INTRODUCTION

Intervertebral disc degeneration (IDD) is the main cause of low back pain and neck pain, and became a worldwide disability that causes low quality of health among the patients. The pathology factors of IDD is versatile, including gene, age, improper lifestyle, and the specific mechanism of which is yet fully unveiled. In recent years, it has been found that inflammation directed nucleus pulposus (NP) extracellular matrix metabolism dysregulation is known to be the main cause of the degeneration process, few is known about the protective factors. Using high-throughput label-free proteomics, we found that inflammation-related autocrine factor Chitinase-3-like protein 1 (CHI3L1, or YKL-40) is highly expressed in the NP cells during degeneration. Immunohistochemical analysis show that the expression of CHI3L1 is NP tissue specific, and increase significantly during degeneration. Overexpression of CHI3L1 significantly decrease the catabolism, and increase the anabolism of extracellular matrix. Knockdown of CHI3L1 using siRNAs show the opposite results, which imply that the protective role of CHI3L1 in IDD. Using high-throughput RNA sequencing and functional analyses, we find that AKT3 expression and its phosphorylation is mainly regulated by CHI3L1. And lastly, the mechanism of which is also validated using human and mouse degenerated NP tissues. In summary, our findings show that the inflammation-related autocrine factor CHI3L1 is NP specific, and it protects IDD by promoting the AKT3 signaling, which may serve as a potential therapeutic target in intervertebral disc degeneration.

KEYWORDS

AKT3, CHI3L1, intervertebral disc degeneration, nucleus pulposus

Received: 16 August 2019 | Revised: 6 October 2019 | Accepted: 23 October 2019
DOI: 10.1096/fj.201902096R

The FASEB Journal. 2020;34:3554–3569.

wileyonlinelibrary.com/journal/fsb2

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Funding information

National Natural Science Foundation of China, Grant/Award Number: 81572096, 81772392, 81672211, 81871802, 81772376, 81601928, 81702149 and 3700147; Shanghai Municipal Commission of Health and Family Planning, Grant/Award Number: 20184Y0181 and 18ZR1438900; Shanghai Education Development Foundation and Shanghai Municipal Education Commission, Grant/Award Number: 17SG35 and 17CG36

Abstract

Intervertebral disc degeneration (IDD) is the main cause of low back pain and the mechanism of which is far from fully revealed. Although inflammation directed nucleus pulposus (NP) extracellular matrix metabolism dysregulation is known to be the main cause of the degeneration process, few is known about the protective factors. Using high-throughput label-free proteomics, we found that inflammation-related autocrine factor Chitinase-3-like protein 1 (CHI3L1, or YKL-40) is highly expressed in the NP cells during degeneration. Immunohistochemical analysis show that the expression of CHI3L1 is NP tissue specific, and increase significantly during degeneration. Overexpression of CHI3L1 significantly decrease the catabolism, and increase the anabolism of extracellular matrix. Knockdown of CHI3L1 using siRNAs show the opposite results, which imply that the protective role of CHI3L1 in IDD. Using high-throughput RNA sequencing and functional analyses, we find that AKT3 expression and its phosphorylation is mainly regulated by CHI3L1. And lastly, the mechanism of which is also validated using human and mouse degenerated NP tissues. In summary, our findings show that the inflammation-related autocrine factor CHI3L1 is NP specific, and it protects IDD by promoting the AKT3 signaling, which may serve as a potential therapeutic target in intervertebral disc degeneration.

KEYWORDS

AKT3, CHI3L1, intervertebral disc degeneration, nucleus pulposus

1 | INTRODUCTION

Intervertebral disc degeneration (IDD) is the main cause of low-back pain and neck pain, and became a worldwide disability that causes low quality of health among the patients. The pathology factors of IDD is versatile, including gene, age, improper lifestyle, and the specific mechanism of which is yet fully unveiled. In recent years, it has been found

Abbreviations: AF, Annulus Fibrosus; cDNA, Complementary DNA; CHI3L1, chitinase 3-like 1; DNA, Deoxyribonucleic acid; ECM, Extracellular matrix; ELISA, Enzyme-linked immunosorbent assay; GO, Gene Ontology; IDD, Intervertebral disc degeneration; IVD, Intervertebral disc; mRNA, Messenger RNA; NP, Nucleus Pulposus; RNA, Ribonucleic acid.

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that many inflammation-related factors played an important role in IDD process. However, their low disease specificity indicates that there might be other IDD specific factors take part in the degeneration process, which may be important for the further understanding of IDD.

Intervertebral disc (IVD) is the fibrous cartilage that connects adjacent vertebral bodies to hold the entire spine. The central area of disc is the hydrogel-like nucleus pulposus (NP). NP mainly consists of proteoglycan and type II collagen fiber, and the hydrogel-like status enables NP to absorb the pressure it received from every direction, which is critical in maintaining the flexibility and stability of the spine. The structure that surrounds NP is the annulus fibrosus (AF), which is a concentric array of type I and type II collagen fibers and elastin fibers. It can undertake the pressure of outside mechanical pressure on spine and maintain the position of NP under mechanical pressure. Since the importance of NP in stabilizing the spine, any degenerative changes will first take place in it.

As many studies reported, IDD is a complicated progress including cell senescence, decreased proliferation ability, self-repair impairment, enhanced inflammation, reduced anabolism, and increased catabolism. Among which, inflammation played the most important role in the IDD progress, many inflammatory factors can lead to IDD, such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and so on. However, inflammatory factors take place in many tissues, they are basically lack of tissue specificity, so the therapy which based on inflammation still need to overcome many problems before applied to a clinical setting. Besides, the metabolism of extracellular matrix (ECM) is of great importance to IDD. Anabolic and catabolic activities are imbalanced during IVD degeneration. Thus, the dysregulation and expression of anabolic factors like type II collagen (Col II), SOX9, transforming growth factor (TGF), insulin-like-growth-factor I (IFG-1) are also critical to the IDD process.

