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Cartilage intermediate layer protein promotes lumbar disc degeneration

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ABSTRACT

Lumbar disc disease (LDD) is one of the most common musculoskeletal disorders, and accompanies intervertebral disc degeneration. *CILP* encodes cartilage intermediate layer protein, which is highly associated with LDD. Moreover, CILP inhibits transcriptional activation of cartilage matrix genes in nucleus pulposus (NP) cells *in vitro* by binding to TGF-β1 and inhibiting the phosphorylation of Smads. However, the aetiology and mechanism of pathogenesis of LDD *in vivo* are unknown. To demonstrate the role of CILP in LDD *in vivo*, we generated transgenic mice that express CILP specifically in the intervertebral disc tissues and assessed whether CILP exacerbates disc degeneration. Degeneration of the intervertebral discs was assessed using magnetic resonance imaging (MRI) and histology. The level of phosphorylation of Smad2/3 in intervertebral discs was measured to determine whether overexpressed CILP suppressed TGF-beta signalling. Although the macroscopic skeletal phenotype of transgenic mice appeared normal, histological findings revealed significant degeneration of lumbar discs. MRI analysis of the lumbar intervertebral discs indicated a significantly lower signal intensity of the nucleus pulposus where CILP was overexpressed. Intervertebral disc degeneration was also observed. The number of phosphorylation of Smad2/3 immuno-positive cells in the NP significantly was decreased in CILP transgenic mice compared with normal mice. In summary, overexpression of CILP in the NP promotes disc degeneration, indicating that CILP plays a direct role in the pathogenesis of LDD.

1. Introduction

Lumbar disc disease (LDD) is one of the most common musculoskeletal disorders, and is accompanied by intervertebral disc degeneration and disc herniation, which contribute to the development of low back and unilateral leg pain. Low back pain affects 70–85% of all people during their lifetimes, and LDD is the most common cause of limitations in activity of patients younger than 45 years of age [1]. However, the aetiology and pathogenesis of LDD are unknown.

The intervertebral disc comprises the annulus fibrosus (AF, the outer layer) and the nucleus pulposus (NP, the interior structure). The nucleus pulposus is a cartilage-like tissue that contains an abundant extracellular matrix (ECM) consisting of proteoglycans, mainly aggrecan, and collagens [2]. CILP is a monomeric glycoprotein that resides in the ECM. It is expressed in the intermediate zone of human articular cartilage [3] and localises to the meniscus articularis [4], tendon, ligament [5], synovial membrane [6], and intervertebral disc [7].

CILP was originally isolated from a chromatographic fraction that also contained nucleotide pyrophosphohydrolase (NTPPHase), which has been implicated in the degeneration of cartilage [8,9]. A single nucleotide polymorphism in *CILP* is highly associated with LDD [7]. In our study of sequence variations in *CILP* in 467 patients with LDD and 654 controls, we found that the most significant association of LDD was with the single nucleotide polymorphism (SNP) c.1184T>C (p.I395T) ($p = 0.0000068$) [7]. CILP binds to transforming growth factor (TGF)-β and prevents it from activating the transcription of cartilage matrix genes in NP cells *in vitro* [7]. Other studies support roles for ECM proteins in regulating growth factors, including TGF-β [10,11], insulin-like growth factor (IGF)-1 [4], and bone morphogenic protein-2 (BMP)-2 [12].

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TGF-β1 induces the synthesis of proteoglycans and cell proliferation in intervertebral discs [13,14] as well as the expression of *CILP* [15]. Further, injection of an adenoviral TGF-β1 expression vector increases proteoglycan synthesis in the human intervertebral disc [16]. These observations indicate that TGF-β and CILP play important roles in maintaining ECM proteins in human intervertebral discs. CILP inhibits TGF-β-induced transcriptional activation of cartilage matrix genes in NP cells *in vitro* by binding to TGF-β1 and inhibiting the phosphorylation of Smads [7]. To demonstrate the functional significance of CILP *in vivo*, we generated transgenic mice that differentially express CILP in the intervertebral disc tissues to determine whether CILP is responsible for the exacerbation of disc degeneration.

