

Sp1 downregulates proinflammatory cytokine-induced catabolic gene expression in nucleus pulposus cells

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Abstract. During the pathogenesis of intervertebral disc degeneration, pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), stimulate the degradation of the extracellular matrix (ECM) of intervertebral discs via the activity of catabolic enzymes including matrix metalloproteinases (MMPs), disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs), and cyclooxygenase 2 (Cox2). The transcriptional promoters of the human catabolic enzymes MMPs, ADAMTS, Cox2 and Syndecan 4 contain at least one specificity protein-1 (Sp1) transcription factor-binding site. The present study investigated the role of Sp1 in the regulation of the mRNA and protein expression of the aforementioned catabolic enzyme genes in nucleus pulposus cells, using reverse transcription-quantitative polymerase chain reaction, western blot, transfection and RNA interference. The data demonstrated that Sp1 transcription factor protein expression is induced by TNF- α and interleukin-1 β . Specific inhibitors of Sp1 DNA binding to its GC-rich consensus site, WP631 and mithramycin A, partially suppressed TNF- α -induced catabolic enzyme expression and activity. Genetic inhibition of Sp1 by small interfering RNA-mediated Sp1 knockdown partially inhibited catabolic enzyme induction by TNF- α . In addition, Sp1 transcription factor inhibitors decreased the activity of MMP3, ADAMTS4 and ADAMTS5 promoters. Furthermore, chromatin immunoprecipitation revealed functional Sp1 binding sites at -577/-567 bp within the

ADAMTS4 promoter and -718/-708 bp within the ADAMTS5 promoter. These results provide pharmacological and genetic evidence of the importance of Sp1 in catabolic enzyme gene regulation during TNF- α stimulation. Thus, Sp1 may represent an effective target in reducing intervertebral disc-associated ECM loss.

Introduction

The intervertebral disc lies between adjacent cartilaginous endplates in the spine and is composed of a gel-like core, termed the nucleus pulposus (NP), and the surrounding annulus fibrosus (1). In the NP, proteoglycan is the primary aggrecan, while collagen II is the primary collagen (2). Decreased levels of aggrecan and collagen II in the NP result in the initiation of intervertebral disc degeneration (3,4). Tumor necrosis factor- α (TNF- α) and interleukin 1- β (IL-1 β) are the pro-inflammatory cytokines principally associated with the progression of intervertebral disc degeneration and have been previously demonstrated to increase the degradation of the extracellular matrix (ECM), including aggrecan and collagen II (5-7). Matrix metalloproteinases (MMPs), disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs) and cyclooxygenase 2 (Cox2) are the major catabolic enzymes associated with ECM degradation induced by pro-inflammatory factors in the intervertebral disc (6-10). Syndecan 4 (SDC4), a cell surface heparin sulfate proteoglycan, serves a major role in matrix catabolism through the activation of MMP3, ADAMTS4 and ADAMTS5 in the intervertebral disc (6,7,10).

Mitogen-activated protein kinase (MAPK) inhibition can decrease the upregulation of MMP3, ADAMTS4 and ADAMTS5 expression induced by TNF- α and IL-1 β (6,7,11). In addition, nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and SDC4 are involved in the signaling pathways in NP cells that mediate TNF- α and IL-1 β induced aggrecan degradation by MMP3, ADAMTS4 and ADAMTS5 (6,7,11). Similarly, the induction of MMP13 and Cox2 expression by TNF- α and IL-1 β is regulated through the MAPK and NF- κ B signaling pathways (12,13). Specificity protein-1 (Sp1) is an important transcription factor that contains a zinc finger DNA-binding domain, and binds to GC-rich regions in the

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promoters of genes to activate transcription (14). It is also involved in the expression of ECM-associated genes in various cells, including renal tubular epithelial cells and melanoma cells (15,16). Inhibition of Sp1 and Sp1 knockdown has been previously demonstrated to partially inhibit ADAMTS4 and Cox2 induction by IL-1 β or Toll-like receptor 4 in chondrocytes (17,18). However, the effect of the Sp1 transcription factor on the expression of catabolic enzymes induced by inflammatory cytokines in NP cells remains to be determined.

The aim of the current study was to examine the role of the Sp1 transcription factor in the regulation of TNF- α -induced catabolic factor, including MMP3 and ADAMTS4, gene expression in NP cells.