Anti-inflammatory factors, on the other hand, is seldomly discussed during IDD. Reports shown SIRT1 could inhibit the degeneration effect of IL-1β through TLR2/SIRT1/NF-κB Pathway, and TIMP3 could inhibit the expression of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Both factors decreased dramatically during degeneration, and are potential protective factors against IVD degeneration. However, their roles as anti-inflammatory factors are not tissue specific. Here, we set out to seek the IDD specific endogenic molecules that can protect degeneration during inflammation-induced IDD process. Using high-throughput proteomic analysis, we found that inflammation-related autocrine factor CHI3L1 is highly regulated during degeneration. However, the functional role of CHI3L1 in IDD has not been elucidated.

In this article, we find CHI3L1 as a potential protective molecule that take part in the inflammation-induced degeneration of nucleus pulposus cells. The overexpression of CHI3L1 protects the NP cells from inflammation-induced degeneration phenotype by significantly decreasing the expression of catabolic factors like MMPs and ADAMTS, and increasing the expression of matrix synthetic genes COL2, ACAN, and CHSY1. Using high-throughput RNA sequencing and functional analyses, we find that AKT3 expression as well as its phosphorylation is mainly regulated by CHI3L1, which proved it protects intervertebral disc NP degeneration by promoting the AKT3 signaling.

2 | MATERIALS AND METHODS

2.1 | Tissue sample collection

Informed consent is provided by the patients and their relatives before obtaining the intervertebral disc tissue in surgery. The experiment was authorized by the ethics committee of Second Military Medical University. Normal intervertebral disc tissue sample were collected from lumbar trauma patients who underwent spinal fusion with no radiological sign of degeneration (Pfirrmann grade I, n = 6, age 30 to 55 years, mean 44 years). Degenerated disc tissue was collected from diagnosed lumbar herniation patients who underwent disc resection and fusion surgery to relieve symptom (Pfirrmann grade IV-V, n = 6, age 40 to 55 years, mean 48 years). MRI T-2 weighted images were collected and the modified Pfirrmann grading system was used to evaluate the degree of IDD. Three samples from either normal or degenerated are proceed to nucleus pulposus cell culture and the other samples are used for proteomic research.

2.2 | Human primary nucleus pulposus cell culture

NP tissue specimens were washed twice with PBS, then minced and digested with 2 U/mL protease in DMEM/F12 medium (Gibco) for 30 minutes at 37°C. NP cells were released from the NP tissues by treating with 0.25 mg/mL type II collagenase (Gibco, Cat. No. 17101-015) for 4 hours at 37°C. The remaining cell suspension was transferred into a 40 μm cell strainer (BD Biosciences) and centrifuged at 800 g for 5 minutes. The NP cells were resuspended in DMEM/F12 containing 10% FBS (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine. The viability of the suspended cells was over 90% when assessed using cell counting kit-8 (Dojindo). Cells were incubated at 37°C in 5% CO₂ and the medium was changed every 3 days. Cells at the second passage were used for subsequent experimental procedures.
For in vitro NP cell degeneration, we treated NP cells with Recombinant Human TNF-α (PEPROTECH, USA) (50 ng/mL) or Recombinant Human IL-1β (PEPROTECH, USA) (25 ng/mL) to establish the degeneration model. For CHI3L1 overexpression, we used either CHI3L1 overexpression plasmid provided by OBiO Technology (Shanghai) Corp., Ltd or synthetic YKL-40 recombinant protein (Sino Biological). For CHI3L1 inhibition, we use either siRNA mediated knockdown or polyclonal blocking antibody (Abclonal, A3166).

2.3 | RNA extraction and Real-time PCR

RNA was collected from human NP samples by RNAiso (TAKARA) according to the manufacturer’s instructions. Concentration of total RNAs are measured at 260 nm with a spectrophotometer (DU-800; Beckman Coulter, Brea, CA). The PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA) are used to conduct reverse transcription with a 20-ul final reaction mixture according to the manufacturer’s instructions. Real-time PCR was performed using TB Green® Premix Ex Taq™ (TAKARA) with a Step One Plus real-time PCR system (Applied Biosystems). GAPDH was used to normalize the gene expression of other mRNAs. The relative amount of each transcript was calculated according to the comparative Ct method. Each experiment was repeated at least three times independently. Primers used in this article are listed in Table 1.

2.4 | Western Blot

Human NP cells were harvested using iced-cold lysis buffer (Cell Signaling Technology). For detection of phosphorylation proteins level, the total protein was extracted at 12 hours after treatment, whereas the other treatment duration was 24 hours. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology). PMSF (Byotime) and protease inhibitor cocktail (Meilune) were used to prevent protein degradation. Proteins transferred to PVDF membranes (Bio-Rad) by electroblotting. The membranes were blocked by 5% no-fat milk in TBST (50 mmol/L Tris, pH 7.6, 150 mmol/L Nacl, 0.1% Tween-20) and then incubated in TBST with antibodies overnight at 4°C. Immunolabeling was detected using the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), Pierce™ECL Western Blotting Substrate (Thermo Fisher Scientific) to detected protein bands. Antibodies used are CHI3L1 (ab77528, 1:1000 dilution), MMP1 (ab52631, 1:2500 dilution), MMP3 (ab53015, 1:1000 dilution), MMP13 (ab39012, 1:1000 dilution), ADAMTS4 (ab185722, 1:500 dilution), ACAN (ab3778, 1:500 dilution), CHSY1 (ab153813, 1:500 dilution), AKT3 (ab189643, 1:1000 dilution), p-AKT3 (ab192623, 1:1000 dilution), GSK3b (ab32391, 1:500 dilution), p-GSK3b (ab75745, 1:500 dilution) all from Abcam and GAPDH (1:5000 dilution) (Proteintech).