2. Materials and methods

2.1. Generation of transgenic mice

Tsumaki et al. reported [17] that the pNASSβ transgene containing the *Col11a2* promoter/intervening sequence (IVS)1 is differentially expressed in the foetal intervertebral disc according to the length of the promoter/IVS1 sequence. We confirmed these findings using the expression vector 453*LacZ*Int (B6, *Tg*(*Col11a2-LacZ*)*3Tt*) (Fig. 1A). A full-length *Cilp* cDNA was originally cloned from a mouse cDNA library (Clontech). Full-length mouse *Cilp* cDNA expression vectors were constructed using the *Col11a2* promoter/IVS1 [17] that is specifically activated in intervertebral disc tissues as follows: The construct designated 453*Cilp*Int (B6, *Tg*(*Col11a2-Cilp*)*3Tt*) expresses CILP specifically in the NP of the foetal intervertebral disc (Fig. 1B). DNA sequencing verified that the influenza virus hemagglutinin (HA) epitope was fused in-frame to the 3′-end of the *Cilp* cDNA open reading frame. Transgenic (*Tg*) mice were generated by microinjecting each of the expression vectors into pronuclei of fertilised eggs of C57BL/6 mice. Founder mice were identified by PCR analysis of genomic DNA extracted from the tail. This study was approved by the Institutional Animal Care and Use Committee of University of Toyama (approval no. S-2009 MED-47).

2.2. Western blotting

The presence of the transgene was confirmed by PCR analysis of DNA extracted from tails. Mouse tissues (day post-coitus (d.p.c.) 13.5) were homogenised and total proteins were isolated using a T-PER Protein Extraction Reagent (Pierce). Proteins were electrophoresed through 12.5% Tris–HCl SDS–PAGE gels (Bio-Rad) and transferred electrophoretically to nitrocellulose membranes (Amersham Bioscience) according to standard protocols. Equal amounts of protein were loaded for each experiment. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween (TBST). The anti-HA antibody was used at a 1:1000 dilution in 5% FBS-TBST. The HA tag sequence was as follows: ACC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT. An anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signalling Technology) was used as the secondary antibody (1:3000 dilution) in 5% FBS-TBST. To determine the levels of phosphorylated Smad2/3, NP cells were isolated from transgenic and normal mice (one week

