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Original Article

Acupuncture at Yanglingquan (GB34) acupoint alleviates lumbar intervertebral disc degeneration in rats

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Abstract: Acupuncture treatment of lumbar intervertebral disc degeneration (LIVD) has been confirmed in clinical, but the exact mechanism of its effectiveness is not known yet and worthy of further study. The present study aimed to investigate the effects and mechanism of acupuncture at Yanglingquan (GB34) acupoint on the LIVD in rats. Rats were randomly divided into four groups, five in each group: normal group, sham group, LIVD model group and acupuncture group. LIVD model was induced by abnormal stress and spine instability. After 4 weeks of acupuncture at Yanglingquan acupoint, the mRNA expression levels of osteoblastic marker genes (ALP, osteocalcin, Osterix and BSP) and LIVD degeneration-related genes (Col1, Col2, COMP, ADAMTS-7, ADAMTS-12, MMP-13, TIMP-1 and PGRN) in lumbar intervertebral discs were detected by qRT-PCR, the protein expression levels of MMP-13, TIMP-1 and PGRN were measured by western blotting. The location and expression of PGRN in lumbar intervertebral discs were observed by immunohistochemistry. *In vitro*, After PGRN targeted siRNA was transfected into cartilage endplate (CEP)-derived primary cells (CEPCs), the mRNA expression levels of MMP-13, TIMP-1, PGRN, TNF-α, COMP, ADAMTS-7 and ADAMTS-12 were tested with qRT-PCR. Acupuncture treatment significantly inhibited the up-regulated osteoblastic marker genes mRNA expression induced by the surgery. Compared with model group, the mRNA levels of Col1, ADAMTS-7 and ADAMTS-12 in acupuncture group were significantly decreased, in contrast, the mRNA levels of Col2 and COMP were increased. We also found that acupuncture treatment significantly improved the mRNA and protein levels of PGRN and TIMP-1, and reduced the levels of MMP13 at both mRNA and protein levels in degenerated lumbar intervertebral discs (IVD). Studies *in vitro* indicated that PGRN may play a regulation on the expression of TIMP-1, MMP-13, TNF-α, Col1, Col2, COMP, ADAMTS-7 and ADAMTS-12, and may play a pivotal role in the amelioration of LIVD by acupuncture treatment. In conclusion, acupuncture Yanglingquan acupoint could effectively alleviate surgery induced LIVD, and the mechanism may be related to upregulation of PGRN expression and inhibition of NF-κB pathway.

Keywords: Lumbar intervertebral disc degeneration, acupuncture, Yanglingquan, PGRN, NF-κB

Introduction

Lumbar disc herniation (LDH) is commonly seen in young and middle-aged person [1], and results in diminished quality of life, loss of working ability, potential psychological distress, and increased health care costs. Although the etiology of LDH has not been fully understood, it is typically associated with lumbar intervertebral disc degeneration (LIVD), which is characterized by increased extracellular matrix (ECM) degradation [2], ectopic bone formation and production of inflammatory mediators [3, 4].

In recent years, the incidence of LIVD-related diseases had obviously increased in China, in addition to drug therapies and surgical methods, the traditional conservative treatment such as acupuncture, moxibustion and herb are also widely used and recognized in clinics. The curative effects of acupuncture had been confirmed in clinical and animal experimental studies. However, the mechanisms remain unknown.

Progranulin (PGRN) is a multifunctional growth factor. PGRN is expressed in various cells and plays a critical role in a wide variety of biological

and disease processes such as: wound healing [5], cancer [6], and inflammation [7]. Studies have also found that PGRN has a close relationship with the nerve and articular cartilage degenerative disease [8, 9]. PGRN, as one of the downstream molecules of BMP-2, plays an important role in the proliferation, differentiation, growth and development of cartilage cells and endochondral ossification [10-12]. In addition, PGRN exhibited anti-inflammatory functions in osteoarthritis through its ability to bind to TNF receptors, TNFR1 and especially TNFR2 [13, 14].

The present study was aimed to investigate the amelioration effect of acupuncture at Yanglingquan points on LIVD in rats and the underlying mechanisms. The LIVD model was induced by abnormal stress and spine instability. The expression of PGRN in normal and degenerated intervertebral discs was evaluated by qRT-PCR, western blotting and immunohistochemistry. The potential effects of PGRN deficiency on ECM degradation and NF-κB alteration were also determined.

