Original Research

The long non-coding RNA *NEAT1* contributes to extracellular matrix degradation in degenerative human nucleus pulposus cells

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Impact statement

For the first time, our study demonstrates that lncRNA *NEAT1* plays a role in the occurrence and development of IDD by participating in extracellular matrix remodeling. This lncRNA regulates catabolic MMP13 and ADAMTS5 and anabolic collagen II and aggrecan by affecting the ERK/MAPK signaling pathway in degenerative human nucleus pulposus (NP) cells. Our research provides a scientific basis for targeting of *NEAT1* for the IDD.

Abstract

Intervertebral disc degeneration is a complex disease involving genetic and environmental factors and multiple cellular processes. The role and expression of the lncRNA *NEAT1* were assessed in intervertebral disc degeneration. *NEAT1* expression was assessed in degenerative and control nucleus pulposus using RT-PCR. Western blotting and RT-PCR were also used to investigate p53 and p21 levels in nucleus pulposus tissues. *NEAT1* function in degenerative nucleus pulposus cells was assessed with gain- and loss-of-function experiments. ERK/MAPK signaling was also examined. *NEAT1*, p53, and p21 were dramatically upregulated in intervertebral disc degeneration. Furthermore, catabolic MMP13 and ADAMTS5 were dysregulated and collagen II and aggrecan were downregulated after *NEAT1* overexpression. This effect was reversed by transfection with si-NEAT1 in degenerative nucleus pulposus cells. In addition, *NEAT1* was found to affect the activation of the ERK/MAPK pathway. The NEAT1-induced ECM degradation may involve ERK1/2/MAPK signaling. LncRNA *NEAT1* may represent a novel molecular target for intervertebral disc degeneration treatment by preventing nucleus pulposus ECM degradation.

Keywords: NEAT1, degeneration, intervertebral disc, nucleus pulposus cells, ERK/MARK

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Introduction

Intervertebral disc degeneration (IDD) is deeply involved in low back pain, which has deleterious health and social consequences,[1] with a cost of more than $100 billion per year in America.[2] The potential etiological factors for IDD include abnormal process of cell degradation associated with genetics and age.[3] The exact pathogenesis of IDD remains poorly understood.

Emerging evidence has indicated that lncRNAs is involved in pathogenesis of IDD by regulating human nucleus pulposus (NP) cells.[4,5] LncRNAs are defined as RNAs at least 200 nucleotides (nt) long that are independently transcribed; they resemble mRNAs at the molecular level, but do not have recognizable potential for encoding functional proteins. Genomic studies based on EST and cDNA sequencing, tiling microarrays, and RNA sequencing have identified thousands of lncRNAs in diverse animal and plant genomes. However, their involvement in human disease is only beginning to be understood.

NEAT1 (nuclear enriched abundant transcript 1) is a lncRNA essential for the integrity of the nuclear paraspeckle substructure.[6] Although *NEAT1* expression is dysregulated in many human malignancies, including downregulation in gastric, prostate, esophageal, and lung cancer and acute promyelocytic leukemia,[7–11] a previous lncRNA–mRNA microarray study showed that anomalous *NEAT1* expression was higher in IDD than in normal discs.[12]

In this study, we verified that *NEAT1*expression increases with the progression of degeneration. Our findings indicate that *NEAT1* is deeply associated with the

generation of IDD by participating in NP ECM degradation. Furthermore, we confirmed that this effect involved the ERK/MAPK signaling pathway.

Materials and methods

Patients and samples

The study protocol was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent was obtained from all participants. Human lumbar NP specimens used as normal controls were collected from six patients ($n = 3$ women and 3 men; mean age, 22 years; range, 18–32 years) with spinal fracture who underwent fusion surgery. Degenerative human lumbar NP specimens were collected from 10 patients ($n = 4$ women and 6 men; mean age, 38.4 years; range, 27–57 years) with IDD who underwent disc excision and spinal fusion surgery. The degree of IDD was assessed according to the modified Pfirrmann grading system using preoperative MRI scans.[13]

Human NP cells

Ten degenerative NP tissue samples were collected from IDD. Human degenerative NP cells were isolated and cultured as described previously.[14] Cells were passaged two to three times for use in the following experiments.

