

Original Article

LincRNA-SLC20A1 (SLC20A1) promotes extracellular matrix degradation in nucleus pulposus cells in human intervertebral disc degeneration by targeting the miR-31-5p/MMP3 axis

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Abstract: Long non-coding RNAs (lncRNAs) are novel players in intervertebral disc degeneration (IDD) and show multiple functions. LncRNA lincRNA-SLC20A1 (SLC20A1) is aberrantly expressed in IDD. However, the role of SLC20A1 in degenerative nucleus pulposus (NP) cells and its underlying mechanism are unclear. The expressions of SLC20A1, miR-31-5p, and MMP3 were determined using RT-qPCR and western blotting. Extracellular matrix (ECM) degradation was evaluated by ECM-related genes collagen II, aggrecan, and ADAMTS4 using western blotting and an enzyme-linked immunosorbent assay (ELISA). The target binding between miR-31-5p and SLC20A1 or matrix metalloproteinase (MMP3) was predicted based on the *miRcode* or *starBase* websites and confirmed using a luciferase reporter assay and an RNA pull-down assay. SLC20A1 expression is upregulated in NP tissues from IDD patients, and this expression promotes ADAMTS5 expression and represses collagen II and aggrecan expression in degenerative NP cells derived from IDD patients. Mechanically, SLC20A1 acts as a competing endogenous RNA (ceRNA) to negatively regulate miRNA-31-5p (miR-31-5p) expression. Moreover, MMP3 is a downstream target for miR-31-5p and is positively modulated by SLC20A1 in degenerative NP cells. Similar to the SLC20A1 effect in human NP cells, the downregulation of miR-31-5p facilitates ECM degradation as well. On the contrary, miR-31-5p upregulation abolishes the promoting role of SLC20A1 in degenerative NP cells, the effect of which is then blocked by the ectopic expression of MMP3. The upregulation of SLC20A1 aggravates ECM degradation in degenerative human NP cells by targeting the miR-31-5p/MMP3 axis, suggesting that the SLC20A1/miR-31-5p/MMP3 pathway can contribute to IDD progression.

Keywords: SLC20A1, miR-31-5p, MMP3, nucleus pulposus (NP), intervertebral disc degeneration (IDD)

Introduction

Intervertebral disc degeneration (IDD) is a chronic, prevalent, and age-related degenerative musculoskeletal disorder that brings an enormous socioeconomic burden worldwide [1]. The incidence of IDD has also been increasing in younger adults in the past decades. Usually, IDD is a common clinical problem and the significant pathological basis of low back pain [2]. Current therapeutic options for IDD are aimed at pain reduction and symptom control rather than disease modification, even though surgery is presently the most effective treatment [3]. IDD is a multifactorial process, and a variety of cellular events are disturbed in the

progression of IDD, including cell proliferation, apoptosis, autophagy, the inflammatory response, as well as extracellular matrix (ECM) degradation [4, 5]. It is well known that intervertebral discs consist of three anatomical substructures, including the nucleus pulposus (NP), the annulus fibrosus, and the cartilaginous endplates [6]; moreover, early IDD arises from degenerative NP due to the loss of active cells and ECM. Therefore, some biological strategies have been developed to prevent early disc degeneration by promoting ECM repair and regeneration [7-9].

With the advances of special gene dysregulation in IDD, several strategies targeting deregulation

Table 1. The clinical characteristics of the study participants

Clinical characteristics	Control (n = 17)	IDD (n = 44)
Age (years)	50.21 ± 11.89	53.45 ± 12.36
Sex		
Female	6	18
Male	11	26
Pfrrmann		
I	/	4
II	/	14
III	/	9
IV	/	10
V	/	7

lated genes, such as long non-coding RNAs (lncRNAs), microRNAs (miRNAs) and protein coding genes, have been suggested [10, 11]. lncRNAs are longer than 200 nucleotides in length and have little protein coding potential. lncRNAs can regulate biological processes through diverse molecular mechanisms. For example, lncRNAs serving as competing endogenous RNAs (ceRNAs) participate in miRNA-dependent crosstalk and then contribute to the complexity of gene regulation, thus resulting in disease occurrence and development [12]. The aberrantly expressed lncRNAs' profile in human IDD was first addressed by Wan *et al.*, and their function annotation has been investigated [13]. Later, Chen *et al.* reviewed lncRNAs as novel players in IDD [10]. Recently, differentially expressed lncRNA and messenger RNAs (mRNAs) in patients with IDD has been reported, and the lncRNA-miRNA-mRNA ceNA regulatory network has been constructed [4]. lncRNA lincRNA-SLC20A1 (SLC20A1) belongs to the SLC20 family which functions as a sodium phosphate co-transporter across the plasma membrane [14]. SLC20A1 is expressed ubiquitously in all tissues. Generally, SLC20A1 is considered to be a housekeeping transport protein; however, its importance was re-defined as the tissue-specific activity, regulatory pathways, and gene-related pathophysiologies. In IDD, SLC20A1 was found to be upregulated to 5.99-fold in the degenerative NP tissues compared to non-degenerative specimens [13]. However, the role of SLC20A1 in degenerative NP cells and its underlying mechanism are unclear.

MiRNA-31-5p (miR-31-5p) is one of the most popular candidates and altered expressions of miR-31-5p that has been reported in diseases

[15]. Emerging evidence has pointed to miR-31-5p as an oncogenic or tumor-suppressor gene in different types of tumors, as well as a useful clinical prognostic biomarker [16]. The expressions of miR-31-5p together with miR-124a and miR-127-5p are commonly down-regulated in IDD tissues [17] and are associated with ECM synthesis in the formation of hypertrophic scars [18]. In this study, the dysregulation of SLC20A1 in NP tissues from IDD patients was confirmed. Subsequently, we acquired degenerative human NP cells derived from IDD patients to investigate the role of SLC20A1 in ECM degradation, as well as its mechanism of acting as a ceRNA.

