

## Original Article

# HtrA1 upregulates the expression of ADAMTS-5 in HNPCs via the ERK/NF- $\kappa$ B/JNK signaling pathway

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**Abstract:** Intervertebral disc degeneration (IDD) is a form of chronic inflammation and is one of the most common disorders reported to be involved in low back pain (LBP). The pathophysiology of degeneration is not completely understood, but the consensus is that the degradation of extracellular matrix (ECM) proteins in the disc is the leading factor contributing to IDD. High temperature requirement A1 (HtrA1) is serine protease that has been shown to be increased in degenerated intervertebral discs as a result of an increase in the expression of matrix metalloproteinases (MMPs), but no study has focused on the effect of HtrA1 on a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTSs). In the present study, we successfully isolated human nucleus pulposus cells (HNPCs) from IDD patients who were our research subjects to elaborate on the potential role of HtrA1 in the pathogenesis of IDD. We confirmed that HtrA1 has the potential to induce the expression of ADAMTS-5 in a dose-dependent manner. Consistently, this was mediated by the ERK, NF- $\kappa$ B and JNK pathways. By using inhibitors of these pathways, the increase in ADAMTS-5 could be reduced. Our findings indicated that HtrA1 can induce the expression of ADAMTS-5 in HNPCs via the ERK/NF- $\kappa$ B/JNK signaling pathway, and our study also elucidated the involved induction mechanisms in HNPCs, which may provide new insights for the treatment of IDD.

**Keywords:** Intervertebral disc degeneration, high temperature requirement A1, a disintegrin-like and metalloproteinase with thrombospondin motifs, extracellular matrix, human nucleus pulposus cells

## Introduction

Low back pain (LBP) is a leading cause of morbidity. It is estimated that nearly 70 percent of the population will experience LBP during their lifetime [1]. Recently, intervertebral disc degeneration (IDD) has been shown to be a significant contributor to LBP and spine-related disability. Disc degeneration is a chronic process that can lead to radiculopathy, herniation, spinal stenosis, myelopathy and degenerative spondylolisthesis by altering the structure and function of the intervertebral discs (IVD) and leading to painful conditions [2].

The IVD is the largest avascular organ in the body that consists of highly differentiated cells and extensive extracellular matrix and is com-

posed of three interrelated structures: a central nucleus pulposus (NP), the surrounding annulus fibrosus (AF), and the cartilaginous and bony endplates [3]. In a healthy disc, the NP is highly hydrated, and adequate encapsulated and proteoglycan-rich extracellular matrix (ECM) maintain the internal pressure of the IVD [4]. During disc degeneration, significant changes are observed in the IVD, among which NP matrix remodeling has also been shown to be an early step in the aging process. There is great interest in understanding the pathogenesis of NP matrix remodeling, and recent reports of the existence of NP cells in IVD have provided new insights into the pathophysiology of disc degeneration. High temperature requirement serine protease A1 (HtrA1) is a conserved PDZ serine protease that belongs to the HTRA family of ser-

ine proteases, which comprises four known members (HtrA1, -2, -3, and -4). Since its initial identification 15 years ago, HtrA1 has been extensively studied and has been shown to be involved in numerous basic biological mechanisms in mammals [5]. It is a secreted enzyme that is widely expressed in human cells and tissues [6]. In humans, HtrA1 has been linked to the pathogenesis of microvascular and aging-related diseases such as age-related macular degeneration [7], arthritis [8], familial small vessel disease [9], CARASIL syndrome [10], and tumor progression [11]. Our previous study [12] found that HtrA1 plays an important role in the pathological process of IDD. However, the mechanism(s) by which it regulates these processes has not been fully elucidated in IDD.

