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Heterodimeric BMP-2/7 for nucleus pulposus regeneration – *in vitro* and *ex vivo* studies

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Abstract

Intervertebral disc (IVD) degeneration is the leading trigger of low back pain, which causes disability and leads to enormous healthcare toll worldwide. Biological treatment with growth factors has evolved as potential therapy for IVD regeneration. Bone morphogenetic protein 2 (BMP-2) and BMP-7 have shown promise in this regard. In the current study, we evaluated the effect of BMP-2/7 heterodimer for disc regeneration both *in vitro* and in organ culture. Nucleus pulposus (NP) cells isolated from bovine caudal disc were cultured in a fibrin-hyaluronan (FBG-HA) hydrogel for up to 14 days. BMP-2/7 heterodimer covalently incorporated within the hydrogel up-regulated the aggrecan and type II collagen gene expression, and glycosaminoglycan synthesis of NP cells. The activity of the BMP-2/7 heterodimer was dose dependent. A higher dose of BMP-2/7 was further assessed in an IVD whole organ system. After 14 days of culture with cyclic dynamic load, the BMP-2/7 heterodimer delivered into the nucleotomized region showed potential to stimulate the gene expression and synthesis of proteoglycan in the remaining NP tissue after partial nucleotomy. The gene expression level of type I collagen and alkaline phosphatase in the native disc tissue were not affected by BMP-2/7 treatment, indicating no adverse fibroblastic or osteogenic effect on the disc tissue. Intradiscal delivery of BMP-2/7 heterodimer may be a promising therapeutic approach for NP regeneration. The current IVD whole organ partial nucleotomy model may be utilized for screening of other biomaterials or drugs to treat early degenerated disc.

Keywords: BMP-2/7 heterodimer, nucleus pulposus regeneration, intervertebral disc, whole organ culture

Introduction

The intervertebral disc (IVD) is composed of the central gel-like nucleus pulposus (NP), the surrounding annulus fibrosus (AF), and the cartilaginous endplates that anchor the discs to the adjacent vertebrae[1]. The main functions of the IVD are to provide flexibility to the spine, and to transmit the mechanical load. Low back pain caused by IVD degeneration is the leading cause of disability worldwide[2]. Biological approaches for treatment of IVD degeneration have evolved in the last decade, including the use of recombinant or natural proteins that can lead to the restoration of the native healthy disc[3; 4].

Bone morphogenetic proteins (BMPs) are involved in a wide variety of developmental processes, including bone, cartilage and IVD formation[5; 6]. In particular, BMP-2 and BMP-7 have been investigated in musculoskeletal research and have demonstrated promising potential for IVD regeneration. BMP-2 can up-regulate the gene expression and protein synthesis of extracellular matrix components in rabbit NP cells[7], and human NP cells[8; 9] cultured *in vitro*. In a rabbit *in vivo* study, treatment with adeno-associated viral vector carrying the gene for BMP-2 delayed the degeneration process in annulotomized discs[10]. BMP-7 was found to stimulate extracellular matrix synthesis in NP cells and AF cells from rabbit[11; 12] and human[13] origin. It also inhibited cellular apoptosis, induced by tumor necrosis factor-alpha, in human NP cells[14]. The effect of BMP-7 for IVD regeneration has furthermore been explored in small animal models, including rat[15; 16], rabbit[17-19] and canine[20]. These studies revealed that intradiscal delivery of BMP-7 could restore disc height, stimulate extracellular matrix formation and prevent degeneration in the IVD.

Several studies have demonstrated that heterodimeric BMP-2/7 had a stronger activity for bone regeneration compared with BMP-2 or BMP-7 homodimers[21; 22]. Thus in this study, we

investigated the effect of heterodimeric BMP-2/7 on the regeneration of the NP. In addition, the BMP-2/7 heterodimer was covalently incorporated within a fibrin-hyaluronan (FBG-HA) conjugate hydrogel[23], in order to obtain a slow release and prevent ectopic leakage. The dose dependent effects of the BMP-2/7 heterodimer on the proliferation and extracellular matrix synthesis of bovine NP cells were first tested *in vitro* in a three-dimensional FBG-HA hydrogel. Then the effect of the BMP-2/7 heterodimer, covalently bound to the FBG-HA hydrogel, on IVD regeneration was evaluated *ex vivo* in a bovine IVD whole organ culture nucleotomy model.

Methods

1. BMP-2/7 heterodimer production

BMP-2/7 heterodimer was produced as described previously [23]. The BMP-2/7 heterodimer contained extra amino acids for transglutaminase crosslinking and plasmin cleavage sites at the N terminus. This manipulation facilitates the covalent binding of BMP-2/7 to fibrin or fibrin-like materials and cell-demanded growth factor release [23]. Briefly, recombinant human BMP-2 including amino acids for transglutaminase and plasmin cleavage sites (TG-BMP-2) was cloned and expressed in *E. coli*. TG-BMP-2 monomers were purified with affinity and size exclusion chromatography. TG-BMP-2 and BMP-7 monomers were then refolded in a buffer with CHAPS (3((3-cholamidopropyl)-dimethylammonio)-propansulfonate) and glutathione. In a final purification step, BMP-2/7 heterodimers were separated from unfolded monomers and BMP-2 homodimers.

2. Fabrication of FBG-HA conjugate hydrogel

A FBG-HA conjugate hydrogel, which had been shown to promote NP cell growth and matrix production[24], was used for BMP-2/7 heterodimer delivery. Fibrinogen-HA conjugate solution was synthesized with 235 KDa HA at FBG/HA w/w ratio of 17:1 via a two-step procedure as described elsewhere [24]. FBG-HA hydrogels were prepared by mixing 2/3 volume of FBG-HA conjugate solution with 1/3 volume of thrombin solution (5.2 U/mL) and allowed to polymerize at 37ºC for 20 min.