Materials and methods

Patients and collection of samples

With the approval of the Research Ethics Committee of West China Hospital, Sichuan University and the written informed consents from 44 patients with IDD and 17 patients with idiopathic scoliosis, NP tissue samples were obtained during operations and immediately frozen in liquid nitrogen and then stored at -80°C. Patients with IDD combined with degenerative spinal stenosis, idiopathic scoliosis, tumors, infections or previous lumbar disc surgery were excluded from this study. All patients underwent a routine MRI of the lumbar spine before surgery, and the disc degeneration degree was analyzed according to T2-weighted images using the modified Pfirrmann classification. The clinical features of all the enrolled patients are shown in **Table 1**.

Cell culture

Human degenerative NP cells were isolated from the NP tissues from the IDD patients. The NP tissues were washed with sterile-free phosphate-buffered saline (PBS) and minced into pieces, then digested with 0.2% collagenase II (Sigma, St Louis, MO, USA) and 0.25% trypsin (Gibco, Carlsbad, CA, USA) for 3 h. After that, the cells were re-suspended in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 mg/mL streptomycin, and 100 U/mL penicillin. The NP cells were incubated at 37°C with 5% CO₂. And the cultivated NP cells between 1-5 generations were stored for use in subsequent experiments.

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Cell transfection

The MiR-31-5p mimic, the miR-NC mimic, anti-miR-31-5p, and anti-miR-NC were obtained from *GenePharma* (Shanghai, China), and SLC20A1 was cloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA). The NP cells were transfected of these oligonucleotides and plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The transfected NP cells were cultured for an additional 48 h prior to further studies.

RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR)

For the examination of the SLC20A1, miR-31-5p, and MMP3 mRNA expressions, total RNAs from the NP tissues and cells were extracted with TRIzol reagent (Invitrogen) and were reverse transcribed into cDNA with a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The quantitative PCR was performed with SYBR green detection (Promega, Madison, WI, USA) on an ABI 7500 real-time PCR system (Applied Biosystems). GAPDH mRNA was used as a reference gene for SLC20A1 and MMP3, and U6 small nuclear RNA (U6) was the internal control to mature miR-31-5p. The reactions were performed in quadruplicate for each sample with at least three independent runs, and the primers involved were as follows: SLC20A1: 5'-CATCCTCCCACCCAATCATC-3' forward and 5'-GGACCTCCAGCAAACACCAG-3' reversed; MMP3: 5'-AGAAGAGAAATTCATGGAGC-3' forward and 5'-CTCCAAGTGTGAAGATCCAGTA-3' reversed; GAPDH: 5'-GCTCTCTGCTCCTCCTGTTC-3' forward and 5'-ACGACCAAATCCGTTGACTC-3' reversed; miR-31-5p: 5'-CGGCGGAGGCAAGATGCTGGCA-3' forward and 5'-CAACTGGTGTCGTGGAGTCGG-3' reversed; U6: 5'-CTCGCTTCGGCAGCACA-3' forward and 5'-AACGCTTCACGAATTTGCGT-3' reversed. The relative expressions of SLC20A1, miR-31-5p, and MMP3 mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH or U6.

Protein isolation and western blotting

Total protein from the cultured NP cells was isolated in a RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland), and the protein concentrations were determined

using the Bradford protein assay reagent (Bio-Rad, Shanghai, China). Equal amounts of protein (20 µg) from each sample were loaded for the standard procedures of the western blot assay. GAPDH on the same membrane was used as an internal standard to normalize the protein levels. The primary antibodies were purchased from Abcam (Cambridge, UK) and were as follows: collagen II (#34712, 1:10000), aggrecan (#3778, 1:100), ADAMTS4 (#185722, 1:1000), MMP3 (#53015, 1:500) and GAPDH (#9485, 1:2500).

Enzyme-linked immunosorbent assay (ELISA)

The ECM-related genes in the culture supernatant of the NP cells were determined using commercial human-specific ELISA kits purchased from *Abcam* and were as follows: collagen II (#34712, 1:5000), aggrecan (#3778, 1:50), ADAMTS4 (#213753, 1:1000). The culture supernatant was acquired from the NP cells that were treated for 48 h. Their levels were measured at a wavelength of 450 nm.

Dual-luciferase reporter assay

The SLC20A1 3'UTR wild and mutant types (SLC20A1-Wt/Mut) were constructed into pmirGLO (Invitrogen), as well as the MMP3 mRNA 3'UTR wild and mutant types (MMP3-Wt/Mut). Human NP cells were co-transfected with an miR-31-5p/NC mimic and either SLC20A1-WT/MUT or MMP3-Wt/Mut. All transfection procedures were performed using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, the luciferase activity was measured using a dual-luciferase reporter system (Promega). The ratio of firefly to Renilla luciferase activity was used as the relative luciferase activity. All operations were repeated three times.

RNA pull-down assay

Biotin-labelled (Bio-) SLC20A1, miR-31-5p, their mutant, and negative controls were synthesized by *GenePharma* and transfected into NP cells. After 48 h, whole cell lysates were harvested and further incubated with streptavidin-coupled agarose beads (Invitrogen) for 1 h. Then, the RNA-protein complexes on the beads were pulled down with bio-SLC20A1, SLC20A1-Mut, and NC or bio-miR-31-5p, miR-31-5p-Mut and miR-NC were for 10 min and treated with a

