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Lactoferricin Enhances BMP7-Stimulated Anabolic Pathways in Intervertebral Disc Cells

Michael B Ellman^{1,2,#}, Jaesung Kim^{1,#}, Howard S An², Di Chen¹, Ranjan Kc¹, Xin Li¹, Guozhi Xiao¹, Dongyao Yan¹, Joon Suh¹, Andre J. van Wijnen⁵, James H-C Wang⁶, Su-Gwan Kim⁷, and Hee-Jeong Im^{1,2,3,4,*}

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

²Department of Orthopedic Surgery, 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, IL 60612

⁵Center of Regenerative Medicine and Departments of Orthopedic Surgery & Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN 55905

⁶MechanoBiology Laboratory, Departments of Orthopedic Surgery and Bioengineering, University of Pittsburgh, PA 15213, USA

⁷Department of Oral and Maxillofacial Surgery, School of Dentistry, Chosun University, Gwangju City, Republic of Korea, 501-759

Abstract

Bone-morphogenetic protein-7 (BMP7) is a well-known anabolic and anti-catabolic growth factor on intervertebral (IVD) matrix and cell homeostasis. Similarly, lactoferricin B (LfcinB) has recently been shown to have pro-anabolic, anti-catabolic, anti-oxidative and/or anti-inflammatory effects in bovine disc cells *in vitro*. In this study, we investigated the potential benefits of using combined peptide therapy with LfcinB and BMP7 for intervertebral disc (IVD) matrix repair and to understand cellular and signaling mechanisms controlled by these factors. We studied the effects of BMP7 and LfcinB as individual treatments and combined therapy on bovine nucleus pulposus (NP) cells by assessing proteoglycan (PG) accumulation and synthesis, and the expression of matrix protein aggrecan and transcription factor SOX-9. We also analyzed the role of noggin, a BMP antagonist, in IVD tissue and examined its effect after stimulation with LfcinB. To understand the molecular mechanisms by which LfcinB synergizes with BMP7, we investigated the ERK-SP1 axis as a downstream intracellular signaling regulator involved in BMP7 and LfcinB-mediated activities. Treatment of bovine NP cells cultured in alginate with LfcinB plus BMP7 synergistically stimulates PG synthesis and accumulation in part by upregulation of aggrecan gene expression. The synergism results from LfcinB-mediated activation of Sp1 and SMAD signaling pathways by (i) phosphorylation of SMAD 1/5/8; (ii) downregulation of SMAD inhibitory factors [i.e., noggin (BMP receptor antagonist) and SMAD6 (inhibitory

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

#Drs. Kim and Ellman equally contributed this study.

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SMAD)]; and (iii) upregulation of SMAD4 (universal co-SMAD). These data indicate that LfcinB-suppression of noggin may eliminate the negative feedback of BMP7, thereby maximizing biological activity of BMP7 and ultimately shifting homeostasis to a pro-anabolic state in disc cells. We propose that combination growth factor therapy using BMP7 and LfcinB may be beneficial for treatment of disc degeneration.

Introduction

Low back pain is associated with degenerative disc disease (DDD) and disc degeneration results from an imbalance between anabolic and catabolic events in the intervertebral disc (IVD) (Andersson, 1999; Freemont et al., 2001). Progressive breakdown of the extracellular matrix (ECM) is associated with DDD (Roughley, 2004). A greater understanding of the pathophysiologic mechanisms of DDD may help identify biological treatments capable of promoting ECM repair and regeneration (An et al., 2003a; An et al., 2003b; Masuda, 2008). Under normal conditions, homeostasis of the IVD is the result of an intricate balance between anabolism and catabolism of cells and ECM turnover, regulated by a complex interplay between a variety of growth factors, cytokines, matrix-degrading enzymes and their inhibitors (Iannone and Lapadula, 2003). In degenerative discs, there is an upregulation of destructive enzymes such as matrix metalloproteases (MMPs) and aggrecanases (ADAMTS), accompanied by a gradual loss of ECM, most notably the proteoglycan (PG) aggrecan (Le Maitre et al., 2007a; Le Maitre et al., 2007b). To develop potential regenerative biological treatments for disc degeneration, studies have assessed the benefits of either upregulating the major components of the ECM (e.g., aggrecan) (Masuda et al., 2004; Le Maitre et al., 2007b), or downregulating the expression of catabolic matrix-degrading proteases (e.g., MMPs and ADAMTS) (Le Maitre et al., 2007b). The latter are induced by various pro-inflammatory cytokines, growth factors, and metabolites, including interleukin-1 (IL-1) (Shinmei et al., 1988; Le Maitre et al., 2004; Le Maitre et al., 2007a), tumor necrosis factor- α (TNF- α) (Seguin et al., 2005), and basic fibroblast growth factor (bFGF or FGF-2) (Ellman et al., 2008).

Lactoferrin (Lf) is an 80kDa iron- and heparin-binding glycoprotein from the transferrin family isolated from bovine milk, synthesized by epithelial cells, and present in most mammalian exocrine secretions (Tsuda et al., 2002). Pepsin digestion of Lf from milk *in vitro* led to the initial isolation of bovine Lactoferricin (LfcinB) (Tsuda et al., 2002; Mader et al., 2007). LfcinB is a 25-amino acid, amphiphatic peptide cleaved from the amino terminus (17-41aa) of full length Lf, and is more potent than equimolar amounts of intact Lf. It is cell membrane-permeable and interacts electrostatically with negatively-charged matrix and cell surface glycosaminoglycans (GAGs), heparin and chondroitin sulfate (Gitay-Goren et al., 1992). The anti-inflammatory, anti-oxidative, and anti-cancer properties of LfcinB have already been reported in a variety of tissues (Gifford et al., 2005). We have recently demonstrated that LfcinB has potent anabolic, anti-catabolic, anti-oxidative and anti-inflammatory effects on bovine disc cells *in vitro* (Kim et al., 2012; Kim et al., 2013).

BMP7, a member of the transforming growth factor- β (TGF- β)/BMP superfamily, is expressed in cartilage and exerts potent anabolic effects on osteocyte and chondrocyte differentiation and metabolism via the SMAD1/5/8 signaling pathways (Sampath et al., 1992; Flechtenmacher et al., 1996). More recently, BMP7 has been found to confer similar anabolic effects on matrix metabolism in human adult articular chondrocytes (Flechtenmacher et al., 1996; Stove et al., 2006), bovine IVD cells (Zhang et al., 2004), rabbit IVD cells (Takegami et al., 2005; Masuda et al., 2006), and human IVD cells (Imai Y, 2003a; Imai et al., 2007). Takegami *et al.* reported that BMP7 was effective in stimulating matrix repair by rabbit NP and AF cells after ECM depletion in response to either IL-1 (Takegami et al., 2002) or chondroitinase-ABC (Takegami et al., 2005). This finding

suggests that administration of BMP7 after PG depletion might facilitate disc regeneration. An and colleagues subsequently established that a single BMP7 injection mitigates rabbit disc degeneration *in vivo* by restoring disc height, PG content and viscoelastic properties in the NP (An et al., 2005) in a puncture animal model (Masuda et al., 2006) and in a C-ABC-induced matrix depletion model (Imai Y, 2003b) of disc degeneration. Therefore, BMP7 has significant potential for use as a therapeutic factor to reverse disc degeneration. In contrast, noggin is a well-known extracellular receptor-antagonist of BMP signaling during skeletogenesis that may play an anti-anabolic role in disc homeostasis. In principle, disc degeneration can be ameliorated by enhancing bioavailability of BMP7 and/or suppressing the antagonistic activity of noggin.