Material and methods

Animals

Twenty male SPF Sprague-Dawley (SD) rats (3 weeks old, weighing 200-250 g) from the Animal laboratory of Shanghai Sixth People's Hospital were randomly divided into 4 groups: normal control group, LIVD model group, sham operation group and acupuncture treatment group. The feeding conditions of these rats were temperature of 22-24°C, humidity of 40%-70% and natural lighting. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Establishment of LIVD model

The rats were anesthetized via intraperitoneal injection of 3% pentobarbital sodium (1 ml/kg b.w.) (Sigma-Aldrich, St. Louis, USA), then were fixed in the prone position on the operating table, disinfected rats back, prepared a 4 cm×8 cm area on the back skin using a depilatory. After incising the skin along the spinous processes of the back waist, a subperiosteal dissection was performed. Cut through bilateral erector spinae muscle, supraspinous ligaments and interspinous ligaments, removed L1-L6 spinous processes and articular processes with a rongeur. Washed and stitched subcutaneous fascia and skin back together layer by layer. The rats were treated with 50,000 U penicillin by intramuscular injection for 7 consecutive days. In the sham group, only the skin was cut open, and then was sutured. Two weeks after operation, when the wound healed up, rats were kept upright in self-made molds for 2 h per day, for 8 weeks.

Acupuncture treatment

The hibateral "Yanglingquan" (GB34, located in the depression anterior and inferior to the fibula capitulum) acupuncture points were punctured once a day with a sterile silver needle (0.25 mm in diameter, 25 mm in length; Shanghai Tai Cheng Technology Development Co., Ltd., Shanghai, China). The needle was inserted with a depth of 0.5~0.8 cm, advanced into the acupoint by twisting and twirling the needle until mild resistance was felt, and retained for 20 minutes. Acupuncture treatment was performed once a day, 6 days constituting a course, continuous treatment of 4 courses, and one day interval between courses.

Separation of lumbar intervertebral discs

One month later, rats in each group were executed by over dosage of anaesthesia through peritoneal injection of 3% pentobarbital sodium and lumbar vertebras were isolated. The whole discs were separated, including a small amount of cancellous bone attached to the cartilaginous end plate, and were placed into freezer storage tube and stored in a liquid nitrogen tank.

qRT-PCR

The disc samples (50 mg) were pulverized under liquid nitrogen, and transfer to a 1.5 mL EP tube. Ground samples were mixed thoroughly with 1 mL TRIzol reagent (Invitrogen, Carlsbad, USA) and incubated on ice for 10 min. Then total RNAs were isolated in accordance with the standard method and dissolved in DEPC-treated water and stored at -80°C.

2 μg total RNA was reverse-transcribed to cDNA using the Takara reverse transcription kit (TaKaRa Biotechnology, Dalian, China), accord-

Table 1. Sequences of primers used in the real-time PCR

	Primer sequence (5'-3')		Primer sequence (5'-3')
ALP	F: AAACTCGCTTATGTCCCGCA	Osteocalcin	F: GACCCTCTCTCTGCTCACTC
	R: GAAGAAGGGGTGCTACGTCC		R: GCTAGCTCGTCACAATTGGG
PGRN	F: GGACAGTACTGAAGACTCTG	Col1a1	F: TAGGAGTCGAGGGACCCAAG
	R: GGATGGCAGCTTGTAATGTG		R: GGCCTTGGAAACCTTGAGGA
Osterix	F: CTTCGTGCCAGACCTCTTGA	Col2a1	F: GCCAGGATGCCCGAAAATTAG
	R: GGACTGGAGCCATAGTGAGC		R: CTCGTCAAATCCTCCAGCCA
COMP	F: CAGGAGACTAATGCGGCGCT	ADAMTS-7	F: AGTTAGGTGCCAGTTTTTGGC
	R: GGAAACAGGGGTGAGCGTTG		R: AGTCAACCCAGGAAAGAGGC
BSP	F: TCTTTAAGTACCGGCCACGC	ADAMTS-12	F: GGTGGCCCAGCTTCTTAACT
	R: CGGTTACCCCTGAGAGTGTG		R: TGGCCACTGGCATCTACTTG
TIMP-1	F: CAGCTTTCTGCAACTCGGAC	MMP-13	F: ACCCTGGAGCACTCATGTTTCCTA
	R: AACCGGAAACCTGTGGCATT		R: TGGCATCAAGGGATAAGGAAGGGT
GAPDH	F: TCTCTGCTCCTCCCTGTTCT		
	R: GATGGTGATGGGTTTCCCGT		

