N-cadherin attenuates nucleus pulposus cell senescence under high-magnitude compression

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Abstract. Mechanical compression is important in disc degeneration. N-cadherin (N-CDH)-mediated signaling contributes to the maintenance of the normal nucleus pulposus (NP) cell phenotype and NP matrix biosynthesis. Our preliminary study demonstrated that a high-magnitude compression (20% deformation) promotes NP cell senescence in a three-dimensional scaffold culture system. The aim of the present study was to investigate whether N-CDH-mediated signaling alleviates NP cell senescence under the above-mentioned high-magnitude compression. NP cells were transfected with recombinant lentiviral vectors to enhance N-CDH expression. All the transfected or un-transfected NP cells were seeded into the scaffolds and subjected to 20% deformation at a frequency of 1.0 Hz for 4 h once per day for 5 days. Results indicated that N-CDH overexpressed NP cells exhibited decreased senescence-associated β-galactosidase activity and downregulated expression levels of senescence-associated markers (p16 and p53). Furthermore, the N-CDH overexpressed NP cells exhibited increased cell proliferation potency, telomerase activity and matrix biosynthesis compared with NP cells without N-CDH overexpression under high-magnitude compression. Thus, N-CDH-mediated signaling contributes to the attenuation of NP cell senescence under high-magnitude compression.

Introduction

Intervertebral disc degeneration (IDD) is regarded as a leading cause of lower back and leg pain (1). Due to a lack of complete understanding of the pathogenesis of IDD, current treatments are effective in symptomatic relief, but not biological regeneration of degenerative disc tissue (2-4). Further studies are required to develop effective regenerative strategies for IDD.

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The intervertebral disc (IVD) functions as a connection structure that absorbs and transmits mechanical load (5). Under physiological conditions, the disc is subjected to various magnitudes of mechanical compression (6,7). In line with previous studies, it was demonstrated that mechanical compression significantly affected disc biology *in vitro* (8,9). Furthermore, our preliminary study identified that a high-magnitude compression (20% deformation) promoted nucleus pulposus (NP) cell senescence in a three-dimensional (3D) scaffold culture system (unpublished data). As NP senescence is a classical cellular characteristic during disc degeneration (10,11), it is proposed that prevention of NP cell senescence may be a potential mechanism to alleviate high-magnitude compression-induced disc degeneration.

N-cadherin (N-CDH) is an adhesion molecule that was initially identified in the nervous system (12,13). Recent studies have indicated that N-CDH is a molecule that is highly expressed in normal NP cells and is gradually downregulated with disc degeneration (14,15). Notably, N-CDH-mediated signaling facilitates with maintaining a normal NP cell phenotype and NP matrix biosynthesis under the stimulation of certain pathological factors (16,17). However, the effects of N-CDH-mediated signaling on NP cell senescence remain unclear.

Therefore, the aim of the present study was to investigate the effects of N-CDH-mediated signaling on NP cell senescence under high-magnitude compression. To achieve this objective, a 3D scaffold culture system based upon a self-developed perfusion bioreactor was involved (18). NP cell senescence was evaluated by senescence-associated β-galactosidase (SA-β-Gal) activity, NP cell proliferation, telomerase activity, senescence marker (p16 and p53) expression levels and the matrix homeostatic phenotype.

Materials and methods

Ethical statement. All experimental animals were used in accordance with the relevant guidelines [SYXK (YU) 2012-0012] of the Ethics Committee at Southwest Hospital affiliated to the Third Military Medical University (Chongqing, China).