Materials and methods

Plasmid constructs and reagents. Sp1 short hairpin RNA (shRNA) and plasmid (pLKO.1 shRNA) were provided by Dr Hyoung-Pyo Kim of Yonsei University College of Medicine (Seoul, Korea) (19). psPAX2, pMD2.G and pRL-TK plasmids were provided by Dr Dong Xiao of Nanfang Medical University (Guangzhou, China) (20). To assay the promoter activity, the 5'-flanking region of the MMP3, ADAMTS4 and ADAMTS5 genes was inserted into the firefly luciferase reporter vector, pGL3-Basic (Promega Corporation, Madison, WI, USA), which contained no eukaryotic promoter or enhancer element. To construct the promoter, genomic DNA was isolated from 293T cells, which were provided by Dr Dong Xiao of Nanfang Medical University (20), using a Gentra genomic DNA isolation kit (Qiagen, Inc., Valencia, CA, USA). Polymerase chain reaction (PCR) was used to amplify the 2.3 kb MMP-3 promoter (MMP3-Luc, F 5'-GGCGCCTCG AGTTGACATTTGCTATGAGC-3'; R 5'-CAAGCTTGCTT GCCTGCCTCCTTGTAGGT-3'); 2.1 kb ADAMTS4 promoter (ADAMTS4-Luc, F 5GCGTGCTAGCCCCGGGCTCGAG GGTGGGTGATCCAGGAAGTG-3, R5'-CAGTACCGGAAT GCCAAGCTTAGTAGCAGAAGCAGCCACAC-3'); 2.2 kb ADAMTS5 promoter (ADAMTS5-Luc, F5'-GCGTGCTAG CCCGGGCTCGAGCCCCAGCGTAGCCAAAGTTA-3', R 5'-CAGTACCGGAATGCCAAGCTTGCTTTATCCTGG GCAGGTGT-3') with *Xho*I- and *Hind*III restriction enzyme digestive sites. The cycling conditions were as follows: An initial denaturation step at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 40 sec, and a final extension step at 72°C for 5 min. The pGL3-SV40 plasmid (Promega Corporation) containing the firefly luciferase gene driven by the SV40 promoter was used as a positive control. WP631 and mithramycin A were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). TNF- α and IL-1 β were obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). Rabbit anti-MMP3 (catalog no. ab52915) and rabbit anti-SDC4 (catalog no. ab24511) antibodies were purchased from Abcam (Cambridge, UK); rabbit anti-Cox2 (catalog no. 12282) and rabbit anti-Sp1 (catalog no. 9389) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA); mouse anti-GAPDH (catalog no. AM4300) antibody was provided by Thermo Fisher Scientific, Inc. (Waltham, MA, USA); and mouse anti- β -tubulin (catalog no. N357) antibody and anti-mouse (catalog no. RPN4201) and anti-rabbit (catalog

no. RPN4301) horseradish peroxidase (HRP)-conjugated IgG were purchased from GE Healthcare Life Sciences (Chalfont, UK).

Isolation, culture and treatment of NP cells. Consistent with the Institutional Review Board guidelines of Sun Yat-sen University (Guangzhou, China), human tissue samples were obtained from patients with thoracolumbar fractures undergoing spine fusion; informed consent for sample collection was obtained from each patient. A total of 8 male Sprague-Dawley rats (age, 2-3 months; weight, 220.3 \pm 13.2 g) were obtained from the Experimental Animal Center of Sun Yat-sen University. Rats were housed in specific pathogen-free conditions, at 19-28°C under a 12-h light/dark cycle and with *ad libitum* access to food and water. The Animal Care and Use Committee of the Sun Yat-sen University approved the experimental procedures.

Following anesthesia with 10% chloral hydrate (Wuhan Hechang Chemical Co., Ltd., Wuhan, China) (>3.5 ml/kg), NP cells were isolated as described previously (21,22). NP cells were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in a 5% CO₂ incubator. The medium was refreshed every 3 days. Following growth to 80% confluence, the NP cells were treated with 50 ng/ml TNF- α or 10 ng/ml IL-1 β for 4, 8 or 24 h, and cell RNA or protein extraction was performed. WP631 (100 μ M) or 1,000 nM mithramycin A were added 1 h prior to addition of cytokines.

Reverse transcription-quantitative PCR (RT-qPCR). Following the manufacturer's instructions, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and single-stranded cDNA was prepared by RT from 2,000 ng total RNA using Superscript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Template cDNA and gene-specific primers were added into the Fast SYBR Green master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). A 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used and the cycling conditions were as follows: An initial denaturation step at 95°C for 5 min was followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 58°C for 10 sec and extension at 72°C for 15 sec, and a final extension step at 72°C for 10 min. mRNA expression was quantified using the standard curve method (23). Hypoxanthine phosphoribosyltransferase 1 and β -actin were used to normalize the expression of rat and human samples, respectively. Each sample was analyzed in duplicate. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and are listed in Table I.

Western blotting. Following treatment, the NP cells and medium were collected and then treated with lysis buffer, containing 1X protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), NaCl (5 mM), NaF (200 μ M), Na₃VO₄ (200 μ M) and dithiothreitol (0.1 mM). Following quantification by bicinchoninic acid assay, total cell proteins (30 ng) were resolved by sodium dodecyl sulfate-polyacrylamide gels

Table I. Primer sequences for quantitative polymerase chain reaction.