ing to the manufacturers' specifications. PCR was performed in a final volume of 20 μL as follows: 10 μL Sybr green, 1 μL forward primer (5 μM), 1 μL reverse primer (5 μM), 0.4 μL RoxDye (II), 2 μL cDNA, 5.6 μL RNase-free H_2O. The PCR amplification procedure: an initial denaturing at 95°C for 30 s, followed by denaturing at 95°C for 3 s, annealing and extension at 60°C for 30 s, 40 cycles. The primer pairs were shown in **Table 1**. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry

The L5-6 intervertebral disc was fixed in formalin, embedded in paraffin, and cut into 5 μm sections. The sections were deparaffinized in xylene, and rehydrated in a reverse-graded series of ethanol. After antigen retrieval, the sections were incubated with 3% hydrogen peroxide solution at room temperature for 25 minutes to quench endogenous peroxidase. The nonspecific binding was blocked by incubation with 3% BSA at room temperature for 30 minutes. Then, the sections were incubated overnight at 4°C with anti-PGRN polyclonal antibody (1:200; ABBIOTEC; 251319). The sections were then incubated at room temperature for 50 min with secondary antibodies labeled with horseradish peroxidase (HRP). Between each of these steps, the sections were washed three times in PBS for 5 minutes. Then, the sections were incubated in the chromogen 3,3'-diaminobenzidine (DAB, Vector, Burlingame, USA) at room temperature and coloration time was controlled through a microscope. Sections were then washed in running tap water and counterstained with hematoxylin.

Western blotting

The intervertebral disks were cut into small pieces and grounded to powder with liquid nitrogen and a precooled grinding machine. Total proteins were isolated using considerable RIPA buffer (200 μL for each intervertebral disk) and protein concentration was examined by BCA assay. The cells cultured for 48 h were collected and directly lysed using RIPA lysis buffer with proteinase inhibitor. For determination of NF-κB2, the cytoplasmic and nuclear proteins were extracted from the primary cortical microglial cells using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China). Samples were heated at 100°C for 5 min in 5× loading sample buffer and resolved by SDS-PAGE, followed by western blot analysis. Briefly, we loaded samples (18 μL), separated them on 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred them to nitrocellulose membranes (Whatman, Maidstone, England). The membranes were blocked for 60 min with nonfat milk and incubated overnight at 4°C with primary antibodies against MMP-13 (1/1000, Invitrogen Antibodies, MA5-14247), TIMP-1 (1/2000, Novus, NBP1-96554), PGRN (1/200; ABBIOTEC; 251-319), NF-κB (1/10000; Abcam; ab175192), Col1 (1/500; Abcam; ab90395), Col2 (1/500; Invitrogen; MA1-37493), COMP (1/1000; Abc-

