UTRECHT UNIVERSITY

The effect of a bone morphogenetic protein (BMP)-2 and/or mesenchymal stromal cell (MSC)-based treatment for canine intervertebral disc degeneration

Sanne Loos 3896692
Supervisors: F.C. Bach, M.A. Tryfonidou
Orthopaedics Research Group, Faculty of Veterinary Medicine, Utrecht University, April 2016

Contents

Abstract

Introduction: Low back pain in humans and dogs is associated with intervertebral disc (IVD) degeneration. The degeneration is accompanied by a reduced glycosaminoglycan (GAG) content and a shift of collagen type II to type I in the nucleus pulposus. Current therapies focus on reducing the clinical symptoms, rather than restoring the original function of the degenerated disc. Regenerative therapies for IVD degeneration include cell-based therapy, the application of growth factors and gene therapy. The growth factor bone morphogenetic protein (BMP) plays a crucial part in all organ systems and belongs to the transforming growth factor (TGF)-β superfamily. Stem cells, like mesenchymal stromal cells (MSC), are able to differentiate into variable cell types, e.g. NPCs and can excrete growth factors that assist in the regeneration of the IVD. The aim of this study was to define the effect of BMP-2 alone and in combination with MSCs on degenerated canine nucleus pulposus cells (NPCs) in a 3D hydrogel culture system. It was hypothesized that BMP-2 has a regenerative effect on canine NCD NPCs and MSCs exert an additional regenerative effect in combination with BMP-2.

Methods: NPCs of non-chondrodystrophic (NCD) dogs were cultured in an albumin-based hydrogel (3,000,000 NPCs/mL hydrogel) in basal culture medium and with 250 ng/mL BMP-2 . The NPCs were also cultured with MSCs with/without BMP-2 (1,500,000 NPCs and 1,500,000 MSCs/mL hydrogel) and in low concentration with/without BMP-2 (1,500,000 NPCs /mL hydrogel). Main readout parameters were cell proliferation, extracellular matrix production and apoptosis. The samples were evaluated for gene expression profiling at day 7 of culture, and glycosaminoglycan (GAG) and DNA content and histology at day 28.

Results: BMP-2 demonstrated to have a proliferative effect and stimulated matrix production, reflected by an increased GAG deposition and upregulation of COL2 and ACAN. The catabolic genes MMP13 and ADAMTS5 and the pro-apoptotic gene caspase 3 were downregulated in the BMP-2 hydrogels. No additive effect of MSCs was demonstrated.

Conclusions: BMP-2 provides a proliferative, anabolic and anti-catabolic effect on the extracellular matrix of degenerated canine NPCs. MSCs appear to have no additive effect.

Key words: Intervertebral disc, degeneration, bone morphogenetic protein, mesenchymal stromal cell

Introduction

A common phenomenon in humans and dogs is low back pain, which is associated with degeneration of the intervertebral disc (IVD) (1). When degeneration of the IVD causes clinical symptoms, it is called IVD disease (2,3). The main function of the IVD is providing mobility and stability to the spine and transmitting compressive forces between vertebral bodies. The healthy IVD consists of a nucleus pulposus (NP), annulus fibrosus (AF), transition zone (TZ) and cartilaginous endplates (EP) and is located between two vertebral bodies. The healthy, gelatinous NP is mainly composed of water and is surrounded by the more fibrocartilagenous AF. Because of the avascularity of the inner part of the disc, the EPs play an important part in nutrient supply by enabling diffusion and osmosis. The healthy outer part of the AF mainly consists of collagen type I, while the inner AF part and NP mainly consist of proteoglycans and collagen type II (4). In the healthy IVD, the NP contains notochordal cells (NC), which produce the extracellular matrix (e.g. proteoglycans). The proteoglycans are composed of a protein backbone with glycosaminoglycan (GAG) side chains, which attract water into the NP. The most common glycoprotein in the IVD is aggrecan (2). Matrix metalloproteinases (MMPs), disintegrins and metalloproteinases with thrombospondin motifs (ADAMTS) and tissue inhibitors of metalloproteinases (TIMPs) regulate the remodeling process of the extracellular matrix (ECM) of the IVD. According to a study of Li *et al.* (2015) the imbalance between MMPs, ADAMTSs and TIMPs plays an important part in the degeneration of the IVD (2,5,6).

The main causes of IVD degeneration are believed to be amongst others, a lack of nutrient supply, genetic predisposition, chronic abnormal mechanical loads and trauma. The degeneration is accompanied by reduced disc height (distance between two EPs) and changes in cell phenotype and cell numbers in the NP and AF. During the normal aging process, the boundary between the NP and AF becomes less clear macroscopically, since the NP becomes more fibrotic and solid, as demonstrated in figure 1 (7). It is believed that IVD degeneration starts in the NP, with subsequently degeneration of the AF. IVD degeneration is characterized by the loss of notochordal cells (NCs) in the NP and replacement by chondrocyte-like nucleus pulposus cells (NPCs), and their associated cartilaginous ECM. This process is called chondrification. In addition, cell proliferation and apoptosis occur during IVD degeneration (2). In healthy IVDs, there is a high expression of the anti-apoptotic gene B-cell lymphoma 2 (BCL-2) and a low expression of the pro-apoptotic genes caspase-3 and B-cell lymphoma 2-associated X (Bax) (8). Changes in the ECM of a degenerated IVD include a decreasing amount of GAGs while the overall collagen amount increases. The collagen content in the NP demonstrates a shift from collagen type II to collagen type I. This process leads to a loss of the ability of the disc to maintain hydration under load. The AF takes over the load bearing function of the NP, the stiffer and weaker AF increases in size, and consequently, the IVD bulges outwards. The degenerated disc is, due to its avascularity and low cellular nature, not able to repair the matrix and becomes more vulnerable to damage and stress. A vicious circle occurs in which the degeneration results into more damage rather than regeneration (figure 2) (2).

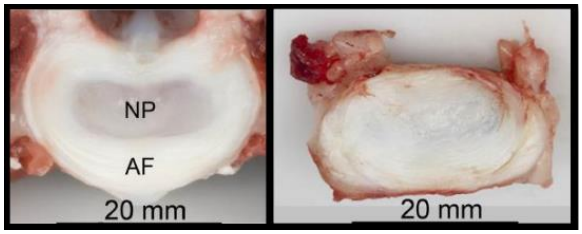


Figure 1 *On the left a healthy IVD, which demonstrates a clear boundary between NP and AF. On the right a degenerated IVD, where the distinction is less clear.* (From Bach *et al.* (14))

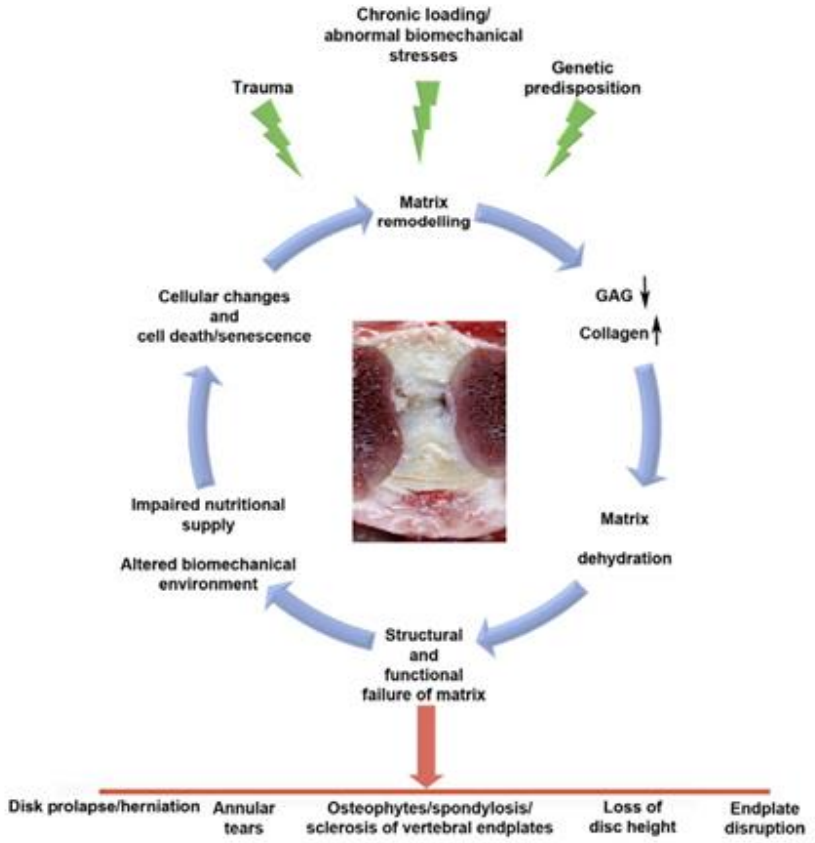


Figure 2 *Schematic representation of intervertebral disc degeneration. The vicious circle of degeneration is demonstrated. Inadequate repair leads to more damage and degeneration rather than regeneration of the disc.* (from Bergknut *et al.* (2))

To facilitate the study on human IVD degeneration, the use of animal models is essential. Animal models can be divided into two categories of degeneration: naturally derived and experimentally induced. Due to their spontaneously occurring IVD degeneration, dogs can be classified into the category naturally derived degeneration. According to Bergknut *et al.* (2012) in all different stages of IVD degeneration the gross pathology, GAG content, histopathology and MMP-2 activity of the canine and human discs are similar, which makes the dog a suitable model for human IVD degeneration (9,10).