3. Effect of BMP-2/7 heterodimer on NP cells *in vitro*

3.1. Experimental set-up

An *in vitro* cell culture experiment was performed to determine the potential of BMP-2/7 at different doses for promoting the metabolic activity and function of NP cells. NP cells were isolated from 4-10 months old bovine caudal discs as previously described [24]. Primary NP cells were encapsulated within FBG-HA conjugate hydrogel beads at a density of 1.2 x 10^5 cells per bead (4 x 10^6 cells/mL). Each bead had a volume of 30 µL and was prepared by mixing 20 µL of FBG-HA conjugate, containing NP cells in suspension, with 10 µL of thrombin containing different concentrations of BMP-2/7. The final concentrations of BMP-2/7 in the FBG-HA hydrogels were 0 (hydrogel control), 1000 ng/mL, and 5000 ng/mL. Cell–gel constructs were cultured in Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L glucose) with 2% fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin (1% Pen/Step) (all products from Gibco, Praisley, UK), 1% ITS+ Premix (Discovery Labware, Inc., Bedford, USA) and 50 µg/mL ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, USA) for 7 or 14 days. The cell culture medium was changed three times per week.

3.2. Gene expression analysis

Total RNA extraction from NP cells in hydrogels was performed with TRI Reagent (Molecular Research Center, OH, USA) after 7 and 14 days of culture. Reverse transcription was carried out with TaqMan™ reverse transcription reagents (Applied Biosystems, CA, USA) using random hexamer primers and 0.5 μg of total RNA. Real-time PCR was performed using the Step-One-Plus instrument (Applied Biosystems). Table 1 shows the sequences of custom designed bovine primers and TaqMan probes (Microsynth, Balgach, Switzerland) for collagen type I (COL1A2), collagen type II (COL2A1) and aggrecan (ACAN). For amplification of 18S ribosomal RNA, a gene expression assay from Applied Biosystems was used (18S, 4310893E). The comparative Ct method was performed for relative quantification of target mRNA with 18S rRNA as endogenous control.

3.3. Glycosaminoglycan (GAG) and DNA content

On day 7 and day 14, the cell–gel constructs were digested with 0.5 mg/mL proteinase K at 56°C overnight. The GAG content in the hydrogels was measured using the 1,9-dimethylmethylene blue dye (DMMB) method [25] and was normalized to the DNA content in respective samples. GAG content in hydrogels on day 0 were subtracted from all the samples. The DNA content in the hydrogels was measured by PicoGreen assay (Invitrogen, Eugene, Oregon, USA) according to the manufacturer's instruction.

4. Effect of BMP-2/7 heterodimer on IVDs with NP replacement under dynamic loading *ex vivo*

4.1 Dissection of bovine caudal IVDs

For assessment of the BMP-2/7 heterodimer in an IVD organ culture system, which supplies a microenvironment close to the *in vivo* situation, bovine caudal spines (4-10 months) were obtained from local abattoirs and harvested aseptically within three hours after death. After removal of the soft tissue, IVDs including the cartilaginous endplates were dissected with a band saw (model 30/833, Exakt Apparatebau, Norderstedt, Germany) and cleaned with a Pulsavac jet-lavage system (Zimmer, IN, USA) [24]. Initial disc height and diameter were measured with a caliper before discs were washed with phosphate buffered saline (PBS) containing 1000 U/mL penicillin and 1000 µg/mL streptomycin for 15 min. Then discs were transferred to a 6-well plate containing disc culture medium (DMEM 4.5g/L glucose supplied with 2% FCS, 1% Pen/Strep, 1% ITS+ Premix, 50 µg/mL ascorbate-2-phosphate and 0.1% Primocin) and incubated overnight at 37°C, 85% humidity and 5% CO_2. After dissection, disc height of all bovine IVDs in this study ranged from 7 to 14 mm and disc diameter ranged from 13 to 20 mm.

4.2 Partial nucleotomy and NP replacement in organ cultured IVDs

A partial nucleotomy was performed mechanically as described previously [24]. To reach a similar nucleotomy ratio of approximately 50% of the total NP volume for different sizes of discs, a NP cavity with diameter of 4 mm was created in small discs with diameter of 13-16.5 mm; in discs with diameter of 16.5-20 mm, a NP cavity with diameter of 6 mm was created. Discs were nucleotomized through the endplate by central incisions using a biopsy punch of 4 or 6 mm diameter and a number 11 blade. After removing an endplate core, the NP tissue below the endplate core until the distal endplate was excised. After partial nucleotomy, 50–80

μL of FBG-HA hydrogel containing 0 or 5000 ng/mL BMP-2/7 were pipetted into the nucleotomized region. Our previous study had shown that the FBG-HA hydrogel alone was not sufficient to restore the axial stiffness of partially nucleotomized discs under dynamic load[24]. Therefore, a polyurethane (PU) scaffold (kindly supplied by Nicast Ltd., Lod, Israel) with swelling ability *in situ*[26] was subsequently placed into the FBG-HA hydrogel in the NP cavity. The PU scaffold was composed of a water absorbable core and an electrospun envelope. It has been used as a NP replacement material in this IVD organ culture nucleotomy model, thereby filling the nucleotomized region and restoring the mechanical property of nucleotomized IVDs [26]. After implantation of the biomaterials, the removed endplate stopper was immediately re-inserted, and the crack between the stopper and the remaining endplate was sealed by polymethyl methacrylate (Vertecem Mixing Kit, Synthes, Teknimed S.A., Bigorre, France) according to manufacturer's instruction. For each disc, approximately 100–150 mg of polymethyl methacrylate was applied.