It is known that degenerative changes in the NP are associated with the degradation of the ECM by locally produced matrix metalloproteinases (MMPs) and aggrecanases, which belong to the ADAMTS family, leading to tissue stiffness and the impairment of the structural integrity and function of the IVD [13]. Collagens and aggrecans are the major components of the ECM in the IVD and are synthesized by the IVD, and they are broken down by MMPs and aggrecanases [13] to maintain dynamic equilibrium. Our previous study [14] demonstrated that HtrA1 upregulated the expression of MMPs via ERK1/2/ROCK-dependent pathways during IDD. Similar to MMPs, ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are key extracellular metalloproteinases involved in ECM turnover that have been implicated in tissue destruction [15] and inflammation [16]. ADAMTS-4 has been reported to participate in the development of vascular diseases [17], and ADAMTS-5 has been implicated in osteoarthritis [18]. However, no study has reported a correlation between HtrA1 and ADAMTS-4 or -5 during the progression of IDD, and the underlying mechanism(s) has not been explained.

### Materials and methods

#### *Isolation and identification of human nucleus pulposus cells (HNPCs)*

NP tissue samples were obtained from five patients who had undergone posterior discectomy and fusion surgeries, and the HNPCs were isolated as previously described [19]. Informed

consent was obtained from each patient, and the study was approved by the Ethics Committee of the Affiliated Hospital of Jiangsu University (JDFYLL-2015016). Briefly, NP tissue was obtained from the inner gelatinous part of the IVD. In a clean bench, the NP tissues were separated and harvested without any visible contamination by other tissues and then mechanically minced into small pieces (<1 mm<sup>3</sup>). These pieces were digested with 0.25% collagenase II (Sigma Aldrich, St Louis, USA) for 4 h at 37°C. Then, the samples were resuspended in phosphate-buffered saline (PBS), filtered through a 200-mesh filter and centrifuged for 5 min at 500 g. The cell pellets were cultured in a monolayer in Dulbecco's modified Eagle medium (DMEM-L; Keygen Biotech, Nanjing, China) containing 10% fetal calf serum (Gibco, Gaithersburg, USA) and 1% penicillin/streptomycin. When the cells reached 80% confluence, the harvested primary cells were subcultured on coverslips in six-well plates for 24 h and rinsed thrice with PBS on the following day. The cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 15 min and blocked with 3% bovine serum albumin for 30 min at room temperature. After incubation with rabbit anti-Col-2, KRT-18, KRT-19, GLUT-1, HIF-1 $\alpha$ , Sox-9, ACAN, and CD24 (Abcam, Cambridge, UK) overnight at 4°C, the coverslips were incubated with secondary antibodies for 20 min and counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) for nuclear imaging. Finally, the cells were examined using confocal laser scanning microscopy (Leica, Wetzlar, Germany).

#### *Cell culture and treatment*

Isolated HNPCs were cultured in 6-well plates using nucleus pulposus cell medium (ScienCell, USA) with 10% fetal bovine serum supplemented with antibiotics to enable the cells to grow to 80% confluence. After the cells reached confluence, the cells were challenged with exogenous recombinant HtrA1 (rHtrA1, CloudClone Corp) alone or in combination with p38, ERK, NF- $\kappa$ B, the JNK signaling pathway inhibitor SB203580 (Sigma-Aldrich, China), SCH772984 (MCE, China), QNZ (Selleck, China), or SP600125 (MCE, China) at a dosage of 10  $\mu$ mol/l, and untreated cells were used as a control. The cells were harvested at different time points (0, 6, 12 and 24 h) after HtrA1 challenge and then

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stored at  $-80^{\circ}\text{C}$  prior to the observation of the temporal expression pattern of mRNAs and proteins. In addition, the cell culture supernatant was collected for the measurement of ADAMTS-4 and ADAMTS-5 by using specific ELISA kits.

