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Ceramide accumulation accelerates nucleus pulposus cells degradation by p38MAPK activation

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Abstract. – OBJECTIVE: Ceramide is a lipid molecule that regulates life activities such as cell differentiation, proliferation, apoptosis, and aging. However, whether ceramide plays a role in the intervertebral disc degeneration (IDD) is not clear. The aim of this study is to explore the effect of ceramide during the nucleus pulposus (NP) cells degeneration.

PATIENTS AND METHODS: We used human NP cells and passaged them until the fourth generation to analyze the content of ceramide. Cell-permeable C6-ceramide was used to upregulat[illegible] ceramide expression, and myriocin was u[illegible] inhibit the accumulation of ceramide. To [illegible]e[illegible] stand the relation between p38MAPK and [illegible]-mide, SB203580 was used to inhibit the activa[illegible] of p38MAPK. We tested the viability of NP cells [illegible] the detection of collagen II and [illegible]xpressio[illegible] the proliferation, and the apo[illegible] cells.

RESULTS: Ceramide con[illegible]was i[illegible]ased in NP cells from passage 1 [illegible]o P4. T[illegible]upregulation of ceramide accele[illegible]the [illegible] generation by the red[illegible]on [illegible]n II produc-tion and proliferati[illegible]lls pop[illegible]n, increased p16 expression, [illegible]poptotic c[illegible]opulation. However, the s[illegible]on of ceram[illegible]delayed the degenera[illegible] of [illegible] cells in the previous aspects. T[illegible] accumula[illegible]f ceramide activated the p[illegible]norylation of [illegible]APK, and the inhibitio[illegible] p38MAPK activat[illegible] also alleviated the C[illegible]eramid[illegible]duced NP cell degeneration.

CONCLUSIONS: Ceramide accumulates during NP c[illegible]ation, the upregulated cera-mide co[illegible]tes to [illegible]NP cells degeneration by [illegible]APK [illegible]ti[illegible].

Key[illegible]rds:
[illegible]ide, Nucleus pulposus cells, Apoptosis, Cell [illegible]3MAPK.

Introduction

Chronic low back pain is a high-prevalence, difficult-to-treat orthopedic disease, which is one of the most serious diseases associated with chronic aging[1]. There [illegible]bout 600 [illegible]ple suffer low back pa[illegible]vide, and 70[illegible]% of adults will expe[illegible]ce di[illegible]t degrees of low back pain throughout their live[illegible]ich will greatly affect the [illegible] own quality [illegible]fe, national medical e[illegible]ses, and social produc[illegible]vity[2]. Most low back p[illegible] is tolerable a[illegible] self-limiting. The acute phase [illegible] about 4 we[illegible] and the subacute phase lasts 4[illegible]2 weeks. H[illegible]ver, some patients will develop [illegible] ch[illegible] phase lasting up to 12 weeks. Clinica[illegible] data based on histopathology and imaging indicate that most of the occurrence of [illegible]k pain begins with degenerative changes [illegible] nucleus pulposus (NP) of the intervertebral disc[3]. The structure and biochemical composition of the NP changes during the progress of intervertebral disc degeneration (IDD). The decrease in proteoglycan (PG) and water content affects the carrying capacity of the intervertebral disc. In addition, the change in the composition of collagens and the decrease in its content has lost its original flexibility and scalability[4]. Therefore, exploring the mechanism of NP degeneration is one of the important directions of medical research on IDD.

Ceramide is a class of lipid molecules that has important regulatory effects on life activities such as cell differentiation, proliferation, apoptosis, and aging. When cells are stimulated by various external conditions such as ultraviolet radiation, heat shock, and chemotherapy drugs, endogenous ceramides begin to synthesize and may induce a series of physiological responses such as cell aging, growth inhibition, and apoptosis. Dadsena et al[5] reported ceramide triggers mitochondrial apoptosis by binding VDAC2. Boon et al[6] found ceramide promotes inflammation in type 2 diabetes. Jadhav et al[7] elucidated ceramide induces C2C12 myoblasts senescence which can be inhibited by Metformin. Gulbins et al[8] stated ceramide regulates neuronal stem cell proliferation in the hippo-

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campus. Ceramide is also proved to have an ability in tumor suppressive action[9]. However, the effect of ceramide in the IDD still remains unknown.

