MUSCULOSKELETAL PATHOLOGY

Caspase 3 Silencing Inhibits Biomechanical Overload—Induced Intervertebral Disk Degeneration

Katsuhisa Yamada,* Hideki Sudo,[†] Koji Iwasaki,* Naoki Sasaki,[‡] Hideaki Higashi,[§] Yusuke Kameda,* Manabu Ito,[†] Masahiko Takahata,* Kuniyoshi Abumi,* Akio Minami,* and Norimasa Iwasaki*

From the Departments of Orthopedic Surgery* and Advanced Medicine for Spine and Spinal Cord Disorders,[†] Hokkaido University Graduate School of Medicine, Hokkaido; the Division of Advanced Interdisciplinary Science,[‡] Faculty of Advance Life Science, Hokkaido University, Hokkaido; and the Division of Infection and Immunity,[§] Hokkaido University Research Center for Zoonosis Control, Sapporo, Hokkaido, Japan

Accepted for publication
November 12, 2013.

Address correspondence to
Hideki Sudo, M.D., Ph.D.,
Department of Advanced Med-
icine for Spine and Spinal Cord
Disorders, Hokkaido University
Graduate School of Medicine,
N-15, W-7, Kita-ku, Sapporo,
Hokkaido 060-8638, Japan.
E-mail: hidekisudo@yahoo.co.jp.

Intervertebral disk (IVD) degeneration causes debilitating low back pain in much of the worldwide population. No efficient treatment exists because of an unclear pathogenesis. One characteristic event early in such degeneration is the apoptosis of nucleus pulposus (NP) cells embedded in IVDs. Excessive biomechanical loading may also be a major etiology of IVD degeneration. The present study used *in vitro* and *in vivo* models of compressive loading to elucidate the underlying mechanism of IVD degeneration. In addition, we investigated whether the inhibition of apoptosis is a potential clinical therapeutic strategy for the treatment of IVD degeneration induced by biomechanical stress. A TUNEL assay showed that NP cell–agarose three-dimensional composite cultures subjected to uniaxial, unconfined, static, compressive loading exhibited a time-dependent increase in apoptosis. Western blot analysis revealed the up-regulation of several extracellular matrix-degrading enzymes and down-regulation of tissue inhibitor of metalloproteinase 1. These responses to compressive loading were all significantly inhibited by caspase 3 siRNA. In the *in vivo* model of compressive loading-induced IVD degeneration, a single local injection of caspase 3 siRNA significantly inhibited IVD degeneration by magnetic resonance imaging, histological findings, IHC, and TUNEL assay. The present study suggests that caspase 3 siRNA attenuates overload-induced IVD degeneration by inhibiting NP cell apoptosis and the expression of matrix-degrading enzymes. (*Am J Pathol* 2014, 184: 753–764; <http://dx.doi.org/10.1016/j.ajpath.2013.11.010>)

Human intervertebral disk (IVD) degeneration is a common cause of low back pain, affecting daily activities.^{1–3} IVD degeneration also leads to most spinal diseases, including spinal canal stenosis, disk herniation, and spinal deformity. To our knowledge, no clinical treatment preventing the development of IVD degeneration exists, and available therapeutic options for spinal complications [namely, analgesics and surgical procedures (ie, discectomy or spinal fusion)] do not focus on the etiology.

The pathogenesis of IVD degeneration remains unclear. One characteristic event early in the degeneration is the apoptosis of nucleus pulposus (NP) cells embedded in IVD.^{4–7} These cells play a critical role in the synthesis of extracellular matrix proteins maintaining lumbar disk integrity and flexibility. Because disk aging and degeneration decreases NP cell numbers,^{4,5,8} leading to diminished extracellular matrix generation,^{5,9–11} therapeutic approaches

aiming to suppress NP cell apoptosis may decrease IVD degeneration.^{6,7,12–14}

Each IVD is composed of a central NP limited by the annulus fibrosus and separated from adjacent IVDs by cartilaginous end plates. These disks continuously transmit the load down the spine during daily activities involving mechanical stress. There is increasing evidence that excessive biomechanical loading is also a major etiology of IVD degeneration.^{11,15–19} Reportedly, mechanical overload induced IVD degeneration through a caspase 9–dependent apoptotic pathway.¹¹ In addition, evidence exists that excessive loading induces apoptotic cell death and raises caspase 3 expression in an IVD organ culture model.¹⁵ Biomechanical overload is at

This work was supported by Ministry of Education, Culture, Sports, Science and Technology of Japan grant 23592150, the Takeda Science Foundation grant, and the Suzuken Memorial Foundation grant.

Disclosures: None declared.

the crux of IVD degeneration through apoptotic death of NP cells.^{11,15–19} In this study, we used *in vitro* and *in vivo* models of compressive loading to elucidate the underlying mechanism of IVD degeneration. Furthermore, we investigated whether the inhibition of apoptosis targeting caspase 3 is a potential clinical therapeutic strategy for the treatment of IVD degeneration induced by biomechanical stress.

Materials and Methods

Human NP Cell Culture

Healthy intact human NP samples were obtained from patients who had undergone spinal fusion for adolescent idiopathic scoliosis.¹² We obtained informed consent from these patients, and the ethics committee of the Hokkaido University Graduate School of Medicine (Hokkaido, Japan) specifically approved this study. The NP cells were isolated, as previously described.¹² All experiments were performed with high-density cultured NP cells in passage 1 or 2.

Preparation of NP Cell-Agarose 3D Composites

The NP cells were detached from the culture plates with 0.25% trypsin/EDTA (Invitrogen, Carlsbad, CA) and collected by centrifugation at $300 \times g$. The cells (2.0×10^6 cells/mL) were suspended in 1.0 mL of Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum. Then, 1.0 mL of 4% (w/w) low melting temperature agarose (Sigma-Aldrich, Deisenhofen, Germany) in PBS was added to obtain a 2.0-mL solution containing 1.0×10^6 cells/mL in 2% (w/w) agarose.²⁰ After gelling at 4°C for 5 minutes, cylinders (8 mm diameter $\times$ 2 mm thick) were carved out of the gel slab using a stainless steel punch.²¹ These NP cell–agarose composites were cultured overnight in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1.25 μ g/mL fungizone before the unconfined uniaxial compression experiments, as previously described.^{21–23} All experiments were performed on three independent agarose cultures from three different individuals.

Preparation and Transfection of siRNA

Human caspase 3 siRNA was constructed (sequences: 5'-GCAUAUCAGUUGAGCUUCATT-3' and 5'-UGAAGC-UCAACUGAUAUGCTT-3') and transfected into NP cells, as described previously.¹² A scrambled siRNA-negative control without specific function was synthesized and transfected into NP cells (sequences: 5'-UCUUAUC-GCGUAUAAGGCTT-3' and 5'-GCCUUAUACGCGAU-UAAGATT-3'). Forty-eight hours after transfection, the cells were harvested to form NP cell–agarose composites.

