

Original Article

TGF- β 1 suppresses syndecan-2 expression through the ERK signaling pathway in nucleus pulposus cells

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Abstract: Intervertebral disc degeneration (IVDD) is the main cause of low back pain and has become a worldwide problem causing enormous economic loss. Thus, mechanisms and treatment of IVDD are attracting great attention from surgeons and physicians. The syndecan (SDC) family has been reported to play important roles in various physiopathologic processes. In this study, we found that SDC2 expression levels were positively correlated with IVDD grades in human samples. Moreover, we demonstrated that transforming growth factor- β 1 inhibited SDC2 expression through ERK1/2 signaling pathway activation in nucleus pulposus cells. Knocking down SDC2 in disc cells significantly suppressed aggrecanase-1 and aggrecanase-2 expression. The results of our study indicate that SDC2 may be a therapeutic target through which extracellular matrix degradation of IVDD can be controlled.

Keywords: Syndecan-2, intervertebral disc, degeneration, transforming growth factor- β 1, ERK signaling

Introduction

Intervertebral discs (IVDs) are an important component of the spinal column and consist of an outer ligamentous annulus fibrosus (AF) and an enclosed gel-like nucleus pulposus (NP). These components play important roles in resisting spinal compression and allowing limited movement [1]. Research published in the Lancet has indicated that IVD degeneration (IVDD) leads to lower back pain that may reduce patient quality of life. Thus, IVDD has become a worldwide problem that causes enormous economic loss [2, 3]. Understanding mechanisms underlying IVDD will be helpful in preventing and treating IVDD-related diseases and lower back pain.

Transforming growth factor- β (TGF- β) is involved in extracellular matrix (ECM) metabolism and cell proliferation in IVD. Recent studies have demonstrated that TGF- β 1 has protective effects against IVDD. These effects include promoting NP cell proliferation, stimulating ECM synthesis, and inhibiting matrix metalloprotein-

ase (MMP) and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) expression [4, 5]. Our previous work has also shown that TGF- β 1 exhibits anti-inflammatory effects by downregulating chemokine CCL4 expression in NP cells and rat IVDD models [6].

Syndecans (SDCs) are a family of 4 transmembrane heparan sulfate proteoglycans (HSPGs) that can act as co-receptors to help bind and modify the action of various growth factors and cytokines [7]. SDC4 plays important roles in osteoarthritis by binding to ADAMTS5 [8]. Our previous work has also reported that pro-inflammatory cytokines IL-1 β and TNF- α could induce SDC4 expression through activating NF- κ B pathways [9]. A recent study suggested that the cytoplasmic domain of syndecan-2 upregulates MMP-7 expression in colon cancer cells via activation of PKC γ -mediated FAK/ERK signaling [10]. Mansouri R et al. found that syndecan-2 functions as an inhibitor of Wnt- β -catenin-T-cell factor signaling pathways. This inhibition activates glycogen synthase kinase 3 and then decreases Wnt-dependent production

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Table 1. Information of human disc samples from 11 patients

Human disc sample	Sex	Age	Diagnosis	Level	Grade
1	F	13 y	AIS	L2/3	I
2	M	17 y	AIS	L3/4	I
3	F	18 y	AIS	T9/10	II
4	F	16 y	AIS	T7/8	II
5	M	25 y	Disc herniation	L4/5	III
6	F	28 y	Disc herniation	L2/3	III
7	M	31 y	Disc herniation	L3/4	III
8	M	46 y	Disc herniation	L4/5	IV
9	F	54 y	Disc herniation	L2/3	IV
10	F	54 y	Disc herniation	L4/5	V
11	F	75 y	Disc herniation	L3/4	V

of Wnt ligands and R-spondin [11]. Although SDC2 is an important member of the SDC family, its role in IVD pathological and physiological changes has drawn little attention. Moreover, its regulation by cytokines or growth factors such as TGF-β1 remains unclear.

Thus, the objective of this study was to investigate regulatory effects of TGF-β1 on SDC2 expression and underlying mechanisms during the process of IVDD. This study also aimed to identify a potential therapeutic target for treating human IVDD.

Materials and methods

All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University and all experiments were performed in accordance with approved guidelines for the care and use of laboratory animals of the above institution.