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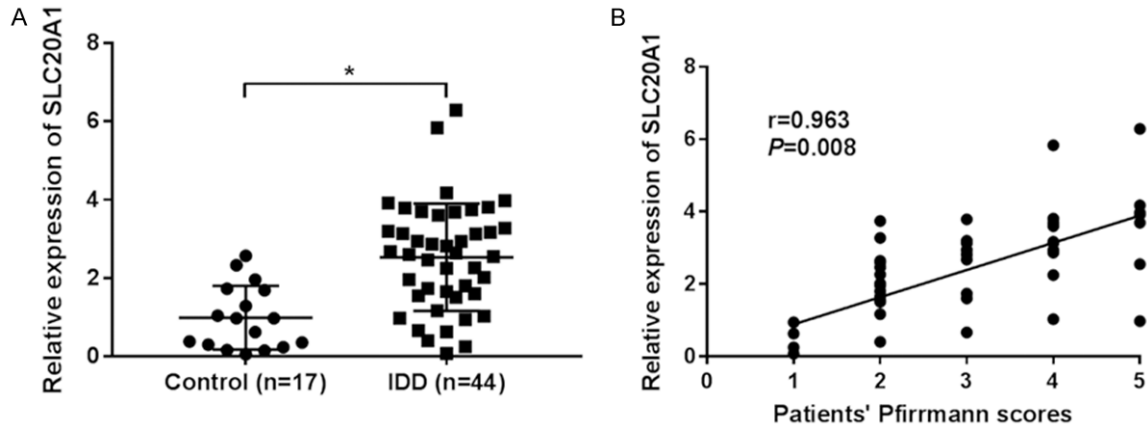


Figure 1. LncRNA-SLC20A1 (SLC20A1) expression was upregulated in the degenerative nucleus pulposus (NP) tissues from patients with intervertebral disc degeneration (IDD). A. Relative expression levels of SLC20A1 were determined by RT-qPCR in NP tissues from IDD patients (n = 44) and normal patients (n = 17). B. The correlation between the expression of SLC20A1 and the patients' Pfirrmann scores. Data are expressed as the mean $\pm$ standard deviation (SD) and * $P < 0.05$.

washing buffer. The isolated RNA was transcribed into cDNA and then the amount of miR-31-5p or SLC20A1 was detected using RT-qPCR.

Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). A Student's *t*-test was performed to compare the differences between the treated groups relative to their controls. The differences between groups were evaluated using a one-way ANOVA. The statistical analyses were performed using Graphpad, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and the *P*-values indicated in the figures were considered to be statistically significant when compared with a value < 0.05 . Spearman's two-tailed correlation test and the two-tailed chi-square test were used for the Pfirrmann scores and SLC20A1 expression.

Results

SLC20A1 expression was upregulated in the degenerative human NP tissues from IDD patients

In order to investigate the expression of SLC20A1 in NP tissues, human degenerative NP tissues and the control NP tissues were obtained, and all the clinical characteristics of the recruited patients are shown in **Table 1**. An RT-qPCR analysis was performed to determine the differential expressions of SLC20A1 in

patients, and SLC20A1 was statistically upregulated in the NP tissues from the IDD patients (**Figure 1A**). Moreover, the correlations between SLC20A1 expression and the severity of IDD were investigated. We observed significant positive linear correlations between the expressions of SLC20A1 and the patients' Pfirrmann scores (**Figure 1B**). These data suggested a potential biomarker of SLC20A1 in the pathogenesis and development of IDD.

The upregulation of SLC20A1 promoted ECM degeneration in degenerative human NP cells

To figure out the role of SLC20A1 in the NP cells, human NP cells were isolated from IDD patients and SLC20A1 was forcibly highly expressed in the cultivated NP cells *in vitro*. The high transfection efficiency was confirmed after the transfection as evidenced by dramatically increased SLC20A1 (**Figure 2A**). Subsequently, RT-qPCR and western blotting demonstrated that the upregulated collagen II and aggrecan expressions both at the mRNA (**Figure 2B**) and protein levels (**Figure 2C** and **2D**) were distinctively decreased when transfected with pcDNA3.1-SLC20A1, but the ADAMTS4 expression was increased. Furthermore, the levels of collagen II and aggrecan (**Figure 2E** and **2F**) secreted in the culture supernatant were also attenuated, and the ADAMTS4 level (**Figure 2G**) was promoted. These results showed that SLC20A1 could aggravate ECM degeneration in degenerative NP cells from IDD patients.

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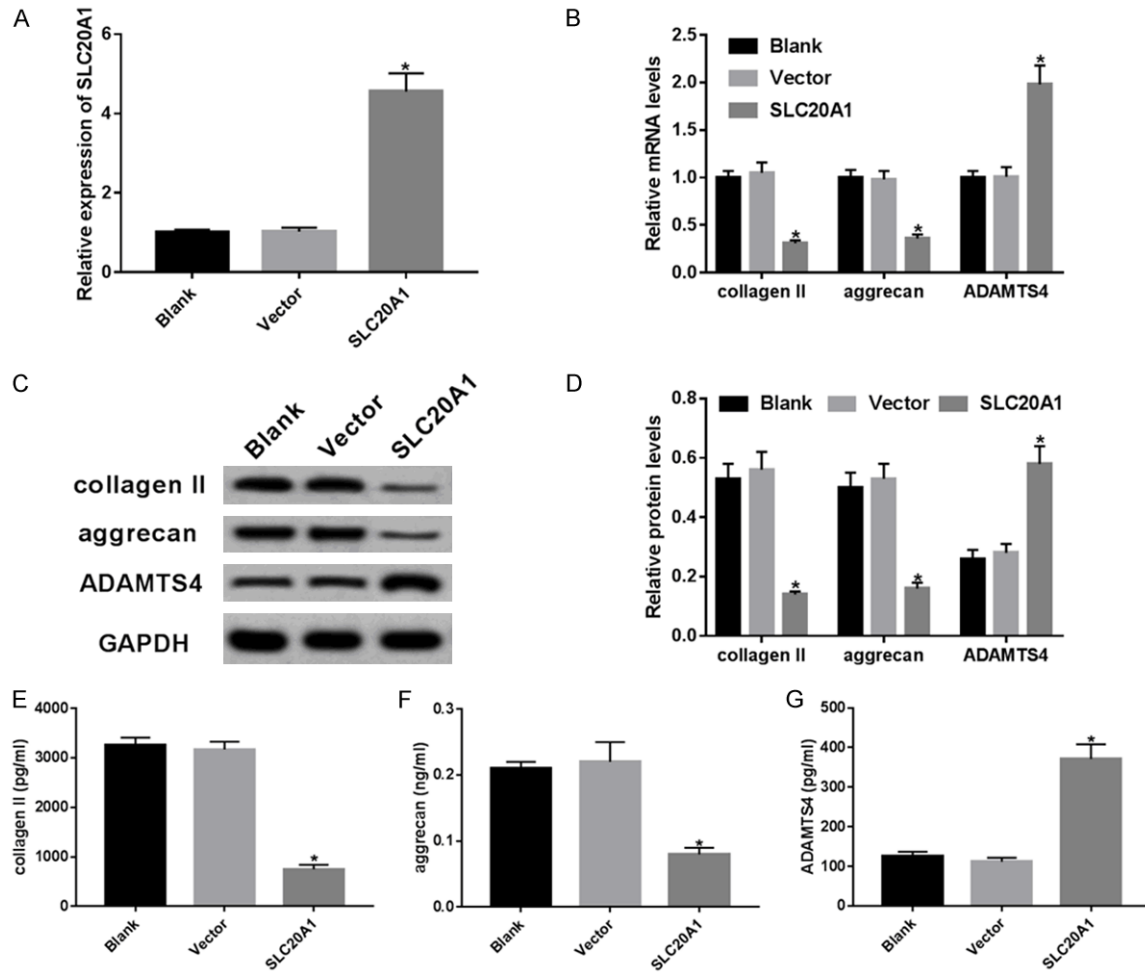


