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Transcriptional regulation of the cartilage intermediate layer protein (CILP) gene ☆

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Abstract

Cartilage intermediate layer protein (CILP) is an extracellular matrix protein abundant in cartilaginous tissues. CILP is implicated in common musculoskeletal disorders, including osteoarthritis and lumbar disc disease. Regulation of the *CILP* gene is largely unknown, however. We have found that *CILP* mRNA expression is induced by TGF-β1 and dependent upon signaling via TGF-β receptors. TGF-β1 induction of *CILP* is mediated by Smad3, which acts directly through *cis*-elements in the *CILP* promoter region. Pathways other than Smad3 also are involved in TGF-β1 induction of *CILP*. These observations, together with the finding that CILP protein binds and inhibits TGF-β1, suggest that CILP and TGF-β1 may form a functional feedback loop that controls chondrocyte metabolism.

Keywords: CILP; Transcription; Responsive element; TGF-β1; Smad3

Cartilage intermediate layer protein (CILP) is a monomeric glycoprotein that resides in the extracellular matrix (ECM). It is expressed chiefly in the middle (intermediate) zone of human articular cartilage [1] but also localizes to meniscus [2], tendon, ligament [3], synovial membrane [4], and intervertebral disc [5]. A pro-form of two polypeptides, CILP, is post-translationally processed into amino (N)- and carboxyl (C)-terminal halves. N-terminal CILP binds TGF-β1 directly in vitro and acts as a negative regulator of TGF-β1 in chondrocytes from rabbit nucleus pulposus [5]. Sequence homology suggests that C-terminal CILP functions as nucleotide pyrophosphohydrolase [6,7], although its enzymatic activity remains uncertain [2,5].

CILP has been implicated in several diseases that affect cartilage. Its expression increases substantially in association with aging in human articular cartilage. In osteoarthritis (OA), CILP is among the few cartilage matrix proteins whose expression is up-regulated in early and late stages of the disease [1,8]. Genetic analysis has shown significant association between a single nucleotide polymorphism (SNP) in *CILP* and OA progression [9]. In mice, injection of recombinant CILP induces arthropathy [10]. *CILP* expression also is up-regulated in articular cartilage from patients with calcium pyrophosphate dihydrate (CPPD) crystal deposition disease [11]. Finally, we have shown that *CILP* is associated with lumbar disc disease (LDD), and its expression is markedly increased in the intervertebral discs

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of LDD patients [5]. These findings indicate that CILP is important in maintaining cartilage homeostasis in humans, and its dysfunction contributes to various diseases. However, regulation of the *CILP* gene remains largely unknown.

In this study, we investigated the transcriptional regulation of *CILP*. We show that *CILP* mRNA expression is induced by TGF-β1, in a manner dependent upon signaling via the type I TGF-β receptor (Tβ-RI). TGF-β signal is mediated directly by Smad3, as evidenced by the induction of *CILP* mRNA expression in cells over-expressing Smad3 and the presence of Smad3-responsive elements in the *CILP* promoter region. We also show that pathways other than Smad are involved in TGF-β1 induction of *CILP*.

Materials and methods

Cell culture. HuH-7 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and HeLa cells from the JCRB Cell Bank (Osaka, Japan). HEK293 cells were purchased from Clontech (Palo Alto, CA). CS-OKB cells were a gift from Dr. Chano (Shiga University of Medical Science). These cell lines and HCS-2/8 cells [12] were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum (FBS). OUMS-27 cells were obtained from the IFO Animal Cell Bank (Osaka, Japan) and cultured in DMEM-high glucose supplemented with kanamycin (50 μg/ml) and 10% FBS. ATDC5 cells were obtained from the RIKEN Cell Bank and maintained in a 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12) (Invitrogen, Carlsbad, CA), supplemented with 5% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin). Cells were plated at 3×10^4 cells per well in 12-well tissue culture plates. After cells reached confluence, the culture medium was replaced with differentiation medium (DMEM/F12 supplemented with 5% FBS, antibiotics, 10 μg/ml bovine insulin, 10 μg/ml human transferrin, and 3×10^{-8} M sodium selenite) and cultured for various experimental time periods. The growth medium was replaced every other day.

Construction of plasmids. CILP promoter-luciferase fusion genes were constructed in the pGL3-basic or pGL3-promoter vector (Promega, Madison, WI). Various *CILP* fragments were amplified by PCR using primer pairs containing *Mlu*I- or *Nhe*I-cleaved restriction sites at the 5′ ends. DNA sequences of PCR products were verified by automated DNA sequencing (model 3700; ABI, Foster City, CA). The SBE4-Lux reporter construct was made by annealing primers 5′-GATCTAAG TCTAGACGGCAGTCTAGAC-3′ and 5′-GATCGTCTAGACTGCCG TCTAGACTTA-3′ then concatemerizing them into the *Bgl*II site of the pGL3 promoter vector (Promega). Full-length cDNAs encoding SOX9, SOX5, and SOX6 were PCR amplified and cloned into the pEGFP-C1 or pShuttle mammalian expression vector (Clontech). The full-length cDNA encoding Smad3 was PCR amplified and cloned into the pcDNA3.1(+) vector.