Biomechanical Loading Experiments

Human NP cell–agarose three-dimensional (3D) composites were subjected to mechanical loading in a custom-designed

bioreactor.^{21,24–26} The composite was placed on a permeable porous metal plate submerged in a testing bath filled with culture medium. The testing bath was attached to a 50-N load cell (LUR-A-SA1; Kyowa Electronic Instruments, Tokyo, Japan) to detect the stress applied to the sample. Compression was applied using an impermeable metal indenter. A 25% uniaxial unconfined static compressive strain,^{25,26} calculated based on sample thickness after preload, was applied (4 seconds) using a micrometer head attached to the sliding rod. All experiments were performed at 37°C and 5% CO₂ in a tissue culture incubator. The human NP cell–agarose composites were divided into four groups: i) unloaded control, non-transfected NP cell–agarose 3D composites cultured for the same duration with an indenter resting on top of them, but without compression; ii) compression-only group, non-transfected cell–agarose 3D composites with compression; iii) control siRNA group, siRNA-negative control siRNA transfected cell–agarose 3D composites with compression; and iv) caspase 3 siRNA group, caspase 3 siRNA transfected cell–agarose 3D composites with compression. All four groups were analyzed after the designated times of compression.

Cell Viability and Apoptosis Analysis

Live and dead cells were detected with Calcein AM and DAPI (Dojindo, Kumamoto, Japan), respectively. At the end of each loading experiment, the NP cell–agarose 3D composite was washed with PBS (three times, 5 minutes), then PBS containing Calcein AM (1 μ g/mL) and DAPI (1 μ g/mL) was added.^{3,9,27} After an incubation (37°C, 1 hour), the composites were washed with PBS (three times, 5 minutes), cut in slices (0.5 mm thick), and examined by fluorescent microscopy. The proportion of green (Calcein AM) and blue (DAPI) cells was counted in five independent, randomly selected fields. Percentage viability was determined as follows: number of Calcein-positive cells/total number of Calcein AM– and DAPI-positive cells. All measurements were performed on three slides for each group and each time point.

Analysis of NP cell apoptosis was performed with paraffin-embedded NP cell–agarose 3D composites subjected to a TUNEL assay using an In Situ Apoptosis Detection Kit, according to the manufacturer's recommendations (TaKaRa Bio, Shiga, Japan). The number of TUNEL-positive cells was counted in five independent, randomly selected fields on a light microscope. The TUNEL-positive cell ratio was measured by obtaining the ratio of the number of TUNEL-positive cells/total number of cells. All measurements were performed on three slides for each group and each time point.

Western Blot Analysis

After compression, the NP cell–agarose 3D composites were washed twice with PBS, and dissolved with 100 U/mL agarase (Sigma-Aldrich) at 37°C for 1 hour. The cell suspension was washed twice with PBS and lysed with 0.1 mL of ice-cold T-PER tissue protein extraction reagent (Pierce, Rockford, IL).

Cell lysate (20 µg protein per lane) was loaded and separated, as described previously.¹² After probing with primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse (Cell Signaling Technology, Danvers, MA), or anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies. The antibodies used were caspase 3 (Cell Signaling Technology), cleaved caspase 3 (Cell Signaling Technology), matrix metalloproteinase (MMP)-3 (Santa Cruz Biotechnology), MMP-13 (R&D Systems, Boston, MA), a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-4 (Abnova, Taipei, Taiwan), ADAMTS-5 (Santa Cruz Biotechnology), tissue inhibitor of metalloproteinases (TIMP)-1 (Abcam, Cambridge, UK), type II collagen (Abcam), aggrecan (Abcam), and integrin $\alpha 5 \beta 1$ (Chemicon, Temecula, CA). β -Actin was used as an internal control to confirm equal protein loading. The density of specific bands was quantified using a densitometer imaging system [ImageJ software version 1.45s (NIH, Bethesda, MD); <http://imagej.nih.gov>, last accessed November 1, 2013], as previously described.²⁸

Animals

We used male Japanese white rabbits weighing 3.2 to 3.5 kg (4 to 5 months old). All animal procedures in this study were specifically approved by the Institutional Animal Care and Use Committee at Hokkaido University.

Induction of IVD Degeneration with an External Compression Model and Caspase 3 siRNA Transfection

To induce intervertebral disk degeneration *in vivo*, a rabbit external compression model was used,^{29–31} with modifications.

Figure 1 Setup used for the application of uniaxial, unconfined, static compressive loading on human NP cell-agarose 3D culture composites. **A:** NP cell-agarose 3D composite (8 mm diameter, 2 mm thick). **B:** Uniaxial, unconfined, static compressive-loading device in a culture incubator. **C:** Load cell measuring pressure changes in the NP cell-agarose 3D composite during compressive stress. **D:** Typical pressure changes in an NP cell-agarose 3D composite during compressive loading.

An animal was attached to the custom-made external-loading device under *i.v.* anesthesia with pentobarbital sodium (30 mg/kg) and local anesthesia with 1% lidocaine. Four percutaneous stainless steel pins tied to two K-wires (diameter, 1.5 mm) were inserted into L4 and L5 bodies parallel to the adjacent study disk using a variable-speed electric drill. After the wound was closed, axial stress to the disk was immediately applied using the calibrated spring of the loading device. A 150-N compressive force generates 1.67-MPa pressure on a rabbit lumbar disk,³² which is the maximum stress the lumbar spine can withstand without damage. Because the spring length is proportional to a compressive force of ≤ 150 N and a pilot study showed that IVD degeneration progressed too slowly under both 50 and 100 N (data not shown), the change in spring length was set at 12.6 mm to generate 150 N during compression. A sham operation was performed with control animals subjected to surgical attachment of the K-wires and the external device, but without loading. After surgery, all animals were allowed free unrestricted activity in the cages. All rabbits were monitored daily.

The rabbit caspase 3 siRNA was constructed, as described previously,⁷ with the following sequences: 5'-CGUCCCCGUCUGGUACAGAUGUAAA-3' and 5'-UUUACAUCUGUACCAGACCGGGACG-3'. A scrambled siRNA-negative control was also synthesized with these sequences: 5'-CGUUGGCGGUCACAUGUAGUCCAAA-3' and 5'-UUUGGACUACAUGUGACCGCCAACG-3'. The *in vivo* administration of caspase 3 or control siRNA was performed with the siRNA-Invivofectamine Reagent (Invitrogen) prepared according to the manufacturer's instructions (final concentration, 1 µg/µL). One week after the compression session, the L4/5 disk was injected with 10 µL of the mixture under fluoroscopy, as described previously.⁷ In brief, caspase 3 or control siRNA was injected into the center of the L4/5 NP using a microsyringe with a 26-gauge needle. The timing of injection was based on a previous study documenting the effect of caspase 3 siRNA injections during the early phase of IVD degeneration.^{7,33} The rabbits were sacrificed 8 or 16 weeks after the injection.