Combination growth factor therapy can have a profound positive synergistic impact on articular cartilage (Loeser et al., 2003) and intervertebral disc cells (Kim et al., 2012). For example, stimulation of bovine NP cells cultured in alginate or monolayer with BMP7 plus the well-known anabolic mediator insulin-like growth factor-1 (IGF-1) has a greater anabolic impact on PG accumulation, PG synthesis, aggrecan expression, and collagen type II expression than treatment with either growth factor alone. Here we investigated whether combination peptide therapy using LfcinB and BMP7 is useful for treatment of disc degeneration. Therefore, we assessed the biological and mechanistic effects of co-administering LfcinB and BMP7 on cartilage homeostasis using bovine IVD as a pre-translational model. Specifically, we examined the effects of co-therapy using LfcinB and BMP7 compared to individual peptide therapy on bovine IVD cartilage homeostasis by assessing PG content and noggin expression. We also investigated the mechanisms by which LfcinB potentiates BMP7 activity, with the goal of determining potential benefits of using combination peptide therapy to retard or reverse the progression of IVD degeneration.

Materials & Methods

IVD Cell Isolation and Culture

Tails from young adult bovine animals (15–18 months old) were commercially acquired from a local vendor. Coccygeal discs were opened en bloc, and the NP of each disc was separated. The cells were released by enzymatic digestion in DMEM/Ham's F-12 (1:1) culture medium with sequential treatments of 0.2% pronase and 0.025% collagenase P, as previously described (Im et al., 2003). Three-dimensional alginate bead culture that maintains chondrocytic phenotype and monolayers were prepared for long-term (21 days) and short-term (1–2 days) studies, respectively as we previously performed (Li et al., 2008a; Li et al., 2008b). Triplicates were performed for each condition for alginate cultures and for monolayers with at least five independent experiments for each condition. For alginate bead culture, isolated disc cells were resuspended in 1.2% alginate, and beads were formed by drop-wise addition into a CaCl₂ solution as previously described (Li et al., 2008a; Li et al., 2008b). Cells were treated with BMP7 100 ng/ml (Stryker Biotech, Hopkinton, MA, USA), LfcinB (Biosynthesis, Lewisville, Texas), and IL-1 1 ng/ml (Amgen, Thousand Oaks, CA), a well-known catabolic cytokine used for control. For inhibition of the ERK-SP1 pathway, pharmacological inhibitors of ERK (PD98059) and Sp1 (WP631, methanesulfonate) were purchased from Calbiochem (Gibbstown, NJ) and Sigma-Aldrich (St. Louis, MO), respectively. Triplicate wells were used for each condition. Media was changed every other day for a 21-day period before dimethylmethylene blue (DMMB) assay.

For monolayer cultures, isolated NP cells were counted and plated onto 12-well plates at 8×10^5 cells/cm² as previously described (Im et al., 2003; Li et al., 2008a). The cells were treated with LfcinB (100 µg/ml), BMP7 (100 ng/ml), and noggin (100 ng/ml; PerproTech Inc., Rocky Hill, NJ, USA), a well-known BMP antagonist, and combinations of the above factors. Supernatants were collected 24 hours after the initiation of each treatment and

subjected to western blotting, as described below. Cells were treated for 24 hrs before total RNA harvesting.

Western blotting

Cell and tissue lysates were prepared using modified RIPA buffer as previously described (Muddasani et al., 2007). Total protein concentrations of cell lysates were determined by BCA protein assays (Pierce, Rockford, IL, USA). Equal amounts of protein (20 µg per lane) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane for western blot analyses as described previously (Im et al., 2007). Immunoreactivity was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

Reverse Transcription and Real-Time Polymerase Chain Reaction

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. Reverse transcription (RT) was carried out with 1µg total RNA using ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA) for first strand cDNA synthesis. For real-time PCR, cDNA was amplified using MyiQ Real-Time PCR Detection System (Bio-Rad Hercules, CA). A threshold cycle (Ct value) was obtained from each amplification curve using iQ5 Optical System Software provided by the manufacturer (Bio-Rad). Relative mRNA expression was determined using the $\Delta\Delta C_T$ method, as detailed by manufacturer (Bio-Rad). The deviations in samples represent three different donors in three separate experiments. The primer sequences and their conditions for use are summarized in Table 1.

DMMB assay for Proteoglycan Production and DNA Assay for Cell Numbers

At day 21 of culture, the alginate beads were collected and processed for PG assays using the DMMB assay, as previously described (Gruber et al., 2006). The PG levels measured in the cell-associated matrix (CM) were quantified in relation to cell number (as measured by DNA content) to determine the relative cellular production and retention of PGs in alginate bead cultures (Im et al., 2003). Cell number based on total DNA content was determined using PicoGreen (Molecular Probes, Carlsbad, CA) as previously described (Im et al., 2003).

³⁵S-sulfate Incorporation into Newly-Synthesized Proteoglycans

At Day 7 of culture in alginate, the medium was removed and replaced by fresh medium. One hour later, this medium was replaced with fresh medium containing [³⁵S]-sulfate at 20 µCi/ml (Amersham Corp, Arlington Heights, IL). After incubation for 4 hr, the labeling medium was removed and beads were rinsed twice in cold 1.5mM sulfate-containing washing media. Beads were dissolved to separate out the CM and digested with papain (20 µg/ml in 0.1 M sodium acetate, 0.05 M EDTA, pH 5.53) at 60 °C for 16 hr. Sulfate incorporation into PGs was measured using the Alcian blue precipitation method (Loeser et al., 2000). All samples were analyzed in duplicate and normalized for DNA content using Hoechst 33258 as previously described (Loeser et al., 2000).

Particle Exclusion Assay for Matrix Assessment

The cells with their pericellular matrix were visualized using the particle exclusion assay, as previously described (Knudson, 1993). Briefly, after day 21 of culture in alginate, the beads were solubilized with sodium citrate. The cells were pelleted by centrifugation, resuspended in DMEM, and then placed in the bottom of a multi-well plate. The cells were allowed to settle and attach to the plates for 6 to 12 hr, and formalin-fixed erythrocytes were then added and allowed to settle for 10 to 15 minutes. Cells were then observed and photographed with an inverted phase-contrast microscope (Nikon, Melville, NY).

Gel Shift Assay

Gel shift assay was performed as previously described (Muddasani et al., 2007). Briefly, the nuclear proteins were isolated using the nuclear extraction kit (Panomics) according to the manufacturer's protocol. Following stimulation, culture media was removed, and the cells were then lysed with 1 ml buffer A, incubated on ice for 10 min, and centrifuged at $15,000 \times g$ at 4°C for 3 min. The supernatants were discarded, and the pellets were resuspended in 150 μl of buffer B. The samples were incubated on ice for 2 h and centrifuged, and the supernatants were aliquoted and stored at -80°C . Gel shift assays of Sp1 were performed by using the commercially available kits (Panomics). Following stimulation, the nuclear extracts (prepared as mentioned above) were incubated with the biotin-labeled double-stranded consensus recognition motif of transcription factor (Sp1) or a Sp1 motif present in the aggrecan gene promoter. The samples were then resolved in a 6% non-denaturing polyacrylamide gel and transferred to Pall Biodyne B membrane, blocked, and incubated with Streptavidin-horseradish conjugate. The bands were visualized with a chemiluminescence-imaging system. Unlabeled, double-stranded wild-type oligonucleotides were used as specific competitor DNAs to establish the specificity of protein/DNA complexes.