### *Quantitative RT-PCR (RT-qPCR)*

The mRNA levels of ADAMTS-4 and ADAMTS-5 were analyzed by RT-qPCR according to a previously described method. Briefly, mRNA was extracted from HNPCs. Cells from 3 wells were pooled to collect sufficient mRNA for each sample. Total RNA was extracted by using Trizol reagent (Life Technologies), and 500 ng total RNA was transcribed into cDNA according to the manufacturer's instructions using an RT reagent kit (TaKaRa, Ohtsu, Japan) with oligo (dT) primers. For quantitative real-time PCR, cDNA (1  $\mu\text{L}$ ) was amplified by real-time PCR with the SYBR Green Premix EX Taq kit (TaKaRa, Ohtsu, Japan) by using the following primer sets: ADAMTS-4 sense 5'-GGTCCTGGGCCCT-AGTTGTATTTA-3' and antisense 5'-AAGCAGG-GCAGGTTGTACCTCATA-3'; ADAMTS-5 sense 5'-CGC AGATAC AGCTGTGGTGAAGA-3' and antisense 5'-ATTGTGTGCTC CTTTGTGTAGGCC-3'. Each sample was analyzed in triplicate with a CFXA96 Thermal Cycler, and the relative mRNA expression was calculated with the comparative threshold cycle (Ct) method.  $\beta$ -actin was used as an internal control.

### *Western blotting analysis*

HNPCs were homogenized and lysed in RIPA buffer supplemented with proteinase inhibitors. Equal amounts of total protein were loaded and separated on a 12% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to a PVDF membrane (Millipore, USA), blocked in 5% (w/v) nonfat milk, and incubated with the primary antibodies. The membranes were incubated with monoclonal antibodies against  $\beta$ -actin (CW BIO, CW0096), ADAMTS-4 (Bioworld Technology, BS1229), ADAMTS-5 (Abcam, ab52915), p38 (Santa Cruz, sc-8832), p-p38 (Santa Cruz, sc-101759), ERK (Santa Cruz, sc-514302), p-ERK (Santa Cruz, sc-101760), p-65 (Abcam, ab16502), p-p-65 (Santa Cruz, sc-136548), JNK (Santa Cruz, sc-7345), and p-JNK (Santa Cruz, sc-6254) at  $4^{\circ}\text{C}$  overnight. After three washes, HRP-conjugated secondary antibody

was added and incubated for 1 h at  $37^{\circ}\text{C}$ . Detection was performed with enhanced chemiluminescence (ECL), and the relevant blots were quantified by densitometry by using a computerized image analysis program.

### *Enzyme-linked immunosorbent assay (ELISA)*

The concentrations of ADAMTS-4 and ADAMTS-5 in the cell culture supernatants were measured according to the manufacturer's protocols (eBioscience, San Diego, CA, USA) by ELISA. All samples were analyzed in batches and in triplicate, and the optical density (OD) value was detected using an ELISA microplate reader (Labequip Ltd, Ontario, Canada) at a wavelength of 450 nm.

### *Statistical analysis*

All statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA, USA). Comparisons between groups were conducted by using the unpaired *t* test. The association between 2 clinicopathological variables was determined using the Spearman test. A *p*-value  $<0.05$  was considered to indicate a statistically significant difference.

## **Results**

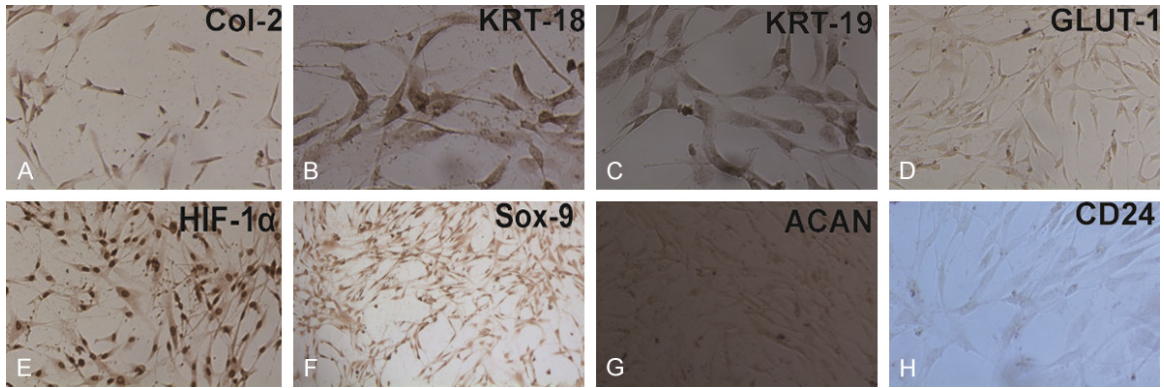
### *Morphology and characteristics of HNPCs*