MRI Analysis

Midsagittal or midaxial images of the treated disks were analyzed qualitatively for evidence of degenerative changes using an ultra-high magnetic field strength, 7.0-T MR scanner [Varian Unity Inova (Varian Medical Systems, Palo Alto, CA)], as described previously.⁷ The degree of IVD was assessed on sagittal images using the Pfirrmann classification.³⁴ Quantitative analysis of the sagittal image slices was also processed using Analyze software version 10.0 (AnalyzeDirect, Overland Park, KS), as reported previously.³⁵ The magnetic resonance imaging (MRI) index (product of NP area and average signal intensity) was used to quantify alterations in NP.^{7,35} The quantitative data were expressed as percentage relative to those obtained with unloaded control disks.⁷ All image assessments were performed by two independent blind

observers (K.Y. and H.S.), and the quantitative data were presented as the mean of the three evaluations ($n = 8$ disks from each treatment group per time point).

Histological and IHC Analysis

After MRI analysis, each intervertebral disk was prepared for histological and immunohistochemical (IHC) staining, as described previously.⁷ Midsagittal sections (5 μ m thick) were stained with H&E, and with safranin O—fast green. Semi-quantitative evaluation of disk degeneration was performed with a grading system including four categories, with the final grade ranging from 4 (normal) to 12 (highly degenerative).³⁶ All image assessments were performed by two independent blind observers (K.Y., H.S.), and the quantitative data were presented as the mean of the three evaluations ($n = 8$ disks from each treatment group per time point).

The IHC was performed for histological detection of cleaved caspase 3, MMP-3, and integrin $\alpha 5 \beta 1$, using anti-cleaved caspase 3 rabbit monoclonal antibody (1:100; Cell Signaling Technology), anti-MMP-3 goat polyclonal antibody (1:50; Santa Cruz Biotechnology), and anti-integrin $\alpha 5 \beta 1$ mouse monoclonal antibody (Chemicon),

respectively. The sections were deparaffinized in xylene and rehydrated. The sections of cleaved caspase 3 were subjected to autoclave treatment, and the sections of integrin $\alpha 5 \beta 1$ were treated with Proteinase K (Dako, Kyoto, Japan) for 20 minutes as pretreatment procedures. Any pretreatments were not necessary for the MMP-3 immunostaining. After PBS wash, the sections were treated 30 minutes with 1% H_2O_2 —methanol, then incubated with primary antibody at room temperature for 60 minutes (cleaved caspase 3 and MMP-3) or at 4°C overnight (integrin $\alpha 5 \beta 1$). The sections were incubated 30 minutes with peroxidase (EnVision + System Kit; Dako) (cleaved caspase 3 and integrin $\alpha 5 \beta 1$) or biotinylated anti-goat IgG (1:100; Vector, Burlingame, CA) and AVC reagent (MMP-3; Vector). Staining was developed with 3,3'-diaminobenzidine hydrochloride (Dako) and Mayer's hematoxylin for counterstaining. In addition, rabbit NP cell apoptosis was analyzed by performing TUNEL assays on sagittal paraffin-embedded sections using an In Situ Apoptosis Detection Kit, according to the manufacturer's recommendations (TaKaRa Bio).⁷ The number of cleaved caspase 3—, MMP-3—, integrin $\alpha 5 \beta 1$ —, or TUNEL-positive cells was counted in five independent, randomly selected

Figure 2 Impact of compressive loading on human NP cells *in vitro*. **A:** Viability and apoptosis assessed after 1, 6, and 24 hours of compressive loading by Calcein AM fluorescence and TUNEL assay. **Arrows** indicate TUNEL-positive cells. **B:** Western blot and densitometry analyses of protein extracts from NP cell—agarose 3D composites after 0 to 24 hours of compressive loading. The expression levels of caspase 3, cleaved caspase 3, MMP-3, MMP-13, ADAMTS-4, ADAMTS-5, TIMP1, type II collagen, aggrecan, integrin $\alpha 5$, and integrin $\beta 1$ were normalized to those of β -actin. * $P < 0.05$, ** $P < 0.01$ versus unloaded control. Data are given as means $\pm$ SD ($N = 3$).

fields.⁷ These values were expressed as the percentage of positive cells relative to the total cell number. All experiments were performed on eight disks from each treatment group and time point.

Statistical Analysis

Statistical analyses of multiple groups were performed with analysis of variance, followed by a Student–Newman–Keul’s test or a Student’s *t*-test. $P < 0.05$ was considered statistically significant.

Results

In Vitro Impact of Compressive Loading on Human NP Cells

The impact of compressive stress on human NP cells was investigated *in vitro* by subjecting NP cell–agarose 3D composites to uniaxial unconfined static loading (Figure 1, A–C). NP cell–agarose constructs exhibited characteristic viscoelastic behavior, as shown by the stress relaxation graphs (Figure 1D).²³ Loading generated a transient pressure of

19.6 ± 2.1 kPa, followed by a rapid reduction to 1.8 ± 0.4 kPa at 2 minutes. The pressure gradually declined to 1.0 ± 0.16 kPa over 1 hour, which was maintained for ≥ 24 hours. The results indicate that there is a fast relaxation process up to 2 minutes, followed by a plateau. The plateau represents the modulus of network chains, and the relaxation is regarded as rearrangements of gel-forming materials by squeezing liquids with which the gel was swollen.²⁰

Cell viability was significantly reduced from 90% to 75% after 1-hour loading, then stabilized at approximately 70%, as indicated by Calcein AM staining (Figure 2A). In contrast, apoptosis significantly increased with loading time, and reached a plateau of 15% at 6 hours, as indicated by the TUNEL-positive cells (Figure 2A). These data were confirmed by Western blot analysis of cell extracts from the NP cell–agarose 3D composites (Figure 2B). Compressive loading caused a twofold increase in cleaved caspase 3 expression over 24 hours. In addition, loading stimulated the expression of MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5, compared with unloaded conditions. In contrast, the expression of TIMP-1, type II collagen, and aggrecan decreased with loading time. These data show that mechanical loading modulates the production of extracellular matrix in NP cells, generating an

Figure 3 Inhibition of caspase 3 signaling in human NP improves cell viability and reduces the production of matrix-degrading enzymes. Cells transfected with caspase 3 siRNA and cultured in 3D agarose gel were subjected to 0 to 48 hours of compressive loading. **A:** Calcein AM fluorescence assay. **B:** TUNEL assay. There was no significant difference between the unloaded control and caspase 3 siRNA groups after 1, 6, 24, and 48 hours of compressive loading. $*P < 0.05$, $**P < 0.01$ (**A** and **B**). **C:** Western blot analysis of NP cell extracts 24 and 48 hours after compression. $^{\dagger}P < 0.05$ compared with unloaded control. Data are given as means $\pm$ SD ($N = 3$).

expression profile typical of IVD degeneration. The effects of compressive force on NP cells at 48 hours generated results comparable with those observed at 24 hours ([Supplemental Figure S1](#)). Finally, compressive loading stimulated the expression of the integrins $\alpha 5$ and $\beta 1$. Incidentally, integrin $\alpha 5 \beta 1$ has been identified in IVD cells and articular chondrocytes, and is believed to be responsible for the initial mechanotransduction response.^{37–40} The present results indicate that our *in vitro* model transmits the mechanical load down to the IVD cells.