Human tissue collection and grading

From April 2015 to August 2017, we collected 11 disc samples from 10 patients (male:female, 3:7) whose average age was 34.3 years (range, 13-75 years). Detailed information regarding these samples is listed in **Table 1**. Each patient provided informed consent for sample collection before participation in the study. Patient disease states were evaluated using the Pfirrmann grading scheme which entails use of T2-weighted magnetic resonance imaging (MRI) and independent image analyses by three observers. Additional data on NP tissue

specimens used for cell isolation and specimen donors can be made available upon request to the corresponding author.

Isolation and culture of primary NP cells

NP cells were isolated from adult Wistar rats (300-350 g) using our previously reported method [12]. NP cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA) and 10% fetal bovine serum (FBS, Invitrogen, CA) supplemented with antibiotics (Invitrogen, CA) at 37°C in a humidified atmosphere containing 5% CO₂.

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from NP cells using TRIzol Reagent (Shenbang, China). Purified DNA-free RNA was transcribed into cDNA using a ReverTra Ace® qRT-PCR kit (TOYOBO, Japan). Reactions were set up in triplicate in 96-well plates using SYBR Green PCR Master Mix (Applied Biosystems). qRT-PCR analyses were performed to detect target mRNA expression using a real-time IQ5 system (Bio-Rad, USA) and β-actin was used as an internal control. The total volume of reaction mixture used for each PCR was 20 μL which included 10 μL of SYBR Green Master Mix, 0.6 μL of each primer (10 μmol/L), 2 μL of cDNA template, and 6.8 μL of ddH₂O. The following PCR conditions were used for experiments: 95°C for 15 minutes, 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 30 seconds for 40 cycles. Relative gene expression levels were normalized to β-actin expression levels and data were presented as fold changes using the formula 2^{-ΔΔCT} as recommended by the manufacturer. Sequences of the primers used herein are listed in **Table 2**.

Immunohistochemical (IHC) staining for SDC2 expression in NP tissue

IHC staining of tissue sections harvested from normal and degenerated NP tissues was performed to determine SDC2 levels. After antigen retrieval, endogenous peroxidase quenching, and nonspecific binding blocking, the sections were incubated with a mouse polyclonal antibody against SDC2 (Santa Cruz Biotechnology; 1:200 dilution) and then an HRP-conjugated secondary antibody. The number of immuno-

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Table 2. Primers used for qRT-PCR analysis of gene expression

Rat-GAPDH-F	GCAAGAGAGAGGCCCTCAG
Rat-GAPDH-R	TGTGAGGGAGATGCTCAGTG
Rat-SDC2-F	ATTCTTCTGCCTCTGGCTCA
Rat-SDC2-R	GACTCGGTCTGAGTGGGTGT
Human-SDC2-F	CATCTCCCCCTTGCTAACGGC
Human-SDC2-R	TAATCCATCTCCTTCCCCAGG
Human-GAPDH-F	GGA AGGTGA AGGTCTGGAGTCA
Human-GAPDH-R	GTCATTGATGGCAAC AATATCCAAT
Rat-ADAMTS4-F	ACAATGGCTATGGACTGCCTCT
Rat-ADAMTS4-R	TGTGGACAATGGCTTGAGTCAGGA
Rat-ADAMTS5-F	GTCCAAATGCACTTCAGCCACGAT
Rat-ADAMTS5-R	AATGTCAAGTTGCACTGCTGGGTG

positive cells in NP tissues was counted in five high-power fields ($\times 400$) by independent researchers blinded to the study information. Then, the percentage of immunopositive cells was calculated by dividing the number of immunopositive cells by total number of NP cells and multiplying that number by 100.

Immunofluorescence confocal microscopy

NP cells were seeded on flat-bottom 96-well plates (5×10^3 /well). After treatment and incubation, the NP cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100 in phosphate-buffered saline (PBS) for 10 minutes, blocked with PBS containing 1% BSA, and incubated with rabbit polyclonal anti-phospho-ERK1/2 antibody (CST, MA) (1:1,000). Then, cells were incubated overnight at 4°C. After being washed, the NP cells were incubated with an Alexa Fluor-488 conjugated anti-rabbit secondary antibody (Invitrogen, CA, USA) (1:1,000) for 1 hour at room temperature. The cells were then imaged with a laser scanning confocal microscope using a 20 \times /0.4 LCPlanFI objective (Olympus, Tokyo, Japan).

siRNA and transfection

Rat SDC2 siRNA used herein was constructed by GenePharma (Suzhou, China). Rat NP cells were seeded in a 6-cm dish (5×10^6 cells) one day before transfection. Lipofectamine 2000 (Invitrogen) served as the transfection reagent. Fifteen microliters of siRNA (10 nM) and 20 μ L of Lipofectamine were separately diluted in 500 μ L of opti-MEM. Five minutes later, they were mixed together and incubated for 20 min-

utes. This mixture and 5 ml of opti-MEM were then added to the above dish. After 6 hours, the medium was replaced with fresh complete medium. Cells were treated and harvested for RNA or protein extraction three days after transfection.

