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Bone morphogenetic protein-2, but not mesenchymal stromal cells, exert regenerative effects on canine and human nucleus pulposus cells

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Abstract
Chronic back pain is related to intervertebral disc (IVD) degeneration and dogs are employed as animal models to develop growth factor- and cell-based regenerative treatments. In this respect, the differential effects of transforming growth factor beta-1 (TGF-β1) and bone morphogenetic protein-2 (BMP2) on canine and human chondrocyte-like cells (CLCs) derived from the nucleus pulposus of degenerated IVDs were studied. Human and canine CLCs were cultured in 3D micro-aggregates in basal culture medium supplemented with/without TGF-β1 (10 ng/mL) or BMP2 (100 or 250 ng/mL). Both TGF-β1 and BMP2 increased proliferation and GAG deposition of human and canine CLCs. TGF-β1 induced collagen type I deposition and fibrotic (re)differentiation, whereas BMP2 induced more collagen type II deposition. In dogs, TGF-β1 induced Smad1 and Smad2 signaling, whereas in humans, it only tended to induce Smad2 signaling. BMP2 supplementation increased Smad1 signaling in both species. This altogether indicates that Smad1 signaling was associated with collagen type II production, whereas Smad2 signaling was associated with fibrotic CLC (re)differentiation. As a step towards preclinical translation, treatment with BMP2 alone and combined with mesenchymal stromal cells (MSCs) was further investigated. Canine male CLCs were seeded in albumin-based hydrogels with/without female bone marrow-derived MSCs (50:50) in basal or 250 ng/mL BMP2-supplemented culture medium. Although the results indicate that a sufficient amount of MSCs survived the culture period, total GAG production was not increased and GAG production per cell was even decreased by the addition of MSCs, implying that MSCs did not exert additive regenerative effects on the CLCs.
**Introduction**

Low back pain is a major cause of disability\(^1\) and has been associated with intervertebral disc (IVD) degeneration\(^2\). The IVD consists of an outer annulus fibrosus and inner nucleus pulposus (NP), and provides flexibility to the spine. The NP cell phenotype changes during maturation of the IVD: a shift from typical large, vacuolated notochordal cells (NCs) towards smaller chondrocyte-like cells (CLCs). During IVD degeneration, the glycosaminoglycan (GAG) and water content of the NP decreases. IVD disease is currently treated by physiotherapy, medication, and surgery to reduce pain, but these therapies only relieve symptoms and do not achieve IVD repair. Therefore, there is increasing interest in regenerative strategies stimulating functional IVD restoration, including growth factor supplementation and/or cell transplantation\(^3\).

Extracellular matrix (ECM) synthesis and cell proliferation can be stimulated with growth factors, such as members of the transforming growth factor beta (TGF-\(\beta\)) superfamily, e.g. TGF-\(\beta\)\(^1\), and bone morphogenetic protein-2 (BMP2). Members of this family transduce their signals by phosphorylation of the type I receptor by the type II receptor. Generally, type I receptors ALK1, ALK2, ALK3, and ALK6 are activated by BMPs and phosphorylate Smad1/5/8, whereas ALK4, ALK5, and ALK7 are activated by TGF-\(\beta\) and phosphorylate Smad2/3\(^4\). After phosphorylation, Smad2/3 and 1/5/8 form complexes with Smad4, translocate to the nucleus, and assemble target gene transcription. Subsequently, Smad2/3 and 1/5/8 target distinctive genes, resulting into differential effects\(^5,6\). TGF-\(\beta\) and BMP2 have already been tested in vitro\(^6,8\) and in vivo in animal models with experimentally induced IVD degeneration\(^9\)–\(^11\).

Growth factor stimulation alone may, however, be insufficient for IVD repair, since cell viability is impaired and cell numbers are decreased in degenerated IVDs\(^12\). Combined cell- and growth factor-based therapy may solve this problem\(^13\)–\(^15\). Mesenchymal stromal cells (MSCs) are an emerging cell source for regenerative treatments. They can be isolated from various tissues, e.g. bone marrow and adipose tissue, and can differentiate into different cell types, e.g. osteoblasts, adipocytes and chondrocytes\(^16,17\). Tissue regeneration can be supported by MSCs because of their immunosuppressive properties and the trophic factors they secrete\(^18\). Affirmatively, MSC transplantation increased ECM synthesis in animal models with experimentally induced IVD degeneration\(^19\)–\(^25\).