Inhibition of Caspase 3 Signaling Improves Human NP Cell Viability and Reduces the Production of Matrix-Degrading Enzymes

Because compressive loading reduced cell viability but increased apoptosis in NP cells, we tested the impact of caspase 3 silencing on their survival. The composites were separated into four groups: unloaded control (non-transfected; not loaded), compression only (non-transfected; loaded), control siRNA (scrambled siRNA; loaded), and caspase 3 siRNA (transfected; loaded). We transfected human NP cells with caspase 3 siRNA using a protocol that reduces caspase 3 mRNA levels to <10%, and protein levels to <30%, *in vitro*.¹² The cell viability was significantly lower in the compression-only and control siRNA groups than the unloaded control group at 1, 6, 24, and 48 hours after loading ([Figure 3A](#)). In contrast, cell viability was significantly improved in the caspase 3 siRNA group compared with the control siRNA group after 1, 6, and 48

hours of loading. Although there was no significant difference between the control and caspase 3 siRNA groups after 24 hours of loading, cell viability was higher in the caspase 3 siRNA group. There was no significant difference between the unloaded control and caspase 3 siRNA groups at 1, 6, and 24 hours after loading. Furthermore, there were significantly fewer TUNEL-positive cells in the caspase 3 siRNA group than in the compression-only and control siRNA groups ([Figure 3B](#)). Finally, there was no significant difference between the unloaded control and caspase 3 siRNA groups after 1, 6, 24, and 48 hours of loading. These data clearly demonstrate the anti-apoptotic effect of caspase 3 siRNA on human NP cells under compressive stress.

Western blot analysis was performed to test whether the anti-apoptotic effect of caspase 3 siRNA may also maintain normal production of extracellular matrix components during 48 hours of loading. [Figure 3C](#) shows that caspase 3 silencing inhibited the expression of cleaved caspase 3, compared with the control siRNA group. Interestingly, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 protein levels were lower in the caspase 3 siRNA than in the compression-only group. Furthermore, there was no significant difference between the unloaded control and caspase 3 siRNA groups after 48 hours of loading. In contrast, the expression levels of TIMP-1, type II collagen, and aggrecan were higher in the caspase 3 siRNA group than in the compression-only group. These data show that apoptosis inhibition targeting caspase 3 is associated with a profound reduction in the production of matrix-degrading enzymes. Similar results were obtained after 48 hours of loading on the NP cells. Also, caspase 3 silencing did not alter the protein expression of integrin $\alpha 5$ and integrin $\beta 1$.

We also investigated the long-term impact of caspase 3 siRNA on compressive loading on NP cells *in vitro*. Although most of the agarose gels showed partial cracking 7 days after loading, cell viability was significantly lower in the compression-only, control siRNA, and caspase 3 siRNA groups than in the unloaded control group ([Supplemental Figure S2](#)). However, cell viability was significantly higher in the caspase 3 siRNA group compared with the control siRNA group. Western blot analysis showed that caspase 3 silencing inhibited the expression of cleaved caspase 3 compared with that in the control siRNA group. In contrast, the expression level of aggrecan was higher in the caspase 3 siRNA group than in the control siRNA group ([Supplemental Figure S2](#)). These results indicated that the *in vitro* system induced degenerative processes after long-term loading, such as a decrease in cell viability and proteoglycan content, whereas caspase 3 siRNA inhibited these alterations.

Impact of Caspase 3 siRNA on Compressive Loading-Induced IVD Degeneration *in Vivo*

We investigated the anti-apoptotic effects of caspase 3 siRNA on biomechanical overload-induced IVD degeneration *in vivo* using the rabbit model. This set of experiments

Figure 4 Compressive loading of rabbit IVD and percutaneous local injection of caspase 3 siRNA *in vivo*. **A:** Loading device with a calibrator. **B:** Calibrated spring within the loading device, generating a 150-N external IVD compressive force. Given the linear relationship between compressive force and spring length of ≤ 150 N, the change in spring length was set to 12.6 mm during the experiment. **C:** Custom-made external-loading device attached to the animal using four stainless steel pins inserted into the L4 and L5 bodies parallel to the disk. Axial stress immediately applied to IVD by a calibrated spring. **D:** One week after the operation, control or caspase 3 siRNA was s.c. injected in the center of the NP under fluoroscopy.

was performed with a custom-made external-loading device (Figure 4A) successfully used to assess therapeutic strategies for IVD degeneration in rabbits.^{29–31} A 150-N axial compressive force was loaded on the L4/5 IVD during the daily activities of the animal (Figure 4, B and C), generating 1.67 MPa on the disk,³² which is equivalent to the lumbar intradiscal pressure experienced by a human lifting 20 kg with knees flexed.⁴¹ One week afterward, control or caspase 3 siRNA was s.c. injected at the center of the L4/5 IVD under fluoroscopy (Figure 4D). In a previous study, we confirmed that caspase 3 siRNA is successfully introduced into the cytoplasm of rabbit NP cells.⁷ This transfection protocol reduces caspase 3 mRNA levels to <25% and protein levels to <40% of control injection.

Evidence of degenerative changes in rabbit lumbar IVDs was analyzed qualitatively by MRI capturing T2-weighted, midsagittal, and midaxial images (Figure 5A). Signal intensities decreased over time in loaded IVDs. However, signal intensities in the MRIs of NP were stronger in the caspase 3 siRNA group than in the control siRNA group at both 8 and 16 weeks after injection. The Pfirrmann grading scores, assessing the extent of structural degeneration,³⁴ were significantly lower in caspase 3 siRNA-treated IVDs than in control siRNA-treated IVDs (Figure 5B). After 8 weeks, there was no significant difference between unloaded (sham) and caspase 3 siRNA groups. After 16 weeks, the Pfirrmann scores were significantly higher in caspase 3

siRNA-treated IVDs than in unloaded IVDs, but significantly lower than in control siRNA-treated IVDs. The MRI index provides quantitative analysis of the midsagittal image.^{7,35} Values were significantly higher in caspase 3 siRNA-treated IVDs than in control siRNA IVDs at both 8 and 16 weeks (Figure 5C). Altogether, these data suggest that caspase 3 siRNA injection protects IVDs from the development of degenerative changes during compressive loading.

