Chronic low-back pain (LBP) has become a major cause of disability in industrialized societies, with consequent economic and health care costs. LBP can originate from several sources, such as intervertebral discs (IVDs), ligaments, muscles, sacroiliac joints, and lumbar facet joint degeneration. In patients with a discogenic component to LBP, IVD degeneration is thought to be the initial step and plays a critical role. Following IVD degeneration, the biomechanical status of the vertebral column changes, and the likelihood of facet joint degeneration, spondylosis, spondylolisthesis, and spinal stenosis increases. At present, the management of LBP consists of a variety of conservative and invasive therapies that are aimed at symptomatic relief. Unfortunately, many of the treatments used currently are not obviously effective.7

Nerve growth factor promotes expression of novel genes in intervertebral disc cells that regulate tissue degradation

Laboratory investigation

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Object. Increased neurotrophin activity in degenerative intervertebral discs (IVDs) is one potential cause of chronic low-back pain (LBP). The aim of the study was to assess if nerve growth factor (NGF) might alter gene expression of IVD cells and contribute to disc degeneration by enhancing expression or activity of factors that cause breakdown of IVD matrix.

Methods. Rat-tail IVD cells were stimulated by NGF and subjected to microarray analysis. Real-time polymerase chain reaction, Western blotting, and immunocytochemistry of rat and human IVD cells and tissues treated with NGF in vitro in the absence or presence of the NGF inhibitor Ro 08-2750 were used to confirm findings of the microarray studies. Phosphorylation of mitogen-activated protein kinase (MAPK) was used to identify cell signaling pathways involved in NGF stimulation in the absence or presence of Ro 08-2750.

Results. Microarray analysis demonstrated increased expression of chitinase 3-like 1 (Chi3l1), lipocalin 2 (Lcn2), and matrix metalloproteinase–3 (Mmp3) following NGF stimulation of rat IVD cells in vitro. Increased gene expression was confirmed by real-time polymerase chain reaction with a relative increase in the Mmp/Timp ratio. Increased expression of Chi3l1, Lcn2, and Mmp3 following NGF stimulation was also demonstrated in rat cells and human tissue in vitro. Effects of NGF on protein expression were blocked by an NGF inhibitor and appear to function through the extracellular-regulation kinase 1/2 (ERK1/2) MAPK pathway.

Conclusions. Nerve growth factor has potential effects on matrix turnover activity and influences the catabolic/anabolic balance of IVD cells in an adverse way that may potentiate IVD degeneration. Anti-NGF treatment might be beneficial to ameliorate progressive tissue breakdown in IVD degeneration and may lead to pain relief. (http://thejns.org/doi/abs/10.3171/2014.6.SPINE13756)

Key Words • intervertebral disc degeneration • chitinase 3-like 1 • lipocalin 2 • MMP3

Abbreviations used in this paper: AF = annulus fibrosus; BSA = bovine serum albumin; Chi3l1 = chitinase 3-like 1; EPK1/2 = extracellular-regulation kinase 1/2; HRP = horseradish peroxidase; IL-1β = interleukin-1β; IVD = intervertebral disc; LBP = low-back pain; Lcn2 = lipocalin 2; MAPK = mitogen-activated protein kinase; Mmp3 = matrix metalloproteinase–3; NF-kB = neurotrophic factor kB; NGF = nerve growth factor; NGFR = nerve growth factor receptor; NP = nucleus pulposus; PBS = phosphate-buffered saline; p75NTR = p75 neurotrophin receptor; TBST = Tris-buffered saline/Tween; VE = vertebral endplate.
pulposus (NP); and the adjacent vertebral endplate (VE). The healthy NP is avascular, and its nutritional supply depends on diffusion via the AF and VE. In a normal disc the NP is also devoid of nerve fibers, whereas the outer AF and VE contain nerve fibers derived from branches of sympathetic trunk and sinuvertebral nerves. During IVD degeneration, increased nerve ingrowth is found in the NP and has been suggested as a potential contributor to LBP. Nociceptive neuropeptides such as calcitonin gene–related peptide and substance P, which are present within the nerve fibers of the outer AF and dorsal root ganglion, have been associated with discogenic pain transmission. Therapies targeting neuronal transmission pathways can reasonably be expected to be useful and relieve pain. Neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor, and neuropeptides (substance P) have a wide range of activities, including roles in tissue development and repair, modulation of inflammatory responses, and bone and cartilage metabolism.

