Link Protein N-terminal Peptide Binds to Bone Morphogenetic Protein (BMP) Type II Receptor and Drives Matrix Protein Expression in Rabbit Intervertebral Disc Cells*

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**Background:** LPP induces synthesis of aggrecan and collagen II; however, the mechanism is unknown.

**Results:** LPP up-regulated expression of aggrecan, collagen II, and SOX9 through binding to BMP-RII, initiating a complex Smad/BMP feedforward circuit.

**Conclusion:** LPP could promote disc matrix production directly or boost the activity of exogenous BMPs.

**Significance:** LPP may have value in the treatment of degenerated discs.

Intervertebral disc (IVD) degeneration and associated spinal disorders are leading sources of morbidity, and they can be responsible for chronic low back pain. Treatments for degenerative disc diseases continue to be a challenge. Intensive research is now focusing on promoting regeneration of degenerated discs by stimulating production of the disc matrix. Link protein N-terminal peptide (LPP) is a proteolytic fragment of link protein, an important cross-linker and stabilizer of the major structural components of cartilage, aggrecan and hyaluronan. In this study we investigated LPP action in rabbit primary intervertebral disc cells cultured *ex vivo* in a three-dimensional alginate matrix. Our data reveal that LPP promotes disc matrix production, which was evidenced by increased expression of the chondrocyte-specific transcription factor SOX9 and the extracellular matrix macromolecules aggrecan and collagen II. Using colocalization and pulldown studies we further document a noggin-insensitive direct peptide-protein association between LPP and BMP-RII. This association mediated Smad signaling that converges on BMP genes leading to expression of BMP-4 and BMP-7. Furthermore, through a cell-autonomous loop BMP-4 and BMP-7 intensified Smad1/5 signaling though a feedforward circuit involving BMP-RI, ultimately promoting expression of SOX9 and downstream aggrecan and collagen II genes. Our data define a complex regulatory signaling cascade initiated by LPP and suggest that LPP may be a useful therapeutic substitute for direct BMP administration to treat IVD degeneration and to ameliorate IVD-associated chronic low back pain.

Chronic low back pain is a common worldwide health concern (1–4). Epidemiological studies have shown that the lifetime prevalence of chronic low back pain is 54–80% in the general population, and the annual prevalence is reported as 15–45% (4, 5). In the United States, low back pain is rated as second only to upper respiratory problems among symptom-related reasons for visits to a physician, the third most common reason for surgical procedures, and the fifth ranking cause of admission to hospitals (2, 4).

IVD degeneration and associated spinal disorders have been implicated as a major cause of chronic low back pain (2, 3, 6). Current treatment for the chronic low back pain related to disc degeneration is usually limited to conservative care, including nonoperative modalities (*e.g.* anti-inflammatory medication, physical therapy, and pain management) and operative methods (*e.g.* spinal fusion, discectomy) (7). Unfortunately, none of these treatment strategies produces reliable outcomes because they aim to relieve acute symptoms but fail to halt the process of IVD degeneration. Advances in the fields of molecular and cellular biology may offer new therapeutic approaches; these involve regeneration of degenerated discs through stimulation of matrix production (8, 9).

IVD degeneration is a multifactorial process involving genetic, mechanical, and biologic factors (10–12). Although the cause and pathophysiology of IVD degeneration remain unclear, hallmarks of IVD degeneration are progressive loss of matrix macromolecular components, aggrecan and collagen II (11–16). It is generally accepted that an increase in catabolic activity and a decrease in anabolic activity lead to a loss of matrix macromolecular components and subsequently IVD degeneration (10, 12). Therefore, research is now focusing on promoting regeneration of degenerated discs by stimulating production of the disc matrix. Bone morphogenetic proteins, including BMP-2 and BMP-7, which have been demonstrated to stimulate the production of IVD matrix *in vitro* and *in vivo*,

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3 The abbreviations used are: IVD, intervertebral disc; Act, activin; BMP, bone morphogenetic protein; BMP-RI, BMP type I receptor; BMP-RII, BMP type II receptor; LP, link protein; LPP, link protein N-terminal peptide.
are under investigation (12, 17–19). However, BMP-2 and BMP-7 are known to be osteoinductive molecules; they have been developed into commercial products used to induce spinal fusion or promote fracture healing. As such, there is concern that BMP-2 and BMP-7 could cause undesirable bone formation around or within the disc. Moreover, the supraphysiologic doses of BMPs required for efficacy are associated with high costs and safety concerns such as osteolysis, intense inflammation, and possibly malignancy (20). Therefore, there is a need to identify new molecules that can induce IVD matrix regeneration.

