Published in final edited form as:

Gene. 2013 March 15; 517(1): 12–18. doi:10.1016/j.gene.2013.01.001.

Bovine lactoferricin induces *TIMP-3* via the ERK1/2-Sp1 axis in human articular chondrocytes

Dongyao Yan¹, Di Chen¹, John R Hawse⁵, Andre J van Wijnen⁶, and Hee-Jeong Im^{1,2,3,4,*}

¹Department of Biochemistry, Section of Rheumatology, Rush University Medical Center, Chicago, IL 60612

²Department of Orthopedic Surgery, Section of Rheumatology, Rush University Medical Center, Chicago, IL 60612

³Department of Internal Medicine, Section of Rheumatology, Rush University Medical Center, Chicago, IL 60612

⁴Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60612

⁵Department of Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN 55905

⁶Department of Orthopedics, Mayo Clinic, Rochester, MN 55905

Abstract

Bovine lactoferricin (LfcinB) is a heparan sulfate-binding peptide with multiple bioactivities. In human articular cartilage, LfcinB antagonizes interleukin-1 β (IL-1 β) and fibroblast growth factor 2 (FGF-2) in proteoglycan metabolism, catabolic protease expression, and induction of pro-inflammatory mediators. LfcinB specifically activates ERK1/2, p38 and Akt, but whether these signaling pathways control the expression of LfcinB target genes remained unknown. In this report, we characterized a novel aspect of LfcinB-mediated genetic response in human articular chondrocytes, tissue inhibitor of metalloproteinase 3 (*TIMP-3*) induction. Inhibition of individual signaling pathways revealed that ERK1/2 functions as the major pathway in *TIMP-3* expression, whereas Akt plays a minor role. Further investigation identified Sp1 as a critical transcriptional activator in *TIMP-3* regulation, and Sp1 activity is modulated by ERK1/2, not Akt. Comparative quantification indicates significant downregulation of *TIMP-3* occurs in OA chondrocytes, suggesting a beneficial role of LfcinB in OA pathogenesis. Our results collectively provide new insights into the mechanism of action of LfcinB, and support the candidacy of LfcinB as a chondroprotective agent.

Keywords

Bovine lactoferricin; Osteoarthritis; Tissue inhibitor of metalloproteinase; Articular cartilage; Chondrocyte

© 2012 Elsevier B.V. All rights reserved.

*Correspondence to: Hee-Jeong Im, Ph.D., Cohn Research BD 516, 1735 W. Harrison, Rush University Medical Center, Chicago, IL 60612, 312-942-3091 (Tel), 312-942-3053 (fax), Hee-Jeong_Sampen@rush.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

COMPETING INTERESTS

The authors declare that there is no competing interest.

1. INTRODUCTION

Articular cartilage homeostasis is maintained by a delicate balance between catabolic and anabolic activities. In osteoarthritis (OA), chondrocytes shift their metabolism to a catabolism-dominant state under the influence of various noxious stimuli. Due to the avascularity of articular cartilage and the spatial separation of resident chondrocytes, articular cartilage has a very limited capacity to heal itself. Under the circumstances where chondral defects are “repaired” *via* intrinsic mechanisms, fibrocartilage occurs and serves as a poor substitute for the original hyaline cartilage. Besides cartilage degeneration, OA also possesses an inflammatory component in synovium, which cooperates to perpetuate disease progression [Kapoor et al., 2011]. It is therefore of importance to devise therapies targeting either or both of these processes.

A wide array of mediators, including cytokines, proteases, and endogenous protease inhibitors, has been implicated in OA pathogenesis. Interleukin-1 β (IL-1 β) is considered as one of the major drivers in OA. In chondrocytes, IL-1 β inhibits the synthesis of extracellular matrix (ECM) proteins and stimulates the production and release of ECM-degrading proteases (e.g., collagenases and aggrecanases) [Kapoor et al., 2011]. IL-1 β also induces other inflammatory mediators (e.g., IL-6 and IL-8) in chondrocytes and synoviocytes, thus further amplifying detrimental cellular responses [Kapoor et al., 2011]. The induced protease activities are mitigated by endogenous inhibitors, most notably tissue inhibitors of metalloproteinases (TIMPs). Among the four TIMP family members, TIMP-3 appears to be particularly relevant to OA, because it is capable of quenching the activities of aggrecanases, gelatinases, a disintegrin and metalloproteinases (ADAMs), and membrane-bound matrix metalloproteinases (MT-MMPs) [Butler et al., 1999; Nagase et al., 2006; Will et al., 1996; Zhao et al., 2004]. The critical role of TIMP-3 in maintaining cartilage homeostasis was explicitly demonstrated in TIMP-3^{-/-} mice, where spontaneous degradation of collagen and aggrecan was evident [Sahebjam et al., 2007]. Hence an imbalance between proteases and TIMPs may account for elevated ECM degeneration in OA.