Disc harvest and NP cell isolation. Twenty-five healthy New Zealand rats (weight, 250 g; age, 6-8 weeks) were obtained

from the Animal Center of Third Military Medical University (Chongqing, China) and sacrificed by excessive carbon dioxide exposure. Briefly, after NP tissues were separated from the harvested thoracic and lumbar discs, NP tissue samples were sequentially digested with Gibco 0.25% trypsin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 3-5 min at 37˚C and Sigma-Aldrich type I collagenase (0.25%; Merck KGaA, Darmstadt, Germany) for 10 min. Subsequently, NP cell pellets were collected by centrifugation (500 x g at 4˚C for 5 min) and cultured in Dulbecco's modified Eagle's medium/F12 (Gibco; Thermo Fisher Scientific, Inc. medium containing Gibco 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% (v/v) penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) under standard conditions (37˚C, 20% O_2 and 5% CO_2).

NP cell transfection. NP cells were seeded in a 24-well plate and grown to 40-50% confluence. Subsequently, NP cells were incubated with 400 μl fresh culture medium containing 40 μl concentrate of recombinant lentiviral vectors (Shanghai GenePharma Co., Ltd., Shanghai, China) for 48 h to overexpress N-CDH in the NP cells (NP-N-CDH). NP cells transfected with negative vectors served as controls (NP-N-CDH-NC). Thereafter, the transfected cells were further selected via puromycin for 4-6 days. N-CDH overexpression in NP cells was verified by quantitative polymerase chain reaction (qPCR) and western blotting assays.

Compression application on NP cells. The transfected or un-transfected NP cells were suspended in collagen solution (1 mg/ml; Shengyou Biotechnology Co., Ltd., Hangzhou, China) and seeded into the prepared bovine decalcified bone matrix scaffold [DBM; 10x10x5 mm ($1x10^7$ cells per DBM)], provided by Tissue Engineering Center of the Third Military Medical University (Chongqing, China). After NP cells seeded in the scaffold were pre-cultured under standard conditions (37˚C, 20% O_2 and 5% CO_2) for 2 days, NP cells seeded in the DBM scaffolds were perfusion-cultured at 37˚C in the tissue culture chambers of the self-developed bioreactor (Fig. 1) for 5 days, and simultaneously subjected to dynamic compression (20% deformation at a frequency of 1.0 Hz for 4 h once per day).

SA-β-Gal activity. Subsequent to compression, NP cells seeded in the scaffold were collected by digestion with 0.05% trypsin and 0.1% collagenase I. The NP cells ($1x10^4$ per group) were allowed to adhere in 6-well plates within 6-8 h. Subsequently, an SA-β-Gal staining assay was performed according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, China). Finally, SA-β-Gal stain-positive cells were observed under an Olympus BX51 light microscope (Olympus Corp., Tokyo, Japan) and SA-β-Gal activity expressed as the percentage of SA-β-Gal stain-positive cells to the total cells was analyzed using the Image-Pro Plus software (Version 5.1.0.20; Media Cybernetics, Inc., Rockville, MD, USA).

Cell proliferation assay. Following compression, NP cells seeded in the scaffold were harvested as described above. NP cells ($3x10^3$ cells per group) were seeded in a 96-well plate and NP cell proliferation was detected at 6, 24 and 48 h using a Cell Counting Kit-8 (CCK-8; C0037; Beyotime Institute of Biotechnology).

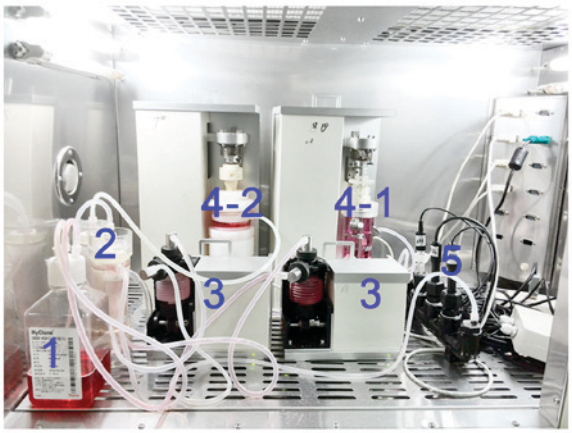

Figure 1. Image of the substance exchanger-based perfusion bioreactor. 1, medium reservoir; 2, substance exchanger; 3, peristaltic pump; 4, tissue culture chamber; 5, pH, PO_2 and PCO_2 sensor.