Species	Gene	Sense (5' to 3')	Anti-sense (5' to 3')
Rat	MMP3	CAGGGAAAGTGACCCACATATT	CGCCAAGTTTCAGAGGAAGA
	MMP13	GAACCACGTGTGGAGTTATGA	GCATCTACTTTGTGCGCCAATTC
	ADAMTS4	GGAGATCGTGTTCAGAGAAG	CAAAGGCTGGTAATCGGTACA
	ADAMTS5	GAATGTAGACCCTACAGCAACTC	CACACTCCACACTTGTCTACT
	Cox2	TCAACCAGCAGTTCCAGTATC	GTGTACTCCTGGTCTTCAATGT
	SDC4,	CCCTTGGTGCCACTAGATAAC	GACCTCATTCTCTCCAGTTCC
	Sp1	GAGGATGAGTAGACCAGCTTTG	TGGAAGAGGGAGTAGGAAGT
	HPRT	GCTGACCTGCTGGATTACAT	CCCGTTGACTGGTCATTACA
Human	MMP3	GCAAGACAGCAAGGCATAGA	CAGCAACAGTAGGATTGGAAGA
	MMP13	GTTTGGTCCGATGTAACCTCCTC	GAAGTCGCCATGCTCCTTAAT
	ADAMTS4	GGTGGTGGTGATAGGTATAAGTG	TCAGGAAGAGGAAAGCAGAAC
	ADAMTS5	CTGCCACCACACTCAAGAA	TCCTCCCGAGTAAACAGGATAG
	Cox2	CTATGTGCTAGCCCACAAAGA	GCATCCACAGATCCCTCAA
	SDC4,	GACTGGGATTGGATCACTTCTT	ACTCCACACAACATCCGTTAG
	Sp1	TCCAAGGCCTGGCTAATAATG	GACAGGTAGCAAGGTGATGTT
	β -actin	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT

on 10% gels, then transferred by electroblotting to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked in 5% non-fat dry milk for 1 h at room temperature in Tris-buffered saline (50 mM Tris, pH 7.6, 150 mM NaCl) plus 0.1% Tween-20 and incubated overnight at 4°C with the primary antibody (1:1,000 anti-MMP3; 1:1,000 anti-Cox2; 1:1,000 anti-SDC4; 1:1,000 anti-Sp1; 1:3,000 anti-GAPDH; or 1:3,000 anti- β -tubulin). Membranes were then incubated with HRP-conjugated anti-rabbit or anti-mouse IgG (1:2,000-1:5,000) for 1 h and the signals detected using enhanced chemiluminescence (GE Healthcare Life Sciences). GAPDH and β -tubulin were used as protein loading controls for rat and human samples, respectively. Protein bands were quantified by densitometric analysis using Kodak 1D software version 3.6 (Kodak, Rochester, NY, USA).

Transfections and dual-luciferase reporter assay. Rat NP cells were seeded in 48-well plates at a density of 3×10^4 cells/ml with 2% Opti-Minimal Essential Medium Reduced Serum (Gibco; Thermo Fisher Scientific, Inc.). The following day, cells were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), 250 ng MMP3, ADAMTS4 or ADAMTS5 promoter luciferase reporter plasmids and 250 ng of the internal control plasmid, pRL-TK. Cells were stimulated with WP631 (100 μ M) or mithramycin A (1,000 nM) 48 h post-transfection. Cells were harvested 24 h later, and firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter Assay Kit (Promega Corporation) according to the manufacturer's instructions.

Lentivirus production and transduction. HEK 293T human embryonic kidney cells were seeded in a humidified incubator at 37°C, 5% CO₂ at a density of 3×10^6 cells per 10 cm plate

in DMEM with 10% heat-inactivated FBS. Following ~24 h incubation, cells were transfected with lentiviral vectors and packaging plasmids (psPAX2 and pMD2.G) using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following 16 h incubation, the transfection medium was replaced with DMEM with 10% heat-inactivated FBS. At 48 and 60 h post-transfection, the supernatant was harvested from HEK 293T human embryonic kidney cells and was introduced into human NP cell cultures (0.5×10^6 cells/plate) with 6 mg/ml Polybrene (EMD Millipore, Billerica, MA, USA). A total of 24 h later, the medium containing viral particles was removed and replaced with DMEM containing 10% FBS and antibiotics. Cells and medium were harvested from NP cells 5 days later for mRNA or protein extraction.