While the aforementioned growth factors have been studied separately, their differential effects on the IVD have not been addressed. Therefore, in this study the effect of TGF-\(\beta\)\(^1\) and BMP2 was determined on canine and human CLCs derived from degenerated IVDs. Since dogs suffer from spontaneous IVD degeneration with similar characteristics as observed in humans, they are considered a valid in vivo animal model for human IVD degeneration\(^26\). Based on physical appearance, dog breeds can be divided into chondrodystrophic (NCD) and non-chondrodystrophic (CD)\(^27\). CD dogs have short bowlegs due to disrupted endochondral ossification. This polygenetic trait has strongly been linked with IVD degeneration. In CD dogs, replacement of NCs by CLCs in the NP starts already before one year of age. IVD disease occurs frequently and develops around 3–7 years of age, usually in the cervical or thoracolumbar spine\(^27\). In contrast, in NCD dogs, NCs remain the predominant cell type until later in life. If IVD disease develops, it usually occurs around 6–8 years of age due to wear-and-tear, in the caudal cervical or lumbosacral spine\(^27\). Since CD and NCD dogs show differences in cause, prevalence, and age of onset of IVD degeneration, the regenerative potential of their CLCs could differ from each other, but also from human CLCs. Therefore, we aimed to define whether CD and/or NCD dogs were, besides a valid in vivo, also a suitable in vitro animal model for growth factor-based treatment of human IVD degeneration. Lastly, as a step towards preclinical translation, the additive regenerative effect of MSCs on growth factor-based treatment of IVD disease was
studied in an albumin-based hydrogel that has already been employed in vivo for intradiscal cell delivery.28

Material and Methods

The effect of TGF-β1 and BMP2 on canine and human CLC micro-aggregates

IVD tissue sources

CLCs of 3 human (2 females, 1 male, 47-63 years), 4 chondrodystrophic (CD; Beagles, male, 3-10 years) and 4 non-chondrodystrophic (NCD; 2 mixed breed dogs, 1 Jack Russell terrier, 1 German Shepherd dog; 3 females, 1 male, 2-11 years) canine donors were obtained from Thompson grade III IVDs. The NPs from all human and canine donors contained approximately 100% CLCs. During standard post mortem diagnostic procedures at the University Medical Centre (UMC) Utrecht, the L2-L5 part of the human spine was collected. Anonymous use of redundant tissue for research purposes is a standard treatment agreement with patients in the UMC Utrecht (Local Medical Ethical Committee number 12-364). The material was used in line with the ‘Proper Secondary Use of Human Tissue’ code installed by the Federation of Biomedical Scientific Societies. Canine spines were collected from dogs euthanized in unrelated research studies (University 3R-policy, approved by the Utrecht University Animal Ethics Committee) and client-owned dogs submitted for necropsy to the Department of Pathobiology (Faculty of Veterinary Medicine, Utrecht University). Briefly, NPs were digested by 0.15% pronase (45 min) and 0.15% collagenase (overnight) treatment. The CLCs were stored in hgeDMEM+Glutamax (1966, Invitrogen) with 10% Fetal Bovine Serum and 10% DMSO (-196°C) until use.

Cell culture

First, the CLCs were expanded at 21% O2, 5% CO2, 37°C. The CLCs were pooled at passage 2 in order to have biological representative samples of the respective populations. Human, NCD, and CD canine micro-aggregates of 35,000 CLCs were formed in low-adherence cell-repellent surface 96-well plates (650970, CELLSTAR® Greiner Bio-one) in 50 µL basal culture medium/well: hgeDMEM+Glutamax, 1% P/S (P11-010, GE Healthcare Life Sciences), 1% ITS+ premix (354352, Corning Life Sciences), 0.04 mg/mL L-proline (P5607, Sigma-Aldrich), 0.1 mM Ascorbic acid 2-phosphate (A8960, Sigma-Aldrich), and 1.25 mg/mL Bovine Serum Albumin (A9418, Sigma-Aldrich). The 96-well plates were centrifuged for 5 min at 50 g to stimulate micro-aggregate formation. The next day, basal culture medium was replaced with basal culture medium either or not supplemented with growth factors at a concentration well-known to be biologically active.6, 29-31: 10 ng/mL human recombinant TGF-β1 (240-B, R&D Systems), 100 ng/mL (BMP2100) or 250 ng/mL (BMP2250) human recombinant BMP2 (pharmaceutical quality, gift from TETEC AG). The micro-aggregates were cultured at 21% O2, 5% CO2, 37°C for 28 days.

Gene expression profiling

Micro-aggregates were collected at day 7 (n=7). RNA isolation, cDNA synthesis, and RT-qPCR were performed as described previously.29 Primer specifications for all canine and human reference and target genes are given in Supplementary File 1.