Western blot analysis

NP cells were placed on ice immediately after treatment and washed twice with ice-cold PBS. Cells were lysed in RIPA buffer and total protein was extracted and quantified using a BCA protein assay kit (Pierce, USA). Total cellular protein samples (20 μ g per well) were resolved via 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore, USA) via electroblotting. The membranes were blocked in 5% non-fat dry milk with TBST and incubated with the following primary antibodies overnight at 4°C: anti-SDC2 antibody (Santa Cruz Biotechnology; 1:500 dilution), anti-ERK1/2 antibody (CST, MA) (1:1,000), anti-phospho-ERK1/2 antibody (CST, MA) (1:1,000), and anti-GAPDH antibody (CST, MA). After being washed with TBST, membranes were incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies, which were subsequently detected with ECL Plus reagent (Millipore, USA). Results of this experiment were quantified using a multi-gauge densitometry system (Fujifilm, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS 18 software (SPSS, Inc., Chicago, IL). One-way analysis of variance (ANOVA) and Student's t-test were used to analyze differences between groups. Results are presented as mean \pm S.E.M. $P < 0.05$ was considered statistically significant.

Results

SDC2 expression in normal and degenerated human IVD tissues

First, we compared expression levels of SDC2 in human NP and AF tissues. SDC2 mRNA and protein expression levels in NP tissue were significantly higher than those in AF tissue (**Figure 1A, 1B**). Representative MRI images of different degenerative discs are shown in **Figure 1C** (from the left, Grades I, II, III, IV, and V). Then, we examined SDC2 expression in different

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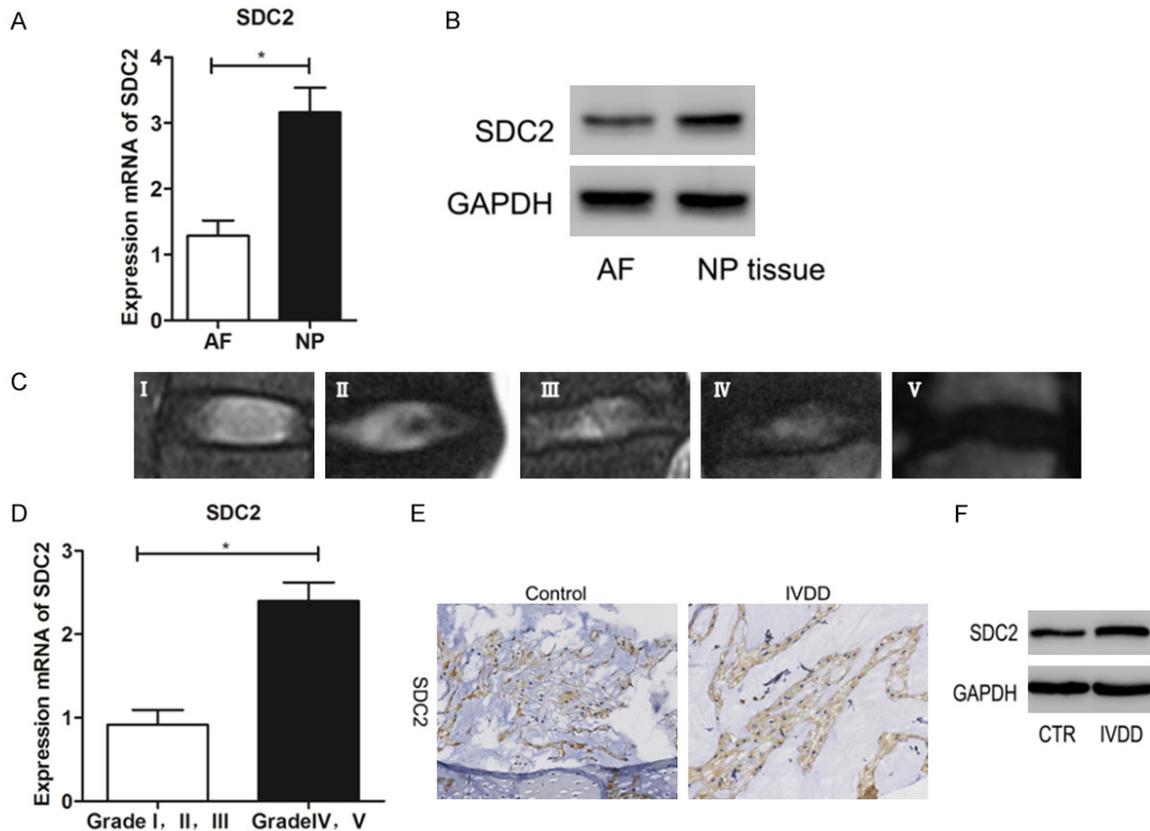