Luciferase reporter assay. Cells were plated at a density of 5×10^4 cells per well in 24-well tissue culture plates. Using FuGENE6 (Roche Applied Science, Mannheim, Germany), luciferase reporter plasmids were co-transfected with the pRL-TK plasmid as an internal control for transfection efficiency. Cells were harvested 48 h after transfection, and luciferase activity was measured using the PG-DUAL-SP Reporter Assay System (Toyo Ink, Tokyo, Japan). Relative transcriptional activity was calculated as the ratio of luciferase activity from the experimental vector to that from the internal control vector.

Real-time quantitative PCR assays. Total RNAs were isolated from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and digested with *DNase*I. Total RNA (500 ng) was reverse transcribed into cDNA using the Taqman Core Reagent Kit (ABI) according to the manufacturer's protocol. An aliquot (2 μl) of the reaction was used as a template for the second step of SYBR Green real-time PCR (Qiagen). Partial cDNAs of *CILP* and *GAPDH* were amplified by PCR using the primer sequences 5′-CCACCATCAAGGCAGAGTTT-3′ and 5′-CTGCACTGG ATCTCCCTTTC-3′, 5′-ACCACAGTCCATGCCATCAC-3′, and 5′-TC CACCACCCTGTTGCTGTA-3′, respectively, and cloned into the pCR-TOPOII vector. In ATDC5 cells, first-strand cDNA was amplified using 5′-AAAGATGCTGACCCGAACAG-3′ and 5′-GCTTTGCAGCA CAGAGACAC-3′ for *Cilp*, 5′-GCCAAGACCTGAAACTCTGC-3′ and 5′-GCCATAGCTGAAGTGGAAGC-3′ for *Col2a1*, 5′-CCAAACC AGCCTGACAACTT-3′ and 5′-TCTAGCATGCTCCACCACTG-3′ for *Agc1*, 5′-CATAAAGGGCCCACTTGCTA-3′ and 5′-TGGCTGATATT CCTGGTGGT-3′ for *Col10a1*, 5′-TTGCTTCAGCTCCACAGAGA-3′ and 5′-GTTGGACAACTGCTCCACCT-3′ for *Tgfb1*, and 5′-ACCAC AGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for *Gapdh*. Primer sequences for each quantitative PCR are available on request. SYBR Green PCR amplification and real-time fluorescence detection were performed using the ABI PRISM 7700 sequence detection system. PCR cycling conditions were as follows: 94 °C for 15 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

Inhibition of intracellular signaling mediators. Downstream signaling from TGF-β/Tβ-RI was examined by measuring the effects of biochemical inhibitors on TGF-β1-stimulated *CILP* induction. Specific inhibitors included SB-431542 for TGF-β type 1-receptor kinase activity, LY-294002 for phosphatidylinositol 3-kinase (PI3 kinase), U-0126 for extracellular signal-regulated kinase (ERK1/2), H-89 for protein kinase A, and SB-203580 for p38 mitogen-activated protein kinase (MAPK).

Huh-7 cells were seeded at 5×10^4 cells per well in 12-well tissue culture plates, in DMEM-high glucose supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% FBS. The following day, the growth medium was replaced with the same mixture containing 0.2% FBS. On the third day, cells were treated with signal inhibitors (dissolved and diluted in 10 mM DMSO) 4 h before adding TGF-β1 (1 ng/ml), at concentrations determined by preliminary experiments (data not shown). *CILP* mRNA expression was measured using real-time quantitative PCR assays. The inhibitor effects were evaluated by measuring relative *CILP* mRNA expression, calculated as the ratio of TGF-β1-stimulated expression in the presence or absence of inhibitors.

Results

CILP mRNA expression in various human cell lines and during chondrocyte differentiation

We measured expression of *CILP* mRNA in various human cell lines using real-time quantitative PCR. Substantial *CILP* expression was detected in chondrogenic cell lines and HEK293 cells (Fig. 1). We also examined *Cilp* expression during chondrocyte differentiation using ATDC5 cells, an in vitro model of chondrogenesis [13] (Fig. 2). *Cilp* expression increased until day 10, then decreased. The increase in *Cilp* expression preceded those of *Agc1* and *Col2a1*, suggesting an earlier role in chondrocyte differentiation. *Cilp* mRNA expression also paralleled the expression of *Tgfb1*.