Cell transfection

NEAT1 has two isoforms, a smaller 3.7 kb (NEAT1–1) and a larger 23 kb isoform (NEAT1–2). A specific vector targeting NEAT1 (pcDNA3.1-NEAT1) and an empty vector (used as a negative control) were purchased from Invitrogen. Human degenerative NP cells were then transfected with the control vector or pcDNA3.1-NEAT1using Lipofectamine, 2000 (Invitrogen). In addition, short interfering (si)RNA against *NEAT1* (si-NEAT1) and si-NC (control group) were purchased from RiboBio (Guangzhou, China), and transfected into NP cells by manufacturer's instructions. The NEAT1 siRNA primer sequences were: siRNA-1:GUGAGAAGUUGCUUA GAAAUU, siRNA-2:UGGUAAUGGUGGAGGAAGAUU, siRNA-3: GGAGGAGUCAGGAGGAAUAUU.

Quantitative real-time PCR

We used TRIzol reagent to extract RNA from NP tissues and cells. Total RNA was used to synthesize cDNA with a cDNA Synthesis Kit (TaKaRa Biotechnology, Otsu, Japan). Real-time PCR was performed and products were analyzed, respectively, with SYBR Green kit Master Mix (Applied Biosystems) and the ABI, 7500 Sequencing Detection System. The primer sequences: NEAT1forward: ATGCCACAACGCAGATTGAT, reverse: CGAGAAACGC ACAAGAAGG. Aggrecan forward: ACTCTGGGTTTTC GTGACTCT, reverse: ACACTCAGCGAGTTGTCATGG. MMP13 forward: ACTGAGAGGCTCCGAGAAATG, reverse: GAACCCCGCATCTTGGCTT. ADAMTS4 forward: ACCCAAGCATCCGCAATC, reverse: TGCCCAC ATCAGCCATAC. Homo β-actin forward: AGCGAGC ATCCCCCAAAGTT, reverse: GGGCACGAAGGCTCATC ATT.

Western blotting

Western blotting was performed as described previously with antibodies specific for type II collagen, aggrecan, MMP-13, ADAMTS-4, p53, p21,phospho-ERK1/2 (p-ERK), ERK1/2, and β-actin (all from Abcam, Cambridge, MA, USA).

Statistical analysis

Data are expressed as the mean ± SD. Data are analyzed with Student's *t*-test or by analysis of variance using SPSS v.18.0 software (SPSS Inc., Chicago, IL, USA). The statistical significance is defined as $P < 0.05$.

Results

NEAT1 is upregulated in degenerative human NP tissues

To assess whether *NEAT1*expression is altered in IDD, *NEAT1* expression was measured in 6 control NP tissues and 10 degenerative tissues. As shown in Figure 1(a), *NEAT1* expression was significantly upregulated in degenerative NP tissues compared with that in controls. Moreover, differential expression of several ECM-related molecules was detected. ADAMTS-4 and MMP-13 levels were increased and aggrecan and type II collagen levels are decreased in IDD (Figure 1(b) and (c)), which is in agreement with findings of previous studies. Because previous experiment showed that NEAT1 is a direct transcriptional target of p53,[15,16] we assessed the expression of p53 and its target gene p21.p53 and p21 levels were increased in degenerative tissues (Figure 1(d) and (e)).

Silencing of *NEAT1* inhibits ECM degradation of degenerative NP cells

We next studied the effect of silencing *NEAT1* on NP ECM metabolism. Three siRNAs were designed to silence *NEAT1* (Figure 2(a)); si-NEAT1–1 showed the best silencing effect and was used for the subsequent experiments. Aggrecan and type II collagen expression was increased in the NEAT1-silenced group compared to the control (Figure 2(b), (c), and (f)). MMPs and ADAMTSs are involved in NP ECM degradation. We thus assessed ADAMTS-4and MMP-13 levels in degenerative NP cells. *NEAT1* inhibition decreased the expression of ADAMTS-4 andMMP-13 relative to that in the control (Figure 2(d) to (f)).