DNA and GAG content and release

Micro-aggregates were collected at day 28 (n=7). DNA and GAG sample preparation was performed as described previously.29 The GAG content of the micro-aggregates was determined using a dimethyl methylene blue (DMMB) assay.12 The DNA content was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Q32851, Invitrogen) according to the manufacturer’s instructions.
Histology
At day 28, the micro-aggregates were fixed in 4% neutral buffered formaldehyde (4286, Klinipath B.V.) with 1% eosin (115935, Merck Millipore) and embedded in 2.4% alginate and paraffin. Five μm sections were mounted on Microscope KP+ slides (KP-3056, Klinipath B.V.). Alizarin Red S\textsuperscript{16}, Safranin O/Fast Green staining and Collagen type I, II, and X immunohistochemistry\textsuperscript{20} was performed as described previously using collagen I mouse monoclonal antibody (0.07 μg/mL (canine) and 0.1 μg/mL (human), ab6308, Abcam), collagen II mouse monoclonal antibody (0.02 μg/mL (canine) and 0.4μg/mL (human), II-ll6B3, DSHB) and collagen X mouse monoclonal antibody (2031501005, Quartett). In isotype controls, normal mouse IgG\textsubscript{1} (3877, Santa Cruz Biotechnology) showed no aspecific staining. The positive control for collagen type X (growth plate) showed specific staining.

pSmad1 and pSmad2 ELISA
200,000 human, CD and NCD canine CLCs (n=4) were plated per well (12-wells plate, 665180, Greiner CELLSTAR\textsuperscript{®}) in expansion medium. After 2 days, the expansion medium was replaced with basal culture medium with/without 10 ng/mL TGF-β, or 250 ng/mL BMP2. After 24 hours of growth factor treatment, cells were homogenized in RIPA buffer containing 0.6 mM phenylmethylsulphonyl fluoride, 17 μg/mL aprotinin and 1 mM sodium orthovanadate (Sigma-Aldrich). Protein concentrations were measured using the Qubit\textsuperscript{®} Protein Assay Kit (Q32851, Invitrogen) and ELISAs for pSmad1 (SER463/465, PEL-SMAD1-S463, RayBiotech) and pSmad2 (S245/250/255, PEL-SMAD2-S245, RayBiotech) were performed according to the manufacturer’s instructions.

The effect of BMP2 on canine CLCs and MSCs in a hydrogel
The same CD and NCD canine CLC donor pools were used as described for the micro-aggregate experiments. Bone marrow-derived MSCs were obtained from a Beagle (CD) and a mixed breed dog (NCD) (both female, 2-years-old), which were euthanized in unrelated research studies. The MSCs were isolated, expanded, and characterized\textsuperscript{16}. Thereafter, CLCs and MSCs were incorporated in 40 μL hydrogels composed of albumin crosslinked by polyethylene glycol spacers to hyaluronic acid\textsuperscript{13} (3*10\textsuperscript{5} CLCs/mL hydrogel (CLC), 1.5*10\textsuperscript{6} CLCs/mL hydrogel + 1.5*10\textsuperscript{6} MSCs/mL hydrogel (CLC:MSC) and 1.5*10\textsuperscript{6} CLCs/mL hydrogel (½ CLC)) and cultured in basal culture medium with/without 250 ng/mL BMP2 at 21% O\textsubscript{2}, 5% CO\textsubscript{2}, 37°C for 28 days. Since the differentiation potential of canine MSCs differs between breeds\textsuperscript{25}, it was decided to only combine CLCs and MSCs of one breed: CD (Beagle) CLCs + CD (Beagle) MSCs and NCD (mixed breed) CLCs + NCD (mixed breed) MSCs.

DNA and GAG content measurements (n=8), Safranin O/Fast Green staining and collagen type I and II immunohistochemistry (n=3) was performed as described above, with adjustment of the pH of the papain and DMMB solution to 6.8 and addition of 2.16 M guanidinium chloride to mask the hyaluronic acid. To determine the fate of the MSCs after 28 days, the CLC (male): MSC (female) ratio was determined by SRY:GAPDH PCR on genomic DNA isolated from 20 μl papain digested sample using the DNEasy Blood and tissue kit (69581, Qiagen). The DNA was diluted 10x and used for SRY or GAPDH qPCR (Supplementary File 1). To determine the samples male DNA percentage, a standard series with known female: male genomic DNA amounts was used. C\textsubscript{q} SRY:C\textsubscript{q} GAPDH was used to interpolate the amount of male DNA in the samples from that of the known standard series.

Statistical analysis
Statistical analyses were performed using IBM SPSS statistics 22. Data were examined for normal distribution using a Shapiro Wilks test. General linear regression models based on ANOVAs were used for normally distributed data and Kruskal Wallis and Mann-Whitney U tests for non-normally distributed data. To correct for multiple comparisons, a Benjamini &
Hochberg False Discovery Rate post-hoc test was performed. p-values < 0.05 were considered significant.

