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CDMP1 promotes type II collagen and aggrecan synthesis of nucleus pulposus cell *via* the mediation of ALK6

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Abstract. – OBJECTIVE: Destruction of extracellular matrix (ECM), especially collagen II and aggrecan, is an essential feature of intervertebral disc degeneration (IDD). This project planned to elucidate the role of cartilage-derived morphogenetic protein-1 (CDMP-1) in the collagen II and aggrecan synthesis of nucleus pulposus (NP) cells under the IL-1β induced degeneration.

PATIENTS AND METHODS: We cultured human primary NP cells in the different concentrations of IL-1β medium and analyzed the CDMP-1 level. Recombinant human CDMP-1 protein was used to co-culture with IL-1β to investigate its effects on collagen II and aggrecan synthesis of NP cells. Additionally, the bone morphogenetic protein type IB receptor (ALK6) gene silenced and upregulated NP cells were used to evaluate the function of ALK6 in the CDMP-1 treated NP cells. Collagen II, aggrecan, MMP9, MMP13, and TIMP4 expression level were analyzed to assess the ECM stability of NP cells.

RESULTS: CDMP-1 gene expression decreased in the IL-1β treated NP cells with a dose-dependent. Appropriate CDMP-1 protein supplement contributed to the collagen II and aggrecan production, the suppression of MMP9 and MMP13, and the upregulation of TIMP4. However, the silencing of ALK6 rejected the positive function of CDMP-1 on the collagen II and aggrecan; on the contrary, ALK6 upregulation magnified the CDMP-1 induced collagen II and aggrecan production.

CONCLUSIONS: CDMP-1 is efficient in promoting the collagen II and aggrecan synthesis of NP cells, which is probably based on the mediation of ALK6.

Key Words:
Nucleus pulposus cells, Type II collagen, Aggrecan, CDMP-1, ALK6.

Introduction

Low back pain is a common and frequently-occurring disease that affects human health, and the most crucial factor of it is intervertebral disc degeneration (IDD)[1]. The intervertebral disc is composed of a nucleus pulposus (NP) located in the center, a fibrous ring surrounded by the periphery, and upper and lower endplates. The three-assist each other and play a physiological role such as buffering load and maintaining spinal mobility as a whole[2]. NP is a jelly-like tissue composed of notochord cells, cartilage-like cells, and a large number of extracellular matrix (ECM), which dominates the physiological function of intervertebral discs.

The most important ingredients of ECM produced by NP cells are aggrecan and type II collagen (collagen II)[3]. Aggrecan can absorb water molecules and keep the water content and hydrostatic pressure of the NP, which contributes to disperse the pressure uniformly. Collagen II can produce monosaccharides and disaccharide derivatives through saccharification, forming a connection with aggrecan, forming a loose network structure in the NP matrix, thereby resisting the tension created by aggrecan and water[4]. At present, it is believed that IDD starts from the NP, and the reduction of NP cells and abnormalities of ECM are the key factors that contribute to the disc degeneration[5]. At the level of cell biochemistry, the decline of the functional ECM reflects the imbalance of anabolic and catabolism of NP cells. With the decrease of collagen II and aggrecan in the matrix, dehydration and the decreased height of the NP affect the biomechanical properties of the spine[6]. Therefore, maintaining the viability of NP cells and the content of collagen II and aggrecan are the focus of current research on IDD.

Cartilage-derived morphogenetic protein-1[7] (CDMP-1, known as growth differentiation factor 5 as well) is a member of the bone morphogenetic protein (BMP) superfamily, and it is the most specific type of growth factor related to cartilage morphogenesis and development. It can continue to maintain normal cartilage tissue growth after

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birth and promote joint cartilage damage repair. It mainly regulates the differentiation of precursor cells and participates in almost all biological processes of cartilage tissue formation, growth, and damage repair[8,9]. CDMP1 is an effective inducer for ECM production. Fibroblasts can differentiate into chondrocytes under the induction of CDMP1, and secrete chondrocyte-specific matrix collagen II and aggrecan[10]. Luo et al[11] reported that GDF-5 could promote ECM secretion by degenerated NP cells. However, the expression of CDMP-1 is also affected by the degenerated degree of NP tissue. Le Maitre et al[12] found CDMP-1 in degenerated NP cells were significantly reduced compared with the healthy NP cells.