In our study, we used the NP cells spontaneous degeneration model by repeated passage to explore the effect of ceramide in the progression of NP cell degradation. The inhibitor of ceramide named myriocin was applied to see whether the suppression of ceramide made sense to prevent the NP cell degeneration. What we found indicated that ceramide accumulated during the repeated passage of NP cells and the intervention of ceramide may be an effective way to alleviate NP cell degeneration.

Patients and Methods

NP Tissue Collection and NP Cells Isolation

We collected degenerative disc samples from patients who underwent NP discectomy due to disc herniation in Ruijin Hospital, Shanghai Jiaotong University School of Medicine. All of the written informed consents from the patients were signed before the surgeries. We divided the NP from the endplates and conserved them in cold cell c... medium for the following NP cell isolati... fore isolation, we washed the samples with ...s-phate-buffered saline (PBS) and then cut ... into pieces as smaller as possible. The fragme... were incubated with 0.15% ty... ollagena... for digestion at 37°C overnig... day, the solution was filtrated using ...0 μm p... strainer and resuspended with B...co's M... Fa... gle's Medium/F12 (D... M/... , Rock... e, MD, USA) contain... 10% f... ovine serum (FBS; Gibco, Ro... MD, USA)... cells were seeded in 6-we... a... 1×10^5 cells/... and passaged till the ...rth ge... on. C6-ceramide and the p38M... inhibitor SB... 80 used for NP cell culture... e purchased from ...ma-Aldrich (St. Loui... O, US... This investigation has been approv... the ...cs Committee of Ruijin Hospital, Shang... ng Uni... ty School of Medicine.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from NP cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's meth... was reverse transcribed to complem... y deoxy-ribose nucleic acid (cDNA) by M... Mix (Applied Biosystems, Foster City, CA, ... RT-PCR was performed using SYBR... en M... plied Biosystems, Foster City, C... USA) to de... IL-1β, TNF-α, MMP-3, ...MTS-... and glyc... dehyde 3-phosphate d... rogen... (GAPDH) (as normalization) RN... le... Fl... imer s... nces used for RT-PCR... listed ... ble I b... on the Primer Bank d... ase. Relati... N... xpression levels were... d by the 2$^{-\Delta}$... hods.

Western Blot (W... alysis

N... were lysed ... adioimmunoprecipit... assay (RIPA) lysate (Beyotime, Shanghai, C... a), centrifug... at 4°C (12000 G for 5 min), t... upernatant w... removed, and the protein concen... ion was d... mined by bicinchoninic acid (BC... thod... yotime, Shanghai, China) to adjust sa... l protein equally. The protein was ...bjected to vertical electrophoresis on a polyacrylel, and then electrotransferred onto a nitro... membrane. The membrane was blocked with 5% skimmed milk for 2 h and incubated with anti-p38 (ab170099, Abcam, Cambridge, MA, USA), anti-Phospho-p38 (p-p38, Cat#44-684G, Thermo Fisher Scientific, Waltham, MA, USA), anti-β-actin (ab179467, Abcam, Cambridge, MA, USA) antibodies at 4°C overnight. After washing, the membrane was incubated with the secondary antibody for 2 h at room temperature. The gray ratio of the bands of the protein was scanned by Scinn Corporation analysis software corresponding to the relative content of β-actin.

Immunofluorescence (IF) Staining

NP cells were fixed with 4% formaldehyde for 15 min at room temperature, washed with PBS, and

Tab... Primer s... ces of the genes for RT-PCR.

	Forward (5′>3′)	Reverse (5′>3′)
...TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
...1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
...3	AGTCTTCCAATCCTACTGTTGCT	TCCCCGTCACCTCCAATCC
A...MTS-5	GAACATCGACCAACTCTACTCCG	CAATGCCCACCGAACCATCT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

then blocked with 5% bovine serum albumin (BSA) for 30 min. NP cells were following incubated with a primary antibody against p16 (ab51243, Abcam, Cambridge, MA, USA) or collagen II (ab34712, Abcam, Cambridge, MA, USA) overnight at 4°C and then incubated by Cy3-conjugated 488 antibody for 1 h in the dark. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the cell nucleus. Finally, the fluorescence intensity of NP cells was visualized using an inverted microscope.

Flow Cytometry

The apoptotic ratio of NP cells was determined by flow cytometry using Annexin V-FITC/PI double-stained apoptosis detection kit (Keygen, Nanjing, China). NP cells were collected, washed with PBS, centrifuged, and labeled with Annexin V-FITC and propidium iodide (PI), and we used the FACS Calibur flow cytometer (BD Corporation, Franklin Lakes, NJ, USA) to detect the distribution of apoptotic rate. The proliferative ratio of NP cells was determined using the EdU assay kit (Keygen, Nanjing, China) according to the manufacturer's protocol.