Link protein (LP) is a glycoprotein that stabilizes the interaction between major matrix structural components, aggrecan and hyaluronan (21, 22). In articular cartilage, LP binds aggrecan along the hyaluronan chain. The resulting aggregates produce a stable macromolecular structure that contributes to compression resistance and shock absorption in the joint. LP exists in three isoforms, LP1, LP2, and LP3, all derived from the same structural gene. LP1 and LP2 are different glycosylated forms of the same intact protein core. LP3 is derived from either LP1 or LP2 and formed by proteolytic cleavage between His^{16}—Ile^{17} residues, resulting in the loss of the N-terminal 16 amino acids (23–25). This cleaved N-terminal 16-amino peptide (DHLSDNYTLDHRAIH) is called the link protein N-terminal peptide (LPP, also referred to as link-N). Studies have shown that both native LP and the biochemically synthesized form can stimulate synthesis of aggrecan and collagen II in cartilaginous cells (26–28) and intervertebral disc cells (29, 30). However, it remains unknown whether or not LPP regulates chondrocyte-specific transcription factors. Moreover, the mechanism by which LPP promotes matrix production is unknown.

Using primary intervertebral disc cells cultured in a three-dimensional alginate bead matrix, our present study demonstrates that the peptide LPP induces expression of the chondrocyte-specific transcription factor SOX9 and the disc matrix macromolecules aggrecan and collagen II. Our studies provide strong evidence demonstrating that the peptide LPP binds directly to BMP-RII. This binding initiates a complex Smad/BMP signaling cascade and consequently promotes production of intervertebral disc matrix. These data support the further development of LPP as a disc-regenerating therapeutic for patients suffering from low back pain as a consequence of IVD degeneration.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Mouse anti-BMP-2, rabbit anti-BMP-4, rabbit anti-BMP-6, mouse anti-BMP-7, and rabbit anti-SOX9 were purchased from Santa Cruz Biotechnology. Antibodies against Smad1, Smad2, ERK1/2, p38, AKT, phosphorylated Smad1/5, phosphorylated Smad2/3, phosphorylated ERK1/2, phosphorylated p38, phosphorylated AKT, and anti-GAPDH were purchased from Cell Signaling Technology. Noggin, protease inhibitor mixture, phosphatase inhibitor mixture I and II, and pharmacological inhibitors of PI3 (LY294002), ERK1/2 (U0126), p38 (SB203580), BMP-RII-induced Smad1/5 (dorsomorphin), and TGFβ-induced Smad2/3 (SB431542) were purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch.

**Peptide Synthesis**—Peptide LPP (DHLSDNYTLDHRAIH) representing the consensus sequence for human LPP and an inactive control peptide LPR (the reverse sequence of LPP called LPR; HIARHDLTYNDSLHD), biotin-labeled LPP (LPPb), and biotin-labeled LPR (LPRb) were synthesized by New England Peptide LLC (Gardner, MA). Biotin was incorporated at the N terminus using HBTU-HOBt/DIPEA in Me$_2$SO. Peptide resins were cleaved by 1-h exposure to 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% H$_2$O solvent. Released peptides were purified to $>$90% by preparative reversed-phase C$_{18}$ high performance liquid chromatography, characterized by electrospray ionization mass spectroscopy, and lyophilized to dryness. They were stored at $-20^\circ$C before use.

**IVD Tissue and Cell Isolation**—All animal studies were conducted in accordance with approved protocols by the Atlanta Veterans Affairs Medical Center IACUC. Rabbit IVD tissues were obtained from eight New Zealand White rabbits 6 months of age. From each rabbit, the nucleus pulposus from each of the six lumbar discs (L1–L2 to L6–L7) was harvested under sterile conditions. The nucleus pulposus material derived from a single rabbit was pooled to make up one sample, and cells were isolated by sequential enzyme digestion for 1 h at 37°C with 0.2% protease. Tissues were further digested overnight with 0.025% collagenase, and released primary IVD cells were cultured in complete DMEM/F12 medium containing 10% fetal bovine serum (FBS; Sigma).

**Cell Culture in Alginate Beads and Treatments**—Confluent primary IVD cells were resuspended in 1.2% low viscosity alginate (Keltone LV; Kelco, Chicago, IL) in 0.15 M sodium chloride at a density of 2 $\times$ 10$^6$ cells/ml. The cell suspension was then gently expressed through a 22-gauge needle and dropped into a 102 mM calcium chloride solution, where each drop was instantly transformed into a semisolid microspheric bead. After 10 min of incubation at 37°C, to allow further polymerization, the newly formed beads were washed to remove excess calcium chloride. Thirty beads were placed in each well of a 6-well plate and cultured for 1 week in complete DMEM/F12 medium with 10% FBS. The growth medium was then replaced with DMEM/F12 medium with 2% FBS and the cells treated for up to 9 days with 200 ng/ml peptide LPP or control peptide LPR. Cultures were treated with or without pharmacological inhibitors of major signal transduction pathways including Smad1/5/8, Smad2/3, ERK1/2, p38, and PI3/AKT. After treatment, the cells were harvested to analyze expression of the target genes.