Safranin O–fast green staining revealed degenerative structural changes in the NPs of the control siRNA group 8 weeks after injection (Figure 6A). The H&E staining of the rabbit lumbar IVDs showed no abnormal finding, such as inflammation or cancerous growth, in any of the groups, even 16 weeks after siRNA injection (Figure 6B). However, after 16 weeks, NPs of the control siRNA group had collapsed and the annulus fibrosis layer was disrupted by wavy lamellar structures (Figure 6B). There was no vacuole and fewer NP cells, and the space was filled with a hypocellular fibrocartilaginous matrix. In contrast, the rabbits who received caspase 3 siRNA exhibited a well-organized round NP with numerous large vacuolated cells and smaller chondrocyte-like cells, even 16 weeks after injection (Figure 6B). In addition, the cell number per square millimeter was significantly lower in the control siRNA group than that in the unloaded (sham) group, indicating that compressive loading significantly decreased the cell

Figure 5 Representative MRI scans and data analysis of Pfirrmann score and MRI index 8 and 16 weeks after injection of caspase 3 or control siRNA into the rabbit NP. **A:** T2-weighted, midsagittal (top panel), and midaxial (bottom panel) images of IVDs. **B:** Pfirrmann grading score of IVD degeneration. **C:** MRI index (product of NP area and average signal intensity) of NP alterations. The numerical values are expressed as percentage compared with unloaded sham control disks. * $P < 0.05$, ** $P < 0.01$. Data are given as means $\pm$ SD ($N = 8$).

number. However, cell numbers were significantly higher in the caspase 3 siRNA group, suggesting that caspase 3 silencing preserves cell integrity during compressive loading. Safranin O—fast green staining revealed degenerative structural changes in the NPs of the control siRNA group 8 weeks after injection. Histological grades³⁶ were significantly higher in the control siRNA group than in the sham group at both 8 and 16 weeks after injection. In contrast, they were significantly lower in the caspase 3 siRNA group than in the control siRNA group, but significantly higher than in the unloaded group.

The IHC revealed cleaved caspase 3—positive NP cells with increasing signal intensity over time after injection, in both the control and caspase 3 siRNA treatment groups. However, the percentage of cleaved caspase 3—positive NP cells was significantly lower in the caspase 3 siRNA group than in the control siRNA group (Figure 7, A and B). Similarly, TUNEL staining showed that NP cell apoptosis intensified with loading time in both the control and caspase 3 siRNA groups. However, apoptotic cells were less abundant in the caspase 3 siRNA group. Quantification of the TUNEL staining indicated that caspase 3 siRNA significantly reduced the ratio of

apoptotic NP cells/total cells, compared with control siRNA (Figure 7, C and D). Likewise, the percentage of MMP-3—positive cells was significantly lower in the caspase 3 siRNA group than in the control siRNA group (Figure 7, E and F). These data confirm that inhibition of apoptosis by targeting endogenous caspase 3 was effective in preventing IVD degeneration induced by compressive loading. The expression of integrin $\alpha 5 \beta 1$ was increased by loading, but not by caspase 3 knockdown (Figure 7, G and H).

Discussion

The development of target-based treatment strategies for diseases dominated by apoptosis requires an understanding of the molecular mechanisms of programmed cell death.⁴² Several studies have demonstrated that activation of mitochondria-dependent apoptosis is an important event in IVD degeneration.^{4,11} Bcl-2 is an anti-apoptotic modulator of mitochondria-regulated cell death. We reported that Bcl-2 overexpression in NP cells prevented apoptotic cell death and decreased the mRNA expression of caspase 3 under serum-starved conditions.⁶ In addition, we recently showed, by microarray analysis, that nutrient deprivation in NP cells results in the activation of signaling events involving DNA damage checkpoint genes regulating the cell cycle.¹² Both G₁ arrest and apoptosis induced by serum starvation were inhibited in cells transfected with caspase 3 siRNA.¹² Because caspase 3 is considered a major player in the apoptotic program, gene silencing could potentially reduce NP cell dysfunction and delay cell death.^{7,12}

NP is an avascular structure encapsulated by an end plate and the annulus fibrosus. Therefore, nutrients are only supplied by capillaries in the vertebral bodies.⁴³ Because IVD has limited regenerative capacity and nutrient availability, it is extremely important to limit the damage caused by NP cell death.^{12,43} Given the chronic and progressive nature of IVD degeneration, caspase inhibition is a plausible approach to the treatment of IVD degeneration. In our previous studies, we induced apoptosis *in vitro* by serum starvation.^{6,7,12} In addition, in an animal model of annulus fibrosus injury, we demonstrated that caspase 3 gene silencing inhibits IVD degeneration *in vivo*.⁷ However, because human IVDs are exposed to high intradiscal pressure during daily activities, it is important to investigate the mechanism of apoptosis in IVD cells during mechanical stress. A static compression animal model has the potential to mimic human IVD degeneration. The present study clearly demonstrates that biomechanical overload induces NP cell apoptosis *in vitro* and *in vivo*.

The importance of apoptosis in human degenerative disk disease remains unclear. Apoptosis may play an important role in preserving normal disk function by eliminating aberrant cells. Therefore, the local injection of therapeutic agents aiming to inhibit apoptosis would allow malfunctioning cells to accumulate in NP and reduce the overall

Figure 6 *In vivo* impact of caspase 3 siRNA on the histological, IHC, and TUNEL staining of IVDs after long-term compressive loading. Rabbits were injected with control or caspase 3 siRNA in the NP center under fluoroscopy, and evaluated after 8 or 16 weeks of compressive loading. **A:** Safranin O—fast green staining was used for semiquantitative histological scores. **B:** H&E staining of NP shows no obvious abnormal findings, such as inflammation and cancerous growth in the control and caspase 3 siRNA groups, even 16 weeks after injection. However, after 16 weeks, the NPs of the control siRNA group collapsed and the annulus fibrosis layer was disrupted by wavy lamellar structures. There was no vacuole and fewer NP cells, and the space was filled with hypocellular fibrocartilaginous matrix (boxed area). In contrast, the rabbits that received caspase 3 siRNA exhibited a well-organized round NP, with numerous large vacuolated cells (arrowheads) and smaller chondrocyte-like cells (arrows), even 16 weeks after injection. In addition, the cell numbers per square millimeter were significantly lower in the control siRNA group than in the unloaded (sham) group. Nonetheless, they were significantly higher in the caspase 3 siRNA group than in the control siRNA group. * $P < 0.05$, ** $P < 0.01$.

tissue function.⁴⁴ However, the apoptotic cascade contains several pathways, and the existence of compensatory pathways of caspase activation has been suggested.^{42,45,46} Furthermore, we have shown that caspase 3 siRNA only partially suppressed the expression of caspase 3,⁷ which may be the key to prevent undesirable adverse effects. For most chronic diseases, the goal of any treatment is to normalize exaggerated cellular responses and to restore homeostasis.