2.5 | siRNA transfection

siRNAs were purchased from GenePharma Co., Ltd. According to the instruction of Lipofectamine RNAiMAX Reagent (Thermo Fisher SCIENTIFIC). 9 μL Lipofectamine RNAiMAX Reagent was diluted in 150 μL DMEM to. Meanwhile, 3 μL (30 pmol) siRNAs were diluted in 150 μL DMEM and incubated for 5 minutes before use. The sequence of siRNAs is listed in Table 2.

2.6 | Label-free proteomics

For proteomic research, three normal or degenerated NP tissues samples were used. They are transferred to a 1.5-mL screw capped tube and centrifuged at 10,000 × g for 30 minutes at 4°C. Next, 100 μL lysis buffer (7 mol/L urea, 2 mol/L thiourea) were added into each sample and then ultrasonic crushed to extract total proteins. Proteins were precipitated with trichloroacetic acid (TCA) for 30 minutes on ice and centrifuged at 40,000 × g for 30 minutes. Protein concentration was determined using the Qubit fluorescent protein quantification kit (Invitrogen) according to the manufacturer’s instructions. The further high-throughput label-free proteomic procedures were performed by Shanghai NovelBio Bio-Pharm Technology Co., Ltd.

2.7 | High-throughput RNA sequencing and informatic analysis

For transcriptome sequencing, total RNA was extracted using the TRIzol solution (Invitrogen), according to the manufacturer’s protocol. Further library construction, RNA quality control check, purification, quantification, and validation steps are conducted by Shanghai NovelBio Bio-Pharm Technology Co., Ltd. For analysis of transcriptome sequencing data, we aligned approximately 100 bp long reads to the human genome (hg19) using TopHat2/Bowtie2. We identified mapped data to gene structures derived from RefSeq using the summarize overlaps function with mode Intersect Strict (Genomic Ranges, Bioconductor). Using the initial raw counts data, we calculated reads per kilobase per million reads mapped (RPKM) values for the same gene set using Cufflinks. The differential analysis was carried out using edgeR, applying TMM (trimmed Mean of M-values) library normalization and a 0.05 false discovery rate (FDR) to select expressed transcripts. All processed and
raw data can be found in GEO database under the Accession No. GSE135219.

Gene Ontology (GO) and KEGG pathway analyses were performed as previously described. In brief, we calculated the $P$-value of each GO term using right-sided hypergeometric tests, and Benjamini-Hochberg adjustment was used for multiple test correction. An adjusted $P$-value that is lower than .05 indicated a statistically significant deviation from the expected distribution, and thus the corresponding GO terms and pathways were enriched in target genes. We analyzed all the differentially expressed mRNAs using GO and KEGG pathway analyses, and we analyzed the mRNAs that were included in the miRNA regulator network using GO analysis.

### 2.8 ELISA

To detect the expression of CHI3L1 between the normal and inflammation status of NP cell, the expression was detected by Human CHI3L1 (YKL-40) ELISA kit (Thermo Fisher Scientific). According to the manufacturer’s instruction of ELISA kit. First, added standard and samples to 96-well plates, then incubated for 2.5 hours at RT. After washed for four times, used antibody and incubated for 1 hour. Then washed four times again, next, added streptavidin-HRP Reagent and incubated for 45 minutes. After washed, used TMB substrate and stopped it, finally, detected absorbance and obtain results.

### 2.9 Immunohistochemistry staining

Male C57BL/6 Mice are purchased from Shanghai Model Organisms Center, Inc (Shanghai, China). Firstly, ketamine (100 mg/kg) was used for general anesthesia. We locate the disc through opening of abdomen where the ventral part of IVD is observed. And a syringe needle was vertically inserted into the lumbar disc through ventrally and rotated 180 degrees along the axis for 10 seconds. The 23-G needle was inserted into the intervertebral disc 1.5 mm parallel to the endplate via AF to NP to decompress the nucleus. Mice are sacrificed at different time point to obtain intervertebral disc specimens. Immunohistochemistry was performed to localize CHI3L1, AKT3, and OCN in human and mice NP samples. The general immunohistochemistry protocol has been described elsewhere. Briefly, antigen retrieval was performed using trypsin for 30 minutes at 37°C, and the sections were blocked with 1% bovine serum albumin for 15 minutes at room temperature. Next, the sections were incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal antibody against CHI3L1 (1:100 dilution), rabbit polyclonal antibody against AKT3 (1:100 dilution), and rabbit polyclonal antibody against OCN (1:100 dilution) (Proteintech). Next, the secondary antibody peroxidase-conjugated affinipure

| TABLE 1 Oligonucleotide sequences of all primers in quantitative RT-PCR analysis |
|-----------------------------|-----------------------------|-----------------------------|
| Gene | Primer sequence, 5'-3'| Primer sequence, 5'-3' |
| Forward | Reverse |
| LYZ | GCCTAGCAGCTGATGACCTAGC | AGTCTCTGCGCAACTCCACACC |
| CHI3L1 | TGTTCCGAGGTCAGGAGGAT | TGCCCCATACCAGCTTACTG |
| AEBP1 | TGCCCATACACCAGCTTACTG | GAGAGGATGGCCTCCTTG |
| ACAN | GAGAGGATGGCCTCCTTG | GCTCTCTTGCTGCTGCTG |
| CHSY1 | CTTCTCTTCTGCTGCTGCTG | TGGAGAGAAGCTGTGAGT |
| ADAMTS4 | TGGAGAGAAGCTGTGAGT | TCTGGTTTGGTAGTGAGT |
| ADAMTS5 | TCTGGTTTGGTAGTGAGT | TCTGGTTTGGTAGTGAGT |
| COL2 | TTCATTTTTCGCCAATCAGC | CACATCTCCCGGTTAGCTC |
| MMP1 | CACATCTCCCGGTTAGCTC | CTCAGGGTCGGTCCCTTGG |
| MMP3 | CTCAGGGTCGGTCCCTTGG | AGACCCCTCAACTATACCTC |
| MMP13 | AGACCCCTCAACTATACCTC | GCCCTCCCAGACGAGTTCC |
| P21 | GCCCTCCCAGACGAGTTCC | TCGTGGTAGGCGAAGAC |
| CCND1 | TCGTGGTAGGCGAAGAC | CATGAGGGCGGCTTGAAG |
| AKT3 | CATGAGGGCGGCTTGAAG | CCGCTTCCACACATCTTAT |
| GAPDH | CCGCTTCCACACATCTTAT | AAGGCGATGTACAGGCT |

