or ERβ protein in the 2000bp promoter regions of CCN5 using the JASPAR core database (http://jaspar.genereg.net/). Luckily, we found several putative binding sites, we chose four (P1 to P4) most likely of which and used ChIP assay to verify the real situation. Surprisingly, there is a region (P4) that can be bound from both ERα and ERβ, which is contained in the P2 sites. We illustrated the location of the four putative binding sites in the diagram of Figure 2A. The orange color (P1, P2) indicated the predicted sites of ERα, and the green color (P3, P4) indicated the ERβ, of which P2 totally covers P4. We collected the chromatin of the NP cells and used anti-ERα, anti-ERβ, and IgG antibody linked beads to pull down the indicted protein. The IgG linked beads were used as a negative control. The total DNA from the cell lysis without immunoprecipitation was pointed to be Input as a positive control. The immunoprecipitated DNA regions were amplificated by PCR and imaged by agarose gels electrophoresis. The information of the four putative sites and PCR sequence were listed in Table II. As shown in Figure 2B, all the

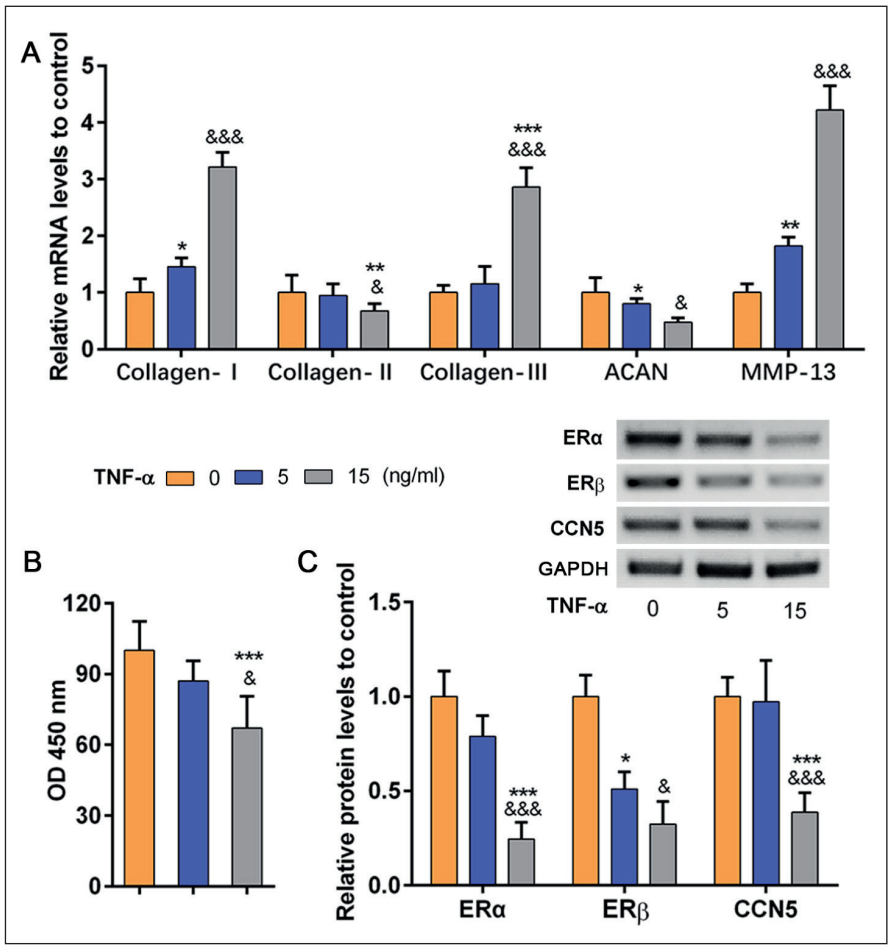

Figure 1. ERs and CCN5 decrease in the TNF-α treated NP cells. NP cells were cultured without or with TNF-α (5 ng/mL or 15 ng/mL) for three days. **A**, Real time-PCR analysis for collagen I/II/III, ACAN, and MMP-13 expression by normalization to GAPDH. **B**, Cell proliferation was determined by CCK8 assay. **C**, WB analysis for ERα, ERβ, and CCN5 expression, and the quantification of blots measured by Image J software. Results are expressed as mean ± SD (*p<0.05, **p<0.01, ***p<0.001 compared to the control; $^{\&}p$<0.05, $^{\&\&\&}p$<0.001 compared to 5 ng/ml TNF-α group).