Anti-cytokine therapy for OA is a feasible strategy which is being actively explored [Calich et al., 2010]. Previously we discovered bovine lactoferricin (LfcinB), a 25-amino acid heparan sulfate-binding peptide derived from bovine lactoferrin, exerts protective biological effects in bovine nucleus pulposus cells and human articular chondrocytes [Kim et al., 2012; Yan et al., 2012]. In human chondrocytes, LfcinB inhibits the catabolic actions mediated by FGF-2 and IL-1 β in proteoglycan accumulation and induction of ECM-degrading proteases and pro-inflammatory mediators [Yan et al., 2012]. LfcinB alone also downregulates these proteases and inflammatory factors, and upregulates anti-inflammatory cytokines (i.e., IL-4 and IL-10) [Yan et al., 2012]. The observation that LfcinB specifically activates ERK1/2, p38 and Akt pathways inspired the question whether LfcinB utilizes these pathways to regulate its target genes in chondrocytes [Yan et al., 2012]. In addition, LfcinB exerts similar protective effects in synovial fibroblasts, suggesting this peptide is a potential candidate for novel OA therapy.

In this study, we uncovered a new mechanism whereby LfcinB promotes anti-catabolism in articular chondrocytes. We determined the signaling pathway and transcription factor responsible for LfcinB-mediated genetic response. We also revealed the pathological relevance of such target gene regulation to OA therapy.

2. MATERIAL AND METHODS

2.1 Materials

LfcinB was purchased from BioSynthesis (Lewisville, TX). TIMP-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody were purchased from Abcam (Cambridge, MA). Specificity protein 1 (Sp1) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, SB203580, LY294002, and Akt inhibitor IV were purchased from EMD Chemicals (Gibbstown, NJ). WP631 was purchased from Sigma (St. Louis, MO). Small interfering RNA (siRNA) targeting *SP1* was acquired from Life Technologies (Carlsbad, CA).

2.2 Tissue acquisition and chondrocyte culture

Normal human femoral articular cartilage (age ranging from 40 to 70) was obtained within 72 hours after death through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) with prior approval by the local ethics committee and consent from families. Before dissection each specimen was graded based on a modified 5-point scale of Collins [Muehleman et al., 1997]. Osteoarthritic femoral and tibial cartilage was obtained from patients (age ranging from 40 to 70) through the Orthopedic Tissue and Implant Repository Study (Chicago, IL) with consent from the patients. Human tissues were processed according to the guidelines of the Human Investigation Committee of Rush University Medical Center. Unless specified, cartilage for this study was graded 0 or 1. After aseptic dissection cartilage was digested in DMEM/Ham's F-12 (1:1) media with 0.2% Pronase for 1 hour, followed by overnight digestion with 0.025% Collagenase P supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO₂ and continuous agitation.

Chondrocytes released from enzymatic digestion were resuspended to a density of 3×10^6 cells/mL in DMEM/Ham's F-12 media (1:1) supplemented with 10% FBS (complete media). For short-term monolayer culture, cells were then plated onto 6-well (2 mL/well), 12-well (1 mL/well) or 24-well (0.5 mL/well) plates. After 3-day culture, the media were replaced with serum-free DMEM/Ham F-12 media (1:1). After another 24 hours, culture media were replaced with fresh serum-free media again, 2 hours prior to treatments. LfcinB concentrations used in monolayer culture were 50 µg/mL and 100 µg/mL. Cells were harvested after 24-hour stimulation, and subjected to downstream analyses as detailed below.

2.3 Immunoblotting

Cell lysates were prepared using a modified RIPA buffer [Im et al., 2007]. Total protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Equal amounts of protein were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane for immunoblotting as described previously [Im et al., 2007]. Immunoreactivity was visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ) and the Signal Visual Enhancer system (Pierce, Rockford, IL) to magnify the signal.

2.4 Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction

Total RNA from normal and osteoarthritic articular chondrocytes was isolated using Trizol reagent (Life Technologies, Carlsbad, CA) according to the instructions provided by the manufacturer. RT was carried out with 1 µg total RNA using ThermoScript™ RT-PCR system (Life Technologies, Carlsbad, CA) for first-strand cDNA synthesis. For real-time PCR, cDNA was amplified using a MyiQ Real-Time PCR Detection System (Bio-Rad,

Hercules, CA). Relative gene expression was determined using the $\Delta\Delta C_T$ method, as detailed by manufacturer guidelines (Bio-Rad, Hercules, CA). 18S rRNA and GAPDH were used as internal controls in the reactions for normalization. *TIMP-3* primer sequences are: F: 5'-TTCTGCAACTCCGACATCGT-3'; R: 5'-TTGATGGTGTAGACCAGCGT-3'. The standard deviations in samples represent at least five different donors from independent experiments.