| TABLE 2 SiRNA Sequences used in this study |
|-----------------------------|-----------------------------|-----------------------------|
| Primer sequence, 5'-3' | Primer sequence, 5'-3' |
| Forward | Reverse |
| siCHI3L1-1 | CCACCCUAAUCAAGGAAAUU | |
| siCHI3L1-2 | GAGCCACAGUCGCAACAUAAUU | |
| siAKT3-1 | CCCUCAGUGAAGGCUAUAUU | |
| siAKT3-2 | GGUGCGUUGGCAUAAUU | |
goat anti-rabbit IgG (1:1000 dilution) (Proteintech) was applied to the sections and they were counterstained with hematoxylin. The image analysis procedure has been previously described, briefly, samples were imaged using a ZEISS microscope (ZEISS Axio Imager A2, Carl Zeiss microscopy GmbH). Immunostained slides were identified independently by three pathologists who were blinded to patients’ data and outcomes. When there were different evaluation results, a consensus result should be achieved after re-examination. Within each sample, 200 cells were counted and the number of immune-positive cells expressed as a proportion of this sample.

2.10 | Transwell assay

The ability of MMPs secretion and migration of NP cells under different treatment were assessed by Matrigel-coated transwell assay. NP cells were planted into the upper chamber coated with Matrigel (BD Biosciences). Inflammatory cytokines IL-1β or TNF-α were treated 8 hours later at a concentration of 50 ng/mL. After 48 hours of incubation, swabs were used to gentle scrub the cells in the Matrigel containing surface of the upper chamber. Next, to visualize the migrated cells in the upper chamber, we fixed cells by 4% paraformaldehyde for 30 minutes, and use 0.1% crystal violet stained for 10 minutes, ddH2O were used to washed it until water is clarified. After natural air drying, samples were observed under microscopy.

2.11 | Flow cytometry

Cell apoptosis was measured by flow cytometry using Annexin V-FITC/PI Double-staining Kit for Apoptotic Cells (Fushen, Shanghai). Cells were collected after 48 hours of intervention, digested by trypsinase without EDTA, washed by PBS, and followed up with the kit’s instruction. Briefly, cells are suspended with 100 μL binding buffer, transferred to centrifugal tube and added with 5 μL dye, then incubate in dark for 15 minutes. Then the tube is added with 400 μL binding buffer and detected by flow cytometry (CyAn ADP, Beckman).

2.12 | Statistical analysis

Data are presented as mean ± standard deviation of at least three independent experiments. GraphPad Prism 6.0 software (GraphPad Software, Inc) was used for statistical analysis. The normality of the data was tested using the D’Agostino-Pearson omnibus normality test. The data of the proportions of immune-positive NP cells in human NP tissues did not pass the normality test; therefore, these data were analyzed by applying the two-tailed Mann-Whitney U test. The remaining data passed the normality test, and the two-tailed Student’s t-test or an analysis of variance (ANOVA) followed by the Turkey’s t-test were performed for comparison of two groups or multiple groups, respectively. The significance threshold was 0.05.

3 | RESULTS

3.1 | CHI3L1 expression is significantly elevated in degenerated nucleus pulposus

Normal nucleus pulposus (NP) and degenerated NP were put to high-throughput label-free proteomic analysis (Data S1). The 125 differentially expressed proteins were further categorized using Gene Ontology (GO) analysis (Data S2, Figure 1A,B). We found that among the most enriched GO categories, most of which are related to extracellular matrix organization (Figure 1B, GO:0031012, GO:0005578). These results are consistent with acknowledged pathological changes of degenerated NP in intervertebral disc.

In order to find the key component during NP degeneration, the top three highly enriched GO groups from either biological process, cellular component and molecular function category of GO analysis were subjected to Venn analysis. Among which, only three genes were present in all six highly enriched GO terms: LYZ, CHI3L1 and AEBP1 (Figure 1C, Figure S1A). We analyzed the mRNA levels of LYZ, CHI3L1, and AEBP1 by Real-time PCR, and found only the mRNA level of CHI3L1 was significantly increased in IDD NP samples (Figure 1D). Further immunohistochemistry analysis of human NP samples also showed consistent results (Figure 1E). These results showed that CHI3L1 expression is elevated in degenerated NP tissue both in mRNA and protein form, which indicate that CHI3L1 may play an important role in the intervertebral disc degeneration.