4.3 Dynamic load on IVDs within bioreactor

To mimic physiologically relevant conditions in organ cultures, dynamic loading was applied on the IVDs with a bioreactor system [27]. IVDs were placed in custom made chambers filled with disc culture medium, and loaded dynamically for 3 hours/day at 0-0.1 MPa and 0.1 Hz. After dynamic loading IVDs were cultured free swelling in 6-well plates overnight. The repetitive dynamic loading and free swelling culture was applied for 14 consecutive days. The experiments were performed using bovine IVDs from 12 tails, with 2 discs from each tail randomly assigned to one of the two experiment groups: FBG-HA without BMP2/7, or FBG-HA with 5000 ng/mL BMP2/7.

4.4 Disc height change

Disc height was measured with a caliper at different time points: day 0 after dissection, day 1 after 1st load, day 2 after 1st recovery, day 7 after 7th load, day 8 after 7th recovery, day 14 after 14th load, and day 15 after 14th recovery. Each disc was measured at four positions and the mean value was used to calculate the percentage of disc height change. Disc height change was normalized to the initial dimension after dissection.

4.5 Biological evaluations

After 14 days of culture with repetitive dynamic loading and free swelling recovery, cartilaginous endplates of each disc were removed, and remaining NP and AF tissues were harvested by using a biopsy punch and scalpel, respectively. Approximately 100 mg of each AF and NP was used for mRNA extraction; 50 mg was digested with 0.5 mg/mL proteinase K at 56°C overnight for glycosaminoglycan (GAG), collagen and DNA content measurement; and 30 mg of NP was used for [^{35}S] sulfate incorporation assay to assess proteoglycan synthesis rate.

Gene expression analysis

Tissue samples were flash frozen, pulverized in liquid N_2, and homogenized using a TissueLyser (Qiagen, Venlo, The Netherlands). Total RNA was extracted with TRI Reagent and reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA). Quantitative real-time PCR was performed using the Step-One-

Plus instrument. The sequences of custom designed bovine primers and TaqMan probes are shown in Table 1. For amplification of 18S ribosomal RNA, Versican (Bt03217632_m1), Biglycan (BGN, Bt 03244532_m1) and Alkaline Phosphatase (ALPL, Bt03244508_m1), gene expression assays from Applied Biosystems were used. The comparative Ct method was performed for relative quantification of target mRNA with 18S rRNA as endogenous control.

GAG, collagen, and DNA content

The GAG content in the NP and AF tissue was determined by using the DMMB method as mentioned above. The total amounts of collagen in NP and AF samples were measured using a quantitative Hydroxy-Proline assay as described earlier[28]. DNA content was measured spectrofluorometrically using Hoechst (33258) dye.

^{35}S sulfate incorporation rate

NP tissue was incubated for 16-20 hours in disc culture medium supplemented with 2.5 µCi ^{35}S-sulfate /mL (Na_2 ^{35}SO4; PerkinElmer, Schwerzenbach, Switzerland). Thereafter NP tissue was digested with 0.5 mg/mL proteinase K at 56°C overnight. Conditioned medium and proteinase K digested tissue samples were analyzed after PD-10 desalting column (GE Healthcare, Glattbrugg, Switzerland) and radioactive counts were measured by Wallac 1414 Liquid Scintillation Counter (PerkinElmer, Schwerzenbach, Switzerland). Finally the counts per minute (cpm) were normalized to the DNA content of the respective samples.

4.6 Safranin O/Fast Green staining

After 14 days of culture with repetitive dynamic loading and free swelling recovery, whole discs with implanted biomaterials were fixed in 70% methanol and transferred into PBS with 5% sucrose at 4˚C overnight before cryosectioning. Transverse sections of IVDs were cut at a thickness of 12 µm. Sections were stained with 0.1% Safranin-O and 0.02% Fast Green to reveal proteoglycan and collagen deposition, respectively, and counterstained with Weigert's Haematoxylin to reveal cell distribution.

4.7 Fourier transform infrared (FTIR) microscopic imaging

After 14 days of culture with repetitive dynamic loading and free swelling recovery, a set of discs were decalcified and subsequently embedded in paraffin. Four micrometre sagittal IVD sections were cut and mounted on custom made reflective 316 stainless steel slides for infrared analysis. FTIR microscopic images were collected using an Agilent 680-IR FTIR spectrometer (ISys50®, Malvern Instruments Limited, Worcestershire, UK) coupled to a FTIR imaging microscope fitted with a liquid nitrogen cooled 64 x 64 mercury-cadmium-telluride focal plane array detector (FPA) and an automated sampling stage. For each sample, an area of 71 x 192 pixels (approximately 6.3 x 16.8 mm) was mapped in transflectance mode. Spectra were attained with a spectral resolution of 4 cm^{-1} over a wavenumber range of 950-1900 cm^{-1}. Data was pre-processed by performing a second derivative (Savitsky-Golay ISys50 software, Filter order 3, filter length 15) on the spectra to reduce baseline effects and assist the resolution of weaker absorption peaks[29]. Pixels within the tissue section where spectra indicated only the presence of paraffin or the substrate were identified and masked using spectral statistics (ISys50, histogram). Regions of interest (ROI; 10 x 192 pixels) across the middle of each sagittal section were cut and compiled to one data matrix. The data set was

analyzed using a multivariate curve resolution-alternating least squares algorithm (MCR-ALS) described by Andrew and Hancewicz [30] and Wang et.al. [31]. MCR-ALS was carried out using the MCR-ALS v1.6 software (MCRv1.6 Copyright © 2003-2004 Unilever) on two wavenumber regions of 950-1600 cm^{-1} and 950-1300 cm^{-1} with the following settings; initial estimates: 4 -10F, NIPALS, ALS; ALS constraints: MALS-2D, none, 1e-005, 500. General tissue, collagen type I, collagen type II and proteoglycan distributions were estimated according to previous studies that have shown good agreement between (immuno-) histological staining and the integrated peak area of the 2nd derivative of the amide III spectral region (1186-1297 cm^{-1}) as well as MCR-ALS score maps [32]. Additionally, average matrix component per tissue distribution across ROIs were calculated and are represented as estimated component per tissue distribution line profiles across the normalized width (0-100%) of each IVD.