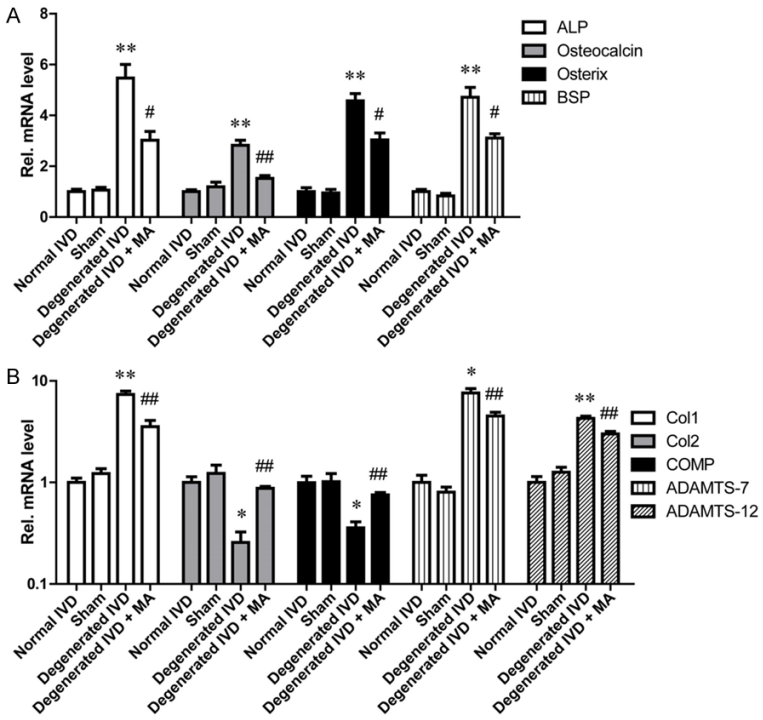

Figure 1. A. mRNA levels of the osteoblastic marker genes ALP, osteocalcin, Osterix and BSP in LIVD from each group. B. mRNA levels of the LIVD degeneration-related genes Col1, Col2, COMP, ADAMTS-7 and ADAMTS-12 in LIVD from each group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with Sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with Degenerated LIVD group. LIVD: lumbar intervertebral disc; MA: manual acupuncture.

am; ab42225), ADAMTS-7 (1/500; Abcam; ab-201083) and ADAMTS-12 (1/2000; Abcam; ab37760). Next, we incubated the membranes for 1 h at room temperature with HRP-conjugated secondary antibodies. The protein bands were detected by chemiluminescence technology and analyzed using the Image J software.

Isolation and culture of cartilage end plate-derived cells

Rats were sacrificed by cervical dislocation and the global lumbar spine was taken out under sterile conditions. The intervertebral discs were exposed after dissection of fascia and muscle around the spine. After the spine was washed by sterile PBS for three times, the cartilage end plates were isolated and minced into small pieces (0.3-0.5 mm^3). After washing by PBS with 1% penicillin-streptomycin solution three times, the cartilage end plate pieces were digestive with 10-15 times volume of 0.25% trypsin for 40 min at 37°C, followed by centrifugation at 1,000 rpm for 5 min. Waste supernatant, the sediment was digestive with 1 mg/mL type II collagenase in DMEM (Gibco, USA) for 1 h at 37°C, followed by centrifugation (1,000 rpm, 5 min). Repeat the previous digestion process once. Finally, the sediment was digestive with 1 mg/mL type II collagenase for 3-4 h at 37°C. During the time, a microscope was used for observation the presence of cell release and cell count.

At the end of the digestion, the remaining sample was resuspended in DMEM containing 10% FCS and 1% penicillin and streptomycin. The cell dumps were mechanically dissociated into single cell suspension with pipette. The suspended cells were filtered through a 0.22 μm cell filter and the filtrate was centrifuged at 1000 rpm for 5 min. Then, the sediment was washed by 3 mL DMEM and centrifugation (1,000 rpm, 5 min) for 3 times. At last, the cells were cultured in DMEM containing 10% FCS and 1% penicillin and streptomycin with a concentration of 2×10^4/mL, and placed in 37°C, 5% CO_2 incubator. The medium was changed every three days. The cell growth and adhesion were observed by inverted microscope.

Small interfering RNA (siRNA) transfection

When the primary cells completed 50% confluence, the silencing experiments with siRNA were performed. Briefly, PGRN-siRNA1, PGRN-siRNA2, scramble siRNA and lipofectamin2000 (Invitrogen, Carlsbad, USA) were used in this study and the final concentration of siRNA was 50 nM. After 4 h, the cells were washed by PBS and cultured in DMEM-F12 medium containing 10% FBS for 48 h. Then, the expression levels of MMP-13, TIMP-1, PGRN, TNF-α, COMP, ADAMTS-7 and ADAMTS-12 were measured by qRT-PCR and western blotting. The siRNA sequences (designed by Shanghai GenePharma