3.2 | CHI3L1 is inflammatory sensitive and located specifically in nucleus pulposus tissue

To find out whether CHI3L1 expression is consistent in mouse IVD tissue, we applied immunohistochemical staining demonstrating the expression of CHI3L1 in mouse IVD at various age and status. Results showed that the expression of CHI3L1 is localized mainly in NP tissues compare with annulus fibrosus tissue, indicating its NP specificity (Figure 2A). Consistent with CHI3L1 expression change found in human tissues, the expression of CHI3L1 significantly increased in degenerated NP tissue, and normally the
FIGURE 1  CHI3L1 expression is significantly elevated in degenerated NP tissue. A, The GO function classification analysis of high-throughput label-free proteomics detecting differentially expressed proteins in IDD. The chart shows the number of differentially expressed proteins that are categorized in each GO term of the three GO categories as biological processes, cellular components, and molecular functions. The listed GO terms have passed the significance examine ($P < .05$). The red bar represents upregulated proteins in IDD in each GO term, whereas the green bar represents the downregulated proteins. B, The top 20 enriched GO terms that are significant in IDD NP samples. The bubble area represents the amount of protein, and the bubble color represents the $P$ value. C, Six-way Venn diagram shows the shared upregulated differentially expressed proteins in IDD among the six GO terms that are the top two of each GO category. D, qPCR detecting the mRNA level of LYZ, CHI3L1, and AEBP1 in normal (IVD) and degenerated NP (IDD) tissues, n = 5. E, Representative images of hematoxylin-eosin staining (HE), Masson staining, Safranin O-fast green (SOFG) staining, and immunohistochemical staining of CHI3L1 expression in normal (IVD) and degenerated (IDD) human NP tissues. Quantifications of positive cells in the immunohistochemical analysis were shown in the right panels. Data are shown as mean ± SD. *$P < .05$; **$P < .01$
expression of CHI3L1 is most high in the NP of newborn mice (Figure 2B).

To find the relationship between CHI3L1 and degeneration, here we compared the expression of CHI3L1 and other NP degeneration-related genes in human primary NP cells. Results showed that CHI3L1 expression is significantly higher in NP primary cells derived from IDD patients, whereas ACAN, CHSY1 decreased significantly and ADAMATS4 increased significantly in IDD primary NP cells (Figure 2C), indicating CHI3L1 is indeed upregulated during degeneration.

Since inflammation is the main cause of IDD, here we tested whether CHI3L1 is upregulated during inflammation induced in vitro NP cell degeneration. By adding either TNF-α or IL-1β to normal human primary NP cells, we found that both the protein and mRNA level of CHI3L1 increased significantly (Figure 2D). And Since the product of CHI3L1, YKL-40 is a secreted protein, we tested its level in supernatant of different dosage of TNF-α or IL-1β agitation. The results showed dose dependent elevation of secreted CHI3L1 level by ELISA assay in NP cell supernatant (Figure 2E).

Combining these results, we found that CHI3L1 expression increased significantly in siCHI3L1 group and fewer in oeCHI3L1 group, indicating CHI3L1 is indeed upregulated during degeneration.

3.3 | CHI3L1 shows protective effects against inflammatory agitation

To explore the function of CHI3L1 in IDD, we first manipulate the expression of CHI3L1 in normal NP cells using either siRNA to knockdown or overexpression by plasmid transfection. The efficiency of CHI3L1 knockdown by its targeting siRNA is tested (Figure S1B). We detected the expression of normal NP ECM-related gene ACAN, CHSY1, and COL2 by qPCR. The results showed that the expression of ACAN, CHSY1, and COL2 had no significant difference after CHI3L1 overexpression (oeCHI3L1) or CHI3L1 knockdown (siCHI3L1) (Figure 3A,B). However, in degenerated model which induced by inflammatory factor IL-1β or TNF-α, we found that the expression of ECM-related genes significantly decreased in siCHI3L1 group, and increased significantly in oeCHI3L1 group (Figure 3C, Figure S1C). On the other hand, IDD-related gene like MMPs and ADAMTS decreased significantly in oeCHI3L1 group and increased significantly in siCHI3L1 group (Figure 3D,E), indicating a degeneration protective and inflammation protective role of CHI3L1 in NP cells. Similar results were found in inflammation-induced degeneration NP cells (Figure 3F, Figure S1D).

Since inflammatory factors can induce degeneration phenotype of NP cells by elevating the expression of ECM catabolic gene like MMPs and ADAMTS, we take advantage of Matrigel-coated Transwell assay to further validate the effect of CHI3L1 on inhibiting inflammation-induced NP cell degeneration (Figure 3G). No obvious NP cells were migrated in the Blank group, which inferred that normally the expressions of ECM catabolic gene are relatively low and insufficient to digest the Matrigel in 48 hours (Figure 3H). More migrated cells were observed in siCHI3L1 group and fewer in oeCHI3L1 group, indicated that the secretion of inflammatory and ECM catabolic factors like MMPs can be significantly reduced by CHI3L1 (Figure 3H, Figure S1E). These results suggesting that CHI3L1 may play a role of inhibiting inflammation in intervertebral disc degeneration.

3.4 | AKT3 is a downstream target of CHI3L1 in degeneration

To elucidate the mechanism of CHI3L1, we performed mRNA sequencing to find the downstream factors of CHI3L1 (Figure 4A, Figure S2A). CHI3L1 overexpressed samples are compared with control samples under either IL-1β or TNF-α induced degeneration environment, and 160 differentially expressed genes were found in IL-1β and TNF-α datasets (Figure 4B,C, Figure S2B). To further unveil the mechanism, we performed Kegg Pathway analysis of these two datasets (Figure 4D, Figure S2C). Among which, we found MAPK signaling, mTOR signaling (Figure 4D) and JAK-STAT signaling (Figure S2C) are mostly enriched and IDD-related pathways. Combining the related differentially expressed genes under these enriched pathways, we found that AKT3, CRK, and RPS6KA6 are the shared differentially expressed genes in both datasets, so we validated their expression change using both IL-1β or TNF-α induced degeneration models. Results showed that the expression of AKT3 significantly upregulated during CHI3L1 overexpression and downregulated under CHI3L1 knockdown (Figure 4E). To further validate the pathway factors, we selectively expanded the candidates and tested their expression change under CHI3L1 modulation. To our expect, only AKT3 showed a significant correlation with CHI3L1 expression (Figure 4F). These results suggested that AKT3 is a potential downstream target of CHI3L1.