Results

The effect of TGF-β1 and BMP2 on CLC proliferation and apoptosis

The effects of 100 and 250 ng/mL BMP2 (BMP2<sub>100</sub> and BMP2<sub>250</sub>, respectively) and 10 ng/mL TGF-β<sub>1</sub> were determined on micro-aggregate cultures of CD and NCD canine and human CLCs from degenerated IVDs. The DNA content of the human, CD and NCD canine micro-aggregates was significantly increased by TGF-β<sub>1</sub>, BMP2<sub>100</sub> and BMP2<sub>250</sub> treatment compared with controls, with the lowest increase in BMP2<sub>100</sub>-treated micro-aggregates (Figure 1a, p<0.01). TGF-β<sub>1</sub> induced the highest DNA content in human (p<0.01) and CD canine (p<0.001) micro-aggregates, whereas in NCD canine micro-aggregates, TGF-β<sub>1</sub> and BMP2<sub>250</sub> were equally potent in this respect. At day 7, expression of proliferation marker CCND1 was not increased by TGF-β<sub>1</sub> in human nor canine CLCs, while both BMP2 concentrations induced CCND1 expression compared with controls in CD canine and human CLCs (p<0.05, Supplementary File 2).

In BMP2<sub>100</sub>-treated CD and NCD canine and in BMP2<sub>250</sub>-treated CD canine micro-aggregates, a central area containing cells with karyorrhexis and pyknosis was observed, while there were no signs of apoptosis in the human micro-aggregates (Figure 2). BMP2<sub>250</sub>, however, significantly reduced apoptosis marker BAX expression in NCD canine CLCs (p<0.05, Supplementary File 2) and tended to decrease BAX expression compared with controls in human and CD canine CLCs (p<0.1). Expression of BAX was significantly decreased by TGF-β<sub>1</sub> in CD and NCD canine CLCs compared with controls (p<0.05).

The effect of TGF-β1 and BMP2 on CLC matrix production and remodeling

ACAN expression was significantly increased by BMP2<sub>250</sub> treatment in CD canine and human CLCs compared with control- and TGF-β<sub>1</sub>-treated CLCs (p<0.05), but not in NCD CLCs (Supplementary File 2). Nevertheless, GAG deposition was significantly increased by BMP2<sub>100</sub>, BMP2<sub>250</sub>, and TGF-β<sub>1</sub> treatment in human and canine CLCs compared with controls (p<0.05, Figure 1b and Figure 2). BMP2 concentration-dependently increased GAG deposition in human and canine CLCs (p<0.05). In CD canine CLCs, TGF-β<sub>1</sub> was significantly more potent than BMP2 in inducing GAG deposition (p<0.05). In contrast, NCD canine micro-aggregates treated with BMP2<sub>250</sub> had a significantly higher GAG content than those treated with TGF-β<sub>1</sub> (p<0.001), whereas TGF-β<sub>1</sub> and BMP2<sub>250</sub> were equally potent in increasing GAG deposition by human CLCs. The GAG/DNA content of the BMP2<sub>250</sub>-treated human and canine micro-aggregates was significantly increased compared with all other conditions (p<0.05, Figure 1c). In human and CD canine CLC micro-aggregates, the GAG/DNA content was significantly more increased by TGF-β<sub>1</sub> than by BMP2<sub>100</sub> treatment, whereas in NCD dogs, the opposite occurred.