Though CDMP1 is indicated to play a positive role in the collagen II and aggrecan production, the mechanism underlies the NP cells remaining unclear. In this study, we explored the effect of CDMP1 and the bone morphogenetic protein (BMP) type IB receptor (ALK6, also known as BMPR1B) on the IL-1β induced NP cell degeneration. The aim is to provide a theoretical reference for finding new evidence to prevent IDD *via* the application of CDMP1.

Patients and Methods

Reagent

The DMEM/F12 medium, penicillin-streptomycin, trypsin, type II collagenase, fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA); Collagen II and aggrecan antibody were purchased from Abcam (Cambridge, MA, USA); Alexa Fluor488 was purchased from Invitrogen (Carlsbad, CA, USA); Lipofectamine 2000, Cell Counting Kit-8 (CCK-8) reagent, radioimmunoprecipitation assay (RIPA) lysis buffer, bicinchoninic acid (BCA) kit, and enhanced chemiluminescence (ECL) substrate were purchased from Beyotime (Shanghai, China); RNAiso Plus RNA extraction reagent, reverse transcription kit, and SYBR Premix kit were purchased from TaKaRa (Kusatsu, Shiga, Japan).

NP Tissue Specimen Collection

We collected six degenerative NP specimens removed from patients with lumbar disc herniation, including 4 males and 2 females, aged 27 to 38 years. The specimens were donated voluntarily by patients. The research protocol was approved by the Ethics Committee of the Caoxian People's Hospital.

NP Cells Isolation and Cell Culture

The NP tissue of the intervertebral disc removed during the operation was minced and washed with PBS solution containing 1% penicillin-streptomycin for 3 times. Then, the specimens were cultured with 2.5 g/L trypsin and 2 g/L type II collagenase at 37°C for 6 h. After filtration and centrifuge, we got NP cells and seeded them with DMEM/F12 medium containing 10% phosphate-buffered saline (PBS). The passage 1 NP cells were reseeded and deserved into the following treatment: 1) cultured with 5/10 ng/mL IL-1β; 2) cultured with 1/2/3 μg/mL recombinant human CDMP-1 protein; 3) transfected with siRNA or plasmid targeting ALK6.

Immunofluorescence (IF)

After treatments, NP cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X. 5 % of bovine serum albumin (BSA) was used to block the cells at room temperature. NP cells were following washed with PBS, and then incubated with collagen II and aggrecan primary antibodies overnight at 4°C. The next day, NP cells were incubated with Alexa Fluor488 conjugated secondary antibody for 1 h in the dark. The staining intensity is used to express its relative expression.

Western Blot Analysis

The NP cells of each group were collected, and total protein was isolated with the radioimmunoprecipitation assay (RIPA) buffer. The protein concentration was determined according to the bicinchoninic acid assay (BCA) method. 40 μg of protein was added to each channel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. After that, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked with freshly prepared skim milk with a volume fraction of 0.05 for 2 h. Primary antibodies against ALK6, CDMP1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were added on the membranes and incubated overnight at 4°C. After washing the membrane, the secondary antibody labeled with horseradish peroxidase (HRP) was added on the membranes for another 1 h at room temperature. Finally, membranes were exposed by enhanced chemiluminescence (ECL). The gray level value of the specific band of the protein is used to express its relative expression.

CCK8 Assay

The viability of NP cells was determined *via* the CCK8 assay. After treatment, NP Cells in 96-well plates were incubated with CCK8 reagent according to the manufacturer's instructions. We observed the intensity at 570 nm using a microplate reader. Data were calculated as a percentage relative to non-treated value.