**RNA Isolation, Reverse Transcription, and Real-time PCR**—Total RNA was extracted from the cell pellets using the RNeasy mini-kit (Qiagen) and reverse-transcribed by oligo(dt) priming using Superscript III reverse transcriptase following the manufacturer’s instructions (Invitrogen). Real-time RT-PCR analyses were performed using the ABI PRISM 7500 Fast Real-Time PCR system (Applied Biosystems) and SYBR Green. Gene expression profiling was completed using the comparative Ct method of relative quantification. Relative RNA quantities were normalized to the amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are expressed as a ratio to untreated control. The sequences of the forward and reverse primers are available from the authors upon request.
primes are as follows: aggrecan forward, 5’-acccaggctcagtggatgtgc-3’ and reverse, 5’-ccagctcagtggatgtgc-3’; collagen II forward, 5’-cccaagacaattggcttc-3’ and reverse, 5’-gctcaccagcttttctgg-3’; GAPDH forward, 5’-acctgaactcaccactc-3’ and reverse, 5’-ggagactctggat-3’. siRNA Knockdown Experiments—To achieve gene knockdown, small interfering RNAs (siRNAs) targeting rabbit BMP-RII, Act-RIIa, ActR-IIb, BMP-RIa, and BMP-RIb were designed down, small interfering RNAs (siRNAs) targeting rabbit BMP-
untreated control. ELISA data. Results are presented as a ratio relative to the
(Bio-Rad), and this value was used to normalize the respective
protein was determined by Coomassie Blue dye-binding assay
ELISA (BioSource). Measurements were performed in tripli-
plates were transfected using OligofectamineTM Reagent
(Lafayette, CO). The primary disc cells cultured in 6-well
siRNA against lamin A/C was obtained from Dharmacon
was obtained from Invitrogen. In addition, validated control
sized duplex siRNA against BMP-RII, Act-RIIa, and ActR-IIb
ensure that these siRNA target sequences did not cross-react
BLAST was used to
ture. The basic alignment search tool (BLAST) was used to
design parameters involving target length, molar percentage
of guanine and cytosine base content, and melting tempera-
ture. The basic alignment search tool (BLAST) was used to
ensure that these siRNA target sequences did not cross-react
with other known rabbit genes. Custom chemically synthe-
sized duplex siRNA against BMP-RII, Act-RIIa, and ActR-IIb
was obtained from Invitrogen. In addition, validated control
siRNA against lamin A/C was obtained from Dharmacon
(Lafayette, CO). The primary disc cells cultured in 6-well
plates were transfected using Oligofectamine™ Reagent
according to the manufacturer’s protocol with a final siRNA
concentration of 100 nM. Knockdown efficiency was eval-
uated by measuring protein levels in cell lysates using Western
blotting.

Western Blot Analysis—The IVD cells were homogenized in lysis buffer (including protease inhibitor mixture) and incubated with papain digest
dolution (1 µg/ml; Sigma) for 14 h at 65 °C. The supernatant,
containing the soluble fraction of IVD cell matrix, was used to
determine the concentrations of aggrecan by commercial
ELISA (BioSource). Measurements were performed in triplic-
ate according to the manufacturer’s instructions. Total soluble
protein was determined by Coomassie Blue dye-binding assay
(Bio-Rad), and this value was used to normalize the respective
ELISA data. Results are presented as a ratio relative to the
untreated control.

siRNA Knockdown Experiments—To achieve gene knock-
don, small interfering RNAs (siRNAs) targeting rabbit BMP-
RII, Act-RIIa, ActR-IIb, BMP-RIa, and BMP-RIb were designed
using the BLOCK-iT™ RNAi Designer based on standard
design parameters involving target length, molar percentage
of guanine and cytosine base content, and melting tempera-
ture. The basic alignment search tool (BLAST) was used to
ensure that these siRNA target sequences did not cross-react
with other known rabbit genes. Custom chemically synthe-
sized duplex siRNA against BMP-RII, Act-RIIa, and ActR-IIb
was obtained from Invitrogen. In addition, validated control
siRNA against lamin A/C was obtained from Dharmacon
(Lafayette, CO). The primary disc cells cultured in 6-well
plates were transfected using Oligofectamine™ Reagent
according to the manufacturer’s protocol with a final siRNA
concentration of 100 nM. Knockdown efficiency was eval-
uated by measuring protein levels in cell lysates using Western
blotting.

Western Blot Analysis—The IVD cells were homogenized in lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml biotinylated peptide LPPb, biotinylated control peptide LPRb, or control medium. Following the incubation, cells were
washed and incubated for 1 h with BS3 cross-linker (Pierce) at 5
µM in PBS (pH 8.0). Excess cross-linker was quenched by adding
50 µl of 1 M Tris buffer (pH 7.5) and further incubating for
15 min at room temperature. Cells were scraped into buffer R
(100 mM KCl, 3 mM NaCl, 1 mM Na2ATP, 3.5 mM MgCl2, and
10 mM HEPES (pH 7.4)) containing freshly prepared 5 mM diiso-
propyl fluoroephosphate, 1.25 mM phenylmethylsulfonyl fluo-
ride, and 10 µg/ml chymostatin. Cells were then disrupted at
4 °C in a nitrogen cavitation bomb (Parr Instruments, Moline,
IL). The unbroken nuclei and cellular debris were removed from the supernatant by a 1000 × g sedimentation at 4 °C.