The successful isolation of HNPCs was confirmed by IHC. The results showed that the NP cells from donors with degeneration had increased expression of Col-2, KRT-18, KRT-19, HIF-1 $\alpha$ , GLUT-1, Sox-9, ACAN, and CD24, and the positive expression rates were nearly 80% (**Figure 1**).

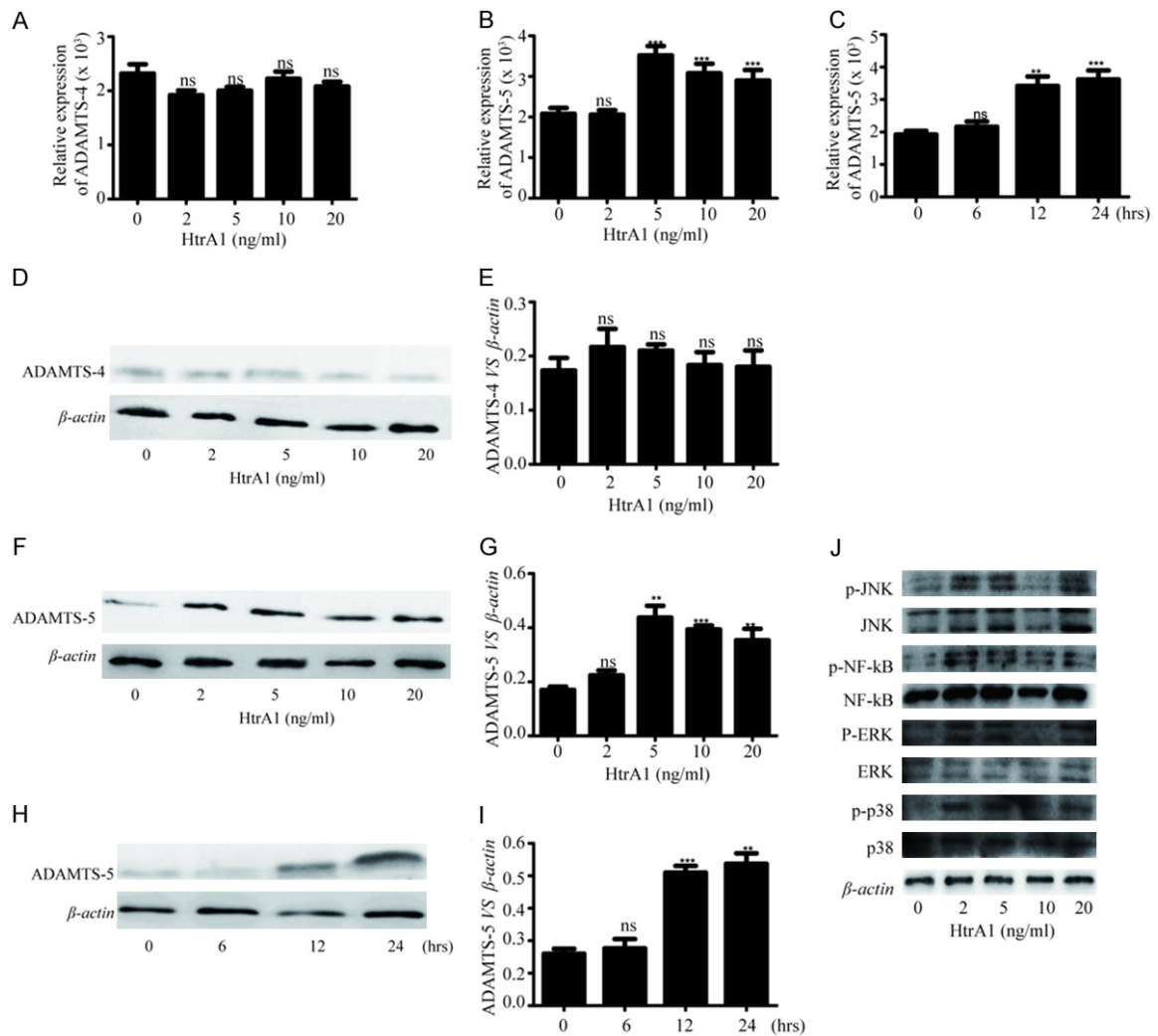
### *HtrA1 upregulated the protein expression of ADAMTS-5*