Identification of core promoter of the CILP gene

To identify the core promoter of the *CILP* gene, we generated luciferase constructs (pGL3 basic vector) containing various lengths of the putative promoter region and the 5′-untranslated region (5′-UTR) (+1 to +46) and measured relative luciferase activity (RLA) in transfected HCS-2/8 cells. Various 5′-deletions from −1014 to −95 retained

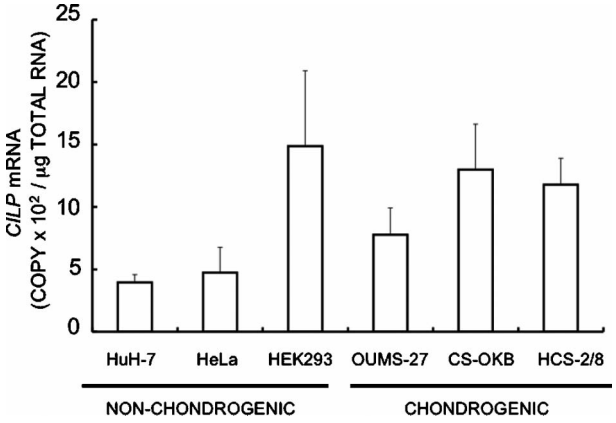

Fig. 1. *CILP* mRNA expression in chondrogenic (OUMS-27, CS-OKB, and HCS-2/8) and non-chondrogenic (HuH-7, HeLa, and HEK293) cell lines. Endogenous *CILP* mRNA levels were quantified by real-time PCR and normalized to total RNA. *CILP* mRNA expression in increased in chondrogenic cells. Values represent means ± standard error of mean (SEM) of triplicate measurements.

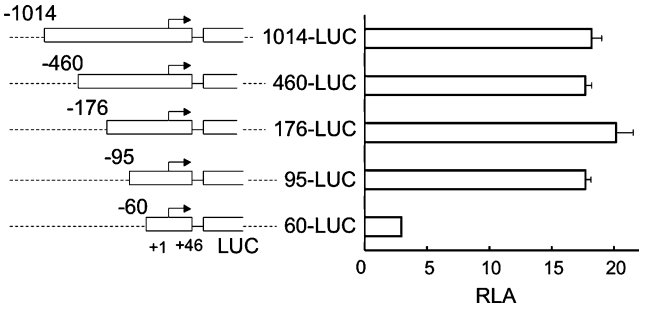

Fig. 3. Localization of the core promoter of the *CILP* gene. *CILP* reporter gene constructs and their luciferase activities in HCS-2/8 cells. Various lengths of promoter fragments (white boxes) were cloned into the pGL3 reporter vector. Positions of the 5′ nucleotides relative to the transcription start site (+1) of the *CILP* gene (GenBank Accession No. NM003613) are indicated above the boxes. Relative luciferase activity (RLA) is calculated as the ratio of luciferase activity from the experimental vector to that from the internal control vector. Sequences between −95 and −60 contain critical elements. Values represent means ± SEM of triplicate measurements.

almost complete promoter activity, while a further deletion to −60 showed remarkably decreased activity (Fig. 3). Consensus sequence analysis localized the TATA box between nucleotides −23 and −29.

Effect of SOX9 on CILP expression

SOX9 is the critical transcription factor in chondrogenesis. In concert with its co-activators SOX5 and SOX6, SOX9 regulates the expression of cartilage matrix genes including *COL2A1*, *COL11A1*, and *AGC1* [14–16]. To examine upstream factors in *CILP* expression, we tested the effects of these *SOX* genes on *CILP* expression in OUMS-27 cells. Expression of *CILP* depended neither on SOX9 alone nor on combinations of SOX9 with SOX5 or SOX6 (Fig. 4).

Effect of TGF-β1 on CILP expression

TGF-β1 is a key regulator of chondrocyte differentiation and proliferation, and it is known to induce *Cilp* expression in adult and young porcine chondrocytes [17]. We examined *CILP* mRNA expression over time in Huh-7 cells treated with TGF-β1. *CILP* expression peaked 24 h after addition of TGF-β1 (Fig. 5A). In various human cell lines, TGF-β1 markedly induced *CILP* mRNA expression in a dose-dependent manner (Figs. 5B–D).