In order to get a better understanding of canine IVD degeneration, dogs can be categorized into two groups: chondrodystrophic (CD) and nonchondrodystrophic (NCD) dogs. These categories are based on the predisposition of chondrodystrophy, which includes disturbed endochondral ossification, i.e. an ossifying process which stopped too early, especially in the areas where the most growth energy occurs (the diaphysis of the long bones). The Dachshund, English Bulldog and Cavalier King Charles Spaniel are examples of CD breeds, while NCD breeds include especially large breeds like the Labrador Retriever, Rottweiler and Dalmatian. In CD dogs, the NCs are completely replaced by NPCs by 1 year of age. Symptoms of IVD disease in CD dogs occur around 3-7 years of age, mostly in the cervical or thoracolumbar spine, whereas in NCD dogs they mostly occur in the caudocervical or lumbosacral spine about an age of 6-8 years. In NCD dogs, the NCs remain the predominant cell type throughout life. Although differences in the degeneration process of CD and NCD dogs can be found, the fundamental process as well as the treatment has a lot of similarities (11,12). The main cause of the IVD

degeneration in CD dogs appears to be genetic, whereas in NCD dogs it appears to be mainly due to trauma (wear and tear) (11).
Current therapies for IVD degeneration focus on reducing the clinical symptoms by performing physiotherapy, surgery (e.g. nuclectomy) or supplying anti-inflammatory medication, which can all have side effects. On the other hand, regenerative therapies try to restore the original function of the degenerated discs. These regenerative therapies include cell-based therapy (mesenchymal stromal cells (MSCs), NPCs and NCs), growth factors and gene therapy (13,14).

The interest in growth factors, like bone morphogenetic protein (BMP), as regenerative therapy for IVD degeneration has increased. All BMPs, except for BMP-1, are a member of the transforming growth factor (TGF)-β superfamily and play a crucial part in all organ systems. They contain seven cysteine residues in the mature domain and are synthesized with their receptors within the cell (15). At this moment, 20 different types of BMPs have been isolated and identified and two of them, recombinant human (rh) BMP-2 and rhBMP-7, have been used in human orthopaedic surgery (16). BMPs appear to have promising effects on differentiation, cell survival, migration and cell death and perform these through binding on its receptor and activation of Smad dependent and independent pathways (15). There are two types of BMP receptors: BMP type I receptors, which includes activin-like kinases (ALK) 1–7, and BMP type II receptors. The type I receptors ALK1, ALK2, ALK3 and ALK6 bind BMPs and activate Smad1 and Smad5, whereas the type I receptors ALK4, ALK5 and ALK7 bind TGF-β and activate Smad2 and Smad3 (15,17). After ligand binding, the BMP II receptor phosphorylates the BMP I receptor, which in turn phosphorylates Smad1 or Smad5, also known as Regulatory Smads or Class I Smads. Subsequently, Class I Smads form complexes with Class II or Common Smads (Smad4). These complexes can directly provide molecular transcriptional responses. Class III or Inhibitory Smads (Smad6 en Smad7) are known to be antagonists of the function of Class I Smads (15,18,19). The BMP signalling cascade is demonstrated in figure 3.

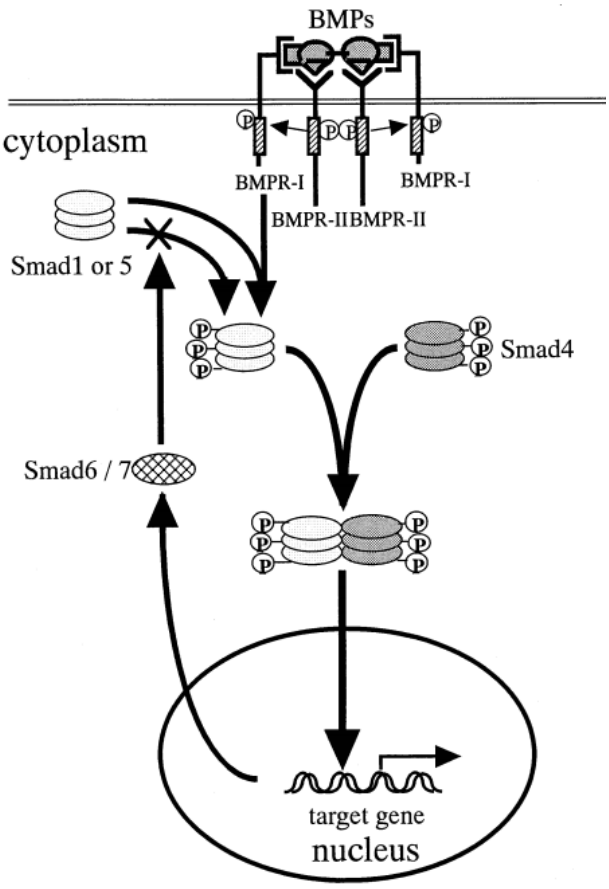


Figure 3 *The BMP signaling cascade with Smad proteins and their function.* (from Sakou *et al.* (15))

In the current study, BMP-2 is used, which is known to stimulate proteoglycan synthesis in the NP (20). A study of Inoue *et al.* (2015) described the effect of a BMP-2 injection *in vivo* on IVD degeneration in a rat tail model. They concluded that injection 4 weeks after puncturing the coccygeal discs induced an improvement in disc grade and stimulated chondrogenic differentiation. These improved discs, however, did not maintain their positive changes (20). Also according to Gilbertson *et al.*

(2008), BMP-2 had a stimulating effect on NPC matrix protein synthesis *in vitro*, while on the other hand it had minimal effects on AF cells (21).

Another possible effective regenerative therapy is the application of MSCs, which can be isolated from amongst others bone marrow, umbilical cord and adipose tissue. MSCs are able to differentiate into variable cell types, e.g. NPCs, depending on their environment. They also excrete growth factors that assist in the regeneration of the IVD (14,22). Furthermore, they have been proposed to exert anti-inflammatory effects by the release of growth factors and cytokines (23). MSCs co-cultured in direct cell-cell contact with NPCs are demonstrated to differentiate and stimulate the degenerated NPCs to regain their non-degenerated phenotype (24). A study of Hu *et al.* (2015) described the role of bone marrow-derived MSCs (BMSCs) in controlling IVD degeneration *in vitro*. NPCs were derived from Sprague-Dawley rats and cultured alone or with IL-1β or/and BMSCs. According to the results, the NPCs co-cultured with BMSCs showed significantly reduced degenerative and apoptotic indexes after inflammatory stimulation, making the addition of BMSCs an interesting regenerative therapy (25).

The specific aim of this experiment was to define the effect of BMP-2 alone and in combination with MSCs on degenerated NCD NPCs in a 3D hydrogel culture system. It was hypothesized that BMP-2 has a regenerative effect on canine NCD NPCs and MSCs exert an additional regenerative effect in combination with BMP-2.

Materials and methods

Cell culture

BMSCs from one mixed breed NCD donor and NPCs from degenerated IVDs of NCD dogs (two mixed breeds, one German Shepard and one Jack Russel; all Thompson grade III) were readily available for cell culture. To study the (regenerative) effect of BMP-2, passage 2 NPCs were cultured for 28 days (5% CO_2, 5% O_2, 37°C) in an albumin-based hydrogel (3,000,000 cells/mL gel) in base culture medium supplemented with or without 250 ng/mL rhBMP-2. This BMP-2 concentration was based upon previous work by the orthopaedics research group. To study the additive effect of MSCs on these NCD NPCs, the NPCs and MSCs were cultured in a 1:1 ratio for 28 days in the same hydrogel (1,500,000 NPCs and 1,500,000 MSCs per mL hydrogel) in base culture medium (negative control) or supplemented with 250 ng/mL BMP-2. The NPCs were also cultured alone in the low concentration (1,500,000 NPCs), without the addition of MSCs, in similar culture media (NPC ½ control) and supplemented with 250 ng/mL rhBMP-2 (NPC ½ BMP).

RNA isolation and Quantitative Polymerase Chain Reaction (qPCR)

At culture day 7, the samples of the NPC control, NPC BMP-treated, NPC MSC control and NPC MSC BMP-treated hydrogels for qPCR were obtained. Before RNA could be isolated, the samples were lysed. After defrosting on ice and vortexing the samples, they were transferred to 2 mL tubes and 400 µL RLT (151012987, Qiagen, Hilden, Germany) + β-mercaptoethanol was added. A metal bead was added, the samples were placed in the tissue lyser (4 min, 20 Hz) and thereafter RNA isolation was performed.

After defrosting on ice and vortexing the samples, 600 µL RLT (151012987, Qiagen, Hilden, Germany) + β-mercaptoethanol was added. By pipetting the samples up and down, they were lysed and homogenised. The samples were centrifuged (5 min, max speed) and the supernatant was transferred without the gel to 2 mL tubes. An equal volume of 70% ethanol (1000 µL) was added, mixed and pipetted into the RNeasy micro spin columns (74004, Qiagen, Valencia, USA) in 3 times through the columns. Thereafter, the columns were centrifuged (30 sec, max speed), flow-through was removed and the columns were washed with 350 µL RW1 buffer (151013997, Qiagen, Hilden, Germany). The columns were centrifuged again (30 sec, max speed) and flow-through was removed. After pipetting 80 µL DNase (RNAse-Free DNase Set, 79254, Qiagen, Valencia, USA) directly on the membrane to assure the genomic DNA was broken down, the columns were incubated for 15 minutes and 350 µL RW1 was added. The columns were centrifuged (30 sec, max speed) and new collection tubes (151032285, Qiagen, Hilden, Germany) were used. Subsequently 500 µL RPE+ (151014626, Qiagen, Hilden, Germany) was added, centrifuged (30 sec, max speed) and flow-through was removed. 500 µL 80% ethanol was added, followed by centrifuging the samples (2 min, max speed) and removal of the collection tubes. Thereafter, the samples were centrifuged (5 min, max speed) with open cover and the 2 mL collection tubes were replaced by 1.5 mL tubes. 17 µL RNase free water (151022581, Qiagen, Hilden, Germany) was pipetted directly on the membrane, incubated (1 min) and centrifuged (1 min, max speed). The flow-through was pipetted on the membrane and the samples were centrifuged (1 min, max speed). After this process cDNA synthesis was performed.

To synthesize cDNA, the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) was used. The samples were defrosted, vortexed and centrifuged. The concentration RNA and required amount of RNA/water was calculated. After pipetting water, the RNA was pipetted into the strips to yield a

total volume of 15 µL. The strips were vortexed, centrifuged and 5 µL iScript mix (4 µL iScript reaction mix + 1 µL iScript reverse transcriptase) was added to each sample, making the total volume 20 µL per sample. Subsequently, the strips were incubated in Dyad Disciple™ Peltier Thermal Cyclers (Bio-Rad, Veenendaal, The Netherlands).