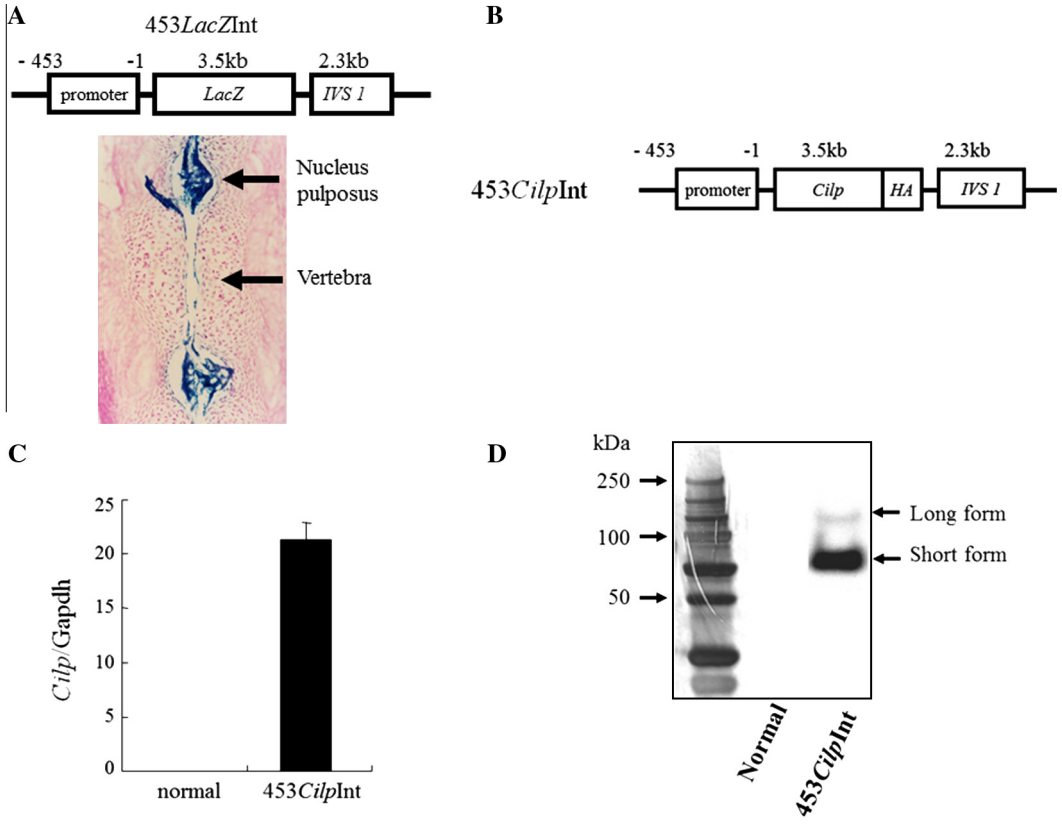

Fig. 1. Expression vectors and CILP expression in *Tg* mice. (A) Expression vectors were constructed using the intervertebral disc-specific promoter *Col11a2* promoter/IVS1 as described previously [17]. (B) The construct designated 453*Cilp*Int expresses CILP specifically in the NP of the intervertebral disc. Expression vectors were constructed using the intervertebral disc-specific promoter *Col11a2* promoter/IVS1 containing mouse *Cilp* and the HA epitope (CILP C-terminal domain). (C) Real-time PCR analysis of expression of the *Cilp* transgene in mice. HA-tagged *Cilp* mRNA was abundantly expressed in all tissues of 453*Cilp*Int mice. Data represent the ratio of HA-tagged *Cilp* to *Gapdh* mRNAs. (D) Western blots probed with an anti-HA antibody. CILP was abundantly expressed in tissues of 453*Cilp*Int mice (13.5 d.p.c.). The arrow indicates the CILP precursor (138 kDa) and its C-terminal (82 kDa) cleavage product.

after birth) using a published proteinase–collagenase digestion method [7]. After one subculture, we prepared cell lysates using the M-PER protein extraction kit (Pierce). SDS–PAGE, electroblotting, and blocking nonspecific binding by the membrane were carried as described above. An equal amount of each sample was analysed. We used primary antibodies against phosphorylated Smad2/3, Smad2/3, and β-actin (all from Cell Signalling Technology) each diluted 1:1000 in TBST containing 5% FBS. We used a horseradish peroxidase-conjugated antibody against rabbit IgG (GE Healthcare) as the secondary antibody, which was diluted 1:3000 in TBST containing 5% FBS.

2.3. Analysis of CILP expression

Transgene expression in each mouse line was analysed using reverse transcription-polymerase chain reaction (RT-PCR). For analysis of CILP, whole mouse tissue (13.5 d.p.c.) was homogenised, and proteins were extracted using the T-PER Protein Extraction Reagent (Pierce). For western blotting, an anti-HA antibody (Roche) was diluted 1:1000 in TBST.

2.4. Real-time PCR analysis

Total RNA was extracted from the tail of each mouse using Isogen (Nippongene) and purified using the SV Total RNA Isolation System (Promega). Random-primed cDNAs were synthesized using MultiScribe™ Reverse Transcriptase (Applied Biosystems). Quantitative real-time PCR was carried out using a PRISM 310 Genetic Analyser with the QuantiTect® SYBR® Green PCR kit (Qiagen) according to the manufacturer's instructions. The forward primer sequence was derived from the C-terminal domain of mouse *Cilp*, and the reverse primer was derived from sequences encoding HA as stated above.