Figure 2. The Role of SLC20A1 in the extracellular matrix (ECM) synthesis in human degenerative NP cells derived from IDD patients. The degenerative human NP cells were isolated from the IDD patients and were transfected with pcDNA3.1-SLC20A1 (SLC20A1) or with a pcDNA3.1-vector (Vector) for 48 h. (A) The levels of SLC20A1 were measured. The expressions of the ECM-related genes collagen II, aggrecan, and ADAMTS4 in the NP cells were examined using RT-qPCR (B) and western blotting (C and D). (E-G) The levels of collagen II, aggrecan, and ADAMTS4 secreted in the culture supernatant were determined using an enzyme-linked immunosorbent assay (ELISA). Data are expressed as the mean $\pm$ SD and * $P < 0.05$.

SLC20A1 negatively regulated miR-31-5p by acting as ceRNA

A possible target gene of SLC20A1 was retrieved and identified as miR-31-5p on the *miRcode* website. The sequences of the putative binding site in the SLC20A1-3'UTR wild type were mutated as the complementary sequences as shown in **Figure 3A**. To confirm this, a dual-luciferase reporter assay was performed, and NP cells were transfected with an miR-31-5p/NC mimic. The transfection efficiency of miR-31-5p was found to be high (**Figure 3B**). Also, **Figure 3C** shows that the miR-31-5p mimic lowered the relative luciferase activity of

the SLC20A1 wild type, but there was little influence on the luciferase activity of the LINC00261 mutant whenever it was transfected with the miR-31-5p mimic or the miR-NC mimic. Moreover, the relative expression of SLC20A1 in the NP cells was greatly lowered only by bio-miR-31-5p and vice versa (**Figure 3D** and **3E**). In addition, the regulatory relationship between LINC00261 and miR-31-5p was determined in the NP cells. We observed a high transfection efficiency of pcDNA3.1-SLC20A1 and si-SLC20A1 (**Figure 3F**), and the miR-31-5p expression level was decreased when it was transfected with pcDNA3.1-SLC20A1 and increased when it was transfected with si-

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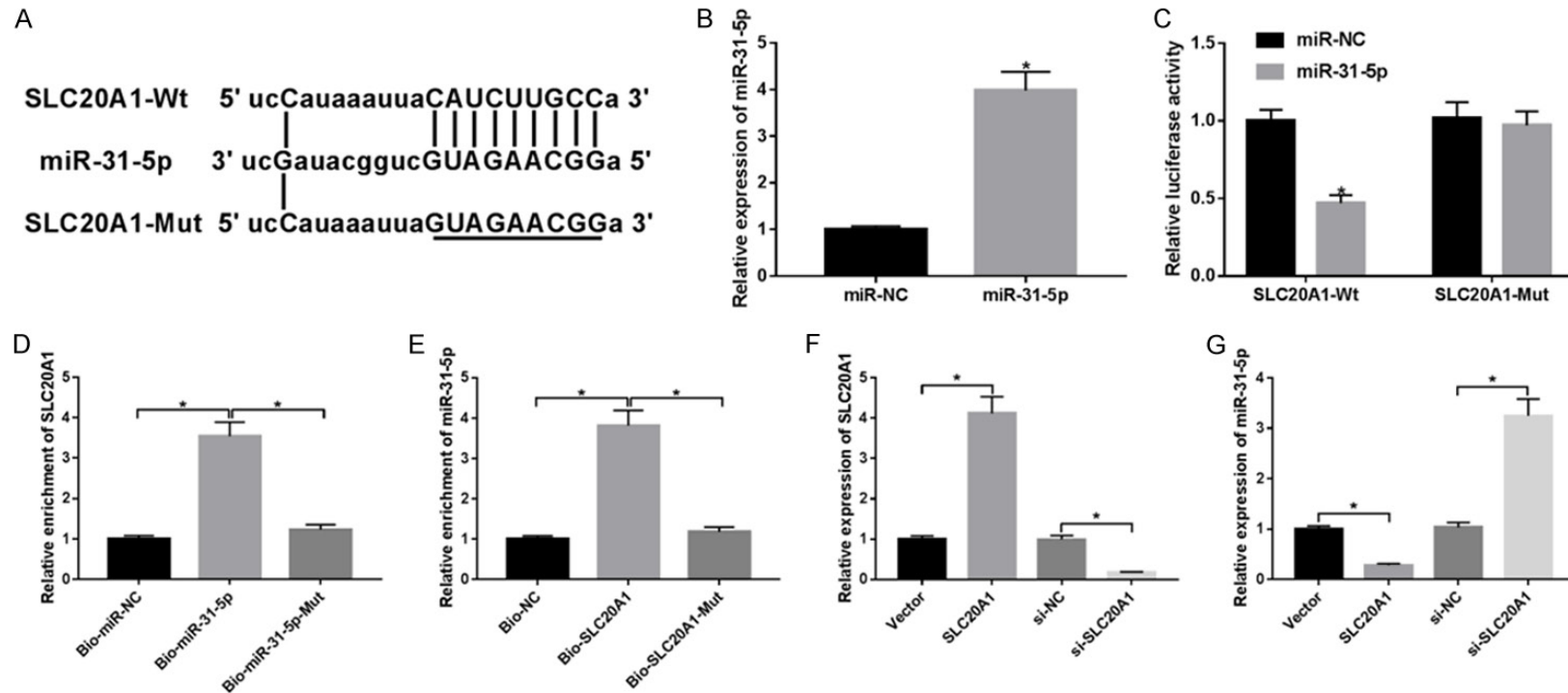