2.5 Preparation of Nuclear Extract

A nuclear extraction kit (Affymetrix, Santa Clara, CA) was used based on the manufacturer's instructions. Upon the completion of stimulation, media were removed, and washed cells were lysed in 1 mL buffer A. After incubation on ice for 10 min, lysates were centrifuged at 14000×g, 4°C for 3 min. Supernatants were discarded, and cell pellets were resuspended in 40 µl buffer B. Then samples were incubated on ice for 1 hour before centrifugation at 14000×g, 4°C for 5 min. The supernatants were aliquoted and stored at -80°C. Total protein concentration in each sample was determined by BCA assay (Pierce, Rockford, IL) right before electrophoretic mobility shift assay (EMSA).

2.6 EMSA

The nuclear extracts were prepared after stimulation as described above. The EMSA kit (Affymetrix, Santa Clara, CA) was used according to the manufacturer's instructions. EMSA was performed by incubating labeled biotin-conjugated probes with 5 µg of nuclear extract. Samples were resolved in 6% non-denaturing polyacrylamide gels. Then proteins were transferred to Pall Biodyne B membrane. The membrane was blocked with 1× blocking buffer, and then incubated with streptavidin-HRP conjugate. After proper washing, the signals were visualized in a chemiluminescence-imaging system. In each experiment, a separate reaction using unlabeled double-stranded DNA (cold probe) was set up to demonstrate binding specificity.

2.7 Transfection of siRNA

Nucleofection was optimized for human articular chondrocytes based on the manual of the Nucleofector™ kit (Lonza, Walkersville, MD) as described previously [Loeser et al., 2005; Pulai et al., 2005]. Chondrocytes were cultivated for 3 days before transfection. Sp1 siRNA at a concentration of 200 nM (20 pmol/sample) was used for transfection. After 48 hours, cell lysates were subjected to quantitative polymerase chain reaction (qPCR) and immunoblotting for validation of successful knockdown. In parallel, stimulations were performed 48 hours after transfection. Cell lysates and total RNA were collected for downstream analyses.

2.8 *TIMP-3* Luc Promoter Reporter Construct and Promoter Activity Assay

A 1620 bp fragment of the mouse *TIMP-3* promoter, spanning from -1473 to +146 relative to the transcriptional start site, was PCR amplified from C57BL/6 mouse tail genomic DNA. PCR primers were engineered to include an XhoI site on the forward primer and a HindIII site on the reverse primer. Primer sequences were as follows: F: 5'-ATACTCGAGACTACTTAATGATACTCTGTCTCAAA-3' and R: 5'-ATAAAGCTTTGTGAGCGAGAGTCCAGGGCTGC-3'. The resulting PCR product was gel purified, subjected to XhoI and HindIII restriction digest overnight, cloned into the PGL4.1 basic luciferase reporter construct and subsequently sequenced to ensure proper orientation and integrity.

For promoter activity assays, 0.5 µg of Renilla luciferase construct and 2 µg of *TIMP-3*-Luc luciferase construct were transfected into each sample using the Nucleofection protocol.

Stimulations were carried out in serum-free condition 48 hours after transfection. Cell lysates were then prepared and assayed for luciferase activity using a dual luciferase reporter assay system according to the manufacturer's instructions (Promega, San Luis Obispo, CA). The luciferase signal was normalized with the Renilla signal from the same sample to yield a relative ratio.

2.9 Statistical Analyses

Statistical significance was determined by Student's t-test, or one-way repeated measures ANOVA followed by Sidak *post-hoc* test, using the SPSS 17 software (IBM Corporation, Somers, NY). P values lower than 0.05 were considered to be statistically significant in each test. Each value in the figures is presented as mean $\pm$ standard deviation.

3. RESULTS

3.1 LfcinB potently induces TIMP-3, a natural inhibitor of multiple cartilage degrading enzymes in human primary articular chondrocytes

Our previous studies revealed that LfcinB represses the basal expression of cartilage-degrading proteases (eg., *MMP-13* and *ADAMTS-5*) and pro-inflammatory mediators (eg., *IL-1 β* , *IL-8* and *TLR2*) [Yan et al., 2012]. Furthermore, LfcinB efficiently counteracts the catabolic and inflammatory activities promoted by IL-1 β and FGF-2 in articular cartilage [Yan et al., 2012]. These anti-catabolic action mediated by LfcinB is *via* activation of the ERK1/2 MAPK and Akt pathways [Yan et al., 2012]. Our promising results motivated us to further investigate other potential target genes that are associated with articular cartilage homeostasis by LfcinB. Because LfcinB exerts potent anti-catabolism in articular cartilage, we hypothesized that LfcinB induces anti-catabolic and anti-inflammatory factors to exert chondroprotective effects. Human chondrocytes incubated with two concentrations of LfcinB (50 and 100 μ g/mL) significantly upregulated *TIMP-3* (4- and 8-fold; $p < 0.05$, $p < 0.01$, respectively) at mRNA and protein levels (Figures 1A & 1B; $p < 0.01$). Our observation is quite exciting because among all TIMP family members (TIMP1~4), TIMP-3 plays a unique role in articular cartilage homeostasis, acting as a potent natural inhibitor of MMPs, ADAMs, ADAMTS4, ADAMTS5 and tumor necrosis factor convertase (TACE, ADAM-17), which are not inhibited by other TIMPs [Apte et al., 1995].