TGF-β<sub>1</sub> and BMP2<sub>250</sub> were equally potent in inducing COL2A1 expression in canine and human CLCs (Supplementary File 2), while collagen type II protein deposition was induced by both TGF-β<sub>1</sub> and BMP2 in canine CLCs, but only by BMP2<sub>250</sub> in human CLCs (Figure 2). COL10A1 expression was only detected in TGF-β<sub>1</sub>-treated human CLCs (data not shown), but collagen type X protein was not detected in canine nor human micro-aggregates regardless the treatment (Supplementary File 3). Additionally, mRNA of the osteogenic marker OSX was not detected in any culture condition, and RUNX2 and BGLAP gene expression was very low (C<sub>r</sub>-values >37) and not significantly different between conditions (data not shown). Moreover, Alizarin Red S staining showed no calcium deposition regardless the treatment (Supplementary File 3).
Figure 1. The effect of TGF-β1 and BMP2 on human and canine chondrocyte-like cell proliferation, matrix production and Smad signaling. Micro-aggregates of chondrocyte-like cells derived from degenerated human/canine intervertebral discs were cultured for 28 days (DNA and GAG content) or 24 hours (phosphorylated (p)Smad1, pSmad2 signaling). (a) DNA content, (b) GAG content, (c) GAG/DNA content, (d) and (e) Relative pSmad1 and pSmad2 expression (controls set at 1), n = 4 (pSmad) - 7 (DNA and GAG content). GAG: glycosaminoglycan, CD: chondrodystrophic, NCD: non-chondrodystrophic. *, **, ***: significantly different from all other conditions (p < 0.05, p < 0.01, p < 0.001, respectively); $$, $$$: significantly different from all other conditions except the one with the same mark type (p < 0.01, p < 0.001, respectively); #: p < 0.05, ##: p < 0.01.
Figure 2. The effect of TGF-β1 and BMP2 on glycosaminoglycan, collagen type I and II deposition of human and canine chondrocyte-like cell (CLC) micro-aggregates. Micro-aggregates of CLCs derived from degenerated human/canine intervertebral discs were cultured for 28 days. Red staining in Safranin O/Fast Green indicates glycosaminoglycan deposition. CD: chondrodystrophic, NCD: non-chondrodystrophic. n = 7.
In all species, TGF-β1-treated micro-aggregates showed signs of fibrotic (re)differentiation. Expression of collagen type I gene (Supplementary File 2) and protein (Figure 2) was increased by TGF-β1 compared with control and BMP2 treatment in both species, except for COL1A1 expression in CD canine CLCs. Morphologically, a GAG-depleted, collagen type I-rich outer rim was formed after TGF-β1 treatment, which contained almost no cells in canine, and fibroblast-like cells in human micro-aggregates (Figure 2).

Expression of the matrix remodeling gene MMP13 was significantly induced by BMP2 in canine CLCs, but only induced by TGF-β1 treatment in human CLCs (p<0.05, Supplementary File 2). ADAMTS5 expression was not significantly affected by the different growth factors in human CLCs (data not shown). In contrast, TGF-β1 decreased CD canine CLC ADAMTS5 expression 10-fold, whereas BMP2 decreased NCD canine CLC ADAMTS5 expression 7-fold compared with controls (p<0.05, data not shown). TIMP1 (inhibitor of matrix metalloproteinase) expression was decreased in TGF-β1- and BMP2-treated human CLCs (2-fold and 1.5-fold, respectively) compared with controls (p<0.05), whereas it was 30-fold decreased by BMP2 in NCD canine CLCs (p<0.05), and not affected by growth factor treatment in CD canine CLCs (data not shown).

The effect of TGF-β1 and BMP2 on Smad signaling

To determine the effect of TGF-β1 and BMP2 on Smad signaling, ELISAs for phosphorylated (p) Smad1 and Smad2 were performed. In CD canine CLCs, TGF-β1 significantly induced Smad1 and Smad2 signaling, whereas BMP2 only induced pSmad1 levels (p<0.05, Figure 1d-e). In NCD canine CLCs, TGF-β1 significantly increased pSmad2 (p<0.05) and tended to increase pSmad1 levels (p=0.1), whereas BMP2 significantly increased pSmad1 levels (p<0.05). TGF-β1-treated human CLCs showed a tendency towards increased pSmad2 levels (p=0.1), whereas BMP2-treated human CLCs showed significantly increased Smad1 signaling (p<0.05).

The effect of BMP2 on canine CLCs and MSCs in 3D hydrogel cultures

The best growth factor was considered the one that induced GAG and collagen type II production and cell proliferation, and inhibited apoptosis and fibrotic (collagen type I-rich) and osteogenic (collagen type X- and calcium-rich) ECM deposition. In this respect, human and canine CLC micro-aggregates responded best to 250 ng/mL BMP2. Therefore, the effect of 250 ng/mL BMP2 alone or in combination with MSCs was determined on canine CLCs using hydrogels employed for intradiscal cell transplantation. Canine CLCs and MSCs from CD and NCD breeds were incorporated at different concentrations: 3*10^6 CLCs/mL (CLC), 1.5*10^6 CLCs/mL + 1.5*10^6 MSCs/mL (CLC:MSC) and 1.5*10^6 CLCs/mL (½ CLC).

In hydrogels containing CD (Figure 3) and NCD (Supplementary File 4) canine ½ CLC, CLC and CLC:MSC, BMP2 treatment significantly increased the DNA content, GAG release, GAG deposition, and collagen type I and II deposition compared with controls (p<0.05). Generally, BMP2 treatment also significantly increased the GAG/DNA content of NCD (Supplementary File 4) and CD (Figure 3d) canine cell containing hydrogels (p<0.01), only not the GAG/DNA content of CD CLC:MSC containing hydrogels.