To determine the effects of HtrA1 on ADAMTS-4 and ADAMTS-5 expression, HNPCs were treated with exogenous rHtrA1 (2  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , 10  $\mu\text{g}/\text{ml}$  and 20  $\mu\text{g}/\text{ml}$ ), and the cells and culture supernatants were harvested at different time points (0, 6, 12 and 24 h). The mRNA and protein expression of ADAMTS-4 and ADAMTS-5 was examined by real time RT-PCR, Western blotting and ELISA. It is noteworthy that we found that the expression of ADAMTS-5 induced by exogenous rHtrA1 was increased in a dose-

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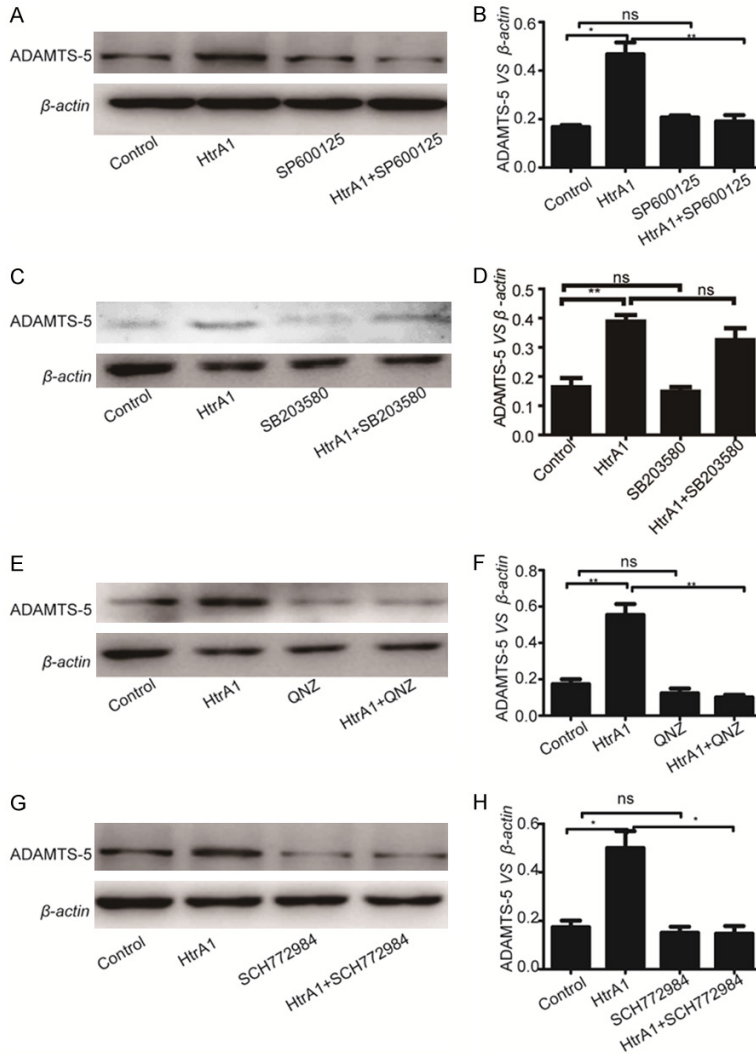


**Figure 1.** Morphology and characteristics of HNPCs. The results showed that NP cells from donors with degeneration have increased expression of Col-2 (A), KRT-18 (B), KRT-19 (C), GLUT-1 (D), HIF-1 $\alpha$  (E), Sox-9 (F), ACAN (G), and CD24 (H).