Figure 1. SDC2 expression levels in normal and degenerated human IVD tissues. A, B. SDC2 expression in human AF and NP tissues. C. Representative MRI images of different degenerative discs. D. SDC2 mRNA expression in different degenerative discs. E, F. SDC2 protein expression in normal and degenerated NP tissues.

degenerative human IVD tissues using real-time RT-PCR analyses. SDC2 mRNA expression levels in severely degenerative NP tissue [Grades IV ($n = 3$) and V ($n = 4$)] were 2.54-fold higher ($P = 0.003$) (**Figure 1D**) than those in mildly degenerative NP tissue [Grades I ($n = 1$), II ($n = 1$), and III ($n = 2$)]. We also evaluated SDC2 expression in normal and degenerated NP tissues via immunohistochemical staining analyses. SDC2 immunoreactivity levels ($31.00 \pm 4.36\%$ vs. $14.67 \pm 2.60\%$, $P = 0.032$) in degenerated NP tissue were significantly higher than those in normal NP tissue (**Figure 1E**).

TGF-β1 suppresses SDC2 expression in NP cells

To investigate the effects of TGF-β1 on SDC2 mRNA and protein expression, we treated NP cells with different doses of TGF-β1 at different time points, as indicated previously. Our RT-PCR results showed that TGF-β1 treatment decreased SDC2 mRNA expression to levels that were 0.75-fold ($P = 0.021$), 0.68-fold ($P =$

0.019), and 0.56-fold ($P = 0.003$) lower than untreated levels at 4 hours, 8 hours, and 24 hours post-treatment, respectively (**Figure 2A**). In addition, TGF-β1 decreased SDC2 mRNA expression levels at different doses (**Figure 2B**). Western blotting results showed that SDC2 protein expression levels were also decreased in a time- and dose-dependent manner after TGF-β1 treatment (**Figure 2C, 2D**).

ERK1/2 signaling pathway inhibition blocks TGF-β1-mediated SDC2 suppression

To confirm that the ERK1/2 signaling pathway is required for TGF-β1-mediated inhibition of SDC2 expression in NP cells, we evaluated ERK1/2 signaling pathway activity by Western blotting after cells were treated with TGF-β1. We noted a rapid increase in ERK1/2 phosphorylation after TGF-β1 treatment. We also noted that ERK1/2 activity levels peaked at 5-15 minutes post-treatment and decreased thereafter (**Figure 3A**). We further confirmed activation of ERK signaling by immunofluores-