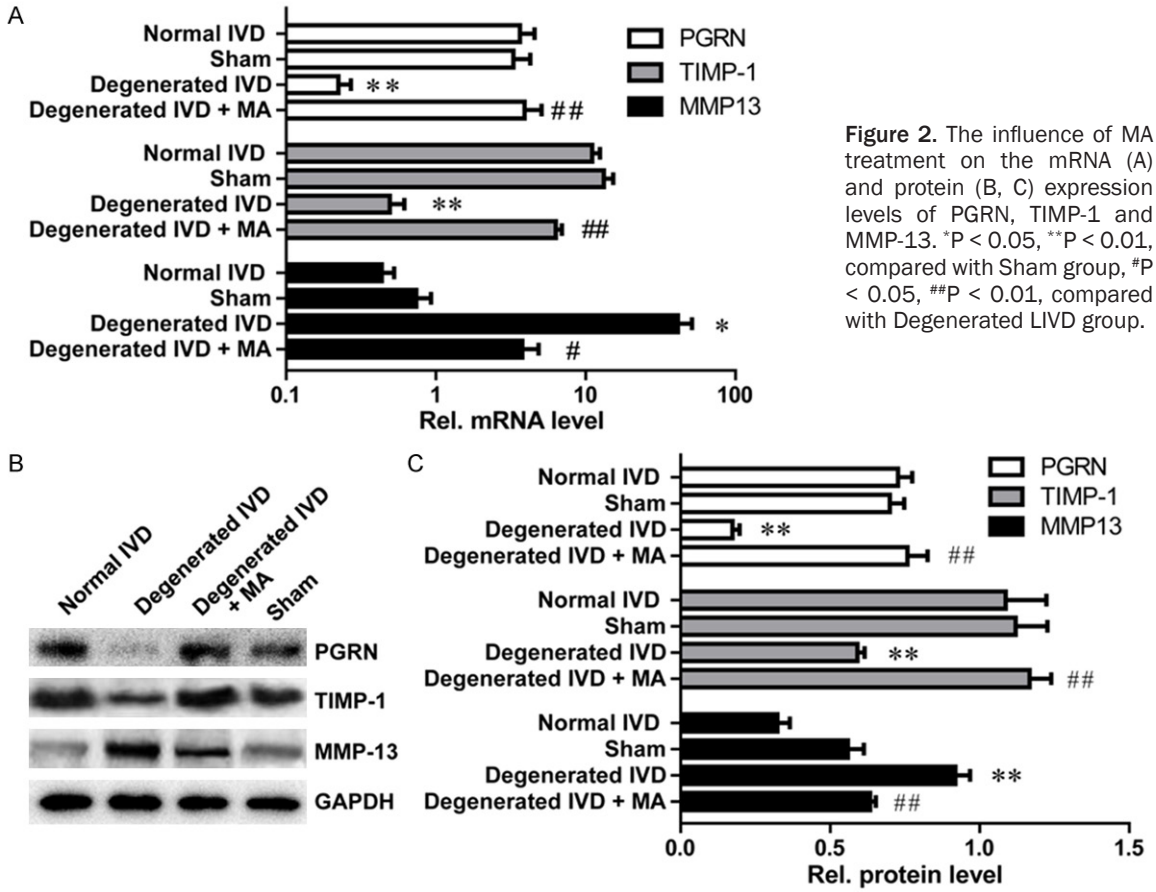

Figure 2. The influence of MA treatment on the mRNA (A) and protein (B, C) expression levels of PGRN, TIMP-1 and MMP-13. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with Sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with Degenerated LIVD group.

Co. Ltd) were as follows: PGRN-siRNA-1 Sense: 5'-GGGUGUAUCUUGUGAUGAUdTdT-3'; PGRN-siRNA-2; Sense: 5'-CUGGACACAUGGCCUAUA-AdTdT-3'.

Statistical analysis

Statistical analyses were performed using SPSS 21.0. All data are expressed as the mean ± standard error (SE). Multiple comparisons between groups were compared with analysis of variance (ANOVA) and pairwise comparison were performed by LSD test when the data are in accord with the normal distribution and homogeneity of variance, if not, the data were logarithmically transformed before analyzed by ANOVA. $P < 0.05$ was considered statistically significant.

