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

MING NIU1, FEI MA1, JUN QIAN2, JUNWEI LI1, TONG WANG1, YUZHEN GAO1 and JIAN JIN3

1The Second Department of Surgery, Ganzhou People's Hospital; 2The First Department of Orthopaedic Surgery, Zhangye People's Hospital Affiliated to Hexi University, Zhangye, Gansu 734000; 3Department of Spine Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China

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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.

The intervertebral disc (VID) 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

Correspondence to: Dr Jian Jin, Department of Spine Surgery, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou, Guangdong 510515, P.R. China
E-mail: 38323580@qq.com

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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 (1x10⁵ 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% O₂ and 5% CO₂) 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 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% O₂ and 5% CO₂).

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^ΔΔCt (19).

Cell cycle analysis. Following compression, NP cells seeded in the scaffold were harvested as described above. NP cells (3x10⁵ 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).

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.
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), aggrated G1 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
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 G1 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%...
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 G1 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 G1 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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References

1. Chen JW, Ni BB, Li B, Yang YH, Jiang SD and Jiang LS: The responses of autophagy and apoptosis to oxidative stress in nucleus pulposus cells: Implications for disc degeneration. Cell Physiol Biochem 34: 1175-1189, 2014.

2. Yang SD, Ma L, Gu TX, Ding WY, Zhang F, Shen Y, Zhang YZ, Yang DL, Zhang D, Sun YP and Song YL: 17β-Estradiol protects against apoptosis induced by levofloxacin in rat nucleus pulposus cells by upregulating integrin αβ1. Apoptosis 19: 789-800, 2014.

3. Yang SD, Ma L, Yang DL and Ding WY: Combined effect of 17β-estradiol and resveratrol against apoptosis induced by interleukin-1β in rat nucleus pulposus cells via PI3K/Akt/caspase-3 pathway. Peerd 4: e1640, 2016.

4. Yang SD, Yang DL, Sun YP, Wang BL, Ma L, Feng SQ and Ding WY: 17β-estradiol protects against apoptosis induced by interleukin-1β in rat nucleus pulposus cells by down-regulating MMP-3 and MMP-13. Apoptosis 20: 348-357, 2015.

5. Lee CR, Iatridis JC, Poveda L and Alini M: In vitro organ culture of the bovine intervertebral disc: Effects of vertebral endplate and potential for mechanobiology studies. Spine (Phila Pa 1976) 31: 515-522, 2006.

6. Neidlinger-Wilke C, Galbusera F, Pratsinis H, Mietsch A, Kletzas D and Wilke HJ: Mechanical loading of the intervertebral disc: From the macroscopic to the cellular level. Eur Spine J 3 (Suppl 23): S333-S343, 2014.

7. Hwang D, Gabai AS, Yu M, Yew AG and Hsieh AH: Role of load history in intervertebral disc mechanics and intradiscal pressure generation. Biomech Model Mechanobiol 11: 95-106, 2012.

8. Li P, Gan Y, Wang H, Zhang C, Wang L, Xu Y, Song L, Li S, Li S, Ou Y and Zhou Q: Dynamic compression effects on immature nucleus pulposus: A study using a novel intelligent and mechanically active bioreactor. Int J Med Sci 13: 225-234, 2016.

9. Li P, Gan Y, Xu Y, Song L, Wang H, Zhang C, Wang L, Zhao C, Luo L and Zhou Q: Matrix homeostasis within the immature annulus fibrosus depends on the frequency of dynamic compression: A study based on the self-developed mechanically active bioreactor. Biomech Model Mechanobiol 16: 385-394, 2017.

10. Gruber HE, Ingram JA, Norton HJ and Hanley EN Jr: Senescence in cells of the aging and degenerating intervertebral disc: Immunolocalization of senescence-associated beta-galactosidase in human and sand rat discs. Spine (Phila Pa 1976) 32: 321-327, 2007.

11. Wang F, Cai F, Shi R, Wang XH and Wu XT: Aging and age related stresses: A senescence mechanism of intervertebral disc degeneration. Osteoarthritis Cartilage 24: 398-408, 2016.

12. Brassé JL: N-cadherin signaling in synapse formation and neuronal physiology. Mol Neurobiol 33: 237-252, 2006.

13. Halbleib JM and Nelson WJ: Cadherins in development: Cell adhesion, sorting, and tissue morphogenesis. Genes Dev 20: 3199-3214, 2006.

14. Lv F, Leung VY, Huang S, Huang Y, Sun Y and Cheung KM: In search of nucleus pulposus-specific molecular markers. Rheumatology (Oxford) 53: 600-610, 2014.

15. Minogue BM, Richardson SM, Zeeff LA, Freemont AJ and Hoyland JA: Transcriptional profiling of bovine intervertebral disc cells: Implications for identification of normal and degenerate human intervertebral disc cell phenotypes. Arthritis Res Ther 12: R22, 2010.

16. Hwang PY, Jing L, Chen J, Lim FL, Tang R, Choi H, Cheung KM, Rishbud MV, Gersbach CA, Guijak F, et al: N-cadherin is key to expression of the nucleus pulposus cell phenotype under selective substrate culture conditions. Sci Rep 6: 28038, 2016.

17. Hwang PY, Jing L, Michael KW, Richardson WJ, Chen J and Setton LA: N-cadherin-mediated signaling regulates cell phenotype for nucleus pulposus cells of the intervertebral disc. Cell Mol Bioeng 8: 51-62, 2015.

18. Li ST, Liu Y, Zhou Q, Lue RF, Song L, Dong SW, Guo P and Kopjar B: A novel axial-stress bioreactor system combined with a substance exchanger for tissue engineering of 3D constructs. Tissue Eng Part C Methods 20: 205-214, 2014.

19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta T) method. Methods 25: 402-408, 2001.

20. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linkens M, Rubelj I, Pereira-Smith O, et al: A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92: 9363-9367, 1995.

21. Gruber HE, Ingram JA, Davis DE and Hanley EN Jr: Increased cell senescence is associated with decreased cell proliferation in vivo in the degenerating human annulus. Spine J 9: 210-215, 2009.

22. Oshima J and Campisi J: Fundamentals of cell proliferation: Control of the cell cycle. J Dairy Sci 74: 2778-2787, 1991.

23. Chatterjee S: Telomeres in health and disease. J Oral Maxillofac Pathol 21: 87-91, 2017.

24. Beauséjour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P and Campisi J: Reversal of human cellular senescence: Roles of the p53 and p16 pathways. EMBO J 22: 4212-4222, 2003.

25. van Deursen JM: The role of senescent cells in ageing. Nature 509: 439-446, 2014.

26. Cristofalo VJ, Lorenzini A, Allen RG, Torres C and Tresini M: Replicative senescence: A critical review. Mech Ageing Dev 125: 827-848, 2004.

27. Gao X, Zhu Q and Gu W: Prediction of glycosaminoglycan synthesis in intervertebral disc under mechanical loading. J Biomech 49: 2655-2661, 2016.

28. Jünger S, Gantenbein-Ritter B, Lezuo P, Alini M, Ferguson SJ and Ito K: Effect of limited nutrition on in situ intervertebral disc cells under simulated-physiological loading. Spine (Phila Pa 1976) 34: 1264-1271, 2009.

29. Chan SC, Ferguson SJ and Gantenbein-Ritter B: The effects of dynamic loading on the intervertebral disc. Eur Spine J 20: 1796-1812, 2011.