Chromatin immunoprecipitation (ChIP). The ChIP assay was performed as described previously (21,22). Briefly, human NP cells were cultured in DMEM media supplemented with 10% FBS to 80% confluence. The cells were then cross-linked, lysed and the chromatin was sheared by sonication. Input DNA was generated by treating aliquots with RNase, proteinase K, and heat, followed by ethanol precipitation. DNA complexes were immunoprecipitated overnight using antibodies for Sp1 and nonspecific rabbit IgG followed by binding to protein G-agarose beads. Cross-links were reversed by treatment with proteinase K and heat, and the DNA was purified using DNA purification elution buffer (Active Motif, Carlsbad, CA, USA). qPCR analysis was performed using a ChIP-IT quantitative PCR analysis kit (catalog no. 53040; Active Motif) using the following primer pairs for putative Sp1 sites within the ADAMTS4 and ADAMTS5 promoters: (-577/-567) ADMATS4 motif, 5'-CTATTATCCATTCGG CTGCTAGA-3' (sense) and 5'-ATGGGTGTATCATCGCTT CC-3' (anti-sense); and -718/-708 ADAMTS5 motif, 5'-TTA

AGAGGCAGCGTGCAG-3' (sense) and 5'-GCGAGCGCTGTGAAGAT-3' (anti-sense). The negative control primers were purchased from Active Motif (catalog no. 71001).

Statistical analysis. All experiments were repeated independently at least three times. Data are presented as the mean \pm standard error. Statistical analyses were performed in SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed by one-way analysis of variance, followed by the post hoc tests Student-Newman-Keuls and least significant difference. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with Sp1 inhibitors attenuates catabolic gene expression induced by TNF- α . To observe the effect of Sp1 and GC-rich island inhibitors on the expression of the catabolic enzyme genes MMP3, ADAMTS4 and Cox2, 1 h prior to TNF- α stimulation, the NP cells were treated with WP631 and mithramycin A, which serve as transcriptional inhibitors by displacing Sp1 from promoters by binding to GC-rich DNA motifs. The data revealed that MMP3, MMP13, ADAMTS4, ADAMTS5, Cox2 and SDC4 mRNA expression was significantly increased following stimulation with TNF- α compared with untreated controls ($P < 0.05$), whereas the mRNA expression was significantly decreased following treatment with WP631 or mithramycin A compared with TNF- α treatment only ($P < 0.05$; Fig. 1A). Furthermore, western blot results revealed that stimulation with TNF- α significantly increased the intracellular protein expression of MMP3, Cox2 and SDC4 and the protein expression of MMP3 in the medium compared with unstimulated cells ($P < 0.05$; Fig. 1B). However, treatment with WP631 or mithramycin A significantly suppressed this increased expression ($P < 0.05$; Fig. 1B).

Sp1 transcription factor controls MMP3, ADAMTS4 and ADAMTS5 promoter activities in NP cells. To further confirm the involvement of Sp1 in the regulation of catabolic factors, the luciferase activity of pGL3 firefly luciferase reporter constructs containing the promoter region of MMP3, ADAMTS4 and ADAMTS5 was assessed following stimulation with TNF- α or IL-1 β , and treatment with WP631 or mithramycin A. Stimulation with TNF- α and IL-1 β resulted in significantly increased MMP3 promoter activity compared with unstimulated rat NP cells ($P < 0.05$), while WP631 and mithramycin A significantly suppressed this MMP3 promoter induction ($P < 0.05$; Fig. 2A). TNF- α and IL-1 β stimulation had no significant effect on ADAMTS4 and ADAMTS5 promoter activities (Fig. 2B and C), despite TNF- α stimulation having been demonstrated to increase ADAMTS4 and ADAMTS5 mRNA and protein expression levels (Fig. 1). However, treatment with WP631 and mithramycin A did result in significantly reduced ADAMTS4 and ADAMTS5 promoter activity compared with untreated cells that had been stimulated with TNF- α or IL-1 β ($P < 0.05$; Fig. 2B and C, respectively).

To determine whether Sp1 binds to the catabolic gene promoters, ChIP analysis was performed to assess Sp1 binding to the cognate Sp1 motifs in the promoter regions of