Ceramide Quantitation

The ceramide content of NP cells was mined by liquid chromatography-mass spec -etry (LC-MS) method. Briefly, cells were har ed and then washed and diluted in 100 µL P Sphingolipid extracts were prep Bligh-D er method and analyzed as pr sly cribed[10]. Quantification of ceramide perfor d by the extracted ion chromatog sing a rupole mass spectrom as .

Statistical Ana

All data w d ed by the ans values ± SD (sta ard de n), and graphs were generated GraphPad 5 Jolla, CA, USA) softwa fferences betwe wo groups were anal by usi the Student's *t*-test. Comparis wee ultiple groups was done using One-w VA tes owed by Post-Hoc Test st S ant I erence). A *p*-value below as co tatistically significant.

Results

amide Accumulated In Spontaneously ssive NP Cells

To determine the content of ceramide in the NP cells of different degenerated degrees, we passaged NP cells from the first generation to the fourth to achieve the spontaneously regressive NP cells. As shown in Figure 1A and 1B, the expression of collagen II decreased significantly with the increased generation of NP cells, which nt NP cells in a higher generation were weak in collagen II synthesis. Add ally, the p16 expression was raised as the N ls going to a higher generation, indicating a se t phenotype gradually appeared. Fo vingly, esult of ceramide content meas ent sugge the ceramide level was gulate from the generation to the fou enera (Figure 1C). As with many oth c g proc [12-14], ceramide accum ed in NP c Apart from this, we analyzed ative and apoptotic r P cells by cytometry (Figure 1 1E). pected, the proliferative NP cell population w creased along with the cell ally passage d the apoptotic cells in sed obviously from passage 1 to passage 4, esponding to degenerated cell phenotype. data indica that the content of ceramide a ulated in degenerated NP cells, which sho ass d with the development of NP cell de n.

ide Overexpression Accelerated s Degeneration

To determine whether the upregulation of ceramide affects the phenotype of NP cells, we treated passage 1 NP cells with a cell-permeable analog of naturally occurring ceramides named C6-ceramide (ceramide)[15]. Under the stimulation of ceramide, the expression of collagen II decreased compared to the control with a dose-dependent. Besides, the p16 level significantly increased due to the ceramide treatment compared to the control (Figure 2A, 2B). The result of the LC-MS showed that ceramide was upregulated resulting from the ceramide analog treatment, especially in the concentration of 20 µM (Figure 2C). Ceramide also affected the viability of NP cells by increasing the apoptosis (Figure 2D) but decreasing the proliferation (Figure 2F) compared to the control. The overexpression of ceramide was proved to be related to the inflammatory response in many cells[16]. Therefore, we also tested the mRNA levels of some inflammatory factors during the NP cell degeneration, such as IL-1β, TNF-α, MMP-3, and ADAMTS-5, which were all increased after ceramide treatment (Figure 2E). The results indicated that the ceramide overexpression by an analog stimulation accelerated the NP cell degradation by the reduction of collagen II synthesis and cell