5. Statistical analysis

SPSS 21.0 statistical software was used for statistical analysis. One sample Kolmogorov–Smirnov test was used to define whether the data were normally distributed (normal distribution at $p > 0.1$). For data that were normally distributed, one-way ANOVA with Tukey post hoc test was used to determine differences among three or more different groups, unpaired T test was used to determine differences between two groups. For data that were not normally distributed, Kruskal-Wallis test was used to determine differences among three or more different groups, Mann-Whitney U test was used to determine differences between two groups. A p-value <0.05 was considered statistically significant; p-value <0.1 was considered to indicate a trend.

Results

1. Effect of BMP-2/7 heterodimer on NP cells *in vitro*

The ACAN, COL1A2 and COL2A1 gene expression levels of NP cells encapsulated in FBG-HA hydrogels after 7 or 14 days of culture were normalized to the expression levels of cells at day 0 (after isolation from NP tissue and before 3D culture, Fig. 1 A). On day 7, there was a trend of higher ACAN expression in hydrogels containing 5000 ng/mL of BMP-2/7 compared with hydrogels without ($p=0.065$) and with 1000 ng/mL ($p=0.067$) of BMP-2/7. On day 14, a trend of higher COL2A1 expression was observed in hydrogels containing 5000 ng/mL of BMP-2/7 compared with hydrogels without ($p=0.085$) and with 1000 ng/mL ($p=0.053$) of BMP-2/7.

The cell proliferation was not affected by BMP-2/7 incorporation into the hydrogel (Fig. 6 B); while the GAG amounts synthesized per cell (GAG/DNA value) were significantly increased in hydrogels with 1000 or 5000 ng/mL BMP-2/7 after 7 days of culture (Fig. 1 C; $p<0.05$).

2. Effect of BMP-2/7 heterodimers on IVDs with NP replacement under dynamic loading *ex vivo*

2.1 Disc height change

A diurnal disc height change pattern was observed during the entire period of 14 days of repetitive dynamic load (Fig. 2 A). Compared with the initial disc height after dissection, a disc height loss of -3.6% to -0.3% was observed after 3 hours of dynamic loading. After

overnight free swelling, the discs height recovered, whereby an increase of 4.7% to 11.7% was observed.

2.2 Gene expression

Gene expression levels after 14 days of culture with dynamic load were compared to the expression levels of disc tissue from respective bovine tails before starting organ culture on day 0 (Fig. 2 B and C). Compared with day 0, the gene expression of ACAN in NP tissue of discs with hydrogel, but without BMP-2/7 implantation, did not change. When FBG-HA hydrogel containing BMP-2/7 was implanted, the gene expression of ACAN in NP tissue increased 4.5-fold ($p = 0.083$, Fig. 2 B). Compared with day 0, gene expression of COL2, COL1, MMP13, and ALPL increased, while gene expression of BGN decreased in the NP tissue of discs without BMP-2/7. The implantation of FBG-HA hydrogel containing BMP-2/7 did not show significant effects on the expression of these genes in the NP tissue (Fig. 2 B).

Compared with day 0, gene expression of ACAN, COL1, MMP13, ALPL and Versican increased, while gene expression of COL2 decreased in the AF tissue of discs without BMP-2/7. The implantation of FBG-HA hydrogel containing BMP-2/7 did not show significant effects on the expression of these genes in the AF tissue (Fig. 2 C).

2.3 Biochemical analysis

GAG content, collagen content and PG synthesis rate per cell of native NP and AF tissue in partially nucleotomized discs treated with hydrogel with/without BMP-2/7 incorporation are shown in Table 2. In the remaining NP tissue in contact with the hydrogel-growth factor

implants, the GAG/DNA ratio increased by 46% when BMP-2/7 was incorporated (p=0.113). The Collagen/DNA ratio and PG synthesis rate/DNA ratio were not affected by addition of BMP-2/7 to the hydrogel. In the AF tissue, neither GAG/DNA ratio nor Collagen/DNA ratio was affected by BMP-2/7 incorporation.

2.4 Histological analysis of whole discs

Images of Safranin O/Fast Green stained transverse sections of discs cultured for 14 days are shown in Figure 3. In line with a previous study on the PU scaffold for NP replacement[26], the swollen PU scaffold filled the nucleotomized region completely (Fig. 3 A, C). The envelope of the PU scaffold was observed as a ring in direct contact with the remaining disc NP tissue. The core of the PU scaffold essentially consists of water and therefore appears as an empty non-stained region in the center of the disc section. Part of the FBG-HA hydrogel with/without BMP-2/7 diffused into the envelope of the PU scaffold, as indicated by the intense red Safranin-O staining due to the HA component in the hydrogel (Fig. 3 B, D). The remaining native disc NP tissue continued to present intense proteoglycan staining with Safranin O after 14 days of culture with dynamic load.