2.5. MRI analysis

We analysed the lumbar spines of mice aged six weeks ($n = 6$ for each mouse line and normal littermates; total = 18) using a Varian Unity INOVA 4.7 T scanner (4.7 T, Matrix: np/nv 512/512, tr/te = 2.5/0.04 s FOV: 40 × 40 mm, slice thickness, 1.1 mm). Signal intensity was evaluated using Adobe Photoshop CS2.

2.6. Safranin-O staining and immunostaining

Safranin-O staining was performed according to the manufacturer's instructions. We also evaluated the staining intensity and area of the NP of the lumbar intervertebral disc of mice aged 24 weeks using Adobe Photoshop CS2 and a VH-analyser VH-H1A5 (Keyence, Osaka). For immunostaining, the sections were deparaffinized, rehydrated, and then digested with 500 U/ml testicular hyaluronidase for 30 min at 37 °C. Endogenous peroxidase activity was inhibited by treating the sections with 3% H_2O_2 containing 0.1% sodium azide for 5 min at room temperature. We incubated the sections with an anti-phospho-Smad2/3 antibody (Cell Signalling Technology) overnight at 4 °C. Immunostaining reactions were detected using an Envision™ ± Kit (DakoCytomation) followed by staining with 3,3′-diaminobenzidine.

3. Results

We first determined that the transgene was expressed in transgenic mice (453*LacZ*Int) generated using the specific *Col11a2* promoter/IVS1 containing the *LacZ* reporter. Transgene expression was detected only in the NP (d.p.c. 13.5, Fig. 1A). Fig. 1B shows the structure of the 453*Cilp*Int expression vector. We determined the levels of HA-tagged mouse *Cilp* mRNA using real-time PCR in the 453*Cilp*Int mice (Fig. 1C). *Cilp* mRNAs were highly expressed by d.p.c. 13.5, and the expression of HA-tagged CILP was readily detected by western blotting (Fig. 3B). The CILP precursor (long form, 138 kDa) is cleaved upon its secretion from cells into 92 kDa (N-terminal domain) and 82 kDa (C-terminal domain, short form) components [3]. Fig. 1D shows the HA-tagged long (full-length CILP) and short (C-terminal CILP) forms. The N-terminal domain was not detected by western blotting, because the HA tag is at the C-terminus.

We initially examined the mice when they were 5 months old. Using soft X-ray analysis, we did not detect any morphological differences in the spine or other skeletal components in transgenic mice (Supplementary Fig. 1). Further, there were no significant differences between the blood-test values and body weights (Supplementary Figs. 2 and 3). Using MRI, we examined the signal intensity of the NP to determine whether overexpressed CILP might promote disc degeneration by suppressing TGF-β signalling. The signal intensity of NP was significantly higher in normal compared with 453*Cilp*Int mice (Fig. 2A). The signals generated by lumbar intervertebral discs (L1/2–L5/6) in 453*Cilp*Int mice were significantly lower in the NP where CILP was overexpressed (Fig. 2A and B), suggesting early deterioration of the intervertebral disc matrix in the presence of CILP expression.

Histological analysis further confirmed the presence of severe lumbar disc degeneration. A histological grading score was used as reported by Masuda et al. [18]. Histological analysis of the disc sections revealed significant differences in the AF, the border between the AF and NP, NP cellularity, and the matrix of NP in the 453*Cilp*Int mice compared with those in normal mice (Fig. 3A–E), again suggesting loss of proteoglycan and enhanced degeneration in the NP of mice overexpressing CILP. The 453*Cilp*Int mice showed shrinkage of the NP and focal loss of the lamellar structure of the annulus with partial disruption of the end plate (Fig. 3F–I). These destructive changes of the AF and endplate might occur subsequent to the degeneration of NP in intervertebral disc of the transgenic mice.