PCR primers could amplify the predicted promoter regions (lane 1), and no nonspecific binding site was observed (lane 4). However, ERα was useful in pulling down the P2 site (lane 2), and ERβ pulled down the P4 (lane 3). Interestingly, ERα and ERβ almost bind to the same promoter regions of CCN5, which might be responsible for the ERs to activate the CCN5 expression.

ERα and ERβ Overexpression Activate CCN5 Promoter Regions

In addition, to confirm the ERα and ERβ binds to the CCN5 promoter, we further verified the efficiency of the binding place using a Dual-Luciferase reporter gene assay. Due to the binding site of ERβ (the green color sequence) is contained in the ERα's (the orange plus green color sequence) (Figure 2C), we cloned the same DNA sequence containing the WT or mutated P4 region (red color, Figure 2D) into the pGL6-TA plasmids (WT-pGL and mutated-pGL), which was used to transiently transfected into NP cells to overexpress WT or mutated P4 region. The empty pGL was used as a negative control. Besides, ERα and ERβ encoded vector was used to upregulate the cellular ERα or ERβ gene expression, and the empty-vector was used as control. The Luciferase activity was normalization to the density of Renilla-pRL plasmid (pRL). Overexpression of either ERα or ERβ gene could significantly active the Luciferase activity of WT-pGL. In contrast, the Luciferase activity was significantly lower in the mutated-sequence even when the ERα or ERβ gene was upregulated. Therefore, we concluded that ERα or ERβ could transcriptionally activate the CCN5 expression by binding to its promoter.

ERα and ERβ Overexpression Upregulate the CCN5 Expression and Alleviate NP Cells Degradation

At the beginning of our study, TNF-α was a valid cause to disrupt the ECM generation and suppressed the ERα, ERβ, and CCN5 expression. Since the ERα and ERβ can regulate CCN5 expression, we continuously explored whether upregulation of ERα or ERβ gene ex-

pression could result in the increase of CCN5 in the degenerated NP cells and saw what would happen to the ECM production. NP cells without TNF-α (15 ng/mL) treatment and vector transfection were divided as control. NP cells with ERα- or ERβ-vector transfection were cultured with TNF-α for three days. As shown in Figure 3A, compared to the only TNF-α treated group, cellular ERα and ERβ significantly remained when transfected with the ERα- or ERβ-vector, respectively. Additionally, the CCN5 protein level was far higher than the non-transfected NP cells under the TNF-α stimulation resulting from the high level of ERα or ERβ expression. In addition, we measured the cell proliferation of the four groups with CCK-8 kit, and the result suggested the maintaining of a higher level of ERs and CCN5 did not significantly improve the cell proliferation under TNF-α simulation compared to the non-transfected group (Figure 3B). Apart from this, we noticed the higher level of ERs and CCN5 suppressed the collagen I, collagen III, and MMP-13 mRNA expression, and contained more ACAN expression compared to the non-transfected TNF-α group (Figure 3C). However, the collagen II expression was not significantly changed in these groups.