Degenerated IVDs present an aberrant expression profile of extracellular matrix components essential for the maintenance of structural integrity.⁴⁷ The molecular changes are dominated by up-regulation of matrix-degrading enzymes, resulting in enhanced matrix destruction, tissue hydration loss, and disk height loss.^{47,48} Several *in vitro* studies showed that static compressive stress up-regulates catabolic metalloproteinases and down-regulates anabolic enzymes (aggrecan and collagen type II). For instance, Neidlinger-Wilke et al⁴⁹ reported that MMPs 1, 3, and 13 were up-regulated, whereas aggrecan was down-regulated, in disk biopsy specimens subjected to hydrostatic pressure. Korecki et al⁵⁰ reported that MMP-3 was up-regulated in response to mechanical loading in bovine 3D cultured IVD cells. Similarly, the *in vivo* study of MacLean et al⁵¹ showed that the protein expression levels of MMP-3 and ADAMTS-4 increased in a rat tail compression model. More recently, Yurube et al⁵² reported that the percentage of immunopositive cells for MMP-3 increased, whereas TIMP-1 immunopositivity decreased, during rat tail static compression. They concluded that the rat tail sustained static compression

model reproduces the extracellular matrix metabolic imbalance of MMPs, aggrecanases, and TIMPs, typical of human degenerative disks, and that the predominant MMP-3/TIMP-1 imbalance identifies an advanced stage of IVD degeneration. These findings are consistent with our *in vitro* data obtained with the NP cell—agarose 3D composite culture model and our *in vivo* data using the rabbit external compression model for spinal disks. More important, for the first time to our knowledge, we demonstrate that caspase 3 silencing maintained normal expression of these matrix enzymes during sustained compressive loading. These data indicate that mechanical overload initiates IVD degeneration through a caspase 3—dependent imbalance in the expression of extracellular matrix enzymes. Several studies demonstrated relationships between matrix-degrading enzymes and apoptosis.^{53–55} MMPs modulate apoptosis by cleaving death ligands [eg, tumor necrosis factor (TNF)- α and Fas ligand] and their receptors, which triggers apoptosis in an autocrine or paracrine manner.^{53,56} The proteolysis of nuclear substrates by MMPs also triggers apoptosis.⁵⁵ Furthermore, MMP-3 up-regulation is involved in apoptosis,^{54–56} through caspase 3 activation.^{55,56} The maintenance of the NP cellular and extracellular matrix is tightly regulated by the MMPs, the ADAMTS family of enzymes, and TIMPs.^{57,58} Inflammatory mediators promote an imbalance of these activities in favor of the catabolic enzymes, which fosters the progression of IVD degeneration.⁵⁹ For instance, TNF- α has been shown to stimulate the production of MMP-3 in IVD cells.⁶⁰ Furthermore, the incubation of human IVD cells with IL-1 was reported to cause

Figure 7 A and B: IHC of the signal intensity of the cleaved caspase 3—positive NP cells (arrows). C and D: Quantification of TUNEL staining, with apoptotic NP cells (arrows). E and F: The percentage of MMP-3—positive cells (arrows). G and H: Biomechanical stress on integrin $\alpha 5 \beta 1$ —positive cells (arrows). Data are given as means $\pm$ SD ($N = 8$). * $P < 0.05$, ** $P < 0.01$.

up-regulation of catabolic enzymes (MMP-3, MMP-13, and ADAMTS-4) and down-regulation of anabolic enzymes (aggrecan and collagen II).⁵⁹ The current study provides the first evidence that caspase 3 activation affects these proteins in IVDs. Our results showed that long-term excessive loading on IVDs promoted tissue damage by a mechanism involving caspase-mediated overexpression of catabolic enzymes that attacked the integrity of the extracellular matrix. Although it is unclear how caspase 3 stimulates expression of catabolic enzymes in NP cells, it has been shown that this enzyme activates NF- κ B pathways in neural cells through protein kinase C δ , leading to transcriptional activation of inflammatory gene expression, such as TNF- α and IL-1 β .^{61,62} However, further experiments are required to elucidate the molecular mechanism in NP cells.

This study has a few limitations. The local injection of caspase 3 siRNA into lumbar disks is unsuitable for late-stage disk degeneration with severely degenerated NP cells. In addition, our *in vitro* model of static compressive loading does not reflect all aspects of human disk degeneration taking place during daily activities,⁵² which warranted validation by our *in vivo* model, allowing the rabbits to move freely in their cage during compressive loading. Furthermore, our previous study showed that the knockdown effects of caspase 3 decreased after day 2 and week 4, but remained stable between week 4 and week 8.⁷ Therefore, it is expected that the silencing effects lasted at least 16 weeks. In the present study, it is difficult to determine the efficiency of caspase 3 silencing after 16 weeks and how long the silencing effect lasted. We have initiated other studies to address this issue.

In summary, the present study offers a promising noninvasive therapy for biomechanical stress-induced IVD degeneration, for which there is no efficient treatment. A single local injection of caspase 3 siRNA protected rabbits against IVD degeneration during at least 16 weeks of sustained compressive overload. This approach is based on a breakthrough discovery on the molecular mechanism of NP destruction. We show that the imbalance in matrix-degrading enzyme production is largely dependent on caspase 3 activation.

Therefore, the manipulation of local apoptotic events may constitute a simple and effective means to prevent and/or reduce IVD degeneration. This noninvasive treatment strategy is expected to significantly affect future clinical research on IVD degeneration diseases.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.11.010>.