The cDNA was diluted 10 times (10 µL cDNA + 90 µL MQ) and the standard line was made by pipetting 15 µL of every 10x diluted cDNA samples into yield S1 as highest standard and a serial dilution of 4x was made up to S7 (253 µL MQ + 84 µL S). To yield 50 times diluted cDNA, the 10x diluted cDNA was diluted another 5 times (160 µL MQ + 40 µL 10x diluted cDNA). The master mix consisted of 575 µL SYBR Green, 4.6 µL forward primer, 4.6 µL reverse primer and 106 µL MQ. In each PCR tube 6 µL master mix and 4 µL 50x diluted cDNA was put and the plate was centrifuged (1 min, 1900 rpm).

qPCR was performed using an iCycler CFX384 Touch thermal cycler, and IQ SYBRGreen Super mix (Bio-Rad, Veenendaal, The Netherlands) to evaluate the effects of BMP-2 on NPCs with or without the addition of MSCs at gene expression levels on: 1) ECM anabolism (aggrecan (*ACAN*), collagen type II (*COL2*), collagen type I (*COL1*) and collagen type 10 (*COL10*)), 2) ECM catabolism (matrix metalloproteinase 13 (*MMP13*), a disintegrin and metalloproteinase with trombospondin motifs (*ADAMTS5*) and tissue inhibitor of metalloproteinase 1 (*TIMP1*)), 3) proliferation (cyclin-D1 (*CCND1*)), 4) apoptosis (caspase 3 (*CASP3*), B-cell lymphoma 2-associated X (*BAX*) and B-cell lymphoma 2 (*BCL2*)), 5) Chondrogenic differentiation (SRY-box 9 (*SOX9*)), 6) BMP signalling (activin receptor-like kinase 1 (*ALK1*) and inhibitor of DNA binding 1 (*ID1*)) and 7) TGF signalling (activin receptor-like kinase 5 (*ALK5*) and plasminogen activator inhibitor 1 (*PAI1*)). Details of the used primers are demonstrated in table 1.

By using the efficiency-corrected deltadelta CT (ΔΔCt) method, the relative gene expression levels were analysed. The CT value represents the number of cycles required for the fluorescent signal to cross the threshold. CT values of each specific gene were normalized by the mean CT value of four reference genes. As reference genes hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S19 (*RPS19*) and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) were used which have been proven to be stably expressed in canine NPCs regardless their treatment. Gene expression in the NPC control hydrogels was set at 1 to relate to the gene expressions in the other conditions.

Table 1 *Details of the primers used in qPCR*

	Primer sequence	Product size	(°C)
HPRT	AGCTTGCTGGTGAAAAGGAC TTATAGTCAAGGGCATATCC	104	56-58
GAPDH	TGTCCCCACCCCCAATGTATC CTCCGATGCCTGCTTCACTACCTT	100	58
Bax	CCTTTTGCTTCAGGGTTTCA CTCAGCTTCTTGGTGGATGC	108	58-59
Alk5	GAGGCAGAGATTTATCAGACC ATGATAATCTGACACCAACCAG	116	59,5
ID1	CTCAACGGCGAGATCAG GAGCACGGGTTCTTCTC	135	59,5
Prnp	CCTGTGTTCCATCCTCCA GATCCTGCTTCTCTTTGTGG	81	58,5-60
Axin2	GGACAAATGCGTGGATACCT TGCTTGGAGACAATGCTGTT	128	60
CCND1	GCCTCGAAGATGAAGGAGAC CAGTTTGTTCACCAGGAGCA	117	60

ADAMTS5	CTACTGCACAGGGAAGAG GAACCCATTCCACAAATGTC	148	61
Alk1	CCTTTGGTCTGGTGCTGTG CGAAGCTGGGATCATTGGG	107	61
Casp3	CGGACTTCTTGTATGCTTACTC CACAAAGTGACTGGATGAACC	89	61
Col 1	GTGTGTACAGAACGGCCTCA TCGCAAATCACGTCATCG	109	61
Col 10	CCAACACCAAGACACAG CAGGAATACCTTGCTCTC	80	61
SDHA	GCCTTGGATCTCTTGATGGA TTCTTGGCTCTTATGCGATG	92	61
Acan	GGACACTCCTTGCAATTTGAG GTCATTCCACTCTCCCTTCTC	110	61-62
Pai1	AAACCTGGCGGACTTCTC ACTGTGCCACTCTCATTCAC	98	61,5
RPS19	CCTTCCTCAAAAAGTCTGGG GTTCTCATCGTAGGGAGCAAG	95	61+63
BCL2	GGATGACTGAGTACCTGAACC CGTACAGTTCCACAAAGGC	80	61,5-63
Sox9	GGAGGCCACCGAACAGACGC GGATTGCCCCGAGTGCTTGCC	124	62+63
Col2	GCAGCAAGAGCAAGGAC TTCTGAGAGCCCTCGGT	150	60,5-65
MMP13	CTGAGGAAGACTTCCAGCTT TTGGACCACTTGAGAGTTCG	250	65
TIMP1	GGCGTTATGAGATCAAGATGAC ACCTGTGCAAGTATCCGC	120	66

GAG and DNA content

The samples of all the conditions for GAG and DNA content were obtained at culture day 28. The samples were digested using 120 µL papain digestion solution with adjusted pH (6.8) (250 µg, P3125, Sigma-Aldrich, Saint-Louis, USA) + 1.57 mg cysteine HCl (C7880, Sigma-Aldrich, Saint-Louis, USA), incubated overnight at 60 °C. The next day, the samples were centrifuged and incubated for an additional hour. To quantify the GAG content of the samples and media the 1,9-dimethylmethylene blue (DMMB) assay was performed. The DMMB stock solution was prepared one day before use by adding 16 mg DMMB to 5 mL 100% ethanol and incubated for 2-16 hours on roller bench. For the final solution, 2.37 g NaCl and 3.04 g Glycine were added to 1L distilled water. After setting the pH at 3.0, 5 mL DMMB in ethanol was added to the solution. The solution was kept away by the light, using aluminium foil and stored at 4 °C in aliquots of 50 mL. Subsequently the chondroitin sulphate C (0.5 mg/mL) stock was diluted in PBS-EDTA (1:50), and to prepare a standard line, dilution series of 1:4 were made. In duplo, 100 µL of the diluted standard line was pipetted in the wells. The hydrogel samples were 5x and the culture medium 25x diluted in PBS-EDTA (5.68g 0.04 M Na2HPO4, 8.28g 0.06 M H2NaPO4*2H2O and 3.72g 0,01 M Na2EDTA*2 H2O added to 1L distilled water). In duplo 100 µL of the diluted samples were pipetted in the 96-wells plate and 200 µL DMMB (pH adjusted to 6.8) was added to each well. Also, 20 µL guanidinium chloride was used to mask the hyaluronic acid present in the hydrogels. The absorbance was read at 540 and 595 nm using a microplate reader and the total GAG in each sample was calculated according to the polynomic formula ($y = ax^2 + bx + c$).

To determine the DNA content per sample, the Quant-iT dsDNA BroadRange assay kit and the Qubit Fluorometer (Q32851, Invitrogen, Paisley, UK) were used. To prepare the Qubit™ Working Solution, per sample 1 µL Qubit Reagent and 199 µL Qubit Buffer were mixed. Two standard assay tubes were made by adding 10 µL of the Standard (from kit) to 190 µL of Working Solution. Samples were 40x diluted prior to measurement. All tubes were vortexed for 2-3 seconds and incubated for 2 minutes at room temperature before inserted in the Qubit Fluorometer.

Histology

Safranin-O/ Fast Green staining

At culture day 28, the samples of the NPC control, NPC BMP-treated, NPC MSC control and NPC MSC BMP-treated hydrogels for histology were obtained. To determine the presence of GAG deposition, Safranin-O/fast green staining was performed. After deparaffinization with xylene (2x 5 min), ethanol (96%, 80%, 70% and 60%) and MQ (each for 5 minutes), the sections were placed in hematoxylin (640505, Klinipath B.V., Duiven, The Netherlands) for a few seconds, followed by washing in demineralized water (5 min). Subsequently, the sections were stained with filtered 0.4% aqueous Fast Green (F7252, Sigma-Adrich, Saint Louis, USA) for 4 minutes, two times subjected to acetic acid 1% (3 and 2 min) and stained with 0.125% aqueous Safranin-O (58884, Sigma-Aldrich, Saint Louis, USA) for 7 minutes. The sections were then subjected to 100% ethanol (2 min), and xylene (2x 3min) and mounted (Vectamount, H5000, Vector Laboratories, Burlingame, USA).

Immunohistochemistry collagen type I and II

For immunohistochemistry on collagen type I and II, the sections were deparaffinized with xylene (2x 5 min), ethanol (100%, 96%, 80% and 70%) (each for 3 min) and washed in PBS (5 min). The slides were blocked with 0.3% H_2O_2 (51008600.9025, Boom B.V., Meppel, the Netherlands) at room temperature (10 min) and washed in PBS-T 0.1% (2x 5 min). With PAP-pen (Vector Laboratories, Burlingame, USA) similar sized circles around the samples were made, so the liquid would remain in place. Antigen retrieval was performed with 1 mg/mL Pronase (11459643001, Roche Diagnostics, Almere, The Netherlands) for 30 minutes at 37 °C and the sections were washed in PBS-T 0.1% (2x 5 min). Subsequently, antigen retrieval was performed with 10 mg/mL hyaluronidase (H3506, Sigma-Aldrich, Saint Louis, USA) for 30 minutes at 37 °C and the sections were washed in PBS-T 0.1% (2x 5 min). Thereafter, sections were blocked with PBS/BSA5% at room temperature for 30 minutes and incubated overnight at 4 °C with collagen type I mouse monoclonal antibody (100 µg/mL, ab6308) in PBS/BSA 5% or with collagen type II, mouse monoclonal antibody (42 µg/mL, II-II6B3, DSHB, Iowa City, IA) in PBS/BSA 5%. As negative control, normal mouse IgG_1 (100 µg/mL, 3877, Santa Cruz Biotechnology) in PBS/BSA 5% was used. The next day, the sections were washed with PBST 0.1% (2x 5 min) and incubated with Secondary Antibody conjugated with HRP (EnVision+ System-HRP Goat Anti-Mouse, K4001, DAKO, Glostrup, Denmark) for 60 minutes at room temperature. After washing the sections with PBS (2x 5 min), the sections were incubated in DAB peroxidase substrate solution (K3468, DAKO, Glostrup Denmark) for 1 minute, rinsed briefly in MQ and demineralized water and counterstained with Hematoxylin QS solution (H3404, Vector Laboratories, Burlingame, USA) for 1 minute. Thereafter, the sections were rinsed in running tap water for 15 minutes and dehydrated through 70% ethanol (3 min), 80% ethanol (3 min), 95% ethanol (2x5 min), 100% ethanol (5 min) and xylene (2x 5 min) and mounted (Vectamount, H5000, Vector Laboratories, Burlingame, USA).