Reverse Transcription-PCR Analysis (RT-PCR)

The NP cells of each group were collected and lysed *via* RNAiso reagent to extract total RNA. The RNA was then reverse-transcribed to complementary deoxyribose nucleic acid (cDNA) *via* the reverse transcription kit. The relative RNA analysis of CDMP1, collagen II, aggrecan, tissue inhibitors of matrix metalloproteinase 4 (TIMP4), and matrix Metalloproteinase 9/13 (MMP9/13) was performed by RT-PCR using the SYBR Premix kit. We used GAPDH as an internal reference to calculate gene expression according to the method of $2^{-\Delta\Delta Ct}$. All primers (Table I) were synthesized by Gima Biotechnology (Shanghai, China).

shRNA and Plasmid Transfection

We silenced and upregulated the ALK6 gene expression by transfecting shRNA (TRCN0000277916, Sigma, St. Louis, MO, USA) and plasmid vector (pcDNA3-ALK6 Q203D, Addgene plasmid #80883). NP cells were seeded into a 6-well plate at a density of 1×10^4 / well and transfected with vector *via* Lipofectamine 2000 according to the manufacturer's instructions. After 24 h culture, we changed the medium was exchanged and cultured for another 72 h.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) Version 20 software (IBM Corp., Armonk, NY, USA). The measurement data results were expressed as mean ± standard deviation (SD). Comparisons between groups were analyzed by one-way analysis of variance ANOVA (followed by Least Significant Difference as the Post-Hoc Test) or Student's *t*-tests. The difference was statistically significant with $p<0.05$.

Results

IL-1β Treatment Decreases Collagen II, Aggrecan, and CDMP-1 Expression of NP Cells

To determine the CDMP-1 expression in the degenerated NP cells, we used IL-1β (5 ng/mL and 10 ng/mL) to induce NP cells degeneration as previous describe[13]. Due to the degenerated NP cell always has the impaired ability in collagen II and aggrecan synthesis[14], we found collagen II and aggrecan level were significantly decreased resulting from the IL-1β treatment, which was in a dose-dependent (Figure 1A and 1B). In addition to this, we noticed IL-1β significantly affected the CDMP-1 protein expression compared to the control, especially in a higher dose (Figure 1C and 1D), which was also verified in an mRNA level (Figure 1E). Therefore, we hypothesized CDMP-1 should take part in the development of NP cell degeneration.

CDMP-1 Increases Collagen II and Aggrecan Synthesis of NP Cells

To explore the effect of CDMP-1 supplement to the NP cells, we firstly tested the security of CDMP-1 to the viability of NP cells. We cultured NP cells with a ranged concentration of CDMP-1 protein from 1 μg/mL to 4 μg/mL for 24 h and analyzed the cell viability with CCK8 assay. Compared to the control group, CDMP-1 did not

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

Gene name	Forward (5′>3′)	Reverse (5′>3′)
CDMP-1	TCCAGACCCTGATGAACTCC	TCCACGACCATGTCCTCATA
collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
aggrecan	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
MMP9	GCCACTACTGTGCCTTT	CCCTCAGAGAATCGCC
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
TIMP4	CCACTCGGCACTTGTGATTC	CATCCTTGACTTTCTCAAACCCT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