Telomerase activity detection. Subsequent to compression, NP cells seeded in the scaffold were harvested as described above. The NP cell pellets were incubated with RIPA lysis buffer (Beyotime Institute of Biotechnology) and centrifuged (12,000 x g at 4˚C for 5 min) to collect the supernatant. Then, a telomerase ELISA kit (ml-003023; Mlbio, Shanghai, China) was used to measure telomerase activity (IU/l) according to the manufacturer's instructions.

Cell cycle analysis. Following compression, NP cells seeded in the scaffold were harvested as described above. The NP cell pellets were fixed with 75% ethanol overnight at 4˚C and stained with propidium iodide solution (50 μg/ml; Beyotime Institute of Biotechnology) and RNase A (100 μg/ml; Beyotime Institute of Biotechnology) for 30 min at 4˚C. Subsequently, NP cells were subjected to flow cytometry assay and the fraction of each cell cycle phase (G_0/G_1, G_2/M and S) was calculated using the multicycle software (FlowJo 7.6.1, Engine 2.79000; Phenix Co., Ltd., Tokyo, Japan).

qPCR analysis. Gene expression of senescence markers (p16 and p53) and matrix macromolecules (aggrecan and collagen II) was analyzed by qPCR assay. Briefly, total RNA was extracted using TriPure Isolation Reagent (11667157001, Roche Applied Science, Penzberg, Germany) and synthesized into cDNA using a First Strand cDNA Synthesis kit (04379012001, Roche Applied Science). Then, qPCR was performed using a reaction system containing cDNA, SYBR Green Mix (Toyobo Life Science, Osaka, Japan) and primers (Table I). The thermal cycling conditions for all reactions were as follows: 5 min at 95˚C, followed by 35 amplification cycles of 30 sec at 95˚C, 20 sec at 56˚C and 15 sec at 72˚C. β-actin served as an internal reference and the relative gene expression was expressed as $2^{-\Delta\Delta Cq}$ (19).

Western blot analysis. Protein expression levels of senescence markers (p16 and p53) and matrix macromolecules (aggrecan and collagen II) were analyzed by western blotting assay.

Table I. Primers of target genes.

Gene	Accession no.	Forward (5'-3')	Reverse (5'-3')
β-actin	NM_031144.3	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
Aggrecan	XM_002723376.1	ATGGCATTGAGGACAGCGAA	GCTCGGTCAAAGTCCAGTGT
Collagen II	NM_012929.1	GCCAGGATGCCCGAAAATTAG	CCAGCCTTCTCGTCAAATCCT
P53	XM_008767773.1	CCTTAAGATCCGTGGGCGT	GCTAGCAGTTTGGGCTTTCC
P16	NM_031550.1	TACCCCGATACAGGTGATGA	TACCGCAAATACCGCACGA