Overexpression of *NEAT1* promotes ECM degradation in degenerative NP cells

We also investigated the effect of *NEAT1* overexpression on NP ECM degradation. As shown in Figure 3(a), *NEAT1* overexpression vector was used to overexpress *NEAT1*, which resulted in increases in ADAMTS-4 and

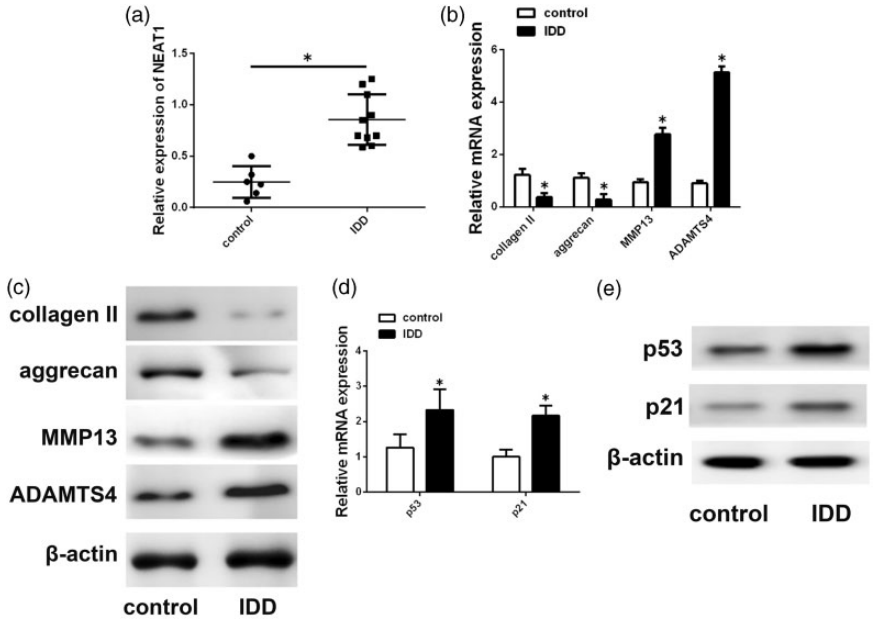

Figure 1. *NEAT1*expression in human NP tissue. (a) *NEAT1* exhibited higher expression in degenerative NP tissues. (b, c) MRNA and protein levels of ADAMTS4, aggrecan, type II collagen andMMP13 were analyzed by Western blotting and qRT-PCR. (d, e) p53 and p21 expression level were analyzed. Data represent the mean ± SD. *$P < 0.05$ vs. control group.