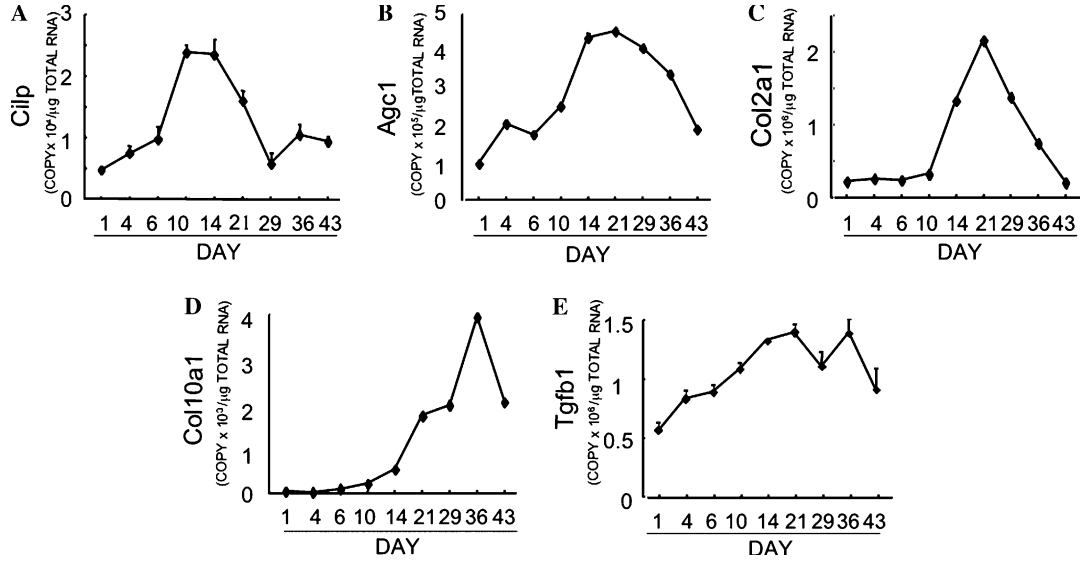

Fig. 2. *CILP* expression during chondrocyte differentiation. mRNA expression of *Cilp* (A), *Agc1* (B), *Col2a1* (C), *Col10a1* (D), and *Tgfb1* (E) during chondrocyte differentiation of ATDC5 cells was quantified by real-time PCR. *Cilp* expression parallels those of early chondrocyte differentiation markers and *Tgfb1*. Values represent means ± SEM of triplicate measurements.

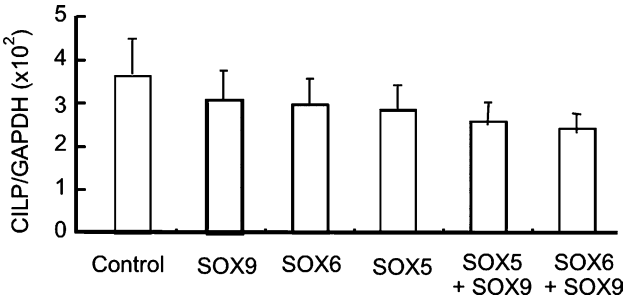

Fig. 4. *CILP* mRNA is not induced by SOX genes. Total RNA was isolated from OUMS-27 cells following transient transfection of SOX5, SOX6, SOX9, and SOX9 with SOX5 or SOX6. *CILP* mRNA levels were quantified by real-time PCR and normalized to *GAPDH* mRNA. The values represent means ± SEM of triplicate measurements.

SB-431542 is a selective inhibitor of TGF-β type 1-receptor kinase activity [18,19] (Fig. 6A). To further evaluate the pathway mediating *CILP* induction, we examined the effect of SB-431542 on TGF-β1-induced *CILP* expression in Huh-7 cells. SB-431542 inhibited the induction of *CILP* in a dose-dependent manner, with complete inhibition observed at 10 μM (Fig. 6B), indicating that induction occurs mainly via the TGF-β receptor.

TGF-β signaling pathways on CILP expression

Smad3 is the cardinal mediator of TGF-β1 signal in chondrogenic cells [20]. To examine whether *CILP* transcription is regulated by Smad3, we measured *CILP* mRNA expression in Huh-7 cells transiently transfected with Smad3. Real-time PCR analysis showed that Smad3 significantly induced *CILP* expression (Fig. 7A). In transient co-transfection assays using various *CILP* promoter constructs, luciferase activity decreased significantly for constructs lacking sequences between −3190 and −1014 (Fig. 7B), suggesting the presence of a Smad3-responsive element in this region. Smad3 specifically recognizes an identical 8-bp palindromic sequence (GTCTAGAC) [21]. Sequences between −1276 and −1269 (GTCTAGAG) and between −1873 and −1880 (CAGATCTA) are very similar to the Smad3 consensus sequence, and sequences between −2139 and −2146 (GACTAGGC) and between −2393 and −2404 (GGCTAGGC) each share 6 bp with the consensus sequence.