2.5 FTIR imaging analysis of disc tissue

An example of an image reflecting the tissue distribution (sagittal direction) of a paraffin embedded partially nucleotomized disc treated with hydrogel without BMP-2/7 (0 ng/mL BMP-2/7) cultured for 14 days under dynamic load is shown in Figure 4 A. Tissue distribution maps of ROI of partially nucleotomized discs treated with hydrogel without BMP-2/7 (top) and with 5000 ng/mL BMP-2/7 (bottom) cultured for 14 days under dynamic

load are shown in Figure 4 B. Both ROI tissue images show a cavity in the center of the disc where the implant was situated, surrounded by native disc tissue. Estimated component / tissue distribution line profiles of proteoglycan, collagen type II and collagen type I across the IVD sections display distinctive trends, with proteoglycan and collagen type II profiles showing an overall increase and collagen type I profiles revealing a decrease from the outer AF region towards the central NP region (Fig. 4 C). A comparison of the line profiles between samples treated with hydrogel with and without BMP-2/7 indicates very similar overall trends for proteoglycan, collagen type II and collagen type I. However, localized differences in proteoglycan distributions can be observed, with the disc treated with 0 ng/mL BMP-2/7 showing a lower proteoglycan per tissue ratio than the disc treated with 5000 ng/mL BMP-2/7 in most areas across the disc, apart from two regions at around 26-30 % and 34-38 % IVD width. Distribution line profiles of collagen type II for discs treated with 0 ng/mL and 5000 ng/mL BMP-2/7 show similar but less prominent trends in comparison to the proteoglycan distributions. The collagen type I line profile of the disc treated with hydrogel and 0 ng/mL BMP, shows more pronounced localized lower component to tissue ratios (sharp negative features) at around 6-12 %, 20-45 % and 84-90 % in comparison to the profile of the disc treated with hydrogel and 5000 ng/mL BMP-2/7.

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Discussion

Fibrinogen-hyaluronan conjugate hydrogel has previously been evaluated as a favorable biomaterial for nucleus pulposus regeneration[24]. In the present study, we first investigated the dose effect of BMP-2/7 covalently incorporated within the FBG-HA hydrogel by encapsulating NP cells in the hydrogel and culturing for up to 14 days. The proliferation rate

of the NP cells was not affected by BMP-2/7. Incorporation of BMP-2/7 at the concentration of 1000 ng/mL and 5000 ng/mL both stimulated the GAG synthesis after 7 days of culture. In addition, NP cells treated with 5000 ng/mL of BMP-2/7 showed higher ACAN gene expression level after 7 days, and higher COL2 gene expression level after 14 days, which indicates a stimulatory effect on the further production of NP like extracellular matrix after another culture period. These results are in line with previous *in vitro* disc cell culture studies performed with BMP-2[7-9] and BMP-7[11-13] and further corroborate that BMP-2/7 can stimulate the extracellular matrix production of NP cells in 3D culture *in vitro*.

Based on the results of the *in vitro* study, the higher dose of 5000 ng/mL BMP-2/7 was selected in the IVD organ culture experiments. After 14 days of culture with repetitive dynamic load, BMP-2/7 delivered into the partially nucleotomized disc up-regulated the ACAN gene expression level in the remaining NP tissue. The FTIR spectrum also showed higher proteoglycan intensity over most areas of the analyzed sagittal IVD sections in the BMP-2/7 treated discs. In biochemical analysis, although not significant, the GAG/DNA ratio was up-regulated 1.5-fold in the NP tissue and 2.3-fold in the AF tissue with the delivery of BMP-2/7. These results indicate that the covalently incorporated BMP-2/7 has the potential to stimulate the gene expression and synthesis of proteoglycan components in the NP tissue of partially nucleotomized discs.

A previous study by Huang *et al.* has reported negative effects after intradiscal injection of 100 µg BMP-2 into rabbit lumbar discs[33]. Fibroblastic disc tissue, osteophyte formation, and hypertrophy of cartilaginous endplate were observed at 12 weeks after surgery. In the current IVD whole organ culture study, the COL1 and ALPL gene expression levels in the native disc tissue were not dysregulated by BMP-2/7 treatment. The Safranin O/Fast Green staining also

revealed intense proteoglycan staining in the remaining NP tissue after partial nucleotomy and BMP-2/7 treatment. These results demonstrate that the BMP-2/7 applied at the current concentration (0.25-0.4 µg / disc) and in the formulation of covalent binding to FBG-HA does not have adverse fibroblastic or osteogenic effects on the disc tissue. This is consistent with a previous study by Kim *et al.*, where the exposure of human disc cells cultured in 3D beads to BMP-2 did not result in an osteogenic response, whereas proteoglycan synthesis was increased in NP cells[9]. The dose, the exposed cell type, and the delivery method of growth factors play an important role in directing the fate of the cell phenotype. Importantly, leakage of growth factor out of the disc space substantially increases the risk for unwanted effects on other cell types. The covalent binding of BMP-2/7 to the FBG-HA conjugate hydrogel provides a depot of growth factor, avoiding the need for repetitive application, and at the same time minimizes the potential for growth factor leakage.