**Figure 2.** HtrA1 upregulated the protein expression of ADAMTS-5 via the ERK, NF- $\kappa$ B and JNK signaling pathways. A. The mRNA expression of ADAMTS-4 after treatment with different dosages of rHtrA1 (2  $\mu$ g/ml, 5  $\mu$ g/ml, 10  $\mu$ g/ml and 20  $\mu$ g/ml). B. The mRNA expression of ADAMTS-5 after treatment with different dosages of rHtrA1 (2  $\mu$ g/ml, 5  $\mu$ g/ml, 10  $\mu$ g/ml and 20  $\mu$ g/ml). C. The mRNA expression of ADAMTS-5 at different time points at a dosage of 5  $\mu$ g/ml. D-I. Similar expression patterns of ADAMTS-4 and ADAMTS-5 were found for the proteins. J. Challenging HNPCs with rHtrA1 resulted in an increase in the phosphorylation of MAPK, ERK, NF- $\kappa$ B and JNK in HNPCs.

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**Figure 3.** Decreased protein levels of ADAMTS-5 in HNPCs. A, B. The protein expression of ADAMTS-5 was significantly reduced by the JNK inhibitor SP600125. C, D. The protein expression of ADAMTS-5 was not significantly reduced by SB203580. E, F. The protein expression of ADAMTS-5 was significantly reduced by QNZ. G, H. The protein expression of ADAMTS-5 was significantly reduced by SCH772984.

dependent manner in HNPCs, while no obvious significant increase was found for ADAMTS-4. Furthermore, we observed that the ADAMTS-5 protein level peaked at 24 h at a dosage of 5  $\mu$ g/ml (Figure 2).

*Increases in the level of ADAMTS-5 induced by rHtrA1 were reduced by ERK, NF- $\kappa$ B and JNK signaling pathway inhibitors*

We found that challenging HNPCs with rHtrA1 resulted in an increase in the phosphorylation of MAPK, ERK, NF- $\kappa$ B and JNK within HNPCs, and the levels of these proteins peaked at a

dosage of 5  $\mu$ g/ml, which was consistent with the expression of ADAMTS-5 (Figure 2J). To further confirm that the increase was due to the activation of these four signaling pathways, we used signaling pathway inhibitors for all these pathways. We performed real-time RT-PCR and Western blotting to determine the expression of ADAMTS-5 and ADAMTS-4 in HNPCs treated with exogenous rHtrA1 with or without inhibitors. Our results showed that the expression of ADAMTS-4 showed no significant change; however, the expression of ADAMTS-5 was significantly decreased after treatment with SCH772984, QNZ and SP600125, which are inhibitors of the ERK, NF- $\kappa$ B and JNK pathways, respectively (Figures 3, 4 and Supplemental Figure 1).