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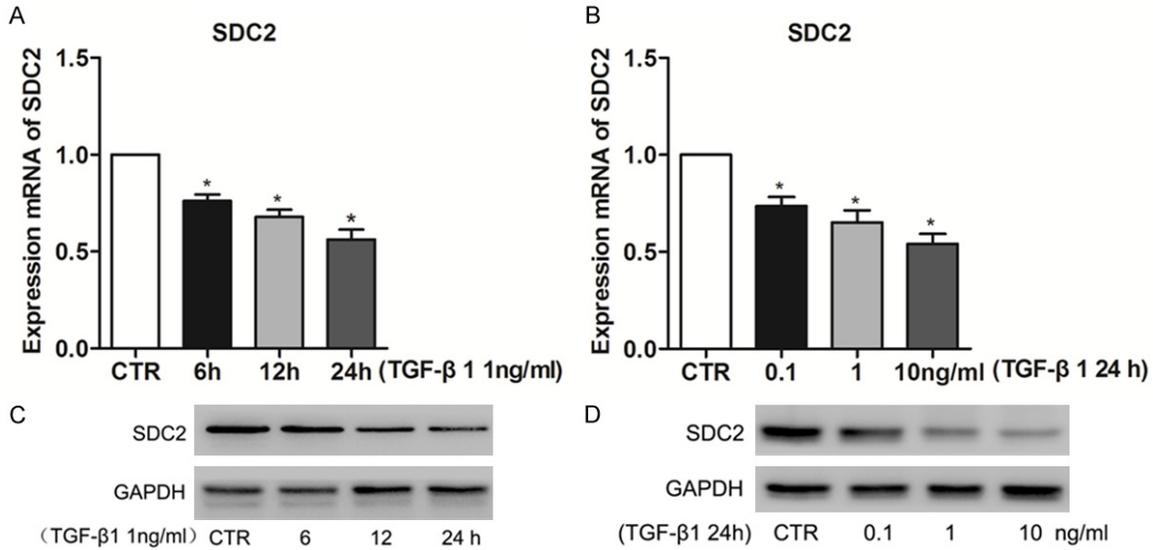


Figure 2. TGF-β1 suppresses SDC2 expression in NP cells. A, B. SDC2 mRNA expression at different doses and time points of TGF-β1 treatment. C, D. SDC2 protein expression at different doses and time points of TGF-β1 treatment.

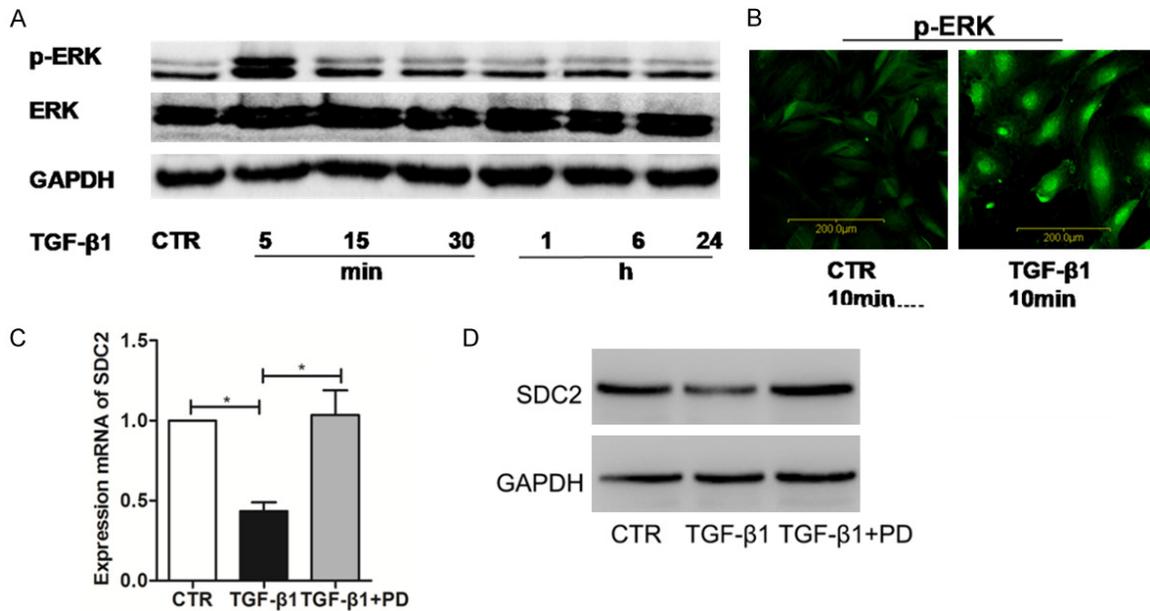


Figure 3. ERK1/2 signaling pathway inhibition blocks TGF-β1-mediated SDC2 suppression. A. ERK1/2 phosphorylation was rapidly increased post-TGF-β1 treatment. B. Phospho-ERK fluorescence intensity was strong at 10 min after TGF-β1 treatment. C, D. TGF-β1-mediated down-regulation of SDC2 mRNA and protein expression was significantly antagonized by PD98059.

cence confocal microscopy (**Figure 3B**). To determine whether TGF-β1 suppresses SDC2 expression through ERK1/2 signaling activation, we treated NP cells with ERK1/2 signaling-specific inhibitor PD98059. We found that downregulation of SDC2 mRNA expression by TGF-β1 was significantly antagonized by PD98059 (**Figure 3C**). Similarly, PD98059 could

also restore SDC2 protein expression levels (**Figure 3D**).