3.2 TIMP-3 expression level is significantly downregulated in osteoarthritic chondrocytes compared to normal and early-stage OA condition

Using age-matched samples (40~70-year-old), we found that *TIMP-3* expression was significantly down-regulated in OA chondrocytes compared to normal or early-stage OA cells (grade 0 or 1) at both mRNA and protein levels (Figures 2A & 2B; $p < 0.01$). The 27 kDa and 24 kDa form represents glycosylated and unmodified TIMP-3, respectively, as previously reported [Apte et al., 1995]. Considering the significant role of TIMP-3 in articular cartilage homeostasis, the significant stimulation of *TIMP-3* by LfcinB serves as a part of the mechanistic basis for its chondroprotective activities, and again suggests the possibility of applying LfcinB in future OA treatment.

3.3 LfcinB induces TIMP-3 via the ERK1/2-Sp1 axis

Considering the dramatic response of *TIMP-3* upon LfcinB treatment, we pursued to delineate the signaling pathway and transcriptional mechanism which govern this process. Previously we identified ERK1/2 and Akt as two major LfcinB-induced specific signaling nodes in human articular chondrocytes [Yan et al., 2012]. In the current study, we also observed short-lived p38 MAPK activation after LfcinB stimulation. In keeping with our previous report [Yan et al., 2012], JNK was not activated by LfcinB in human articular

chondrocytes (data not shown). To associate the *TIMP-3* response with its responsible signaling pathway in the presence of LfcinB, we pretreated chondrocytes with individual pathway-specific pharmacological inhibitors of ERK, p38 and Akt before LfcinB stimulation. Our data show that ERK1/2 inhibition resulted in a marked reversal of the *TIMP-3* induction (Figure 3A; $p < 0.01$). Compared with the effect of ERK1/2 inhibition, inhibition of Akt led to only a moderate reversal of *TIMP-3* induction by LfcinB, though it was statistically significant (Figure 3A; $p < 0.05$). On the other hand, pharmacological inhibitor study suggests that p38 pathway had no influence on *TIMP-3* induction by LfcinB. These findings suggest that LfcinB-mediated *TIMP-3* upregulation is mainly controlled by the ERK1/2 MAPK pathway, while Akt plays a minor role, in human adult articular chondrocytes.

We then endeavored to determine the critical transcription factor responsible for the *TIMP-3* induction by LfcinB. It was reported that TGF- β induces *TIMP-3* in an Sp1-dependent manner [Qureshi et al., 2005]. Analysis of *TIMP-3* promoter revealed four Sp1 elements located within the -1 to -120 region (Figure 3B), which had been reported to be functional [Wick et al., 1995]. We therefore hypothesized that Sp1 participates in *TIMP-3* induction by LfcinB. To determine the involvement of Sp1, we knocked down Sp1 activity in human articular chondrocytes by either pre-incubation with Sp1 pharmacological inhibitor WP631 (50 nM) or transfection with siRNA targeting *Sp1*. The siRNA efficiently suppressed Sp1 expression on protein level (Figure 3C). Then cells were subjected to LfcinB (50 μ g/mL) stimulation for 24 hour. Both Sp1 inhibitor (WP631) and siRNA abolished LfcinB-mediated stimulation of *TIMP-3* on mRNA level, confirming the participation of Sp1 (Figure 3D; $p < 0.05$). A pharmacological inhibitor of ERK1/2 also resulted in a similar effect, suggesting a connection between ERK1/2 and Sp1 in this particular context (Figure 3D; $p < 0.05$). The changes in *TIMP-3* mRNA expression after ERK1/2 or Sp1 inhibition also translated to changes in its protein levels, which further supports the hypothetical roles of ERK1/2 and Sp1 in *TIMP-3* induction (Figure 3E).