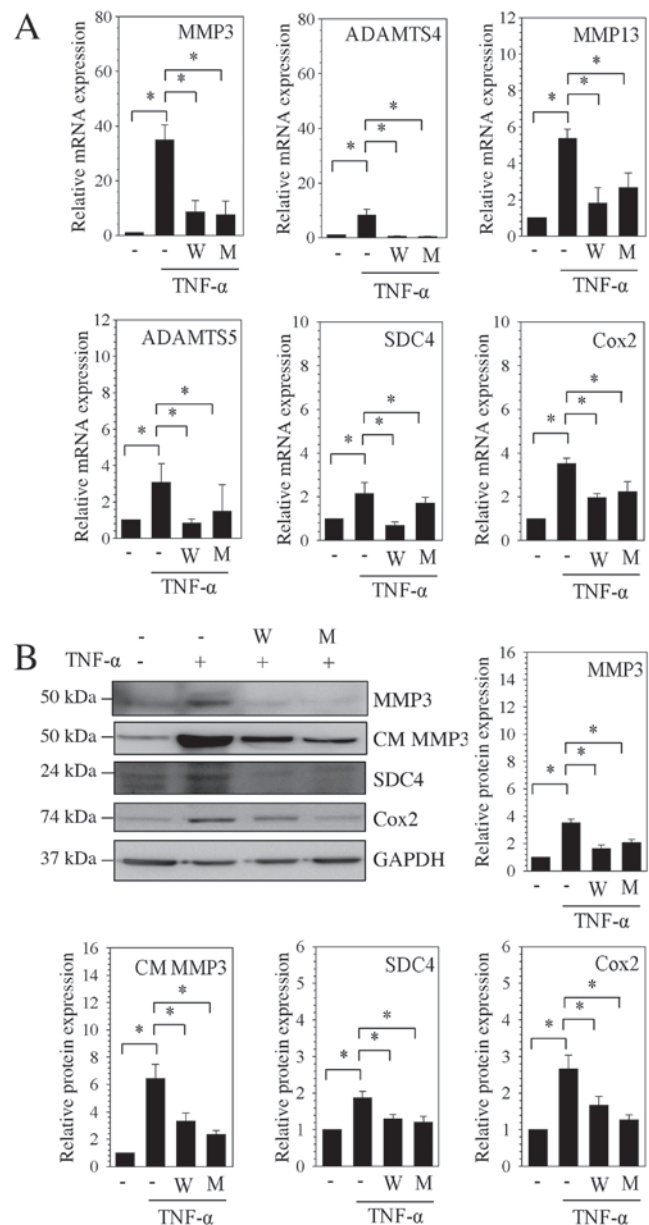


Figure 1. Expression of catabolic factors induced by TNF- α in nucleus pulposus cells was suppressed by specificity protein-1 transcription factor inhibitors. (A) mRNA expression levels of MMP3, ADAMTS4, MMP13, ADAMTS5, SDC4 and Cox2 were assessed by reverse transcription-quantitative polymerase chain reaction following stimulation with TNF- α , and treatment with WP631 or mithramycin A. (B) Western blot analysis of protein expression levels of MMP3, CM MMP3, Cox2 and SDC4, following stimulation with TNF- α and treatment with WP631 or mithramycin A. Densitometric quantification was relative to GAPDH. Data are presented as the mean \pm standard error of three independent experiments performed in triplicate, relative to the unstimulated control; * $P < 0.05$, comparison indicated by brackets. MMP, matrix metalloproteinases; W, WP631; M, mithramycin A; TNF- α , tumor necrosis factor- α ; ADAMTSs, disintegrins and metalloproteinases with thrombospondin motifs; SDC4, syndecan 4; Cox2, cyclooxygenase 2; CM, conditioned medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

ADAMTS4 and ADAMTS5. As presented in Fig. 2D, using the Sp1 antibody for ChIP resulted in ~ 3.6 -fold enrichment in binding of Sp1 to a site located at -577/-567 bp in the human ADAMTS4 promoter compared with the IgG control ($P = 0.039$; Fig. 2D), while ~ 6.1 -fold enrichment of Sp1 binding compared with IgG control was detected at the motif located