Although the biochemical analysis revealed higher matrix synthesis and content after incorporation of growth factor compared to pure hydrogel delivery, these results did not reach statistical significance. An explanation might be the relatively short observation time of two weeks in combination with a delivery system that requires hydrogel degradation for growth factor release[23]. Longer culture periods may be needed to detect a significant impact of sustained release BMP-2/7 delivery on the tissue matrix level. Furthermore, combinations of different growth factors might show superior efficacy. The synergistic effects of BMP and other growth factors on disc cells have been investigated by several groups with *in vitro* studies[34-37]. BMP-7 and insulin like growth factor 1 potentiated anabolic stimulation through complementary mechanisms on matrix formation of bovine NP cells compared with treatment with either growth factor alone[36]. Porcine AF cells treated with the combination of BMP-2

and transforming growth factor beta 1 caused a greater decrease in MMP-1 and increase in aggrecan than either growth factor alone[34]. In another study, bovine NP cells were cultured with BMP-7 and lactoferricin B in alginate[35]. It was found that lactoferricin B maximizes the regenerative activity of BMP-7 by inhibiting noggin. Human degenerative NP cells co-transfection with BMP-7 and Sox9 also showed a synergistic effect on type II collagen transcription[37]. However, all these studies have been performed using disc cells cultured *in vitro*, thereby lacking the disc tissue and organ specific microenvironment. Further studies will be required to elucidate the synergistic effect of BMP and other growth factors for disc regeneration. The use of the described IVD whole organ nucleotomy model could bring information at higher relevance level for clinical transplantation.

Conclusion

Covalently incorporated BMP-2/7 heterodimer stimulated the ACAN and COL2 gene expression, and GAG synthesis of bovine NP cells cultured in FBG-HA hydrogel. In an IVD whole organ model, BMP-2/7 heterodimer delivered into the nucleotomized region has the potential to promote the gene expression and synthesis of proteoglycan in the remaining NP tissue, without causing adverse fibroblastic or osteogenic induction. Intradiscal delivery of BMP-2/7 heterodimer may be a promising therapeutic approach for NP regeneration. The current IVD whole organ partial nucleotomy model may be utilized for screening of other biomaterials or bioactive factors to treat early degenerated disc.

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Table 1. Oligonucleotide primers and probes (bovine) used for real-time PCR.

Gene	Primer/probe type	Sequence
COL1A2	Primer fw (5'-3') Primer rev (5'-3') Probe (5'FAM/3'TAMRA)	TGC AGT AAC TTC GTG CCT AGC A CGC GTG GTC CTC TAT CTC CA CAT GCC AAT CCT TAC AAG AGG CAA CTG C
COL2A1	Primer fw (5'-3') Primer rev (5'-3') Probe (5'FAM/3'TAMRA)	AAG AAA CAC ATC TGG TTT GGA GAA A TGG GAG CCA GGT TGT CAT C CAA CGG TGG CTT CCA CTT CAG CTA TGG
ACAN	Primer fw (5'-3') Primer rev (5'-3') Probe (5'FAM/3'TAMRA)	CCA ACG AAA CCT ATG ACG TGT ACT GCA CTC GTT GGC TGC CTC ATG TTG CAT AGA AGA CCT CGC CCT CCA T
MMP13	Primer fw (5'-3') Primer rev (5'-3') Probe (5'FAM/3'TAMRA)	CCA TCT ACA CCT ACA CTG GCA AAA GTC TGG CGT TTT GGG ATG TT TCT CTC TAT GGT CCA GGA GAT GAA GAC CCC
ACAN: aggrecan; COL1A2: collagen type I; COL2A1: collagen type II; MMP13: matrix metalloproteinase 13; FAM: Carboxyfluorescein; fw: Forward; rev: Reverse; TAMRA: Tetramethylrhodamine		

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Table 2. Biochemical analysis of native disc tissue in partially nucleotomized discs implanted with PU scaffolds and FBG-HA hydrogel with/without BMP-2/7 after 14 days of dynamic load. Total glycosaminoglycan (GAG) content, total collagen content and proteoglycan (PG) synthesis rate were measured and normalized to the DNA content of the respective samples. GAG/DNA ratio in µg/µg; Collagen/DNA ratio in µg/µg, PG/DNA in counts per minute (CPM)/µg; PG/DNA was measured for NP tissue only. Mean ± SEM, n=6.

Biochemical analysis of IVD tissue		
NP tissue		
Group	0 ng/mL BMP-2/7	5000 ng/mL BMP-2/7
GAG / DNA	837.7 ± 172.3	1226.5 ± 129.6
Collagen / DNA	578.4 ± 313.3	1034.9 ± 277.9
PG / DNA	62.3 ± 4.6	75.6 ± 15.0
AF tissue		
Group	0 ng/mL BMP-2/7	5000 ng/mL BMP-2/7
GAG / DNA	305.4 ± 44.9	707.2 ± 142.1
Collagen / DNA	957.5 ± 119.5	957.1 ± 336.3