Transient Transfection by Nucleofection (Electroporation)

Commercially validated double-stranded siRNAs targeting Sp1 were purchased (Invitrogen, 5'-GCA GACACAGCAGCAACAAAUUCUU-3' and 3'-AAGAAUUUGUUGCUGCUGUGUCUGC-5'). siRNA was introduced into cells using Nucleofector™ kit (Amaxa Biosystems) and Chondrocyte Kit (Lonza, Walkersville, MD) as previously described (Muddasani et al., 2007). NP cells isolated from bovine disc tissues were washed in cold PBS and resuspended in chondrocyte nucleofector solution that was pre-warmed to room temperature to a final concentration of 5×10^6 cells/100 μl . The cell-nucleofector solution complexes and the siRNA (300 nM) were then transferred into an Amaxa certified cuvette and gently mixed by tapping followed by electroporation, using the nucleofection program U-28. Immediately, 500 μl of pre-warmed culture media, containing 20 % serum and supplements, was added to the cells, mixed and then transferred into 6-well plates. The cells were then incubated in humidified $37^\circ\text{C}/5\% \text{CO}_2$ incubator. The medium was removed 24 hrs post nucleofection and replenished with fresh culture medium, containing 10 % serum and supplements followed by further incubation for 24 hrs. The cells then change to serum free medium for another 24 hrs before treatment.

Statistical Analysis

Analysis of variance was performed using StatView 5.0 software (SAS Institute, Cary, NC). P-values lower than 0.05 were considered significant.

Results

Anabolism mediated by LfcinB on IVD cells is markedly enhanced when combined with BMP7

Previously, we reported that LfcinB has potent anabolic and anti-catabolic effects on disc-derived NP cells (Kim et al., 2012; Kim et al., 2013). Although LfcinB and BMP7 both are anabolic peptides for discs, properties of these two anabolic peptides may complement each other. For example, LfcinB acts as a potent anti-inflammatory agent, whereas BMP7 promotes cell proliferation and cell survival. Therefore, we hypothesize that combination of these peptides provide a more ideal biological environment for enhanced disc tissue regeneration. Dose-dependent cellular response experiments previously revealed that 10 $\mu\text{g}/\text{ml}$ of LfcinB is a minimal dose for maximum anabolic effects in bovine NP cells cultured in

alginate (Kim et al., 2012) so this dose was used throughout the experiments. To test our hypothesis, we cultured bovine NP cells in alginate beads for 21 days in the presence of LfcinB (10 μ g/ml) and BMP7 (100 ng/ml) and analyzed accumulation of PG using DMMB assay (Fig 1A). Incubation of cells with LfcinB or BMP7 alone results in an increase of, respectively, 1.5-fold ($p<0.05$; *lane 2*) and 2-fold ($p<0.05$; *lane 3*) in PG accumulation per cell compared to control. In combination, cells treated with both LfcinB and BMP7 synergistically increase PG accumulation per cell by > 3 -fold compared to control ($p<0.01$; *lane 4*), or twice that of LfcinB treatment alone ($p<0.01$). This result reveals that the combination of LfcinB and BMP7 has a more potent anabolic impact on PG production in bovine NP cells. than either growth factor alone.

To determine if the increase in PG accumulation is mediated by stimulation of PG synthesis, the incorporation of 35 S-sulfate by bovine NP cells into PGs was quantified. NP cells incubated with either LfcinB or BMP7 alone significantly increase PG synthesis (both $p<0.05$), with BMP7 demonstrating a greater anabolic effect. Again, the combination of LfcinB plus BMP7 strikingly enhances PG synthesis, stimulating a 4.5-fold and 2-fold increase, respectively, compared to either treatment alone (Fig 1B; *lane 4*), suggesting that the BMP7 plus LfcinB-mediated increase in PG accumulation is due, at least in part, to increased PG synthesis.

Similar findings are obtained using particle exclusion assays in which the pericellular matrix formed by NP cells is visualized (Fig 1C). After 21 days of culture, alginate beads were solubilized to isolate cells and exclusion of erythrocytes by the pericellular matrix formed by the NP cells was examined using an inverted phase-contrast microscope. Consistent with other quantitative results (Fig 1A&B), treatment with the combination of LfcinB plus BMP7 results in significantly more pericellular matrix formation compared to individual treatments alone (Fig 1C).

We next examined whether the effects of PG accumulation by these ligands are due to upregulation of aggrecan, a key component of the PG in bovine NP cells. Real-time qPCR results demonstrate that stimulation of bovine NP cells cultured in monolayer with LfcinB (100 μ g/ml) and BMP7 (100 ng/ml) induces a 3-fold and 4.5 fold increase in aggrecan gene expression, respectively (Fig 1D, $p<0.01$). When given in combination, LfcinB significantly enhances BMP7-mediated aggrecan gene expression compared to either growth factor alone ($p<0.05$, Fig 1D, *lane 4*). Taken together, these results suggest that the combination effect by LfcinB plus BMP7 on PG production is, in part, due to the upregulation of PG synthesis as well as the transcriptional stimulation of aggrecan.

LfcinB enhances BMP7-mediated anabolism by a potent inhibitory effect on noggin expression

Previously, we have shown that noggin, a natural BMP7 antagonist, inhibits the BMP7-mediated upregulation of anabolic matrix proteins in bovine NP cells, and IGF-1 (anabolic growth factor) suppresses BMP7-mediated induction of noggin gene expression, suggesting a possible mechanistic explanation for the synergistic effects of IGF-1 and BMP7 (Kim et al., 2012). In bovine disc cells, BMP7 markedly upregulates noggin at both mRNA and protein levels in a dose-dependent fashion, with a maximal stimulatory effect at 100 ng/ml (Fig 2A). This upregulation of noggin reflects BMP7-mediated negative feedback mechanisms that attenuate BMP7-SMAD signaling in NP cells.

Addition of exogenous noggin significantly suppresses the BMP7-mediated upregulation of anabolic genes such as aggrecan and SOX-9, a well-known transcription factor involved in cartilage formation and the expression of collagen type-II (the most abundant collagen found in the IVD) (Bi et al., 1999) ($p<0.05$, Fig 2B), consistent with the known antagonistic effects

of noggin on BMP7 at the receptor level. Therefore, we examined whether LfcinB modulates noggin expression, thereby enhances BMP7-induced anabolic activity. Serum-starved NP cells were cultured in the presence of varying concentrations of LfcinB (1, 10, and 100 $\mu\text{g/ml}$) for 24 hours, and the levels of noggin mRNA were quantified by real-time PCR. Our data revealed that LfcinB dose-dependently suppresses noggin expression and, the concentration of 100 $\mu\text{g/ml}$ of LfcinB significantly ($p < 0.05$) suppresses noggin expression. Hence, LfcinB-mediated suppression of noggin contributes to the co-stimulatory anabolic effects of LfcinB and BMP7 in NP cells (Fig 2C).