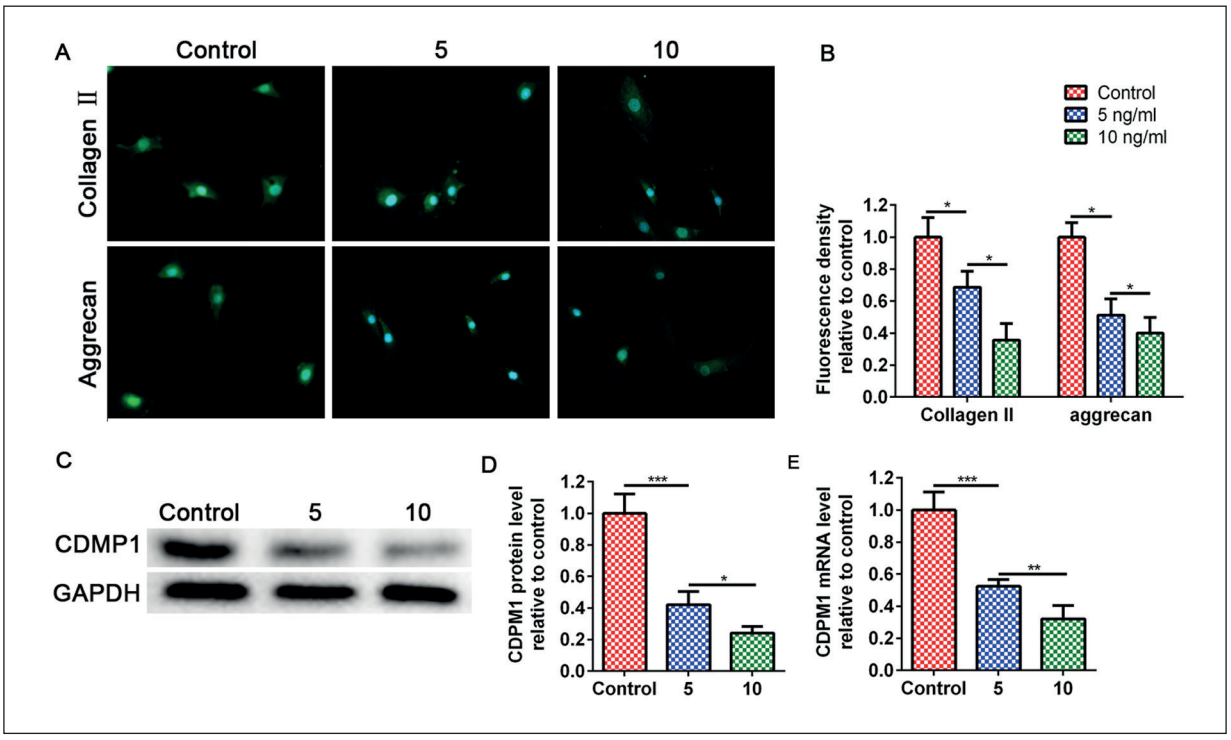

Figure 1. CDMP-1 decreases due to the degeneration of intervertebral disc tissues. **A,** The protein expression level of collagen II and aggrecan were determined by IF (magnification: 400×) **B,** quantification analysis. **C,** The protein levels of CDMP-1 was determined by Western blot and **D,** quantification analysis. **E,** mRNA level of CDMP-1 was determined by RT-PCR. The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

affect the viability until 4 μg/mL, which means high-dose CDMP-1 was not secure for the NP cell culture (Figure 2A). Therefore, we used the concentration of 1, 2, and 3 μg/mL CDMP-1 to treat NP cells and analyze the related gene expression of the mRNA level. After exogenous CDMP-1 treatment, the expression of CDMP-1 was gradually increased, and the concentration of 3 μg/mL was the most effective one that promoted collagen II and aggrecan mRNA expression (Figure 2B). In general, MMPs and TIMPs maintain a balanced state in tissues. The interaction between them determines whether the ECM is degraded or aggregated, to affect the process of intervertebral disc ECM degradation[15]. Surprisingly, CMDP-1 supplement played a role in the suppression of MMP9 and MMP13 and contributed to the expression of TIMP4 in the concentration of 2 and 3 ***μg/m***L (Figure 2C). These data suggested that appropriate CDMP-1 supplement protected the collagen II and aggrecan expression of NP cells; meanwhile, it also suppressed MMP9 and MMP13 and upregulated TIMP4 level leading to protection of the stability of the ECM.

ALK6 Deficiency Weakens CDMP-1 Induced Collagen II and Aggrecan Synthesis of NP Cells

As shown above, CDMP-1 was efficient in promoting collagen II and aggrecan production. Therefore, we thought the CDMP-1 supplement should play a decisive role in the IL-1β induced NP cell degeneration. As a subtype of the BMP family and a member of the TGF subgroup, CDMP-1 has been reported to share BMP receptors with other subtypes, and the binding between CDMP-1 and ALK6 is more efficient than the other type receptors[16]. However, whether the function of CDMP-1 of NP cells is associated with ALK6 remains unknown. To explore the role of ALK6 in the CDMP-1 induced collagen II and aggrecan synthesis, we silenced the ALK6 gene in NP cells and co-cultured the cells with IL-1β and CDMP-1 for 24 h. As shown in Figure 3A and B, IL-1β significantly decreased ALK6 and CDMP-1 protein expression after 24 h treatment comparing to the control. After adding CDMP-1, the procession of ALK6 and CDMP-1 were increased compared to the IL-1β group. ALK6 protein was significantly