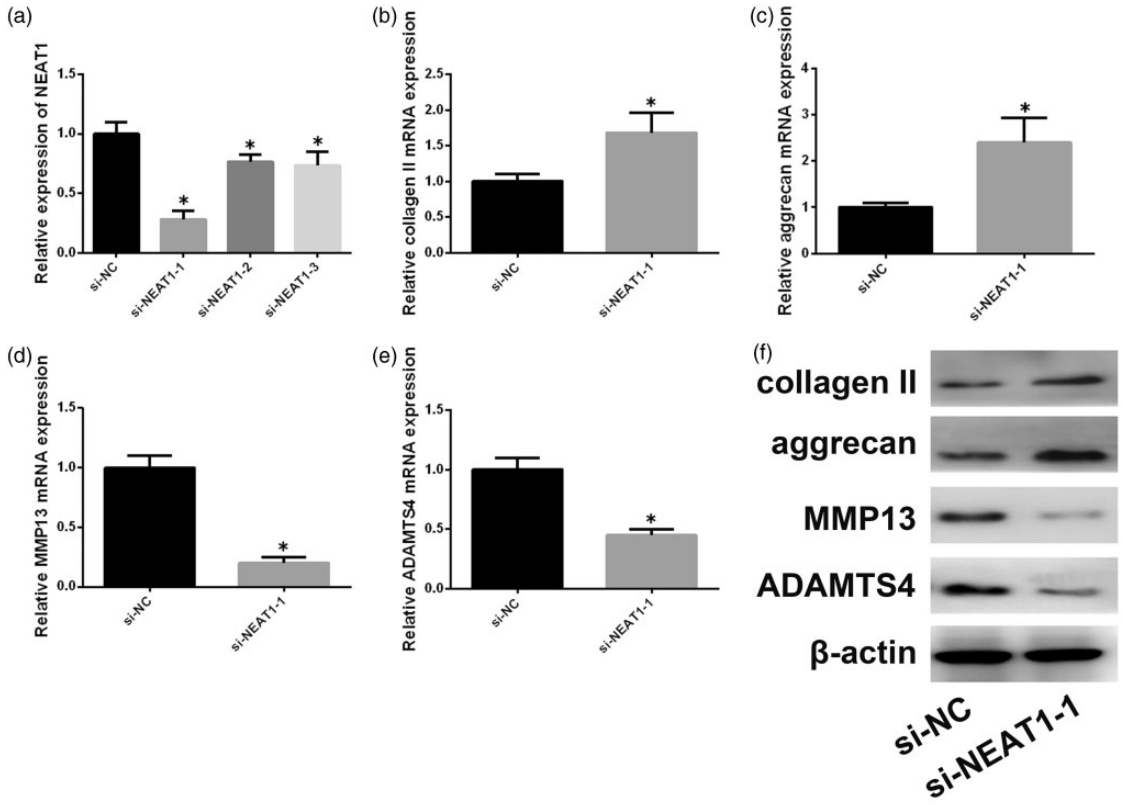

Figure 2. *NEAT1* inhibition affects ECM degradation in degenerative NP cells. (a) Effects of three different siRNAs against *NEAT1*were assessed by qRT-PCR. (b–e) mRNA expression levels of ADAMTS-4, aggrecan, MMP-13, and type II collagen were determined after silencing of *NEAT1* by siRNA. (f) Effect of silencing of*NEAT1* onADAMTS-4, aggrecan, MMP-13, and type II collagen protein expression. Data represent the mean ± SD. *$P < 0.05$ vs. control group, $n = 3$.

MMP-13 and decreases in aggrecan, type II collagen at the mRNA, protein levels (Figure 3(b) to (f)). These findings show that *NEAT1* is related to NP ECM degradation in IDD.

NEAT1 affects MAPK pathway activation

To obtain a preliminary understanding of the exact molecular mechanism by which *NEAT1* promotes NP ECM degradation, Western blotting was performed to assess the