*Decreased protein level of ADAMTS-5 in cell culture supernatants*

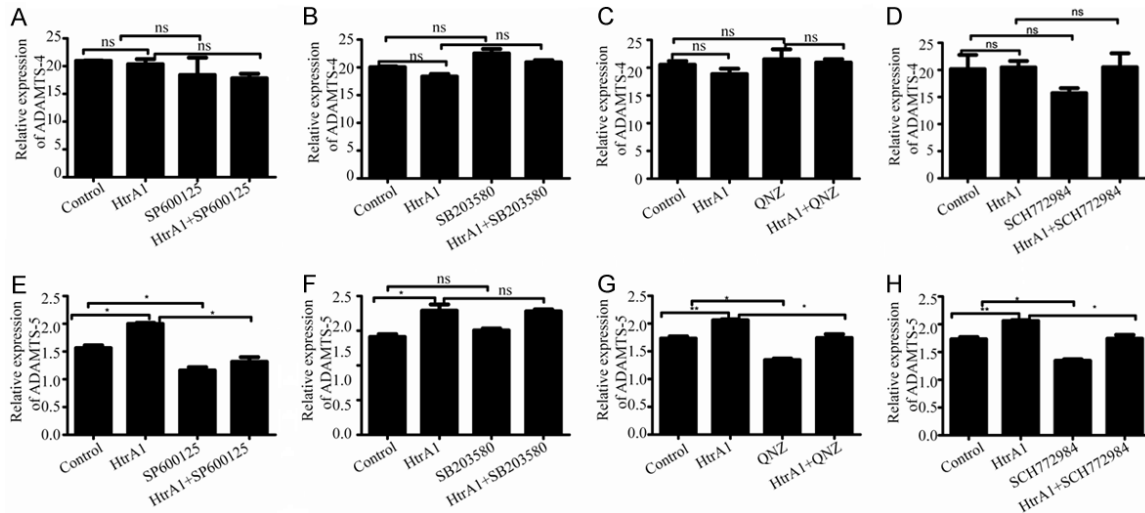
Our results showed that exogenous rHtrA1 could upregulate the expression of ADAMTS-5 in cell culture supernatants. Next, we analyzed the protein levels of ADAMTS-4 and ADAMTS-5 in cell culture supernatants. Exogenous rHtrA1-treated HNPCs were exposed to ERK/NF- $\kappa$ B/JNK signaling pathway inhibitors.

Our data showed that the levels of ADAMTS-5 in the supernatants were obviously decreased, while no change was found in the level of ADAMTS-4 (Figure 4). These results demonstrated that HtrA1 may contribute to ADAMTS-5 upregulation via the ERK/NF- $\kappa$ B/JNK signaling pathways.

### Discussion

IDC is a chronic disease that alters the structure and function of the IVD, leading to painful conditions [20]. Many studies have focused on the process and pathophysiology of IDC; how-

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**Figure 4.** Increases in the levels of ADAMTS-5 induced by rHtrA1 were reduced by ERK, NF- $\kappa$ B and JNK signaling pathway inhibitors. A-D. The protein expression of ADAMTS-4 in cell culture supernatants showed no significant changes in the presence of the signaling pathway inhibitors. E, G and H. The protein expression of ADAMTS-5 in cell culture supernatants was significantly reduced by ERK, NF- $\kappa$ B and JNK signaling pathway inhibitors. F. The protein expression of ADAMTS-5 in cell culture supernatants showed no significant changes in the presence of a p38 signaling pathway inhibitor.

ever, the mechanism underlying degeneration is not well understood. Previous studies have suggested that the decreased anabolism or increased catabolism of extracellular matrix (ECM) proteins expressed in the NP may be an important contributor to IDD [21]. It is commonly accepted that matrix-degrading enzymes play essential roles in the degradation of ECM [22]. There are two predominant families of matrix-degrading enzymes that can hydrolyze aggrecan core proteins: the MMP and ADAMTS families. ADAMTSs comprise a newly discovered family of metalloproteinases that have been implicated in the degradation of aggrecan in IDD [23, 24], among which ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, and ADAMTS-9 have been shown to degrade proteoglycans in joint tissues of patients with osteoarthritis [25]. Previous research has shown that ADAMTS-4 may be associated with lumbar IDD [26]. Caijun Liu et al. reported that elevated ADAMTS-5 expression has the potential to promote the degeneration of IVD [27]. HtrA1 is a serine protease that was shown to promote the expression of MMPs and the progression of IDD in our previous study [14], but the intrinsic mechanisms involved in its effects on the expression of ADAMTS-4 and ADAMTS-5 still need further study.