Next, we examined whether LfcinB counteracts BMP7 mediated upregulation of noggin in bovine NP cells. Stimulation of cells with BMP7 significantly ($p < 0.01$) induces noggin gene expression reaching almost 15-fold induction (Fig 2D, *lane 3*). Importantly, co-stimulation with LfcinB significantly alleviates the dramatic induction of noggin by BMP7 ($p < 0.05$; *lane 4*). Our findings suggest that LfcinB blocks the negative feedback regulation of BMP7 by noggin in bovine NP cells, providing a plausible explanation for the synergistic action between the two factors in matrix homeostasis.

Synergism of LfcinB and BMP7 is, in part, regulated *via* modulation of regulatory R-SMADs, co-SMAD, and the inhibitory I-SMAD

SMAD complexes are transcriptional co-factors that control gene expression in response to TGF β /BMP2 signaling (Motazed et al., 2008). After phosphorylation, R-SMADs translocate from the cytoplasm into the nucleus to help initiate gene transcription (Schmierer and Hill, 2005). Previously, we have shown that co-treatment with IGF-1 and BMP7 results in the phosphorylation and nuclear translocation of both TGF β and BMP7-responsive SMAD proteins (SMAD1/5/8, SMAD2, and SMAD3) that modulate TGF β -SMAD2/3-, and BMP7-SMAD1/5/8-dependent anabolic target genes in bovine NP cells (Kim et al., 2012). Here, we investigated the intracellular signaling cascades utilized by BMP7 and LfcinB in bovine NP cells.

To determine the complex interplay between LfcinB, BMP7, and the intracellular SMAD complexes, serum-starved NP cells were stimulated with LfcinB (100 $\mu\text{g/ml}$) and BMP7 (100 ng/ml) for the indicated periods of time. Cell lysates were then prepared and analyzed by western blotting using anti-phospho-specific SMAD1/5/8 as well as anti-SMAD4 (co-SMAD) and anti-SMAD6 (inhibitory SMAD) antibodies. Anti- β -actin antibody was used for normalization. We found that individual stimulation of cells with either LfcinB (Fig 3A; upper panel) or BMP7 (Fig 3A; lower panel) activates SMAD1/5/8 within 15 min and activity was sustained for > 60 min as evidenced by the phosphorylation status. Furthermore, LfcinB significantly stimulates SMAD4 while suppresses SMAD6, an inhibitory SMAD that blocks the SMAD1/5/8-related cell signaling in a dose dependent manner with maximal effects at a concentration of 100 $\mu\text{g/ml}$ LfcinB (Fig 3B). Our findings suggest that the synergistic anabolism by co-administration with LfcinB and BMP7 is achieved by LfcinB-mediated induction of the BMP7-SMAD1/5/8-SMAD4 signaling cascade and simultaneous suppression of its inhibitor SMAD6 in NP cells.

The ERK/MAPK-SP1 axis regulates the LfcinB-driven SMAD pathway

We next investigated molecular mechanisms by which the combination of LfcinB and BMP7 enhances anabolic pathways in disc NP cells. Previously, we reported that LfcinB-initiated activation of ERK/MAPK signaling pathway is mainly involved in the expression of aggrecan in bovine NP cells (Kim et al., 2012). In many different types of tissue and cells, ERK/MAPK regulates activity of transcription factor Sp1 (Li et al.; Trisicuglio et al., 2004) by which the ERK-SP1 axis controls cartilage metabolism by regulating SMAD signaling

(Schmierer and Hill, 2005). Thus, we investigated whether LfcinB induction of SMAD activity occurs through ERK activation of Sp1 in human primary articular chondrocytes.

To test LfcinB-induced activation of Sp1 protein-DNA interaction, we performed gel shift assay using nuclear extracts from primary NP cells that were stimulated with different concentrations of LfcinB (10, 50 and 100 $\mu\text{g/ml}$) and commercially available double-stranded Sp1 DNA response element. Markedly increased Sp1 protein-DNA interaction was observed in the presence of LfcinB in a dose-dependent fashion compared to untreated cells (Fig 4A; *lane 1*). Maximum intensity of Sp1 protein-DNA interaction was observed at a concentration of LfcinB 100 $\mu\text{g/ml}$ (*lane 4*). In the presence of ERK pathway-specific inhibitor (ERKi), LfcinB-mediated Sp1-DNA binding activity was abolished, suggesting that the LfcinB-ERK/MAPK axis activates Sp1 in human articular chondrocytes (Fig 4A; *lane 7*). On the other hand, cells treated with BMP7 (100 ng/ml) show no differences in the DNA binding activity of Sp1 (data not shown). Nuclear extract incubated with excess amount of unlabeled probe (cold competition) was used as a control for specificity (Fig 4A; *lanes 5 and 6*).

Given that LfcinB highly upregulates the expression of aggrecan and further potentiates aggrecan expression when combined with BMP7, and that LfcinB significantly suppresses noggin, we tested if inhibiting LfcinB-enhanced Sp1 activity would attenuate these biological effects. NP cells in monolayer were co-incubated with LfcinB and a pharmacological inhibitor of Sp1 (WP631, 50 nM) for 24 hours followed by analyses of gene expression. Individual administration of LfcinB or BMP7 significantly stimulates expression of aggrecan gene (Fig 4B; *lanes 3 and 5*; $p<0.05$ and $p<0.01$ respectively). Consistently, combination of LfcinB and BMP7 markedly enhanced aggrecan gene expression with a 2-fold induction compared to LfcinB alone (Fig 4B; *lane 3 versus 7*; $p<0.05$). Importantly, the presence of Sp1 inhibitor prevents upregulation of aggrecan gene expression by LfcinB ($p<0.05$; *lane 4*) as well as co-stimulation by the combination of LfcinB plus BMP7 ($p<0.001$; *lane 8*). Inhibition of Sp1 has no effect on BMP7-induced aggrecan gene expression (*lanes 5 versus 6*).

Next, we examined the role of Sp1 in the suppressive action on noggin by LfcinB. The presence of Sp1 inhibitor reverses LfcinB-suppression of noggin expression (Fig 4C; $p<0.05$; *lanes 3 versus 4*). Again, Sp1 inhibitor has no impact on BMP7-induced stimulation of noggin (Fig 4C; *lanes 5 versus 6*). We further validated our pharmacological inhibitor studies with siRNA targeting Sp1. First, we ensured that the siRNA efficiently suppressed Sp1 expression on protein level in human primary articular chondrocytes [Fig 4D(a)]. Transient expression of siRNA targeting Sp1 significantly reduces LfcinB-induced aggrecan gene expression [Fig 4D(b); $p<0.01$; *lanes 3 versus 4*). Similarly, Sp1 siRNA abrogates markedly enhanced aggrecan expression by the combination of LfcinB and BMP7 ($p<0.001$; *lanes 7 versus 8*). Again, Sp1 inhibitor has no impact on BMP7-induced stimulation of aggrecan expression (*lanes 5 versus 6*).