To capture biotinylated LPP-bound protein, the supernatant
containing biotinylated peptide-protein conjugates was passed
through a monomeric avidin-Sepharose column (Pierce). Eluted
peptide-protein conjugates were subjected to SDS-PAGE and
transferred to PVDF membranes, and proteins were detected
using peroxidase-conjugated streptavidin. Alternately, 200 µg of
protein lysates containing biotinylated peptide-protein conjugate
was immunoprecipitated in buffer R containing 1% n-octyl gluco-
side with anti-BMP-RII antibody and protein G-Sepharose over-
night at 4 °C. The precipitated pellets were washed three times
with 1 ml of washing buffer containing 50 mM Tris-HCl, pH 7.5,
150 mM NaCl, 1 mM EDTA, 0.25% gelatin, and 0.1% Nonidet P-40.
The pellets were resuspended in SDS sample buffer, resolved by
SDS-PAGE, and immunoblotted. Each immunoprecipitation was
repeated at least three times.

Immunofluorescence Microscopy—The primary IVD cells were
incubated on Labtek Permanox chamber slides (Nunc,
Naperville, IL) and treated with biotinylated peptide LPPb or
biotinylated control peptide LPRb for 2 days. Cells were rinsed
twice with ice-cold 10 mM HEPES-buffered Hanks’ balanced
salt solution, pH 7.4. The cells were fixed for 30 min in 3.7%
paraformaldehyde, permeabilized for 30 min in 0.5% Triton
X-100, and incubated for 1 h in Hanks’ balanced salt solution +
containing 5% normal goat serum. The bound biotinylated pep-
tides were localized by labeling with Alexa Fluor 568-conju-
gated streptavidin (red). BMP-RII was labeled with Alexa Fluor
488-conjugated BMP-RII antibody (green). Then, cells were
washed with Hanks’ balanced salt solution and mounted in Pro-
long Antifade Agent and analyzed using a Zeiss LSM510 laser
scanning confocal microscope (Zeiss Microimaging).

Docking of LPP Peptide and BMP-RII Ectodomain—To dock
the LPP peptide onto the BMP-RII ectodomain, a combination
of interactive and automatic docking was employed using the
SwarmDock server. Solvation properties were assessed for dif-
ferent combinations of receptor, LPP peptide, and receptor-LPP
peptide complex. A difference in solvation between the receptor-
peptide complex and the individual binding partner points to the
key side chain residues involved in direct interaction. The frac-
tional surface of a residue buried in the contact (differential acces-
sible surface area: % DASA = (SASres−SASbound)/SASfree) × 100;
solvent-accessible surface, SAS) indicates the location of a residue
within the structural epitope. The surface-accessible area of resi-
dues in bound and unbound receptor-peptide complex identified
the specific residues that were involved in the interaction between
the LPP peptide and the ectodomain structure of BMP-RII.
Statistical Analyses—All experiments were performed three times independently unless indicated, and representative data are shown. Data are expressed as the mean ± S.D. A two-tailed unpaired Student’s t test was used for group comparisons. A value of $p < 0.01$ was considered statistically significant.

RESULTS

LPP Induces Production of Disc Matrix in IVD Cells—The peptide sequence chosen for this study (DHLSDNYTLDHRRAIH) is in fact the human consensus sequence for LPP. This was chosen because our long term aim is to develop LPP into a possible therapeutic agent for treating human disc degeneration. The rabbit consensus sequence for LPP is 75% homologous to the human LPP, although the core sequence motif (YTLDHRRA) is 88% homologous to human, suggesting a high probability of functionality in the rabbit system. To ratify the rabbit primary chondrocyte culture model and the efficacy of human LPP we examined the activity of LPP on rabbit IVD cells by quantifying the expression of the transcription factor SOX9 and the major extracellular matrix macromolecules, aggrecan and collagen (31–33). Previous studies (34, 35) demonstrate that SOX9 activates expression of aggrecan and collagen II. Therefore, primary IVD cells were treated for 9 days in three-dimensional cultures with peptide LPP, or a control inactive peptide LPR, and compared with untreated cells. Real-time RT-PCR demonstrated that LPP treatment significantly up-regulated the expression of aggrecan, collagen II, and SOX9 in IVD cells (Fig. 1A). As mRNA expression may not necessarily correlate with protein synthesis we next examined aggrecan protein production using a commercial ELISA, and collagen II and SOX9 proteins were analyzed by Western blotting (Fig. 1B). Aggrecan, collagen II, and SOX9 were significantly elevated by LPP treatment. Taken together, our data demonstrate that LPP induces expression of the chondrocyte-specific transcription factor SOX9 and the chondrocyte-specific matrix macromolecules aggrecan and collagen II. The results suggest that human LPP is effective in inducing disc matrix production in rabbit IVD cells. Together, these data confirm previous findings (29, 30) regarding chondrocyte matrix production by LPP and ratify the rabbit primary chondrocyte culture model for further mechanistic studies.
We next performed a time course experiment to determine when maximum up-regulation of disc matrix genes is achieved. Primary disc cells were cultured in alginate beads and treated for 0, 4, 7, 9, 14, and 21 days in the presence of LPP. As shown in Fig. 1C, LPP treatment significantly increased expression of aggrecan and collagen II by day 7. The maximum up-regulation of aggrecan and collagen II by LPP occurred at 9 days and remained significantly up-regulated for up to 21 days.