Although at day 0, the DNA content of CLC and CLC:MSC containing hydrogels was comparable, after 28 days, the DNA content of NCD CLC:MSC containing control hydrogels was higher than that of NCD CLC containing control hydrogels (p<0.01, Supplementary File 4). The DNA content of the BMP2-treated CLC:MSC containing hydrogels was higher than that of the BMP2-treated CLC containing hydrogels in both CD and NCD dogs (p<0.05, Figure 3a and Supplementary File 4). At day 0, the CLC (male)-derived DNA content of CD CLC:MSC containing hydrogels was 50% (0.45 µg) of the total DNA content. After 28 days of culture, 57% (0.30 µg) of cells in CD CLC:MSC containing control hydrogels appeared female (MSC)-derived, whereas this was 27% (0.34 µg) in CD BMP2-treated CLC:MSC containing hydrogels (Figure 3a), indicating that in
In absolute terms, the MSC-derived DNA content remained relatively unchanged throughout culture. At day 28, the CLC (male)-derived DNA content of CD CLC:MSC containing hydrogels was comparable with the total DNA content of CD CLC containing hydrogels, both in the presence and absence of BMP2 (Figure 3a).

GAG release and deposition was comparable in all BMP2-treated cell-hydrogel combinations. The GAG/DNA content was highest in the hydrogels containing BMP2-treated ½ CLC, followed by BMP2-treated CLC hydrogels (Figure 3d, Supplementary File 4). The GAG/DNA content of NCD and CD BMP2-treated CLC:MSC containing hydrogels was significantly lower than that of BMP-treated ½ CLC and CLC containing hydrogels (p<0.01). A schematic overview of the observed effects of TGF-β1 and BMP2 on CD and NCD canine and human CLCs is given in Supplementary File 5.

Figure 3. The effect of BMP2 on matrix production and cell proliferation of chondrodystrophic (CD) canine chondrocyte-like cells (CLCs) and mesenchymal stromal cells (MSCs). CLC (3*10^6/mL hydrogel), ½ CLC (1.5*10^6/mL) or CLC:MSC (1:1, both 1.5*10^6/mL) hydrogels were cultured for 28 days with or without 250 ng/mL BMP2. (a) Total and male (CLC-derived) DNA content, (b) GAG release, (c) GAG content, (d) GAG/DNA content, (e) Safranin O/Fast green staining and collagen type I and II immunohistochemistry. Red staining in Safranin O/Fast Green indicates glycosaminoglycan (GAG) deposition. n = 8. #, ##, ###: p<0.05, p<0.01 and p<0.001, respectively; *, **: significantly different from all other conditions (p<0.05, p<0.01, respectively).
Discussion
Both humans and dogs experience chronic back pain related to IVD degeneration. As current treatments do not achieve IVD repair, there is need for cell- and growth factor-based regenerative treatments leading to functional IVD restoration. This study demonstrates that both growth factors BMP2 and TGF-β1 increased proliferation and matrix production of human and canine CLCs, but that TGF-β1 induced a fibrotic phenotype, whereas BMP2 did not. To determine whether MSCs exerted additional regenerative effects, canine CLCs and MSCs were cultured in a hydrogel that has already been shown to facilitate IVD repair. Although the results indicate that a considerable amount of MSCs survived the culture period, they did not exert additive regenerative effects on the CLCs.

The effect of TGF-β1 and BMP2 on human and canine CLCs
It has already been shown that BMP2 and TGF-β1 are able to induce GAG deposition in CLCs. In the current study, the response of CD and NCD canine CLCs to these growth factors was in a similar direction as observed in human CLCs, but sometimes with a different intensity. Both BMP2 and TGF-β increased cell proliferation and ECM production, while they decreased the expression of apoptotic-related genes in human and canine CLCs. These effects were reflected in a high DNA and GAG content of the CLC micro-aggregates at the end of the study. TGF-β1 stimulated Smad1 and Smad2 signaling in canine CLCs (as also shown in chondrocytes), and induced GAG, collagen type I and II deposition and a cell/GAG-depleted fibrotic rim. In contrast, BMP2 only stimulated Smad1 signaling in canine CLCs, and induced GAG and collagen type II deposition, but no collagen type I deposition or fibrotic phenotype. In human CLCs, TGF-β1 tended to stimulate only Smad2 signaling, and induced a fibroblast-rich construct, GAG and collagen type I, but no collagen type II deposition. Moreover, BMP2 only stimulated Smad1 signaling in human CLCs, and induced GAG and collagen type II but no collagen type I deposition. Altogether, these results suggest that Smad1 signaling was associated with collagen type II production, whereas Smad2 signaling accompanied fibrotic (re)differentiation in human and canine CLCs. In line with our results, TGF-β is a well-known, collagen type I-inducing, fibrotic agent, whereas BMP2 did not increase COL1A1 expression in human CLCs. In chondrocytes, however, TGF-β1-mediated Smad2/3 signaling results in protective, and Smad1/5/8 signaling in deleterious responses, indicating that CLCs respond distinctively different to (TGF-β1-mediated) Smad2/3 signaling than chondrocytes.