We asked whether the difference in the ability to repair the damage to the NP or to form the NP of intervertebral discs and the endplate causes progressive degeneration. Therefore, we

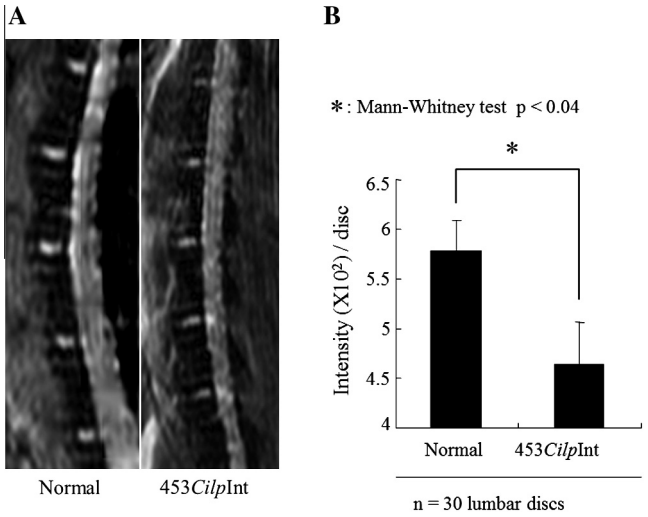

Fig. 2. MRI analysis of 6-week-old transgenic mice. (A) MRI data acquired from scans of the lumbar spine (4.7 T, Matrix: np/nv 512/512, tr/te = 2.5/0.04 s FOV: 40 × 40 mm, Slice thickness: 1.1 mm). (B) Densitometric analysis. The signal intensity of CILP in the NP of normal mice is significantly higher than that of 453*Cilp*Int mice. ($n = 30$ discs, Mann–Whitney test, $p < 0.05$).