Figure 3. SLC20A1 negatively regulated miR-31-5p by sponging. A. The prediction of the potential binding site of miR-31-5p on SLC20A1 wild type (SLC20A1-Wt) found on the *miRcode* website. The putative targeting site was mutated as SLC20A1-Mut. B. Human degenerative NP cells were transfected with an miR-31-5p/NC mimic (miR-31-5p/NC) and the levels of miR-31-5p were confirmed by RT-qPCR after transfection. C. The relative luciferase activity of SLC20A1-Wt/Mut was examined in the NP cells transfected with miR-31-5p/NC using the dual-luciferase reporter system. D. Relative enrichment of SLC20A1 in NP cells transfected with biotin-labelled miR-NC (bio-miR-NC), miR-31-5p (bio-miR-31-5p), and a mutant of miR-31-5p (bio-miR-31-5p-Mut). E. Relative enrichment of miR-31-5p in NP cells transfected with biotin-labelled NC (bio-mNC), SLC20A1 (bio-SLC20A1), and a mutant of (bio-SLC20A1-Mut). F, G. NP cells were transfected with SLC20A1, siRNA against SLC20A1 (si-SLC20A1), and its negative controls, and the expression of SLC20A1 and miR-31-5p was measured by RT-qPCR. Data are expressed as the mean $\pm$ SD and * $P < 0.05$.

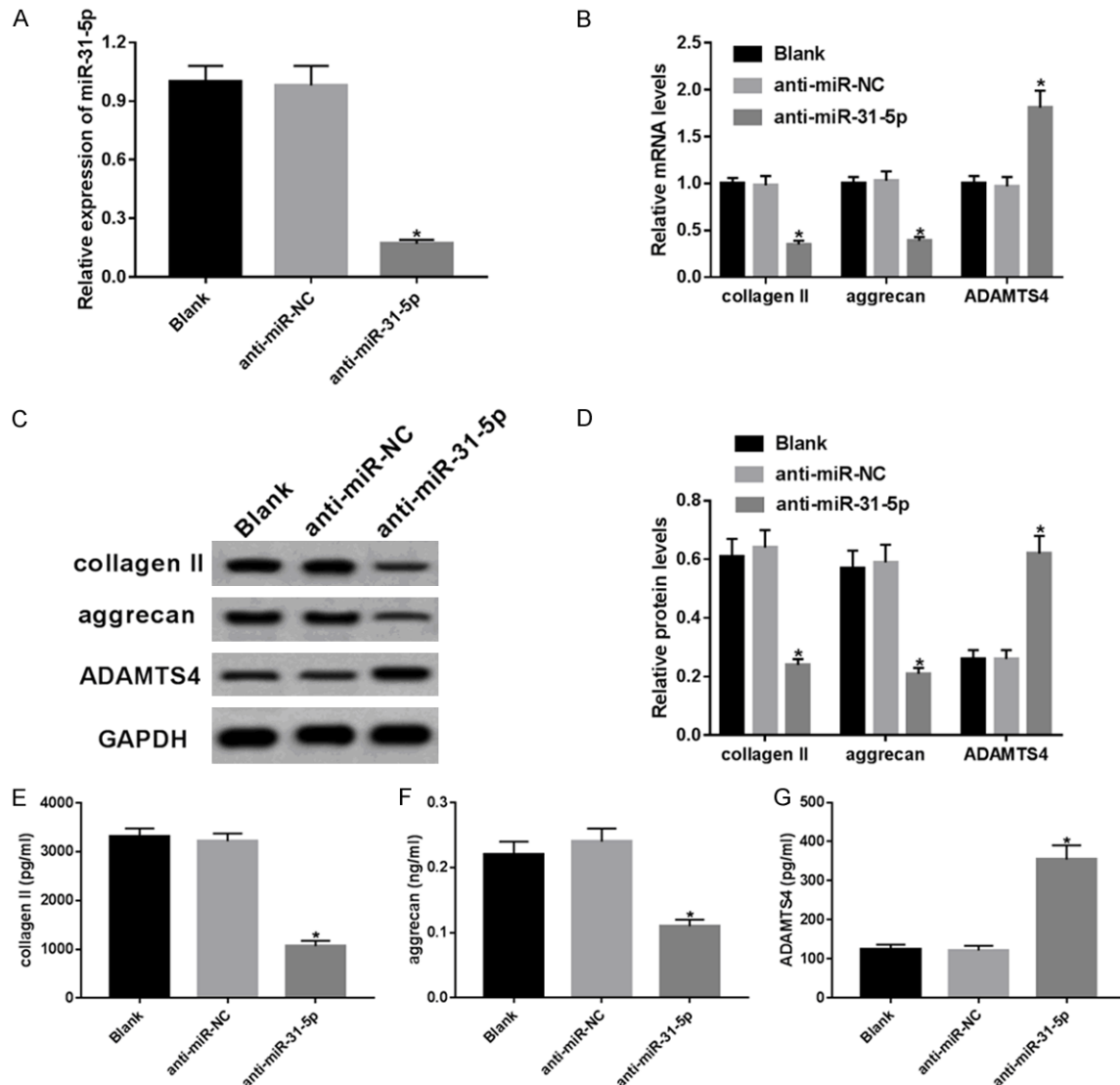


Figure 4. The role of miR-31-5p in ECM synthesis in human degenerative NP cells derived from the IDD patients. The NP cells were transfected with anti-miR-31-5p/NC for 48 h. (A) The levels of miR-31-5p were measured. The expressions of collagen II, aggrecan, and ADAMTS4 in NP cells were examined using RT-qPCR (B) and western blotting (C and D). (E-G) The levels of collagen II, aggrecan and ADAMTS4 secreted in the culture supernatant were determined by ELISA. Data are expressed as the mean $\pm$ SD and * P < 0.05.