Hypertrophic differentiation is known to precede BMP (Smad1/5/8)-induced bone formation in articular chondrocytes. In the current study, however, no signs for hypertrophic differentiation or osteogenic matrix production were detected in BMP2-treated canine or human CLCs. In line with our results, others showed only chondrogenic, but no osteogenic effects of BMP2 on rabbit NCs, human CLCs and degenerated goat IVDs, indicating that CLCs may also respond rather different to BMP2 (Smad1/5/8 signaling) than chondrocytes. Altogether, the results of this study suggest that BMP2 can be a valuable candidate for IVD regeneration, since it induced chondrogenic, but no osteogenic or fibrotic ECM production in canine and human CLCs. Future studies should, however, investigate long-term exposure to BMP2. Lastly, CD and NCD canine CLCs responded rather comparably to growth factor treatment as human CLCs, indicating that CLCs from both CD and NCD dogs can be used as in vitro animal model for human IVD degeneration.
The effect of MSCs on canine CLCs

Hydrogels that are already applied in clinical studies for intradiscal cell transplantation were used to determine the possible additive regenerative effect of MSCs on BMP2-treated CLCs. The results of the current study indicate that a considerable amount of MSCs survived the 28-day CLC:MSC co-culture period. Also in vivo, injected MSCs maintain their viability for at least 28 days within rat IVDs. In co-cultures, MSCs have been shown to stimulate chondrocyte proliferation and ECM production. Although previous work on human CLC:MSC co-cultures also showed (modest) trophic effects of MSCs, the present study on canine CLC:MSC co-cultures did not. Comparable results as observed in this canine co-culture study have been demonstrated for human chondrocyte:MSC co-cultures, stressing the importance of using proper controls. Cells should be seeded at comparable densities, but also at lower densities to account for possible differences in available nutrition or cells producing factors inhibiting their own proliferation.

Taken together, MSCs did not exert regenerative effects on CD or NCD canine CLCs in vitro. The discrepancy between these results and the results of previous work could be due to species differences. Additionally, our experiments were performed in normoxia, whereas the NP is an hypoxic tissue. Hypoxia produces a favorable microenvironment that improves MSC cell viability, as well as differentiation into NP-like cells and ECM synthesis. Furthermore, previous work indicated that the optimal CLC:MSC ratio for MSC differentiation appeared 75:25. Therefore, future studies should test different CLC:MSC ratios under hypoxic culture conditions. The results of the current study do not directly imply that MSCs will not have regenerative effects on degenerated IVDs in vivo. Given that regenerative effects of MSCs were encountered in different animal models with experimentally induced IVD degeneration, future studies should investigate whether MSCs also have beneficial effects in dogs with spontaneously degenerated IVDs, a valid in vivo animal model for human IVD degeneration.

Conclusion

This study demonstrates that while both TGF-β1 and BMP2 exerted regenerative effects on human and canine CLCs, BMP2 induced a more healthy NP matrix than TGF-β1. (BMP2-mediated) Smad1 signaling increased collagen type II production, whereas (TGF-β1-mediated) Smad2 signaling induced fibrotic CLC (re)differentiation. MSCs did not exert additive regenerative effects on control- or BMP2-treated canine CLCs. Since CD and NCD canine CLCs responded rather comparable to TGF-β1 and BMP2 as human CLCs, support for the dog as a valid in vitro model for human IVD degeneration was provided.

Acknowledgments

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References


40. Wu L, Prins HJ, Helder MN, van Blitterswijk CA, Karperien M. 2012. Trophic effects of mesenchymal stem cells in chondrocyte co-cultures are independent of culture conditions and cell sources. Tissue Eng Part A 18: 1542-1551.


### Supplementary File 1. Primers used for quantitative PCR

#### Primers used for quantitative PCR of canine samples

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward sequence 5′ → 3′</th>
<th>Reverse sequence 5′ → 3′</th>
<th>Amplicon size</th>
<th>Annealing temp (°C)</th>
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<td><strong>Reference genes</strong></td>
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All primers were designed in-house using Primer3 except for MMP13³ and BAX².