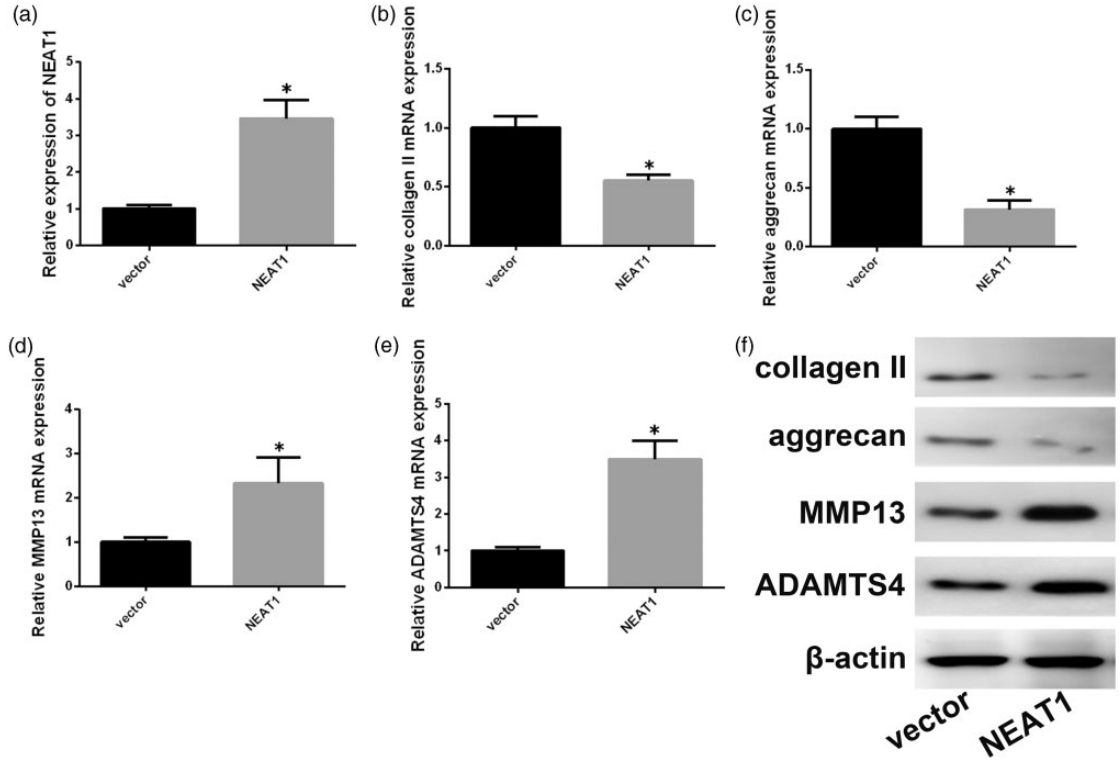

Figure 3. *NEAT1* affects NP ECM metabolism. (a) Expression of *NEAT1* in degenerative NP cells transfected with pcDNA3.1-NEAT1 (NEAT1) or the control plasmid was determined with qRT-PCR. (b–e) mRNA expression levels of ADAMTS-4, aggrecan, MMP-13, and type II collagen were determined after *NEAT1* overexpression. (f) Effects of *NEAT1* overexpression on ADAMTS-4, aggrecan, MMP-13, and type II collagen protein expression. Data represent the mean ± SD. *$P < 0.05$ vs. control group, $n = 3$.

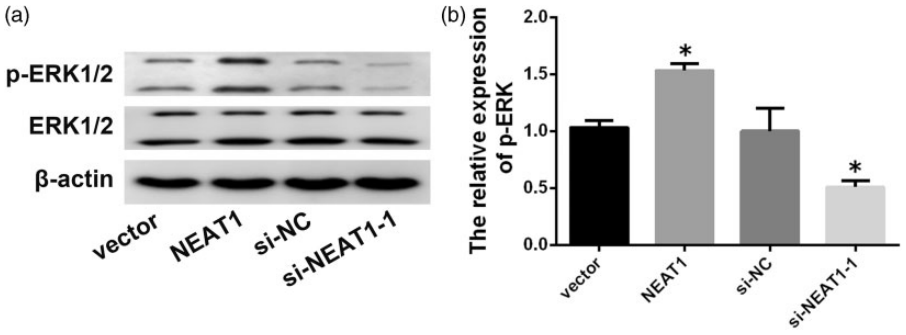

Figure 4. *NEAT1* activates ERK/MAPK in NP cells. (a, b) Protein levels of p-ERK and total protein levels of ERK in degenerative NP cells transfected using empty vector, pcDNA3.1-NEAT1, si-NC, or si-NEAT1–1 for 48 h. Data represent the mean ± SD. *$P < 0.05$ vs. control group.

effects of *NEAT1* down- and up-regulation on the ERK/MAPK pathway, which is often aberrantly activated in IDD and contributes to NP ECM degradation. Western blotting analysis showed that downregulation of *NEAT1* significantly reduced the levels of phosphorylated ERK1/2, whereas upregulation of *NEAT1* promoted the phosphorylation (Figure 4(a) and (b)). These results show that the ERK/MAPK pathway activation by *NEAT1* overexpression may contribute to degenerative NP ECM degradation.