SLC20A1 (Figure 3G). Taken together, our data suggest that SLC20A1 negatively regulated miR-31-5p in degenerative human NP cells through target binding.

The downregulation of miR-31-5p facilitates ECM degeneration in degenerative human NP cells

To figure out the role of miR-31-5p in NP cells, human degenerative NP cells were forcibly lowly expressed with miR-31-5p by the trans-

fection of anti-miR-31-5p *in vitro*. The high transfection efficiency was confirmed after the transfection as evidenced by a remarkably decreased miR-31-5p level (Figure 4A). Subsequently, the expressions of collagen II and aggrecan both on the mRNA (Figure 4B) and protein levels (Figure 4C and 4D) were distinctively lower when transfected with anti-miR-31-5p, but the ADAMTS4 expression was increased. Furthermore, the levels of collagen II and aggrecan (Figure 4E and 4F) secreted in the culture supernatant were also attenuated,

and the ADAMTS4 level (**Figure 4G**) was promoted. These results show that miR-31-5p could aggravate ECM degeneration in degenerative NP cells from IDD patients.

MMP3 is a direct target of miR-31-5p and is regulated by SLC20A1

This study observed a potential binding site between miR-31-5p and MMP3 3'UTR as predicted on the *starBase* website (**Figure 5A**). The MMP3 3'UTR wild type containing the putative miR-31-5p target site and its corresponding mutant were cloned into the luciferase reporter pmirGLO vector, and the relative luciferase activity of the MMP3 wild type was significantly decreased in the NP cells when transfected with the miR-31-5p mimic (**Figure 5B**); meanwhile, there was no difference in the MMP3 mutant groups. As demonstrated in **Figure 5C**, the expression of MMP3 was downregulated when transfected with the miR-31-5p mimic and upregulated when expressed with anti-miR-31-5p (**Figure 5C** and **5D**). Moreover, the MMP3 expression was found to be elevated by ectopic SLC20A1 and then was blocked by the miR-31-5p transfection (**Figure 5E** and **5F**). These data show that MMP3 is a downstream target for miR-31-5p and is positively regulated by SLC20A1 in NP cells.

The upregulation of MMP3 contributes to the promotion effect of SLC20A1 on ECM degeneration through miR-31-5p

Based on our results, we hypothesized that the SLC20A1/miR-31-5p/MMP3 pathway might contribute to ECM degeneration in degenerative human NP cells. To further confirm this, transfected NP cells were divided into 6 groups: Vector, SLC20A1, SLC20A1+miR-NC, SLC20A1+miR-31-5p, SLC20A1+miR-31-5p+Vector, and SLC20A1+miR-31-5p+MMP3. First, we measured the MMP3 mRNA expression in the transfected NP cells. It was found that the upregulation of MMP3 mediated by SLC20A1 was reversed by the miR-31-5p mimic, which was further rescued in the presence of pcDNA3.1-MMP3 (**Figure 6A**). At the same time, the expression of collagen II and aggrecan in both the degenerative NP cells (**Figure 6A** and **6B**) and the culture supernatant (**Figure 6C** and **6D**) was inhibited when SLC20A1 was upregulated and was improved with the co-transfection of pcDNA3.1-SLC20A1 and

miR-31-5p. Moreover, the ectopic expression of MMP3 could abolish the impact of miR-31-5p upregulation on SLC20A1's role in ECM degeneration. Also, the ADAMTS4 expression (**Figure 6A**, **6B** and **6E**) was consistent with MMP3 and was just the opposite of the collagen II and aggrecan expressions. These results revealed that the upregulation of the miR-31-5p/PTEN axis contributed to the promotion effect of SLC20A1 on ECM degeneration in degenerative human NP cells.

Discussion

LncRNAs, including SLC20A1, play a pivotal role in IDD through different mechanisms. For example, the ectopic expression of SNHG1 [19] and RMRP [20] induced cell growth and enhanced the expression of ki-67, PCNA and cyclin D1 in NP cells by targeting miRNA-326 and miRNA-206, respectively; the HCG18 [21] sponging of miRNA-146-5p retarded the growth of NP cells by decreasing the S phase of the cell cycle, inducing cell apoptosis, the recruitment of macrophages and hypercalcification. GAS5 overexpression [22] promoted, and HOTAIR overexpression [23] suppressed, NP cell apoptosis due to the altered apoptosis rate and the expression of Bcl-2, Bax, and caspase-3, which may be directly associated with miRNA-155 and miRNA-34a/Bcl-2. The upregulation of linc00641 could regulate autophagic cell death by targeting miRNA-153-3p and the autophagy-related gene 5 (ATG5) [24]. Moreover, ECM expression was modulated by multiple lncRNAs, such as FAM83H-AS1 [25], RMRP [20], NEAT1 [26], H19 [27], and linc00641 [24], as evidenced by the promoted expression of MMP2/3/9/13 and ADAMTS4/5, and the suppressed expression of collagen I/II and aggrecan. The association between the dysregulation of lncRNAs and the signaling pathways including notch [25], Wnt/ β -catenin [27, 28], and Erk [29] was also confirmed in degenerative NP cells. FAM83H-AS1, RMRP, SNHG1, HCG18, and NEAT1 expressions were positively correlated with the degree of disc degeneration grade [19-21, 25, 26]. Here, we investigated expression and role of SLC20A1 in degenerative human NP tissues and cells. As a result, the upregulation of SLC20A1 was found to be consistent with a previous study [13], and this expression could aggravate ECM degradation by increasing ADAMTS4 and decreasing colla-