**LPP Promotes Production of the Anabolic Factors BMP-4 and BMP-7**—Having demonstrated that LPP induces the up-regulation of aggrecan, collagen II, and SOX9, further experiments were performed to elucidate the potential molecular mechanisms. Members of the BMP family can elicit potent anabolic responses in IVD cells including synthesis of IVD matrix proteins (10, 12, 19). Therefore, experiments were performed to determine whether LPP promotes BMP transcription and protein production. Primary IVD cells were treated for 9 days in three-dimensional alginate cultures with LPP peptide or control LPR peptide and compared with untreated cells. Real-time PCR quantification of major BMP family members including BMP-2, -4, -6, and -7 revealed significant up-regulation of BMP-4 and BMP-7, but not BMP-2 or BMP-6 (Fig. 2A). Similarly, protein production quantified by Western blotting demonstrated that LPP treatment significantly increased the protein production of BMP-4 and BMP-7, but not BMP-2 and BMP-6 (Fig. 2B).

**Inhibition of BMP Activity Reduces LPP-induced Production of the Disc Matrix**—To further confirm that LPP induced up-regulation of aggrecan, collagen II, and SOX9 by a mechanism involving induction of BMPs, a BMP antagonist noggin was
used to block the BMP signaling pathway. Noggin is a decoy molecule that binds directly to the BMPs preventing their association with the BMP receptors (36). Primary IVD cells were treated for 9 days in three-dimensional alginate cultures with LPP in the absence or in the presence of noggin (0, 200, and 600 ng/ml). Whereas LPP treatment increased expression of aggrecan, collagen II, and SOX9 mRNAs, assessed by real-time RT-PCR, high dose noggin treatment completely inhibited LPP-induced expression of aggrecan, collagen II, and SOX9 (Fig. 3A). These data were further confirmed by quantification of aggrecan protein concentration using commercial ELISA and SOX9 and collagen II by Western blotting (Fig. 3B). Because no exogenous BMPs were added to the culture system this result suggests that LPP induces up-regulation of aggrecan, collagen II, and SOX9 by a mechanism involving cell-autonomous production of BMPs (specifically BMP-4 and BMP-7).

**LPP Promotes Disc Matrix Production by BMP-RI-induced Smad1/5 Phosphorylation**—The Smad signal pathway is a primary mediator of BMP activity. Our data above suggest a specific involvement of BMPs in LPP-mediated responses. We therefore carried out a time course experiment to examine the effect of LPP on Smad phosphorylation. LPP increased phosphorylation of classical BMP-induced Smad species (Smads 1 and 5) within 2 h (Fig. 4A), peaking by 1 day of LPP stimulation, and remaining at peak steady-state levels for at least 2 days. No significant effect on TGFβ-induced Smad2/3 was observed at any time point. Furthermore, LPP failed to significantly promote activation of ERK1/2 and AKT signal transduction pathways, but a modest activation of p38 was observed (Fig. 4B).

To prove a specific role for BMP signaling pathways in LPP-mediated cell responses, we employed a specific BMP type I receptor (BMP-RI) antagonist, dorsomorphin, which specifically inhibits BMP-RI-mediated signal transduction and thus blocks BMP-induced Smad1/5/8 phosphorylation (37, 38). As a control we utilized SB-431542, a specific inhibitor that blocks TGFβ-mediated Smad2/3 phosphorylation. As shown in Fig. 5A, LPP-induced aggrecan protein production, as quantified by ELISA, was significantly inhibited by dorsomorphin, but not by SB431542 (39). Similarly, dorsomorphin, but not SB431542, potently inhibited LPP-induced SOX9 and collagen II production, as quantified by Western blotting (Fig. 5A). By contrast, cells treated with the PI3K/AKT inhibitor LY294002, the p38 MAPK inhibitor SB203580, or the ERK1/2 inhibitor U0126 failed to suppress LPP-induced production of aggrecan, collagen II, and SOX9 (figure 5B). Identical data were observed at the transcriptional level using real time RT-PCR (Fig. 5C). Collectively, these data support the concept that BMP-RI-mediated activation of Smad1/5 is required for LPP promoted disc matrix production.

Further, to verify the function of BMP-RI in LPP-mediated responses, we utilized a small interfering RNA (siRNA)-based approach to knockdown two major BMP type I receptors, namely BMP-Rla (ALK3) and BMP-Rlb (ALK6). We used Western blotting to validate the effective knockdown of BMP-Rla and BMP-Rlb (Fig. 6A). Importantly, BMP-Rlb knockdown dramatically reduced LPP-mediated up-regulation of aggrecan, collagen II, and SOX9 compared with the control (Fig. 6B). Moreover, knockdown of BMP-Rlb dramatically reduced LPP-mediated Smad1/5 phosphorylation compared with the control (Fig. 6C). These data confirmed a critical role of BMP-Rlb in LPP-mediated disc matrix production.