We hypothesized that NGF, whose local production is increased in degenerated IVD, could modify gene expression of IVD cells and thereby influence tissue remodeling and contribute to enhanced matrix breakdown. Increased knowledge of the basic mechanisms involved in IVD degeneration and chronic LBP will allow development of therapies that may delay or obviate surgical intervention and may improve postsurgical outcomes.

Methods

Experimental Animals and Ethics Statement

Two-month-old skeletally mature male Sprague-Dawley rats (380–420 g) were obtained from the National Applied Research Laboratories and National Laboratory Animal Center. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Taichung Veterans General Hospital. Human IVD tissue was obtained with full informed consent under approval from the ethics committee of Taichung Veterans General Hospital from patients undergoing discectomy.

Reagents

Human and rat beta-nerve growth factor (NGF) were purchased from R&D System Inc. Stock solution of NGF (100 μg/ml) was prepared in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). The NGF inhibitor Ro 08-2750 was purchased from Tocris Bioscience; a 10-mM stock solution was prepared in dimethyl sulfoxide. Tetrasisole methylthiotetrazole assay. A total of 1 × 10⁴ cells were seeded onto 96-well microtiter plates in 0.2 ml of growth medium. In triplicate experiments, NGF (100 ng/ml) was added to cell-seeded wells and incubated for 0, 1, 3, and 5 days. Tetrazolium (100 μl; Sigma) was added to each well and incubated for 3 hours at 37°C, followed by the addition of 150 μl dimethyl sulfoxide. The plate was shaken for 10 minutes, and then optical density at 570 nm was determined using a microplate reader (Dynatech Laboratories Inc.).

Microarray Assays

Gene expression of rat-tail disc cells with or without NGF stimulation for 5 days was analyzed. Briefly, fluorescent-amplified RNA targets were hybridized to the Rat Whole Genome OneArray (Phalanx Biotech Group), and the signals were scanned by an Axon 4000 scanner (Molecular Devices, LLC). The fluorescent intensity of each spot was analyzed by GenePix 4.1 software (Molecular Devices). The signal intensity of each spot was corrected by subtracting background signal. Spots with a signal-to-noise ratio < 1 or from control probes were filtered out. Spots that passed these criteria were normalized by the R program (TIBOC Software Inc.). The fold change of gene expression was calculated by dividing the normalized signal intensities of genes in NGF-treated cells by those in untreated cells. Genes with more than 2-fold or less than 2-fold changes were analyzed by Kyoto Encyclopedia of Gene and Genomes pathways on the Gene Ontology Tree Machine website, a respected Web-based and tree-based data mining environment for gene sets. The gene Set Test function implemented in the limma R package was used to test significant Kyoto Encyclopedia of Gene and Genomes pathways. The Web Gestalt tool was used to test significant Gene Ontology terms. The microarray data comply with MIAME (Minimum Information About a Microarray Experiment) guidelines, and the raw data have been deposited in a MIAME-compliant database.

T. H. Kao et al.
Nerve growth factor promotes intervertebral disc degradation

**Extraction of RNA and Real-Time Polymerase Chain Reaction**

Total RNA from cell cultures was extracted using TRIzol RNA isolation reagents (Invitrogen). For first-strand cDNA synthesis, 2 μg total RNA was used in a single-round reverse-transcription reaction with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complementary DNAs were amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and a StepOne Real-Time PCR System (Applied Biosystems) under the following conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 minutes, and 60°C for 60 seconds. Amplification of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) simultaneously served as an internal control and allowed normalization of the various mRNA levels against the total mRNA content in the samples, which was used to calculate changes in gene expression by 2^−ΔΔCt. Table 1 lists the specific primers used.