Discussion

IDD is characterized by abnormal NP apoptosis, the increased expression of various inflammatory factors, and disordered ECM metabolism in NP cells.[17–19] The molecular mechanisms of IDD remain unclear, although various factors are associated with IDD etiology. Accumulating evidence has shown that non-coding RNAs, including miRNAs and lncRNAs, play a vital role in IDD. One experiment revealed that the lncRNA RP11–296A18.3 promotes HIF1A expression by sequesteringmiR-138, thus promoting human NP cell proliferation and ECM synthesis.[4] Another recent study showed that silencing of*TUG1* may not only protect human NP cells from TNF-α-induced apoptosis and senescence but may also promote cell proliferation by blocking the Wnt/β-catenin pathway.[20] In this regard, the data presented in this study are consistent with previous studies indicating that the lncRNA *NEAT1* may be a

causative factor in the development of IDD. Increased *NEAT1* expression decreased aggrecan and collagen levels and increased ADAMTS4 and MMP-13 levels.

NEAT1 exerts a regulatory function in proliferation and differentiation, and it is upregulated and plays an oncogenic role in most types of solid tumor. In contrast, *NEAT1* is downregulated in acute promyelocytic leukemia, where it promotes leukocyte differentiation.[21] In osteoarthritis (OA) tissues, *NEAT1* is upregulated and represses synoviocyte proliferation. After *NEAT1* knockdown, MMP13, IL-6, and IL-8 expression is reduced.[22] In our study, the expression of *NEAT1* in NP tissues from IDD patients was higher than that in controls. Further studies showed levels of p53 and p21, a canonical p53 target gene, were higher than those in controls. These findings suggest that NEAT1 might be upregulated in a p53-dependent manner. This is consistent with published reports that NEAT1 expression was induced by p53 activation.[15,16]

Accumulating studies have highlighted the role of NP cell degeneration and senescence in IDD.[23] Anabolic and catabolic processes control the homeostasis between ECM synthesis and degradation.[24] A recent study showed that epoxy eicosanoids prevent intervertebral disc ECM degeneration, perhaps by inhibiting the matrix remodeling response of NP cells to TNF-α.[14] Therefore, we investigated the function of *NEAT1* in ECM degradation in degenerative NP cells, *NEAT1* was shown to participate in ECM degradation. The levels of catabolic MMP-13 and ADAMTS-5 and anabolic collagen II and aggrecan were affected by *NEAT1*expression, further indicating that high expression of *NEAT1* may have significant impacts on the occurrence and development of IDD. *NEAT1* has thus been demonstrated to participate in ECM remodeling.

Multiple studies have shown an important role of the ERK/MAPK pathway in IDD. Beta1 integrin was shown to decrease apoptosis in annulus fibrosus (AF) cells via the ERK1/2 MAPK pathway.[25] Tensile stress promotes MAPK activation in AF cells, which is associated with proinflammatory gene expression and drives an inflammatory reaction.[26] The tolerance of NP cells to hyperosmotic milieu is associated with ERK/MAPK pathway activation.[27] Loss-of-function studies have confirmed that ERK/MAPK signaling contributes to the cytokine-dependent induction promoter activity of MMP3, which is important in IDD.[28] Inhibition of this pathway reduces or abrogates IL-1β-mediated catabolic effects in disc degeneration. Furthermore, a previous study indicated that ERK/MAPK signaling increases with the progression of IDD,[29] which is in line with our findings. In addition, the level of phosphorylated ERK1/2 was regulated by*NEAT1* in degenerative NP cells from IDD patients, further indicating that high expression of *NEAT1* may have significant impacts on the occurrence and development of IDD by specifically activating the ERK/MAPK signaling pathway.

In conclusion, we demonstrated the effects of *NEAT1* on IDD. The data from the current study provide new evidence showing that *NEAT1* may activate ERK/MARK signaling to exacerbate the imbalance in ECM metabolism in degenerative human NP cells. This is important in the treatment of IDD, which induces the progression of LBP. Further studies are needed to demonstrate the effect of *NEAT1* expression in normal NP cells to promote the clinical treatment of IDD.

Authors' contributions: FL designed the study. FL and ZR wrote the manuscript. ZR, HM and JL conducted the experiments. HYL and HRJ analyzed the data. FL reviewed and revised the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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