LfcinB activates SMAD1/5/8 and simultaneously downregulates SMAD6, an inhibitory SMAD of BMP pathway, suggesting LfcinB-induced SMAD signaling pathway. Thus, we next tested whether the resulting enhancement of SMAD signaling proceeds through the activation of the ERK-Sp1 axis in NP cells. Based on data presented elsewhere in this study (see Fig 3), we selected a specific time point (60 min and 24 hrs) and concentration of LfcinB (100 $\mu\text{g/ml}$) that have shown a greater SMAD activity in the presence of LfcinB. To investigate the role of ERK-Sp1 axis in the SMAD pathway induced by LfcinB, NP cells were co-incubated with either Sp1i or ERKi in the presence of LfcinB for 60 min (phospho-SMAD1/5/8) or for 24 hrs (Smad6 expression) followed by immunoblot analyses [Fig 4E(a) and (b), respectively]. Co-incubation of cells with pharmacological inhibitors of either Sp1

or ERK significantly diminishes LfcinB-induced phosphorylation of SMAD1/5/8, and simultaneously abolishes LfcinB-mediated suppression of Smad6 production. Collectively, our findings suggest that LfcinB enhances the SMAD activity *via* the ERK-Sp1 signaling pathway and plays a critical role in enhancement of anabolic pathways in disc cells when combined together with BMP7.

Discussion

Previously we reported that ERK and/or p38 MAPK pathways are the key signaling cascades that exert the biological effects of LfcinB in NP cells, regulating both anabolic genes (e.g., aggrecan, SOX-9, TIMP-1, TIMP-2, TIMP-3) as well as catabolic genes (iNOS, cartilage degrading enzymes including MMPs and ADAMTS) at the transcriptional level, suggesting that LfcinB may have considerable promise in the treatment of disc degeneration in the future (Kim et al., 2012). This study indicates that LfcinB and BMP7 co-stimulate anabolic pathways in bovine IVD derived cells reflected by significantly greater overall PG accumulation, synthesis, and aggrecan gene induction compared to treatment with either factor alone. Synergism results at least in part from LfcinB-enhanced SMAD signaling pathways by (i) activation of SMAD 1/5/8; (ii) upregulation of co-SMAD, SMAD4; and (iii) downregulation of an anti-SMAD molecules such as noggin and inhibitory SMAD (I-SMAD) SMAD6.

Our molecular mechanistic studies suggest that LfcinB-mediated activation of the ERK/MAPK-Sp1 axis is the key signaling components governing the SMAD pathway including the activation and production of SMAD1/5/8, SMAD6 and noggin as well as downstream ECM genes in disc NP cells. Suppression of noggin by LfcinB may eliminate the negative feedback of BMP7, thereby maximizing biological activity of BMP7 and ultimately shifting homeostasis to a pro-anabolic state in disc cells. The effect of combination of LfcinB and BMP7 on Smad6 and phospho-Smad1/5/8 has not been compared with individual treatment. However, we expect that the combination of LfcinB and BMP7 increases the activity of Smad1/5/8 and nuclear trans-localization of Smad4. Our results demonstrate the potential therapeutic benefit of combined treatment with BMP7 and LfcinB on disc homeostasis, and ultimately for the treatment of degenerative disc disease and associated low back pain.

Promising results from several studies suggest that therapies with individual growth factors such as lactoferrin (Lf), LfcinB, and BMP7, have potential in the treatment of musculoskeletal diseases. *In vivo* animal studies using mice (Guillen et al., 2000) and rat (Hayashida et al., 2004) have shown that injections with, respectively, human or bovine Lf invokes anti-inflammatory, anti-catabolic and anti-analgesic responses in joint, thus suggesting that Lf may have preventative and therapeutic effects on cartilage degeneration and pain. LfcinB blocks bFGF- and IL-1 β -mediated catabolic effects in various tissues/cells such as articular cartilage (Yan and Im, 2011; Yan et al., 2013) as well as endothelial cells (Mader et al., 2006), suggesting a possible anabolic, chondroprotective biological role in joint diseases. In addition, our group and others have previously demonstrated the anabolic potential of BMP7 to stimulate PG and collagen synthesis in human adult articular chondrocytes (Loeser et al., 2005), rabbit IVD cells (Masuda et al., 2003; An et al., 2005; Takegami et al., 2005), and human NP and AF cells (Imai Y, 2003a; Imai et al., 2007) *in vitro*. Our previous studies revealed the *in vivo* potential of BMP7 (An et al., 2005) injection therapy for use in disc degeneration: a single injection of recombinant BMP7 (2 μ g/disc) restores the biochemical, histological, and biomechanical properties of rabbit IVDs after induction of disc degeneration by anular puncture (Masuda et al., 2006; Miyamoto et al., 2006), C-ABC chemonucleolysis (Imai Y, 2003b; Takegami et al., 2005) or IL-1 (Takegami et al., 2002). The current study reveals that a combination anabolic growth factor therapy based on synergism between two anabolic mediators of cartilage homeostasis,

LfcinB and BMP7, may be useful in the treatment of disc disease. This finding complements previous studies showing biological synergism between BMP7 and IGF-1 in human articular chondrocytes (Loeser et al., 2005) and the IVD (Kim et al.), further suggesting the therapeutic beneficial effects by combination of complementary peptides (anti-inflammatory and proliferation) for disc regeneration.

In the IVD, IGF-1 suppresses noggin expression, thus eliminating the negative feedback regulation of BMP7, enhancing BMP7 activity and ultimately shifting homeostasis to a pro-anabolic state (Kim et al., 2010). We have also found that IGF-1 inhibits noggin expression in human NP tissue but fails to suppress noggin expression in human articular cartilage (unpublished data) suggesting the spine cartilage tissue-specific biological impact of IGF-1. Our data suggest that LfcinB-mediated suppression of noggin is via the ERK-Sp1 axis based on results: (i) The ERK-Sp1 axis is one of the key signals generated by LfcinB in disc cells (Kim et al., 2012 and this study); (ii) The pathway-specific chemical inhibitor of Sp1 or siRNA targeting Sp1 significantly inhibits LfcinB-induced aggrecan gene expression, without any influence on BMP7-induced aggrecan gene expression, suggesting the specific action of Sp1 by LfcinB; (iii) Simultaneously, the chemical inhibitor of Sp1 or siRNA targeting Sp1 significantly interferes with LfcinB-inhibition of noggin without any influence on BMP7-induced noggin expression. Also, (iv) the pathway-specific inhibitors of either Sp1 or ERK significantly antagonize LfcinB-mediated activation of SMAD1/5/8 while efficiently restoring LfcinB-suppressed SMAD6 production. Interestingly, we obtained similar results using LfcinB, in which LfcinB activates SMAD1/5/8 and suppresses noggin in both human and bovine NP cells, but not in human knee joint articular chondrocytes (unpublished data), suggesting that the biological impact mediated by LfcinB may be tissue-specific for spine cartilage.

We elucidated the molecular mechanisms by which LfcinB exerts the anti-catabolic and anabolic effects as well as enhances biological anabolism when combined with BMP7. Our data reveal that downregulation of Sp1 axis abolishes the synergistic effects mediated by LfcinB on BMP7, suggesting a critical functional role of this transcription factor in enabling the biological effects of LfcinB. Further, we demonstrate the LfcinB-modulation of SMAD signaling molecules in disc cells. Studies have previously revealed that TGF- β activates the SMAD2 and 3 proteins via phosphorylation in mammary epithelial cells (Gajewska and Motyl, 2004), human articular cartilage (Qureshi et al., 2008), and human IVD tissue (Chen et al., 2006), which join together to form a complex and translocate to the nucleus to mediate transcription. Qureshi and colleagues noted that TGF- β induced SMAD2/3 and SMAD4 to mediate anti-catabolic effects (inhibit MMPs) in human articular chondrocytes (Qureshi et al., 2008). Others have demonstrated that BMPs activate SMAD1/5/8 in transgenic mice (Okamoto et al., 2006). Importantly, we previously demonstrated that BMP7 may exert its anabolic effects via SMAD1/5/8 as well as SMAD3 in bovine NP cells, and there may be crosstalk between the two pathways to form a SMAD2/3 complex, similar to that seen after induction with TGF- β (Hiyama et al., 2008), leading to potent synergistic anabolic effects on IVD cells (Kim et al.). Here, we show that BMP7 and LfcinB each independently activate SMAD1/5/8, demonstrating a shared signaling pathway intracellularly. LfcinB also stimulate SMAD4 and downregulates SMAD6, a well-known inhibitory factor of SMAD1/5/8, suggesting that the synergism between Lfcin and BMP7 is mediated via the SMAD1/5/8 pathway, and interactions with other SMAD proteins (anti-SMAD6 and SMAD4) that together regulate expression of important matrix proteins such as aggrecan and collagen II. Negative regulation of noggin by LfcinB contributes to the synergistic anabolism by LfcinB and BMP7.