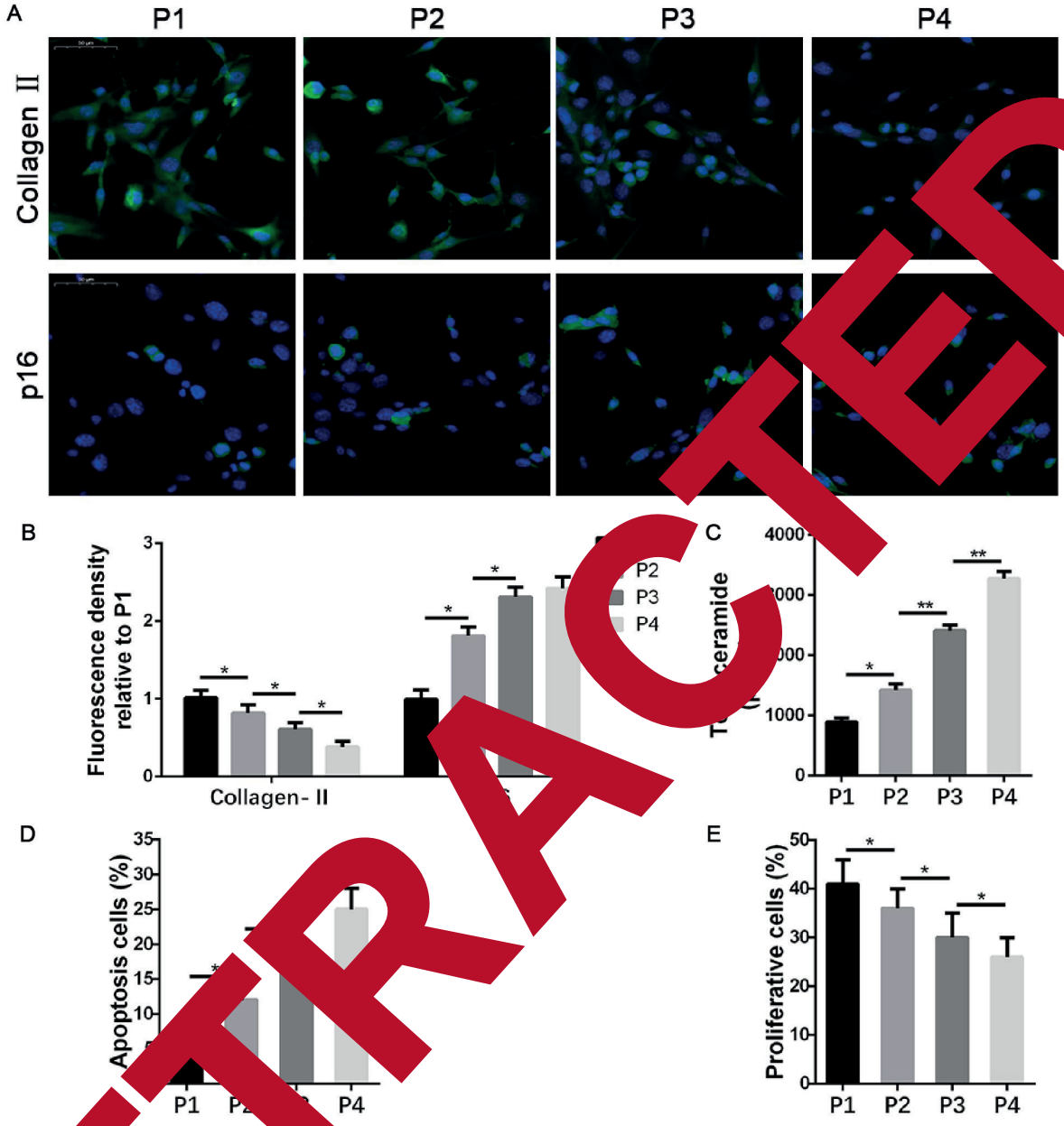
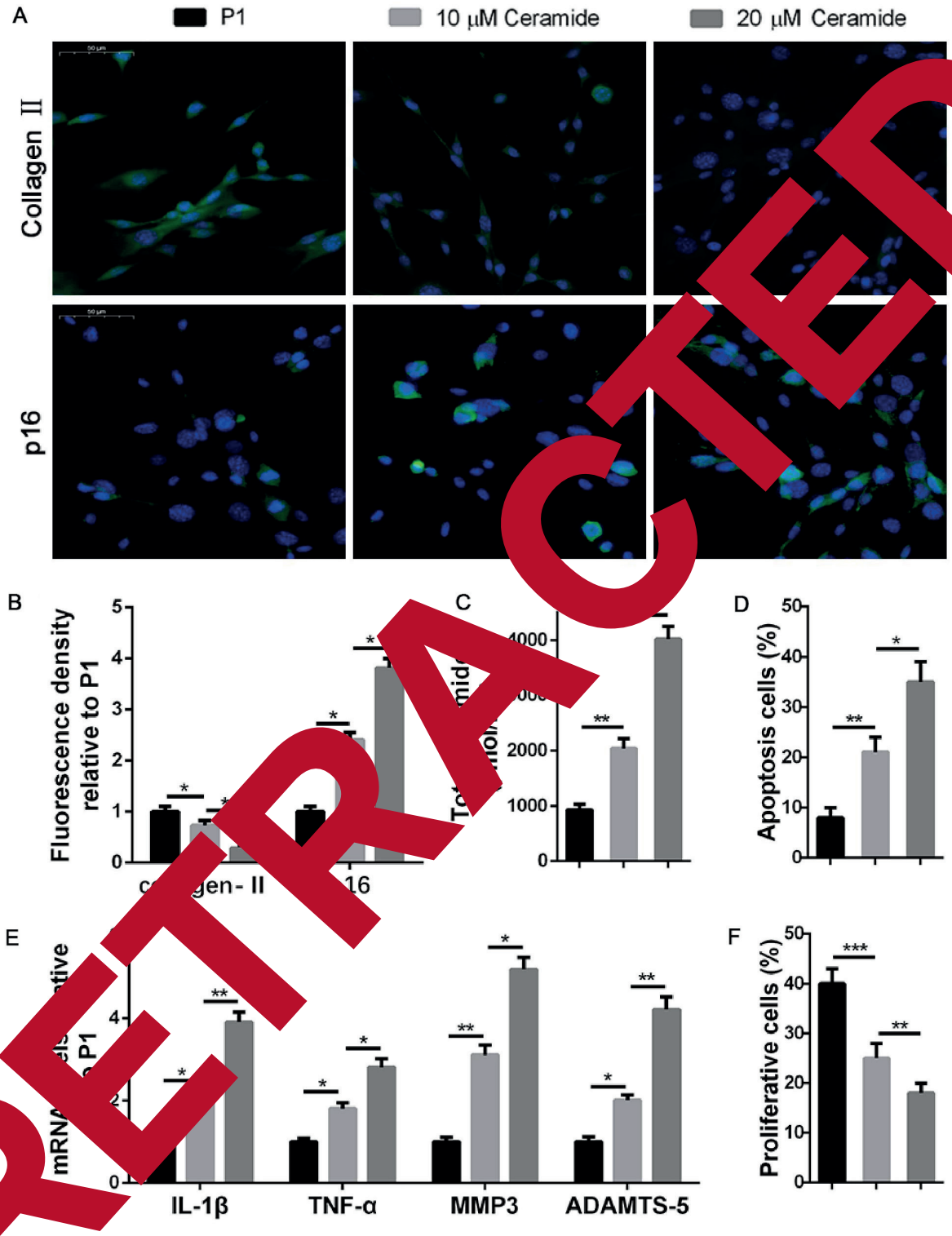