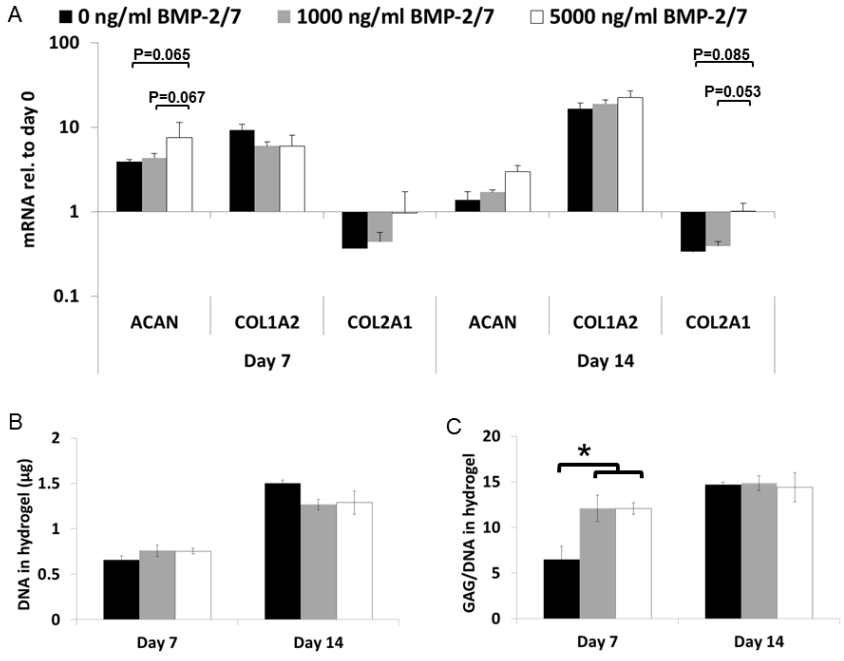
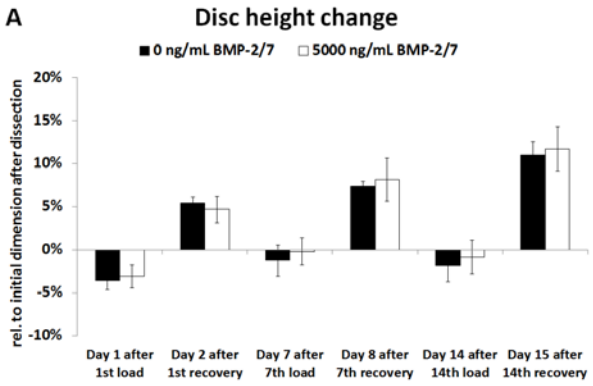
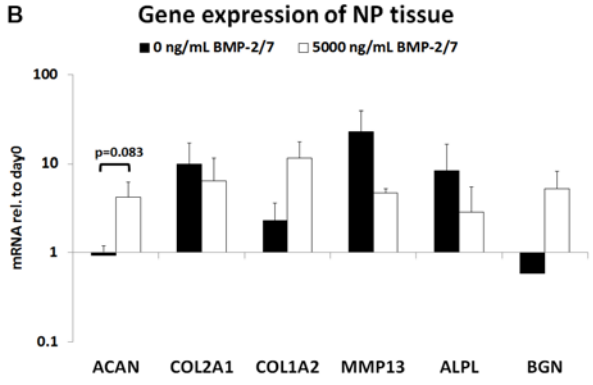


Figure 1. (A) Relative mRNA expression of ACAN, COL1A2, and COL2A1 of nucleus pulposus cells encapsulated in FBG-HA hydrogels with 0, 1000 or 5000 ng/mL BMP-2/7, after 7 or 14 days of culture. Data were normalized to the expression level of cells before hydrogel culture (day 0). Mean + SEM, n=6. (B) DNA content, and (C) GAG/DNA value within nucleus pulposus cells seeded FBG-HA hydrogels with 0, 1000 or 5000 ng/mL BMP-2/7, after 7 or 14 days of culture. Mean ± SEM, n=6, *$p<0.05$.


A
Disc height change
0 ng/mL BMP-2/7
5000 ng/mL BMP-2/7
rel. to initial dimension after dissection
20%
15%
10%
5%
0%
-5%
-10%
Day 1 after 1st load
Day 2 after 1st recovery
Day 7 after 7th load
Day 8 after 7th recovery
Day 14 after 14th load
Day 15 after 14th recovery


B
Gene expression of NP tissue
0 ng/mL BMP-2/7
5000 ng/mL BMP-2/7
mRNA rel. to day0
100
10
1
0.1
p=0.083
ACAN
COL2A1
COL1A2
MMP13
ALPL
BGN

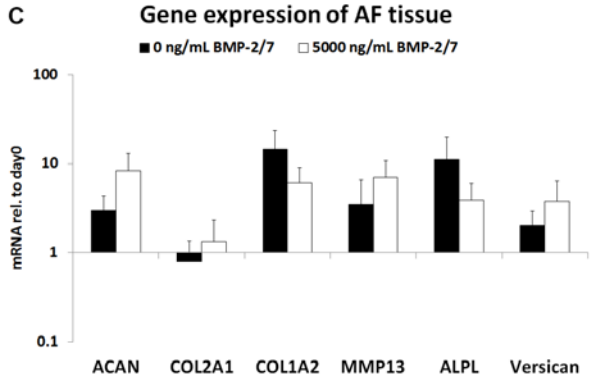

C
Gene expression of AF tissue
0 ng/mL BMP-2/7
5000 ng/mL BMP-2/7
mRNA rel. to day0
100
10
1
0.1
ACAN
COL2A1
COL1A2
MMP13
ALPL
Versican

Figure 2. (A) Disc height change of partially nucleotomized discs implanted with PU scaffolds and FBG-HA hydrogel with/without BMP-2/7 under repetitive dynamic load. Mean ± SEM, n=6. (B-C) Relative mRNA expression of cells in remaining native NP tissue and AF tissue in nucleotomized discs implanted with PU scaffolds and FBG-HA hydrogel with/without BMP-2/7 after 14 days of dynamic load. Data were normalized to the gene expression level of disc tissue from respective bovine tail before starting organ culture on day 0. Mean + SEM, n=8.