To directly assess the contribution of ERK1/2 and Sp1 to *TIMP-3* transcription, human articular chondrocytes were transfected with a *TIMP-3* luciferase reporter construct by Nuclofection. Cells were then pre-incubated with the pharmacological inhibitor of Sp1 (WP631) or ERK1/2, followed by LfcinB stimulation. After normalization with renilla signal, our data show that both Sp1 inhibitor and ERK1/2 inhibitor were able to significantly suppress *TIMP-3* promoter-driven luciferase activity enhanced by LfcinB (Figure 4; $p < 0.01$). Next, we performed EMSA to assess changes in the DNA binding affinity of Sp1 under different conditions. Human primary articular chondrocytes were pre-incubated with individual pharmacological inhibitors of ERK1/2, p38, and Akt, followed by LfcinB stimulation. We observed that the binding of Sp1 protein to the Sp1 consensus DNA sequences was markedly enhanced 1 hour after LfcinB treatment, and such activation was blocked by ERK1/2 inhibition (*lane 3*) but not by p38 (*lane 4*) or Akt inhibition (*lane 5*) (Figure 5). The binding specificity was confirmed by successful cold competition using unlabeled Sp1 probes (*lane 6*). Taken together, our results demonstrate that LfcinB induces *TIMP-3* via the ERK1/2-Sp1 axis in articular chondrocytes.

4. Discussion

Previously we showed that LfcinB regulates the basal expression of multiple targets, and it is able to activate three signaling pathways, ERK1/2, p38 and Akt [Yan et al., 2012]. In the current study, we identified that *TIMP-3* is markedly regulated by LfcinB in human primary adult articular chondrocytes. We demonstrate that *TIMP-3* induction by LfcinB is controlled by the ERK1/2-Sp1 axis, which highlights the significance of this pathway. Comparative analysis of *TIMP-3* expression between normal and OA chondrocytes revealed that *TIMP-3*

is significantly repressed in OA cells, thus, apart from the role of LfcinB-induced anti-inflammatory action [Yan et al., 2012], *TIMP-3* induction may add additional benefits to cartilage repair in this regard.

MMPs and aggrecanases produced by chondrocytes cleave a predominant fraction of ECM components. Despite the augmented proteolytic activities in OA cartilage, there are a very limited number of endogenous inhibitors that target these ECM-degrading enzymes. TIMPs play a central role in restricting the enzymatic activities due to their complimentary target spectrums. In particular, *TIMP-3* inhibits MT1-MMP, which is essential to proMMP-13 activation [Knauper et al., 1996]. Moreover, *TIMP-3* is the only endogenous inhibitor of aggrecanases known to date [Kashiwagi et al., 2001]. Accordingly, *TIMP-3* prevents glycosaminoglycan release from cartilage *ex vivo* [Gendron et al., 2003]. However, previous studies suggest that an imbalance exists between MMPs and TIMPs in arthritides, which favors the MMPs [Tchetverikov et al., 2005]. Our data also indicate that *TIMP-3* is downregulated on both mRNA and protein levels in OA chondrocytes, which may contribute to the unrestrained activities of MMPs in OA. Therefore, LfcinB-mediated *TIMP-3* induction may, at least in part, restore the balance between catabolic and anti-catabolic processes in articular cartilage, thus conferring chondroprotection. Such dramatic induction of *TIMP-3* can also explain why LfcinB is able to preserve the proteoglycan content in the presence of IL-1 β or FGF-2 in cartilage explants in *ex vivo* [Yan et al., 2012].

We previously hypothesized that signaling pathways triggered by LfcinB (i.e., ERK1/2, p38 and Akt) participate in the regulation of its target genes [Yan et al., 2012]. The data presented herein provide direct evidence that ERK1/2 functions as the major pathway controlling *TIMP-3* expression by LfcinB. The robustness of ERK1/2 activation upon LfcinB stimulation can partially explain the striking magnitude of *TIMP-3* induction. It is worth noting that LfcinB may not activate the canonical ERK1/2 pathway, as seen in the case of FGF-2, in human articular chondrocytes. For example, LfcinB-induced ERK1/2 activation does not change the phosphorylation level of Elk-1, a well-established transcription factor downstream of ERK1/2 [Ellman et al., 2008; Muddasani et al., 2007]. FGF-2-induced ERK1/2 activation is associated with *MMP-13* expression [Im et al., 2007], whereas LfcinB significantly downregulates *MMP-13* [Kim et al., 2012; Yan et al., 2012]. Although it remains poorly characterized how LfcinB utilizes ERK1/2 signaling to promote protective processes in chondrocytes, we conjecture that LfcinB uses a subset of heparan sulfate proteoglycans and signaling cofactors to achieve specificity. Future identification of these mediators would shed more light on this topic.

Our findings connect Sp1 transcription factor with ERK1/2 in the context of LfcinB-mediated *TIMP-3* induction. Sp1 is a well known target of ERK1/2. Two residues of Sp1, Thr453 and Thr739, are bona fide phosphorylation sites of ERK1/2 [Milanini-Mongiat et al., 2002]. Unexpectedly, when we examined Sp1 phosphorylation after LfcinB stimulation, we found that there was no notable change at the Thr453 residue (data not shown). Nonetheless, Sp1 binding to DNA was markedly enhanced in an ERK1/2-dependent manner upon LfcinB stimulation, suggesting that Sp1 activity in this scenario is independent of Thr453. It remains a possibility that Thr739, a residue critical for synergistic activation of Sp1-mediated transcription and interaction with cofactors [Chu and Ferro, 2005], is regulated by LfcinB. Whether ERK1/2 further activates other kinases to modulate Sp1 activity also requires investigation.