Figure eramid accumulated in degenerated NP cells. NP cells from passage 1 (P1) cultured until the fusion rate up to 80% passaged 4. **A,** IF staining of collagen-II, and p16 (magnification: 400×). **B,** Quantification analysis. **C,** Total ceramide quantitated by LC-MS. The ratio of **D,** apoptotic or **E,** proliferative NP cells was determined by flow cytometry. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01).

via y, and the increase of senescent cells and the matory response.

eramide Suppression Alleviated NP ls Degeneration

etermine whether the suppression of ceramide protects the NP cells naturally degeneration, we used myriocin to suppress ceramide levels by de novo synthetic pathway. We passaged NP cells from the first generation to the fourth generation with or without myriocin (1 mM or 2 mM). Under the treatment of myriocin, we found the collagen II expression was mainly maintained compared to the cells without myriocin treatment, and 2 mM was effective to alleviate the degenerative progress of NP cells. The marker of senescent cell p16 was inhibited in a dose-dependent compared to the control with no myriocin intervention (Figure



ure 2. Ceramide overexpression promoted NP cells degeneration. NP cells in P1 were treated with C6-ceramide (10 μM M) for 24 h and changed the medium. **A,** IF staining of collagen-II, and p16 (magnification: 400×). **B,** Quantification an . **C,** Total ceramide quantitation by LC-MS. **D,** Ratio of apoptotic NP cells was determined by flow cytometry. **E,** mRNA expression levels were assayed by RT-PCR. **F,** Ratio of proliferative NP cells was determined by flow cytometry. Data are presented as mean ± S.D. of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

3A, 3B). As expected, the ceramide content was decreased in passage 4 resulting from the stimulation of myriocin (Figure 3C). The result of flow cytometry showed that myriocin meanwhile prevented NP cells apoptosis and protected their proliferation (Figure 3D, 3F). Then, we detected