Results

MA restrained the upregulation of osteoblastic marker and LIVD related genes in degenerated IVD

As shown in **Figure 1**, compared with sham group, the mRNA levels of osteoblastic marker genes ALP, Osteocalcin, Osterix and BSP in model group were significantly up-regulated ($P < 0.01$), which could be effectively restrained by manual acupuncture (MA) treatment ($P < 0.05, 0.01$). The mRNA expression of LIVD related genes Col1, ADAMTS-7 and ADAMTS-12 was also up-regulated in the degenerative lumbar intervertebral discs ($P < 0.05, 0.01$). In contrast, mRNA levels of the other two LIVD degeneration-related genes Col2 and COMP were significantly decreased ($P < 0.05$), and MA treatment could apparently increase the mRNA expression levels of Col2 and COMP.

MA restored the expression of PGRN, TIMP-1 and MMP-13 to a normal level in degenerated IVD

As shown in **Figure 2**, the expression levels of PGRN and TIMP-1 in model group were significantly lower than that in sham group and MMP13 was significant higher, at mRNA and protein levels ($P < 0.05, 0.01$). MA treatment could effectively increase PGRN and TIMP-1 expression and reduce MMP13 expression at both mRNA and protein levels ($P < 0.05, 0.01$).

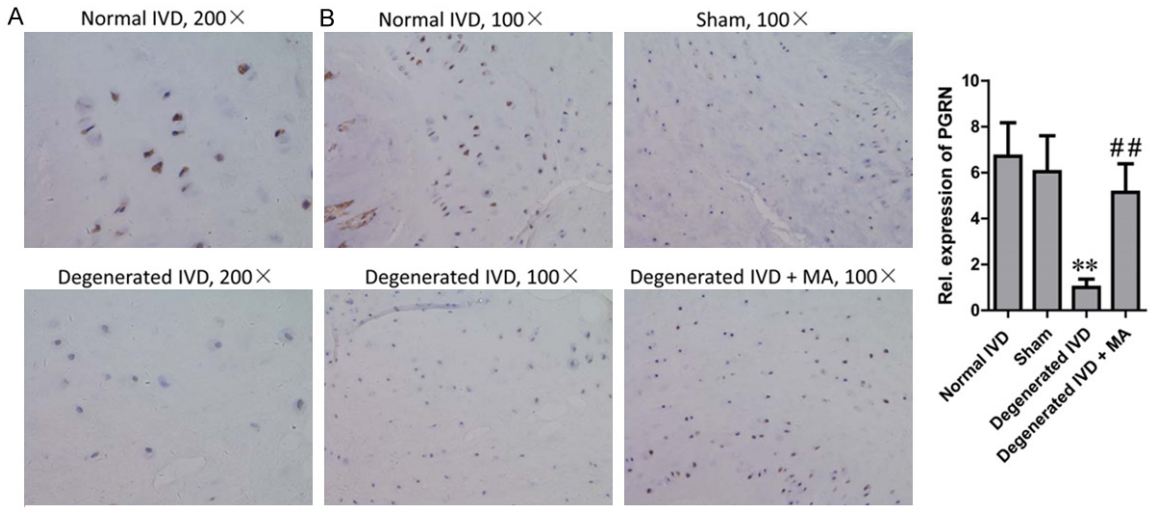

Figure 3. A. The expression of PGRN in normal and degenerated IVD was detected using immunohistochemical analysis and observed under 200× magnification. B. PGRN expression in IVD from different group (Normal, Sham, Model and MA) was detected using immunohistochemical analysis, observed under 100× magnification and semi-quantified using Image-Pro Plus 6.0 software. Rel. expression of PGRN = $IOD_{experiment}/IOD_{normal\ LIVD}$. IOD: integrated optical density. $^{*}P < 0.05$, compared with Sham group, $^{\#}P < 0.05$, compared with Degenerated LIVD group.

MA restored the decreased expression of PGRN to a normal level in degenerated IVD

Immunohistochemical staining showed that PGRN was detectable in the end plate and fiber ring of intervertebral disc, but not in the nucleus pulposus. It was observed with high-power microscope (200×) that PGRN was mainly distributed in the extracellular matrix, not in the nucleus, indicates that PGRN may play important roles in the process of disc degeneration outside the cell. As shown in the **Figure 3**, the expression level of PGRN was markedly reduced in the degenerated LIVD ($P < 0.01$), and MA treatment could effectively promote the PGRN expression ($P < 0.01$).