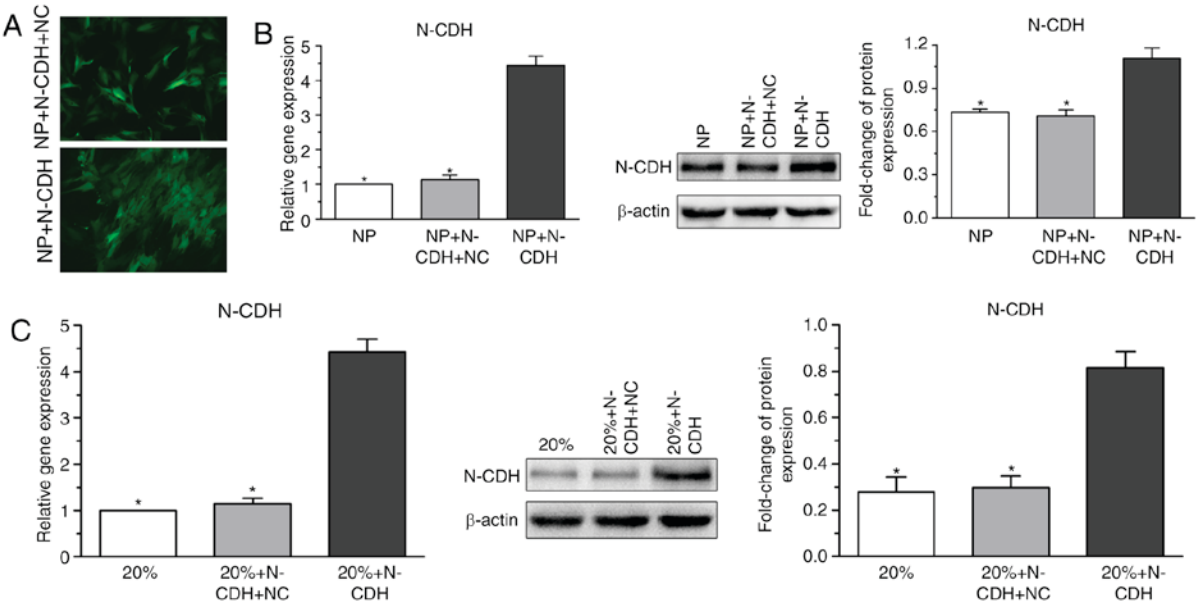

Figure 2. Verification of N-CDH overexpression in NP cells. (A) Successfully transfected NP cells were tagged with green fluorescent protein. Magnification, x200. (B) Quantitative polymerase chain reaction and western blotting assays indicated that N-CDH expression levels were upregulated in NP cells following incubation with recombinant lentiviral vectors. (C) N-CDH expression was increased in NP cells under the high-magnitude compression (20% compressive deformation) following N-CDH overexpression. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, indicates a significant difference between two groups. N-CDH, N-cadherin; NP, nucleus pulposus; NC, negative control.

Briefly, after the total protein was extracted using RIPA lysis solution (Beyotime Institute of Biotechnology) and the protein concentration was measured using a BCA kit (P0009, Beyotime Institute of Biotechnology), protein samples were subjected to an 12% SDS-PAGE system and transferred to a polyvinylidene difluoride (PVDF) membrane (100 V for 60 min). Then, the PVDF membrane was incubated with primary antibodies [β-actin: ProteinTech Group, Inc., Chicago, IL, USA (cat. no. 60008-1-Ig); p16: Novus Biologicals, LLC, Littleton, CO, USA (cat. no. NBP2-37740); p53: ProteinTech Group, Inc. (cat. no. 10442-1-AP); aggrecan: Santa Cruz Biotechnology Inc. (cat. no. sc-16492); collagen II: Abcam, Cambridge, MA, USA. (cat. no. ab34712); all diluted 1:1,000] at 4˚C overnight and the corresponding secondary antibodies (OriGene Technologies, Inc., Beijing, China; 1:2,000) at 37˚C for 2 h. Protein bands were developed using the SuperSignal West Pico Trial kit (34080, Pierce; Thermo Fisher Scientific, Inc.) and analyzed using Image J software (v Java 1.6.0_20 32-bit, National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. All data are expressed as means ± standard deviation and each experiment was performed in triplicate. After the homogeneity test for variance, comparisons between groups were performed by one-way analysis of variance using SPSS 13.0 software, and the post hoc test was determined by the least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Verification of N-CDH overexpression in NP cells. To investigate the role of N-CDH in regulating NP cell senescence under high-magnitude compression, N-CDH expression in NP cells was enhanced by recombinant lentiviral vectors. Predictably, N-CDH expression in NP cells under high-magnitude compression also increased following N-CDH overexpression (Fig. 2).