To investigate further how cell-autonomous BMP-induced signals amplify and sustain LPP-initiated responses, we next examined Smad1/5 phosphorylation by cell-autonomous BMPs (BMP-4 and BMP-7). As shown in Fig. 6D, blockage of BMP activity by noggin effectively inhibited the phosphorylation of Smad1/5 induced by LPP. Furthermore, blockade of BMP-4, by neutralizing antibody, weakly reduced Smad signaling whereas BMP-7 ablation potently prevented LPP-induced phosphorylation of Smad1/5 (Fig. 6E). This suggests a key role for cell-autonomous BMP-7 in LPP action. These results are consistent with the data above showing that both BMP-RI-mediated Smad1/5 phosphorylation and cell-autonomous BMPs are necessary for LPP-induced disc matrix production.

**BMP-RII Mediated LPP-induced Up-regulation of IVD Matrix Production**—Our data above suggest involvement of BMP-RI in the activation of the BMP-Smad signal pathway by LPP as a likely consequence of indirect BMP synthesis. To extend these studies we next investigated the possible involvement of type II BMP receptors in LPP action. There are three distinct type II receptors, namely BMP-RII and activin type II
receptors A and B (Act-RIIa and Act-RIIb). IVD cells were transfected with BMP-RII siRNA (siBMP-RII), combined Act-RIIa siRNA and Act-RIIb siRNA (siActR-IIa and siActR-IIb), or specific control lamin A/C siRNA (siLamin) in the presence or absence of 200 ng/ml LPP peptide. Because the anti-ActR-II antibody recognizes both Act-RIIa and Act-RIIb, siActR-IIa and siActR-IIb were combined to knock down these two proteins. A, after a 3-day incubation, knockdown efficiency of BMP-RII and Act-R-I by siRNA was evaluated by Western blotting using an anti-BMP-RII antibody, or an anti-ActR-II antibody that recognizes both Act-RIIa and Act-RIIb. The top panels reflect densitometry values normalized to GAPDH from three different experiments. The bottom panels show representative Western blots. B, the level of aggrecan protein was determined by ELISA (top panel), and levels of phosphorylated Smad1/5 (pSmad1/5), phosphorylated Smad2/3 (pSmad2/3), collagen II, and SOX9 proteins were measured by Western blotting (bottom panel) in LPP-stimulated cells following BMP-RII or ActR-II knockdown. C, real-time PCR for expression of aggrecan, collagen II, and SOX9 mRNAs in LPP-stimulated cells with BMP-RII knockdown. Data are normalized with GAPDH and are expressed as ratio relative to untreated controls. All experiments were performed three times independently, and representative data are shown. Error bars, S.D.

FIGURE 7. BMP-RII is required for LPP-mediated phosphorylation of Smad1/5 and up-regulation of aggrecan, collagen II, and SOX9 in IVD cells. Cells were transfected with BMP-RII siRNA (siBMP-RII), combined Act-RIIa siRNA and Act-RIIb siRNA (siActR-IIa and siActR-IIb), or specific control lamin A/C siRNA (siLamin) in the presence or absence of 200 ng/ml LPP peptide. Because the anti-Act-R-II antibody recognizes both Act-RIIa and Act-RIIb, siActR-IIa and siActR-IIb were combined to knock down these two proteins. A, after a 3-day incubation, knockdown efficiency of BMP-RII and Act-R-I by siRNA was evaluated by Western blotting using an anti-BMP-RII antibody, or an anti-ActR-II antibody that recognizes both Act-RIIa and Act-RIIb. The top panels reflect densitometry values normalized to GAPDH from three different experiments. The bottom panels show representative Western blots. B, the level of aggrecan protein was determined by ELISA (top panel), and levels of phosphorylated Smad1/5 (pSmad1/5), phosphorylated Smad2/3 (pSmad2/3), collagen II, and SOX9 proteins were measured by Western blotting (bottom panel) in LPP-stimulated cells following BMP-RII or ActR-II knockdown. C, real-time PCR for expression of aggrecan, collagen II, and SOX9 mRNAs in LPP-stimulated cells with BMP-RII knockdown. Data are normalized with GAPDH and are expressed as ratio relative to untreated controls. All experiments were performed three times independently, and representative data are shown. Error bars, S.D.

receptors A and B (Act-RIIa and Act-RIIb). IVD cells were transfected with BMP-RII siRNA (siBMP-RII) and combined Act-RIIa siRNA and Act-RIIb siRNA (siAct-R-IIa and siAct-R-IIb), or specific control lamin A/C siRNA (siLamin) in the presence or absence of 200 ng/ml LPP peptide. Because the anti-ActR-II antibody recognizes both Act-RIIa and Act-RIIb, siActR-IIa and siActR-IIb were combined to knock down these two proteins. A, after a 3-day incubation, knockdown efficiency of BMP-RII and Act-R-I by siRNA was evaluated by Western blotting using an anti-BMP-RII antibody, or an anti-ActR-II antibody that recognizes both Act-RIIa and Act-RIIb. The top panels reflect densitometry values normalized to GAPDH from three different experiments. The bottom panels show representative Western blots. B, the level of aggrecan protein was determined by ELISA (top panel), and levels of phosphorylated Smad1/5 (pSmad1/5), phosphorylated Smad2/3 (pSmad2/3), collagen II, and SOX9 proteins were measured by Western blotting (bottom panel) in LPP-stimulated cells following BMP-RII or ActR-II knockdown. C, real-time PCR for expression of aggrecan, collagen II, and SOX9 mRNAs in LPP-stimulated cells with BMP-RII knockdown. Data are normalized with GAPDH and are expressed as ratio relative to untreated controls. All experiments were performed three times independently, and representative data are shown. Error bars, S.D.