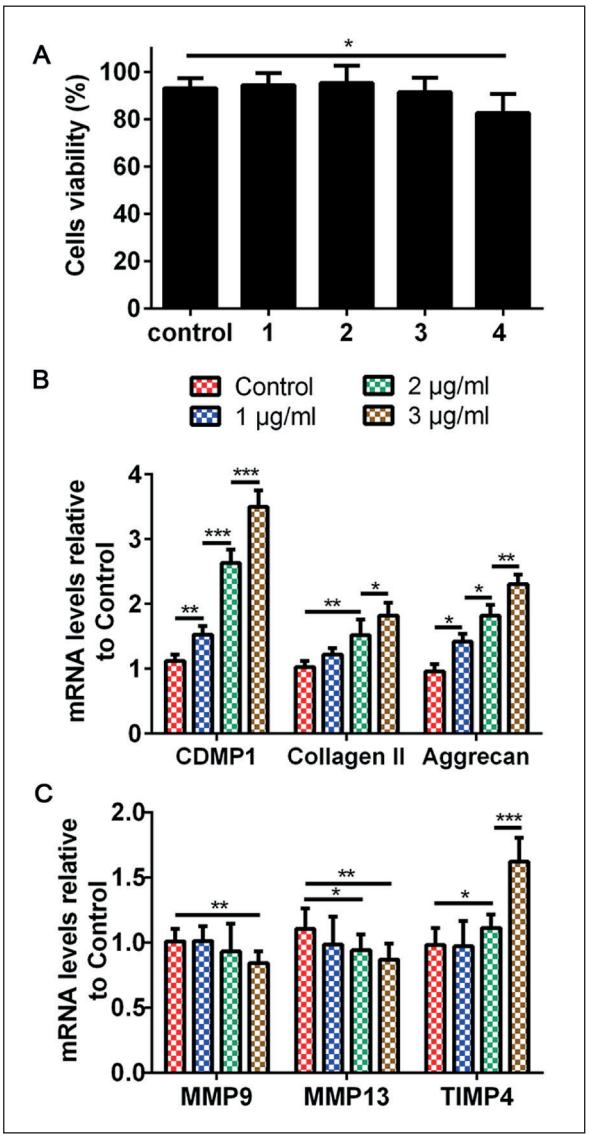

Figure 2. CDMP-1 promotes the ECM stability of NP cells. **A,** Cells viability was analyzed by CCK8 assay. **B, C,** The mRNA expression levels of CDMP1, collagen II, aggrecan, MMP9, MMP13, and TIMP4 were assayed by RT-PCR. The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

decreased after ALK6-shRNA transfection even under the simulation of CDMP1, and the ALK6 silencing did not affect the CDMP-1 expression (Figure 3A and 3B). For the mRNA analysis of MMP9, MMP13, and TIMP4, CDMP-1 rejected the IL-1β induced MMP9 and MMP13 upregulation and TIMP4 downregulation; however, the effect of exogenic CDMP-1 supplement was alleviated in the ALK6 silenced NP cells compared to the non-silenced cells (Figure 3C). Furthermore, we also stained the collagen II and aggrecan in the four groups and found CDMP-1 could protect the collagen II and aggrecan content under the condition of IL-1β. Compared to the non-silenced NP cells, the deficient ALK6 weakened the effect of CDMP-1 in the collagen II and aggrecan synthesis (Figure 3D, 3E). These data indicated CDMP-1 was effective in the protection of collagen II and aggrecan of degenerated NP cells, which was potentially based on the mediation of ALK6.

ALK6 Overexpression Promotes CDMP-1 Induced Collagen II and Aggrecan Synthesis of NP Cells

To confirm the significance of ALK6 to CDMP-1 in degenerated NP cells, we also upregulated the ALK6 expression by plasmid transfection. We also divided NP cells into four groups: non-treated as control; 24 h IL-1β (10 ng/mL) treatment as degenerated group; 24 h CDMP-1(3 μg/mL) and IL-1β (10 ng/mL) co-treatment group as positive control; 24 h CDMP-1(3 μg/mL) and IL-1β (10 ng/mL) co-treatment of ALK6 overexpressed group. The result of Western blot showed ALK6 and CDMP-1 protein expression were remarkably increased after CDMP-1 stimulation, which was aggravated by ALK6-plasmid transfection (Figure 4A and 4B). Additionally, we noticed the overexpression of ALK6 cooperated with CDMP-1 suppressing the MMP9 and promoting TIMP4 mRNA level compared to the positive control; however, there was no significance between positive control and ALK6-plasmid transfected group (Figure 4C). Finally, we continuously tested the collagen II and aggrecan protein expression with IF staining. As shown in Figure 4D and 4E, CDMP-1 upregulation contributed to the synthesis of collagen II and aggrecan compared to the IL-1 induced degenerated group, furthermore, which was magnified by the overexpression of ALK6. In all, ALK6 could aggravate the effect of CDMP-1 in the promotion of collagen II and aggrecan synthesis of NP cells under the IL-1β induced degenerated condition.