SDC2 is essential for ADAMTS4 and ADAMTS5 expression

To explore the role of SDC2 in NP cells, we constructed SDC2-specific siRNA. Our qRT-PCR

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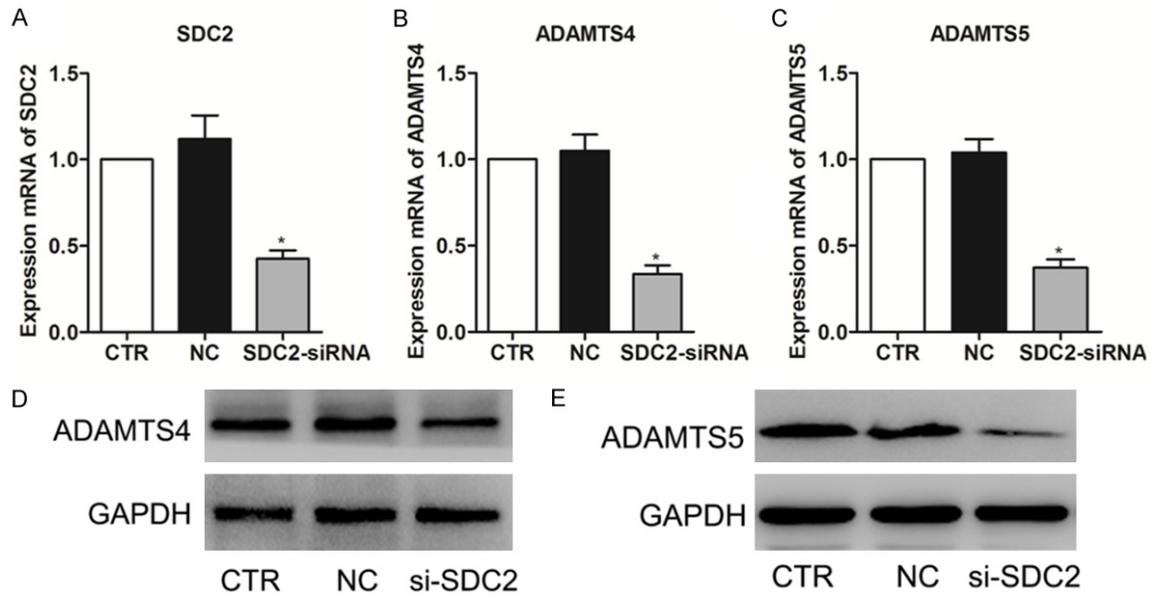


Figure 4. SDC2 is essential for ADAMTS4 and ADAMTS 5 expression. A. SDC2 mRNA expression levels were decreased significantly after siRNA pretreatment. B, C. ADAMTS4 and ADAMTS5 mRNA expression levels were significantly reduced by SDC2 siRNA transfection. D, E. ADAMTS4 and ADAMTS5 protein expression levels were significantly reduced by SDC2 siRNA transfection.

results showed that SDC2 mRNA expression levels decreased significantly after siRNA pretreatment (**Figure 4A**). We found that compared to those in the control group, ADAMTS4 and ADAMTS 5 expression levels were significantly reduced by SDC2 siRNA transfection (**Figure 4B, 4C**). Confirming this finding, Western blotting revealed that ADAMTS4 and ADAMTS5 protein levels were significantly suppressed by SDC2 siRNA transfection (**Figure 4D, 4E**).

Discussion

In this study, we demonstrated that SDC2 expression levels are higher in NP tissues and positively correlate with IVDD grades in human samples. Moreover, for the first time, we demonstrated the regulatory mechanism underlying effects of TGF- β 1 on SDC2 expression in NP cells. Specifically, we demonstrated that TGF- β 1 inhibited SDC2 expression through activating the ERK1/2 signaling pathway in NP cells.

SDCs are a family of transmembrane sulfated proteoglycans found in most organs such as cartilage and liver [13]. We show, for the first time, that SDC2 is expressed in intervertebral disc tissue. In addition, NP tissue had much higher SDC2 expression levels than AF tissue. NP and AF tissues come from distinct embryological origins. Thus, differential expression by

these two tissues may indicate a tissue-specific role for SDC2. Moreover, higher expression levels of SDC2 in degenerated NP tissues reflect an intimate relationship between SDC2 and IVDD.