TGF-β signal can be mediated inside the cell by factors other than Smad3, including PI3 kinase, ERK1/2, protein kinase A, and p38 [22]. Therefore, we examined the contributions of these mediators to TGF-β1-stimulated *CILP* expression using their specific inhibitors. LY-294002 (PI3 kinase inhibitor), U-0126 (ERK1/2 inhibitor), H-89 (protein kinase A inhibitor), and SB-203580 (p38 inhibitor) showed maximal inhibition at concentrations of 0.1, 0.1, 1, and 10 μM, respectively. At these concentrations, the inhibitors decreased *CILP* mRNA expression by about half (Fig. 8). These results suggested that signaling pathways other than Smad are involved in *CILP* induction by TGF-β1.

Discussion

A key growth factor in chondrocyte metabolism, TGF-β influences matrix production, proliferation, and differentiation [20]. TGF-β1 induces *CILP* mRNA expression in porcine chondrocytes [17], and we have shown similar induction in human cells. *Cilp* mRNA expression parallels those of the early chondrocyte differentiation markers *Agc1* and *Col2a1*. Notably, each of these cartilage genes is regulated by SOX9, the cardinal transcription factor in chondrocytes [14–16], whereas *CILP* is not. *Tgfb1* mRNA

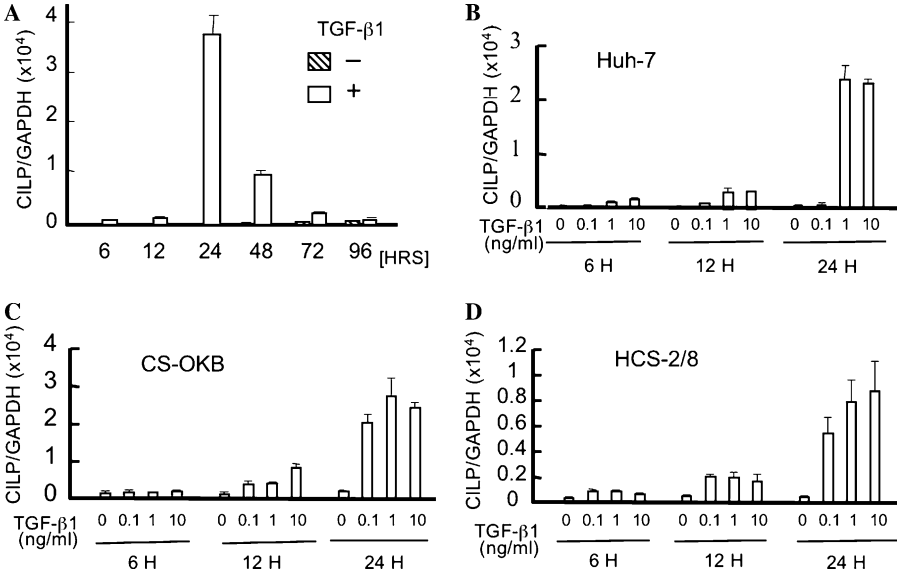

Fig. 5. *CILP* is induced by TGF-β1. (A) Induction of *CILP* mRNA after addition of TGF-β1 (1 ng/ml) in Huh-7 cells. (B–D) TGF-β1 induction of *CILP* mRNA in various cell lines. Values were normalized to *GAPDH* mRNA. The values represent means ± SEM of triplicate measurements.

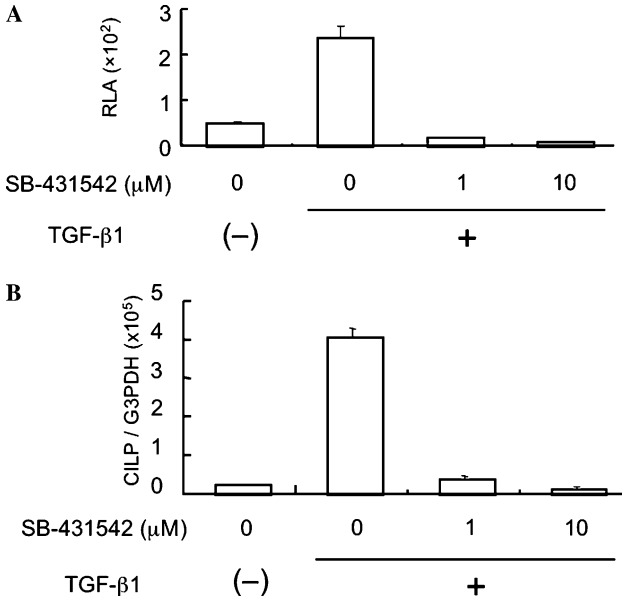

Fig. 6. TGF-β1-induction of *CILP* mRNA is mediated by the canonical TGF-β signal. (A) type I TGF-β receptor kinase inhibitor (SB-431542) inhibited the activity of the TGF-β-responsive reporter (SBE4-lux) in Huh-7 cells. RLA, relative reporter activity. (B) SB-431542 inhibited the TGF-β1 induction of *CILP*. Values represent means ± SEM of triplicate measurements.