17β-E2 Protects NP Cells Degradation Through CCN5 Activation

Estrogen is widely reported to be used as the activator of ERα and ERβ in various cell types. Therefore, we used the highly potent estrogen (17β-E2) to induce the ERα and ERβ expression in addition to the gene-editing method. To confirm CCN5 involving in the ERs mediated ECM stability, we compared the normal NP cells and the CCN5-silenced NP cells under the co-culture of TNF-α and 17β-E2. NP cells without any treatment were divided as control. TNF-α (15 ng/mL) and 17β-E2 (10 nM) treatment of NP cells remained three days. We measured the cellular 17β-E2 content using the ELISA method and found the normal NP cells originally contained a low level of 17β-E2, and it markedly increased after stimulated with exogenic 17β-E2 no matter the CCN5 was silenced or not (Figure 4A). The 17β-E2 supplement also protected the proliferation of degenerated NP cells caused by TNF-α, which was not affected even NP cells were transfected with CCN5-siRNA (Figure 4B). As shown in Figure 4C, 17β-E2 supplement sufficiently increased the ERα and ERβ protein level, as well as the CCN5 expression compared to the TNF-α stimuli only group. The silencing of CCN5 significantly

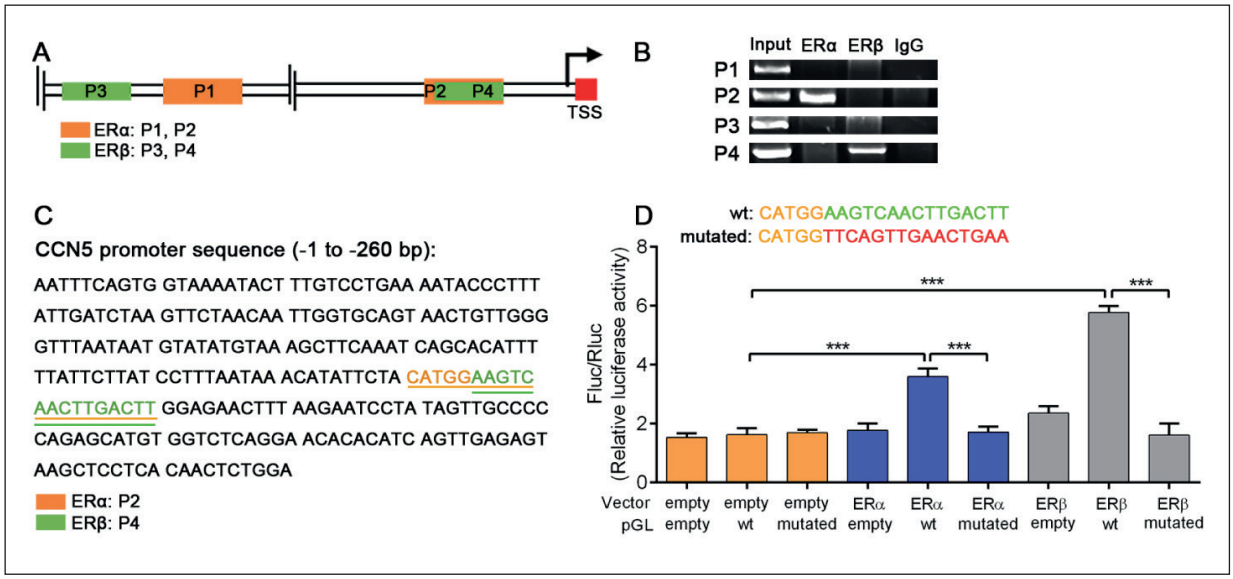

Figure 2. ERs protein binds to the promoter regions of CCN5 and activates its expression. **A**, Two putative sites for ERα and two for ERβ to bind with the CCN5 promoter. **B**, Agarose gels electrophoresis for the PCR after immunoprecipitation. Lane 1 is the positive control of total DNA fragments; lane 2 is the amplification of the DNA from the ERα; lane 3 is the amplification of the DNA from the ERβ linked beads; lane 4 is the amplification of the DNA from the IgG inked beads. **C**, Binding sites for the ERβ (the green color sequence) and ERα (the orange plus green color sequence) on the promoter of CCN5, that for inserting into the pGL reporter plasmid. **D**, Luciferase activity was more dramatic following the ERs-vector transfection compared to the WT-pGL overexpression, and no significant difference in luciferase activity was observed with empty-vector or mutated sequence transfection. Results are expressed as mean ± SD. (***p<0.001).