3.5 | CHI3L1 attenuates NP cell degeneration via AKT3 signaling

Next, in order to study the role of AKT3 signaling in CHI3L1 mediated degeneration protective effect, we first designed AKT3 knockdown siRNAs and test their efficiency (Figure S3A). Using AKT3 siRNAs, we found that AKT3 knockdown
FIGURE 2  CHI3L1 is inflammatory sensitive and located mainly in NP tissue. A, Representative histological images of mouse intervertebral discs in HE, SOFG staining and immunohistochemical staining of CHI3L1 expression at various age point. Degenerated disc samples are collected from needle punctured IDD model mice. n = 5. Quantifications of positive cells in the immunohistochemical analysis were shown in (B). C, qPCR detection of CHI3L1, ACAN, CHSY1, and ADAMTS4 mRNA expression in human IDD and IVD tissue sample derived primary NP cells, n = 5. D, The expression of CHI3L1 during inflammation-induced in vitro NP cell degeneration is tested using qPCR and Western Blot. IL-1β 25 μg/mL or TNF-α 50 μg/mL are treated for 48 hours before detection. E, ELISA assay is used to detect the expression of CHI3L1 in NP cell supernatant after addition of IL-1β or TNF-α, n = 3. All experiments are repeated at least three times, and GAPDH is used as an internal control. Data are shown as mean ± SD. *P < .05; **P < .01
can generate similar phenotype of CHI3L1 knockdown in inflammation-induced NP cell degeneration model (Figure S3B,C). Next, we tested whether AKT3 knockdown can reverse the effect of CHI3L1 overexpression. By qPCR analysis, we found that AKT3 knockdown can significantly reverse the effect of CHI3L1 overexpression in inflammation-induced NP cells (Figure 5A,D). To inhibit AKT3 phosphorylation, we use AKT3-sensitive inhibitor GSK2141795 (50 nmol/L) to inhibit the AKT3 signaling. Results showed that GSK2141795 treatment showed similar effect to AKT3 knockdown (Figure 5B,E). An AKT3-insensitive pan-AKT inhibitor MK-2206 treatment showed less significant effect of reversing the effect of CHI3L1 overexpression, which indicated that AKT3, not AKT1 & AKT2 is a main downstream factor of CHI3L1 (Figure S3D). Again, we applied an AKT signaling activator (SC79) to the CHI3L1 knockdown NP-degenerated cells. And by qPCR, we found that AKT signaling activation could significantly rescue the phenotype brought by CHI3L1 knockdown (Figure 5C,F). Western Blot analysis also confirmed these changes in the protein level (Figure 5G).

In Matrigel-coated Transwell assay, the number of migrated cells increased remarkably in AKT3 knockdown and AKT3 inhibition when compared with CHI3L1 overexpression alone. While AKT3 activation significantly decreased migrated cells compared with CHI3L1 knockdown alone (Figure 5H). Indicating that CHI3L1 indeed exert its inflammation protective and degeneration protective effect through AKT3 signaling. Taken together, these data showed that CHI3L1 may attenuate the inflammation-induced NP cell degeneration through AKT3 signaling pathway.

**FIGURE 3** CHI3L1 shows protective effects against inflammatory agitation. A-C, qPCR analysis showing the relative mRNA level of ACAN, CHSY1, and COL2 expression under each treatment groups. D-F, qPCR analysis showing the relative mRNA level of MMP1, MMP3, MMP13, ADAMTS4, and ADAMTS5 expression under each treatment groups. G, Flow chart of Matrigel-coated transwell assay. H, The microscopy evaluation of cell migration in different groups. Relative migrated cell level was evaluated and shown in the right panel. NC was used as a control. All experiments were repeated at least three times, and GAPDH is used as an internal control. Data are shown as mean ± SD. *P < .05; **P < .01
FIGURE 4  High-throughput RNA sequencing showing AKT3 is a main target of CHI3L1. A, RNA-seq was used to detect the differential expressed genes of CHI3L1 overexpressed degenerated NP cells induced by IL-1β or TNF-α (see also Figure S2A), the heatmap of unsupervised hierarchical cluster analysis showing the differentially expressed genes in IL-1β dataset. Each row represents the relative expression level of each gene in different samples. B, The Pearson coefficient between each sample based on the sequencing results. The greater the color depth, the closer R² approaches 1, the stronger the correlation between the two groups. C, The volcano plot showing the significantly differentially expressed genes between CHI3L1 overexpressed and normal degenerated NP cells induced by IL-1β. D, The KEGG pathway enrichment analysis showing the most abundant GO terms in the IL-1β dataset, the bubble area represented the number of genes enriched in each term, and the color represents the significance. E-F, qPCR analysis showing the relative mRNA expression levels of the candidate CHI3L1 target gene (E) or related pathway gene (F). The qPCR experiments were repeated three times, and GAPDH is used as an internal control. Data are shown as mean ± SD. *P < .05; **P < .01.
3.6 CHI3L1 autocrine is vital to the survival and anti-degeneration effect of NP cells

Since CHI3L1 is an autocrine factor, we next tested how its secretion affects its protective effect in NP degeneration. Using synthetic YKL-40 recombinant protein, we found that adding more CHI3L1 product to the medium would significantly upregulate the expression of CCND1 and downregulate the expression of MMP13 and P21, which is all reported AKT3 downstream targets.\textsuperscript{21,22} (Figure 6A). Further Western Blot analysis confirmed the activation of AKT3 and its downstream targets (Figure 6B). To test the importance of cell-free CHI3L1, we applied polyclonal CHI3L1 antibody to block the CHI3L1 product in the supernatant of degenerated NP cells. Results showed that a significant reduction of CCND1 and upregulation of P21 and MMP13 even at the lowest dose of blocking antibody treated to the inflammation-induced degenerated NP cells (Figure 6C). And Western Blot analysis confirmed a gradual reduction in AKT3 expression as well as its phosphorylation level, indicating that autocrine CHI3L1 is important to sustain AKT3 activation in NP degeneration (Figure 6D). Further qPCR analysis confirmed the cell-free CHI3L1 level is also important to the expression of degeneration-related and ECM-related genes in degenerated NP cells (Figure 6E,F).