Discussion

The imbalance between the synthesis and decomposition of ECM may be the critical pathological and physiological changes that cause IDD. The promotion of collagen II and aggrecan synthesis is the vital foundation for IDD reverse[17]. CDMP-1 is reported to be involved in embryonic

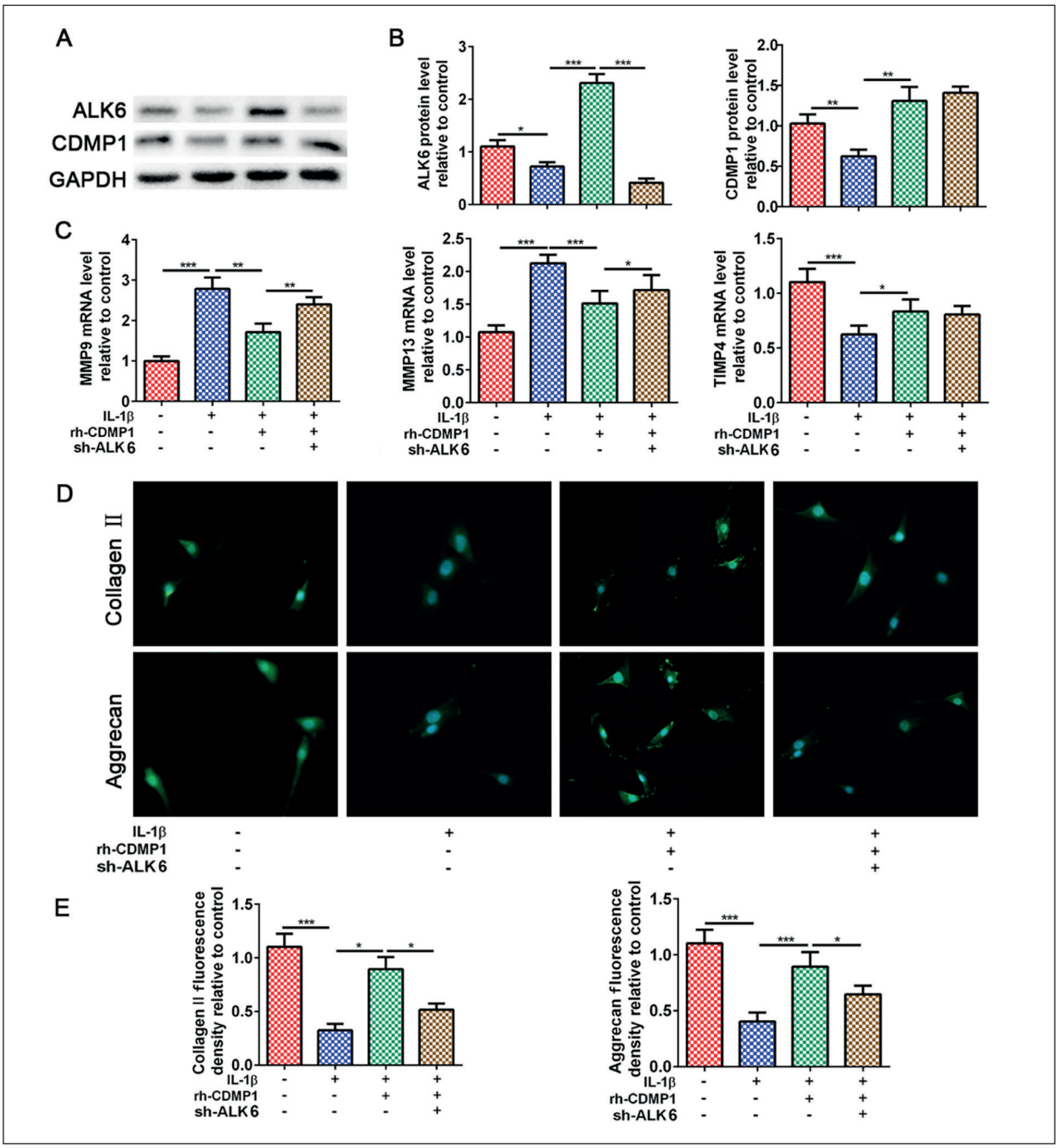

Figure 3. ALK3 deficiency weakens CDMP-1 induced collagen II and aggrecan synthesis. **A,** The protein expression levels of ALK3 and CDMP-1 were determined by Western blot and **B,** quantification analysis. **C,** The mRNA expression levels of MMP9, MMP13, and TIMP4 were assayed by RT-PCR. **D,** The protein expression level of collagen II and aggrecan were determined by IF (magnification: 400×). **E,** Quantification analysis. The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

development, regulating bone marrow differentiation, promoting limb development, and repairing tissue damage (tendons, ligaments, skin, nerves, bones, and cartilage)[18]. However, the research about CDMP-1 in the IDD is limited. In our study, we firstly indicated that CDMP-1 was decreased in degenerated NP cells, and the supplement of exogenic CDMP-1 was efficient to promoted collagen II and aggrecan expression accompanying with suppression of MMP9 and MMP13. MMPs