Analysis of NP cell senescence phenotype following N-CDH overexpression under high-magnitude compression. Senescent cells often exhibit increased SA-β-Gal activity (20), decreased cell proliferation potency (21), aggravated G_1 cell cycle arrest (22), decreased telomerase activity (23) and upregulated expression levels of senescence markers (p16 and p53) (24). Compared with NP cells without N-CDH overexpression under

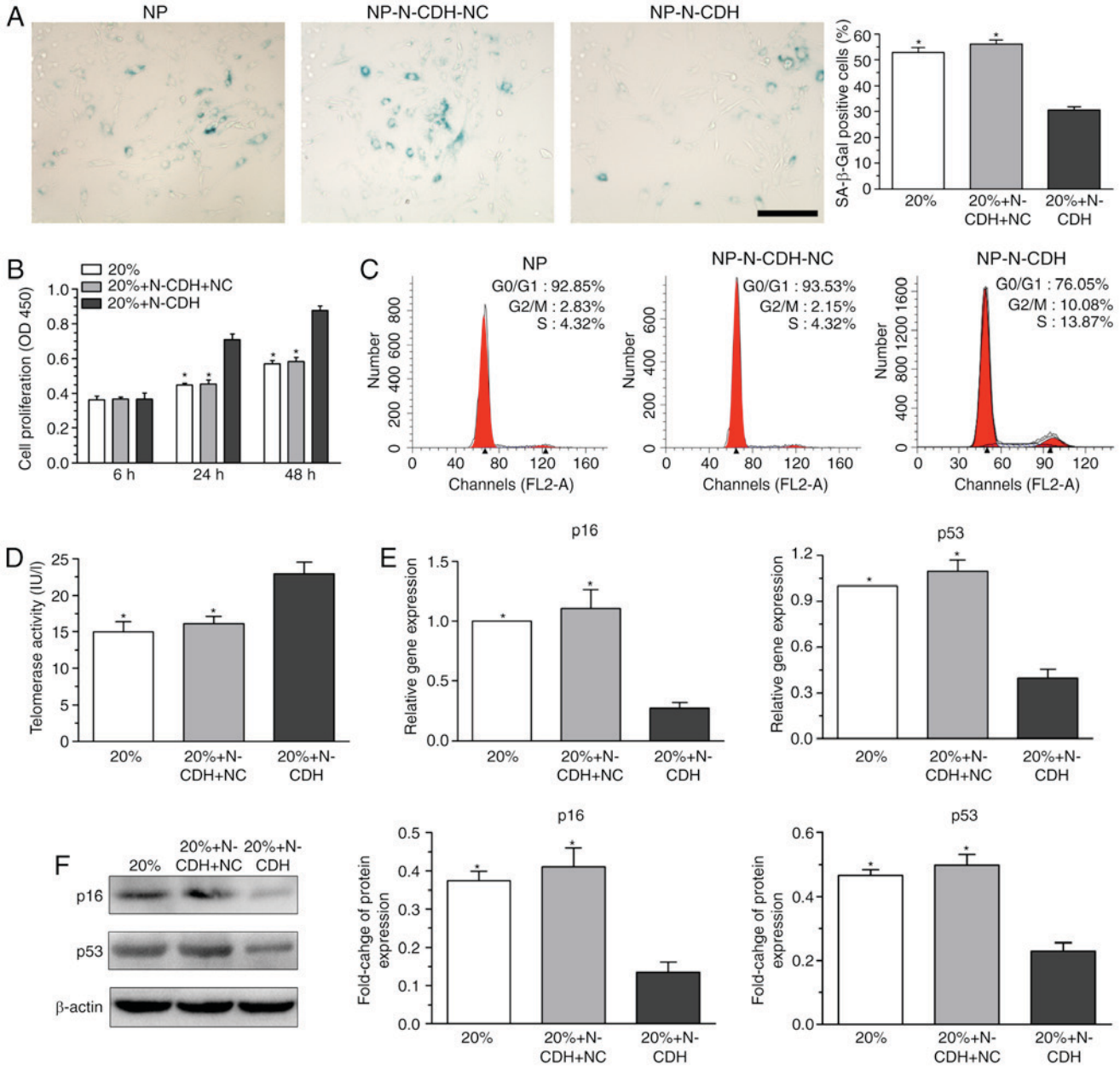

Figure 3. N-CDH overexpression attenuated NP cell senescence under the high-magnitude compression (20% compressive deformation). (A) SA-β-Gal activity analysis (scale bar, 100 μm). (B) Cell proliferation analysis. (C) Cell cycle analysis. (D) Telomerase activity measurement. (E) Quantitative polymerase chain reaction analysis of gene expression of senescence markers (p16 and p53). (F) Western blot analysis of protein expression levels of senescence markers (p16 and p53). Data are expressed as the mean ± standard deviation (n=3). *P<0.05, indicates a significant difference between two groups. N-CDH, N-cadherin; NP, nucleus pulposus; NC, negative control.

high-magnitude compression, N-CDH overexpressed NP cells exhibited significantly decreased SA-β-Gal activity (Fig. 3A), increased cell proliferation potency (Fig. 3B), decreased percentage of cells arrested in the G_1 phase of the cell cycle (Fig. 3C), increased telomerase activity (Fig. 3D), and down-regulated gene (Fig. 3E) and protein (Fig. 3F) expression levels of senescence markers (p16 and p53).

Analysis of the expression levels of matrix macromolecules in NP cells following N-CDH overexpression under high-magnitude compression. Senescent cells demonstrate altered matrix metabolism and matrix catabolism is often promoted in senescent cells (25,26). qPCR indicated that the gene expression of matrix macromolecules (aggrecan and collagen II) in N-CDH overexpressed NP cells was higher than that in NP cells without N-CDH overexpression under high-magnitude compression (Fig. 4A). Additionally, protein expression levels of these matrix macromolecules presented a similar trend (Fig. 4B).

Discussion

It is well established that mechanical load has important effects on disc biology, and that the un-physiological load is a validated risk factor that initiates and aggravates disc degeneration (27-29). Disc cell senescence is a type of typical pathology during disc degeneration (10,11). Our preliminary study demonstrated that high-magnitude compression (20%