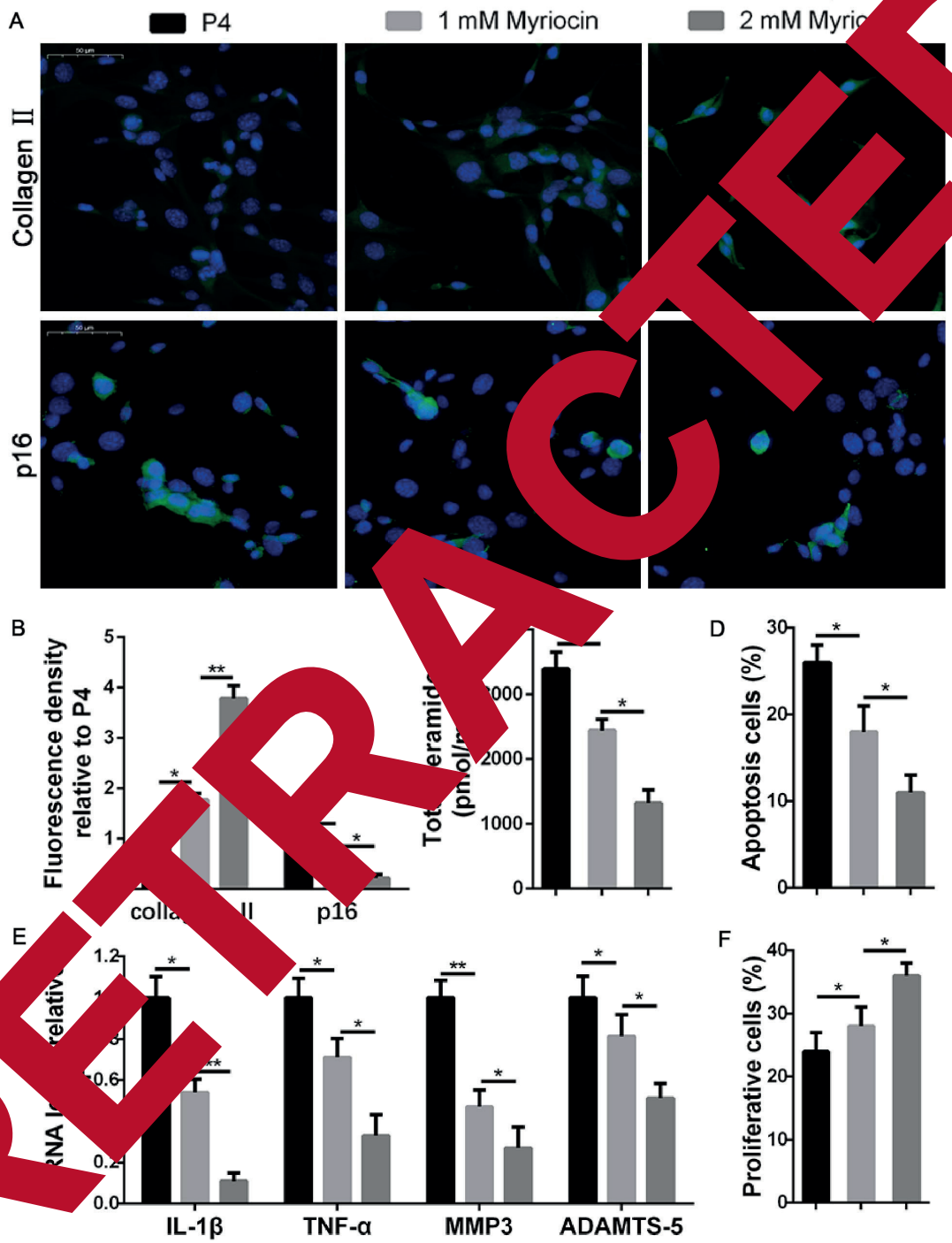


Figure 3. Ceramide inhibition alleviated NP cells degeneration. NP cells were treated with or without myriocin (10 mM or 2 mM) from P1 to P4. **A,** IF staining of collagen-II, and p16 (magnification: 400×). **B,** Quantification analysis. **C,** Total ceramide quantitation by LC-MS. **D,** Ratio of apoptotic NP cells was determined by flow cytometry. **E,** mRNA expression levels were assayed by RT-PCR. **F,** Ratio of proliferative NP cells was determined by flow cytometry. Data are presented as mean ± SD of three independent experiments. (*p<0.05, **p<0.01).