Cell apoptosis is also a phenotype of IDD. Here, we tested whether CHI3L1 autocrine can affect the cell survival of
degenerated NP cells. Using Annexin V-FITC/PI Double-staining flow cytometry assay, we found that recombinant CHI3L1 treatment significantly reduced the percentage of apoptotic NP cells, and CHI3L1 blocking increased the percentage of apoptotic NP cells (Figure 6G,H). While adding AKT3 inhibitor to CHI3L1 overexpression group significantly increased the percentage of apoptotic cells, and AKT3 activation significantly decreased the percentage of apoptotic cells in CHI3L1 knockdown group (Figure 6I). Taken together, these results showed that CHI3L1 secretion is important to the survival and degeneration process of NP cells, and its mainly work through AKT3 signaling.

Lastly, we examined the expression of CHI3L1, AKT3, and AKT3 activation level using human and mice disc samples to confirm previous findings in vivo. Results showed that the expression of AKT3 and CHI3L1 as well as AKT3 activation level significantly upregulated in degenerated NP samples of both human and mice (Figure 7A,B), implying that CHI3L1-AKT3 axis is functional in both human and mice NP cells. Taken together, our findings showed that NP cell specific autocrine factor CHI3L1 could act as a degeneration protective secretion protein that functions through AKT3 signaling in degenerated NP cells (Figure 7C).

FIGURE 6 CHI3L1 autocrine is vital to the survival and anti-degeneration effect of NP cells. A, qPCR analysis showing the mRNA levels of MMP13, P21, and CCND1 expression after different doses of recombinant CHI3L1 treatment (YKL-40) in degenerated NP cell induced by IL-1β. B, The Western Blot analysis showing the protein level changes of AKT3, p-AKT3 (phospho S472), GSK3b, p-GSK3b (phospho Y216), MMP13, and GAPDH expression according to (A). C, The mRNA levels of MMP13, P21 and CCND1 expression were measured by qPCR analysis after different doses of polyclonal anti-CHI3L1 antibody treatment in degenerated NP cell induced by IL-1β. D, The protein levels of AKT3, p-AKT3 (phospho S472), GSK3b, p-GSK3b (phospho Y216), MMP13, and GAPDH expression were measured by Western Blot analysis. E-F, The qPCR analysis detecting the mRNA expression of degeneration-related genes under different doses of recombinant CHI3L1 (E) and polyclonal anti-CHI3L1 antibody treatment (F). Annexin V-FITC/PI Double-staining apoptosis assay showing the percentage of apoptotic cell percentage of different doses of recombinant CHI3L1 (G) and polyclonal anti-CHI3L1 antibody (H) treated degenerated NP cell induced by IL-1β. The effect of AKT3 activation (SC79) and inhibition (GSK2141795) on NP cell apoptosis is also tested using Annexin V-FITC/PI Double-staining apoptosis assay (I) combined with recombinant CHI3L1 treatment (rcCHI3L1) and CHI3L1 knockdown using siRNA (siCHI3L1). The mean percentage of early apoptotic cells and late apoptotic cells in each group were shown in the left panel (G-I). All experiments were repeated three times, and GAPDH is used as an internal control. Data are shown as mean ± SD. *P < .05; **P < .01
DISCUSSION

In our findings, we demonstrated that CHI3L1 is a critical factor that protect NP degeneration from inflammatory factors. In 1990, the 40 kDa CHI3L1 was first described as a highly conserved heparin-binding glycoprotein secreted by synovial cells. It is composed of 10 exons, spanning 8 KB genomic DNA and located on chromosome 1q31-q32, and the product of which is also known as YKL-40, because of its three N-terminal amino acids, tyrosine (Y) - lysine (K) - leucine (L). CHI3L1 protein belongs to 18 families of mammalian glycosylhydrolases containing bacterial chitinase and chitinase from vertebrates and invertebrates. Functionally, chitinase in chitin containing pathogens can

FIGURE 7  CHI3L1-AKT3 axis is functional in both human and mice IDD. A, Representative immunohistochemistry images of intervertebral disc tissues showing the expression of CHI3L1, AKT3 and p-AKT3 (phospho S472) expression. The degenerative grade was classified using the Pfirrmann grading system. Five samples from different individual was analyzed in each group. B, Quantitative analysis of the proportion of CHI3L1-, AKT3-, and p-AKT3-positive cells in the immunohistochemistry staining in (A). NC was used as a control. C, Summery on the mechanism of CHI3L1 in intervertebral disc degeneration. Data are shown as mean ± SD. *P < .05, **P < .01
play an antifungal or antiviral role by cutting off the β-1,4 bonds between adjacent N-N-acetylglucosamine residues of chitin polymers. However, CHI3L1 lacks chitin solubility due to the substitution of amino acids in the catalytic region, nevertheless, CHI3L1 can still bind chitin, heparin, hyaluronic acid, and collagen through the carbohydrate-binding module (CBM). Due to its varied structure, its function is still not clear. So here, we set out to find its function and mechanism in IDD.