The *TIMP-3* promoter is configured in such a way that it is responsive to input from Sp1 [Wick et al., 1995]. The functional significance of Sp1 elements within the *TIMP-3* promoter has been demonstrated in TGF- β 1-stimulated chondrocytes, where Sp1 depletion abolished *TIMP-3* expression [Qureshi et al., 2005]. In our current study, inhibition or

depletion of Sp1 largely negated LfcinB-mediated *TIMP-3* response, suggesting a strong dependence on this transcription factor. It is probable that LfcinB concomitantly activates other transcription factors or cofactors to not only cooperate with Sp1, but also achieve transcriptional specificity. As an Sp1-responsive gene, *COL2A1* expression is not regulated by LfcinB [Chadjichristos et al., 2002], suggesting additional nuclear factors may be involved. In addition, our data suggest that, although the Akt pathway participates in the regulation of *TIMP-3* by LfcinB, it is not associated with Sp1 activation on the transcriptional level. Akt has been previously shown to activate mammalian target of rapamycin (mTOR), which in turn activates p70S6 kinase to induce *TIMP-3* upon TGF- β stimulation [Qureshi et al., 2007]. It is therefore of interest to determine whether this pathway is utilized by LfcinB. For example, further studies are warranted to define whether LfcinB stimulates TGF- β pathways, thereby activating consecutive signaling events (Akt-mTOR-p70S6K) to upregulate *TIMP-3* in human articular cartilage.

In addition to protease inhibition, anti-cytokine therapy appears to be an attractive alternative for OA. Much effort has been made towards the inhibition of IL-1 action using IL-1 receptor antagonist (IL-1ra), based on abundant evidence from *in vitro* and *in vivo* studies. However, randomized trials with IL-1ra produced negative outcomes [Chevalier et al., 2011]. Although pharmacological caveats exist in these trials, these findings demand development of novel strategies to target critical cytokines in OA. Our characterization of LfcinB revealed that this peptide represents another viable way to counteract IL-1 in human synovial joint. LfcinB effectively inhibits IL-1 β -mediated processes in cartilage and synovium, including ECM depletion, cartilage-degrading protease induction, and local inflammation [Yan et al., 2012]. LfcinB also antagonizes FGF-2, which is another pro-catabolic inducer upregulated in OA [Im et al., 2007; Yan et al., 2012]. Based on our current study, LfcinB-mediated *TIMP-3* expression may further dampen the proteolytic activities in articular cartilage, thus conferring additional protection. Importantly, the molecular weight of LfcinB is considerably lower than that of IL-1ra, which likely results in better tissue penetration and hence higher bioavailability. The relatively simple structure of LfcinB also renders it amenable to chemical modifications, such as depeptidization, which would potentially prolong its half-life *in vivo*.

5. CONCLUSION

In this study, we further characterized the bioactivities of LfcinB in human articular cartilage. By means of upregulating *TIMP-3*, LfcinB attenuates endogenous collagenolytic and aggrecanolytic activities and exerts chondroprotection. Such *TIMP-3* induction depends on the functional ERK1/2-Sp1 signaling axis, which directly utilizes the Sp1 elements within the *TIMP-3* promoter. Our findings delineate an important aspect of LfcinB-mediated genetic response, and lend further support to the hypothesis that LfcinB can be used as a therapeutic agent in OA.

Acknowledgments

This work was supported by NIH NIAMS R01 grants (to HJI) from AR053220 and AR062136, and R01 grant from the National Institute of Dental and Craniofacial Research: DE014036 (to JRH). We thank the Gift of Hope Organ Tissue Donor Network and Dr. Margulis for making human tissues available, and we also extend our appreciation to the tissue donor families who made it possible. We thank Dr. Gabriella Cs-Szabo and David Gerard for their time and efforts in OA tissue acquisition.

ABBREVIATIONS

OA osteoarthritis

IL-1β	interleukin-1 β
FGF-2	fibroblast growth factor 2
ECM	extracellular matrix
TIMP	tissue inhibitor of metalloproteinase
ADAM	a disintegrin and metalloproteinase
MMP	matrix metalloproteinase
LfcinB	bovine lactoferricin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
siRNA	small interfering RNA
BCA	bicinchoninic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
EMSA	electrophoretic mobility shift assay
IL-1ra	IL-1 receptor antagonist