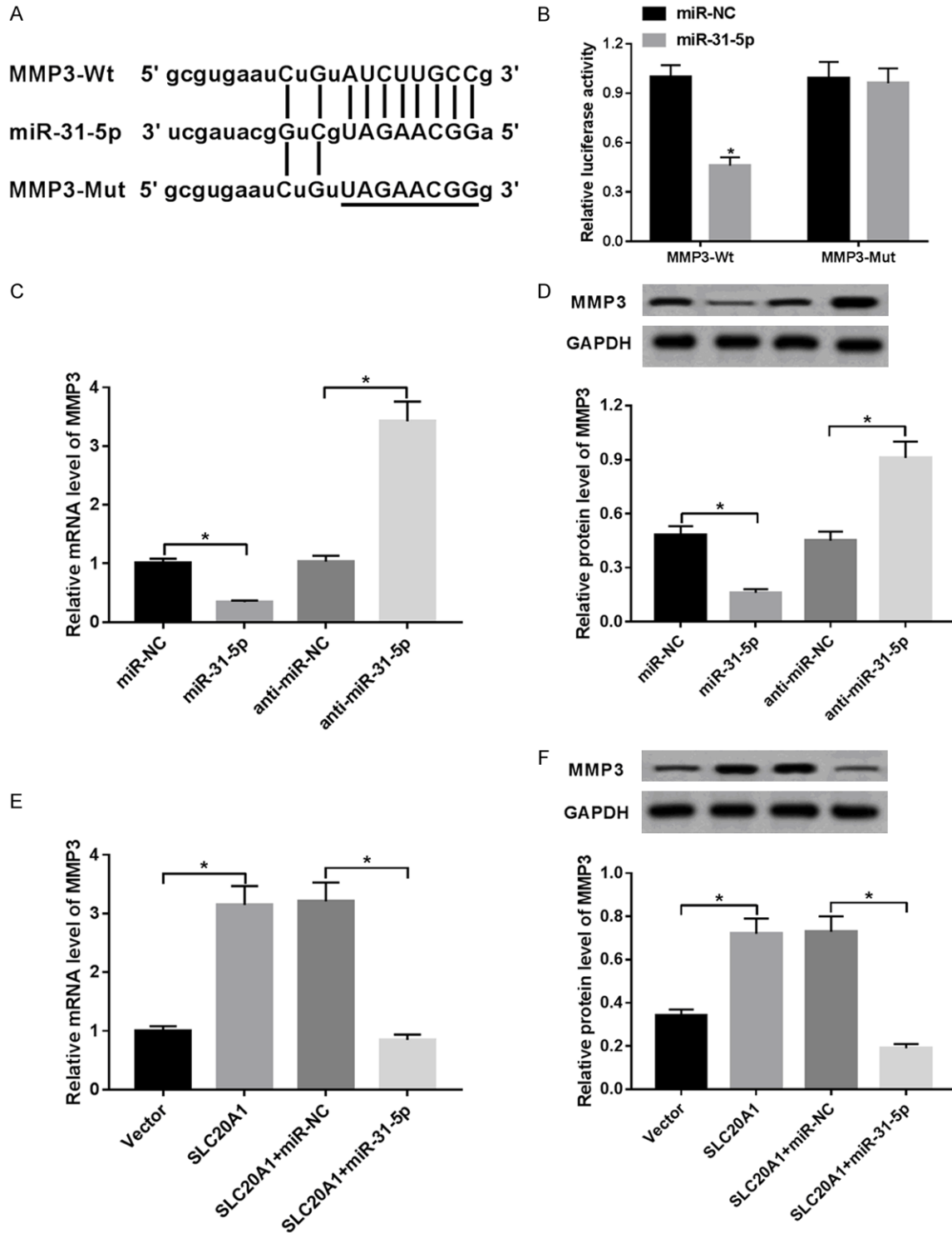


Figure 5. MMP3 was a direct target of miR-31-5p and regulated by SLC20A1. **A.** The potential binding site between miR-31-5p and MMP3 wild type (MMP3-Wt) was predicted on the *starBase* website. The putative targeting site was mutated as MMP3-Mut. **B.** The relative Luciferase activity of MMP3-Wt/Mut was examined using a dual-luciferase reporter system in NP cells transfected with miR-31-5p/NC. **C, D.** The expression of MMP3 mRNA and protein was detected in NP cells when transfected with miR-31-5p/NC or anti-miR-31-5p/NC. **E, F.** The expression of MMP3 mRNA and protein was detected in NP cells when transfected or SLC20A1 or a vector and co-transfected with SLC20A1 and miR-31-5p/NC. Data are expressed as the mean $\pm$ SD and * $P < 0.05$.