From a clinical perspective, the current findings are limited to cell culture studies in vitro, which do not model the complex tissue microenvironment that influences the onset and

progression of disc degeneration *in vivo*. Future studies are warranted to elucidate the long-term effects of direct injections of LfcinB plus BMP7 on the IVD and other organ systems, as well as to clarify the optimal dose and concentration of the two growth factors *in vivo*. Examination of the combined effects of LfcinB plus BMP7 in tandem in AF cells or human IVD tissue *in vitro* or *in vivo* may validate our findings and permit translation of our results to a clinical setting. The results of this study reveal the considerable promise of combined peptide therapy using BMP7 plus LfcinB for the treatment of IVD degeneration.

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References

- An H, Boden SD, Kang J, Sandhu HS, Abdu W, Weinstein J. Summary statement: emerging techniques for treatment of degenerative lumbar disc disease. *Spine* (Phila Pa 1976). 2003a; 28:S24–5. [PubMed: 12897470]
- An HS, Takegami K, Kamada H, Nguyen CM, Thonar EJ, Singh K, Andersson GB, Masuda K. Intradiscal administration of osteogenic protein-1 increases intervertebral disc height and proteoglycan content in the nucleus pulposus in normal adolescent rabbits. *Spine*. 2005; 30:25–31. discussion 31–2. [PubMed: 15626976]
- An HS, Thonar EJ, Masuda K. Biological repair of intervertebral disc. *Spine* (Phila Pa 1976). 2003b; 28:S86–92. [PubMed: 12897480]
- Andersson GB. Epidemiological features of chronic low-back pain. *Lancet*. 1999; 354:581–5. [PubMed: 10470716]
- Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Sox9 is required for cartilage formation. *Nat Genet*. 1999; 22:85–9. [PubMed: 10319868]
- Chen WH, Lo WC, Lee JJ, Su CH, Lin CT, Liu HY, Lin TW, Lin WC, Huang TY, Deng WP. Tissue-engineered intervertebral disc and chondrogenesis using human nucleus pulposus regulated through TGF-beta1 in platelet-rich plasma. *J Cell Physiol*. 2006; 209:744–54. [PubMed: 16955489]
- 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; 420:82–9. [PubMed: 18565695]
- Flechtenmacher J, Huch K, Thonar EJ, Mollenhauer JA, Davies SR, Schmid TM, Puhl W, Sampath TK, Aydelotte MB, Kuettner KE. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. *Arthritis Rheum*. 1996; 39:1896–904. [PubMed: 8912513]
- Freemont TJ, LeMaitre C, Watkins A, Hoyland JA. Degeneration of intervertebral discs: current understanding of cellular and molecular events, and implications for novel therapies. *Expert Rev Mol Med*. 2001; 2001:1–10. [PubMed: 14987359]
- Gajewska M, Motyl T. IGF-binding proteins mediate TGF-beta 1-induced apoptosis in bovine mammary epithelial BME-UV1 cells. *Comp Biochem Physiol C Toxicol Pharmacol*. 2004; 139:65–75. [PubMed: 15556067]
- Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol Life Sci*. 2005; 62:2588–98. [PubMed: 16261252]
- Gitay-Goren H, Soker S, Vlodavsky I, Neufeld G. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J Biol Chem*. 1992; 267:6093–8. [PubMed: 1556117]
- Gruber HE, Hoelscher GL, Leslie K, Ingram JA, Hanley EN Jr. Three-dimensional culture of human disc cells within agarose or a collagen sponge: assessment of proteoglycan production. *Biomaterials*. 2006; 27:371–6. [PubMed: 16098581]
- Guillen C, McInnes IB, Vaughan D, Speekenbrink AB, Brock JH. The effects of local administration of lactoferrin on inflammation in murine autoimmune and infectious arthritis. *Arthritis Rheum*. 2000; 43:2073–80. [PubMed: 11014359]

- Hayashida K, Kaneko T, Takeuchi T, Shimizu H, Ando K, Harada E. Oral administration of lactoferrin inhibits inflammation and nociception in rat adjuvant-induced arthritis. *J Vet Med Sci*. 2004; 66:149–54. [PubMed: 15031542]
- Hiyama A, Mochida J, Omi H, Serigano K, Sakai D. Cross talk between Smad transcription factors and TNF- α in intervertebral disc degeneration. *Biochem Biophys Res Commun*. 2008; 369:679–85. [PubMed: 18307974]
- Iannone F, Lapadula G. The pathophysiology of osteoarthritis. *Aging Clin Exp Res*. 2003; 15:364–72. [PubMed: 14703002]
- 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 C δ pathways in human adult articular chondrocytes. *J Biol Chem*. 2007; 282:11110–21. [PubMed: 17311929]
- Im HJ, Pacione C, Chubinskaya S, Van Wijnen AJ, Sun Y, Loeser RF. Inhibitory effects of insulin-like growth factor-1 and osteogenic protein-1 on fibronectin fragment- and interleukin-1 β -stimulated matrix metalloproteinase-13 expression in human chondrocytes. *J Biol Chem*. 2003; 278:25386–94. [PubMed: 12734180]
- Imai YAH, Pichika R, et al. Recombinant human osteogenic protein-1 upregulates extracellular matrix metabolism by human annulus fibrosus and nucleus pulposus cells. *Orthop Res Soc Trans*. 2003a; 28:1140.
- Imai YAH, Thonar E, et al. Co-injected recombinant human osteogenic protein-1 minimizes chondroitinase-ABC induced intervertebral disc degeneration: an in vivo study using a rabbit model. *Orthop Res Soc Trans*. 2003b:1143.
- Imai Y, Okuma M, An HS, Nakagawa K, Yamada M, Muehleman C, Thonar E, Masuda K. Restoration of disc height loss by recombinant human osteogenic protein-1 injection into intervertebral discs undergoing degeneration induced by an intradiscal injection of chondroitinase ABC. *Spine*. 2007; 32:1197–205. [PubMed: 17495776]
- Kim JS, Ellman MB, An HS, van Wijnen AJ, Borgia JA, Im HJ. Insulin-like growth factor 1 synergizes with bone morphogenetic protein 7-mediated anabolism in bovine intervertebral disc cells. *Arthritis Rheum*. 2010; 62:3706–15. [PubMed: 20812336]
- 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; 227:1512–20. [PubMed: 21678402]
- Kim JS, Ellman MB, Yan D, An HS, Kc R, Li X, Chen D, Xiao G, Cs-Zabo G, Hoskin DW, Buechter DD, van Wijnen AJ, Im HJ. Lactoferricin mediates anti-inflammatory and anti-catabolic effects via inhibition of IL-1 and LPS activity in the intervertebral disc. *Journal of cellular physiology*. 2013
- Knudson CB. Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. *J Cell Biol*. 1993; 120:825–34. [PubMed: 7678838]
- Le Maitre CL, Freemont AJ, Hoyland JA. Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. *J Pathol*. 2004; 204:47–54. [PubMed: 15307137]
- Le Maitre CL, Hoyland JA, Freemont AJ. Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1 β and TNF α expression profile. *Arthritis Res Ther*. 2007a; 9:R77. [PubMed: 17688691]
- Le Maitre CL, Pockert A, Buttle DJ, Freemont AJ, Hoyland JA. Matrix synthesis and degradation in human intervertebral disc degeneration. *Biochem Soc Trans*. 2007b; 35:652–5. [PubMed: 17635113]
- Li L, Zhang K, Cai XJ, Feng M, Zhang Y, Zhang M. Adiponectin upregulates prolyl-4-hydroxylase α 1 expression in interleukin 6-stimulated human aortic smooth muscle cells by regulating ERK 1/2 and Sp1. *PLoS One*. 6:e22819. [PubMed: 21829524]
- Li X, An HS, Ellman M, Phillips F, Thonar EJ, Park DK, Udayakumar RK, Im HJ. Action of fibroblast growth factor-2 on the intervertebral disc. *Arthritis Res Ther*. 2008a; 10:R48. [PubMed: 18435858]