**Immunocytochemistry**

After incubation with NGF for 5 days, cells were washed twice with ice-cold PBS and then fixed using 2 ml of a 1:1 methanol/acetone mixture for 5 minutes at −20°C. For immunocytochemistry, cells were washed twice with PBS before incubation with 1% BSA for 30 minutes at room temperature. The solution was removed, and cells were incubated sequentially with primary antibody anti-Chi3l1 (1:100; AVIVA Systems Biology Corporation), anti-MMP3 (1:100; Epitomics), or anti-Lcn2 (1:100; Abcam plc) for 60 minutes, biotinylated secondary antibody (1:200) for 45 minutes, and horseradish peroxidase (HRP)–conjugated streptavidin for 20 minutes. Following each incubation, cells were washed 3 times with Tris-buffered saline/Tween 20 (TBST; 2.5 mM Tris/HCl, pH 7.6; 137 mM NaCl; 0.1% Tween 20). Diaminobenzidine (Invitrogen) was then added for 15 minutes before counterstaining with Mayer’s hematoxylin and visualizing under light microscopy.

**Protein Extraction and Western Blotting**

Following stimulation, cells were immediately washed with ice-cold PBS containing 100 μM Na3VO4 (Sigma) and lysed in situ with ice-cold lysis buffer at 4°C for 15 min. Lysis buffer contained 1% Igepal (Sigma), 100 μM Na3VO4, and protease inhibitor cocktail tablet (Roche Diagnostics Corporation). Whole-cell lysates were collected after centrifugation at 13,000 rpm for 15 min. Protein concentrations were determined by the Lowry method. Equal amounts of protein were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gels and subsequently transferred to polyvinylidene fluoride membranes (EMD Millipore). Membranes were blocked overnight at 4°C with 2% BSA in TBST. After washing 3 times with TBST, blots were incubated overnight with primary antibody (anti-Chi3l1, 1/1,000; anti-Lcn2, 1/1,000; anti-MMP3, 1/1,000) diluted in 2% BSA. After washing 6 times with TBST, blots were then incubated with the HRP-labeled secondary antibody for 1 hour at room temperature. Membranes were rewashed extensively, and antibody binding was detected using the Amersham Bioscience Enhanced Chemiluminescence plus Western Blotting Detection System according to the manufacturer’s instructions. The membranes were scanned and analyzed by densitometry (model LAS 3000; Fujifilm Corporation). Tubulin expression assessed with the mouse monoclonal antibody tubulin Ab-4 (1/5,000; secondary antibody, 1/20,000) served as internal control.

**Immunohistochemistry**

Annulus fibrosus tissue from 3 human IVDs was incubated in DMEM/F12 serum-free medium with either 100 ng/ml NGF or 10 nM Ro 08-2750 for 12 hours. The tissues were embedded in OCT, and frozen sections cut and fixed. Sections were rinsed in PBS and incubated in 0.3% H2O2 at room temperature for 10 min before incubation with 1% BSA for 30 minutes at room temperature. Sections were then incubated sequentially with anti-Chi3l1 (1:100), anti-MMP3 (1:100), or anti-Lcn2 (1:100) for 60 minutes, biotinylated secondary antibody (1:200) for 45 minutes, and HRP-conjugated streptavidin for 20 minutes. Between each incubation, slides were washed 3 times with TBST. Diaminobenzidine was then added for 15 minutes, and sections were then counterstained with Mayer’s hematoxylin.

**Statistical Analysis**

All values were expressed as mean ± standard deviation. Statistical evaluation of the quantification data of mRNA and protein expression levels was performed by Student t-tests. For the ratios of Timp1 to Mmp3, Lcn2 to Mmp9, and Chi3l1 to Colla1, Wilcoxon matched pairs test was used, allowing for the nonsymmetrical distribution data. The results were considered significant at a p value < 0.05.

**Results**

**Effect of NGF on AF Cell Gene Expression**

Nerve growth factor had no effect on cell viability over the time course of the experiment at the concentration used, as assessed by a tetrazolium methylthiotetrazole assay (data not shown).
Using purified RNA samples from the AF cells, we found that 97 of the 24,358 transcripts (< 0.4%) covered by the Rat OneArray showed > 2-fold change after NGF stimulation. After the initial screen, 3 genes were chosen for further study because of their involvement in matrix turnover regulation: Chi3l1 (chitinase 3-like 1), Lcn2 (lipo-calcin 2), and Mmp3 (matrix metalloproteinase 3) (Table 2).