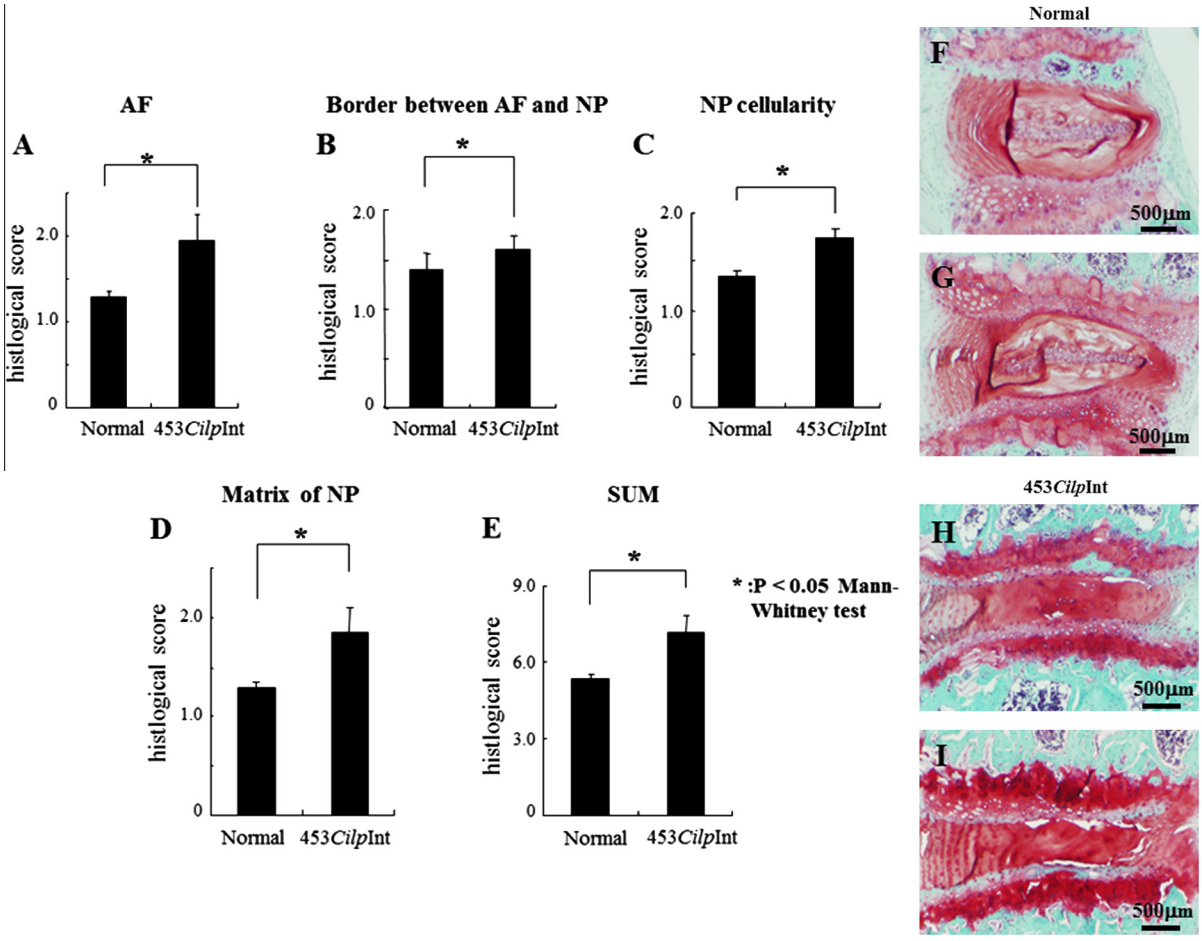

Fig. 3. Histological grading of Safranin-O staining of the lumbar intervertebral discs of transgenic mice. (A) AF. (B) Border between the AF and NP. (C) Cellularity of the NP. (D) The NP matrix. (E) Sum of histological scores. (F, G) Safranin-O staining of the intervertebral disc of a normal mouse. (H, I) Safranin-O staining of the intervertebral disc of a 453*Cilp*Int mouse. (*$p < 0.05$ Mann–Whitney test.)

determined whether CILP inhibited TGF-β-induced transcriptional activation of cartilage matrix genes in NP cells by inhibiting the phosphorylation of Smads *in vivo*. Immuno-staining using an anti-phospho-Smad2/3 antibody was performed. Immuno-positive cells were identified in normal mice, but were detected infrequently in 453*Cilp*Int mice (Fig. 4A and B). There was a significant difference in the number of immuno-positive cells in NP between normal and 453*Cilp*Int mice (Fig. 4C). We conducted western blotting analysis to determine the difference in levels of phospho-Smad2/3 between 453*Cilp*Int and normal mice. Phosphorylation of Smad2/3 was clearly decreased in 453*Cilp*Int mice while the levels of total Smad2/3 were not significantly different (Fig. 4D). These data indicate, for the first time to our knowledge, that *CILP*, which is a susceptibility gene for the LDD, is indeed responsible for the enhanced degeneration of the intervertebral disc by inhibiting the phosphorylation of Smads *in vivo*.

4. Discussion

CILP consists of 1184 amino acid residues, and its predicted molecular mass is 132.5 kDa. It contains a putative N-terminal signal peptide of 21 amino acid residues and is cleaved into a 78.5 kDa polypeptide chain derived from the N-terminus and a C-terminal 51.8 kDa polypeptide that is homologous to a porcine NTPPHase (not including posttranslational modification) [3,8]. Using an antibody against the HA tag in the present study, we detected two forms of CILP expressed by transgenic mice; however, the N-terminal cleavage product was not detected, because it was not tagged with HA. The roles of the CILP precursor and its mature forms in the intervertebral disc remain to be determined.

We generated transgenic mice that specifically overexpressed CILP in cartilage. These mice showed a loss of MRI signal intensity of the NP, a loss of NP proteoglycan, and enhanced NP degeneration detected by Safranin-O staining. Moreover, immunohistochemical analysis revealed a loss of Smad phosphorylation in the NP. Therefore, we conclude that the overexpression of *Cilp* plays an important role in promoting intervertebral disc degeneration by suppressing TGF-β signalling. These finding suggests that CILP controls the expression of other matrix genes by suppressing TGF-β signalling in the nucleus pulposus or that when CILP is overexpressed, it disturbs homeostasis of the protein composition of the ECM.