Statistics

All statistical analysis were performed using SPSS Statistics 22. The data were examined for normal distribution by the Shapiro Wilks test, due to the amount of samples less than 2000. On non-normally distributed data, the non-parametric Kruskal Wallis test was performed, followed by the Mann Whitney U-Test if significance was found. The one-way ANOVA test was performed on normally distributed data. The p-value < 0.05 was considered significant.

Results

qPCR

Effects on the ECM anabolism and catabolism

In the BMP-treated samples, the expression of the extracellular matrix genes *ACAN* and *COL2* was significantly upregulated compared to the negative controls at day 7 (Figure 4b and 4c, $p < 0.01$). A significant increase in the expression of *COL1* in the NPC BMP hydrogels and the NPC MSC control hydrogel compared to the NPC control hydrogel was observed. In the NPC MSC BMP hydrogel the expression of *COL1* was demonstrated to be significantly upregulated compared to the NPC BMP hydrogel, while it was significantly downregulated compared to the NPC MSC control hydrogel (Figure 4a, $p < 0.05$). No expression of *COL10* was measured with the qPCR.

In all the conditions except for the NPC control hydrogels, no expression of *MMP13* was detected (Figure 4d). The relative expression of the catabolic gene *ADAMTS5* and the anti-catabolic gene *TIMP1* in the BMP hydrogels was significantly downregulated compared to the negative controls (Figure 4e and 4f, $p < 0.05$).

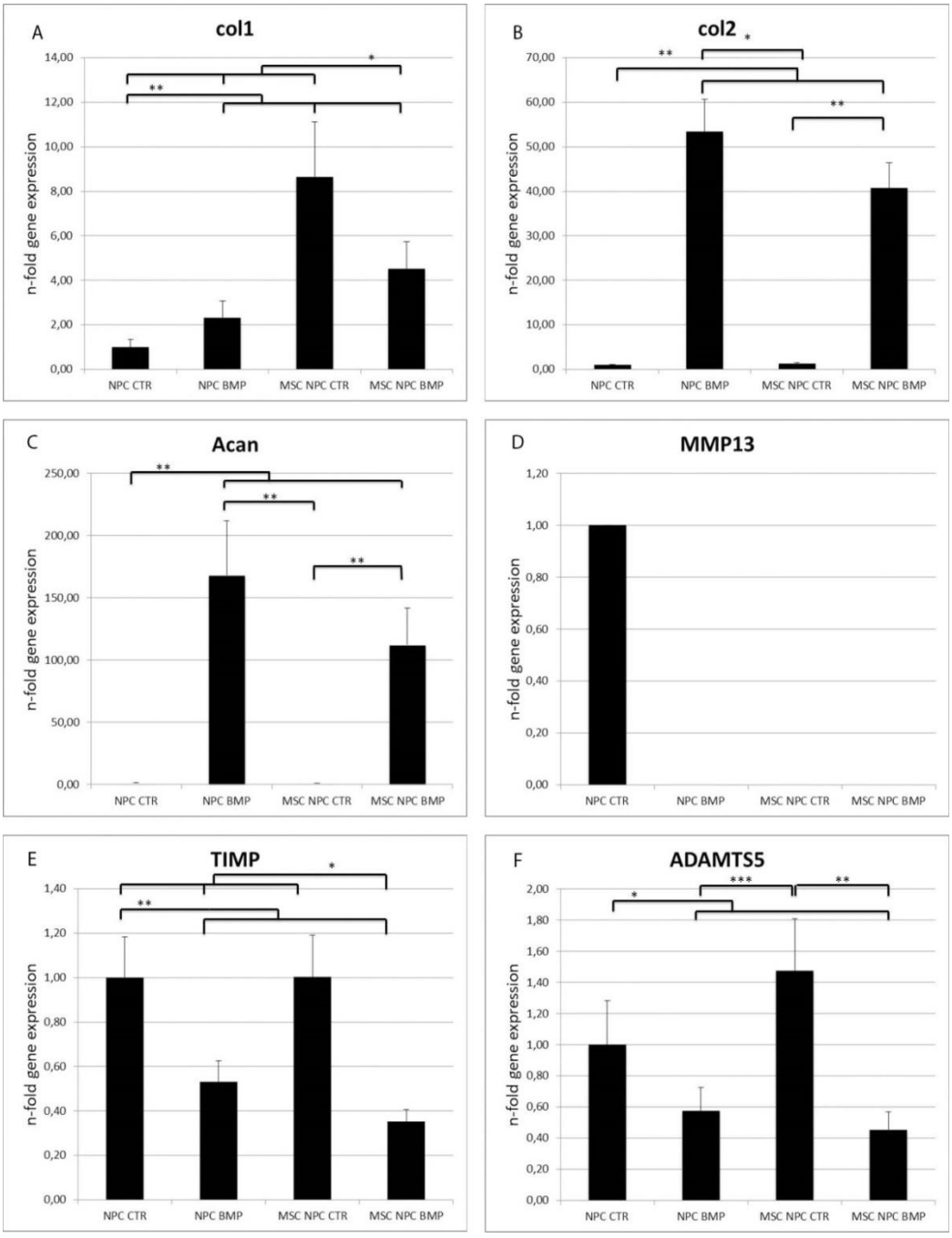


Figure 4 *n-fold gene expression (±stdev) of target genes in nucleus pulposus cell-containing hydrogels at culture day 7. The NPCs were cultured in an albumin-based hydrogel (alone or at a 1:1 ration with MSCs) and treated with basal culture medium (controls) or BMP2-supplemented basal culture medium. This figure demonstrates the effect on ECM anabolism* ***(A, B, C)****, ECM catabolism* ***(D, F)*** *and anti-catabolism* ***(E)****. Gene expression in the NPC control hydrogels was set at 1. NPC: nucleus pulposus cells, MSC: mesenchymal stromal cells, CRT: control, BMP: bone morphogenetic protein 2. * P<0.05, ** P<0.01, *** P<0.001*

Effects on proliferation, apoptosis and chondrocyte differentiation

The expression of proliferation marker *CCND1* was significantly upregulated in the NPC BMP and NPC MSC control hydrogels compared to the negative NPC control hydrogel (Figure 5a, $p < 0.05$).

No significant differences were observed in the expression of the pro-apoptotic gene *BAX* (Figure 5c). The expression of the anti-apoptotic gene *BCL2,* however, was significantly upregulated in the NPC MSC control group compared to the NPC control hydrogel, while it was significantly downregulated in the BMP-treated NPC MSC hydrogel compared to the NPC MSC control hydrogel (Figure 5d, $p < 0.05$). Gene expression of pro-apoptotic *CASP3* was significantly lower in the BMP-treated hydrogels compared to their negative controls. In the NPC MSC control hydrogel gene expression of *CASP3* was significantly increased compared to the NPC control hydrogel and in the BMP-treated NPC MSC hydrogel compared to the BMP-treated NPC hydrogel (Figure 5e, $p < 0.05$). The expression of the chondrocyte differentiation marker *SOX9* was significantly upregulated in the BMP-treated hydrogels compared to their negative controls. In the BMP-treated NPC MSC hydrogel, the expression of *SOX9* was significantly downregulated compared to the BMP-treated NPC hydrogel (Figure 5b, $p < 0.05$).