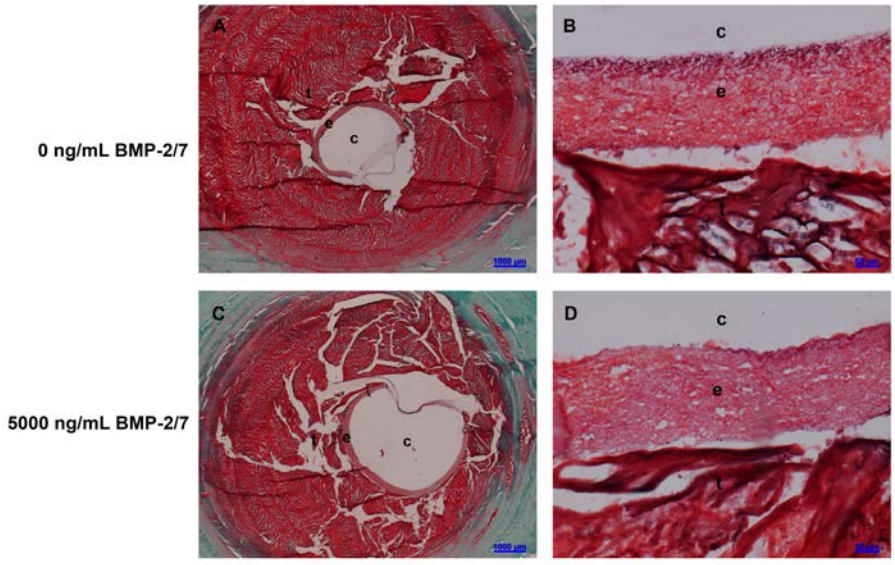


Figure 3. Representative Safranin O/Fast Green stained transverse sections of partially nucleotomized discs implanted with PU scaffolds and FBG-HA hydrogel with/without BMP-2/7 after 14 days of dynamic load. (A, C) Overview of disc, scale bar: 1000 μm. (B, D) Interface between remaining NP tissue and implanted biomaterials with/without BMP-2/7,

scale bar: 50 µm. t – native disc NP tissue, e – the envelope of implanted PU scaffold, c – the core of implanted PU scaffold.

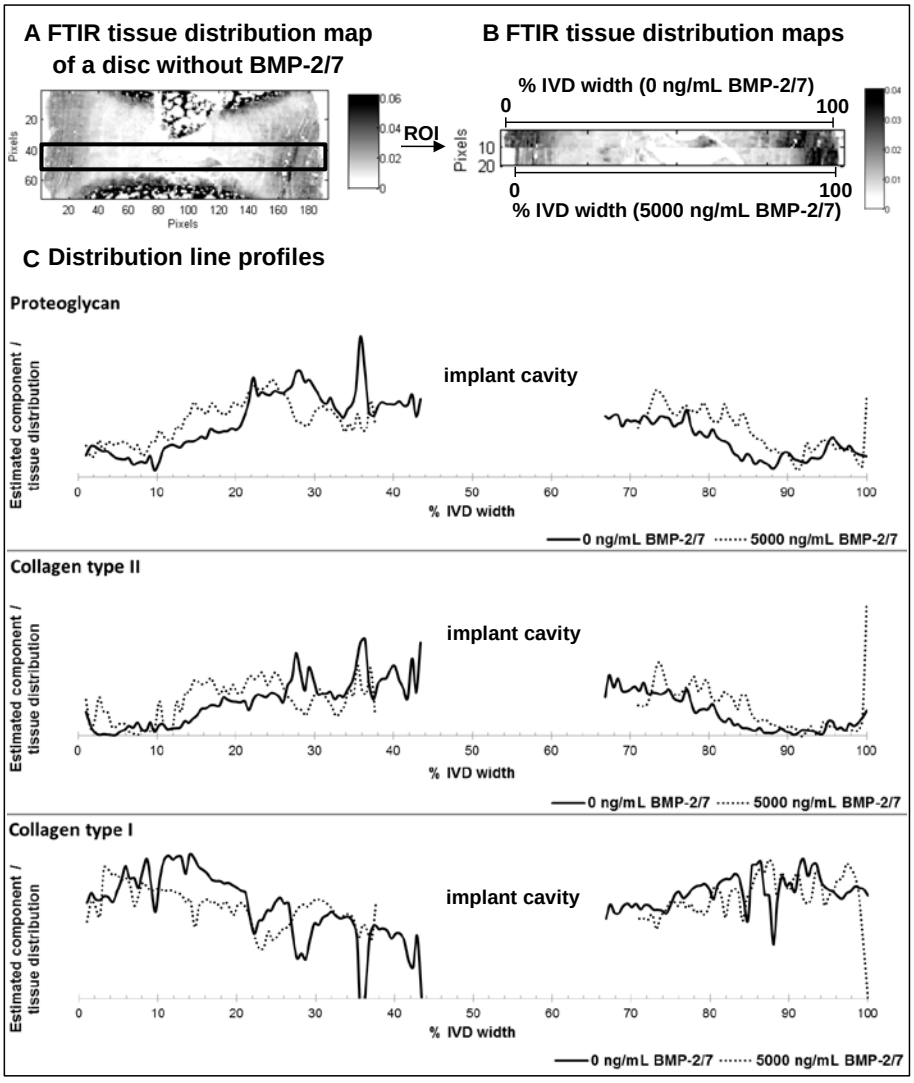


Figure 4. (A) Representative FTIR tissue distribution map of a paraffin embedded sagittal section of a partially nucleotomized disc implanted with PU scaffolds treated with hydrogel without BMP-2/7 (0 ng/mL BMP-2/7) cultured for 14 days under dynamic load. Image contrast for tissue distribution map is generated by integration of the 2nd derivative of the Amide III peak (1186-1297 cm^{-1}). The black square indicates a region of interest (ROI) which was used to investigate the matrix distribution of native disc tissue surrounding the implant cavity. (B) FTIR tissue distribution maps of ROIs of a disc implanted with PU scaffolds treated with hydrogel without BMP-2/7 (top) and treated with 5000 ng/mL BMP-2/7 (bottom) cultured for 14 days under dynamic load (scale bar: white to black indicative of a low to high tissue content). (C) Estimated component per tissue distribution line profiles across the ROI normalised to 100 % IVD width for proteoglycan, collagen type II and collagen type I.