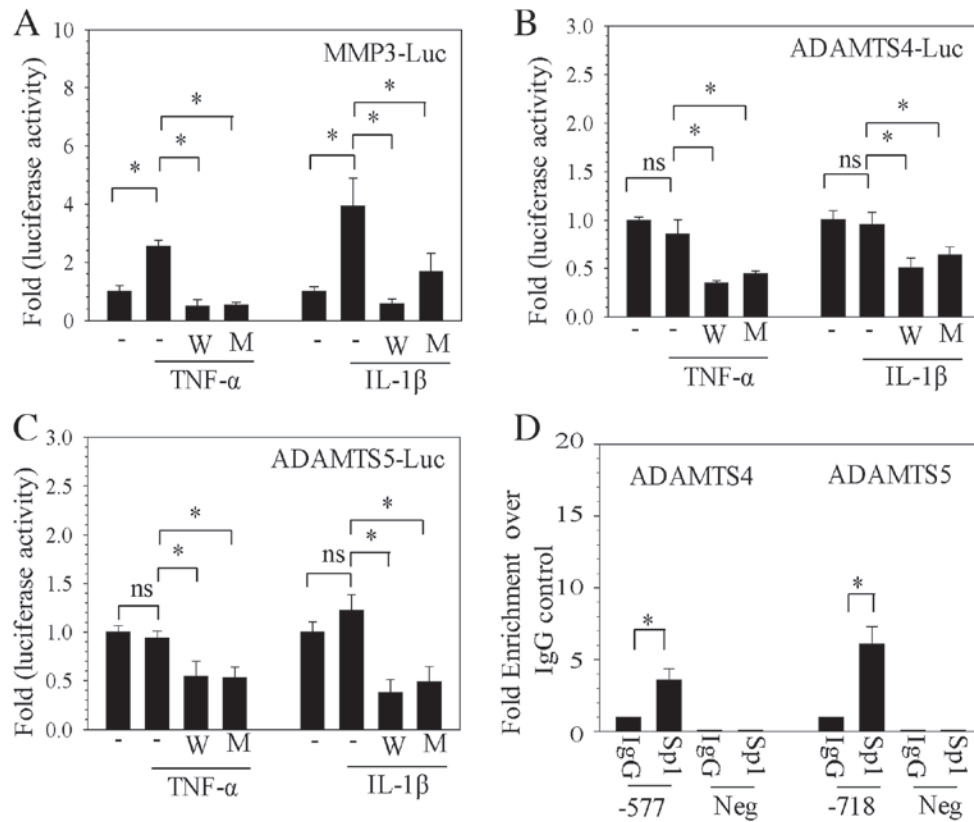


Figure 2. Sp1 controls catabolic factor promoter activity in NP cells. Firefly luciferase was used as a reporter of transcriptional regulation of the promoter regions of (A) MMP3, (B) ADAMTS4 and (C) ADAMTS5, following stimulation with TNF- α or IL-1 β and treatment with WP631 or mithramycin A. (D) Sp1 binding to Sp1 motifs at -577/-567 bp of ADAMTS4 and -718/-708 bp ADAMTS5 promoters in human NP cells was analyzed by genomic chromatin immunoprecipitation analysis with Sp1 and IgG antibody pulldowns. Negative primers revealed minimal amplification from experimental and IgG control samples. Data are presented as the mean \pm standard error of three independent experiments performed in triplicate. * $P < 0.05$, comparison indicated by brackets. Sp1, specificity protein-1 transcription factor; NP, nucleus pulposus; MMP, matrix metalloproteinases; Luc, luciferase; W, WP631; M, mithramycin A; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; ADAMTSs, disintegrins and metalloproteinases with thrombospondin motifs; IgG, immunoglobulin G; Neg, negative control primers.

at -718/-708 bp in the human ADAMTS5 promoter ($P = 0.027$; Fig. 2D). Negative control primers demonstrated almost undetectable amplification (Fig. 2D).

Regulation of Sp1 mRNA and protein following TNF- α and IL-1 β stimulation. To determine the effect of cytokine stimulation on the expression of Sp1 mRNA and protein, NP cells were stimulated with TNF- α and IL-1 β . Sp1 mRNA expression levels remained unchanged at 4, 8 and 24 h following stimulation with TNF- α (Fig. 3A) and IL-1 β (Fig. 3B), compared with unstimulated control cells. However, Sp1 protein expression levels were significantly higher following 24 h TNF- α stimulation compared with the unstimulated control ($P = 0.040$; Fig. 3C and D), and were significantly higher following 8 and 24 h of IL-1 β stimulation compared with the unstimulated control ($P = 0.039$ and $P = 0.048$, respectively; Fig. 3C and D).

Stable silencing of Sp1 decreases catabolic gene expression induced by TNF- α . To further examine whether the Sp1 transcription factor controls catabolic gene expression in human NP cells, Sp1 loss-of-function studies were performed using lentiviral delivery of gene-targeting shRNA. As observed in rat NP cells, no significant difference in Sp1 mRNA expression was observed in response to stimulation with TNF- α in

human NP cells transduced with control shRNA compared with unstimulated cells transduced with control shRNA (Fig. 4A). mRNA expression levels in human NP cells stimulated with TNF- α and transduced with Sp1 shRNA were ~78% reduced, compared with TNF- α -stimulated cells transduced with control shRNA ($P = 0.001$; Fig. 4A). In TNF- α -stimulated NP cells in which Sp1 was suppressed (cells transduced with Sp1 shRNA), MMP3, MMP13, ADAMTS4, ADAMTS5 and SDC4 mRNA expression was significantly reduced compared with TNF- α -stimulated cells transduced with control shRNA ($P < 0.05$; Fig. 4A). Furthermore, western blot analysis was used to investigate protein expression levels of Sp1, the cell protein, Cox2, and condition medium MMP3 in Sp1-silenced cells (cells transduced with Sp1 shRNA). Sp1 protein expression was significantly reduced in cells transduced with Sp1 shRNA and stimulated with TNF- α compared with TNF- α -stimulated cells transduced with control shRNA ($P = 0.001$; Fig. 4B). Consistent with the changes in mRNA expression levels of catabolic genes, including MMP3 and ADAMTS4, Cox2 expression and the level of MMP3 protein in the medium in cells transduced with Sp1 shRNA and stimulated with TNF- α was ~58 and ~44% lower than in TNF- α -stimulated cells transduced with control shRNA ($P = 0.023$ and $P = 0.032$, respectively; Fig. 4B).