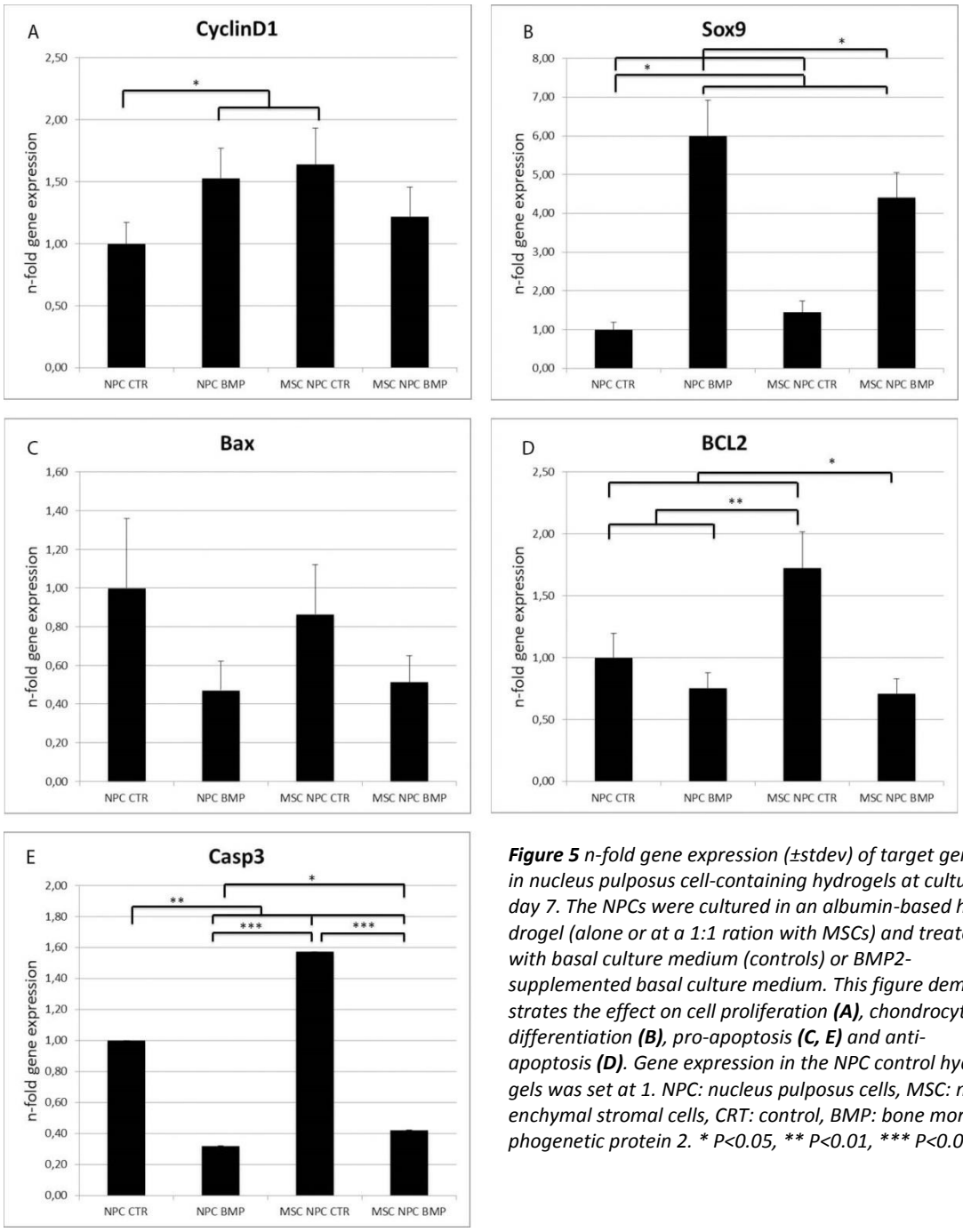


Figure 5 *n-fold gene expression (±stdev) of target genes in nucleus pulposus cell-containing hydrogels at culture day 7. The NPCs were cultured in an albumin-based hydrogel (alone or at a 1:1 ration with MSCs) and treated with basal culture medium (controls) or BMP2-supplemented basal culture medium. This figure demonstrates the effect on cell proliferation* ***(A)****, chondrocyte differentiation* ***(B)****, pro-apoptosis* ***(C, E)*** *and anti-apoptosis* ***(D)****. Gene expression in the NPC control hydrogels was set at 1. NPC: nucleus pulposus cells, MSC: mesenchymal stromal cells, CRT: control, BMP: bone morphogenetic protein 2. * P<0.05, ** P<0.01, *** P<0.001*

Effects on BMP and TGF signalling

The relative gene expression of *ALK1* in the NPC MSC control hydrogel was significantly higher compared to the NPC control hydrogel at day 7 (Figure 6a, p <0.05). The expression of *ID1*, the readout parameter of *ALK1,* was significantly upregulated in the BMP-treated hydrogels compared to their negative controls. In the NPC MSC control hydrogel expression of *ID1* was significantly downregulated compared to the negative NPC control hydrogel (Figure 6b, p <0.05). Gene expression of *ALK5* was significantly upregulated in the NPC MSC control hydrogel compared to the NPC control hydrogel, whereas in the BMP-treated NPC MSC hydrogel it was significantly downregulated compared to the NPC MSC control hydrogel (Figure 5c, p <0.05). No significant differences were found in the expression of *PAI1*, the readout parameter of *ALK5* (Figure 5d).

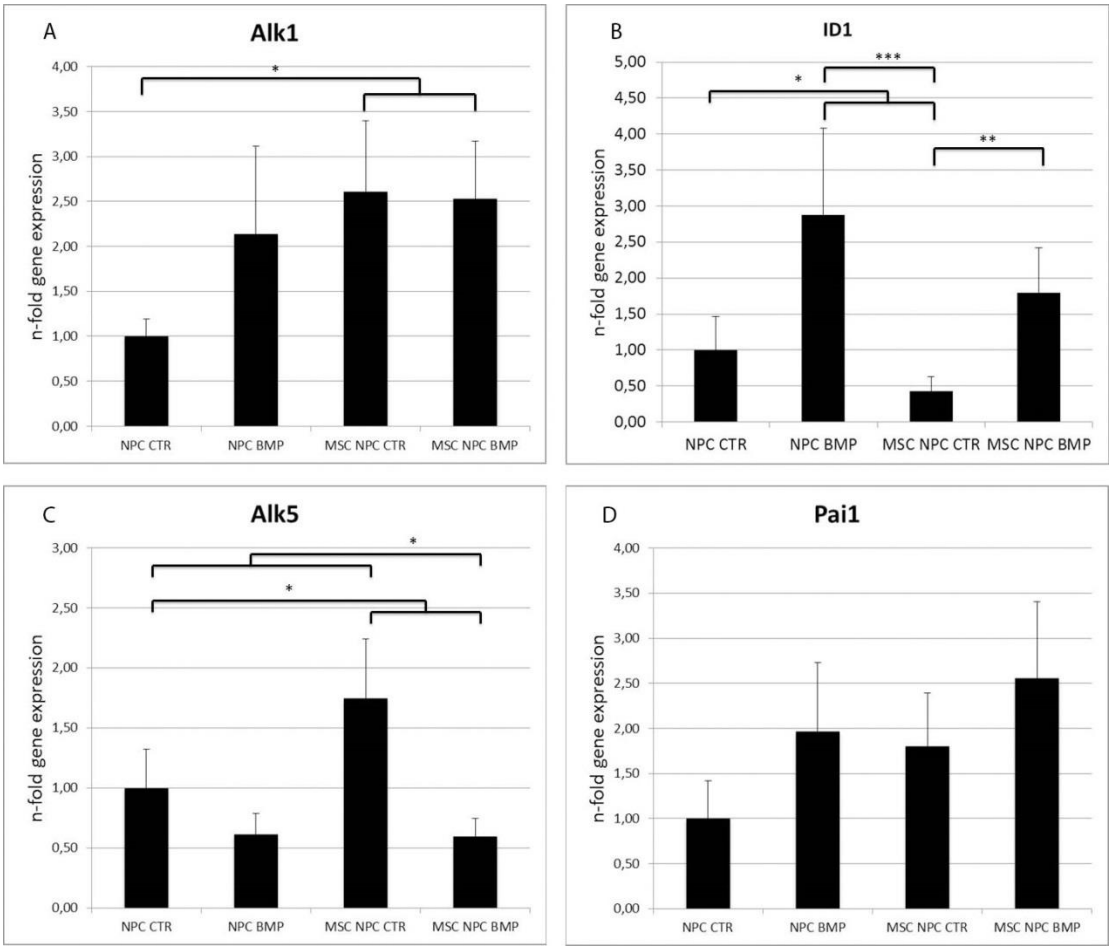
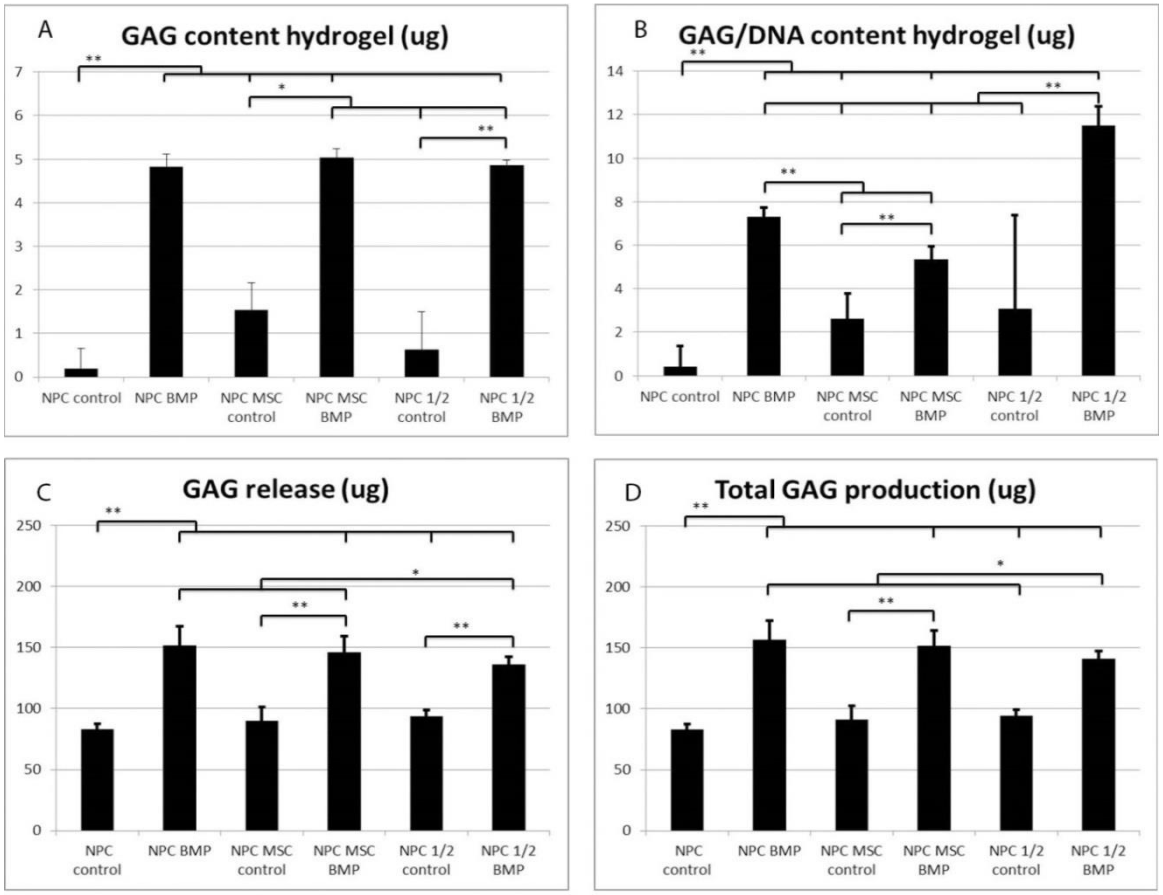