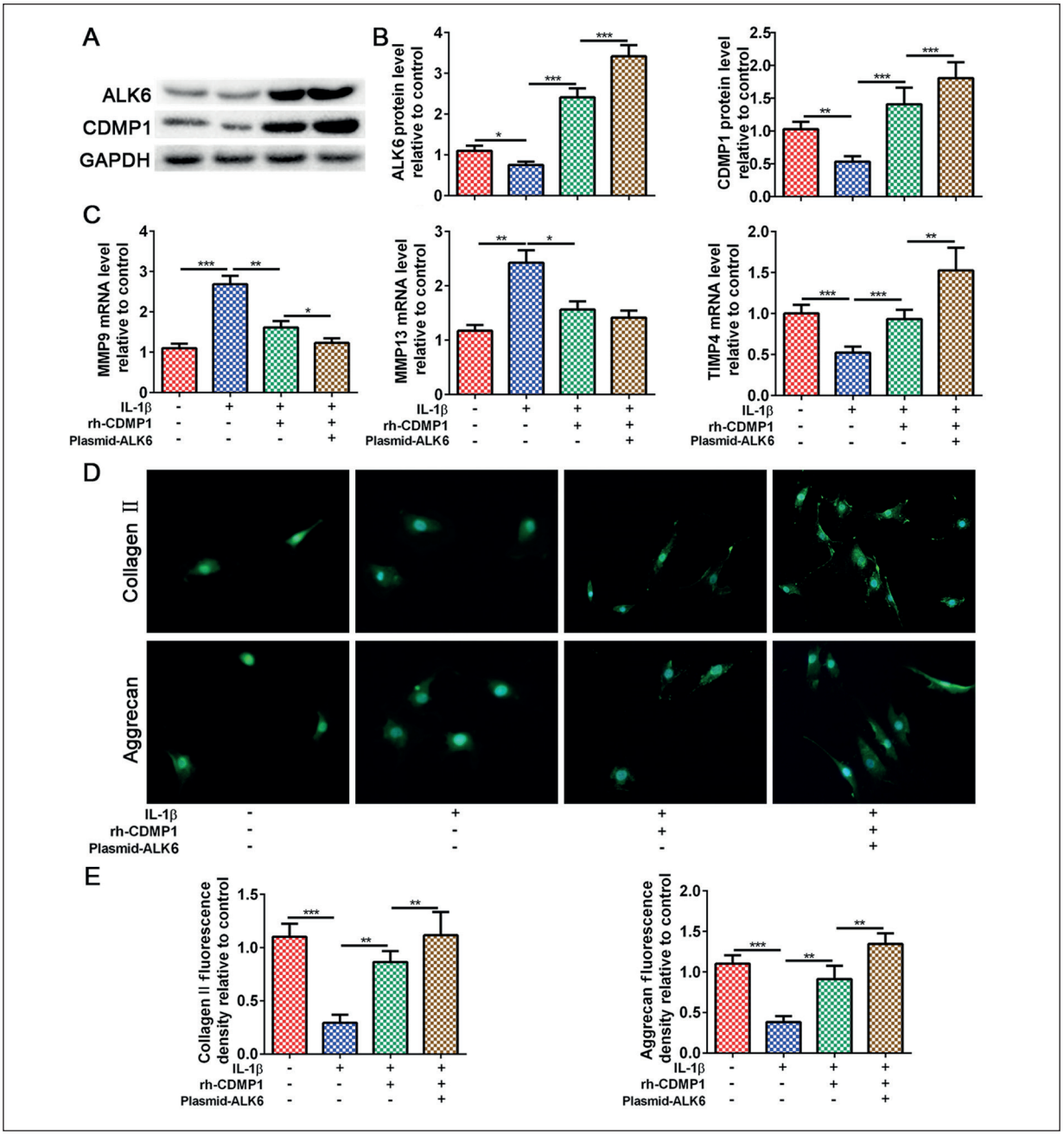

Figure 4. ALK3 overexpression promotes CDMP-1 induced collagen II and aggrecan synthesis. **A,** The protein expression levels of ALK3 and CDMP-1 were determined by Western blot and **B,** quantification analysis. **C,** The mRNA expression levels of MMP9, MMP13, and TIMP4 were assayed by RT-PCR. **D,** The protein expression level of collagen II and aggrecan were determined by IF (magnification: 400×). **E,** Quantification analysis. The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

are one of the most crucial matrix-degrading enzymes, which can degrade almost all components of ECM. It can cause the degradation of collagen aggregates in the disc matrix, reduce the content of proteoglycans, cause dehydration of the NP and lose its inherent elasticity, which can directly lead to the decline and loss of the biomechanical function of the intervertebral disc, resulting in IDD. Preventing IDD by inhibiting the synthesis and activity of MMPs is a meaningful approach[19]. As Wang et al[20] found, overexpression of CDMP-1 and TGF-β biologically promote rabbit and

human intervertebral disc cell proliferation, which suggests it is feasible to treat IDD by CDMP-1 intervention. Li et al[21] also reported that CDMP-1 gene knockout mice had disordered fibrous structures in the disc with decreased collagen II and aggrecan gene expression, and recombinant human CDMP-1 could promote collagen II and aggrecan production of the mouse intervertebral disc cells with a dose-dependent.