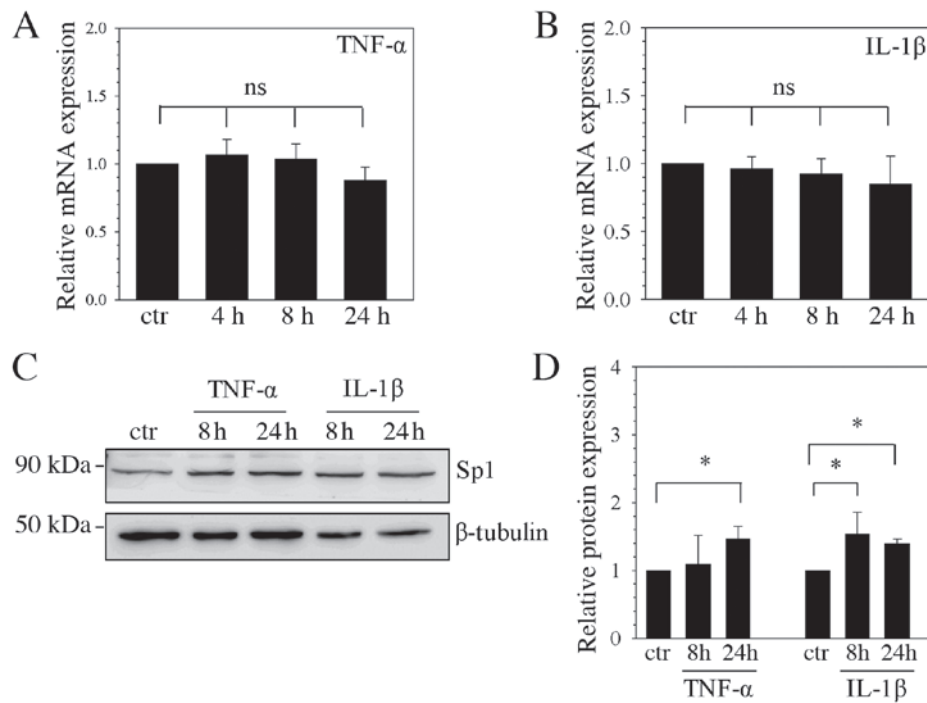


Figure 3. Sp1 mRNA and protein expression levels following the cytokines stimulation of nucleus pulposus cells. Sp1 mRNA expression levels following stimulation with (A) TNF- α and (B) IL-1 β . (C) Sp1 protein expression was assessed by western blot following stimulation with TNF- α and IL-1 β , and (D) quantification normalized to β -tubulin. Data are presented as the mean \pm standard error of three independent experiments performed in triplicate, relative to the unstimulated control; * P <0.05, comparison indicated by brackets. Sp1, specificity protein-1 transcription factor; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; ns, no significance.

Discussion

The experiments described in the present study demonstrated that in NP cells, Sp1 transcription factor inhibitors and knockdown suppressed the TNF- α -induced expression of the catabolic factors MMP3, ADAMTS-4, Cox2 and SDC4. Furthermore, Sp1 was essential for the maintenance of catabolic factor transcriptional expression due to direct interaction with cognate binding sites in their promoters. In addition, Sp1 transcription factor protein expression is induced by stimulation with inflammatory cytokines in NP cells.

Intervertebral disc degeneration is generally believed to be a consequence of increased catabolism of the ECM. Over the last decade, the research on intervertebral disc degeneration has emphasized the mechanism of ECM degradation. MMPs, ADAMTSs and Cox2 have been proposed to be the major catabolic enzymes in the intervertebral disc (8,24). SDC4, a cell-surface heparan sulfate proteoglycan, participates in inflammation and mediates the expression of MMP3, ADAMTS4 and ADAMTS5 in NP cells (4,5,8). TNF- α induces the expression of catabolic factors including MMP3, MMP13, ADAMTS4, ADAMTS5, Cox2 and SDC4 in NP cells and articular chondrocytes (3,5,6,8,9,21,25,26). Sp1 is also involved in the expression of ECM-related genes in various cells (15,16). The present study revealed that the expression of Sp1 mRNA was unaffected by TNF- α and IL-1 β stimulation in human and rat NP cells, as previously reported in bovine NP cells (27). However, Sp1 protein expression was induced by TNF- α and IL-1 β stimulation, as reported in articular chondrocytes (28). In articular chondrocytes, Sp1 mediates the inductive activity of TNF- α (29-31). To the best

of our knowledge, the present study presents the first evidence that the Sp1 inhibitors, WP631 and mithramycin A, suppress the TNF- α -induced expression of catabolic factors in NP cells. Furthermore, loss of function experiments also revealed that Sp1 knockdown decreased cytokine-induced expression of MMP3, MMP13, ADAMTS4, ADAMTS5, Cox2 and SDC4 in NP cells.