References

- Marras WS, Ferguson SA, Burr D, Schabo P, Maronitis A: Low back pain recurrence in occupational environments. *Spine (Phila Pa 1976)* 2007, 32:2387–2397
- Di Iorio A, Abate M, Guralnik JM, Bandinelli S, Cecchi F, Cherubini A, Corsonello A, Foschini N, Guglielmi M, Lauretani F, Volpato S, Abate G, Ferrucci L: From chronic low back pain to disability, a multifactorial mediated pathway: the InCHIANTI study. *Spine (Phila Pa 1976)* 2007, 32:E809–E815
- Mielenz TJ, Garrett JM, Carey TS: Association of psychosocial work characteristics with low back pain outcomes. *Spine (Phila Pa 1976)* 2008, 33:1270–1275
- Park JB, Lee JK, Park SJ, Kim KW, Riew KD: Mitochondrial involvement in fas-mediated apoptosis of human lumbar disc cells. *J Bone Joint Surg Am* 2005, 87:1338–1342
- Gruber HE, Hanley EN Jr.: Analysis of aging and degeneration of the human intervertebral disc: comparison of surgical specimens with normal controls. *Spine (Phila Pa 1976)* 1998, 23:751–757
- Sudo H, Minami A: Regulation of apoptosis in nucleus pulposus cells by optimized exogenous Bcl-2 overexpression. *J Orthop Res* 2010, 28:1608–1613
- Sudo H, Minami A: Caspase 3 as a therapeutic target for regulation of intervertebral disc degeneration in rabbits. *Arthritis Rheum* 2011, 63:1648–1657
- Kim KW, Ha KY, Lee JS, Rhyu KW, An HS, Woo YK: The apoptotic effects of oxidative stress and antiapoptotic effects of caspase inhibitors on rat notochordal cells. *Spine (Phila Pa 1976)* 2007, 32:2443–2448
- Lotz JC, Chin JR: Intervertebral disc cell death is dependent on the magnitude and duration of spinal loading. *Spine (Phila Pa 1976)* 2000, 25:1477–1483
- Buckwalter JA: Aging and degeneration of the human intervertebral disc. *Spine (Phila Pa 1976)* 1995, 20:1307–1314
- Rannou F, Lee TS, Zhou RH, Chin J, Lotz JC, Mayoux-Benhamou MA, Barbet JP, Chevrot A, Shyy JY: Intervertebral disc degeneration: the role of the mitochondrial pathway in annulus fibrosus cell apoptosis induced by overload. *Am J Pathol* 2004, 164:915–924
- Sudo H, Yamada K, Iwasaki K, Higashi H, Ito M, Minami A, Iwasaki N: Global identification of genes related to nutrient deficiency in intervertebral disc cells in an experimental nutrient deprivation model. *PLoS One* 2013, 8:e58806
- Reddi AH: Inhibition of cell death in the intervertebral disc by caspase 3 small interfering RNA. *Arthritis Rheum* 2011, 63:1477–1478
- Evans C: Potential biologic therapies for the intervertebral disc. *J Bone Joint Surg Am* 2006, 88(Suppl 2):95–98
- Walter BA, Korecki CL, Purmessur D, Roughley PJ, Michalek AJ, Iatridis JC: Complex loading affects intervertebral disc mechanics and biology. *Osteoarthritis Cartilage* 2011, 19:1011–1018
- Paul CP, Schoorl T, Zuiderbaan HA, Zandieh Doulabi B, van der Veen AJ, van de Ven PM, Smit TH, van Royen BJ, Helder MN, Mullender MG: Dynamic and static overloading induce early degenerative processes in caprine lumbar intervertebral discs. *PLoS One* 2013, 8:e62411
- Paul CP, Zuiderbaan HA, Zandieh Doulabi B, van der Veen AJ, van de Ven PM, Smit TH, van Royen BJ, Helder MN, Mullender MG: Simulated-physiological loading conditions preserve biological and mechanical properties of caprine lumbar intervertebral discs in ex vivo culture. *PLoS One* 2012, 7:e33147
- Sudo H, Oda I, Abumi K, Ito M, Kotani Y, Hojo Y, Minami A: In vitro biomechanical effects of reconstruction on adjacent motion segment: comparison of aligned/kyphotic posterolateral fusion with aligned posterior lumbar interbody fusion/posterolateral fusion. *J Neurosurg* 2003, 99:221–228
- Sudo H, Oda I, Abumi K, Ito M, Kotani Y, Minami A: Biomechanical study on the effect of five different lumbar reconstruction techniques on adjacent-level intradiscal pressure and lamina strain. *J Neurosurg Spine* 2006, 5:150–155
- Sasaki N, Imai T, Hashimoto A, Yasuda H: Effect of pericellular matrix formation by chondrocytes cultured in agarose gel on the viscoelastic properties of agarose gel matrix. *J Biorheol* 2009, 23:95–101