LDD has a strong familial component [19], because a positive family history increases the risk for juvenile lumbar disc herniation by a factor as much as 5.6 [20]. MRI studies of twins demonstrate the genetic contribution to degeneration of intervertebral discs of the lumbar spine [21,22]. Other studies reported potential susceptibility genes harbouring polymorphisms that are associated with LDD, such as an amino acid residue substitution (*Trp2* allele) in *COL9A2* [23] and *COL9A3* [24]. However, our published analysis did not detect the *Trp2* allele in *COL9A2* in the Japanese population [25]. In contrast, association analysis of *CILP* (c.1184T>C) has been replicated in the Japanese population [26,27]. For example, Min et al. reported that the *CILP* I395T allele is a significant risk factor

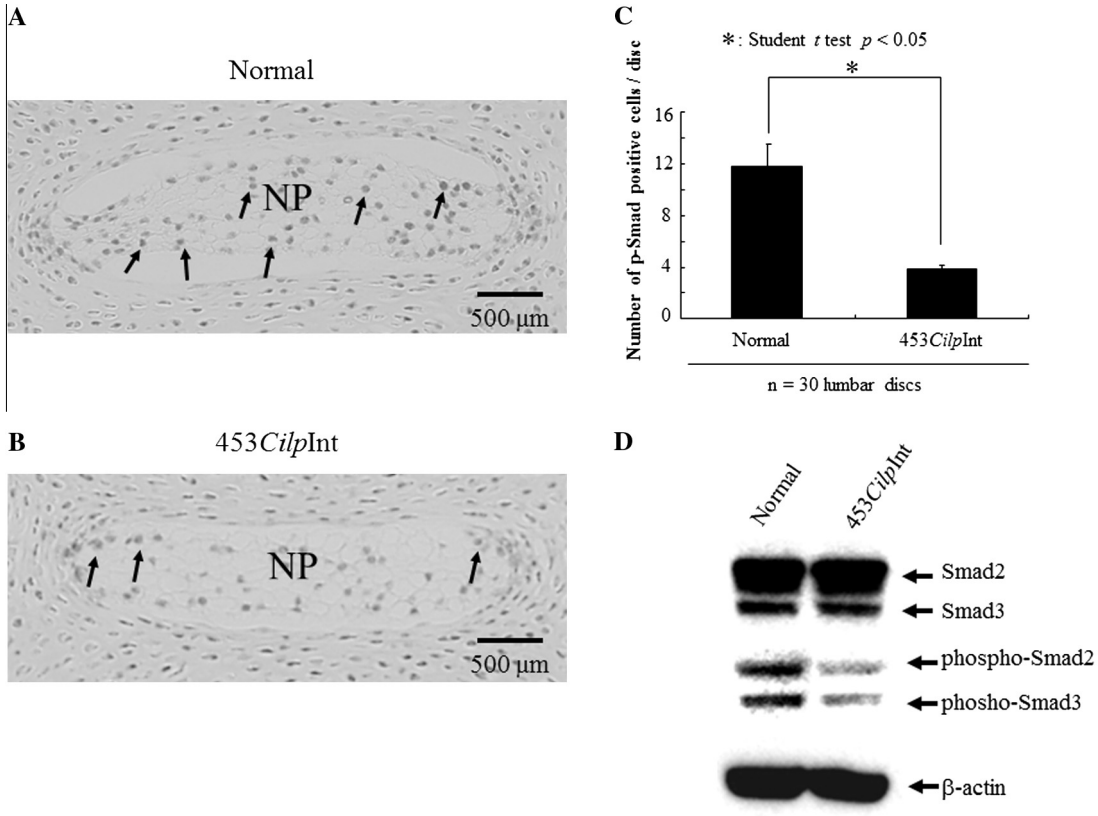

Fig. 4. Immunodetection analysis of Smad2/3 phosphorylation of NP in transgenic mice. (A, B) Immunostaining of phospho-Smad2/3 in the NP. The arrow indicates positive cells. (A) Normal, (B) 453*Cilp*Int. (C) the figure shows the average number of p-Smad-positive cells in normal and 453*Cilp*Int-NP cells. The difference between normal and 453 *Cilp*Int was significant (*Student *t* test, $p \leqslant 0.05$). (D) Western blotting of phospho-Smad2/3, Smad2/3, and β-actin in the NP. The figure shows decreased phosphorylation of phospho-Smad2/3 in 453*Cilp*Int-NP cells.

for lumbar disc degeneration in Japanese collegiate judo athletes [26]. Eighty-nine athletes diagnosed with lumbar disc degeneration were evaluated by MRI using Pfirrmann's classification [27]. Both weight (odds ratio 1.06) and the *CILP* C allele (odds ratio 4.1) were significant risk factors for LDD. Further, LDD suffered by 601 collegiate judo practitioners and other athletes was evaluated using MRI and by genotyping *CILP* (c.1184T>C) [28]. The data indicate that *CILP* (c.1184T>C) is a risk factor for LDD in male athletes. Considering our association study [7] and these data, we conclude that *CILP* is likely to be involved in the pathogenesis of LDD.

The NP is a cartilage-like tissue that contains an abundant ECM consisting of proteoglycans and collagens [2]. Several lines of evidence indicate that ECM proteins play crucial roles in intervertebral disc homeostasis. For example, patients harbouring mutations in genes encoding matrix proteins, including type II collagen [29,30] and COMP [31], present with severe spinal disorders characterised