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To visualize LPP (red) and probed with Alexa Fluor 488-conjugated BMP-RII antibody to visualize BMP-RII (green). As shown in Fig. 8a, strong staining was observed for both LPPb and BMP-RII but not for the LPRb control. Importantly, significant colocalization (yellow) between LPPb and BMP-RII was observed.

To verify a bona fide peptide-protein association, we performed biotinylated peptide pulldown studies to investigate potential peptide-protein interaction. IVD cells were treated with either a biotinylated peptide LPPb or a biotinylated control peptide LPRb. The biotinylated peptide-protein complexes were captured using avidin-Sepharose columns and visualized on Western blots using peroxidase-conjugated avidin. Strong recovery of LPPb was visualized by staining with peroxidase-conjugated avidin whereas control peptide LPRb was not recovered (Fig. 8b). Next, the avidin-captured peptide-protein conjugates were immunoprecipitated with anti-BMP-RII antibody. The coprecipitation of LPPb and BMP-RII was confirmed by probing Western blots with streptavidin-conjugated HRP or anti-BMP-RII antibody (Fig. 8c).

LPP Interacts with BMP-RII by Means of a Direct Peptide-Protein Interaction—Whereas the data above reveal that LPP induces intracellular signal transduction in the IVD cells, the mechanism involved remains unclear. One possible explanation for our data is a direct association of LPP with BMP receptors. To investigate this possibility we examined colocalization between LPP and BMP-RII using confocal microscopy. IVD cells were treated with a biotinylated variant of LPP (LPPb) or a biotinylated variant of LPR (LPRb) as control. After treatment, cells were labeled with Alexa Fluor 568-conjugated streptavidin to visualize LPP (red) and probed with Alexa Fluor 488-conjugated BMP-RII antibody to visualize BMP-RII (green). As shown in Fig. 8a, strong staining was observed for both LPPb and BMP-RII but not for the LPRb control. Importantly, significant colocalization (yellow) between LPPb and BMP-RII was observed.

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basis for the specificity and affinity of this binding, we performed “docking” of the LPP peptide structure onto the BMP-RII ectodomain structure as shown in Fig. 8D. Although LPP did not show any sequence motif similar to any motifs in BMP based on homology, we identified a region within the ectodomain of BMP-RII that shows structural complementarity toward the LPP peptide. This complementary site is uniquely present in BMP-RII and is absent in BMP-RI. These structural inferences support our biochemical data showing binding specificity between LPP peptide and BMP-RII.

Finally, because our prior data demonstrated noggin sensitivity of LPP activity we repeated our LPPb pulldown studies in the absence or presence of noggin. As shown in Fig. 9, the data further confirmed that the peptide LPP specifically associated with BMP-RII. Moreover, noggin failed to impact the peptide-protein association of LPP and BMP-RII, suggesting that in our system noggin was targeting LPP-induced BMPs rather than LPP associations with the BMP receptor. Taken together, these data provide strong evidence of a direct peptide-protein association between LPP and the BMP-RII. The data further support a mode of action involving BMP-RI, but not Act-RII species in LPP-mediated anabolic effects on IVD cells.

DISCUSSION

In this present study, we investigated the role of LPP in primary intervertebral disc cells cultured in a three-dimensional alginate matrix. Our data demonstrate that LPP stimulates the production of the chondrocyte-specific matrix macromolecular components, aggrecan and collagen II, and promotes expression of the key chondrocyte transcription factor SOX9. The results suggest that LPP can stimulate the production of intervertebral disc matrix. Therefore, LPP may be a potentially
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FIGURE 9. Noggin does not affect association of LPP with BMP-RII in IVD cells. Cells were incubated with a biotinylated peptide LPPb in the absence or presence of noggin (600 ng/ml) and compared with control cells. After a 48-h incubation, cells were washed, UV-cross-linked, and homogenized. Biotin-bound peptide/cellular protein conjugates were captured by passing the supernatant through a monomeric avidin-Sepharose column. A, the avidin-captured biotinylated peptide-protein conjugate was detected by Western blotting (wb) using anti-BMP-RII antibody. B, avidin-captured peptide-protein conjugate was immunoprecipitated (IP) with anti-BMP-RII antibody. The precipitates were then probed with HRP-streptavidin or anti-BMP-RII antibody. The data are representative of three independent experiments.