References

- Apte SS, Olsen BR, Murphy G. The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. *J Biol Chem.* 1995; 270:14313–14318. [PubMed: 7782289]
- Butler GS, Apte SS, Willenbrock F, Murphy G. Human tissue inhibitor of metalloproteinases 3 interacts with both the N- and C-terminal domains of gelatinases A and B. Regulation by polyanions. *J Biol Chem.* 1999; 274:10846–10851. [PubMed: 10196161]
- Calich AL, Domiciano DS, Fuller R. Osteoarthritis: can anti-cytokine therapy play a role in treatment? *Clin Rheumatol.* 2010; 29:451–455. [PubMed: 20108016]
- Chadjichristos C, Ghayor C, Herrouin JF, Ala-Kokko L, Suske G, Pujol JP, Galera P. Down-regulation of human type II collagen gene expression by transforming growth factor-beta 1 (TGF-beta 1) in articular chondrocytes involves SP3/SP1 ratio. *J Biol Chem.* 2002; 277:43903–43917. [PubMed: 12186868]
- Chevalier X, Conrozier T, Richette P. Desperately looking for the right target in osteoarthritis: the anti-IL-1 strategy. *Arthritis Res Ther.* 2011; 13:124. [PubMed: 21888686]
- Chu S, Ferro TJ. Sp1: regulation of gene expression by phosphorylation. *Gene.* 2005; 348:1–11. [PubMed: 15777659]
- Ellman MB, An HS, Muddasani P, Im HJ. Biological impact of the fibroblast growth factor family on articular cartilage and intervertebral disc homeostasis. *Gene.* 2008; 420:82–89. [PubMed: 18565695]
- Gendron C, Kashiwagi M, Hughes C, Caterson B, Nagase H. TIMP-3 inhibits aggrecanase-mediated glycosaminoglycan release from cartilage explants stimulated by catabolic factors. *FEBS Lett.* 2003; 555:431–436. [PubMed: 14675751]
- Im HJ, Muddasani P, Natarajan V, Schmid TM, Block JA, Davis F, van Wijnen AJ, Loeser RF. Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase Cdelta pathways in human adult articular chondrocytes. *J Biol Chem.* 2007; 282:11110–11121. [PubMed: 17311929]
- Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol.* 2011; 7:33–42. [PubMed: 21119608]
- Kashiwagi M, Tortorella M, Nagase H, Brew K. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). *J Biol Chem.* 2001; 276:12501–12504. [PubMed: 11278243]

- Kim JS, Ellman MB, An HS, Yan D, van Wijnen AJ, Murphy G, Hoskin DW, Im HJ. Lactoferricin mediates anabolic and anti-catabolic effects in the intervertebral disc. *J Cell Physiol.* 2012; 227:1512–1520. [PubMed: 21678402]
- Knauper V, Will H, Lopez-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM, Murphy G. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem.* 1996; 271:17124–17131. [PubMed: 8663255]
- Loeser RF, Chubinskaya S, Pacione C, Im HJ. Basic fibroblast growth factor inhibits the anabolic activity of insulin-like growth factor 1 and osteogenic protein 1 in adult human articular chondrocytes. *Arthritis Rheum.* 2005; 52:3910–3917. [PubMed: 16320338]
- Milanini-Mongiat J, Pouyssegur J, Pages G. Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription. *J Biol Chem.* 2002; 277:20631–20639. [PubMed: 11904305]
- Muddasani P, Norman JC, Ellman M, van Wijnen AJ, Im HJ. Basic fibroblast growth factor activates the MAPK and NFkappaB pathways that converge on Elk-1 to control production of matrix metalloproteinase-13 by human adult articular chondrocytes. *J Biol Chem.* 2007; 282:31409–31421. [PubMed: 17724016]
- Muehleman C, Bareither D, Huch K, Cole AA, Kuettner KE. Prevalence of degenerative morphological changes in the joints of the lower extremity. *Osteoarthritis Cartilage.* 1997; 5:23–37. [PubMed: 9010876]
- Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res.* 2006; 69:562–573. [PubMed: 16405877]
- Pulai JJ, Chen H, Im HJ, Kumar S, Hanning C, Hegde PS, Loeser RF. NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. *J Immunol.* 2005; 174:5781–5788. [PubMed: 15843581]
- Qureshi HY, Ahmad R, Sylvester J, Zafarullah M. Requirement of phosphatidylinositol 3-kinase/Akt signaling pathway for regulation of tissue inhibitor of metalloproteinases-3 gene expression by TGF-beta in human chondrocytes. *Cell Signal.* 2007; 19:1643–1651. [PubMed: 17376651]
- Qureshi HY, Sylvester J, El Mabrouk M, Zafarullah M. TGF-beta-induced expression of tissue inhibitor of metalloproteinases-3 gene in chondrocytes is mediated by extracellular signal-regulated kinase pathway and Sp1 transcription factor. *J Cell Physiol.* 2005; 203:345–352. [PubMed: 15468069]
- Sahebjam S, Khokha R, Mort JS. Increased collagen and aggrecan degradation with age in the joints of Timp3(-/-) mice. *Arthritis Rheum.* 2007; 56:905–909. [PubMed: 17328064]
- Tchetverikov I, Lohmander LS, Verzijl N, Huizinga TW, TeKoppele JM, Hanemaaijer R, DeGroot J. MMP protein and activity levels in synovial fluid from patients with joint injury, inflammatory arthritis, and osteoarthritis. *Ann Rheum Dis.* 2005; 64:694–698. [PubMed: 15834054]
- Wick M, Haronen R, Mumberg D, Burger C, Olsen BR, Budarf ML, Apte SS, Muller R. Structure of the human TIMP-3 gene and its cell cycle-regulated promoter. *Biochem J.* 1995; 311(Pt 2):549–554. [PubMed: 7487894]
- Will H, Atkinson SJ, Butler GS, Smith B, Murphy G. The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem.* 1996; 271:17119–17123. [PubMed: 8663332]
- Yan D, Chen D, Shen J, Xiao G, van Wijnen AJ, Im HJ. Bovine lactoferricin, an antimicrobial peptide, is anti-inflammatory and anti-catabolic in human articular cartilage and synovium. *J Cell Physiol.* 2012
- Zhao H, Bernardo MM, Osenkowski P, Sohail A, Pei D, Nagase H, Kashiwagi M, Soloway PD, DeClerck YA, Fridman R. Differential inhibition of membrane type 3 (MT3)-matrix metalloproteinase (MMP) and MT1-MMP by tissue inhibitor of metalloproteinase (TIMP)-2 and TIMP-3 regulates pro-MMP-2 activation. *J Biol Chem.* 2004; 279:8592–8601. [PubMed: 14681236]