There are numerous Sp1 binding sites in MMPs, ADAMTSs, Cox2 and SDC4 promoters (17,18,32,33), but the present study limited its investigations to MMP3, ADAMTS4 and ADAMTS5 promoter constructs containing Sp1 sites. Stimulation of NP cells with TNF- α and IL-1 β resulted in increased expression from MMP3 promoter constructs, as previously reported (10), however, Sp1 inhibitors suppressed the induction by TNF- α or IL-1 β . Although TNF- α and IL-1 β stimulation had no effect on transcription from the ADAMTS4 and ADAMTS5 promoters, this may have been due to the promoter constructs being too short or lacking effective binding sites for TNF- α downstream mediators. However, Sp1 inhibitors reduced the baseline activity of ADAMTS4 and ADAMTS5 promoter constructs during stimulation with TNF- α and IL-1 β . To further support the functional studies of ADAMTS4 and ADAMTS5 promoter constructs, genomic ChIP analysis demonstrated that the enriched binding of Sp1 to the ADAMTS4 and ADAMTS5 promoter fragment that contained the -577/-567 and 718/-708 bp binding sites, respectively, controls its basal transcription. The present study therefore revealed that TNF- α induces increased ADAMTS4 and ADAMTS5 mRNA expression, but did not affect transcription from the ADAMTS4 and ADAMTS5 promoter reporter constructs.

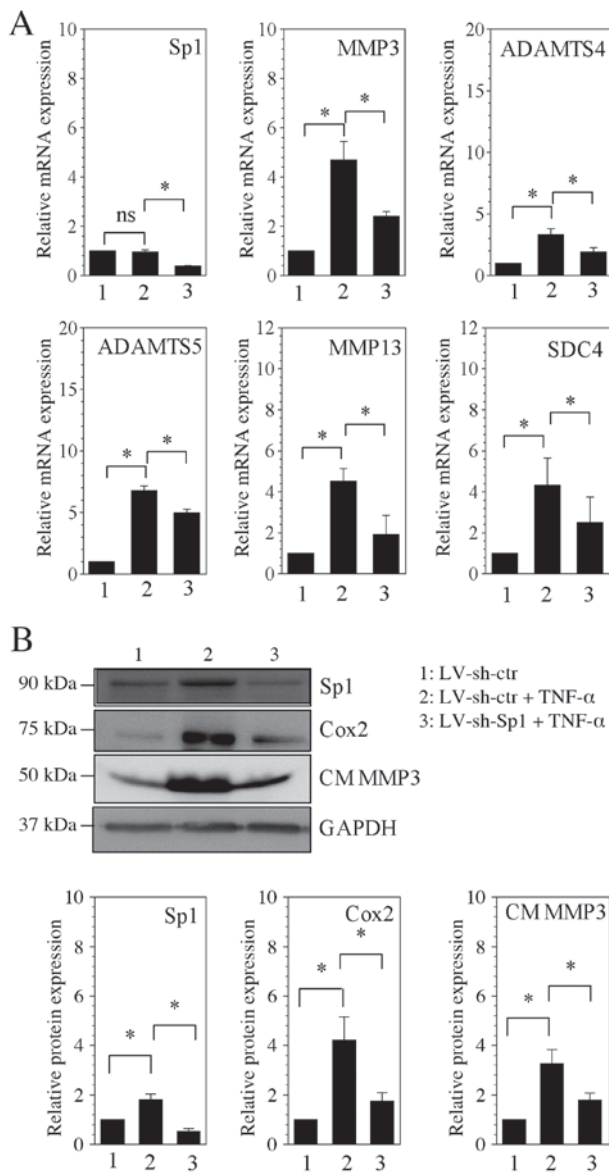


Figure 4. Stable silencing of Sp1 decreases catabolic factors induction by TNF- α in human NP cells. (A) mRNA expression levels of Sp1, MMP3, ADAMTS4, ADAMTS5, MMP13 and SDC4 were assessed by reverse transcription-quantitative polymerase chain reaction following lentiviral transfection and stimulation with TNF- α . (B) Western blot analysis of protein expression levels of Sp1, Cox2 and CM MMP3 following lentiviral transfection and stimulation with TNF- α , with quantification relative to GAPDH. Data are presented as the mean \pm standard error of three independent experiments performed in triplicate, relative to LV-sh-ctr. * P <0.05, comparison indicated by brackets. Sp1, specificity protein-1 transcription factor; ns, no significance; MMP, matrix metalloproteinase; ADAMTSs, disintegrins and metalloproteinases with thrombospondin motifs; SDC4, syndecan 4; LV, lentivirus; sh, short hairpin RNA; ctr, control; TNF- α , tumor necrosis factor- α ; Cox2, cyclooxygenase 2; CM, conditioned medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The pharmacological and genetic evidence presented in the present study demonstrated that Sp1 is involved in the TNF- α -induced expression of catabolic factors, including MMP3 and ADAMTS4. Therapeutic targeting of the Sp1 transcription factor to block the activity these catabolic factors may therefore present a novel strategy to prevent degradation of intervertebral discs by catabolic factors during the pathogenesis of intervertebral disc degeneration.

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