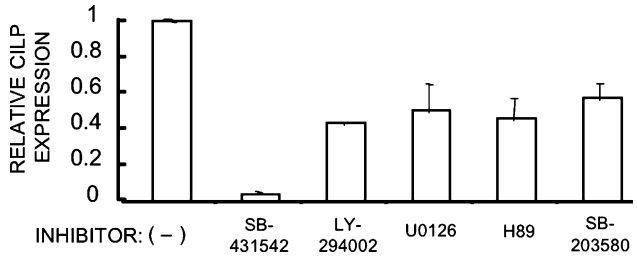

Fig. 8. Effects of TGF-β signaling inhibitors on *CILP* transcription. *CILP* mRNA levels in Huh-7 cells treated with inhibitors of TGF-β1 signaling were determined by quantitative real-time PCR. The expression level relative to the value in the absence of inhibitor is indicated. Values were normalized to *GAPDH* mRNA and represent means ± SEM of triplicate measurements.

expression also parallels that of *Cilp* during chondrocyte differentiation in ATDC5 cells, suggesting that their expression is linked in chondrogenesis. Conversely, CILP acts as a negative regulator of TGF-β1 in rabbit nucleus pulposus cells of the intervertebral disc and CILP protein binds TGF-β1 directly in vitro [5]. These lines of evidence suggest that CILP and TGF-β1 form a functional negative feedback loop. Therefore, production of CILP at appropriate levels is essential for chondrocyte homeostasis and important in the pathogenesis of bone and joint diseases.

We have shown that Smad3 directly induces *CILP*. Smad2 and Smad3 are major mediators of TGF-β signaling and associate directly with the TGF-β receptor complex. Activation of Tβ-RI by the ligand-bound type II TGF-β receptor (Tβ-RII) results in phosphorylation of associated Smad molecules by Tβ-RI [22]. Smad3 also mediates inhibitory effects of TGF-β1 on chondrocyte maturation [23]. Targeted disruption of *Smad3* in mice produces degenerative joint disease, as characterized by a progressive loss of

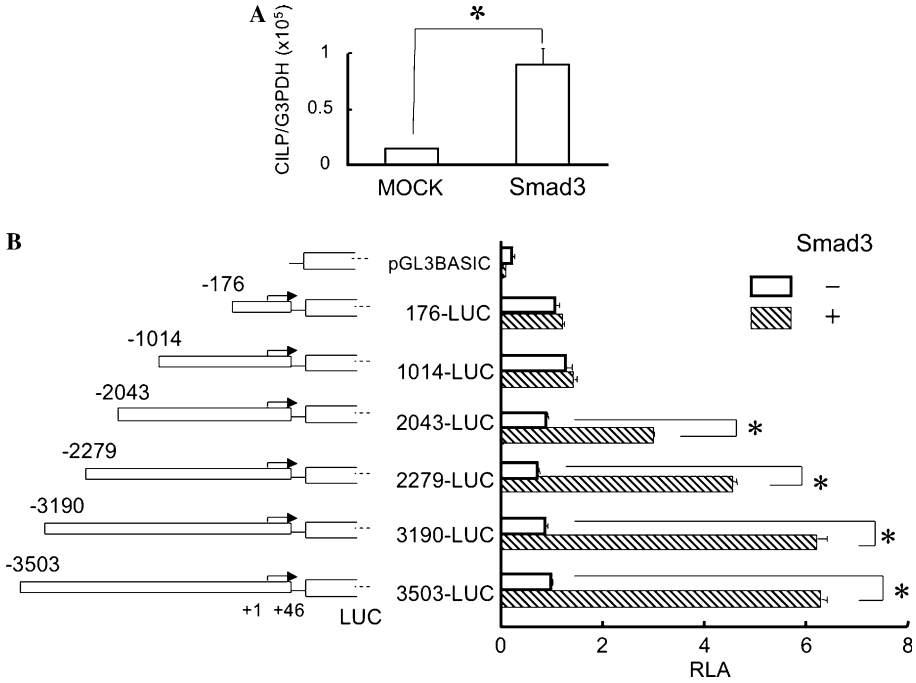

Fig. 7. *CILP* is regulated by Smad3. (A) *CILP* mRNA is induced by Smad3. Real-time PCR assay 48 h after Smad3 transfection in Huh-7 cells. Values represent means ± SEM of triplicate measurements. $^{*}P < 0.05$ (Mann–Whitney *U* test). (B) Smad3-responsive elements in the *CILP* promoter region. Smad3 induction of various 5′-deletion constructs of *CILP* promoter in Huh-7 cells. Smad3 (200 ng/well) was added to cells transfected with the deletion plasmids. Values represent means ± SEM of triplicate measurements. $^{*}P < 0.05$ (Mann–Whitney *U* test).

articular cartilage and decreased production of proteoglycans [24], indicating that Smad3 is a cardinal mediator of TGF-β signaling pathways that affect matrix gene expression in chondrocytes.