Figure 6 *n-fold gene expression (±stdev) of target genes in nucleus pulposus cell-containing hydrogels at culture day 7. The NPCs were cultured in an albumin-based hydrogel (alone or at a 1:1 ration with MSCs) and treated with basal culture medium (controls) or BMP2-supplemented basal culture medium. This figure demonstrates the effect on BMP receptor synthesis* ***(A)*** *including its readout parameter* ***(B)*** *and the effect on TGF receptor synthesis* ***(C)*** *including its readout parameter* ***(D)****. Gene expression in the NPC control hydrogels was set at 1. NPC: nucleus pulposus cells, MSC: mesenchymal stromal cells, CRT: control, BMP: bone morphogenetic protein 2. * P<0.05, ** P<0.01, *** P<0.001*

GAG and DNA content

At day 28 of culture, the GAG content was significantly increased in the BMP-treated hydrogels compared to their controls, in the NPC MSC control hydrogels compared to the NPC control hydrogels and in the NPC MSC BMP-treated hydrogels compared to the NPC BMP-treated hydrogels. A significant decrease in GAG content was demonstrated in the NPC ½ control hydrogels (which contained only $1.5*10^6$ NPCs/mL hydrogel instead of $3*10^6$ NPCs/mL hydrogel in the NPC (1x) hydrogels) compared to the NPC MSC control hydrogels (Figure 7a, p <0.05). The GAG/DNA content was significantly

increased in the BMP-treated hydrogels compared to their controls, in the NPC MSC control hydrogels compared to the NPC control hydrogels and in the NPC ½ BMP-treated hydrogels compared to the other BMP-treated hydrogels (Figure 7b, p <0.01). The BMP-treated hydrogels showed a significant increase in total GAG production and GAG release into the medium compared to their negative controls. The GAG release and total GAG production in the NPC MSC control hydrogel was significantly increased compared to the NPC control hydrogel and in the NPC MSC control hydrogel compared to the NPC ½ control hydrogel (Figure 7c and 7d, p <0.01). The NPC ½ BMP-treated hydrogel showed a significant decrease in GAG release compared to the NPC BMP-treated and NPC MSC BMP hydrogel (Figure 7c, p <0.05). The NPC ½ BMP-treated hydrogel showed a significant lower total GAG production compared to the NPC BMP hydrogel (Figure 7d, p <0.05). The DNA content in the BMP-treated hydrogels was significantly increased compared to their controls. After 28 days, the DNA content of the NPC control and NPC BMP-treated hydrogels was significantly decreased compared to the NPC hydrogels at day 0. Also after 28 days, the DNA content of the NPC MSC control hydrogels was significantly decreased compared to the NPC MSC hydrogels day 0. The DNA content in the NPC ½ control hydrogels was significantly decreased compared to the NPC ½ hydrogels day 0. The NPC MSC control hydrogels showed a significant increase in DNA content compared to the NPC controls (Figure 7e, p <0.01).



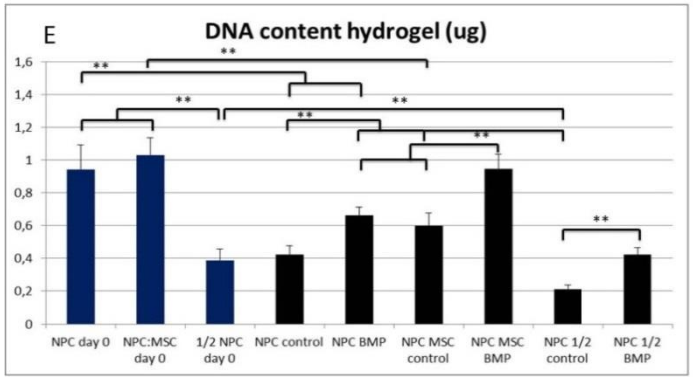


Figure 7 *This figure demonstrates the glycosaminoglycan (GAG) release* ***(C)****, total GAG production* ***(D)****, DNA and GAG/DNA content of the NPC-containing hydrogels* ***(A, B, E)*** *at day 28. The NPCs were cultured in an albumin-based hydrogel (alone or at a 1:1 ration with MSCs) and treated with basal culture medium (controls) or BMP2-supplemented basal culture medium. NPC: nucleus pulposus cells, MSC: mesenchymal stromal cells, CRT: control, BMP: bone morphogenetic protein 2. * P<0.05, ** P<0.01, *** P<0.001*

Histology

Safranin-O/ Fast Green staining

Safranin-O/fast green staining at day 28 showed a higher GAG deposition (more intense red staining) in the matrix of the hydrogels treated with BMP-2 compared to the NPC and NPC MSC controls. This was in line with the gene expression of *ACAN* measured with qPCR at day 7. No differences between the NPC MSC control and the NPC control hydrogels and between the NPC BMP-treated and NPC MSC BMP-treated hydrogels were found with the Safranin-O/ fast green staining (Figure 8).

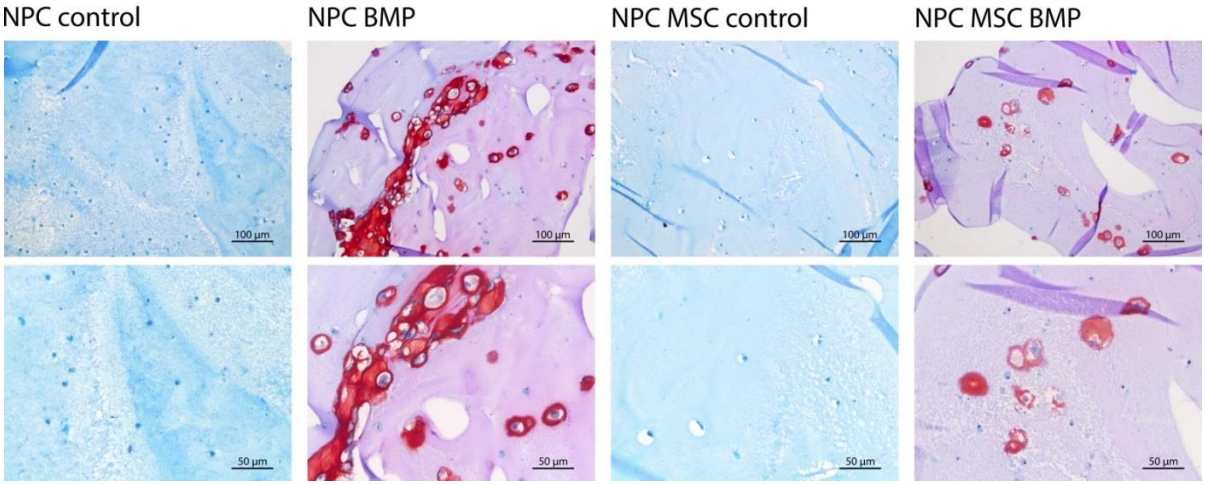


Figure 8 *Demonstration of the GAG content in the different hydrogels at culture day 28. The NPCs were cultured in an albumin-based hydrogel(alone or at a 1:1 ration with MSCs) and treated with basal culture medium (controls) or BMP2-supplemented basal culture medium. The NPC and NPC MSC control hydrogels showed negative Safranin-O/ fast green staining, while the hydrogels treated with BMP showed a red staining due to the GAG deposition in the matrix. The lower panel shows the conditions in more detail. NPC: nucleus pulposus cells, MSC: mesenchymal stromal cells, BMP: bone mor-*

Immunohistochemistry collagen type I and II

All the conditions demonstrated some collagen I protein expression. The BMP-treated NPC MSC hydrogels showed the highest collagen I deposition compared to the other conditions. The collagen II content in the BMP-treated hydrogels was increased compared to their controls. This was in line with the gene expression of collagen II measured with the qPCR at day 7. No differences were demonstrated in collagen II content between the NPC MSC and the NPC controls and between the NPC and NPC MSC hydrogels treated with BMP-2 (Figure 9).

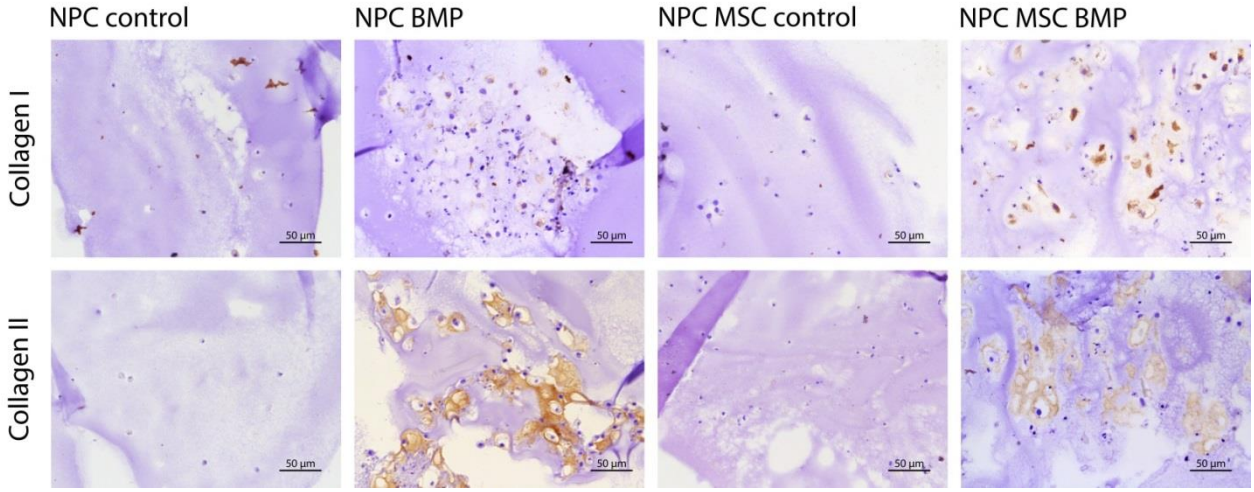


Figure 9 *Demonstration of the collagen I and II content in the different hydrogels at culture day 28.The NPCs were cultured in an albumin-based hydrogel (alone or at 1:1 ration with MSCs) and treated with basal culture medium (controls) or BMP2-supplemented basal culture medium. All the conditions showed some collagen I protein expression. The BMP-treated hydrogels showed an increased collagen II protein expression compared to their negative controls. NPC: nucleus pulposus cells, MSC: mesenchymal stromal cells, BMP: bone morphogenetic protein 2.*

Discussion

Dogs (as well as humans) experience low back pain caused by spontaneous IVD degeneration. As current treatments do not biologically repair the IVD, there is need for regenerative (cell- or growth factor-based) treatments that lead to functional IVD restoration. The aim of this study was to determine the effect of BMP-2 on NCD canine NPCs alone and combined with MSCs. The present study demonstrates that BMP-2 increased proliferation and extracellular matrix production of canine NPCs derived from degenerated IVDs. To determine whether MSCs exerted an additional regenerative effect, canine NPCs and MSCs were cultured in hydrogels. The current study, however, shows that MSCs did not exert additive regenerative effects on NPCs in terms of increased GAG deposition.