Numerous studies have reported that TGF- β 1 has multiple roles in the process of IVDD. Risbud MV et al. showed that TGF- β 1 regulated galectin-3 expression through the canonical Smad3 signaling pathway in NP cells. This mechanism is thought to occur in intervertebral disc degeneration [14]. Furthermore, another study demonstrated that TGF- β 1 can suppress expression of proinflammatory cytokines, such as IL-1 β and TNF- α , and thus inhibit inflammatory response in degenerative discs [15]. Colombier P et al. showed that TGF- β 1 and GDF5 act synergistically to drive the differentiation of human adipose stromal cells toward NP-like cells [16]. These findings provide valuable insights for developing biologically inspired treatments for IVDD such as generating adapted and exhaustively characterized autologous regenerative cells. The unknown effects of TGF- β 1 on SDC2 expression were our inspiration for completing this study.

Although SDC2 has diverse physiological functions in different organs, how its expression is regulated is not yet completely understood.

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SDCs may interact with several ECM molecules, growth factors, and cytokines [17]. Interestingly, cytokines and growth factors could also regulate SDC expression. Regulation of SDC expression by cytokines and growth factors occurs in a cell type-specific manner. Hara T et al. found that TGF- β 1 first upregulates and then downregulates expression of syndecan-4 via the TGF- β receptor ALK5 in cultured vascular endothelial cells [18]. In human periodontal ligament fibroblasts (PDLFs), osteoblasts (OBs), and gingival fibroblasts (GFs), Worapamorn W et al. found increased syndecan-1 mRNA levels in response to either PDGF-BB or TGF- β 1 and decreased levels in response to IL-1 β . The effect of IL-1 β on syndecan-1 mRNA synthesis was partially reversed after adding PDGF-BB and TGF- β 1 [19]. Our study clearly shows that treating NP cells with TGF- β 1 significantly suppresses SDC2 mRNA and protein expression levels in a time- and dose-dependent manner.

MAPKs are a particular class of serine/threonine kinases that includes the p38 MAPK, ERK, and JNK families. In mammalian cells, these families respond to extracellular stimuli such as growth factors, neurotransmitters, hormones, cell stress, and cell adhesion [20]. It has been reported that MAPK signaling plays important roles in the inflammatory response associated with IVDD [21]. Our previous work found that the ERK pathway is a main regulator of the effects of TGF- β 1 on CCL4 expression in NP cells [6]. Consistent with these results, we also demonstrate that TGF- β 1 treatment can activate ERK signaling via phosphorylated-ERK overexpression. Our silencing studies, using an ERK inhibitor, showed that TGF- β 1-dependent SDC2 suppression is attenuated. This finding highlights the function of ERK signaling pathway in regulating SDC2 expression via TGF- β 1.

Among several members of the ADAMTS family that cleave aggrecan *in vitro*, ADAMTS4 (aggrecanase-1) and ADAMTS5 (aggrecanase-2) are the most likely to play a role in degradation of aggrecan and the subsequent pathogenesis of disc degeneration [21]. Interestingly, we found that knocking down SDC2 by using SDC2 siRNA in NP cells significantly suppresses ADAMTS4 and ADAMTS5 expression. This finding indicates a close relationship between SDC2 and ECM degradation. The role of the SDC family in

this process has been well-studied. In an osteoarthritis animal model, SDC4 controls ADAMTS5 activation through direct interaction with protease and through regulating MAPK-dependent synthesis of matrix metalloproteinase-3 (MMP-3) [8]. Another study found that synthetic cannabinoid WIN-55 inhibits ADAMTS4 activity in unstimulated and IL-1 β -stimulated human osteoarthritis articular chondrocytes by decreasing mRNA stability/expression of SDC1 via the cannabinoid receptor [22].

Based on our findings, we propose that TGF- β 1 downregulates SDC2 expression in NP cells through activating the ERK signaling pathway. Furthermore, we suggest that SDC2 plays a role in ADAMTS4 and ADAMTS5 expression. The results of our study indicate that SDC2 is a therapeutic target through which ECM degradation of IVDD can be controlled.

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Disclosure of conflict of interest

None.

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