Real-time polymerase chain reaction was performed and protein expression was analyzed in rat and human IVD cells focusing on these genes and related pathways (Fig. 1). Real-time polymerase chain reaction showed increased expression of Chi3l1, Lcn2, and Mmp3 following NGF stimulation (Fig. 1A). Similarly, Western blotting showed increased expression of Chi3l1, Lcn2, and MMP3 in NGF-stimulated cells (Fig. 1B), which was also shown by immunocytochemistry (Fig. 1C).

Nerve Growth Factor Influences the Balance of Catabolic/Anabolic Activity of AF Cells

Because NGF appears to influence expression of genes thought to have an influence on matrix integrity, we next assessed effects on the catabolic/anabolic activity of AF cells. Because Chi3l1 modulates the rate of type I collagen fibril formation and Lcn2 has been shown to protect Mmp9 from degradation by forming an Lcn2/Mmp9 complex, we focused on expression of these genes/molecules in addition to Mmp3/Timp1. The results are shown in Fig. 2. There was no significant change in the ratio of Chi3l1/Col1a1 (Fig. 2A) or Lcn2/Mmp9 (Fig. 2B) gene expression following NGF stimulation. In contrast, the ratio of Mmp3/Timp1 expression was increased (Fig. 2C), suggesting a trend to a more catabolic phenotype.

Nerve Growth Factor Signals Through ERK1/2 in AF Cells

To determine the signal pathway by which NGF may be influencing gene expression, we stimulated rat AF cells with NGF for 0, 6, 12, or 24 hours and examined activation of neurotrophic factor kB (NF-κB), p38, and extracellular-regulation kinase (ERK1/2) mitogen-activated protein kinase (MAPK) by Western immunoblotting with 24-hour incubation. The results showed that NGF significantly increased ERK1/2 phosphorylation at 12 hours after stimulation (Fig. 3A), while there was no significant activation of either NF-κB or p38 (protein level of the molecular signal examined showed no change). Activation of ERK1/2 by NGF was inhibited by Ro 08-2750 (Fig. 3B), a nonpeptide inhibitor of NGF that binds the NGF dimer.

Effects of NGF on Human AF Cells

To assess whether similar activity of NGF was present in human tissue, we collected AF tissue from 3 patients undergoing discectomy (Pfirrmann Grade 2 and 3), stimulated these ex vivo with human NGF, and undertook immunohistochemical analysis for CHI3L1, LCN2, and MMP3. NGF-treated tissue showed increased expression of CHI3L1, LCN2, and MMP3, which was inhibited by the presence of Ro 08-2750 (Fig. 4).

Discussion

Discogenic back pain has been associated with IVD degenerative changes, including desiccation, space narrowing, and annular tears.35 Advanced changes such as Modic lesions have been associated with an increased incidence of LBP.36 The pathophysiology of disc degeneration has been linked to an imbalance of catabolic and anabolic proteins that leads to matrix degradation and subsequent height loss. Many recent investigations have postulated that altering the biochemical factors within disc tissue can slow the progression of IVD degeneration.

In this study we tested the hypothesis that NGF, a neurotrophin that stimulates neurite outgrowth from neuronal cells,38 influences expression of genes involved in degradation of the IVD matrix and thereby promotes IVD degeneration and chronic LBP. The results demonstrate previously unreported effects of NGF on AF cells that would lead to enhanced tissue breakdown and progressive IVD degeneration. If these observations are replicated in vivo, NGF or downstream mediators of its potential adverse effects on IVD structure would be candidate molecules that could be targeted to slow down IVD degeneration and delay the need for surgical intervention. In addition, targeting NGF may have benefit in the postoperative period by decreasing pain and enhancing tissue regeneration.

Treatment of AF cells with NGF led to altered expression of less than 0.4% of genes, indicating likely specific effects. Importantly, among these significantly upregulated genes, Lcn2 (lipocalcin 2), Mmp3 (matrix metalloproteinase–3), and Chi3l1 (chitinase 3–like 1) are of particular interest because of their novelty and known activity in regulating matrix turnover. We are the first to report that Lcn2 is upregulated by NGF in AF cells. The Chi3l1 protein, also known as YKL-40, has previously been shown to be released following in vitro culture of degenerate IVD tissue35 but has never been associated with dependence on NGF for expression.