Our results indicate that intracellular effectors other than Smad3 are involved in *CILP* induction by TGF-β1. TGF-β regulates the expression of target genes through Smad-independent pathways, including ERK1/2, p38, p85-PI3 kinases and PKA. We have shown that Tβ-RI kinase activity is critical to induction of *CILP* by TGF-β. Smads are direct targets of the kinase activity, but ERK1/2, p38, and p85 are not [19,25]. Therefore, these intracellular signal mediators might associate with Smad or its downstream signaling cascade. ERK-dependent activation of Smad3 occurs through linker region phosphorylation of Smad3 in certain cell types [26]. The p38 pathway also affects linker region phosphorylation, and activation of the p38 MAP kinase pathway is necessary for TGF-β to induce the full transcriptional activation potential of Smad3 in a human breast cancer cell line [27]. The activated TβRI serine-threonine kinase can induce PI3 kinase activity through indirect association with p85 [25], and PI3 kinase modulates TGF-β signaling through a direct interaction with Smad3 [28]. TGF-β activates PKA independent of increased cAMP through formation of a complex between a PKA subunit and Smad [29]. Clarification of the cross-talk between these molecules and Smad in chondrocytes will be the focus of future research.

Acknowledgments

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References

[1] P. Lorenzo, P. Neame, Y. Sommarin, D. Heinegård, Cloning and deduced amino acid sequence of a novel cartilage protein (CILP) identifies a proform including a nucleotide pyrophosphohydrolase, J. Biol. Chem. 273 (1998) 23469–23475.

[2] K. Johnson, D. Farley, S.I. Hu, R. Terkeltaub, One of two chondrocyte-expressed isoforms of cartilage intermediate-layer protein function as an insulin-like growth factor 1 antagonist, Arthritis Rheum. 48 (2003) 1302–1314.

[3] A. Cardenal, I. Masuda, A.L. Haas, D.J. McCarty, Specificity of a porcine 127-kDa nucleotide pyrophosphohydrolase for articular tissues, Arthritis Rheum. 39 (1996) 245–251.

[4] I. Masuda, A. Cardenal, W. Ono, J. Hamada, A.L. Haas, D.J. McCarty, Nucleotide pyrophosphohydrolase in human synovial fluid, J. Rheumatol. 24 (1997) 1588–1594.

[5] S. Seki, Y. Kawaguchi, K. Chiba, Y. Mikami, H. Kizawa, T. Oya, F. Mio, M. Mori, Y. Miyamoto, I. Masuda, T. Tsunoda, M. Kamata, T. Kubo, Y. Toyama, T. Kimura, Y. Nakamura, S. Ikegawa, A functional SNP in CILP, encoding cartilage intermediate layer protein, is associated with susceptibility to lumber disc disease, Nat. Genet. 37 (2005) 607–612.

[6] I. Masuda, J. Hamada, A.L. Haas, L.M. Ryan, D.J. McCarthy, A unique ectonucleotide pyrophosphohydrolase associated with porcine chondrocyte-derived vesicles, J. Clin. Invest. 95 (1995) 699–704.

[7] I. Masuda, B.D. Halligan, J.T. Barbieri, A.L. Haas, L.M. Ryan, D.J. McCarty, Molecular cloning and expression of a porcine chondrocyte nucleotide pyrophosphohydrolase, Gene 197 (1997) 277–287.

[8] P. Lorenzo, M.T. Bayliss, D. Heinegård, Altered patterns and synthesis of extracellular matrix macromolecules in early osteoarthritis, Matrix Biol. 23 (2004) 381–391.

[9] A.M. Valdes, D.J. Hart, K.A. Jones, G. Surdulescu, P. Swarbrick, D.V. Doyle, A.J. Schafer, T.D. Spector, Association study of candidate gene for the prevalence and progression of knee osteoarthritis, Arthritis Rheum. 50 (2004) 2497–2507.

[10] Z. Yao, H. Nakamura, K. Masuko-Hongo, M. Suzuki-Kurokawa, K. Nishioka, T. Kato, Characterisation of cartilage intermediate layer protein (CILP)-induced arthropathy in mice, Ann. Rheum. Dis. 63 (2004) 252–258.