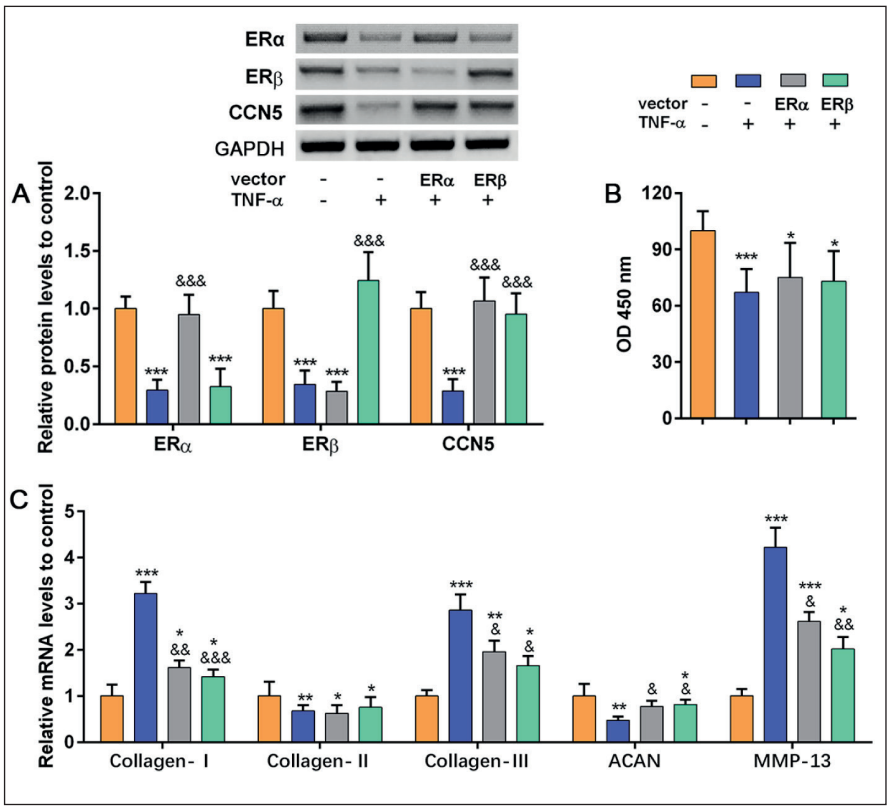

Figure 3. ERα and ERβ overexpression upregulate the CCN5 expression and alleviate ECM degradation. The ERα or ERβ vector-transfected NP cells and non-transfected NP cells were subjected to 15 ng/mL TNF-α for 3 days. **A**, WB analysis for ERα, ERβ, and CCN5 expression, and the quantification of blots measured by Image J software. **B**, Cell proliferation was determined by CCK8 assay. **C**, Real time-PCR analysis for collagen I/II/III, ACAN, and MMP-13 expression by normalization to GAPDH. Results are expressed as mean ± SD. (*p<0.05, **p<0.01, ***p<0.001 compared to the control; $^{\&}p$<0.05, $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 compared to the non-transfected NP cells subjected to TNF-α).

reduced the cellular CCN5 protein expression, but not observably affected the ERα and ERβ expression (Figure 4C). Finally, the mRNA of the related ECM components was also measured. 17β-E2 showed a protective effect on the maintaining of collagen II and ACAN and the suppression of collagen I, collagen III, and MMP-13 expression. However, the silencing of CCN5 partly weakened the effect of 17β-E2 on keeping the balance of ECM synthesis, indicating that CCN5 is essential for the ERs mediated ECM stability (Figure 4D).