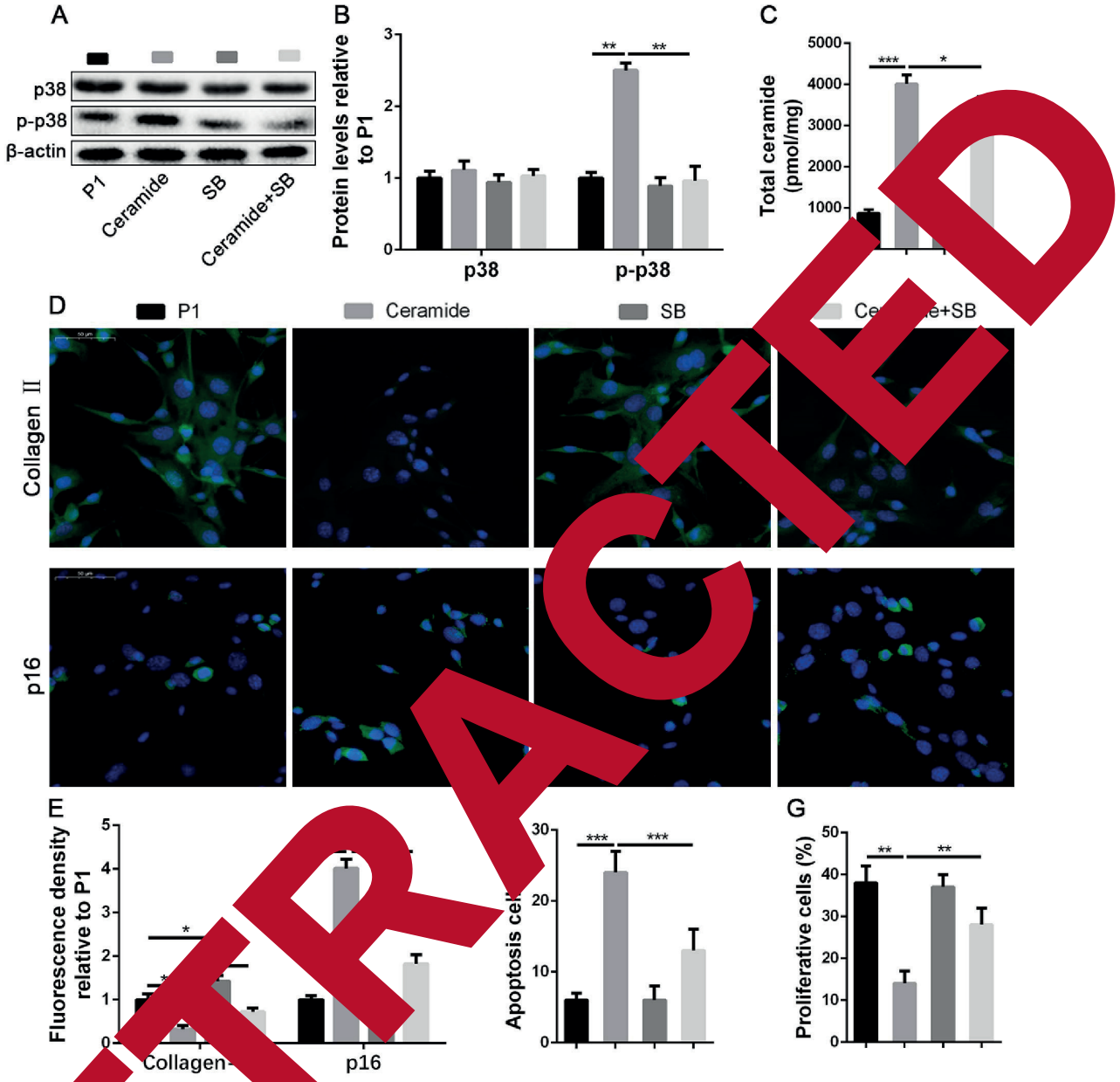


Figure ppress p38MAPK activ rejected ceramide-induced NP cell degeneration. P1 NP cells were treated with C6-ceramid μM) or 0 nM) for 24 h and then changed the medium. Besides, P1 NP cells were pre-treated with C6-ceramide (20 or 24 h then treated with SB (50 nM) for another 24 h. **A,** Protein expression of p38 and p-p38 was determined by W ntificati alysis. **C,** Total ceramide quantitation by LC-MS. **D,** IF staining of collagen-II, and p16 (magfication **E,** Qu cation analysis. The ratio of **F,** apoptotic or **G,** proliferative NP cells was determined by flow try. ed as mean ± SD of three independent experiments. (*p<0.05, **p<0.01, ***p<0.001).

th levels about the inflammatory factors suppression of ceramide by myrio- decreased the IL-1β, TNF-α, MMP-3, and AMTS-5 expression (Figure 3E). In this part, w sidered that the suppression of ceramide alleviated NP cell degeneration by the protected collagen II content and cell viability, and reduced senescent cells and the inflammatory response.