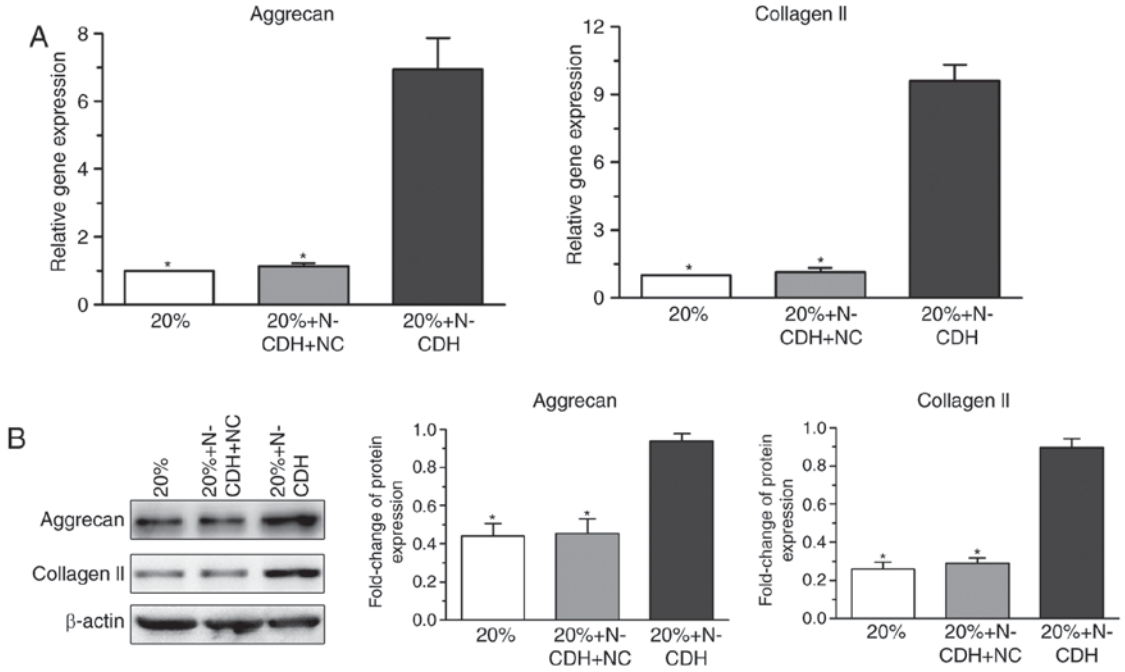

Figure 4. N-CDH overexpression upregulated the expression levels of NP matrix macromolecules (aggrecan and collagen II) under the high-magnitude compression (20% compressive deformation). (A and B) Quantitative polymerase chain reaction and western blot analysis of the expression levels of aggrecan and collagen II, respectively. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, indicates a significant difference between two groups. N-CDH, N-cadherin; NP, nucleus pulposus; NC, negative control.

compressive deformation) promoted NP cell senescence in a 3D scaffold culture system (unpublished data). The present results demonstrated for the first time, to the best of our knowledge, that N-CDH-mediated signaling attenuated NP cell senescence under high-magnitude compression.

N-CDH is a molecular marker of normal juvenile disc NP cells (14,15). Previous studies have indicated that N-CDH-mediated signaling was helpful for promoting NP matrix biosynthesis and maintaining a normal NP cell phenotype (16,17). Here, to investigate whether N-CDH-mediated signaling attenuates NP cell senescence under a high-magnitude compression, N-CDH expression was enhanced during the current study using recombinant lentiviral vectors (Fig. 2).

There are various parameters for evaluating cell senescence, such as SA-β-Gal activity and telomerase activity (20,23). The present results demonstrated that N-CDH overexpression decreased SA-β-Gal activity, whereas it increased telomerase activity in NP cells under high-magnitude compression. In addition, senescent cells are often arrested in the G_1 phase of the cell cycle, which lead to a limited cell proliferation potency (21,22). Consistently, the present result indicated that NP cells exhibited a decrease in the percentage of G_1 phase fractions and an increase in cell proliferation potency under the high-magnitude compression following N-CDH overexpression. Thus, these findings indicate that N-CDH overexpression attenuates NP cell senescence under high-magnitude compression.

Disc cell senescence results from the natural disc aging process, as well as possibly being induced by various stresses, including growth factor insufficiency, oxidative damage, inflammation reaction and mechanical injury (11). There are two approaches responsible for the transduction senescence signal: Replicative senescence (RS) mediated by the p53-p21-pRB signaling pathway and stress-induced premature senescence (SIPS) mediated by the p16-pRB signaling pathway (24). The current results demonstrated that N-CDH overexpression downregulated expression levels of senescence markers (p16 and p53) under high-magnitude compression, indicating that N-CDH overexpression attenuates mechanical overloading-induced NP cell senescence by targeting RS and SIPS. The matrix homeostatic phenotype is an indirect indicator for evaluating cell senescence. The current study identified that expression levels of matrix macromolecules in N-CDH overexpressed NP cells were significantly increased under high-magnitude compression, further indicating that N-CDH overexpression attenuates NP cell senescence under high-magnitude compression.

In conclusion, N-CDH overexpression attenuated NP cell senescence under high-magnitude compression. Although this study provides an improved understanding of NP senescence, the potential signaling transduction behind this process requires further investigation. However, the current study provides an experimental basis for the protective effects of N-CDH on disc biology under high-magnitude compression and contributes to developing novel strategies to alleviate mechanical overload-induced disc degeneration.

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