- Li X, Phillips FM, An HS, Ellman M, Thonar EJ, Wu W, Park D, Im HJ. The action of resveratrol, a phytoestrogen found in grapes, on the intervertebral disc. *Spine (Phila Pa 1976)*. 2008b; 33:2586–95. [PubMed: 19011540]
- 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; 52:3910–7. [PubMed: 16320338]
- Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. *Arthritis Rheum*. 2003; 48:2188–96. [PubMed: 12905472]
- Loeser RF, Shanker G, Carlson CS, Gardin JF, Shelton BJ, Sonntag WE. Reduction in the chondrocyte response to insulin-like growth factor 1 in aging and osteoarthritis: studies in a non-human primate model of naturally occurring disease. *Arthritis Rheum*. 2000; 43:2110–20. [PubMed: 11014363]
- Mader JS, Richardson A, Salsman J, Top D, de Antueno R, Duncan R, Hoskin DW. Bovine lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell membrane and targeting of mitochondria. *Exp Cell Res*. 2007; 313:2634–50. [PubMed: 17570361]
- Mader JS, Smyth D, Marshall J, Hoskin DW. Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor165-induced angiogenesis by competing for heparin-like binding sites on endothelial cells. *Am J Pathol*. 2006; 169:1753–66. [PubMed: 17071598]
- Masuda K. Biological repair of the degenerated intervertebral disc by the injection of growth factors. *Eur Spine J*. 2008; 17(Suppl 4):441–51. [PubMed: 19005698]
- Masuda K, Imai Y, Okuma M, Muehleman C, Nakagawa K, Akeda K, Thonar E, Andersson G, An HS. Osteogenic protein-1 injection into a degenerated disc induces the restoration of disc height and structural changes in the rabbit annular puncture model. *Spine*. 2006; 31:742–54. [PubMed: 16582847]
- Masuda K, Oegema TR Jr, An HS. Growth factors and treatment of intervertebral disc degeneration. *Spine*. 2004; 29:2757–69. [PubMed: 15564925]
- Masuda K, Takegami K, An H, Kumano F, Chiba K, Andersson GB, Schmid T, Thonar E. Recombinant osteogenic protein-1 upregulates extracellular matrix metabolism by rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads. *J Orthop Res*. 2003; 21:922–30. [PubMed: 12919882]
- Miyamoto K, Masuda K, Kim JG, Inoue N, Akeda K, Andersson GB, An HS. Intradiscal injections of osteogenic protein-1 restore the viscoelastic properties of degenerated intervertebral discs. *Spine J*. 2006; 6:692–703. [PubMed: 17088200]
- Motazed R, Colville-Nash P, Kwan JT, Dockrell ME. BMP-7 and proximal tubule epithelial cells: activation of multiple signaling pathways reveals a novel anti-fibrotic mechanism. *Pharm Res*. 2008; 25:2440–6. [PubMed: 18288447]
- 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; 282:31409–21. [PubMed: 17724016]
- Okamoto M, Murai J, Yoshikawa H, Tsumaki N. Bone morphogenetic proteins in bone stimulate osteoclasts and osteoblasts during bone development. *J Bone Miner Res*. 2006; 21:1022–33. [PubMed: 16813523]
- Qureshi HY, Ricci G, Zafarullah M. Smad signaling pathway is a pivotal component of tissue inhibitor of metalloproteinases-3 regulation by transforming growth factor beta in human chondrocytes. *Biochim Biophys Acta*. 2008; 1783:1605–12. [PubMed: 18471442]
- Roughley PJ. Biology of intervertebral disc aging and degeneration: involvement of the extracellular matrix. *Spine (Phila Pa 1976)*. 2004; 29:2691–9. [PubMed: 15564918]
- Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH, et al. Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J Biol Chem*. 1992; 267:20352–62. [PubMed: 1328198]

- Schmierer B, Hill CS. Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads. *Mol Cell Biol.* 2005; 25:9845–58. [PubMed: 16260601]
- Seguin CA, Pilliar RM, Roughley PJ, Kandel RA. Tumor necrosis factor-alpha modulates matrix production and catabolism in nucleus pulposus tissue. *Spine (Phila Pa 1976).* 2005; 30:1940–8. [PubMed: 16135983]
- Shinmei M, Kikuchi T, Yamagishi M, Shimomura Y. The role of interleukin-1 on proteoglycan metabolism of rabbit annulus fibrosus cells cultured in vitro. *Spine.* 1988; 13:1284–90. [PubMed: 3264623]
- Stove J, Schneider-Wald B, Scharf HP, Schwarz ML. Bone morphogenetic protein 7 (bmp-7) stimulates proteoglycan synthesis in human osteoarthritic chondrocytes in vitro. *Biomed Pharmacother.* 2006; 60:639–43. [PubMed: 17056223]
- Takegami K, An HS, Kumano F, Chiba K, Thonar EJ, Singh K, Masuda K. Osteogenic protein-1 is most effective in stimulating nucleus pulposus and annulus fibrosus cells to repair their matrix after chondroitinase ABC-induced in vitro chemonucleolysis. *Spine J.* 2005; 5:231–8. [PubMed: 15863076]
- Takegami K, Thonar EJ, An HS, Kamada H, Masuda K. Osteogenic protein-1 enhances matrix replenishment by intervertebral disc cells previously exposed to interleukin-1. *Spine.* 2002; 27:1318–25. [PubMed: 12065981]
- Trisciuglio D, Iervolino A, Candiloro A, Fibbi G, Fanciulli M, Zangemeister-Wittke U, Zupi G, Del Bufalo D. bcl-2 induction of urokinase plasminogen activator receptor expression in human cancer cells through Sp1 activation: involvement of ERK1/ERK2 activity. *J Biol Chem.* 2004; 279:6737–45. [PubMed: 14660675]
- Tsuda H, Sekine K, Fujita K, Ligo M. Cancer prevention by bovine lactoferrin and underlying mechanisms--a review of experimental and clinical studies. *Biochem Cell Biol.* 2002; 80:131–6. [PubMed: 11908637]
- Yan D, Im HJ. Bovine Lactoferricin Exerts Anti-catabolic Effect *via* an Endocytic Pathway in Human Articular Chondrocytes. 57th Orthopaedic Research Society Annual Meeting Ortho. Trans. 2011:480.
- Yan D, Chen D, Hawse JR, van Wijnen AJ, Im HJ. Bovine lactoferricin induces TIMP-3 via the ERK1/2-Sp1 axis in human articular chondrocytes. *Gene.* 2013; 517:12–8. [PubMed: 23313877]
- Zhang Y, An HS, Song S, Toofanfar M, Masuda K, Andersson GB, Thonar EJ. Growth factor osteogenic protein-1: differing effects on cells from three distinct zones in the bovine intervertebral disc. *Am J Phys Med Rehabil.* 2004; 83:515–21. [PubMed: 15213475]