FIGURE 10. Proposed model for LPP-induced chondrocyte matrix production. A, we propose that LPP initiates a signaling cascade through direct association with BMP-RII. This association initiates Smad signaling that converges on BMP genes leading to expression and production of BMP proteins, including BMP-4 and BMP-7. Through a cell-autonomous loop BMP-4 and BMP-7 engage BMP-RI to sustain or amplify Smad signal transduction, ultimately promoting expression of SOX9 and downstream aggrecan and collagen II genes. B, in a feedforward circuit A activates B, and A and B activate C (42). C, we propose that LPP initiates a feedforward circuit in which A is represented by BMP-RII, B by BMP-RI, and C by Smad1/5. This feedforward circuit acts to sustain Smad1/5 signal transduction necessary for initiation of SOX9 and matrix gene activation.

useful clinical adjunct for rejuvenating disc matrix and repairing IVD degeneration either on its own or to boost the activity of exogenous BMPs.

The molecular mechanism by which LPP promotes matrix production is unknown. Understanding the mechanism could significantly facilitate the future development of this agent for clinical applications. In this study we have intensively investigated the mechanisms by which LPP drives matrix formation. Our studies reveal that LPP targets the IVD cells through a direct peptide-protein association between LPP and BMP-RII but not with BMP-RI. Noggin is a potent antagonist of BMP activity and functions by binding to several BMPs with very high affinities, preventing BMPs from binding to their receptors (40). Our data show that LPP activity is potently suppressed by the addition of noggin; however, the peptide–protein interaction between BMP-RRII and LPP was not itself directly impacted by noggin. These data support a mechanism involving BMP production distal to LPP association with the BMP-RII. Consistent with this notion, LPP was found to potently up-regulate gene expression and protein production of a number of BMPs including BMP-4 and BMP-7. Moreover, blockade of BMP-4 modestly reduced LPP activity whereas BMP-7 virtually ablated it, suggesting a dominant role of BMP-7, with possible lesser contributions by BMP-4.

Another interesting finding was that both BMP-RI and BMP-RRII appear to play critical roles in LPP action although LPP does not associate with BMP-RI. Ablation of BMP-RRII completely eliminated LPP-induced activity consistent with a direct association and initiation of Smad signal transaction from this receptor. However, disruption of BMP-RRI-induced Smad activation by dorsomorphin, a specific BMP-RII antagonist (37), likewise abrogated Smad signaling and downstream LPP-induced activity on SOX9 and disc matrix production. Moreover, the specific knockdown of BMP-RI by siRNA inhibits LPP-mediated Smad1/5 phosphorylation and reduced LPP-mediated up-regulation of aggrecan, collagen II, and SOX9. These data define a crucial role for BMP-RI and suggest that BMP-RI signaling is essential for mediating the noggin-sensitive BMP-4/7-induced segment of the signaling loop. Because dorsomorphin, which targets BMP-RI/Smad signaling, prevented up-regulation of aggrecan, collagen II, and SOX9 in the face of intact BMP-RRII signaling, these data suggest that BMP-RI, driven by BMP4/7, rather than BMP-RI activated by LPP is responsible for driving up-regulation of aggrecan, collagen II, and SOX9.

Because BMPs would be expected to also bind to BMP-RII, one explanation for the specificity of BMP4/7 for BMP-RI is that the relatively high doses of LPP used in these studies may have saturated the BMP-RII binding sites preventing or displacing BMPs. Physiologically in vivo, the lower endogenous concentrations of LPP may allow both receptors to respond directly to cell-autonomous BMPs. In the context of a therapeutic agent, however, pharmacological doses of LPP would likely function more closely to that modeled in our studies.

TGFβ is another potent inducer of Smad signal transduction (41); however, our data suggest that TGFβ is not involved in LPP responses as BMP-specific Smads (1 and 5) downstream of BMP receptors were specifically activated in preference to TGFβ receptor Smads (2 and 3).

Taken together we propose a model (Fig. 10) in which LPP initiates signaling through direct association with BMP-RII that initiates Smad signaling that converges on BMP genes leading to expression and production of BMP proteins, including BMP-4 and BMP-7. Furthermore, through a cell-autonomous loop BMP-4 and BMP-7 engage BMP-RI (and/or II) to sustain or amplify Smad signal transduction, ultimately promoting expression of SOX9 and downstream aggrecan and collagen II genes. This system is outlined diagrammatically in Fig. 10A. Interestingly, LPP establishes a classical feedforward circuit converging on Smad activation. In a feedforward circuit A activates B, and A and B activate C (42). In our system, A is represented by BMP-RII, B by BMP-RI, and C by Smad (see Fig. 10, B and C), and consequently LPP association with BMP-RII initiates Smad1/5 signal transduction that produces BMP-4/7 synthesis that binds to BMP-RI intensifying the Smad1/5 signal.
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This feedforward circuit acts to sustain Smad1/5 signal transduction necessary for disc matrix gene activation.

In summary, our studies provide strong evidence demonstrating that the peptide LPP binds to BMP-RII on chondrocytes initiating a complex Smad/BMP signaling cascade and consequently promotes the production of intervertebral disc matrix. Thus, LPP may have value in the treatment of degenerated discs.

REFERENCES