by irregular endplates, scoliosis, and severe disc degeneration. Similarly, transgenic mice deficient in collagen IX suffer from disc degeneration that precedes herniation [32]. Mice carrying mutations in *AGC* experience herniation of intervertebral discs and deformation of vertebral bodies [33]. These observations underscore the importance of matrix protein metabolism in the aetiology of LDD.

The cartilage-specific promoter *Col11a2* is widely used to generate transgenic mice to determine the effects of gene products of interest on cartilage, skeletal tissue, and intervertebral discs [17,34,35]. In the present study, *Cilp*-transgenic mice exhibited increased lumbar disc degeneration but were otherwise unaffected (Supplementary Figs. 2 and 3) [17]. For example, their skeletal phenotype and external appearance were normal (Supplementary Fig. 1). Although overexpression of *Cilp* does not exclude the possibility that other genes were involved, we conclude that it is likely that *Cilp* contributed to the degeneration of lumbar discs.

The primary function of the intervertebral disc ECM is to ensure physical and biomechanical strength; however, ECM molecules also play important roles in chondrocyte metabolism by regulating the expression of growth factors, particularly TGF-β [11,15,36]. We speculate that CILP modulates the activity of TGF-β in intervertebral disc tissue. The ECM proteins decorin and biglycan bind TGF-β and control accessibility to its receptors [37,38]. Similarly, our study *in vitro* shows that binding of CILP to TGF-β may physically interfere with the binding of TGF-β to its receptor or may render TGF-β inaccessible to its receptor by sequestration [7]. Our data acquired from studies of transgenic mice suggest that the overexpression of CILP perturbed the balance of the control of TGF-β in chondrocyte metabolism and intervertebral-disc tissue maintenance, leading to susceptibility to LDD caused by an inadequate response of intervertebral disc cells to injury and mechanical stress. To our knowledge, our data are the first to demonstrate that the overexpression of CILP directly suppresses TGF-β signalling and promotes intervertebral disc degeneration *in vivo*.

We conclude that overexpression of CILP in the NP promotes intervertebral disc degeneration, suggesting that CILP is directly involved in the pathogenesis of LDD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.025.

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