21. Fernando HN, Czamanski J, Yuan TY, Gu W, Salahadin A, Huang CY: Mechanical loading affects the energy metabolism of intervertebral disc cells. *J Orthop Res* 2011, 29:1634–1641
22. Salvatierra JC, Yuan TY, Fernando H, Castillo A, Gu WY, Cheung HS, Huan CY: Difference in energy metabolism of annulus fibrosus and nucleus pulposus cells of the intervertebral disc. *Cell Mol Bioeng* 2011, 4:302–310
23. Knight MM, van de Breevaart Bravenboer J, Lee DA, van Osch GJ, Weinans H, Bader DL: Cell and nucleus deformation in compressed chondrocyte-alginate constructs: temporal changes and calculation of cell modulus. *Biochim Biophys Acta* 2002, 1570:1–8
24. Wang P, Yang L, Hsieh AH: Nucleus pulposus cell response to confined and unconfined compression implicates mechanoregulation by fluid shear stress. *Ann Biomed Eng* 2011, 39:1101–1111
25. Chen J, Yan W, Setton LA: Static compression induces zonal-specific changes in gene expression for extracellular matrix and cytoskeletal proteins in intervertebral disc cells in vitro. *Matrix Biol* 2004, 22:573–583
26. Ragan PM, Chin VI, Hung HH, Masuda K, Thonar EJ, Arner EC, Grodzinsky AJ, Sandy JD: Chondrocyte extracellular matrix synthesis and turnover are influenced by static compression in a new alginate disk culture system. *Arch Biochem Biophys* 2000, 383:256–264
27. Horner HA, Urban JP: 2001 Volvo Award Winner in Basic Science Studies: effect of nutrient supply on the viability of cells from the nucleus pulposus of the intervertebral disc. *Spine (Phila Pa 1976)* 2001, 26:2543–2549
28. Martin S, Fernandez-Rojo MA, Stanley AC, Bastiani M, Okano S, Nixon SJ, Thomas G, Stow JL, Parton RG: Caveolin-1 deficiency leads to increased susceptibility to cell death and fibrosis in white adipose tissue: characterization of a lipodystrophic model. *PLoS One* 2012, 7:e46242
29. Guehring T, Omlor GW, Lorenz H, Bertram H, Steck E, Richter W, Carstens C, Kroeber M: Stimulation of gene expression and loss of annular architecture caused by experimental disc degeneration: an in vivo animal study. *Spine (Phila Pa 1976)* 2005, 30:2510–2515
30. Omlor GW, Lorenz H, Engelleiter K, Richter W, Carstens C, Kroeber MW, Guehring T: Changes in gene expression and protein distribution at different stages of mechanically induced disc degeneration: an in vivo study on the New Zealand white rabbit. *J Orthop Res* 2006, 24:385–392
31. Hee HT, Chuah YJ, Tan BH, Setiobudi T, Wong HK: Vascularization and morphological changes of the endplate after axial compression and distraction of the intervertebral disc. *Spine (Phila Pa 1976)* 2011, 36:505–511
32. Beckstein JC, Sen S, Schaer TP, Vresilovic EJ, Elliott DM: Comparison of animal discs used in disc research to human lumbar disc: axial compression mechanics and glycosaminoglycan content. *Spine (Phila Pa 1976)* 2008, 33:E166–E173
33. Seki S, Asanuma-Abe Y, Masuda K, Kawaguchi Y, Asanuma K, Muehleman C, Iwai A, Kimura T: Effect of small interference RNA (siRNA) for ADAMTS5 on intervertebral disc degeneration in the rabbit annular needle-puncture model. *Arthritis Res Ther* 2009, 11:R166
34. Pfirrmann CW, Metzger A, Zanetti M, Hodler J, Boos N: Magnetic resonance classification of lumbar intervertebral disc degeneration. *Spine (Phila Pa 1976)* 2001, 26:1873–1878
35. Sobajima S, Kompel JF, Kim JS, Wallach CJ, Robertson DD, Vogt MT, Kang JD, Gilbertson LG: A slowly progressive and reproducible animal model of intervertebral disc degeneration characterized by MRI, X-ray, and histology. *Spine (Phila Pa 1976)* 2005, 30:15–24
36. Masuda K, Aota Y, Muehleman C, Imai Y, Okuma M, Thonar EJ, Andersson GB, An HS: A novel rabbit model of mild, reproducible disc degeneration by an annulus needle puncture: correlation between the degree of disc injury and radiological and histological appearances of disc degeneration. *Spine (Phila Pa 1976)* 2005, 30:5–14
37. Loeser RF: Chondrocyte integrin expression and function. *Bio-rheology* 2000, 37:109–116
38. Millward-Sadler SJ, Salter DM: Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Ann Biomed Eng* 2004, 32:435–446
39. Le Maitre CL, Frain J, Millward-Sadler J, Fotheringham AP, Freemont AJ, Hoyland JA: Altered integrin mechanotransduction in human nucleus pulposus cells derived from degenerated discs. *Arthritis Rheum* 2009, 60:460–469
40. Gilchrist CL, Chen J, Richardson WJ, Loeser RF, Setton LA: Functional integrin subunits regulating cell-matrix interactions in the intervertebral disc. *J Orthop Res* 2007, 25:829–840
41. Wilke HJ, Neef P, Caimi M, Hoogland T, Claes LE: New in vivo measurements of pressures in the intervertebral disc in daily life. *Spine (Phila Pa 1976)* 1999, 24:755–762
42. Friedlander RM: Apoptosis and caspases in neurodegenerative diseases. *N Engl J Med* 2003, 348:1365–1375
43. Guehring T, Wilde G, Sumner M, Grunhagen T, Karney GB, Tirlapur UK, Urban JP: Notochordal intervertebral disc cells: sensitivity to nutrient deprivation. *Arthritis Rheum* 2009, 60:1026–1034
44. Demjen D, Klusmann S, Kleber S, Zuliani C, Stieltjes B, Metzger C, Hirt UA, Walczak H, Falk W, Essig M, Edler L, Krammer PH, Martin-Villalba A: Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury. *Nat Med* 2004, 10:389–395
45. Zheng TS, Hunot S, Kuida K, Momoi T, Srinivasan A, Nicholson DW, Lazebnik Y, Flavell RA: Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. *Nat Med* 2000, 6:1241–1247
46. Adrain C, Martin SJ: Cell biology: double knockout blow for caspases. *Science* 2006, 311:785–786
47. Pockert AJ, Richardson SM, Le Maitre CL, Lyon M, Deakin JA, Buttle DJ, Freemont AJ, Hoyland JA: Modified expression of the ADAMTS enzymes and tissue inhibitor of metalloproteinases 3 during human intervertebral disc degeneration. *Arthritis Rheum* 2009, 60:482–491
48. Millward-Sadler SJ, Costello PW, Freemont AJ, Hoyland JA: Regulation of catabolic gene expression in normal and degenerate human intervertebral disc cells: implications for the pathogenesis of intervertebral disc degeneration. *Arthritis Res Ther* 2009, 11:R65
49. Neidlinger-Wilke C, Würtz K, Urban JP, Börm W, Arand M, Ignatius A, Wilke HJ, Claes LE: Regulation of gene expression in intervertebral disc cells by low and high hydrostatic pressure. *Eur Spine J* 2006;(Suppl 3):S372–S378
50. Korecki CL, Kuo CK, Tuan RS, Iatridis JC: Intervertebral disc cell response to dynamic compression is age and frequency dependent. *J Orthop Res* 2009, 27:800–806
51. MacLean JJ, Lee CR, Alini M, Iatridis JC: The effects of short-term load duration on anabolic and catabolic gene expression in the rat tail intervertebral disc. *J Orthop Res* 2005, 23:1120–1127
52. Yurube T, Takada T, Suzuki T, Kakutani K, Maeno K, Doita M, Kurosaka M, Nishida K: Rat tail static compression model mimics extracellular matrix metabolic imbalances of matrix metalloproteinases, aggrecanases, and tissue inhibitors of metalloproteinases in intervertebral disc degeneration. *Arthritis Res Ther* 2012, 14:R51
53. Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003, 92:827–839
54. Kim YS, Kim SS, Cho JJ, Choi DH, Hwang O, Shin DH, Chun HS, Beal MF, Joh TH: Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. *J Neurosci* 2005, 25:3701–3711

55. Si-Tayeb K, Monvoisin A, Mazzocco C, Lepreux S, Decossas M, Cubel G, Taras D, Blanc JF, Robinson DR, Rosenbaum J: Matrix metalloproteinase 3 is present in the cell nucleus and is involved in apoptosis. *Am J Pathol* 2006, 169:1390–1401
56. Lai MY, Leung HW, Yang WH, Chen WH, Lee HZ: Up-regulation of matrix metalloproteinase family gene involvement in ursolic acid-induced human lung non-small carcinoma cell apoptosis. *Anti-cancer Res* 2007, 27:145–153
57. Le Maitre C, Pockert A, Buttle DA, Freemont AJ, Hoyland JA: Matrix synthesis and degradation in human intervertebral disc degeneration. *Biochem Soc Trans* 2007, 35:652–655
58. Erwin WM, Islam D, Inman RD, Fehlings MG, Tsui FW: Notochordal cells protect nucleus pulposus cells from degradation and apoptosis: implications for the mechanisms of intervertebral disc degeneration. *Arthritis Res Ther* 2011, 13:R215
59. Le Maitre CL, Freemont AJ, Hoyland JA: The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. *Arthritis Res Ther* 2005, 7:R732–R745
60. Haro H, Crawford HC, Fingleton B, MacDougall JR, Shinomiya K, Spengler DM, Matrisian LM: Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. *J Clin Invest* 2000, 105:133–141
61. Venero JL, Burguillos MA, Brundin P, Joseph B: The executioners sing a new song: killer caspases activate microglia. *Cell Death Differ* 2011, 18:1679–1691
62. Burguillos MA, Deierborg T, Kavanagh E, Persson A, Hajji N, Garcia-Quintanilla A, Cano J, Brundin P, Englund E, Venero JL, Joseph B: Caspase signalling controls microglia activation and neurotoxicity. *Nature* 2011, 472:319–324