Discussion

As a complete structural unit, the intervertebral disc comprises three parts: annulus fibrosus, nucleus pulposus, and cartilage endplate. The degeneration of the intervertebral disc is considered to be the pathological basis of disc herniation, spondylolisthesis, and other spinal diseases[17]. IDD is mainly manifested by the aging of NP cells and changes in the composition of ECM: NP cells change from chondrocyte phenotype to fibro-chondrocyte phenotype, with the reduction of water, type II collagen, and ACAN in the ECM, leading to the loss of mechanical characteristics of spine[18,19]. Some studies[20,21] indicated that risk of IDD is approximately twice as common in women than in men. Actually, it exists more commonly in postmenopausal women, suggesting the potential involvement of sex hormones, important estrogens in the pathology of IDD[10]. Furthermore, the ERs, both ERα and ERβ are known to be important involving in the mediation of estrogen in many cell types, containing intervertebral disc cells. Sheng et al[22] elucidated 17β-E2 protects cartilaginous endplates cells during IDD through ERα, which can be attenuated by miR-221. Yang et al[23] found that 17β-E2 increases NP cell proliferation *via* ERβ. Besides, an ovariectomy model of rat also suggests the supplement of 17β-E2 maintains ECM stability of the disc by increasing the type II collagen and inhibiting MMPs expression. At the same time, it can be abolished when the ERs are suppressed[24]. Therefore, the activity of ERs plays an essential role in the estrogen-dependent protection of IDD. However, the cues of how ERs regulate IDD is limited.

ERs is a steroid hormone nuclear receptor that transcriptionally regulates many downstream targets, such as human ovalbumin, c-fos, vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF)[25]. Proteins of the