The LCN2 protein is multifunctional, with roles in innate immunity and tumorigenesis. Also known as neutrophil gelatinase-associated lipocalcin, LCN2 is now recognized as an adipokine because it is produced predominantly by white adipose tissue.39 It has recently been identified in growth plate chondrocytes,36 where ex-

**TABLE 2: Microarray analysis**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Log Ratio (2)</th>
<th>Gene Name</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi3l1</td>
<td>6.643856</td>
<td>chitinase 3-like 1</td>
<td>remodeling or degradation of the extracellular matrix</td>
</tr>
<tr>
<td>Lcn2</td>
<td>6.643856</td>
<td>lipocalcin 2</td>
<td>iron trafficking</td>
</tr>
<tr>
<td>Mmp3</td>
<td>5.930552</td>
<td>matrix metalloproteinase–3</td>
<td>can degrade fibronectin, laminin, gelatins of types I, III, IV, &amp; V; collagens III, IV, X, &amp; IX; &amp; cartilage proteoglycans; activates procollagenase</td>
</tr>
</tbody>
</table>

T. H. Kao et al.
Nerve growth factor promotes intervertebral disc degradation

pression is modulated by interleukin-1β (IL-1β), leptin, adiponectin, lipopolysaccharide, and dexamethasone. The function of LCN2 in cartilage and IVD tissue is not known. Dimerization of LCN2 with pro-MMP9 appears to enhance activation of the enzyme with plasma kallikrein and protects the enzyme from degradation.41 In patients with osteoarthritis, LCN2 levels are increased in the synovial fluid, where it also acts to protect MMP9 from degradation and enhance MMP9-induced cartilage breakdown.15 Although the function of LCN2 in IVD tissue is unknown, similar activity, if present, would be expected to enhance tissue degeneration.

The function of CHI3L1 in the IVD is likely to be similar to that in cartilage. The CHI3L1 protein is abundantly produced by cultured chondrocytes and is generated in cultures of degenerate IVD explants.16,35 It is overexpressed in osteoarthritic cartilage,19 but expression in noncultured IVD tissues has not been previously reported. The CHI3L1 protein is a recognized biomarker in osteoarthritis, with expression correlating with disease progression,19 but the role of CHI3L1 in cartilage and IVD tissues has not yet been clarified. Both tumor necrosis factor-α and IL-1β stimulate the expression of CHI3L1 in articular chondrocytes.36 Interestingly, CHI3L1 inhibits cellular responses induced by IL-1β and tumor necrosis factor-α,30 raising the possibilities that the induction of

Fig. 1. Confirmation of microarray findings. A: Real-time polymerase chain reaction showed that selected expression of Chi3l1 (31.82 ± 7.57-fold, p = 0.0001), Lcn2 (29.86 ± 9.06-fold, p = 0.0001), and Mmp3 (24.67 ± 3.63-fold, p = 0.0001) significantly increased expression after NGF stimulation for 5 days (n = 8). B: Western blot verification of microarray data. The genes Chi3l1 (5.46 ± 0.87-fold, p = 0.0001), Lcn2 (5.1216 ± 1.09-fold, p = 0.0001), and Mmp3 (4.66 ± 1.23-fold, p = 0.0001) were significantly upregulated by NGF stimulation (n = 5). ***Significant at p < 0.001. C: Immunocytochemical detection of Chi3l1, Lcn2, and Mmp3 showing increased expression in cells following NGF stimulation (n = 3). No stimulation (i) and NGF treatment (ii) (100 ng/ml for 5 days), original magnification ×200.
Fig. 2. NGF influences balance of catabolic/anabolic activity of AF cells. Time-course analysis of expression ratios of Chi3l1/Col1a1, Lcn2/Mmp9, and Mmp3/Timp1 from Day 1 to Day 5. The cells were incubated in the absence or presence of NGF for 1, 3, or 5 days (n = 8). 
A: Ratio of Chi3l1 to Col1a1 (p = 0.81). B: Ratio of Lcn2 to Mmp9 (p = 0.074). C: Ratio of Mmp3 to Timp1 revealed balance significantly shifted toward catabolic potential on Day 5 (3.79 ± 0.53-fold, p = 0.022). *Significant at p < 0.05.