The previous studies do not fully explain how the CDMP-1 mediates collagen and aggrecan synthesis. CDMP1, like other members of the TGF-β superfamily, transduces signals through the membrane *via* receptors. However, CDMP1 only binds to ALK6, BMPR-II, and ActR-II, and the activated receptor transmits the message to the nucleus through Smad to regulate downstream gene expression[22]. In addition to the Smad pathway, CDMP1 can also play a signal transduction role by activating p38 mitogen-activated protein kinase[23]. Therefore, we hypothesized ALK6 should play a vital role in the CDMP-1 mediated collagen II and aggrecan synthesis. Exogenic CDMP-1 stimulation reversed IL-1β induced NP cells collagen II and aggrecan reduction. The blocking of ALK6 alleviated the effect of CDMP-1, indicating the ALK6 was possibly responsible for the CDMP-1 induced collagen II and aggrecan synthesis. Additionally, the overexpression of ALK6 magnified the impact of CDMP-1 suggested the promotion of collagen II and aggrecan by CDMP-1 relying on the mediation of ALK6. To balance the ECM stability, CDMP-1 also presented the ability of MMPs suppression and TIMP4 production, which was meaningful to the collagen II and aggrecan maintenance.

Conclusions

Our study, for the first time, elucidates the protective effect of CDMP-1 in the ECM protection by enhancing collagen II and aggrecan synthesis of degenerated NP cells, which is related to the mediation of ALK6. Targeting the activation of CDMP-1 and ALK6 in the degenerated NP can regulate the synthesis of ECM, which is a novel strategy in the biological treatment for disc degeneration diseases in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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