Canine NPCs perform well in an albumin-based hydrogel

The canine NPCs performed well in the albumin-based hydrogels used in this study, in contrast to alginate beads, in which canine NP cells do not thrive (26). Also in canine NPC pellets and micro-aggregates after 28 days of culture, about 80-90 % of the cells die when no growth factor is supplemented to the cells (unpublished *in vitro* data by the Orthopaedics research group, Faculty of Veterinary Medicine, Utrecht University). The albumin-based hydrogel, however, facilitates deposition of the cartilaginous ECM and stabilize the chondrogenic phenotype of the cells. The results of the current study imply that this Albugel could also be tested to inject cells in the IVD *in vivo* (27).

BMP-2 treatment increased extracellular matrix production and cell proliferation of canine NPCs

BMPs provide their function through the Smad cascade by binding to the BMP I receptor. Relative gene expression of the BMP I receptor, ALK1, was not upregulated in the BMP-treated hydrogels compared to their controls. However, BMP-2 treatment induced *ID1* expression (readout for activated BMP Smad 1/5/8 signalling) and thus, increased BMP-receptor synthesis did not appeared to be necessary for BMP-2 induced Smad1/5/8 signalling (28).

Treatment with 250 ng/mL BMP-2 stimulated extracellular matrix production in degenerated canine NPCs, reflected by a significantly increased expression of *COL2* and *ACAN* at day 7 and a significant increase in collagen type II deposition, GAG release and deposition and GAG/DNA content at day 28 of culture. The SOX9 transcription factor is an important regulator of chondrogenesis and controls the expression of *COL2* and *ACAN* by binding to the promotor elements of these genes (29). The BMP-treated hydrogels showed a significant increased expression of *SOX9*, leading to increased collagen type II and aggrecan production. The expression of the catabolic genes *MMP13* and *ADAMTS5* was decreased by BMP-2 treatment, which is consistent with previous studies on the effect of BMP-2 and BMP-7 on ECM catabolism (30-33). The anti-catabolic gene *TIMP1*, however, showed a conflicting significant decrease in the BMP-treated hydrogels, which could be due to active matrix remodelling of the NPCs under the influence of BMP-2. The increased expression of collagen type I in the NPC BMP-treated hydrogels could be explained by the fact that BMPs provide an upregulation of all matrix-related genes, including collagen type I (34,35).Previous studies showed that treating NPCs with TGF strongly upregulates collagen I expression, while treatment with BMP-2 demonstrated a much moderate increase in collagen type I production (36). Lastly, it is known that the supplementation of BMPs could not only result in chondrogenic activity, but also in bone formation (15,37). In this study bone formation (hypertrophic chondrocyte differentiation) was determined by the gene expression of collagen type X, which was not detected after BMP-2 treatment. Before BMP-2 should be tested *in vivo*, however, it should be studied (*in vitro*) whether BMP induces bone formation in other parts of

the IVD, like the AF. In future studies, also alkaline phosphatase assays and Alizarin red-S staining could be performed to detect, respectively, osteogenic marker and bone nodule formation besides an IHC for collagen type X (34).
Gene expression of the proliferation marker cyclin D1 was significantly upregulated in the NPC BMP-treated hydrogels compared with the NPC control hydrogels, but, not in the NPC MSC BMP hydrogel. This is most likely due to the fact that cyclin D1 is expressed in specific cell cycle parts. Cyclin D1 is an activator of cell cycle progression and is expressed in cell cycle phase G1 (38). To get a better understanding of cell proliferation, in future studies the expression of the proliferation marker c-myc could also be determined or a Ki67 staining could be performed (39,40). Ki67 demonstrates the proliferative activity of the cell, and is present in all phases of the cell cycle, except for G0 (40).
Lastly, the expression of the pro-apoptotic gene caspase-3 was significantly downregulated in the BMP-treated hydrogels, which was consistent with the demonstrated anti-apoptotic effect of BMPs in previous studies (41). Overall, BMP-2 appeared to have an anabolic, proliferative and anti-apoptotic effect on NCD canine NPCs.

MSCs do not exert an additive effect on BMP-2 treatment of canine NPCs

To study the additive effect of MSCs, the NPCs were cultured alone (3,000,000 NPCs/mL hydrogel), with MSCs (NPC:MSC - 1,500,000 NPCs/mL hydrogel and 1,500,000 MSCs/mL hydrogel) and alone in low concentration (1,500,000 NPCs/mL hydrogel). Although previous studies found positive effects of MSCs on NPCs of humans and rats (25,42), in this study, MSCs did not exert an additive effect on NPCs, reflected by the fact that total GAG production was not increased and GAG production per cell was even decreased by the addition of MSCs to BMP2-treated canine NPCs. Also according to the qPCR, MSCs appear to have no additive effect on the ECM anabolism: the pro-apoptotic marker caspase 3 was significantly increased in the NPC MSC control compared to the NPC control hydrogel. This non-additive effect of MSCs is also described in a study of Detiger *et al.* (2015), in which MSCs even appear to have a degenerative effect on the IVD in a goat model (43). Increased expression of *COL1* in the NPC MSC hydrogels compared with the NPC hydrogels is most likely due to a higher basal collagen type I production of MSCs (unpublished *in vitro* data by the Orthopaedics research group, Faculty of Veterinary Medicine, Utrecht University). The results of the current study demonstrate that proper control conditions, such as the low concentration NPCs without MSCs, are essential in order to draw the right conclusions with regard to the effects of MSCs. Several other studies, however, did find a positive effect of MSCs on canine IVD degeneration (44,45). This could be due to species differences, but also the fact those studies were performed in hypoxia (as the NP is a hypoxic tissue), while in this study a normoxic condition was used (46). MSCs appear to favour hypoxia for NP-like differentiation, matrix synthesis and maintaining viability and have little tolerance for hypertonicity and the disc-like acidity (47).

Limitations and future studies

The first limitation of this study is that the qPCR only measures gene expression at one specific moment in time, in this study at day 7 of culture. Further research must demonstrate at which time point qPCR should be performed in order to optimize the results or qPCR should be performed at multiple time points. Secondly, of the hydrogels with a low NPC concentration (1,500,000 NPCs/mL hydrogel) only the GAG/DNA content was determined, whereas RTqPCR and immunohistochemistry should be performed as well in the future. This study was performed only on degenerated NPCs of NCD dogs. Further research should also determine the effect of BMP-2 and/or MSCs on NPCs of hu-

mans and CD dogs. The results will indicate if the canine species is also a proper model for human IVD degeneration *in vitro*. In addition, in this study the culture was performed in normoxia, while the NP appears to be hypoxic (46). A future plan would be to culture under hypoxic conditions. Lastly, according to Richardson *et al.* (2006) the optimal ratio for stimulating MSC differentiation was 75:25 NPCs:MSCs, most likely due to the fact that a greater population of NPCs increases the cellular signals received by the MSCs (48). In future studies, other NPC:MSC ratios should be tested as well and the faith of the MSCs should be tracked (whether they survive the 28-day culture period) with GAPDH:SRY PCR analysis using female MSCs and male NPCs.

Conclusions

In conclusion, this study showed that treatment of degenerated NPCs of NCD dogs with 250 ng/mL BMP-2 provides a proliferative, anabolic, anti-apoptotic and anti-catabolic effect on the NCD canine NPCs *in vitro.* No additive regenerative effects of adding MSCs was observed.

References

(1) Luoma K, Riihimaki H, Luukkonen R, Raininko R, Viikari-Juntura E, Lamminen A. Low back pain in relation to lumbar disc degeneration. Spine (Phila Pa 1976) 2000 Feb 15;25(4):487-492.

(2) Bergknut N, Smolders LA, Grinwis GCM, Hagman R, Lagerstedt A, Hazewinkel HAW, et al. Intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. The Veterinary Journal 2013 3;195(3):282-291.

(3) Erwin WM, DeSouza L, Funabashi M, Kawchuk G, Karim MZ, Kim S, et al. The biological basis of degenerative disc disease: proteomic and biomechanical analysis of the canine intervertebral disc. Arthritis Res Ther 2015 Sep 5;17:240-015-0733-z.

(4) Pelle DW, Peacock JD, Schmidt CL, Kampfschulte K, Scholten DJ,2nd, Russo SS, et al. Genetic and functional studies of the intervertebral disc: a novel murine intervertebral disc model. PLoS One 2014 Dec 4;9(12):e112454.

(5) Roughley PJ. Biology of intervertebral disc aging and degeneration: involvement of the extracellular matrix. Spine (Phila Pa 1976) 2004 Dec 1;29(23):2691-2699.