Fig. 3. Nerve growth factor induced ERK1/2 phosphorylation in rat-tail disc cells. A: Increased phosphorylation of ERK1/2 is seen at 12 hours (1.5 ± 0.095-fold, p = 0.027) after NGF treatment (n = 5). *Significant at p < 0.05. B: Phosphorylation of ERK1/2 induced by NGF was inhibited by Ro 08-2750 treatment (0.64 ± 0.14-fold, p = 0.01; n = 4). *Significant at p < 0.05 compared with control. #Significant at p < 0.05 compared with NGF.
Nerve growth factor promotes intervertebral disc degradation

CHI3L1 feeds back to control local tissue responses and that increased production is an attempt at tissue repair. The CHI3L1 protein inhibits the degradation of type I collagen by MMP1 and increases the rate of type I collagen fibril formation, a potentially beneficial response to IVD damage.

Overexpression of MMPs is clearly associated with both osteoarthritis and IVD degeneration. Increased expression of proteinases is presumably predominantly through the activity of catabolic cytokines such as IL-1, but our observations suggest a role for NGF in the production of MMP3 by AF cells. Indeed, NGF has been shown to increase expression of MMPs in other tissues and cell systems. Thus, because NGF may not only regulate neuronal in-growth but also influence the production of MMPs and neoangiogenesis, it may be a prime target for intervention in the course of IVD degeneration to help decrease pain and the rate of disease progression.

Ongoing clinical trials for the treatment of degenerative disc disease, involving intradiscal therapies with genes, growth factors, and stem cells, aim to halt the progression of this condition that can lead to ankylosis and loss of motion at the segment. These therapies seek to alter the balance of catabolic and anabolic factors to help preserve and regenerate tissue when possible. In vitro studies that lead to a reduction in catabolic proteinases deserve consideration when formulating a clinical solution to degenerative disc disease. We believe that the data in this study support the further investigation of NGF as a potential treatment target in this realm.

Fig. 4. Human IVD samples stimulated ex vivo by NGF and analyzed by immunohistochemistry (n = 3). A: No stimulation. B: IVD tissue incubated with NGF. C: Human IVD tissue incubated with Ro 08-2750. D: Human IVD tissue incubated with NGF and Ro 08-2750. Clear staining of CHI3L1, LCN2, and MMP3 was found following NGF treatment for 12 hours that was inhibited by pretreatment of Ro 08-2750. Original magnification x200.
The observation that Ro 08-2750, a nonpeptide inhibitor of NGF that binds the NGF dimer and inhibits binding to both p75NTR and trkA, prevented both activation of ERK1/2 and increased expression of Chi3l1 and Lcn2 in NGF-treated AF cells supports a direct role for NGF in regulating AF function that may be amenable to therapeutic intervention.

Conclusions

We have shown, for the first time, that NGF increases expression of a range of genes in AF cells, including Chi3l1, Lcn2, and Mmp3, promoting a procatabolic phenotype that, when replicated in vivo, would lead to IVD degeneration. The effects appear to be via the high-affinity trkA NGFR. Anti-NGF therapies are currently tested in clinical trials for chronic LBP and pain from osteoarthritis of hip or knee joints, with good preliminary results. Our study shows that NGF potentially influences the process of IVD matrix breakdown, in addition to contributing to pain pathways, and that these activities may be blocked by specific NGF inhibitors. Novel treatments targeting NGF/NGFRs have the potential to influence a range of adverse biological responses, including neurite and vascular infiltration and tissue degeneration, that result in the clinical symptoms and pathology of IVD disease. Adding outcome measurements that assess the extent of IVD degradation in clinical trials of anti-NGF therapies is indicated to establish whether these treatments have effects on disease progression.

Disclosure

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Author contributions to the study and manuscript preparation include the following. Conception and design: Lee, Kao. Acquisition of data: Kao. Analysis and interpretation of data: Kao. Drafting the article: Kao. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Lee. Statistical analysis: Kao. Administrative/technical/material support: Peng, Tsou. Study supervision: Salter.

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T. H. Kao et al.
Nerve growth factor promotes intervertebral disc degradation