PGRN knockdown altered the expression of NF-κB, TIMP-1, MMP-13 and LIVD degeneration related genes

As shown in **Figure 4A**, PGRN expression in CEPCs was knocked down by specific PGRN siRNA. PGRN mRNA expression was significantly inhibited in the PGRN siRNA groups compared with that in the scramble group ($P < 0.01$). The data showed that PGRN siRNA significantly decreased the mRNA and expression of NF-κB, TIMP-1, Col2 and COMP ($P < 0.01$, 0.05), and definitely increased the expression of MMP-13, Col1, ADAMTS-7, and ADAMTS-12 at the protein and mRNA level ($P < 0.01$) (**Figure 4B** and **4C**).

Discussion

The typical features of degenerative intervertebral discs (IVDs) have been derived from various studies concerning the structural, cellular, and molecular changes in IVDs during LIVD. These typical characters include: increased production of Col1, especially in the nucleus pulposus; excessive degradation of Col2 and aggrecan; upregulated expression of matrix degrading enzymes, such as the MMPs and ADAMTS families and increased release of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). TIMPs are inhibitors of MMPs. In normal IVDs, expression of MMPs and TIMPs is in a certain proportion, and they maintain the balance of the synthesis and degradation of ECM. Abnormal mechanical loading could cause the imbalance of MMPs/TIMP, which leads to the degradation of ECM [15]. Cartilage oligomeric matrix protein (COMP) is an essential collagen-binding, structurally stabilizing component of the ECM, and can be degraded by ADAMTS-7 and ADAMTS-12. In present work, the changes in these LIVD related indicators were all coincidental to the identification criteria of degenerative IVDs. Abnormal bone formation was also