p38 Suppression Rejected Ceramide Induced NP Cells Degeneration

Like ceramide, the p38MAPK signaling pathway can be activated during extracellular stimulation such as ultraviolet irradiation, heat shock, high osmotic pressure, and inflammatory factors, and regulates various physiological processes, such as cell differentiation, cell cycle, and inflam-

matory response. Therefore, we wondered whether ceramide affected NP cells metabolism crosslinking with p38 activity. We used SB203580 (SB) to suppress p38MAPK activation. As shown in Figure 4A and 4B, the expression of total p38 was no significantly different among these groups. However, the 20 μM ceramide treatments markedly upregulated the p-p38 expression, which was suppressed by SB again. So, the overexpression of ceramide accompanied by the activation of p38. In addition, SB decreased the accumulation of ceramide even a small drop (Figure 4C). The result of IF showed that SB could partly reverse the ceramide-induced collagen II downregulation and p16 upregulation (Figure 4D, 4E). Finally, apoptosis and proliferation were also measured and the result suggested that SB treatment protected the NP cell proliferation and prevented the apoptosis progress compared to the ceramide treated group (Figure 4F, 4G). We found the suppression of p38MAPK phosphorylation could alleviate the upregulation of ceramide-induced NP cell degeneration, indicating that ceramide might accelerate the NP cell degeneration by p38MAPK activation.

Discussion

Ceramide belongs to a class of highly hyd phobic families and is the centr ule of t sphingomyelin signaling p eramide acts as a second messeng fector participates in the activation ultipl nases and protein ph hat t regu s signal processes su s apopt roliferation, differentiation, a owth arrest g an important physi e in cell h ostasis[18]. NP degenera of t ervertebral disc is a multi-fact omplex dise nd it is currently difficul achieve the delay even reverse the proc of dege ation. Since now, the relation be cer e and IDD is not clear. In our study, ced th e content of ceramide mul ithin degeneration of NP cells P1 to ations[19]. Therefore, we held the wpoint ceramide played a role in the de nt of IDD.

NP cells in the P1 generation with ogenous ceramide to made it overexpressed. eramide can rapidly induce human umbilic ve ndothelial cells senescence[20]. Chan et al[21] found ceramide promoted inflammatory processes and apoptosis in the epithelium of the lung. Ceramide also triggers oxidative stress and nitric oxide synthase on human endothelial cells[22]. Therefore, ceramide accelerates different cell types of degradation with different manners. In our study, the cell-permeable analog effic ly increased the ceramide content an phenotype of P1 NP cells similar e P4, in dicating the accumulation of cer promotes the degenerated degree of NP cell the contrary, the suppression of cer de ac ation by myriocin could preven cell dege containing the aspects c llagen synthes inflammation respo apopt senescence, and proliferation. M is ved to press inflammatory a ngal hibitin amide de novo synth in chroni mmation with comp immunity rek et al[24] stated my in d ed ceramide *de novo* synthesis mediating lipi abolism in the liver of stre in treated ra

mechanism under ies ceramide in the i vention of c l metabolism is complicated, ceramides activate c-Jun N-terminal k (JNK)[25]. lear transcription factor κB (N to e receptor (TLR)[27] signaling pathwa 38MAPK pathway[28]. p38MAPK ignaling pathway regulates various physiological s es such as cell differentiation and apop- si cell cycle, and inflammatory response. We suggested that ceramide contributed to NP cell degeneration by the activation of p38MAPK. We found the p-p38 level was upregulated by the treatment of C6-ceramide. Additionally, the suppression of p38MAPK activation partly reversed the pro-degenerated function of ceramide accumulation. This result indicates that ceramide promotes the activation of p38MAPK contributing to the NP cell degeneration. However, the detailed mechanism of p38MAPK crosslinking with ceramide and the relation between other signaling pathways with ceramide need further exploration.

Conclusions

In summary we elucidated that ceramide accumulated during the development of NP cell degeneration. The overexpressed ceramide accelerates NP cells degeneration in decreasing proliferation, advancing the apoptosis, promoting senescence, and suppressing inflammation of NP cells. Besides, the suppression of ceramide can alleviate NP cells natural degeneration, and these effects underlining ceramide may be associated with the activation of p38MAPK. We believe cer-

amide could be a potential target for the treatment strategy of IDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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