[11] J. Hirose, L.M. Ryan, I. Masuda, Up-regulated expression of cartilage intermediate-layer protein and ANK in articular hyaline cartilage from patients with calcium pyrophosphate dihydrate crystal deposition disease, Arthritis Rheum. 46 (2002) 3218–3229.

[12] M. Takigawa, K. Tajima, H.O. Pan, M. Enomoto, A. Kinoshita, F. Suzuki, Y. Takano, Y. Mori, Establishment of a clonal human chondrosarcoma cell line with cartilage phenotypes, Cancer Res. 49 (1989) 3996–4002.

[13] C. Shukunami, C. Shigeno, T. Atsumi, K. Ishizeki, F. Suzuki, Y. Hiraki, Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor, J. Cell. Biol. 133 (1996) 457–468.

[14] V. Lefebvre, P. Li, B. de Crombrugghe, A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activated the type II collagen gene, EMBO J. 17 (1998) 5718–5733.

[15] B. de Crombrugghe, V. Lefebvre, R.R. Behringer, W. Bi, S. Murakami, W. Huang, Transcriptional mechanisms of chondrocyte differentiation, Matrix Biol. 19 (2000) 389–394.

[16] T. Ikeda, S. Kamekura, A. Mabuchi, I. Kou, S. Seki, T. Takato, K. Nakamura, H. Kawaguchi, S. Ikegawa, U.I. Chung, The combination of Sox5, Sox6, and Sox9 (the Sox trio) provides signals sufficient for induction of permanent cartilage, Arthritis Rheum. 50 (2004) 3561–3573.

[17] J. Hirose, I. Masuda, L.M. Ryan, Expression of cartilage intermediate layer protein / nucleotide pyrophosphohydrolase parallels the production of extracellular inorganic pyrophosphate in response to growth factors and with aging, Arthritis Rheum. 43 (2000) 2703–2711.

[18] N.J. Laping, E. Grygielko, A. Mathur, S. Butter, J. Bomberger, C. Tweed, W. Martin, J. Fornwald, R. Lehr, J. Harling, L. Gaster, J.F. Callahan, B.A. Olson, Inhibition of transforming growth factor (TGF)-β1-induced extracellular matrix with a novel inhibitor of the TGF-β type I receptor kinase activity: SB-431542, Mol. Pharmacol. 62 (2002) 58–64.

[19] G.J. Inman, F.J. Nicolás, J.F. Callahan, J.D. Harling, L.M. Gaster, A.D. Reith, N.J. Laping, C.S. Hill, SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7, Mol. Pharmacol. 62 (2002) 65–74.

[20] E. Grimaud, D. Heymann, F. Rédini, Recent advances in TGF-β effects on chondrocyte metabolism. Potential therapeutic roles of TGF-β in cartilage disorders, Cytokine Growth Factor Rev. 13 (2002) 241–257.

[21] L. Zawel, J.L. Dai, P. Buckhaults, S. Zhou, K.W. Kinzler, B. Vogelstein, S.E. Kern, Human Smad3 and Smad4 are sequence-specific transcription activators, Mol. Cell 1 (1998) 611–617.

[22] R. Derynck, Y.E. Zhang, Smad-dependent and Smad-independent pathways in TGF-β family signaling, Nature 425 (2003) 577–584.

[23] C.M. Ferguson, E.M. Schwart, P.R. Reynolds, J.E. Puzas, R.N. Rosier, R.J. O'keefe, Smad2 and 3 mediate transforming factor-β1-induced inhibition of chondrocyte maturation, Endocrinology 141 (2000) 4728–4735.
[24] X. Yang, L. Chen, X. Xu, C. Li, C. Huang, C.X. Deng, TGF-β/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage, J. Cell Biol. 153 (2001) 35–46.
[25] J.Y. Yi, I. Shin, C.L. Arteaga, Type I transforming growth factor β receptor binds to and activates phosphatidylinositol 3-kinase, J. Biol. Chem. 280 (2005) 10870–10876.
[26] T. Hayashida, M. deCaestecker, H.W. Schnaper, Cross-talk between ERK MAP kinase and Smad-signaling pathway enhances TGF-β dependent responses in human mesangial cells, FASEB J. 17 (2003) 1576–1578.
[27] A.K. Kamaraju, A.B. Roberts, Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-β-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo, J. Biol. Chem. 280 (2005) 1024–1036.
[28] I. Remy, A. Montmarquette, S.W. Michnick, PKB/Akt modulates TGF-β signalling through a direct interaction with Smad3, Nat. Cell Biol. 6 (2004) 358–365.
[29] L. Zhang, C.J. Duan, C. Binkley, G. Li, M.D. Uhler, C.D. Logsdon, D.M. Simeone, A transforming growth factor β-induced Smad3/Smad4 complex directly activates protein kinase A, Mol. Cell. Biol. 24 (2004) 2169–2180.