Stably expressed reference genes (TATAA-box binding protein (TBP), hypoxanthine-guanine phosphoribosyltransferase (HPRT), succinate dehydrogenase subunit A (SDHA) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein and zeta polypeptide (YWHAZ) for the human samples and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S19 (RPS19), SDHA and HPRT for the canine samples) were chosen to normalize gene expression of the target genes: aggrecan (ACAN), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), Bcl2-like-protein (BAX), bone gamma-carboxyglutamate protein/osteocalcin (BGLAP), cyclin D1 (CCND1), collagen type I (COL1A1), collagen type II (COL2A1), collagen type X (COL10A1), matrix metalloproteinase 13 (MMP13), osterix (OSX), runt-related transcription factor 2 (RUNX2), sex determining region Y (SRY), and a tissue inhibitor of metalloproteinases (TIMP1).
### Primers used for quantitative PCR of human samples

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward sequence 5’ → 3’</th>
<th>Reverse sequence 5’ → 3’</th>
<th>Amplicon size</th>
<th>Annealing temp (°C)</th>
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<tr>
<td><strong>Reference genes</strong></td>
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<td>HPRT</td>
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<td><strong>Target genes</strong></td>
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<tr>
<td>ACAN</td>
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<td>BAX</td>
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All reference gene primers were designed in-house using Perprimer. Primers for the target genes ACAN, ADAMTS5, COL1A1, and COL2A1 were kindly provided by UMC Utrecht, the Netherlands. Primers for BAX, CCND1, and MMP13 were obtained from the literature.

### References

Supplementary File 2. The effect of Transforming Growth Factor beta 1 (TGF-β1) and Bone Morphogenetic Protein 2 (BMP2) on gene expression of human and canine chondrocyte-like cells (CLCs) derived from degenerated intervertebral discs (IVDs)

Relative CCND1, BAX, ACAN, COL2A1, COL1A1 and MMP13 gene expression (mean±SD) of the CLC micro-aggregates at day 7. The control micro-aggregates were set at 1. \( n = 7 \). CD: chondrodystrophic, NCD: non-chondrodystrophic. *, **, ***: significantly different from all other conditions (\( p < 0.05, p < 0.01, p < 0.001 \), respectively); $, $$, $$$: significantly different from all other conditions except the one with the other $, $$, or $$$ mark (\( p < 0.05, p < 0.01, p < 0.001 \), respectively); #: \( p < 0.05 \), ##: \( p < 0.01 \).
Supplementary File 3. Transforming Growth Factor beta 1 (TGF-β₁) and Bone Morphogenetic Protein 2 (BMP2) do not induce collagen type X and calcium (identified by Alizarin Red S staining) deposition in human and canine chondrocyte-like cell (CLC) micro-aggregates.

Micro-aggregates of CLCs derived from degenerated human/canine intervertebral discs (IVDs) were cultured for 28 days. \( n = 7 \) per condition. CD: chondrodystrophic, NCD: non-chondrodystrophic.
Supplementary File 4. The effect of Bone Morphogenetic Protein 2 (BMP2) on matrix production and cell proliferation of non-chondrodystrophic (NCD) canine chondrocyte-like cells (CLCs) and mesenchymal stromal cells (MSCs)

CLC (3*10^6/mL hydrogel), ½ CLC (1.5*10^6/mL) or CLC:MSC (1:1, both 1.5*10^6/mL) hydrogels were cultured for 28 days with or without 250 ng/mL BMP2. (a) DNA content, (b) GAG release, (c) GAG content, (d) GAG/DNA content, (e) Safranin O/Fast green staining and collagen type I and II immunohistochemistry. n = 8. #, ###: p < 0.05 and p < 0.001, respectively; **, ***: significantly different from all other conditions (p < 0.01, p < 0.001, respectively); †††: significantly different from all other conditions except for the one with the same ††† mark (p < 0.001); @@: significantly different from all other conditions except for the one with the same @@ mark (p < 0.01); $$, $$$: significantly different from all other conditions except for the one with the same $$ or $$$ mark (p < 0.01, p < 0.001, respectively).
Supplementary File 5. Schematic overview of the observed effects of Transforming Growth Factor beta 1 (TGF-β₁) and Bone Morphogenetic Protein 2 (BMP2) on chondrodystrophic (CD) and non-chondrodystrophic (NCD) canine and human chondrocyte-like cells (CLCs) derived from degenerated intervertebral discs

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<tr>
<th></th>
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<td></td>
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<td>NCD canine</td>
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<td>+</td>
<td>+/- (trend; ( p = 0.1 ))</td>
</tr>
<tr>
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<td>+</td>
</tr>
<tr>
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</tr>
<tr>
<td>Collagen X</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

++: more increased compared with controls than under influence of the other growth factor (TGF-β₁/BMP2); +: increased compared with controls; +/- moderately/mildly increased compared with controls; - not increased compared with controls.