In the present study, we successfully isolated HNPCs from IDD patients, and according to the

measurements of surface markers, the positive expression rates of Col-2, KRT-18, KRT-19, HIF-1 $\alpha$ , GLUT-1, Sox-9, ACAN, and CD24 were nearly 80% (Figure 1).

To further verify the potential role of HtrA1 in promoting the expression of ADAMTS-4 and ADAMTS-5, we used exogenous rHtrA1 to demonstrate that exogenous rHtrA1 can upregulate the protein level of ADAMTS-5 in HNPCs and in cell culture supernatants. We also found that the increase was dose-dependent and peaked at a dosage of 5  $\mu$ g/ml. However, our data did not show any changes in the level of ADAMTS-4 (Figures 2A, 2D, 4A-D), which was consistent with the findings of Furtwängler T, who injected ADAMTS-4 and HtrA1 into human IVD and found that ADAMTS-4 did not induce visible matrix degradation or major shifts in gene expression in IVD. HtrA1 induced a decrease in disc height that was positively correlated with changes in GAG/DNA content [28].

Mitogen-activated protein kinases (MAPKs) have been shown to play important roles in mediating synovial inflammation [29]. Based on this, the effects of ADAMTS-5 on the HtrA1-induced phosphorylation of ERK, NF- $\kappa$ B, JNK and p38 were examined. We postulated that the ERK, NF- $\kappa$ B, JNK and p38 signaling pathways may be critical for the development of ADAMTS-5-induced IDD. Indeed, our results

showed that HtrA1 directly induced the expression of ADAMTS-5, which was associated with the ERK, NF- $\kappa$ B and JNK signaling pathways, and by using inhibitors of these three signaling pathways, the increase in ADAMTS-5 was reduced. Furthermore, we showed that there were increased levels of ADAMTS-5 in the cell culture supernatants, which suggests a more critical role of ADAMTS-5. No obvious change was found in the level of ADAMTS-4.

Our previous study confirmed the potential role of HtrA1 in regulating the expression of MMPs [14]. In the present study, we proved that ADAMTS-5, belonging to one of the predominant matrix-degrading enzyme families that can hydrolyze aggrecan core proteins, can also be induced by HtrA1. Thus, it is of great importance to perform more studies to identify other members of the MMP and ADAMTS families that can be regulated by HtrA1, and the exploration of the potential role of HtrA1 in regulating the expression of MMPs and ADAMTSs is crucial for the search for a promising therapeutic target for IDD in future studies.

### Conclusions

The present study demonstrated that HtrA1 can upregulate the expression of ADAMTS-5 via the ERK, NF- $\kappa$ B and JNK signaling pathways. The use of inhibitors of these signaling pathways can reduce this increase. Our study illustrates that HtrA1 can contribute to the pathogenesis of IDD by inducing the expression of MMPs and ADAMTSs, and the suppression of HtrA1 may be a useful therapeutic strategy for the treatment of IDD.

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### Disclosure of conflict of interest

None.

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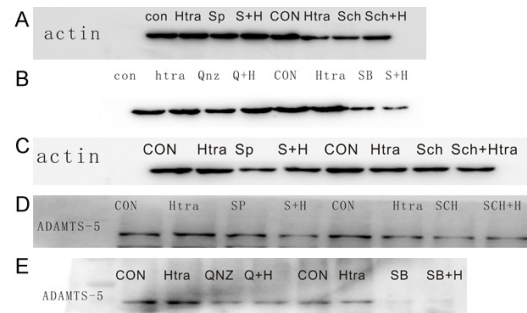
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## HtrA1 up-regulates expression of ADAMTS-5



**Supplemental Figure 1.** Original WB data. A. ACTIN for **Figure 3A**. B. ACTIN for **Figures 3C** and **4E**. C. ACTIN for **Figure 3G**. D. ADAMTS-5 for **Figure 3A** and **3G**. E. ADAMTS-5 for **Figure 3C** and **3E**.