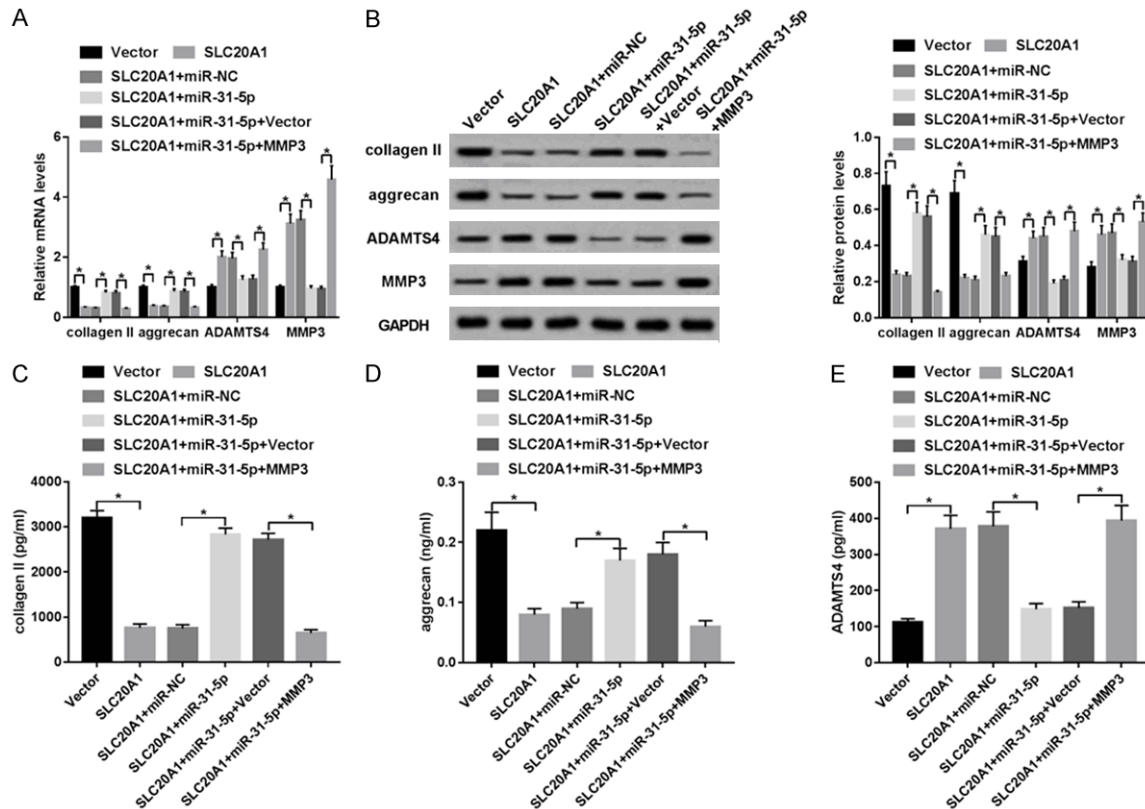


Figure 6. The influence of miR-31-5p and MMP3 on the promotion effect of SLC20A1 on ECM degradation in human degenerative NP cells. Human NP cells were transfected with SLC20A1 or a vector, or the co-transfection of SLC20A1 and miR-31-5p/NC, or the co-transfection of SLC20A1 and miR-31-5p and MMP3. A, B. The expression of MMP3, collagen II, aggrecan and ADAMTS4 in degenerative human NP cells was determined by RT-qPCR and western blotting. C-E. Expression of collagen II, aggrecan, and ADAMTS4 secreted in the culture supernatant was determined by ELISA. Data are expressed as the mean $\pm$ SD and $*P < 0.05$.

gen II and aggrecan, along with directly down-regulating miR-31-5p and upregulating MMP3. Moreover, the target regulatory relationship between miR-31-5p and MMP3 was also verified. Taken together, our study suggests that the SLC20A1/miR-31-5p/MMP pathways are a novel ceRNA network in regulating ECM degradation during IDD pathogenesis and development. Clinically, there was a positive linear correlation between SLC20A1 high expression and the patients' Pfirrmann scores, indicating SLC20A1 might serve as a potential biomarker in the progression of IDD.

Genetic heredity is the predominant risk factor for degenerative disc disease and is estimated to cause over 70% of cases. Besides lncRNAs, miRNAs also represent a new player in IDD by regulating the expression of MMPs. For instance, Zhang *et al.* [30] addressed the miRNA-155-mediated pathological impact on IDD

in vivo using a classic puncture mouse model and found that decreased miRNA-155 occurred in degenerative NP tissues and that this dysregulation could upregulate MMP16 expression and further degrade aggrecan and collagen type II expression, thus leading to the dehydration and degeneration of discs. The overexpression of miRNA-486-5p repressed lipopolysaccharide (LPS)-stimulated NP cells in the attenuated expression of inflammatory cytokines (IL-1 β , IL-6 and TNF- α) and matrix degrading enzymes (MMP3, MMP13, ADAMTS4 and ADAMTS5) and promoted the expressions of ECM-related genes (aggrecan and collagen II) [31]. However, there are very few miRNAs unveiled to take part in IDD by targeting MMPs except two miRNAs. Hua *et al.* [32] indicated that miRNA-127-5p was significantly downregulated in human NP tissues of degenerative discs and that this expression contributed to collagen II degradation by targeting MMP13.

Shi *et al.* [33] declared that miRNA-202-3p's targeting of MMP1 mediated the IL-1 β -induced decrease in the fluorescence intensity of collagen II. To date, our data demonstrated miR-31-5p was abnormally expressed, and it negatively regulated gene expression in IDD, which is consistent with a previous study [17]. Moreover, we not only discovered the promotion effect of miR-31-5p knockdown on ECM gradation (ADAMTS4, aggrecan and collagen II) in degenerative human NP cells, but we also found its molecular mechanism by targeting MMP3. Together with the outcomes of Wang *et al.* [18], we hypothesize that miR-31-5p might be closely related to ECM homeostasis, but this assumption should be further verified.

In this study, we discovered a novel SLC20A1/miR-31-5p/MMP pathway in ECM degradation in NP cells from patients with IDD. However, the role of SLC20A1 in cell growth, cell death, and inflammation remains to be further uncovered, as well as its involvement in signaling pathways. Furthermore, a better understanding of SLC20A1's role and molecular mechanisms in IDD needs to be urgently addressed before it can be an attractive therapeutic target for IDD.

To conclude, SLC20A1 is highly expressed in degenerative NP tissues from IDD patients, and this expression is positively correlated with the degree of disc degeneration. The *in vitro*, upregulation of SLC20A1 can aggravate ECM degradation in degenerative human NP cells by targeting the miR-31-5p/MMP axis. This work demonstrates for the first time the target regulatory relationship between miR-31-5p and either SLC20A1 or MMP3. Our results provide limited but primary data about the biological functions of SLC20A1 in human IDD.

Disclosure of conflict of interest

None.

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