Highlights

LfcinB induces *TIMP-3* in human articular chondrocytes.

LfcinB induces *TIMP-3* mainly *via* the ERK1/2-Sp1 signaling axis.

TIMP-3 is downregulated in human OA chondrocytes.

Figure 1. LfcinB potently induces *TIMP-3* in articular chondrocytes

(A) Chondrocytes in monolayer were incubated with LfcinB (50 and 100 μg/mL) for 24 hours. *TIMP-3* transcripts were quantified by qPCR. (B) Membrane-bound *TIMP-3* was analyzed by immunoblotting. The 27 kDa and 24 kDa form represents glycosylated and unmodified *TIMP-3*, respectively. To assess loading accuracy, immunoblots were reprobed with GAPDH antibody. * $p < 0.05$; ** $p < 0.01$.

Figure 2. *TIMP-3* expression is repressed in OA chondrocytes

(A) *TIMP-3* transcript quantification was performed using age-matched (40 to 70) normal and OA chondrocytes (n=6 in each group). (B) *TIMP-3* protein expression in these individuals was assessed by immunoblotting. ** p<0.01.

Figure 3. LfcinB-mediated *TIMP-3* induction requires ERK1/2 and Sp1

(A) Chondrocytes in monolayer were pre-incubated with individual pathway-specific pharmacological inhibitors (PD98059, a MEK1/2-specific inhibitor, 50 µM; SB203580, a p38-specific inhibitor, 10 µM; Akt inhibitor IV, an Akt inhibitor, 3 µM) for 1 hour, before stimulation with LfcinB (50 µg/mL) for another 24 hours. *TIMP-3* transcripts were quantified by qPCR. (B) Structure of *TIMP-3* promoter. Four Sp1 elements are present between +1 and -120. (C) Chondrocytes were transfected with siRNA targeting *Sp1*, and siRNA with scrambled sequences as a control. Immunoblotting of Sp1 was performed to assess knockdown efficiency. GAPDH was used as loading control. (D) Chondrocytes in monolayer were either transfected with Sp1 siRNA or pre-incubated with individual pharmacological inhibitors before LfcinB stimulation (50 µg/mL). *TIMP-3* transcripts were measured by qPCR. (E) Chondrocytes were treated as described above. *TIMP-3* protein expression was analyzed using cell lysates. Scr: scrambled. * p<0.05; ** p<0.01.

Figure 4. LfcinB stimulates *TIMP-3* transcription via ERK1/2 and Sp1

Chondrocytes were transfected with 0.5 µg of Renilla luciferase construct and 2 µg of *TIMP-3*-Luc luciferase construct. Stimulations were performed in serum-free condition 48 hours after transfection. Cell lysates were then prepared and assayed for luciferase activity using a dual luciferase reporter assay system. The luciferase signal was normalized with the Renilla signal from the same sample to yield a relative ratio. ** p<0.01.

Figure 5. LfcinB activates Sp1 in an ERK1/2-dependent manner

Chondrocytes were pre-incubated with individual pharmacological inhibitors for 1 hour before LfcinB (50 $\mu\text{g/mL}$) stimulation for another hour. A binding reaction using unlabeled (cold) Sp1 probe was set up for each EMSA assay to demonstrate binding specificity. The Sp1-DNA complex was visualized using a chemiluminescence-imaging system.