(6) Li Y, Li K, Han X, Mao C, Zhang K, Zhao T, et al. The imbalance between TIMP3 and matrix-degrading enzymes plays an important role in intervertebral disc degeneration. Biochem Biophys Res Commun 2016 Jan 15;469(3):507-514.

(7) Urban JP, Roberts S. Degeneration of the intervertebral disc. Arthritis Res Ther 2003;5(3):120-130.

(8) Wang H, Liu H, Zheng ZM, Zhang KB, Wang TP, Sribastav SS, et al. Role of death receptor, mitochondrial and endoplasmic reticulum pathways in different stages of degenerative human lumbar disc. Apoptosis 2011 Oct;16(10):990-1003.

(9) Singh K, Masuda K, An HS. Animal models for human disc degeneration. The Spine Journal 2005 0;5(6, Supplement):S267-S279.

(10) Bergknut N, Rutges JP, Kranenburg HJ, Smolders LA, Hagman R, Smidt HJ, et al. The dog as an animal model for intervertebral disc degeneration? Spine (Phila Pa 1976) 2012 Mar 1;37(5):351-358.

(11) Smolders LA, Bergknut N, Grinwis GCM, Hagman R, Lagerstedt A, Hazewinkel HAW, et al. Intervertebral disc degeneration in the dog. Part 2: Chondrodystrophic and non-chondrodystrophic breeds. The Veterinary Journal 2013 3;195(3):292-299.

(12) Hansen HJ. A pathologic-anatomical study on disc degeneration in dog, with special reference to the so-called enchondrosis intervertebralis. Acta Orthop Scand Suppl 1952;11:1-117.

(13) Wang SZ, Chang Q, Lu J, Wang C. Growth factors and platelet-rich plasma: promising biological strategies for early intervertebral disc degeneration. Int Orthop 2015 Feb 5.

(14) Bach FC, Willems N, Penning LC, Ito K, Meij BP, Tryfonidou MA. Potential regenerative treatment strategies for intervertebral disc degeneration in dogs. BMC Veterinary Research 2014;10.

(15) Sakou T. Bone Morphogenetic Proteins: From Basic Studies to Clinical Approaches. Bone 1998 6;22(6):591-603.

(16) Hustedt JW, Blizzard DJ. The controversy surrounding bone morphogenetic proteins in the spine: a review of current research. Yale J Biol Med 2014 Dec 12;87(4):549-561.

(17) Mahlawat P, Ilangovan U, Biswas T, Sun LZ, Hinck AP. Structure of the Alk1 extracellular domain and characterization of its bone morphogenetic protein (BMP) binding properties. Biochemistry 2012 Aug 14;51(32):6328-6341.

(18) Padgett RW, Cho S, Evangelista C. Smads Are the Central Component in Transforming Growth Factor-β Signaling. Pharmacol Ther 1998 4;78(1):47-52.

(19) Jain AP, Pundir S, Sharma A. Bone morphogenetic proteins: The anomalous molecules. J Indian Soc Periodontol 2013 Sep-Oct;17(5):583-586.

(20) Inoue H, Montgomery SR, Aghdasi B, Kaner T, Tan Y, Tian H, et al. The effect of bone morphogenetic protein-2 injection at different time points on intervertebral disk degeneration in a rat tail model. J Spinal Disord Tech 2015 Feb;28(1):E35-44.

(21) Gilbertson L, Ahn SH, Teng PN, Studer RK, Niyibizi C, Kang JD. The effects of recombinant human bone morphogenetic protein-2, recombinant human bone morphogenetic protein-12, and adenoviral bone morphogenetic protein-12 on matrix synthesis in human annulus fibrosis and nucleus pulposus cells. Spine J 2008 May-Jun;8(3):449-456.

(22) Richardson SM, Kalamegam G, Pushparaj PN, Matta C, Memic A, Khademhosseini A, et al. Mesenchymal Stem Cells in Regenerative Medicine: Focus on Articular Cartilage and Intervertebral Disc Regeneration. Methods .

(23) Ennis WJ, Sui A, Bartholomew A. Stem Cells and Healing: Impact on Inflammation. Adv Wound Care (New Rochelle) 2013 Sep;2(7):369-378.

(24) Strassburg S, Hodson NW, Hill PI, Richardson SM, Hoyland JA. Bi-directional exchange of membrane components occurs during co-culture of mesenchymal stem cells and nucleus pulposus cells. PLoS One 2012;7(3):e33739.

(25) Hu J, Deng G, Tian Y, Pu Y, Cao P, Yuan W. An in vitro investigation into the role of bone marrowderived mesenchymal stem cells in the control of disc degeneration. Mol Med Rep 2015 Oct;12(4):5701-5708.

(26) Arkesteijn IT, Smolders LA, Spillekom S, Riemers FM, Potier E, Meij BP, et al. Effect of coculturing canine notochordal, nucleus pulposus and mesenchymal stromal cells for intervertebral disc regeneration. Arthritis Res Ther 2015 Mar 14;17:60-015-0569-6.

(27) Benz K, Stippich C, Osswald C, Gaissmaier C, Lembert N, Badke A, et al. Rheological and biological properties of a hydrogel support for cells intended for intervertebral disc repair. BMC Musculoskelet Disord 2012 Apr 10;13:54-2474-13-54.

(28) Lewis TC, Prywes R. Serum regulation of Id1 expression by a BMP pathway and BMP responsive element. Biochim Biophys Acta 2013 Oct;1829(10):1147-1159.

(29) Hardingham TE, Oldershaw RA, Tew SR. Cartilage, SOX9 and Notch signals in chondrogenesis. J Anat 2006 Oct;209(4):469-480.

(30) Gavenis K, Pufe T, Brandenburg LO, Schiffl K, Schmidt-Rohlfing B. Effects of controlled released BMP-7 on markers of inflammation and degradation during the cultivation of human osteoarthritic chondrocytes. J Biomater Appl 2011 Nov;26(4):419-433.

(31) 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-1beta-stimulated matrix metalloproteinase-13 expression in human chondrocytes. J Biol Chem 2003 Jul 11;278(28):25386-25394.

(32) Johansson N, Saarialho-Kere U, Airola K, Herva R, Nissinen L, Westermarck J, et al. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. Dev Dyn 1997 Mar;208(3):387-397.

(33) Majumdar MK, Chockalingam PS, Bhat RA, Sheldon R, Keohan C, Blanchet T, et al. Immortalized mouse articular cartilage cell lines retain chondrocyte phenotype and respond to both anabolic factor BMP-2 and pro-inflammatory factor IL-1. J Cell Physiol 2008 Apr;215(1):68-76.

(34) Kim DJ, Moon SH, Kim H, Kwon UH, Park MS, Han KJ, et al. Bone morphogenetic protein-2 facilitates expression of chondrogenic, not osteogenic, phenotype of human intervertebral disc cells. Spine (Phila Pa 1976) 2003 Dec 15;28(24):2679-2684.

(35) Haschtmann D, Ferguson SJ, Stoyanov JV. BMP-2 and TGF-beta3 do not prevent spontaneous degeneration in rabbit disc explants but induce ossification of the annulus fibrosus. Eur Spine J 2012 Sep;21(9):1724-1733.

(36) Kim SH, Kuh SU, Kim KN, Park JY, Cho KH, Chin DK, et al. Biologic response of degenerative living human nucleus pulposus cells to treatment with cytokines. Yonsei Med J 2015 Jan;56(1):277-286.

(37) Chen NF, Smith ZA, Stiner E, Armin S, Sheikh H, Khoo LT. Symptomatic ectopic bone formation after off-label use of recombinant human bone morphogenetic protein-2 in transforaminal lumbar interbody fusion. J Neurosurg Spine 2010 Jan;12(1):40-46.

(38) Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev 1993 May;7(5):812-821.

(39) Smolders LA, Meij BP, Riemers FM, Licht R, Wubbolts R, Heuvel D, et al. Canonical Wnt signaling in the notochordal cell is upregulated in early intervertebral disk degeneration. J Orthop Res 2012 Jun;30(6):950-957.

(40) Lalor PA, Mapp PI, Hall PA, Revell PA. Proliferative activity of cells in the synovium as demonstrated by a monoclonal antibody, Ki67. Rheumatol Int 1987;7(5):183-186.

(41) Wei A, Brisby H, Chung SA, Diwan AD. Bone morphogenetic protein-7 protects human intervertebral disc cells in vitro from apoptosis. The Spine Journal 2008 0;8(3):466-474.

(42) Vadala G, Studer RK, Sowa G, Spiezia F, Iucu C, Denaro V, et al. Coculture of bone marrow mesenchymal stem cells and nucleus pulposus cells modulate gene expression profile without cell fusion. Spine (Phila Pa 1976) 2008 Apr 15;33(8):870-876.

(43) Detiger SE, Helder MN, Smit TH, Hoogendoorn RJ. Adverse effects of stromal vascular fraction during regenerative treatment of the intervertebral disc: observations in a goat model. Eur Spine J 2015 Sep;24(9):1992-2000.

(44) Ganey T, Hutton WC, Moseley T, Hedrick M, Meisel HJ. Intervertebral disc repair using adipose tissue-derived stem and regenerative cells: experiments in a canine model. Spine (Phila Pa 1976) 2009 Oct 1;34(21):2297-2304.

(45) Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K, et al. Transplantation of mesenchymal stem cells in a canine disc degeneration model. J Orthop Res 2008 May;26(5):589-600.

(46) Risbud MV, Schipani E, Shapiro IM. Hypoxic Regulation of Nucleus Pulposus Cell Survival: From Niche to Notch. The American Journal of Pathology 2010 4;176(4):1577-1583.

(47) Wang F, Shi R, Cai F, Wang YT, Wu XT. Stem Cell Approaches to Intervertebral Disc Regeneration: Obstacles from the Disc Microenvironment. Stem Cells Dev 2015 Nov 1;24(21):2479-2495.

(48) Richardson SM, Walker RV, Parker S, Rhodes NP, Hunt JA, Freemont AJ, et al. Intervertebral disc cell-mediated mesenchymal stem cell differentiation. Stem Cells 2006 Mar;24(3):707-716.