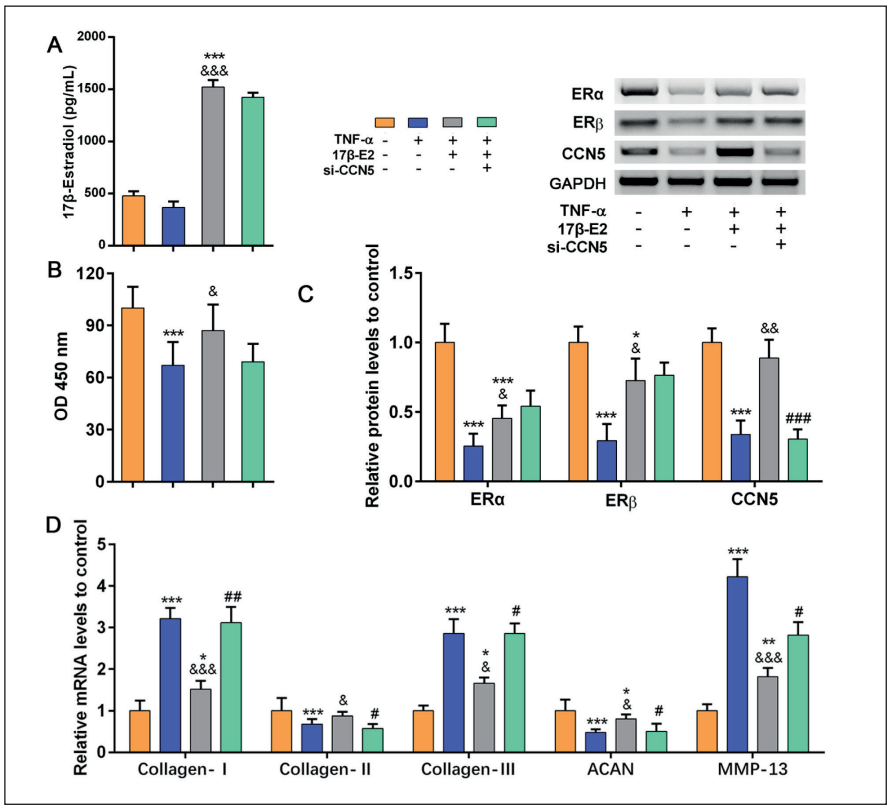

Figure 4. 17β-E2 protects NP cells degradation through CCN5 activation NP cells were cultured with 15 ng/ml TNF-α with or without the presence of 17β-E2. Besides, the CCN5-silenced NP cells were also cultured with TNF-α and 17β-E2 (10 nM). A, ELISA assay of the cellular 17β-E2 content. B, Cell proliferation was determined by CCK8 assay. C, WB analysis for ERα, ERβ, and CCN5 expression, and the quantification of blots measured by Image J software. D, Real time-PCR analysis for collagen I/II/III, ACAN, and MMP-13 expression by normalization to GAPDH. expression. Results are expressed as mean ± SD. (*p<0.05, **p<0.01, ***p<0.001 compared to the control; $^{\&}p$<0.05, $^{\&\&}p$<0.01, $^{\&\&\&}p$<0.001 compared to the non-silenced NP cells subjected to 15 ng/mL TNF-α; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 compared to the non-silenced NP cells subjected to TNF-α and 17β-E2).

CCN family (CCN1-6) are considered to have essentially matricellular action in the ECM[26]. Thereinto, CCN5 is reported directly regulated by the ER and also triggered by stimulation with estrogens[27]. Johnsen et al[28] found that ERα can interact with the CCN5 promoter in human breast cancer cells. However, no report mentioned how ERα interacts with CCN5 in NP cells and whether it happens to ERβ, as well. In this study, we found that the expression of ERα, ERβ, and CCN5 were significantly suppressed during the degradation of NP cells. We hypothesized that CCN5 might be regulated by ERα, or ERβ, or both in NP cells. In the 2000bp upstream of CCN5 promoter regions, we found several putative binding sites of both ERα and ERβ. Surprisingly, there is a region that can be bound by both ERα and ERβ, indicating that CCN5 might be regulated by both two ER in the transcriptional level. Form the results of ChIP and Luciferase assay, we confirmed the region 'AAGTCAACTTGACTT' in the CCN5 promoter is the binding site and can be activated by both ERα and ERβ.

As we all know, the process of IDD involves the low cell proliferation and remodeling of ECM, including the transformation of collagen type and the degradation of ECM by various cytokines represented by MMPs[29]. To further verify whether ERs and CCN5 play a protective role in the NP cells degeneration, we overexpressed the ERα and ERβ in the NP cells and observed the maintains of CCN5 expression, cell proliferation ability, and ECM stability, reflecting in the protection of collagen II, ACAN, and inhibition of collagen I/III and MMP-13. There is some more evidence to support that CCN5 is functional to be regulated by ERs. The ERα-silenced human mammary epithelial cells do not respond to 17β-E2 stimulation with an increase of CCN5 expression. In contrast, stable ERα transfection into the cells promotes the 17β-E2 to upregulate CCN5 expression[30]. Our research, for the first time, announced that the overexpression of ERβ also contributes to the expression of CCN5.

To confirm the CCN5 to be primarily responsible for ER's induction of ECM stability, we silenced the CCN5 expression and upregulated the ERα and ERβ simultaneously with the stimulation of 17β-E2. The results showed the deficiency of CCN5 partly recalled the protection of 17β-E2 in NP cells degeneration by a lower proliferation, lower collagen II and ACAN, higher collagen I/III, and MMP-13 compared to the non-CCN5 silenced NP cells. Hence, CCN5 is an essential

mediator in the ERs-dependent protection of NP cells. Many researches mentioned the high level of CCN5 to suppress the fibrotic phenotypes of tissue, such as pulmonary fibroblasts, which is consistent with the protection of CCN5 in the ECM synthesis in our findings.

Conclusions

Taken together, our study extends the mechanism of the observation to the protection of ERs in the NP cells degeneration. The novelty of this research is proving that a high level of ERα and ERβ maintain the stable ECM synthesis of NP cells involving the activation of CCN5. Due to the critical physiologic roles of ERs and CCN5, the strategy of activating ER or CCN5 could be a therapeutic method to treat IDD, especially the postmenopausal IDD of women.

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Conflict of Interests

The authors declared no conflict of interest.

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