Although CHI3L1 can be expressed by a variety of cells, such as chondrocytes, smooth muscle cells and osteosarcoma cells, its function is usually related to inflammatory effect and tissue remodeling. Carole Ober suggested that CHI3L1 significantly promotes inflammation and inhibits lung function in asthma. EMIKO MIZOGUCHI indicated that CHI3L1 in colon epithelial cells can promote the development of inflammation by increasing bacterial adhesion. However, its role in inflammation remains controversial. Charles S. Dela Cruz et al. found that CHI3L1 knockout mice had significantly increased lung inflammation, bleeding, injury, and mortality, whereas Anneliese D. RECKLIES et al. demonstrated that CHI3L1 could significantly inhibit the expression of inflammation-related genes such as MMPs in fibroblasts. And K Huang et al. suggested that CHI3L1 not only limits the degradation of connective tissue and regulate the response of cells to proinflammatory factors, but also it may play a role in inhibiting inflammation in osteoarthritis. Some studies demonstrated that the expression of CHI3L1 in intervertebral disc degeneration is significantly increased, but none explored the relationship between CHI3L1 and intervertebral disc degeneration. Therefore, by systematic analysis, we propose the effect and mechanism of CHI3L1 as a degeneration protective autocrine factor that act by inhibiting inflammatory cascade specific to intervertebral disc degeneration in this study.

The downstream mechanism of CHI3L1 or YKL-40 is seldom reported. Some researchers demonstrated that TMEM219, also can be named as Insulin-like growth factor-binding protein 3 receptor (IGFBP-3R), is essential for tissue responses which is targeted by CHI3L1. They also indicated that it can provide such effects by activating AKT signaling. These conclusions provide evidence that support our findings. In our study, we also find AKT3 is the potential target of CHI3L1 using high-throughput RNA sequencing and bioinformatic analysis. After over expression CHI3L1 in inflammation model, the expression of AKT3 was significantly increased. Furthermore, silencing of AKT3 and AKT3 inhibitor both can block the effect of CHI3L1 overexpression, which indicated that AKT3 is indeed the potential target of CHI3L1.

According to the published literature, the activation of AKT3/PKB signaling mainly causes reduced cell apoptosis and increased cell proliferation. Furthermore, there are reports shown that AKT3 can significantly inhibit MMP13 in normal human dermal fibroblasts, and the mechanism of which is partly related to suppressing IL-1 and ADAMTS4 expression. AKT3 is also associated with Oncostatin M (OSM) to protect against cardiac injury, which is also related to its inflammatory inhibition effect. These publications further support our findings that AKT3 can modulate intervertebral disc degeneration in nucleus pulposus cells by suppressing the inflammatory effectors like ADAMTS and MMPs. On the other hand, report has shown that YKL-40 can reduce MMP1, MMP3, and MMP13 expression through AKT pathway in human skin fibroblasts, these data greatly support our findings that CHI3L1 can reduce degeneration phenotype through modulating AKT3 signaling. Besides, there exist a study about CHI3L1 indicating that cartilage-derived CHI3L1 has the effect of inhibiting type I collagen expression, which further emphasize the functional role of CHI3L1 in inhibiting IDD. In our findings, we showed that silencing CHI3L1 can decrease the expression of matrix-related genes which including ACAN, CHSY3, and COL2, and increase the expression of inflammation-related genes which including MMPs, ADAMTS4, and ADAMTS5, and the mechanism of which is through AKT3 signaling. Besides, the CHI3L1 expression is highly localized in the NP region compared to annulus fibrosus and other mesenchymal tissues, which indicated that CHI3L1 expression and its function may be NP specific proved by our immunohistochemistry analysis. And thus, we identify CHIL31-AKT3 axis as a NP specific degeneration-protecting mechanism during IDD.

Reports have shown that CHI3L1 is associated with many basic processes in highly conserved species including inflammation, cell apoptosis, tumor metabolism, or even in inflammatory chemotaxis. Here, we propose that CHI3L1 can exert a protective effect during IDD, and the mechanism of which is also conserved between human and mice. Since CHI3L1 is an endogenous factor, it can be a good therapeutic target in developing non-surgical treatment for IDD. In our data, we showed that CHI3L1 expression is elevated almost three times than normal, and its upregulation is important to maintain the normal function of NP cells. However, additional CHI3L1 overexpression or YKL-40 addition can greatly reverse the effect of inflammation induced degeneration, which implies that more YKL-40 during early degeneration may be helpful in stop IDD progression or even reverse it. Moreover, according to the research, inflammation is also the source of discogenic low back pain. Thus, in treating non-herniated discogenic low back pain, CHI3L1 may also effectively alleviate the symptom. Thus, here we provided various evidence supporting CHI3L1, an autocrine and degeneration protective secretion protein, may have a potential therapeutic value which require further studying.
ACKNOWLEDGMENTS
This research was supported by grants from the National Natural Science Foundation of China (81572096, 81772392, 81672211, 81871802, 81772376, 81601928, 81702149), Shanghai Municipal Commission of Health and Family Planning (2018Y0181 and 18ZR1438900), Shanghai Education Development Foundation and Shanghai Municipal Education Commission “Shu Guang” Program (17SG35), “Chen Guang” Program (17CG36, 17CG36) and Shanghai "Rising Stars of Medical Talent" Youth Development Program.

CONFLICT OF INTEREST
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

AUTHOR CONTRIBUTIONS
R. Wang, C. Xu, H. Zhong, and W. Yuan designed research; R. Wang, C. Xu, H. Zhong, B. Hu, L. Wei, N. Liu, and Y. Zhang analyzed data; R. Wang, C. Xu, H. Zhong, Q. Shi, C. Wang, M. Qi, Y. Gu, X. Shen, Ye, T. Tian, Y. Liu, and P. Cao performed research; P. Cao, H. Chen, W. Yuan, and C. Xu wrote the paper; H. Zhong, B. Hu contributed to the proof reading of the paper.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.