Highlights

- We studied the combined effects of BMP7 and LfcinB on bovine nucleus pulposus (NP) cells.
- The synergism results in part from LfcinB-mediated activation of Sp1 and SMAD signaling
- LfcinB maximizes activity of BMP7 by inhibiting noggin.
- Potential therapeutic benefit of BMP7 and LfcinB combination in degenerative disc disease

Figure 1. Co-treatment with Lactoferricin (LfcinB) and bone morphogenetic protein 7 (BMP7) stimulates matrix formation by nucleus pulposus (NP) cells

NP cells were cultured in 1.2% alginate in serum-free medium with mini-ITS (insulin–transferrin–selenium) (control) or the control medium plus 10 µg/ml LfcinB, 100 ng/ml BMP7, a cocktail of LfcinB and BMP7. **A**, NP cells were stimulated for 21 days with the indicated reagents. At the end of the culture period, the amount of proteoglycan (PG) in the cell-associated matrix was measured by dimethylmethylene blue (DMMB) assay and normalized to cell numbers based on DNA content. Samples were measured in triplicate and presented as a percentage of the day 21 control culture values. **B**, NP cells were stimulated for 7 days with BMP7 and/or LfcinB as indicated. At the end of the culture period, PG synthesis was measured during the last 4 hours of culture using ³⁵S-sulfate incorporation, and results were normalized to cell numbers based on DNA content. **C**, NP cell pericellular matrix production after alginate culture for 21 days under the indicated treatment conditions was measured using an exclusion assay. Representative cells were photographed using an inverted phase-contrast microscope. The cell-associated matrix excludes erythrocytes from the cell plasma membrane. Four independent experiments were performed in triplicate (original magnification X400). **D**, The monolayer of NP cells were stimulated with 100 µg/ml LfcinB, 100 ng/ml BMP7, a cocktail of LfcinB and BMP7 for 24 hr. After stimulation, cells were harvested and total RNA was extracted for real-time reverse transcription–

polymerase chain reaction to measure gene expression (e.g., aggrecan). Values are the mean and SD and are representative of 3 different donors in 3 separate experiments.

Figure 2. Lactoferricin prevents BMP7-induced negative feedback by blocking expression of the BMP receptor antagonist noggin

A, BMP7-induced noggin in a dose-dependent manner. NP cells were stimulated with 10, 50, or 100 ng/ml BMP7 for 24 hr. After stimulation, cells were harvested. Total protein and total RNA was extracted for western blot analysis and real-time reverse transcription polymerase chain reaction (qRT-PCR) of noggin. **B,** Noggin antagonizes BMP-7 mediated induction of aggrecan and Sox9 gene expression. NP cells were stimulated with 100 ng/ml BMP7, 100 ng/ml noggin, or a cocktail of LfcinB and BMP7 for 24 hr. After stimulation, cells were harvested and total RNA was extracted. The mRNA induction levels of aggrecan and the transcription factor Sox9 were then measured by qRT-PCR. **C,** LfcinB down-regulates the noggin expression in NP cells. To measure LfcinB-mediated noggin down-regulation, serum-starved NP cells were cultivated for 24 hr in the presence of 1, 10, or 100 ng/ml LfcinB. After stimulation, cells were harvested and total RNA was extracted. Expression of noggin was then measured by qRT-PCR. **D,** LfcinB inhibits BMP7 mediated noggin gene induction in NP cells. To determine whether LfcinB inhibits BMP7-mediated noggin in NP cells induction in bovine disc cells, serum-starved NP monolayer cultured cells were stimulated for 24 hr with LfcinB (100 μ g/ml), BMP7 (100 ng/ml), or a cocktail of LfcinB and BMP7. After cells were harvested and total RNA was extracted. Expression of noggin was then measured by qRT-PCR. Three independent experiments were performed.

Figure 3. Synergism of LfcinB and BMP7 accounts for modulation of R-SMADs, co-SMAD and I-SMAD by LfcinB

A, NP monolayer cultured cells were stimulated with either LfcinB (100 µg/ml) or BMP7 (100 ng/ml) for the indicated periods of time (0, 15, 30 and 60 mins). After stimulation, cell lysates were then analyzed by western blotting with specific anti-phospho-specific Smad 1/5/8 (R-SMADs) antibody. **B**, NP cells were incubated with different concentrations of LfcinB (1, 50 and 100 µg/ml) for 24 hrs, and the resulting cell lysates were analyzed by western blotting using anti-Smad4 (Co-SMAD) and anti-Smad6 (I-SMAD) antibodies. Anti-β-actin antibody was used for normalization.

Figure 4. ERK/MAPK-Sp1 axis regulates the LfcinB-driven SMAD pathway

A, LfcinB regulates the ERK/MAPK-Sp1 axis in NP cells. SP-1 protein-DNA interactions were examined by gel shift assays using biotin-labeled double-strand SP-1 DNA motif present in the aggrecan gene promoter. **B-D**, The anabolic effects of LfcinB are mediated through transcriptional factor Sp1. NP cells were stimulated with 100 μg/ml LfcinB, 100 ng/ml BMP7, 50 nM Sp1 chemical inhibitor [**B**, **C** & **E**(a) and (b)] or 50 ng/ml Sp1 siRNA [**D**(a) and (b)], a cocktail of LfcinB and BMP7 with or without Sp1 inhibitor or Sp1 siRNA for 24 hr. After stimulation, cells were harvested and total RNA was extracted. The mRNA induction levels of aggrecan and noggin were then measured by qRT-PCR. **E**, ERK/MAPK-Sp1 axis regulates the LfcinB-stimulated SMAD pathway. NP monolayer cultured cells were stimulated with LfcinB (100 μg/ml), 50 ng/ml Sp1 inhibitor or 50 μM ERK inhibitor for 1 hr. After stimulation, cell lysates were prepared and analyzed by western blotting with specific anti-phospho-Smad1/5/8 (R-SMAD), and anti-Smad6 (I-SMAD) antibodies.

Figure 5. Schematic diagram of LfcinB-mediated biological effects on the BMP7-SMAD signaling pathways

LfcinB inhibits the production of noggin and inhibitor SMAD (Smad6) mainly through activation of the ERK-Sp1 axis, and simultaneously upregulates SMAD4 by which LfcinB enhances BMP7-mediated anabolism in disc NP cells.