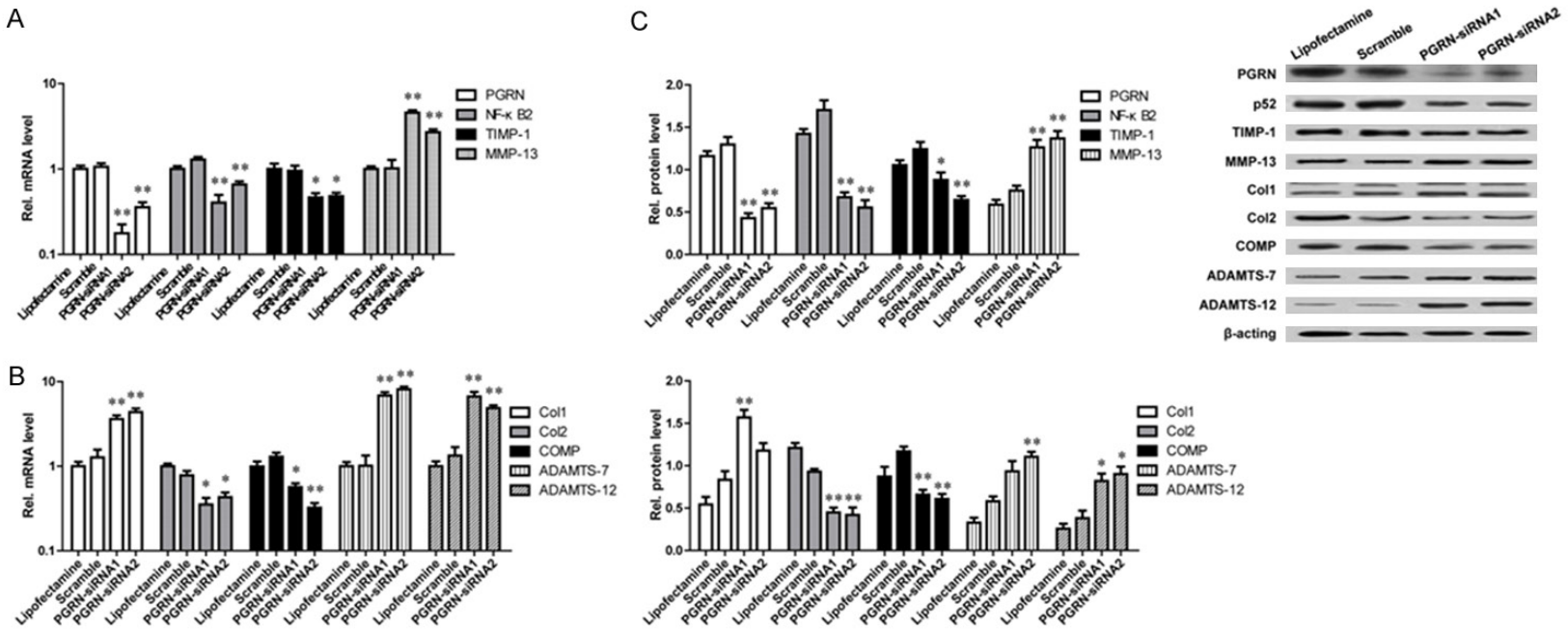

Figure 4. A. The specific siRNAs targeting PGRN altered mRNA expression of NF-κB, TIMP-1 and MMP-13. B. PGRN knockdown altered the mRNA expression of LIVD degeneration-related genes (Col1, Col2, COMP, ADAMTS-7, and ADAMTS-12). C. PGRN knockdown altered the protein levels of NF-κB, TIMP-1, MMP-13 and LIVD degeneration-related genes. $*P < 0.05$, $**P < 0.01$, compared with Scramble group.

one of the characteristics of LIVD. The mRNA expression levels of osteoblastic marker genes, including alkaline phosphatase (ALP), osteocalcin, osterix, and bone sialoprotein (BSP) were all increased in degenerative IVDs, indicates that ectopic bone formation was increased in degenerative IVDs. Compared with model group, the expression levels of osteoblastic marker genes and matrix degrading enzymes in MA treatment group were significantly decreased, the mRNA levels of Col2, COMP and TIMP-1 were apparently increased. The results indicate that MA treatment could alleviate LIVD through inhibition ECM degradation and abnormal bone formation.

PGRN is a multifunctional growth factor and plays an important role in chondrocyte proliferation, differentiation and endochondral ossification of growth plate during development [10-12]. Moreover, PGRN can inhibit the degradation of COMP by ADAMTS-7 and ADAMTS-12 through direct protein-protein interactions and inhibition of TNF-α induced ADAMTS-7 and ADAMTS-12 expression, thus providing protection for cartilage formation and function [16]. It was also reported that PGRN has important actions in maintaining homeostasis cartilage and antagonizing osteoarthritis [13, 14]. PGRN deficient has been associated with rheumatoid arthritis, osteoarthritis and degenerative diseases of the nervous system [17, 18]. It has been demonstrated PGRN knockout would promote LIVD process in aging mice [4]. In present study, PGRN knockdown in CEPCs significantly inhibited the expression of TIMP-1, Col2 and COMP and promoted the expression of MMP-13, Col1, ADAMTS-7, and ADAMTS-12, suggested PGRN may play a vital role in LIVD. It has been confirmed that TNF-α mediated NF-κB pathway activation can be suppressed by PGRN through competitively binding to TNFR1/2 [19]. In this work, PGRN knockdown dramatically promoted NF-κB2 mRNA expression, indicates PGRN knockdown induced NF-κB activity in CEPCs. Stimulation of NF-κB signaling can accelerate age-associated disc degeneration in mice [20]. Moreover, NF-κB pathway was involved in ECM degradation through influencing the expression of matrix degrading enzymes [21]. Altogether, PGRN deficient and subsequent excessive activation of the NF-κB signaling pathway may be a significant contributor to LIVD.

The therapeutic effect of acupuncture on intervertebral disk disease has been confirmed by clinical and animal experiments [22-25], however, the underlying mechanisms were poorly understood. In present study, acupuncture treatment significantly alleviated LIVD in rats through upregulation of PGRN expression and inhibition of NF-κB pathway. The mechanism of acupuncture regulating the expression of PGRN remains to be further studied.

In conclusion, at present, clinical symptoms of LIVD are improved by conservative or surgical treatment, which cannot reverse the process of LIVD, and have many negative effects. The present study demonstrated that PGRN deficient and NF-κB pathway activation may play an important role in LIVD, and provided a potential target for the treatment of LIVD.

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Disclosure of conflict of interest

None.

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